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Safety and efficacy of a feed additive consisting of *Lentilactobacillus diolivorans* (formerly *Lactobacillus diolivorans*) DSM 33625 as a silage additive for all animal species (Lactosan GmbH & Co.KG)

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), Vasileios Bampidis, Giovanna Azimonti, Maria de Lourdes Bastos, Henrik Christensen, Birgit Dusemund, Mojca Fašmon Durjava, Maryline Kouba, Marta López-Alonso, Secundino López Puente, Francesca Marcon, Baltasar Mayo, Alena Pechová, Mariana Petkova, Fernando Ramos, Yolanda Sanz, Roberto Edoardo Villa, Ruud Woutersen, Montserrat Anguita, Rosella Brozzi, Jordi Ortuño and Yolanda García-Cazorla

Abstract

Following a request from the European Commission, EFSA was asked to deliver a scientific opinion on *Lentilactobacillus diolivorans* (formerly *Lactobacillus diolivorans*) DSM 33625 when used as a technological additive to improve ensiling of forage. The additive is intended for use with all forages and for all animal species at a proposed minimum concentration of 1×10^8 colony forming units (CFU)/kg forage. The bacterial species *L. diolivorans* 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 a skin or an eye irritant. In the absence of data, no conclusion can be drawn on the skin sensitisation of the additive. Given the proteinaceous nature of the active agent, the additive should be considered a respiratory sensitiser. The FEEDAP Panel concluded that *Lentilactobacillus diolivorans* DSM 33625 at a minimum concentration of 1×10^8 CFU/kg forage may extend the aerobic stability of silage prepared from easy and moderately difficult to ensile forage material with a DM range of 32–65%.

Keywords: technological additive, silage additive, *Lentilactobacillus diolivorans* DSM 33625, safety, efficacy, QPS

Requestor: European Commission

Question number: EFSA-Q-2021-00590

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Table of contents

1. Introduction

1.1. Background and Terms of Reference

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 feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from Lactosan GmbH & Co.KG[2] for the authorisation of the additive consisting of *Lentilactobacillus diolivorans* DSM 33625, 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). EFSA received directly from the applicant the technical dossier in support of this application. The particulars and documents in support of the application were considered valid by EFSA as of 12 May 2022.

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 feed additive consisting of *Lentilactobacillus diolivorans* DSM 33625, 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 *Lentilactobacillus diolivorans* (formerly *Lactobacillus diolivorans*) DSM 33625. It is not currently authorised 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 *Lentilactobacillus diolivorans* DSM 33625 as a feed additive.

In accordance with Art. 38 of the Regulation (EC) No 178/2002[4] and taking into account the protection of confidential information and of personal data in accordance with Articles 39 to 39 e 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 dossier has been published on Open.EFSA.[6]

According to Art. 32c(2) of Regulation (EC) No 178/2002 and to the Decision of EFSA's Executive Director laying down the practical arrangements on pre-submission phase and public consultations[2], EFSA carried out a public consultation on the non-confidential version of the technical dossier from 7 to 28 December 2022 for which no comments were received.

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

[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] Lactosan GmbH & Co.KG, Industriestrabe West 5, A-8605 Kapfenberg.
[3] FEED dossier reference: FEED-2021-0662.
[4] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, pp. 1–48.
[5] Decision available at: https://www.efsa.europa.eu/en/corporate-pubs/transparency-regulation-practical-arrangements
[6] The non-confidential version of the dossier has been published on Open.EFSA and is available at the following link: https://open.efsa.europa.eu/dossier/FEED-2021-0662
[7] The full report is available on the EURL website: https://joint-research-centre.ec.europa.eu/eurl-fa-eurl-feed-additives/eurl-fa-authorisation/eurl-fa-evaluation-reports_en

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of the active agent is in line with the principles laid down in Regulation (EC) No 429/2008[8] 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 FEEDAP Panel, 2017a), Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017b), Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018a), Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018b), Guidance on the assessment of the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019).

3. Assessment

The present additive is based on a preparation of viable cells of a single strain of *Lentilactobacillus diolivorans* (formerly *Lactobacillus diolivorans*) intended to be added to forages to promote ensiling (technological additive, functional group: silage additive) with the eventual use of the silage for all animal species.

3.1. Characterisation

3.1.1. Characterisation of the active agent

The strain of *L. diolivorans* was originally isolated from silage and is deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) with the accession number DSM 33625.[9] It has not been genetically modified.[10]

The full genome of the strain DSM 33625 was sequenced.[11] The taxonomic identification of the strain was confirmed [redacted][12]

The antimicrobial susceptibility of the strain DSM 33625 was tested against the battery of antibiotics recommended by the FEEDAP Panel (EFSA FEEDAP Panel, 2018b) for obligate heterofermentative lactobacilli. All the minimum inhibitory concentration (MIC) values for the strain were equal to or fell below the corresponding cut-off values, [redacted][13]. Therefore, the strain is considered to be susceptible to all relevant antibiotics.

The whole genome sequence (WGS) of the strain DSM 33625, [redacted] was interrogated for the presence of antimicrobial resistance (AMR) genes [redacted].[14] No hits of concern were identified.

3.1.2. Characterisation of the additive

[redacted]. The final feed additive consists of [redacted] cell concentrate and [redacted] cryoprotectants/carriers to

[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] Annex_II_6_Deposit.
[10] Detailed identification.
[11] Annex_II_7a_WGS_gbk.
[12] Annex_II_7_WGS.
[13] Annex_II_9_Antimicro.
[14] Annex_II_10_AMR.

guarantee a minimum concentration of active agent of 2×10^{11} CFU/g of additive. The applicant states that no antimicrobial substances are used during the manufacturing process.[15]

Analysis of five batches of the additive showed a mean value of 4.1×10^{11} CFU/g (range $3.1–4.9 \times 10^{11}$ CFU/g).[16]

The same five batches were analysed for *Enterobacteriaceae*, yeasts and filamentous fungi (< 1,000 CFU/g) and *Salmonella* spp. was not detected in 25 g.[17] Results of the analyses of three batches for detection of aflatoxins (B1, B2, G1, and G2), zearalenone and deoxynivalenol showed levels below the respective limits of quantification (LOQs) of the analytical methods.[18,19] Arsenic, cadmium, mercury and lead concentrations were below the respective LOQ in all three batches.[20,21]

The levels of the detected impurities do not raise concerns.

The dusting potential of three batches of the additive containing whey powder as carrier tested using the Stauber–Heubach method showed values of 2.33–2.90 g/m^3. The particle size distribution of the additive in the same three batches measured by laser diffraction, showed that approximately 35% of the particles have diameters < 50 μm and 5% of the particles have diameters < 10 μm.[22]

3.1.3. Stability and homogeneity

The shelf-life of the additive containing whey powder as carrier (three batches) was tested when stored in its original packaging (Alu-PE bag) at 20°C for up to 12 months.[23] Losses < 0.5 log of the initial value was observed under the above-mentioned conditions.

The stability of the additive in water was studied in three other batches produced with whey powder as carrier. One gram of the bacterial concentrate (lyophilised powder) was suspended in 19 mL of tap water and half of the sample was stored for 48 h at 20°C, while the other half for 7 days at 4°C.[24] Losses < 0.5 log of the initial value was observed at the end of the respective storage periods.

3.1.4. Conditions of use

The additive is intended for use with all forages and for all animal species at a proposed minimum concentration of 1×10^8 CFU/kg forage.

It is to be applied as such or as an aqueous suspension.[25]

3.2. Safety

3.2.1. Safety for the target species, consumers and environment

The species *L. diolivorans* 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 does not carry acquired resistance to antibiotics of human and veterinary importance. In the view of the FEEDAP Panel, the identity of the strain has been established as *L. diolivorans* and the antibiotic resistance qualification met. Consequently, *L. diolivorans* DSM 33625 is considered safe for the target species, consumers and the environment.

3.2.2. Safety for the user

No specific studies investigating the effects of the additive on the respiratory system were submitted. The dusting potential reported (2.33–2.90 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.

[15] Manufacturing, Annex_II_11_Manuf_active and Annex_II_12_Manuf_add.
[16] Annex_II_2_Batch.
[17] Annex_II_3_Purity.
[18] Annex_II_4_Mycotox.
[19] LOQ for aflatoxins (B1, B2, G1, and G2) 0.03 μg/kg, zearalenone (ZEA) 10 μg/kg and deoxynivalenol (DON) 5 μg/kg.
[20] Annex_II_5_HeavyMet.
[21] LOQ for arsenic, lead and mercury 0.1 mg/kg and for cadmium 0.03 mg/kg.
[22] Annex_III_1_Dust_PSD.
[23] Physico_chemical and Annex_II_13_Stab.
[24] Annex_II_14_Stab_water.
[25] Conditions of use of the additive.

The skin[26] and eye[27] irritation potential of the additive was tested following the principles of Good Laboratory Practice (GLP) 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.

3.3. Efficacy

Three laboratory experiments were made with different forage samples representing materials easy to ensile (studies 1 and 2) and moderately difficult to ensile (study 3), as specified by Regulation (EC) No 429/2008 (Table 1).[28] All the studies included a control and a group inoculated with *L. diolivorans* DSM 33625. The additive was dissolved in water and sprayed on the forage at an intended concentration of 1×10^8 CFU/kg fresh matter (not confirmed by analysis). Forage for the control silos were sprayed with an equal volume of water, but without the additive. Samples of forages were ensiled for 90 days in 6.5 L mini-silos (three replicates per treatment). The ambient temperature during ensiling was controlled at 20 ± 2°C.

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

Study	Test material	Dry matter content (%)	Water-soluble carbohydrate content (% fresh matter)
1[29]	Grass(1)	34.3	4.05
2[30]	Maize whole plant	32.9	3.28
3[31]	Maize Cob Mix	65.0	1.52

(1): 3rd cut of grass, 100% *Festuca arundinacea*.

After 90 days, the silos were opened and the contents were analysed for dry matter (DM), pH, lactic, formic, butyric, acetic and propionic acid as well as ethanol, and ammonia-N content. The aerobic stability of the silage was also determined. At the end of each experiment, samples were taken from each silo and exposed to air. A rise of 3°C above room temperature was considered as 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. The DM loss was corrected for volatiles.

Statistical evaluation of data was done by a one-sided non-parametric test (Mann–Whitney U-test), comparing treated versus control silos. Significance declared at $p = 0.05$. Results are shown in Table 2.

Table 2: Effects of *L. diolivorans* DSM 33625 on the characteristics of ensiled material recovered at the end of the ensiling period (90 days)

Study	Application rate (CFU/kg forage)	Dry matter loss (%)	pH	Lactic acid (% fresh matter)	Acetic acid (% fresh matter)	Ammonia-N (% of total N)	Aerobic stability (days)
1	0	9.02	4.39	1.60	0.33	8.44	4.6
	1×10^8	5.21*	4.05*	1.85*	0.98*	4.75*	> 10*
2	0	3.32	3.97	1.47	0.77	3.18	3.8
	1×10^8	5.35	3.83*	1.37	1.85*	3.18	> 11*

[26] Annex_III_2_Skin.
[27] Annex_III_3_Eye.
[28] Efficacy.
[29] Annex_IV_1_Efficacy1.
[30] Annex_IV_2_Efficacy2.
[31] Annex_IV_3_Efficacy3.

Study	Application rate (CFU/kg forage)	Dry matter loss (%)	pH	Lactic acid (% fresh matter)	Acetic acid (% fresh matter)	Ammonia-N (% of total N)	Aerobic stability (days)
3	0	1.08	3.98	1.00	0.18	3.48	1.4
	1×10^8	1.19	4.21	0.08	1.42*	5.23	> 12*

CFU: colony forming unit.
*: Values in a column within a given trial are considered as significantly different when p = 0.05 (one-tailed).

In the three studies, aerobic stability was improved by the addition of the additive, extending the time with no deterioration of silage exposed to air for longer than 2 days. However, the tested material was restricted to a range of 32–65% DM content.

Regarding the DM loss, only in study 1 a positive outcome was observed, while DM loss increased in the other two studies. Similarly, the addition of the additive resulted in a reduction in ammonia-N production and increased lactic acid concentration in study 1, but there were no differences in study 2 and the results obtained in study 3 were opposite to those expected after an improved ensiling process. The concentration of acetic acid increased in the three studies after the addition of the additive.

3.3.1. Conclusions on efficacy

The use of *L. diolivorans* DSM 33625 at the proposed inclusion rate has the potential to improve the aerobic stability of silage from forages with a DM range of 32–65%.

4. Conclusions

Lentilactobacillus diolivorans DSM 33625 is safe for target species, consumers and the environment.

The additive is not a skin or an eye irritant, but should be considered a respiratory sensitiser. In the absence of data, no conclusion can be drawn on the skin sensitisation potential of the additive.

The addition of *Lentilactobacillus diolivorans* DSM 33625 at a minimum concentration of 1×10^8 CFU/kg forage has the potential to improve the aerobic stability of silage from easy and moderately difficult to ensile forage material with a DM range of 32–65%.

5. Documentation provided to EFSA/Chronology

Date	Event
19/10/2021	Dossier received by EFSA. *Lactobacillus diolivorans* DSM 33625. Submitted by Lactosan GmbH & Co.KG
24/06/2021	Reception mandate from the European Commission
12/05/2022	Application validated by EFSA – Start of the scientific assessment
28/07/2022	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: identification and characterisation of the additive*
05/08/2022	Reception of supplementary information from the applicant - Scientific assessment re-started
12/08/2022	Comments received from Member States
29/08/2022	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
27/09/2022	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: efficacy*
07/10/2022	Reception of supplementary information from the applicant - Scientific assessment re-started
17/01/2023	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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Abbreviations

AMR	antimicrobial resistance
CFU	colony forming unit
DM	dry matter
EURL	European Union Reference Laboratory
FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
GLP	Good Laboratory Practice
LOQ	limit of quantification
MIC	minimum inhibitory concentration
OECD	Organisation for Economic Co-operation and Development
PFGE	pulsed-field gel electrophoresis
QPS	Qualified Presumption of Safety
WGS	whole genome sequence

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of the Analysis for *Lactobacillus diolivorans* DSM 33625

In the current application an authorisation is sought under Article 4 for *Lactobacillus diolivorans* DSM 33625 under the category/functional group 1(k) 'technological additives'/'silage additives', according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the feed additive for all animal species.

According to the Applicant, the *feed additive* contains as active substance viable cells of the non-genetically modified strain *Lactobacillus diolivorans* DSM 33625 at a minimum level of 2×10^{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/g fresh silage.

For the enumeration of *Lactobacillus diolivorans* DSM 33625 in the *feed additive per se* the Applicant proposed for official control the ring-trial validated spread plate (or pour plate) method EN 15787.

Based on the performance characteristics, as already concluded for similar Lactobacilli reports, the EURL recommends for official control the ring-trial validated EN 15787 method for the enumeration of *Lactobacillus diolivorans* DSM 33625 in the *feed additive per se*.

Furthermore, for the identification of *Lactobacillus diolivorans* DSM 33625 at a strain level, the EURL recommends for official control (i) DNA sequencing methods (e.g. Whole Genome Sequencing (WGS)) or (ii) Pulsed-Field Gel Electrophoresis (PFGE).

As the enumeration of added *Lactobacillus diolivorans* DSM 33625 in *silage* is not achievable by analysis, the EURL cannot recommend any method for official control.

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.