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Safety and efficacy of *Lactobacillus plantarum* NCIMB 42150 as a silage additive for all animal species

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

Abstract

The EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the safety and efficacy of a specific strain of *Lactobacillus plantarum* when used as a technological additive intended to improve ensiling at a proposed application rate of 1×10^8 colony forming unit (CFU)/kg fresh material. *L. plantarum* is considered by EFSA to be suitable for the qualified presumption of safety approach to safety assessment and not to require specific demonstration of safety other than demonstrating the absence of resistance to antibiotics of human and veterinary significance. As the identity of the strain was clearly established and as no antibiotic resistance was detected, the use of the strain in the production of silage is presumed safe for livestock species, consumers of products from animals fed treated silage and 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. Five studies with laboratory-scale silos were made using forage of differing water-soluble carbohydrate content. Replicate silos containing forages treated at the proposed application rate were compared to identical silos containing the same but untreated forage. In addition, in four studies, formic acid was included as positive control. The mini-silos were then stored for 90–103 days at 20–24°C. After opening, the contents of the silos were analysed. Results showed that this strain of *L. plantarum* has the potential to improve the production of silage from easy, moderately difficult and difficult to ensile forage species by increasing the production of lactic acid, reducing the pH and increasing the preservation of dry matter when used at an application rate of 1×10^8 CFU/kg.

Keywords: technological additive, silage additive, *Lactobacillus plantarum*, 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 Bio-Competence Centre of Healthy Dairy Products LLC[2] for authorisation of the product *Lactobacillus plantarum* NCIMB 42150, when used as a feed additive for all animals species (category: technological additives; functional group: silage additives). According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded to the European Food Safety Authority (EFSA) 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 dossiers in support of this application. The particulars and documents in support of the application were considered valid by EFSA as of 4 August 2015.

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 plantarum* NCIMB 42150, when used under the proposed conditions of use (see Section 3.1.4).

1.2. Additional information

The present additive is based on a preparation of a single strain of *Lactobacillus plantarum*. The additive *Lactobacillus plantarum* NCIMB 42150 has not been previously authorised as a feed additive in the European Union.

The species *L. plantarum* 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 acquired 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 plantarum* NCIMB 42150 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 substance in animal feed. The Executive Summary of the EURL report can be found in Annex A.[5]

2.2. Methodologies

The approach followed by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) to assess the safety and the efficacy of *Lactobacillus plantarum* NCIMB 42150 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 on the tolerance and efficacy studies in target animals (EFSA FEEDAP Panel, 2011), Guidance on studies concerning 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] Bio-Competence Centre of Healthy Dairy Products LLC, Kreutzwaldi 1, 51014, Tartu, Estonia.
[3] FEED dossier reference: FAD-2015-0013.
[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.
[5] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/default/files/finrep-FAD-2015-0013-lactobacillus%20plantarum.pdf

safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012b), and Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance (EFSA FEEDAP Panel, 2012c).

3. Assessment

The additive under assessment is a preparation containing viable cells of *Lactobacillus plantarum* NCIMB 42150. It is intended to be authorised as a technological additive (functional group: silage additive) for all animal species.

3.1. Characterisation

3.1.1. Characterisation of the active agent

The strain of *L. plantarum* was isolated from grass silage and is deposited with the National Collection of Industrial, Food and Marine Bacteria (UK) with the accession number NCIMB 42150.[6] It has not been genetically modified. Strain identity was established by its phenotypic properties (analytical profile index) and by the full 16S rRNA gene sequence which, by comparison with sequences recorded in databases, gave an unambiguous identification.[7] No method for strain-specific detection was presented. Genetic stability was examined by comparison of the enterobacterial repetitive intergenic consensus region using polymerase chain reaction amplification.[7] Using this method, the master culture was compared with production lots produced in 2011 and 2013. No differences in the resultant patterns were observed.

The strain was tested for antibiotic susceptibility using a serial twofold dilution procedure in agar. The battery of antibiotics tested was that recommended by EFSA (EFSA FEEDAP Panel, 2012c), excluding streptomycin and vancomycin which are not required for this species.[8] None of the minimum inhibitory concentration (MIC) values for the *L. plantarum* exceeded the corresponding cut-off values defined by the FEEDAP Panel, with the exception of kanamycin. As the MIC for kanamycin was within one dilution step of the cut-off value and within the normal variation, no further investigation is considered necessary.

3.1.2. Characterisation of the product[9]

The manufacturing process is detailed in the dossier. The additive consists of approximately 50% freeze-dried cell mass and 50% carrier (equal amounts of dextrose and yeast extract). Material safety datasheets are provided for all components of the medium and carrier.

The minimum content of *L. plantarum* in the final product is specified as 1×10^{11} colony forming unit (CFU)/g additive. Analysis of five production batches made showed a mean value of 2.2×10^{11} CFU/g additive (range $1.3–3.7 \times 10^{11}$ CFU/g additive).[10]

The additive is routinely monitored for microbial contamination at various points in the manufacturing process as specified in the hazard analysis and critical control points (HACCP) plan. Maximum limits and methods used are described for filamentous fungi and yeasts (10^2 CFU/g), sulfite-reducing clostridia (10^2 CFU/g), coagulase-positive staphylococci (10^2 CFU/g), enterobacteriaceae (10^2 CFU/g), enteroccoci (10^2 CFU/g), *Listeria* and *Salmonella* (none detected in 25 g additive).[10] The results of the monitoring of three production batches were provided which showed compliance for all the microorganisms listed with the exception of *Salmonella* and clostridia which were not measured.

Heavy metals (lead, cadmium, and mercury) and arsenic were also analysed.[11] Arsenic and mercury were below the limit of detections (LODs[12]); cadmium was < 0.01 mg/kg and lead 0.03 mg/kg additive. Aflatoxins B1, B2, G1, and G2, zearalenone and deoxynivalenol were all below the LODs.[13]

A single batch of the additive was examined for particle size distribution by a laser diffraction.[14] The mean particle size was ~ 125 μm with approximately 40% of the additive (by volume) consisting of

[6] Technical dossier/Section II/Annex II.9.
[7] Technical dossier/Section II/Annex II.1.
[8] Technical dossier/Section II.
[9] This section has been amended following the confidentiality claims made by the applicant.
[10] Technical dossier/Section II/Annexes II.2 and II.4.
[11] Technical dossier/Section II/Annex II.4.
[12] Limit of detection not specified.
[13] Limit of detections: Aflatoxins B1, B2, G1 and G2: 0.4 μg/kg, zearalenone: 15 μg/kg and deoxynivalenol: 115 μg/kg
[14] Technical dossier/Section II/2.1.5.1.

particles with diameters below 100 μm, 18% below 50 μm and 5% below 10 μm. Dusting potential, using a Heubach dustometer, was measured using the same batch of additive.[15] A mean value of 0.16 g/m^3 was obtained.

3.1.3. Stability

Two sets of stability results are described, the first based on two batches of the product and the second on a single batch.[16] In the first study, the microbial counts were made at time 0 and after 3 days storage at 37°C, 2 months at 20°C, and up to 24 months at +3°C or −21.5°C. Any differences in counts between the initial and final values were within the variation expected for the assay. In the second study, the additive was also stored under similar conditions but for longer periods. Counts indicated that the additive was stable when stored at 20°C for at least 6 months but the activity was substantially reduced (> 1 Log) after 2 days storage at 37°C. Counts were unaffected when the additive was stored under refrigeration (+4 or −18°C) for up to 24 months.

Samples from three batches of additive were individually 'dissolved' in water at a rate of 1 g product/L water to give a minimum count of 1 × 10^8 CFU/L and maintained at 20°C for 48 h.[17] Counts of lactobacilli remained constant over this period. It was noted in a separate experiment that there was a tendency to precipitation after 48 h and stirring before application was advised.[18]

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 1.0 × 10^8 CFU/kg fresh material, to be applied as an aqueous suspension.

3.2. Safety

In the view of the FEEDAP Panel, the antibiotic resistance qualification has been met and the identity of the strain established as *L. plantarum*. Therefore, *Lactobacillus plantarum* NCIMB 42150 is considered by EFSA 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.

3.2.1. Safety for the user

No data were submitted on skin/eye irritation or skin sensitisation. Therefore, no conclusions can be drawn on the skin and eye irritancy or skin sensitisation of the additive. The particle size distribution of the single preparation tested indicated a possibility of users to be exposed via inhalation, despite the dusting potential of only 0.16 g/m^3. Given the proteinaceous nature of the active agent, the additive should be considered to have the potential to be a 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 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

Five laboratory experiments were made with different forage samples. The duration of the experiments was 90 days or longer (90–103 days). All of the studies used 3 L mini-silos capable of holding approximately 1.3 kg chopped forage material with the capacity to vent gas. In each case, the contents of five replicate silos were sprayed with the additive at an intended dose of 1 × 10^8 CFU/kg forage suspended in water. Each suspension was then analysed for the actual cell count and confirmed the intended dose. Forage for the negative control silos were sprayed with an equal volume of water but without the additive. Positive controls were included (except study 5) in which forage was sprayed with 3 g formic acid/kg. Laboratory silos were maintained at 20–24°C for the duration of the experiment. The forages samples used (see Table 1) represented material difficult to ensile (study 1),

[15] Technical dossier/Section II/Annex II.5.
[16] Technical dossier/Section II/Annex II.20.
[17] Technical dossier/Section II/Annex II.21.
[18] Technical dossier/Section II/Annex II.7.

moderately difficult to ensile (studies 2 and 3) and easy to ensile (studies 4 and 5) 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[(a)]	Red clover, second cut	20.3	0.6
2[(b)]	Wilted ryegrass, first cut	23.8	1.5
3[(c)]	Ryegrass, second cut	26.6	2.9
4[(d)]	Grass-legume mixture (50% festulolium, 15% white clover, 20% timothy, 15% perennial ryegrass), first cut	48.8	3.3
5[(e)]	Festulolium[(f)], second cut	30.4	6.4

(a): Annex IV.2.
(b): Annex IV.1.
(c): Annex IV.3.
(d): Annex IV.4.
(e): Annex IV.5.
(f): A hybrid cross of *Festuca pratensis* and a *Lolium* sp.

Replicate silos were opened at the end of the experiment and the contents were analysed for proximate composition, dry matter content, pH, lactic and volatile fatty acid concentrations, ethanol, ammonia and total nitrogen. Counts were also made of total filamentous fungi, yeasts and *Clostridium* spores. Statistical evaluation of data was made by comparison with the negative control using a one-sided non-parametric analysis (Wilcoxon Kruskal–Wallis test) with significance assumed at $p < 0.05$.

The results of the studies are shown in Table 2.

Table 2: Summary of the analysis of ensiled material recovered at the end of the ensiling period with *Lactobacillus plantarum* NCIMB 42150 at a dose of 1×10^8 CFU/kg

Study	Treatment	Dry matter loss (%)	pH	Lactic acid (% dry matter)	Acetic acid (% dry matter)	Ammonia-N (% total N)
1	0	10.3	5.9	2.2	2.2	11.6
	L. plantarum	5.8*	5.0*	7.2*	2.7	7.2*
	Formic acid	5.1*	4.8*	7.3*	2.4	7.1*
2	0	7.1	5.5	2.5	1.2	10.5
	L. plantarum	2.7*	4.4*	6.5*	1.5	5.0*
	Formic acid	3.1*	4.5*	4.1	0.9*	4.1*
3	0	8.5	5.3	4.9	0.6	9.7
	L. plantarum	4.4*	3.9*	13.3*	1.5*	4.6*
	Formic acid	2.7*	4.0*	10.8*	1.0*	5.5*
4	0	3.2	4.6	6.3	0.7	3.0
	L. plantarum	2.5	4.2*	9.5*	0.7	2.0*
	Formic acid	2.6	4.8*	1.8*	0.4*	2.1*
5	0	8.2	3.9	9.8	0.9	6.0
	L. plantarum	8.7	3.8*	11.3*	0.9	4.3

CFU: colony forming unit.
*: Significantly different from the control value at $p < 0.05$.

Application of *Lactobacillus plantarum* NCIMB 42150 at the recommended rate consistently increased the concentration of lactic acid in the ensiled material, reduced the pH and, in the case of difficult and moderately difficult to ensile materials, reduced dry matter losses. Although these changes were also seen in the easy to ensile material, results from the untreated control indicated a successful ensiling without a need for an additive. Consequently, there was little room for improvement. This was confirmed by the results of the positive control in study 4, where the application of formic acid offered no benefits. In four studies, application of the additive significantly reduced the amount of ammonia as a proportion of total nitrogen, implying a reduction in protein degradation.

Counts made of filamentous fungi, yeasts and clostridia before and after ensiling showed no discernible and consistent pattern which could be attributed to an effect of the *L. plantarum* addition.

4. Conclusions

As the identity of the strain *Lactobacillus plantarum* NCIMB 42150 has been established and no antibiotic resistance of concern detected, following the QPS approach to safety assessment, the use of this strain in the production of silage is presumed safe for target species, 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 as a potential respiratory sensitiser.

The additive containing *Lactobacillus plantarum* NCIMB 42150 forage has the potential to improve the production of silage from easy, moderately difficult and difficult to ensile forage species when used at an application rate of 1×10^8 CFU/kg.

Documentation provided to EFSA

1) *Lactobacillus plantarum* TAK 59 NCIMB 42150. Request for authorization according to Regulation (EC) 1831/2003 article 4(1). April 2015. Submitted by Bio-Competence Centre of Healthy Dairy Products LLC.
2) *Lactobacillus plantarum* TAK 59 NCIMB 42150. Request for authorization according to Regulation (EC) 1831/2003 article 4(1). Supplementary information February 2016. Submitted by Bio-Competence Centre of Healthy Dairy Products LLC.
3) Evaluation report of the European Union Reference Laboratory for Feed Additives on the Methods(s) of Analysis for *Lactobacillus plantarum* NCIMB 42150.
4) Comments from 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(12):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. Technical guidance – Update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. EFSA Journal 2012;10(6):2740, 10 pp. doi:10.2903/j.efsa.2012.2740

Abbreviations

BIOHAZ Panel	EFSA Panel on Biological Hazards
CFU	colony forming unit
EURL	European Union Reference Laboratory
FEEDAP Panel	EFSA Panel on Additives and Products or Substances used in Animal Feed
HACCP	hazard analysis and critical control points
LOD	limit of detection
MIC	minimum inhibitory concentration
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 plantarum* NCIMB 42150

In the current application authorisation is sought under Article 4(1) for *Lactobacillus plantarum* TAK 59 NCIMB 42150 under the category/functional group 1(k) 'technological additives'/'silage additives', according to Annex I of Regulation (EC) No 1831/2003. Authorization 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 plantarum* TAK 59 NCIMB 42150. The feed additive is to be marketed as a powder containing a minimum *Lactobacillus plantarum* TAK 59 NCIMB 42150 concentration of 1×10^{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 identification of *Lactobacillus plantarum* TAK 59 NCIMB 42150, 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 a European Standard.

For enumeration of *Lactobacillus plantarum* TAK 59 NCIMB 42150, the Applicant submitted the ring-trial validated spread plate method EN 15787 which was already evaluated by EURL in the frame of previous *Lactobacillus plantarum* dossiers. Based on the performance characteristics available, the EURL recommends for official control this ring-trial validated EN 15787 method for the enumeration of *Lactobacillus plantarum* TAK 59 NCIMB 42150 in the feed additive per se.

The Applicant did not provide any experimental method or data for the determination *of Lactobacillus plantarum* TAK 59 NCIMB 42150 in silage. Furthermore, the unambiguous determination of the content of *Lactobacillus plantarum* TAK 59 NCIMB 42150 added to silage is not achievable by analysis. Therefore, the EURL cannot evaluate nor recommend any method for official control to determine *Lactobacillus plantarum* TAK 59 NCIMB 42150 in silage.

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.