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Safety and efficacy of *Lactobacillus rhamnosus* DSM 29226 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 a strain of *Lactobacillus rhamnosus* when used as a technological additive intended to improve ensiling at a proposed application rate of 5.0×10^7 colony-forming units (CFU)/kg fresh material. The bacterial species *L. rhamnosus* 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 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. In the absence of data, no conclusion can be drawn on the skin and eye irritancy or skin sensitisation of the additive. The additive should be considered as a potential respiratory sensitiser. Seven 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. The results showed that the additive has the potential to improve the production of silage from easy and moderately difficult to ensile forage species by reducing dry matter loss and enhancing protein preservation. This was shown at the proposed application rate of 5×10^7 CFU/kg forage.

Keywords: technological additive, silage additive, *Lactobacillus rhamnosus*, 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 Microferm Limited[2] for the authorisation of *Lactobacillus rhamnosus* DSM 29226, 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 21 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 rhamnosus* DSM 29226, 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. rhamnosus* DSM 29226. It has not been previously authorised as a feed additive in the European Union (EU).

The species *L. rhamnosus* 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 *L. rhamnosus* DSM 29226 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 *L. rhamnosus* DSM 29226 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 assessment of

[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] Microferm Limited, Spring Lane North, Malvern Link WR141BU Worcestershire United Kingdom.
[3] FEED dossier reference: FAD-2015-0033.
[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.

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. rhamnosus* DSM 29226 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 grass. It is deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) with the accession number DSM 29226.[5] It has not been genetically modified.

Species identity was established by phenotypic methods and by the nearly complete 16S rRNA gene sequence, which, by comparison with sequences recorded in databases, enabled the strain to be identified as *L. rhamnosus*. Multilocus sequence typing based on sequencing four specific genes (*rpoA, pheS, atpA* and *dnaK*) was proposed as a means of strain-specific detection.[6] Although the method is suitable for the discrimination of closely related strains, its effectiveness depends on the selection of sequences to be compared. No data were provided to illustrate that comparison of the four gene fragments chosen in this case is able to distinguish between DSM 29226 and other *L. rhamnosus* strains.

The genetic stability was examined by comparing working cultures with the culture collection stock using randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) amplification (RAPD-PCR).[7] No differences in the resultant patterns were observed.

The bacterial strain was tested for antibiotic susceptibility using broth microdilution techniques. The battery of antibiotics used included those recommended by EFSA (EFSA FEEDAP Panel, 2012c).[8] As all the minimum inhibitory concentration values were equal or below the corresponding EFSA cut-off values, no further investigation is required and the additive is considered susceptible to all relevant antibiotics.

3.1.2. Manufacturing process and characterisation of the product[9]

The manufacturing process is detailed in the dossier. The additive is produced with a minimum declared content of 1×10^{10} colony-forming units (CFU)/g.[10] Material safety datasheets are provided for all medium components and cryoprotectants but no purity criteria are included.[11]

The strain is also intended for use in grow-up formulations in which numbers of bacteria are increased by incubation before application to forage. Since the growth of the strain is encouraged, the product is also available in a formulation which contains (feed-grade) nitrogen sources and buffer salts.

Analysis of five freeze-dried cell batches (before blending) showed a mean value of 1.5×10^{11} CFU/g (range $1.3–1.7 \times 10^{11}$ CFU/g).[12]

Microbial contamination is routinely monitored at various points in the manufacturing process and in the final product. Limits are set for yeasts and filamentous fungi (< 10 CFU/g), presumptive coliforms and *Escherichia coli* (< 10 CFU/g) and *Salmonella* spp. (absent in 25 g). Compliance with specifications was proved in five batches.[13] Given the nature of the fermentation medium and the excipients, the probability of contamination with heavy metals or mycotoxins is considered to be low and consequently not included in routine monitoring of batches. Three batches of corn steep liquor powder (medium component) and five batches of *L. rhamnosus* (excipient not given) were tested for heavy metals (lead, cadmium and mercury), arsenic and aflatoxins B1, B2, G1, and G2.[14] Aflatoxins B1, B2,

[5] Technical dossier/Section II/Annex_II_8_safedeposit_29226.
[6] Technical dossier/Section II/Annex_II_2_5_ID_29226.
[7] Technical dossier/Section II/Annex_II_2_genetic_stability_29226.
[8] Technical dossier/Section II/Annex_II_1_antibioticresistance_29226.
[9] This section has been amended following the provisions of Article 8(6) and Article 18 of Regulation (EC) No 1831/2003
[10] Technical dossier/Supplementary information April 2016.
[11] Technical dossier/Section III/Annex MSDS Raw materials.
[12] Technical dossier/Section II.
[13] Technical dossier/Section II/Annex_II_4_contamination.
[14] Technical dossier/Section II/Annex_II_6_mycotoxins_heavymetals.

G1 and G2 were not detected (< 0.01 μg/kg) with two exceptions in which concentration of B2 was < 0.03 μg/kg. Contamination with heavy metals and arsenic was low and of no concern (lead ≤ 1 mg/kg, cadmium ≤ 0.06 mg/kg, mercury < 0.01 mg/kg and arsenic < 0.1 mg/kg).

No specific data were provided on the particle size distribution or dusting potential of the additive under assessment.

3.1.3. Stability

Three batches of the product standardised with maltodextrin to give a count of 1×10^{11} CFU/g and another three batches with dextrose to a level of 2.5×10^{10} CFU/g were stored in sealed aluminium foil bags at ambient temperature.[15] Viability losses were insignificant for both formulations over 6 months but reached approximately 10–15% after 12 months.

A batch of product was standardised to give a count of 1×10^{11} CFU/g using dextrose and ammonium and potassium phosphates as buffer salts. An experiment was designed to mirror practical conditions in which, typically, 10 g of product would be dissolved in 2 L of water and applied to 1 tonne of forage to deliver 1×10^{9} CFU/kg.[16] Three replicates of *L. rhamnosus* in solution were stored at room temperature and samples removed over 7 days. Viable cell counts made indicated that the strain was fully stable for at least 3 days under these conditions. Viability losses (up to 30%) were observed at 7 days.

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 5×10^{7} CFU/kg forage if applied with other microorganisms or 1×10^{8} CFU/kg, if applied alone. It is 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 as *L. rhamnosus*. Consequently, *L. rhamnosus* DSM 29226 is considered to be suitable for the QPS approach to safety assessment, and consequently, 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

No specific data on skin/eye irritation or skin sensitisation were provided for the additive under application. Therefore, no conclusions can be drawn on the skin/eye irritancy or skin sensitisation of the additive. 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 safety issues. For this specific product, the excipients used in the preparation of the final formulation do not introduce additional risks.

3.3. Efficacy

Seven laboratory experiments were made with different forage samples. The duration of the experiments was 90 days (87 in study 3). In all the studies, forage was ensiled in 4.5-L minisilos, fitted with air locks to vent gas. The ambient temperature during ensiling was 20 ± 2°C. The additive was dissolved in water and sprayed on the forage material at an intended concentration of 5×10^{7} 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. Four replicate silos were prepared for each experimental

[15] Technical dossier/Section II/2.4.1.1.
[16] Technical dossier/Section II/2.4.1.2.

treatment (without or with the additive). The forages used were grass/legume mixtures with different botanical composition and different dry matter (DM) and water-soluble carbohydrate (WSC) contents (see Table 1) to represent material easy to ensile (studies 1[17], 2[18] and 3[19]), moderately difficult to ensile (studies 4[20], 5[18] and 6[18]) and difficult to ensile (study 7[21]), as specified by Regulation (EC) No 429/2008.

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

Study	Test material	Dry matter content (%)	Water-soluble carbohydrate content (% fresh matter)
1	Grass/legume mixture (68:32)(1)	43.4	3.4
2	Timothy and perennial ryegrass	38.1	4.7
3	Perennial ryegrass (1st cut)	22.8	3.4
4	Grass/legume mixture (72:28)(1)	40.8	2.3
5	Grass/legume mixture (79:21)(1)	19.8	2.5
6	Grass/legume mixture (74:26)(1)	25.0	2.6
7	Grass/legume mixture (33:67)(1)	21.8	1.2

(1): Grass and legume percentages in the mixture, where the predominant legumes were red clover and lucerne, and the grasses were predominately timothy, meadow fescue and perennial ryegrass.

Silos were opened at the end of the experiment and the contents were analysed by conventional methods to determine silage DM and WSC contents, pH, lactic and volatile fatty acid concentrations, ethanol, ammonia and total nitrogen. DM loss during ensiling was calculated in all cases except for study 3.

Statistical evaluation of data was by a non-parametric test (Wilcoxon Kruskal–Wallis test), comparing treated versus 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 *L. rhamnosus* DSM 29226

Study	Application rate (CFU/kg forage)	Dry matter loss (%)	pH	Lactic acid (% dry matter)	Acetic acid (% dry matter)	Ammonia-N (% total N)
1	0	2.2	4.6	7.1	1.6	6.4
	5×10^7	1.7*	4.3*	8.0	0.9*	5.7*
2	0	3.2	5.0	5.0	0.8	6.4
	5×10^7	2.4*	3.9*	11.3*	0.3*	3.4*
3	0	–	4.0	11.2	2.6	11.3
	5×10^7	–	3.8*	16.9*	2.5*	8.1*
4	0	1.7	4.8	4.5	1.1	7.3
	5×10^7	1.2*	4.1*	7.2*	0.4*	5.2*
5	0	10.4	5.2	3.7	1.5	21.5
	5×10^7	7.4*	4.2*	6.7*	0.9*	12.6*
6	0	8.0	5.4	2.1	0.8	16.0
	5×10^7	3.1*	4.2*	7.9*	0.8	8.3*
7	0	3.9	4.6	7.8	3.6	8.8
	5×10^7	3.8	4.6	8.1*	3.5	9.0

CFU: colony-forming units.
*: Means in a column within a given trial are significantly different $p < 0.05$.

[17] Technical dossier/Section IV/Annexes IV.1 and IV.4.
[18] Technical dossier/Supplementary information September 2016/Annexes 1.1, 1.3 and 1.5.
[19] Technical dossier/Supplementary information September 2016/Annexes 1.2 and 1.4.
[20] Technical dossier/Section IV/Annexes IV.1 and IV.3.
[21] Technical dossier/Section IV/Annexes IV.1 and IV.2.

The addition of *L. rhamnosus* at 5 × 10^7 CFU/kg fresh material decreased pH and ammonia-N as a percentage of total N in the three easy to ensile forages (studies 1, 2 and 3, Table 2), increased lactic acid concentration in two of the materials (studies 2 and 3) and decreased DM loss in other two studies (studies 1 and 2). With all the moderately difficult to ensile materials (studies 4, 5 and 6), the additive decreased pH, DM loss during ensiling and ammonia-N as a percentage of total N and increased lactic acid concentration. With a difficult to ensile clover–lucerne–grass mixture (study 7), the additive significantly increased lactic acid concentration but had no effect on DM loss or ammonia-N concentration.

Considering the effects on DM loss and ammonia-N as percentage of total N, it can be concluded that the additive has the potential to improve the preservation of nutrients in silage prepared from easy and moderately difficult to ensile material.

4. Conclusions

As the identity of the strain has been established as *L. rhamnosus* DSM 29226 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, for consumers of products from animals fed treated silage and for the environment.

In the absence of data, no conclusion can be drawn on the skin and eye irritancy or skin sensitisation of the additive. The additive should be considered to have the potential to be a respiratory sensitiser.

The addition of *L. rhamnosus* DSM 29226 at 5 × 10^7 CFU/kg forage has the potential to improve the production of silage from easy and moderately difficult to ensile forage species by reducing DM loss and enhancing protein preservation.

Documentation provided to EFSA

1) *Lactobacillus rhamnosus* (DSMZ 29226) October 2015. Submitted by Microferm Limited.
2) *Lactobacillus rhamnosus* (DSMZ 29226). Supplementary information February 2016. Submitted by Microferm Limited.
3) Evaluation report of the European Union Reference Laboratory for Feed Additives on the Methods(s) of Analysis for *Lactobacillus rhamnosus* DSM 29226.
4) Comments from the Member States.

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. EFSA Journal 2007;5(11):587, 16 pp. doi:10.2903/j.efsa.2007.587

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 FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2011. Technical guidance: Tolerance and efficacy studies in target animals. EFSA Journal 2011;9(5):2175, 15 pp. doi:10.2903/j.efsa.2011.2175

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012a. Guidance for the preparation of dossiers for technological additives. EFSA Journal 2012;10(1):2528, 23 pp. doi:10.2903/j.efsa.2012.2528

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

Abbreviations

CFU	colony-forming unit
DM	dry matter
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen

EURL	European Union Reference Laboratory
QPS	Qualified Presumption of Safety
RAPD-PCR	randomly amplified polymorphic DNA-polymerase chain reaction
WSC	water-soluble carbohydrate

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* DSM 29226[22]

In the current application authorisation is sought under Article 4(1) for *Lactobacillus rhamnosus* DSM 29226 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 rhamnosus* DSM 29226. The feed additive is to be marketed as a powder or granules containing a minimum *Lactobacillus rhamnosus* DSM 29226 concentration of 1×10^{10} Colony Forming Unit (CFU)/g. The *feed additive* is intended to be added to silage at a minimum dose of 5×10^4 CFU/g fresh silage.

For the identification of *Lactobacillus rhamnosus* DSM 29226, 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 rhamnosus* DSM 29226 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 rhamnosus* DSM 29226 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.

[22] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad_2015_0033_lacto_rhamn.pdf