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Safety and efficacy of *Lactobacillus brevis* NCIMB 42149 as a silage additive for all animal species

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Abstract

Lactobacillus brevis is a technological additive intended to improve the ensiling process at a minimum proposed dose of 1×10^8 colony-forming units (CFU)/kg fresh material. The species *L. brevis* 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 the susceptibility to antibiotics of human and veterinary significance. Although identity was established, the strain was found resistant to ampicillin and clindamycin and may pose a risk for the spread of genes coding for resistance to these antibiotics. Therefore, the use of this strain as a silage additive is not considered safe for target animals, and consumers of products from animals fed the treated silage. 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. Although *L. brevis* is ubiquitous in the environment, due to its antibiotic resistance, the FEEDAP Panel cannot conclude on the extent of the risk of horizontal gene transfer to other bacteria in the environment. Six 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. After opening and exposure to air, an increase of 3°C over ambient was taken to indicate aerobic deterioration. The additive showed a potential to significantly improve the aerobic stability of silage produced from easy, moderately difficult and difficult to ensile forage at a minimum application rate of 1×10^8 CFU/kg plant material.

Keywords: *Lactobacillus brevis*, silage, safety, QPS, efficacy, aerobic stability

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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 brevis* NCIMB 42149,[3] 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 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 4 August 2015.

According to Article 8 of Regulation (EC) No 1831/2003, EFSA shall 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 brevis* NCIMB 42149, 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. brevis* NCIMB 42149. It has not been previously authorised as a feed additive in the European Union.

The species *L. brevis* 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[4] in support of the authorisation request for the use of *L. brevis* NCIMB 42149 as a feed additive. The technical dossier was prepared following the provisions of Article 7 of Regulation (EC) No 1831/2003 and Regulation (EC) No 429/2008[5] 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 FEEDAP Panel to assess the safety and the efficacy of *L. brevis* NCIMB 42149 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 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 Technical guidance on the update of the criteria used in the assessment of bacterial resistance to antibiotics of human or 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] Bio-Competence Centre of Healthy Dairy Products LLC., Kreutzwaldi 1, 51014 Tartu, Estonia.
[3] In the mandate, the applicant identified the strain also with in-house identifier TAK 124-1.
[4] FEED dossier reference: FAD-2015-0014.
[5] 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.

3. Assessment

The additive under assessment is a preparation containing viable cells of *L. brevis* NCIMB 42149. 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. brevis* was isolated from grass silage and is deposited with the National Collection of Industrial, Food and Marine Bacteria (UK) with the accession number NCIMB 42149.[6] It has not been genetically modified. Strain identity was established by its phenotypic properties and by the full 16S rRNA gene sequence which by comparison with sequences recorded in databases gave an unambiguous identification.[7]

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 contains three plasmids (10 kb, 7 kb and 6 kb).

The strain was initially tested for antibiotic susceptibility using the E-test. The battery of antibiotics tested was that recommended by EFSA for obligate heterofermentative lactobacilli (EFSA FEEDAP Panel, 2012c) excluding streptomycin and vancomycin which are not required for this species.[8] Using this method, all minimum inhibitory concentration (MIC) values for the *L. brevis* strain fell below the corresponding cut-off values defined by the FEEDAP Panel. However, when antibiotic susceptibility was tested using a more reliable serial twofold dilution method in agar, the MIC values for tetracycline, clindamycin and ampicillin exceeded the cut-off values used by EFSA.[9] The MIC for tetracycline was within one-dilution step and within the normal variation for the method and thus is not considered a cause for concern. The MIC values for the remaining two antibiotics were outside the expected variation (8 vs 2 mg/L for ampicillin and 8 vs 1 mg/L for clindamycin). Consequently, further studies were made to elucidate the molecular basis of the resistance shown to these antibiotics. Genomic and plasmid fractions were sequenced and searched for known resistance determinants in a consolidated database derived from four public databases (CARD, ARDB, ResFinder and Lahey β-lactamase database). A gene, *LmrB*, known to confer resistance to lincomycin was identified in the genomic fraction but not in plasmid DNA, together with a number of genes encoding transport proteins/effluent pumps. Clindamycin belongs to the same class of antibiotics and is directly derived from lincomycin and it seems probable that *LmrB* is responsible for the resistance shown to clindamycin as is the case for *Corynebacterium glutamicum* (Kim et al., 2001). No genes encoding β-lactamases were found although one chromosomal gene elsewhere annotated as a serine hydrolase was found to contain a domain homologous with β-lactamase. The molecular basis for the resistance shown to ampicillin remains unclear.

3.1.2. Characterisation of the product[10]

The manufacturing process is detailed in the dossier.[11,12]

The minimum content of *L. brevis* in the final product is specified as 1×10^{11} colony-forming units (CFU)/g additive. Analysis of five production batches showed a value of 1.4×10^{11} CFU/g additive for each batch tested.[13]

Microbiological testing showed that filamentous fungi, yeasts, *Escherichia coli, Clostridium perfringens*, total Enterobacteriaceae and *Salmonella* in three batches of the additive were absent or below the set action limits.[14] Routine microbiological testing is included at several stages in the

[6] Technical dossier/Section II/Annex II.2-9.
[7] Technical dossier/Section II/Annex II.2-1.
[8] Technical dossier/Section II.
[9] Technical dossier/Supplementary information August 2016/Annex II.2.
[10] This section has been amended following the confidentiality claims made by the applicant.
[11] Technical dossier/Section II/Annex II.10.
[12] Technical dossier/Section II/Annex II.3.
[13] Technical dossier/Section II/Annex II.2.
[14] Technical dossier and Supplementary information August 2016/Annexes II.4 and II.3.

production process and for the final product. The methods used are specified and action limits described.[15] However, the protocol for routine testing differs slightly from the data initially provided and also includes *Staphylococcus aureus* and *Listeria monocytogenes*, but apparently excludes *Salmonella*. Heavy metals (Pb, Cd and Hg) and arsenic were also analysed. Arsenic, mercury and lead were below the limits of detection (LOD),[16] cadmium was < 0.01 mg/kg. Aflatoxins B1, B2, G1 and G2, zearalenone and deoxynivalenol were all below the LOD.[17] On the basis of these results and considering the medium constituents and excipients used in the production process, contamination by heavy metals, arsenic or mycotoxins is not routinely monitored.

Dusting potential was measured using a Heubach dustometer for four batches of the additive.[18] Each batch was tested once and a mean value of 1.82 g/m^3 was obtained. One of the four batches tested for dusting potential was also examined for particle size distribution by laser diffraction.[18] The mean particle size was ~ 130 μm with approximately 38% of the additive consisting of particles with diameters below 100 μm, 19% below 50 μm and 4% below 10 μm.

3.1.3. Stability

Three batches of additive were examined for shelf-life when stored under different conditions.[19] The microbial count was made at time zero and after 3 days storage at 37°C/relative humidity (RH) 75%, 2 months at 20°C/RH 60, up to 21 months at 3°C and 24 months at −21.5°C. Counts indicated that the additive was stable when stored at 20°C for at least 2 months but activity was reduced by one log after 3 days storage at 37°C. Counts were unaffected when the additive was stored under refrigeration (+3°C) or frozen (−21.5°C). The applicant recommends storage under refrigerated conditions before use.

Samples from three batches of additive were individually suspended 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. Counts of lactobacilli remained constant over this period.[20]

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.

4. Safety

4.1. Safety for the target species and consumers

L. brevis NCIMB 42129 is resistant to ampicillin and clindamycin, two antibiotics of clinical importance, and consequently, the QPS approach to safety assessment cannot be applied. As the resistance of the *L. brevis* strain to ampicillin and clindamycin is not established as intrinsic and as the genetic basis of the observed resistance remains unclear, a potential for horizontal gene transfer among bacteria cannot be excluded. Consequently, the use of *L. brevis* NCIMB 42129 is not considered safe for the target animals and consumers of products from animals fed the treated silage.

4.2. 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 and the dusting potential of the single preparation tested indicated a possibility of users to be exposed via inhalation. Given the proteinaceous nature of the active agent, the additive should be considered 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 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

[15] Action limits: yeasts and filamentous fungi < 10^2 CFU/g, *E. coli* < 10^2 CFU/g, total Enterobacteriaceae < 10^2 CFU/g, *Cl. perfringens* absent in 1 g, and *Salmonella* absent in 25 g additive.
[16] Limits of detection not specified.
[17] Limits of detection: Aflatoxins B1, B2, G1 and G2: 0.4 μg/kg, zearalenone: 15 μg/kg and deoxynivalenol: 115 μg/kg.
[18] Technical dossier/Section II/II.5.
[19] Technical dossier/Section II and Supplementary information August 2016/II.22.
[20] Technical dossier/Section II/Annex II.23.

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.

4.3. Safety for the environment

L. brevis is ubiquitous in the environment. However, due to the antibiotic resistance of this specific strain, the FEEDAP Panel cannot conclude on the extent of the risk of horizontal gene transfer to other bacteria in the environment.

5. Efficacy

Six 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 minisilos 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 in five of the six studies. However, in study 4, the actual dose (4×10^6 CFU/kg forage) was substantially less than that intended. Forage for the negative control silos were sprayed with an equal volume of water but without the additive. In addition, in five of the studies, replicate forage samples were treated with formic acid as a positive control (studies 1–4 at 3 g/kg formic acid and study 5 at 5 mg/kg). Laboratory silos were maintained at 20–24°C for the duration of the experiment. The forages samples (see Table 1) used represented material difficult to ensile (studies 1[21] and 2[22]), moderately difficult to ensile (studies 3[23] and 4[24]) and easy to ensile (studies 5[25] and 6[26]) as specified by Regulation (EC) No 429/2008.

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

Study	Test material	Dry matter content (%)	Water-soluble carbohydrate content (% fresh matter)
1	Red clover, second cut	20.3	0.6
2	Timothy, first cut	29.1	1.1
3	Ryegrass, first cut	23.8	1.5
4	Ryegrass, second cut	26.6	2.9
5	Grass-legume mixture (50% festulolium[(a)], 15% white clover, 20% timothy, 15% perennial ryegrass),first cut	48.8	3.3
6	Festulolium[(a)], second cut	30.4	6.4

(a): 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 acid 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 shown in Table 2 was made by comparison with the negative control using a one-sided non-parametric analysis (Wilcoxon Kruskal–Wallis test) with significance assumed at $p < 0.05$.

At the end of the ensiling period, the content of opened silos after sampling were transferred to an insulated box and continuously monitored for temperature change. Temperature changes were measured for either 216 h (9 days) in the case of studies 1–4 or 279 h (ca. 12 days) in studies 5 and 6. An increase of 3°C over ambient temperature was taken to indicate aerobic deterioration.

The results of the studies are shown in Table 2.

[21] Technical dossier/Section IV/Annex IV.3.
[22] Technical dossier/Section IV/Annex IV.4.
[23] Technical dossier/Section IV/Annex IV.1.
[24] Technical dossier/Section IV/Annex IV.2.
[25] Technical dossier/Section IV/Annex IV.5.
[26] Technical dossier/Section IV/Annex IV.6.

Table 2: Summary of the analysis of ensiled material recovered at the end of the ensiling period with *Lactobacillus brevis* NCIMB 42149 at 1×10^8 CFU/kg forage

Study	Additive	Dry matter loss (%)	pH	Lactic acid (% DM)	Acetic acid (% DM)	NH_3-N (% total N)	Aerobic stability (h)[(a)]
1	0	10.3	5.9	2.2	2.1	11.6	113
	L. brevis	8.2*	5.5*	4.4*	2.3	9.0*	197*
	Formic acid	5.1*	4.8*	7.3*	2.4	7.1*	> 216*
2	0	2.5	4.5	6.3	1.2	5.5	164
	L. brevis	3.0*	4.5	6.4	2.4*	5.6	> 216*
	Formic acid	2.0*	4.2*	6.6	1.1	7.4*	126
3	0	7.1	5.5	2.8	1.2	10.5	32
	L. brevis	5.9*	4.8*	6.0*	2.8*	8.5*	99*
	Formic acid	3.1*	4.5*	4.1*	0.9*	6.7*	59*
4	0	8.5	5.3	4.9	0.6	9.7	26
	L. brevis	9.5*	4.7*	8.8*	1.9*	9.5	> 216*
	Formic acid	2.7*	4.0*	10.8*	1.0*	5.5*	4.3*
5	0	3.2	4.6	6.3	0.7	3.0	186
	L. brevis	4.1*	4.4*	8.8*	1.1*	2.8*	279
	Formic acid	2.6	4.5*	1.8*	0.4*	2.1*	181
6	0	8.2	3.9	9.8	0.9	6.0	22
	L. brevis	9.3*	3.9	9.3	1.8*	5.8*	261*

CFU: colony-forming units; DM: dry matter.
*: Significantly different from the control value at $p < 0.05$.
(a): Period to reach 3°C rise over the ambient temperature (h).

Addition of the additive at the recommended dose produced the effects expected of a heterofermentative lactobacillus. Lactic acid production was significantly increased in four of the six studies and, more importantly, acetic acid in five of the six. This resulted in a significantly lower pH in four studies. However, this had little or no benefit in terms of the direct preservation of nutritional value. The value of addition was seen in an increase in aerobic stability of the silage after exposure to air, which was seen in all studies. The time to detectable deterioration was significantly increased in five studies. The remaining study (study 5) also showed a numerical benefit (from 186 to 279 h) but this failed to reach significance in the non-parametric test used.

Numbers of yeasts were significantly reduced in treated silage in three of the studies. No significant differences were seen in counts of filamentous fungi and yeasts in the remaining studies between treated and untreated silage. Silage from five of the six studies showed very low numbers of clostridia and no treatment related effects were detectable. In the remaining study, clostridial numbers were significantly reduced in the *L. brevis* treated group.

Conclusions

L. brevis NCIMB 42149 is resistant to ampicillin and clindamycin and might pose a risk for the spread of genes coding for resistance to these antibiotics of clinical importance. Therefore, the use of this strain as a silage additive is not considered safe for target animals or consumers of products from animals fed the treated silage.

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.

L. brevis is ubiquitous in the environment. However, due to the antibiotic resistance of this specific strain, the FEEDAP Panel cannot conclude on the extent of the risk of horizontal gene transfer to other bacteria in the environment.

L. brevis NCIMB 42149 showed a potential to significantly improve the aerobic stability of silage produced from easy, moderately difficult and difficult to ensile forage at an application rate of 1×10^8 CFU/kg plant material.

Documentation provided to EFSA

1) *Lactobacillus brevis* TAK 124-1 NCIMB 42149. 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 brevis* TAK 124-1 NCIMB 42149. Request for authorization according to Regulation (EC) 1831/2003 Article 4(1). Supplementary information August 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 brevis* NCIMB 42149.
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, 16pp. 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

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Kim HJ, Kim Y, Lee MS and Lee HS, 2001. Gene *lmrB* of *Corynebacterium glutamicum* confers efflux-mediated resistance to lincomycin. Molecules and Cells, 12, 112–116.

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
LOD	limit of detection
MIC	minimum inhibitory concentration
QPS	qualified presumption of safety
RH	relative humidity

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 brevis* NCIMB 42149[27]

In the current application authorisation is sought under Article 4(1) for *Lactobacillus brevis* TAK 124-1 NCIMB 42149 under the category/functional group 1(k) "technological additives"/"silage additives", according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of the feed additive for all animal species. The feed additive is to be marketed as a powder containing a minimum concentration of 1.0×10^{11} colony forming units (CFU)/g *Lactobacillus brevis* TAK 124-1 NCIMB 42149. The original strain is deposited at NCIMB Ltd. (National Collection of Industrial, Food and Marine Bacteria, Scotland). The feed additive is intended to be added to silage via a water suspension at a minimum dose of 1.0×10^{8} CFU/kg fresh silage.

For the identification of *Lactobacillus brevis* TAK 124-1 NCIMB 42149, the Applicant submitted the carbohydrate fermentation patterns (API system) and molecular methods: Internal Transcribed Spacer Polymerase Chain Reaction (ITS-PCR) and Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR). However, the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for microbial identification.

For enumeration of *Lactobacillus brevis* TAK 124-1 NCIMB 42149 in feed additive, the Applicant submitted a pour plate method based on the ring-trial validated CEN method (EN 15787). Based on the performance characteristics available the EURL recommends for official control the CEN method for the enumeration of *Lactobacillus brevis* TAK 124-1 NCIMB 42149 in the feed additive.

Since the accurate quantification of *Lactobacillus brevis* TAK 124-1 NCIMB 42149 added to silage is not experimentally achievable, the Applicant did not provide any experimental method or data. Therefore, the EURL cannot evaluate or recommend any method for official control to quantify the active substance 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.

[27] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-FAD-2015-0014%20AerobEst.pdf