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Safety and efficacy of *Lactobacillus buchneri* NRRL B-50733 as a silage additive for all animal species

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Abstract

Following a request from the European Commission, 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 strain of *Lactobacillus buchneri* when used as a technological additive intended to improve ensiling at a proposed application rate of 1×10^8 colony forming units (CFU)/kg fresh material. The bacterial species *L. buchneri* 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 a potential respiratory sensitiser. Three studies with laboratory-scale silos were made using samples of easy, moderately difficult and difficult to ensile material. In each case, replicate silos containing untreated forage were compared with identical silos containing the same forage treated with the combination of *Lactobacillus buchneri* NRRL B-50733 at an intended concentration of 1×10^8 CFU/kg fresh matter. The results showed that the addition of *Lactobacillus buchneri* NRRL B-50733 at 1×10^8 CFU/kg fresh material has the potential to improve the aerobic stability of silage with a dry matter content ranging from 30% to 73%.

Keywords: technological additive, silage additive, *Lactobacillus buchneri* NRRL B-50733, 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 Pioneer Hi-Bred Northern Europe[2] for authorisation of the product *Lactobacillus buchneri* NRRL B-50733, when used as a feed additive for all animal species (category: Technological additives; 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). The particulars and documents in support of the application were considered valid by EFSA as of 24 January 2017.

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 buchneri* NRRL B-50733, when used under the proposed conditions of use (see Section 3.1.3).

1.2. Additional information

The additive is a preparation containing viable cells of *Lactobacillus buchneri* NRRL B-50733. It has not been previously authorised as a feed additive in the European Union.

The species *L. buchneri* is considered by EFSA to be suitable for the Qualified Presumption of Safety (QPS) approach to safety assessment (EFSA, 2007, EFSA BIOHAZ Panel, 2017). 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 buchneri* NRRL B-50733 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 FEEDAP Panel to assess the safety and the efficacy of *Lactobacillus buchneri* NRRL B-50733 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), Guidance on studies concerning the 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).

[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] Pioneer Hi-Bred Northern Europe, Apensener Straße 198, 21614, Buxtehude, Germany.
[3] FEED dossier reference: FAD-2016-0060.
[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/en/eurl/feed-additives/evaluation-reports

3. Assessment

The additive is a preparation of viable cells of *Lactobacillus buchneri* NRRL B-50733 intended for use as a technological additive (silage additive) for all animal species to improve the ensiling process.

3.1. Characterisation

3.1.1. Characterisation of the active agent

The strain was isolated from whole plant maize silage. It is deposited in the United States Department of Agriculture Agricultural Research Culture Collection (NRRL Collection) with the accession number NRRL B-50733.[6] It has not been genetically modified.

Taxonomical identification of the product strain as *L. buchneri* was established by analysing the partial sequence (561 bp) of the 16S rRNA gene.[7] For strain-specific identification, restriction endonuclease analysis (REA) with *Eco*RI enzyme of total DNA was used.[8] However, REA patterns were not provided for any other *L. buchneri* strains, and thus, the specificity of the method remains unsure.

The bacterial strain was tested for antibiotic susceptibility using broth microdilution techniques. The battery of antibiotics used included those recommended by EFSA for 'obligately heterofermentative *Lactobacillus*' (EFSA FEEDAP Panel, 2012c).[9] All the minimum inhibitory concentration (MIC) values were below or equal to the corresponding EFSA cut-off values, with the exception of tetracycline which exceeded the cut-off value by two dilutions (32 vs 8 μg/mL). Since 'obligately heterofermentative *Lactobacillus*' covers several different species of lactobacilli, *L. buchneri* group specific MIC information was used in the assessment. The study of Feichtinger et al. (2016) contains the largest number of *L. buchneri* group strains analysed so far (128 of which 15 were *L. buchneri sensu stricto*). Based on this study, the specific tetracycline cut-off for *L. buchneri* group would be 128 μg/mL. The tetracycline MIC of 32 μg/mL of *Lactobacillus buchneri* NRRL B-50733 is within the unimodal distribution of *L. buchneri* group MIC values and does not raise any safety concerns. Consequently, the strain can be considered susceptible to the relevant antibiotics.

To investigate further the nature of the resistance, the applicant sequenced the whole genome of the strain and searched for known tetracycline resistance genes using an in-house developed database consisting of the known tetracycline resistance gene sequences.[10] No significant homologies were found.

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, then separated from the growth medium by centrifugation and concentrated by microfiltration. Cryoprotectants (e.g. maltodextrin, sodium aluminium silicate) are added and the cell mix is freeze-dried and ground. The product consists of approximately 25% cells, cryoprotectants (64%), water (5%) and spent medium (6%) and has a minimum declared content of 1×10^{10} CFU/g.[11]

Analysis of five batches showed a mean value of 3.1×10^{11} CFU/g (range 1.2–4.9 $\times 10^{11}$ CFU/g).[12]

Microbial and aflatoxin B1 contamination is routinely monitored at various points in the manufacturing process and in the final product. Limits are set for coliforms (10^3 CFU/g), *Escherichia coli* (10 CFU/g), yeasts and filamentous fungi (10^3 CFU/g), *Salmonella* spp. (absent in 25 g) and aflatoxin B1 (5 μg/kg). Compliance with action levels was confirmed in seven production batches for microbial contaminants[13] and in three batches for aflatoxin B1.[14] Analysis of the same three batches showed absence of other aflatoxins (B2, G1 and G2 < 5 μg/kg).[15]

[6] Technical dossier/Section II/Annex II.2.2.3.
[7] Technical dossier/Section II/Annex II.2.2.1.
[8] Technical dossier/Section II/Annex II.2.2.2.
[9] Technical dossier/Section II/Annex II.2.2.4.
[10] Technical dossier/Section II/Annex II.2.2.6.
[11] Technical dossier/Section II/Annex II.2.1.5.
[12] Technical dossier/Section II/Annex II.2.1.6.
[13] Technical dossier/Section II/Annex II.2.4.7.
[14] Technical dossier/Section II/Annex II.2.1.9 and Supplementary information April 2017/Annex 1.
[15] Limit of quantification: aflatoxins < 5.0 μg/kg.

Two batches of a granular and two of a water-soluble form of the additive (excipients not described) were examined for particle size distribution by laser diffraction.[16] In average, 28% (v/v) of the water soluble formulation consisted of particles with diameter below 50 μm, while the granular formulation showed a negligible fraction (0.4%) of particles smaller than 50 μm. Three batches of each of the same formulations were examined for dusting potential using a Heubach dustometer.[17] The mean dusting potential of the three batches was 38 g/m^3 for the water-soluble form and 4.2 g/m^3 for the granular formulation.

3.1.3. Stability

Three batches of the additive were stored in sealed aluminium foil bags at 27°C for 15 months.[18] Only small viability losses (< 0.5 log) were observed over this period.

As the additive is intended to be distributed by the spraying of an aqueous suspension, the short-term stability in water was measured simulating the practical conditions of use.[18] This showed that bacterial numbers were maintained (losses < 0.5 log) for two days under ambient conditions.

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.

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. buchneri*. Therefore, *Lactobacillus buchneri* NRRL B-50733 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

Three laboratory studies are described with different forage materials and lasting 90 days. All of the studies used 2.75 L microsilos with the capacity to vent gas. Each microsilo was filled with 1.3 kg of chopped fresh forage (studies 1[19] and 2[19]) or 1.6 kg of maize grain (study 3[19]). Eight micro-silos were used in each study. The contents of four silos per study were treated with *Lactobacillus buchneri* NRRL B-50733 applied at a rate of 1×10^8 CFU per kg of fresh material. Concentration of *L. buchneri* in the additive or silage was not confirmed. An aqueous suspension of the additive was prepared and then sprayed on the forage prior to ensiling. Forage for the four control silos were sprayed with an equal volume of water but without the additive. Ambient temperature was controlled at 20 ± 2°C. The forage used was of different origins with dry matter (DM) and water-soluble carbohydrate contents

[16] Technical dossier/Section II/Annex II.2.1.9.
[17] Technical dossier/Section II/Annex II.2.1.10.
[18] Technical dossier/Section II/Annex II.2.4.1.
[19] Technical dossier/Section IV/Annex IV.4.3.1.

representing material easy to ensile (study 1), moderately difficult to ensile (2) and difficult to ensile (3), as specified by Regulation (EC) No 429/2008 (Table 1).

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	Italian rye grass	34.7	5.3
2	Alfalfa	33.9	2.2
3	Maize grain	73.2	0.9

Silos were opened at the end of the experiment and the contents were analysed to determine silage DM content, pH, lactic, acetic, butyric and propionic acid concentrations, ethanol and total and ammonia-N. Fermentation DM loss was also determined. Aerobic stability of the silage was determined with a modified Honig method where a measured quantity of silage was placed into a plastic container introduced into a polystyrene cooler, and then left in a temperature controlled room (20°C). To follow aerobic stability, a temperature probe was placed into the centre of the silage mass, measuring temperature every 3 h for 1 week. A rise of 3°C above 20°C was considered as indicator of silage deterioration. Non-parametric tests (Wilcoxon/Kruskal–Wallis) were used for the statistical analyses to compare treated versus control silos.[20] Significance was declared at $p < 0.05$. Results are shown in Table 2.

Table 2: Summary of the analysis of ensiled material recovered at the end of the ensiling period with *Lactobacillus buchneri* NRRL B-50733

Study	Application rate (CFU/kg forage)	Dry matter loss (%)	pH	Lactic acid (% silage)	Acetic acid (% dry matter)	Ammonia-N (% total N)	Aerobic stability (days)
1	0	1.6	4.3	1.9	0.3	10.5	2.7
	1×10^8	2.0*	4.3	1.4*	1.0*	12.0*	7.0*
2	0	6.7	4.1	2.2	0.5	1.2	2.9
	1×10^8	1.0*	4.2	1.9	1.2*	1.3	6.7*
3	0	3.3	4.5	0.2	0.04	0.1	1.4
	1×10^8	2.6*	4.5	0.3	0.15*	0.2	6.7*

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

In the three studies, aerobic stability was significantly improved by the addition of *Lactobacillus buchneri* NRRL B-50733. DM losses during ensiling were significantly reduced in studies 2 and 3. Silage pH was not affected by the additive in any of the studies, lactic acid and ammonia-N were decreased by the additive only in study 1, and acetic acid content of the silage was increased in the three studies.

Lactobacillus buchneri NRRL B-50733 added at 1.0×10^8 CFU/kg forage has the potential to improve the aerobic stability of silage with a DM content ranging from 30% to 73%.

4. Conclusions

As the identity of the strain has been established as *Lactobacillus buchneri* NRRL B-50733 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.

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 a potential respiratory sensitiser.

The addition of *Lactobacillus buchneri* NRRL B-50733 at 1×10^8 CFU/kg fresh material has the potential to improve the aerobic stability of silage with a DM content ranging from 30% to 73%.

[20] Technical dossier/Section IV/Annexes IV.4.2.1 and IV.4.2.2 and Supplementary information April 2017/Annex 2.

Documentation provided to EFSA

1) *Lactobacillus buchneri* NRRL B-50733. September 2016. Submitted by Pioneer Hi-Bred Northern Europe.
2) *Lactobacillus buchneri* NRRL B-50733. Supplementary information April 2017. Submitted by Pioneer Hi-Bred Northern Europe.
3) Evaluation report of the European Union Reference Laboratory for Feed Additives on the Methods(s) of Analysis for *Lactobacillus buchneri* NRRL B-50733.
4) Comments from Member States.

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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. https://doi.org/10.2903/j.efsa.2012.2740

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Abbreviations

CFU	colony forming unit
DM	dry matter
EURL	European Union Reference Laboratory
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP
MIC	minimum inhibitory concentration
REA	restriction endonuclease analysis

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 buchneri* NRRL B-50733

In the current application authorisation is sought under Article 4(1) for *Lactobacillus buchneri NRRL B-50733* 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 buchneri NRRL B-50733*. The *feed additive* is to be marketed as a powder, with a minimum *Lactobacillus buchneri NRRL B-50733* content of 1×10^{10} Colony Forming Units (CFU)/g product. The *feed additive* is intended to be added dry or sprayed onto *silage* at a minimum dose of 1×10^{8} CFU/kg fresh *silage*.

For the identification of *Lactobacillus buchneri NRRL B-50733*, the EURL recommends for official control the Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for genetic identification.

For the enumeration of *Lactobacillus buchneri NRRL B-50733* in the feed additive, the Applicant submitted the ring-trial validated spread plate method EN 15787, dedicated specifically for the analysis of Lactobacillus spp. Based on the performance characteristics available, the EURL recommends for official control this method for the enumeration of *Lactobacillus buchneri NRRL B-50733* in the *feed additive per se*.

Since the unambiguous enumeration of *Lactobacillus buchneri NRRL B-50733* initially added to silage is not achievable by analysis, the EURL cannot evaluate nor 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.