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Safety and efficacy of *Lactobacillus diolivorans* DSM 32074 as a silage additive for all animal species

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP)

Abstract

Lactobacillus diolivorans DSM 32074 is a technological additive intended to improve the ensiling process at a minimum proposed dose of 1.0 x 10^8 colony forming units (CFU)/kg fresh material. The bacterial species *L. diolivorans* is considered by EFSA to be suitable for the qualified presumption of safety (QPS) approach to safety assessment. As the identity of the strain has been clearly established and as no antibiotic resistance of concern was 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 eye irritant but should be considered to have the potential to be a respiratory sensitiser. In the absence of data, no conclusion can be drawn on the dermal sensitisation potential of the additive. Four studies with laboratory-scale silos were made using samples of forage of differing dry matter and water-soluble carbohydrate content. In each case, replicate silos containing treated forage were compared with identical silos containing the same but untreated forage. *Lactobacillus diolivorans* DSM 32074 added at 1.0 x 10^8 CFU/kg forage has the potential to extend the aerobic stability of silage prepared from easy, moderately difficult and difficult to ensile material.

Keywords: technological additive, silage additive, *Lactobacillus diolivorans* DSM 32074, safety, QPS, efficacy

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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 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 the authorisation of *Lactobacillus diolivorans* DSM 32074, when used as a feed additive for all animal species (category: Technological additive; 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). 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 6 January 2016.

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 diolivorans* DSM 32074, 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 *L. diolivorans* DSM 32074. It has not been previously authorised as a feed additive in the European Union (EU).

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, 2013). This approach requires the identity of the strain to be conclusively established and evidence that the strain does not show resistance to antibiotics of human and veterinary importance.

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 diolivorans* DSM 32074 as a feed additive. The technical dossier was prepared following the provisions of Article 7 of Regulation (EC) No 1831/2003, Regulation (EC) No 429/2008[4] and the applicable EFSA guidance documents.

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.

2.2. Methodologies

The approach followed by the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) to assess the safety and the efficacy of *Lactobacillus diolivorans* DSM 32074 is in line with the principles laid down in Regulation (EC) No 429/2008 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

[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-2015-0028.
[4] 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.

assessment of bacterial susceptibility to antimicrobials of human and veterinary importance (EFSA FEEDAP Panel, 2012c).

3. Assessment

The additive is a preparation of viable cells of *L. diolivorans* DSM 32074 intended for use as a technological additive (silage additive) for all animal species.

3.1. Characterisation

3.1.1. Characterisation of the active agent

The strain was isolated from maize silage and is deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) with the accession number DSM 32074.[5] It has not been genetically modified.

The strain was identified by sequencing 880 bp of the 16S rRNA gene.[6] The genetic stability was examined by comparing the master culture with the working cell bank and two post-fermentative samples using RAPD-PCR with nine different primers.[7] No differences in the resultant patterns were observed.

The bacterial strain was tested for antibiotic susceptibility using broth microdilution. The battery of antibiotics used included those recommended by EFSA (EFSA FEEDAP Panel, 2012c).[8] The minimum inhibitory concentration (MIC) values were compared with the cut-off values for obligate heterofermentative lactobacilli. They were all below or equal to the EFSA cut-off values except for tetracycline (16 vs 8 mg/L) which was exceeded by a single dilution. This is within the normal variation around the mean, and thus, does not raise concerns for safety.

3.1.2. Manufacturing process and characterisation of the product

The active agent is grown in a sterilised medium typical of those used for lactic acid bacteria and then separated from the growth medium by centrifugation.[9] Cryoprotectants (selected from ascorbic acid, lactose, mannite, monosodium glutamate, sodium citrate, whey powder or polyethylene glycol) are added and the cell mix is freeze-dried and ground. Numbers of lactic acid bacteria are determined in each batch and numbers adjusted to meet a minimum specification by addition of a carrier (whey powder in the product referred to in the dossier but the applicant also lists dextrose anhydrous and maltodextrin). The resultant additive consists of approximately 35–50% freeze-dried cell mass and 50–65% cryoprotectants/carrier.

The minimum content of *L. diolivorans* in the final product is specified as of 3.0×10^{11} colony forming unit (CFU) per gram of additive. Analysis of five production batches showed a mean value of 4.1×10^{11} CFU/g additive (range = $3.3–4.7 \times 10^{11}$ CFU/g additive).[10]

The additive is routinely monitored for microbial contamination at various points in the manufacturing process and in the final product. Limits are set for Enterobacteriaceae (< 1,000 CFU/g), yeasts and filamentous fungi (< 1,000 CFU/g), *Salmonella* (absent in 25 g) and *Escherichia coli* (< 10 CFU/g). Analyses of five batches of the additive (three for *Salmonella* and *E. coli*) demonstrated compliance with these limits.[11] The applicant claims that as raw materials used in the fermentation process are food grade, no contamination with mycotoxins, heavy metals or arsenic is expected and no routine monitoring is carried out. To support this claim, three batches of the additive were tested and no contaminants (aflatoxins B1, B2, G1, G2, zearalenone, desoxynivalenol, lead, mercury, cadmium and arsenic) were detected.[12,13]

[5] Technical dossier/Section II/Annex II.2-4.
[6] Technical dossier/Section II/Annex II.2-5.
[7] Technical dossier/Section II/Annex II.2-7.
[8] Technical dossier/Section II/Annex II.2-8.
[9] Technical dossier/Section II/Annex II.3-1.
[10] Technical dossier/Section II/Annex II.1-2.
[11] Technical dossier/Section II/Annexes II.1-3 and 4.
[12] Technical dossier/Section II/Annex II.1-5.
[13] Limits of detection: aflatoxin B1 < 0.03 μg/kg, aflatoxin B2 < 0.03 μg/kg, aflatoxin G1 < 0.03 μg/kg, aflatoxin G2 < 0.03 μg/kg, zearalenone < 10 μg/kg, desoxynivalenol < 5 μg/kg, Pb < 1.5 mg/kg, Hg < 1.0 mg/kg, Cd < 0.1 mg/kg and As < 0.05 mg/kg.

One formulation of the additive was examined for particle size distribution by laser diffraction[14] and dusting potential with a Heubach dustometer.[15] Results showed that the mean particle size is 103.5 μm and that approximately 29% and 9% by volume of the additive consists of particles with diameters below 50 μm and below 10 μm, respectively. The dusting potential of the formulation tested was 4.4 g/m^3, which is considered high.

3.1.3. Stability

For the purpose of testing shelf life, three batches of the additive were stored at 4°C for 24 months, at 25°C for 12 months and at 40°C for 2 months in the original package that protects it against environmental conditions (light, moisture, and oxygen).[16] Lactobacilli counts remained unvaried (losses < 0.5 log) demonstrating the stability of the product at these conditions over these periods.

One batch of the additive was dissolved in water at a rate of 1 g of product in 20 mL of water to give a minimum count of 1.5 x 10^{10} CFU/mL and maintained at 20°C for 48 h and at 4°C for 7 days.[17] Counts of lactobacilli remained constant (losses < 0.5 log) over this period.

3.1.4. Conditions of use

The additive is intended for use as a silage additive with all forages and for all animal species at a proposed minimum concentration of 1.0 x 10^8 CFU/kg fresh material, to be applied as an aqueous suspension.

3.2. Safety

3.2.1. Safety for the target species, consumers and environment

In the view of the FEEDAP Panel, the antibiotic resistance qualification has been met and the identity of the strain established. Consequently, *L. diolivorans* DSM 32074 is considered to be suitable for the QPS approach to safety assessment and is presumed safe for the target species, consumers of products from animals fed treated silage and the environment.

3.2.2. Safety for the user

A study of acute dermal irritation with *Lactobacillus diolivorans* DSM 32074 (described as 80% of cell mass and 20% of unspecified carrier) was performed following the Organisation for Economic Co-operation and Development (OECD) Guideline 404.[18] The test item caused a very slight erythema in the three New Zealand rabbits treated, which were reversible between day 1 and 3. A very slight oedema, totally reversible on day 1, was noted in one animal. Thus, it is concluded that the additive is not a skin irritant.

A study of acute eye irritation with *Lactobacillus diolivorans* DSM 32074 (described as 80% of cell mass and 20% of unspecified carrier) was performed using three male New Zealand White rabbits and following a protocol that conformed to OECD Guideline 405.[19] The test item produced a slight redness 1 h after test item instillation and totally reversible between day 1 and day 2 in two animals. Overall results indicate that the additive is not to be considered an eye irritant.

In the absence of data, the FEEDAP Panel cannot conclude on the dermal sensitisation potential of the additive.

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 be a potential respiratory sensitiser.

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

[14] Technical dossier/Section II/Annex II.1-6.
[15] Technical dossier/Section II/Annex II.1-7.
[16] Technical dossier/Section II/Annex II.4-1.
[17] Technical dossier/Section II/Annex II.4-2.
[18] Technical dossier/Section III/ Annex III.3-3.
[19] Technical dossier/Section III/ Annex III.3-4.

safety issues. For this specific product, the excipients used in the preparation of the final formulation do not introduce additional risks.

3.3. Efficacy

Four laboratory experiments using different forages were conducted. The duration of the experiments was 90–92 days, with measurements (i.e., pH, lactic acid content) taken during the first and second weeks after ensiling, and at the end of the experiment. Forages were sprayed with the additive at an intended dose of 1 x 10^8 CFU/kg forage suspended in water (doses not confirmed by analysis) and ensiled in containers of different capacity (1.5 L for intermediate samples in all studies, 6.5 L for the final samples in studies 1,[20] 3[21] and 4[22]; or 5 L in study 2[23]) sealed with a plastic lid. Forages for the negative control silos were sprayed with an equal volume of water but without the additive. Three replicate silos were used in treated and control forages. Laboratory silos were maintained at 20°C during the experiment. The forages were different in each study with varying dry matter (DM) and water-soluble carbohydrates contents (see Table 1) to represent material easy to ensile (study 1), moderately difficult to ensile (studies 2 and 3) and difficult to ensile (study 4), as specified by Regulation (EC) No 429/2008.

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

Study	Test material	Dry matter content (%)	Water-soluble carbohydrate content (% fresh matter)
1	Grass, first cut	45.2	4.1
2	Grass, first cut	28.1	2.5
3	Maize, whole plant	35.8	2.4
4	Corn cob mix	68.8	0.6

Microbial counts (lactic acid bacteria, total yeasts and filamentous fungi) of forages at ensiling were reported. Replicate silos were opened at the end of the experiment and the contents were analysed for chemical composition, DM content, pH, lactic and volatile fatty acid concentrations, ethanol, ammonia and total nitrogen. DM losses during the ensiling period were calculated. Aerobic stability of ensiled material at the end of the study was determined as the elapsed time until the temperature of a sample of silage placed in an insulated box was 3°C above ambient temperature (assumed to indicate aerobic deterioration; Honig, 1990). Statistical evaluation of data was by comparison of treated versus control silage using a non-parametric test (Wilcoxon rank sum test), with significance assumed at $p < 0.05$. Results are shown in Table 2.

Table 2: Summary of the analysis of control and treated (by the addition of *Lactobacillus diolivorans* DSM 32074) silage recovered at the end of the studies

Study (duration)	Application rate (CFU/kg forage)	Dry matter loss (%)	pH	Lactic acid (% fresh matter)	Acetic acid (% fresh matter)	NH_3-N (% total N)	Aerobic stability (days)[(a)]
1	0	7.1	5.3	0.9	0.3	8.7	3.5
(90 days)	1 x 10^8	5.4	4.1*	1.8*	1.6*	7.5*	> 12.5*
2	0	12.3	4.8	0.4	0.7	19.4	7.7
(90 days)	1 x 10^8	7.8*	4.4*	0.7*	2.2*	13.5*	> 11.7*
3	0	3.3	3.8	1.7	0.7	14.8	2.0
(90 days)	1 x 10^8	3.8	3.9	0.8*	2.1*	13.4*	> 12.5*
4	0	1.4	3.9	1.5	0.3	7.7	2.2
(92 days)	1 x 10^8	1.5*	4.2*	0.03*	1.8*	7.9	> 10.4*

CFU: colony forming unit.
(a): Period to reach 3°C rise over the ambient temperature (days).
*Significantly different from the control value at $p < 0.05$.

[20] Technical dossier/Section IV/Annex IV.1.
[21] Technical dossier/Section IV/Annex IV.3.
[22] Technical dossier/Section IV/Annex IV.4.
[23] Technical dossier/Section IV/Annex IV.2.

In all the studies, the addition of *Lactobacillus diolivorans* DSM 32074 to the forage material increased acetic acid concentration in silage and extended aerobic stability. Treated silages showed stability in all studies for at least 2 days longer than that observed in the untreated control. Additionally, ammonia-N was reduced in treated silages from easy and moderately difficult to ensile material.

4. Conclusions

As the identity of *Lactobacillus diolivorans* DSM 32074 has been established and no antibiotic resistance of concern has been detected, following the QPS approach to safety assessment, the use of this 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 eye irritant but should be considered to have the potential to be a respiratory sensitiser. In the absence of data, no conclusion can be drawn on the dermal sensitisation potential of the additive.

Lactobacillus diolivorans DSM 32074 has the potential to extend the aerobic stability of silage by increasing acetic acid production. This is shown in forage from easy, moderately difficult and difficult to ensile material at the application rate of 1.0×10^8 CFU/kg forage.

Documentation provided to EFSA

1) Request for authorisation of *Lactobacillus diolivorans* DSM 32074. November 2015. Submitted by Lactosan GmbH & Co.KG.
2) Evaluation report of the European Union Reference Laboratory for Feed Additives on the Methods(s) of Analysis for *Lactobacillus diolivorans* DSM 32074.
3) Comments from Member States.

References

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EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2013. Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2013 update). EFSA Journal 2013;11(11):3449, 108 pp. doi:10.2903/j.efsa.2013.3449

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2016. Statement on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 4: Suitability of taxonomic units notified to EFSA until March 2016. EFSA Journal 2016;14(7):4522, 37 pp. doi:10.2903/j.efsa.2016.4522

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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. doi:10.2903/j.efsa.2012.2539

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012c. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA Journal 2012;10(6):2740, 10 pp. doi:10.2903/j.efsa.2012.2740

Honig H, 1990. Evaluation of aerobic stability. Grass Forage Report. Special Issue, 3, 76–82.

Abbreviations

CEN	European Committee for Standardization
CFU	colony forming unit
DM	dry matter
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
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
RAPD-PCR	random amplification of polymorphic DNA-polymerase chain reaction

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Methods of Analysis for *Lactobacillus diolivorans* DSM 32074[1]

In the current application authorisation is sought under Article 4(1) for *Lactobacillus diolivorans DSM 32074* 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.

According to the Applicant, the *feed additive* contains as *active substance* viable cells of the non-genetically modified strain *Lactobacillus diolivorans* DSM 32074. The *feed additive* is to be marketed as a powder containing a minimum *Lactobacillus diolivorans* DSM 32074 concentration of 3 x 10^{11} colony forming unit (CFU)/g. The *feed additive* is intended to be added to *silage* at a minimum dose of 1 x 10^{5} CFU/g fresh *silage.*

For the identification of *Lactobacillus diolivorans* DSM 32074, the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for genetic identification. This standard methodology for microbial identification is currently being evaluated by the CEN Technical Committee 327 to become European Standard.

For the enumeration of *Lactobacillus diolivorans* DSM 32074 in the *feed additive per se*, the Applicant submitted the ring-trial validated spread plate method EN 15787. Based on the performance characteristics available, the EURL recommends this method for official control.

Since the enumeration of added *Lactobacillus diolivorans* DSM 32074 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) is not considered necessary.

[1] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/default/files/finrep-fad-2015-0028-lacto_dioliv.pdf