SCIENTIFIC OPINION

Scientific Opinion on the safety and efficacy of *Lactobacillus brevis* (DSM 23231), *Lactobacillus buchneri* (DSM 22501), *Lactobacillus buchneri* (NCIMB 40788—CNCM I-4323), *Lactobacillus buchneri* (ATCC PTA-6138) and *Lactobacillus buchneri* (ATCC PTA-2494) as silage additives for all species[1]

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP)[2,3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

One strain of *Lactobacillus brevis* and four strains of *Lactobacillus buchneri* are each intended to improve ensiling at proposed doses ranging from 5×10^7 to 1×10^8 CFU/kg fresh material. Both bacterial species are considered by EFSA to be suitable for the Qualified Presumption of Safety approach to safety assessment. As the identity of all strains was clearly established and as no antibiotic resistance of concern was detected, the use of these strains in silage production is presumed safe for livestock species, consumers of products from animals and the environment. Given the proteinaceous nature of the active agents and the high dusting potential of the products tested, the FEEDAP Panel considers it prudent to treat these additives as skin and respiratory sensitisers. They are also considered irritants. The efficacy of *L. brevis* to improve the preservation of nutritive value or increase the aerobic stability of silage was not demonstrated.. One strain of *L. buchneri* has the potential to improve the production of silage from easy, moderately difficult and difficult to ensile materials by reducing the pH and ammonia nitrogen and by increasing the preservation of dry matter. The remaining three strains of *L. buchneri* showed the potential to improve the aerobic stability, one in all forages and two in easy to ensile materials.

KEY WORDS

Technological additive, silage additive, *Lactobacillus brevis*, *Lactobacillus buchneri*, QPS, safety, efficacy

[1] On request from the European Commission, Question No EFSA-Q-2011-00382, adopted on 12 March 2013.

[2] Panel members: Gabriele Aquilina, Alex Bach, Vasileios Bampidis, Maria De Lourdes Bastos, Gerhard Flachowsky, Mikolaj Antoni Gralak, Christer Hogstrand, Lubomir Leng, Secundino López-Puente, Giovanna Martelli, Baltasar Mayo, Derek Renshaw, Guido Rychen, Maria Saarela, Kristen Sejrsen, Patrick Van Beelen, Robert John Wallace and Johannes Westendorf. Correspondence: FEEDAP@efsa.europa.eu

[3] Acknowledgement: The Panel wishes to thank the members of the Working Group on Silage, including Andrew Chesson, Pier Sandro Cocconcelli and Miklós Mézes, for the preparatory work on this scientific opinion.

Suggested citation: EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Scientific Opinion on the safety and efficacy of *Lactobacillus brevis* (DSM 23231), *Lactobacillus buchneri* (DSM 22501), *Lactobacillus buchneri* (NCIMB 40788—CNCM I-4323), *Lactobacillus buchneri* (ATCC PTA-6138) and *Lactobacillus buchneri* (ATCC PTA-2494) as silage additives for all species. EFSA Journal 2013;11(4):3168. [16 pp.] doi:10.2903/j.efsa.2013.3168. Available online: www.efsa.europa.eu/efsajournal

SUMMARY

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the safety for the target animals, consumer, user and the environment and on the efficacy of the one strain of *Lactobacillus brevis* and four strains of *Lactobacillus buchneri*, when used as technological additives intended to improve the ensiling process at a proposed dose of 5×10^7 CFU/kg fresh material for *Lactobacillus brevis* and 1×10^8 CFU/kg fresh material for *Lactobacillus buchneri*.

The bacterial species *L. brevis* and *L. buchneri* are considered by EFSA to be suitable for the Qualified Presumption of Safety (QPS) approach to safety assessment. Therefore, strains belonging to these species do not require any specific demonstration of safety, other than confirming their susceptibility to antibiotics of human or veterinary clinical significance and safety for the user. As the identity of all five strains was clearly established, and as no antibiotic resistance of concern was detected, the use of the five strains in the production of silage is presumed safe for livestock species, consumers of products from animals fed the treated silage and the environment.

Although users at the farm level are exposed to the additives for only a short period of time when preparing the aqueous suspension, in the absence of data, the irritant potential of the additives cannot be excluded. Given the proteinaceous nature of the active agents and the high dusting potential of the products tested, the FEEDAP Panel considers it prudent to treat these additives as skin and respiratory sensitisers.

Studies with laboratory-scale silos are described; each lasted at least 90 days and used samples of forage of differing water-soluble carbohydrate content and representing material easy, moderately difficult and difficult to ensile. In each case, replicate silos containing treated forage were compared with identical silos containing the same but untreated forage. Silos were opened and contents were analysed for dry matter content, pH, lactic acid and volatile fatty acid concentration, ethanol, ammonia and total nitrogen, as well as aerobic stability in four strains.

The efficacy of *L. brevis* to improve the preservation of nutritive value or increase the aerobic stability of silage was not demonstrated. One strain of *L. buchneri* has the potential to improve the production of silage from easy, moderately difficult and difficult to ensile materials by reducing the pH and ammonia nitrogen and by increasing the preservation of dry matter. The remaining three strains of *L. buchneri* showed the potential to improve the aerobic stability, one in all forages and two in easy to ensile materials.

TABLE OF CONTENTS

BACKGROUND

Regulation (EC) No 1831/2003[4] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular Article 10(2)/(7) of that Regulation specifies that for existing products within the meaning of Article 10(1), an application shall be submitted in accordance with Article 7, within a maximum of seven years after the entry into force of this Regulation.

The European Commission received a request from the company SILAC-EEIG-Silage Additives[5] for re-evaluation of the products *Lactobacillus brevis* (DSM 23231), *Lactobacillus buchneri* (DSM 22501), *Lactobacillus buchneri* (NCIMB 40788 − CNCM I-4323), *Lactobacillus buchneri* (ATTC PTA-6138) and *Lactobacillus buchneri* (ATTC PTA-2494) to be used as feed additives for all animal species (category: technological additive; functional group: silage additive) under the conditions mentioned in Table 1.

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 10(2)/(7) (re-evaluation of an authorised feed additive). EFSA received directly from the applicant the technical dossier in support of this application.[6] According to Article 8 of that Regulation, 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. The particulars and documents in support of the application were considered valid by EFSA as of 24 May 2011.

These products were included in the European Union Register of Feed Additives following the provisions of Article 10(1) of Regulation (EC) No 1831/2003.

TERMS OF REFERENCE

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 the efficacy of the products *Lactobacillus brevis* (DSM 23231), *Lactobacillus buchneri* (DSM 22501), *Lactobacillus buchneri* (NCIMB 40788 − CNCM I-4323), *Lactobacillus buchneri* (PTA-6138) and *Lactobacillus buchneri* (PTA-2494), when used under the conditions described in Table 1.

[4] 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.
[5] SILAC-EEIG-Silage Additives (in such case composed by Biomin GmbH, CHR Hansen, Lallemand SAS and Pioneer) Avenue Louise, 120-Box 13, 1050, Brussels, Belgium.
[6] EFSA Dossier reference: FAD-2010-0108.

Table 1: Description and conditions of use of the additive as proposed by the applicant

Additive	Lactobacilli obligately heterofermentative: *Lactobacillus brevis* (DSM 23231), *Lactobacillus buchneri* (DSM 22501), *Lactobacillus buchneri* (NCIMB 40788 − CNCM I-4323), *Lactobacillus buchneri* (PTA-6138) and *Lactobacillus buchneri* (PTA-2494)			
Registration number/EC No/No	-			
Category(ies) of additive	Technological			
Functional group(s) of additive	Silage additive			
Description				
Composition, description	Chemical formula	Purity criteria	Method of analysis	
L. brevis (DSM 23231), *L. buchneri* (PTA-6138) *L. buchneri* (PTA-2494) each with a minimum content of 1×10^{10} CFU/g, *L. buchneri* (DSM 22501) with a minimum content of 5×10^{10} CFU/g *L. buchneri* (NCIMB 40788 - CNCM I-4323) with a minimum content of 3×10^{9} CFU/g		Significant impurities: - Coliforms: <1000 CFU/g - Yeast and molds: <1000 CFU/g Relevant impurities: - *E. coli*: <10 CFU/g - *Salmonella*: absence in 25g - Aflatoxin B1: <1µg/kg	Enumeration method EN 15787:2009 Identification method (genetic): PFGE	
Trade name	Not applicable			
Name of the holder of authorisation	Not applicable			
Conditions of use				
Species or category of animal	Maximum Age	Minimum content	Maximum content	Withdrawal period
		CFU/kg of complete feedingstuffs		
All species and categories	n.a.	5×10^{7} (moderate and difficult forage) for *L. brevis* (DSM 23231) 1×10^{8} (easy, moderate and difficult forage) for *L. buchneri* (DSM 22501) and for *L.buchneri* (NCIMB 40788 - CNCM I-4323) 1×10^{8} (easy forage) for *L. buchneri* (PTA-6138) and *L. buchneri* (PTA-2494)	n.a.	n.a.

Other provisions and additional requirements for the labelling	
Specific conditions or restrictions for use	In the direction for use indicate the storage temperature and storage life.
Specific conditions or restrictions for handling	For safety: eye protection and gloves shall be used during handling
Post-market monitoring	n.a.
Specific conditions for use in complementary feedingstuffs	n.a.

Maximum Residue Limit (MRL)			
Marker residue	Species or category of animal	Target tissue(s) or food products	Maximum content in tissues
n.a.	n.a.	n.a.	n.a.

ASSESSMENT

1. Introduction

Six genera of lactic acid-producing bacteria are commonly associated with forage species and collectively contribute to the natural ensiling process. This joint application made by a consortium of companies concerns five strains of two species of one of these six genera, one strain of *Lactobacillus brevis* and four strains of *Lactobacillus buchneri*. All are intended to be individually added to forages to promote ensiling (technological additives; functional group: silage additive) for eventual use of the silage in any animal species.

Both species *L. brevis* and *L. buchneri* are considered by EFSA to be suitable for the Qualified Presumption of Safety (QPS) approach to safety assessment (EFSA, 2007, 2012a). This approach requires the identity of the strain to be conclusively established and evidence that the strains do not show resistance to antibiotics of human and veterinary importance.

2. Characterisation

The five strains included in this application are listed in Table 2 together with their accession numbers in internationally recognised culture collections. Each strain has been given a reference letter which, for convenience, will be used throughout this opinion. Accession numbers for which a copy of the certificate of deposition is provided are shown in bold.

Table 2: The strains of *Lactobacillus brevis* and *Lactobacillus buchneri* and their accession numbers

Reference letter	Accession numbers[1]
A[7]	*Lactobacillus brevis* **DSM 23231**
B[8]	*Lactobacillus buchneri* **CCM 1819—DSM 22501**
C[9]	*Lactobacillus buchneri* **NCIMB 40788—CNCM I-4323**
D[10]	*Lactobacillus buchneri* **LN 40177—ATCC PTA-6138**
E[10]	*Lactobacillus buchneri* **LN 4637—ATCC PTA-2494**

[1] DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; ATCC, American Type Culture Collection; CNCM, Collection Nationale de Culture de Microorganismes; NCIMB, National Collection of Industrial and Marine Bacteria.

2.1. Identity and properties of the active agents

The strain of *L. brevis* was isolated from untreated silage. *L. buchneri* strains were isolated from tomato pulp (B), maize silage (C), sorghum silage (D) and high-moisture maize (E). None of the strains has been genetically modified.

Taxonomical identification of all strains was established by 16S rRNA gene analysis and phenotypical tests. Strain-specific identification and genetic stability analysis are based on the use of pulsed-field gel electrophoresis.[11]

Each strain was tested for antibiotic susceptibility using two-fold broth dilutions.[12] The battery of antibiotics tested included all of those recommended by EFSA (EFSA, 2012b). As all minimum inhibitory concentration values for the five strains were equal to or lower than the corresponding cut-off values defined by the FEEDAP Panel, no further investigation is required.

[7] Technical dossier/Section II/Annex II 2-2-8.
[8] Technical dossier/Section II/Annex II 2-2-9.
[9] Technical dossier/Section II/Annex II 2-2-10.
[10] Technical dossier/Section II/Annex II 2-2-11.
[11] Technical dossier/Section II/Annexes II 2-2-1 to 7.
[12] Technical dossier/Section II and Supplementary info Jul 2012/Annexes II 2-2-13 to 16 and Qi and Qii.

2.2. Production and characteristics of the additives

The active agents are grown in sterilised media typical of those used for lactic acid bacteria. Typical ingredients are listed and in most cases material safety data sheets are provided. Cells are then separated from the growth medium by centrifugation or microfiltration, cryoprotectants (e.g. ascorbic acid, dipotassium phosphate) are added and the cell mix is freeze-dried and ground. The ground powder is then blended with sufficient carrier to meet the minimum specified concentration for each additive. The composition and minimum specified content of the active agent are shown for each additive in Table 3. Analysed values were also provided for multiple batches of each strain. However, some of these related to the cell concentrate to be variously blended according to the nature of the final product and, as such, could not be related to the declared minimum count. Where analysed values could be clearly related to a final product, then numbers always exceeded the declared minimum count. Maximum values for the spent medium and cryoprotectants appear to be expressed as a percentage of the product existing at various stages in the manufacturing process, not necessarily the final product.

Table 3: Composition of the five additives and the minimum guaranteed content of the active agent

Strain	Formulation	Minimum guaranteed cell count (CFU/g)
A[13]	Inulin (60–90 %)	1×10^{10}
B[14]	Maltodextrin (50–75 %), silica (8 %)	5×10^{10}
C[15]	Sucrose (30–60%);, silica (2 %)	3×10^{9}
D[16]	Maltodextrin (40–60 %), silica (8-10 %)	1×10^{10}
E[10]	Maltodextrin (40–60 %); , silica (8–10 %)	1×10^{10}

Additives B to E are also manufactured as granules with calcium carbonate (92%) and silica (1%) as carriers.

The additives are routinely monitored for microbial contamination. Limits are set for yeasts and filamentous fungi ($<10^3$ CFU/g additive), coliforms ($<10^3$ CFU/g additive), *Escherichia coli* (<10 CFU/g additive) and *Salmonella* (absence in 25 g of additive). Data from three batches of each additive confirmed compliance with these limits.[17] Given the nature of the fermentation medium and the food-grade excipients, the probability of contamination with heavy metals or mycotoxins is low and apparently not included in routine monitoring. Three batches of products C and D, two batches of E and one batch of A and B were, however, sent for the analysis of aflatoxin B_1. The values obtained were either below the detection limit of the analytical method or were substantially lower (0.05 µg/kg) than the action limit set (1.0 µg/kg additive).[18]

The available measurements of particle size distribution, made by laser diffraction (A–D) and sieve analysis (E), and of dusting potential as determined using a Heubach dustometer are summarised in Table 4. However, it should be noted that, as it is envisaged that many of the products will contribute only to a silage "premix", no final formulation exists as such. As a result, measurements are made on the dry cell mass obtained after mixing with cryoprotectants, or on the formulation used to prepare the premix. In one case, as both strains (D and E) originate from the same company, analysis of a single strain is considered by the applicant to be representative of the other strain.

[13] Technical dossier/Section II/Annex II 2-1-6.
[14] Technical dossier/Section II/Annex II 2-1-7.
[15] Technical dossier/Section II/Annex II 2-1-8.
[16] Technical dossier/Section II/Annex II 2-1-9.
[17] Technical dossier/Section II/Annexes II 2-1-10 to 13.
[18] Technical dossier/Section II/Annexes II 2-1-14 to 17.

Table 4: Particle size and dusting potential

Strain	Particle size	Dusting potential (g/m^3)
A[19]	1.5 % <10 µm; 18% < 50 µm	10.55 and 13.15 (two batches)
B[20]	n.d[1]	n.d[a]
C[21]	10% < 10 µm; 35% < 50 µm	n.d.
D or E[22]	28% < 50 µm[b]	37.9[b]
	0% < 50 µm[c]	4.2[c] (three batches)

[a] n.d., not determined. The data provided did not relate to the strain under consideration.
[b] Water miscible formulation.
[c] Granular form.

2.3. Stability

The shelf-life of strains A, D and E in the sealed moisture-tight containers in which they are supplied was shown to be at least 12 months when stored at ambient temperature of 22–27 °C while the shelf-life of strains A and D was shown to be 18 months under refrigeration (4–5 °C).[23] The shelf-life of strain C measured in two premixtures was 18 months under refrigeration, but this was reduced to approximately three months when stored at 15–21 °C. Strain B showed no loss of viability after 24 months' storage at temperatures up to 25 °C.[24]

All five strains showed good stability in water at ambient temperatures (20–27 °C) for a minimum period of 48 hours.[25]

2.4. Conditions of use

The additives are intended for use with all or a selected range of forages at the recommended doses shown in Table 5. Granulated products are intended to be directly applied to material for ensiling while all other formulations are intended to be first suspended in water and then distributed by spraying.

Table 5: Application and recommended dose

Strain	Type of forage (easy, moderately difficult or difficult to ensile)	Recommended dose (CFU/kg fresh silage)
A	Moderately difficult, difficult	5×10^7
B	All forages	1×10^8
C	All forages	1×10^8
D	Easy	1×10^8
E	Easy	1×10^8

2.5. Evaluation of the analytical methods by the European Union Reference Laboratory (EURL)

EFSA has verified the EURL report as it relates to the methods used for the control of the active agents in animal feed. The Executive Summary of the EURL report can be found in the Appendix.

3. Safety

In the view of the FEEDAP Panel, the antibiotic susceptibility qualification has been met and the identity of the strains established. Consequently, the *L. brevis* strain and the four strains of *L. buchneri*

[19] Technical dossier/Section II/Annex II 2-1-18.
[20] Technical dossier/Section II/Annex II 2-1-19.
[21] Technical dossier/Section II/Annex II 2-1-20.
[22] Technical dossier/Section II/Annexes II 2-1-21 and 2.
[23] Technical dossier/Section II/Annex II 2-4-1 to 3.
[24] Technical dossier/Section II /Annex II 2-4-3.
[25] Technical dossier/Supplementary info Jul 12/Annexes Qiv.

are considered suitable for the QPS approach to safety assessment; no further assessment of safety, other than user safety, is required, and they are presumed safe for the target species, for consumers of products from animals fed treated silage and for the environment.

No data are available on skin or eye irritation for any of the strains in any formulation. However, the generic material safety data sheet proposed for the five strains indicates that preparations containing the strains may cause irritation on prolonged contact with skin and eyes.

The dusting potential of commercial formulations tested was high. This, coupled with the significant fraction of these products that is potentially inhalable, means that exposure via a respiratory route is a significant possibility and hazard. Although users at the farm level are exposed to the additive for only a short period of time when preparing the aqueous suspension, given the proteinaceous nature of the active agents, their potential to be skin/respiratory sensitisers should be considered.

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 focus provided that other components do not introduce concerns. The excipients listed (Section 2.2) would not introduce additional risks to their conventional use.

4. Efficacy

4.1. Lactobacillus brevis DSM 23231 (strain A)

Three studies carried out using 1.8-L (early samplings) and 5.8-L (mid-trial and final samplings) mini-silos are described.[26] The duration of the studies was 90 days (studies 1 and 2) or 91 days (study 3). In each case, the contents of three replicate silos were sprayed with the additive at 5×10^7 CFU/kg forage (not confirmed by analysis). Forage in the control silos was sprayed with an equal volume of water but without the additive. Ambient temperature was controlled at 22 ± 2 °C. Different types of forages were used in the three studies, representing material moderately difficult to ensile (two grass samples from permanent grasslands with water-soluble carbohydrate (WSC) contents of 2.82 % and 2.26 %) and difficult to ensile (crushed maize with WSC 1.03 %), as defined in Regulation (EC) No 429/2008.

Replicate silos were opened 3, 7, 45 ± 3 and 90 days after ensiling and the contents were analysed for dry matter content, pH, lactic acid and volatile fatty acid (VFA) concentration, ethanol, ammonia and total nitrogen, as well as aerobic stability (by continuous measurement of temperature for at least 11 days, considering an increase of 2 °C above ambient temperature as indicative of deterioration) at the end of the experiment (Table 6).

Normal distribution of data was confirmed by the Kolmogorov–Smirnov test and homogeneity of variance by Levene's test. For silage fermentation parameters, the significance of additive effects was assessed using a *t*-test, comparing data from a single test with those from the corresponding control silos. The statistical significance of the difference in aerobic stability between control and treated silages was established by the non-parametric Mann–Whitney test.

[26] Technical dossier/Section IV/Annex IV-1.

Table 6: Summary of the analysis of ensiled material recovered at the end of the experiments with *L. brevis* DSM 23231 (strain A)

Forage	Treatment (CFU/kg forage)	Dry matter loss (%)	pH	Lactic acid (% ensiled matter)	Acetic acid (% ensiled matter)	Ammonia-N (% total N)	Aerobic stability (days)
Permanent grassland	0	2.14	4.1	2.82	0.76	n.d.	11.0
	5×10^7	3.41*	4.2*	2.42*	1.00	n.d.	13.2
Permanent grassland	0	4.26	4.3	1.31	0.94	9.1	7.7
	5×10^7	2.29*	3.9*	2.43*	0.89	6.5*	10.6
Maize	0	3.62	4.4	1.01	0.46	3.6	14.6
	5×10^7	2.98*	4.3	1.22*	0.48	4.0	17.8

n.d. not determined.
* Significantly different to the control at $P < 0.05$.

The potential of. *L. brevis* DSM 23231 to improve the nutritive value of silage was not demonstrated. The mean values for aerobic stability were increased by two or more days in treated compared to control ensiled materials. However, these differences were not significant.

4.2. *Lactobacillus buchneri* DSM 22501 (strain B)

A total of four laboratory studies are described with different forage materials and lasting 90 days. All of the studies used 3-L mini-silos with the capacity to vent gas. In each case, the contents of five replicate silos were sprayed with the additive at 1×10^8 CFU/kg forage (confirmed by analysis). Forage for the control silos were sprayed with an equal volume of water but without the additive. Ambient temperature was controlled at 20 °C. The four studies involved different forages representing easy, moderately difficult and difficult to ensile material as defined in Regulation (EC) No 429/2008 (Table 7).

Table 7: Characteristics of the forage samples used in the ensiling experiments

Study No	Test material	Dry matter content (% fresh material)	Water-soluble carbohydrate content (% fresh material)
1[27]	Whole crop maize	32.8	3.6
2[28]	Red clover/ryegrass	31.7	2.9
3[29]	*Medicago sativa* L. var. Europa	31.4	1.2
	Medicago sativa L. var. Birute	32.2	1.2
	Medicago sativa L. var. Verko	35.0	1.2
4[30]	*Medicago sativa* L. var. FSG4 08	17.2	0.2
	Medicago sativa L. var. Jögeva	13.5	0.1
	Medicago sativa L. var. Galega	14.3	0.1

Replicate silos were opened at the end of the experiment and the contents were analysed for dry matter content, pH, lactic acid and VFA concentration, ethanol, ammonia and total nitrogen, as well as aerobic stability (using a rise of 3 °C as indicative of spoilage) at the end of the experiment (only for maize silage; Table 8).

Statistical evaluation of data was by non-parametric tests, using the Wilcoxon signed-rank test followed by the Kruskal–Wallis chi-squared test.

[27] Technical dossier/Section IV/Annexes IV-2-1 and 2.
[28] Technical dossier/Section IV/Annexes IV-2-2 and 3.
[29] Technical dossier/Section IV/Annexes IV-2-3, 4 and 5.
[30] Technical dossier/Section IV/Annex IV-2-5.

Table 8: Summary of the analysis of ensiled material recovered at the end of the experiments with *L. buchneri* DSM 22501 (strain B)

Forage	Treatment (CFU/kg forage)	Dry matter loss (%)	pH	Lactic acid (% ensiled matter)	Acetic acid (% ensiled matter)	Ammonia-N (% total N)	Aerobic stability (days)
Maize	0	7.4	4.0	0.86	0.81	6.1	2.7
	1×10^8	5.8*	3.7*	1.08*	1.14*	5.2*	6.0*
Red clover/ryegrass	0	10.2	4.7	0.79	0.82	5.7	n.d.
	1×10^8	6.7*	4.3*	0.96	1.14*	4.0*	n.d.
Medicago sativa L. var. Europa	0	6.7	5.5	0.25	0.76	11.1	n.d.
	1×10^8	5.0*	5.3*	1.16*	1.47*	9.0*	n.d.
Medicago sativa L. var. Birute	0	6.9	5.2	0.56	1.08	10.4	n.d.
	1×10^8	4.9*	4.9*	1.44*	1.89*	7.7*	n.d.
Medicago sativa L. var. Verko	0	6.8	5.3	0.75	1.34	9.1	n.d.
	1×10^8	3.9*	4.9*	1.15*	1.30	7.1*	n.d.
Medicago sativa L. var. FSG4 08	0	9.9	6.0	0.21	0.59	18.5	n.d.
	1×10^8	8.8	5.9*	0.48*	0.65	18.0*	n.d.
Medicago sativa L. var. Jögeva	0	10.1	6.5	0.25	0.73	32.7	n.d.
	1×10^8	10.5	6.5	0.17*	0.72	29.0*	n.d.
Medicago sativa L. var. Galega	0	9.5	6.0	0.18	0.44	23.7	n.d.
	1×10^8	10.5*	5.9*	0.10	0.68*	25.4*	n.d.

n.d., not determined.
*Significantly different to the control at $P < 0.05$.

Strains of *L. buchneri* are normally added to silage to increase aerobic stability. Generally, this is achieved by increasing acetic acid production but at a cost of increased dry matter loss, as is shown by the other *L. buchneri* strains included in this application (strains C, D and E). *L. buchneri* DSM 22501 shows an entirely different pattern.

Study 4 was performed with forage materials with an extremely low WSC content (≤ 0.2 %). Significant differences in the measured parameters would not be expected with these extremely difficult to ensile materials. For the remaining studies, dry matter loss, pH and ammonia nitrogen were significantly reduced in all five forage materials tested and lactic and acetic acids significantly increased in four out of five forages. Aerobic stability was not measured.

The results indicate that *L. buchneri* DSM 22501 has the potential to improve the production of silage from easy, moderately difficult and difficult to ensile materials by reducing the pH and ammonia nitrogen and by increasing the preservation of dry matter.

4.3. *Lactobacillus buchneri* NCIMB 40788—CNCM I-4323 (strain C)

Three laboratory experiments were carried out using three different forage samples and lasting 107 days (study 1), 91 days (study 2) or 90 days (study 3).[31] The studies used 60-L (studies 1 and 3) and 2.75-L (study 2) mini-silos with the capacity to vent gas. In each case, the contents of three replicate silos were sprayed with the additive at 1×10^8 CFU/kg or 3×10^8 CFU/kg forage (confirmed by analysis). Forage in the control silos was sprayed with an equal volume of water but without the additive. Ambient temperature was controlled at 20 °C (studies 1 and 2) or ranged between 15 and 25 °C (study 3). The three studies involved different forages representing material easy, moderately difficult and difficult to ensile, as defined in Regulation (EC) No 429/2008 (Table 9).

[31] Technical dossier/Section IV/Annex IV-3.

Table 9: Characteristics of the forage samples used in the ensiling experiments

Study No	Test material	Dry matter content (% fresh material)	Water-soluble carbohydrate content (% fresh material)
1	Clover/ryegrass	36.1	3.3
2	Whole crop wheat	40.0	1.9
3	Alfalfa/ryegrass	38.3	1.4

Replicate silos were opened at the end of the experiments and the contents were analysed for dry matter content, pH, lactic acid and VFA concentration, ethanol, ammonia and total nitrogen and aerobic stability (time to a 3 °C rise in silage temperature above ambient temperature; Table 10). Normal distribution of data was confirmed and then treatment effects were examined within each forage type by one-way analysis of variance (ANOVA).

Table 10: Summary of the analysis of ensiled material recovered at the end of the experiments with *L. buchneri* NCIMB 40788—CNCM I-4323 (strain C)

Forage	Treatment (CFU/kg)	Dry matter loss (%)	pH	Lactic acid (% ensiled matter)	Acetic acid (% ensiled matter)	Ammonia (% total N)	Aerobic stability (days)
Red clover/ryegrass	0	0.9	3.9	3.54	0.83	8.3	14.7
	1×10^8	1.4	4.1	3.07	0.90	9.3	>20.0*
	3×10^8	0.8	4.0	3.39	0.69	8.7	>20.0*
Wheat whole crop	0	1.5	4.4	1.95	0.70	12.0	3.8
	1×10^8	4.4*	5.0*	1.97	0.90*	11.5	>9.0*
	3×10^8	4.9*	4.8*	2.02	0.88*	10.8	>9.0*
Alfalfa/ryegrass	0	n.d.	4.8	1.85	0.71	13.8	6.0
	1×10^8	n.d.	4.1*	1.58*	1.16*	14.8*	>15.0*
	3×10^8	n.d.	4.1*	1.56*	1.19*	15.1*	>15.0*

*Significantly different to the control at $P < 0.05$.

The results indicate that *L. buchneri* NCIMB 40788—CNCM I-4323 has the potential to improve the aerobic stability of silage from easy, moderately difficult and difficult to ensile forage materials (DM content ranging between 36 % and 40 %).

4.4. *Lactobacillus buchneri* ATCC PTA-6138 (strain D) and ATCC PTA-2494 (strain E)

Three laboratory studies lasting 90 days are described.[32] In all the studies the forage ensiled was Italian ryegrass with various WSC contents representing easy to ensile material as defined in Regulation (EC) No 429/2008 (Table 11).

Table 11: Characteristics of the forage samples used in the ensiling experiments

Study No	Test material	Dry matter content (% fresh material)	Water-soluble carbohydrate content (% fresh material)
1	Italian ryegrass	37.7	4.4
2	Italian ryegrass	46.3	6.4
3	Italian ryegrass	39.0	8.4

[32] Technical dossier/Section IV and Supplementary info Jul 2012 /Annexes IV-4 and Qv.

The studies used 2.75-L mini-silos. In each case, the contents of four replicate silos were sprayed with the additive (D or E) at 1×10^8 CFU/kg (not confirmed by analysis). Forage in the control silos was sprayed with an equal volume of water but without the additive. Ambient temperature was controlled at 20 °C (study 1), 21 °C (study 2) or 18 °C (study 3).

Replicate silos were opened at the end of the experiment and silage was analysed for dry matter content, pH, lactic acid and VFA concentration, ethanol and aerobic stability (Table 12). Aerobic stability was measured using the Honig method (Honig, 1986). Normal distribution of data was confirmed (histograms and Q–Q plots), and then statistical evaluation of data was performed by one-way ANOVA (mixed model) using pair-wise comparison of least-square means.

Table 12: Summary of the analysis of material from easy to ensile forage recovered at the end of the experiments with *L. buchneri* ATCC PTA 6138 and ATCC PTA-2494 (strains D and E, respectively)

Forage	Treatment (CFU/kg)	Dry matter loss (%)	pH	Lactic acid (% ensiled matter)	Acetic acid (% ensiled matter)	Aerobic stability (days)
Italian ryegrass	0 D: 1×10^8 E: 1×10^8	1.8 2.2* 2.6*	4.3 4.2 4.3	1.64 1.68 1.12*	0.7 1.4* 2.0*	4.1 7.0* 7.0*
Italian ryegrass	0 D: 1×10^8 E: 1×10^8	2.0 1.9 1.9	4.8 4.6* 4.5*	1.01 1.13 1.84*	0.2 0.8* 0.6*	3.6 7.0* 7.0*
Italian ryegrass	0 D: 1×10^8 E: 1×10^8	2.7 3.0 3.5	4.7 4.4* 4.3*	2.15 1.80* 1.51*	0.3 1.5* 2.3*	3.7 7.0* 7.0*

*Significantly different to the control at $P < 0.05$.

Both strains significantly decreased pH in two of the ensiled materials and increased acetic acid concentration in all three ensiled materials. As a consequence, *L. buchneri* ATCC PTA 6138 and ATCC PTA-2494 have the potential to improve the aerobic stability of silage from easy to ensile forage material with DM content ranging between 37 % and 46 %.

CONCLUSIONS

As the identity of the four strains of *Lactobacillus buchneri* and the strain of *Lactobacillus brevis* has been established and no antibiotic resistance detected, following the QPS approach to safety assessment, the use of these strains in the production of silage is considered safe for the target species, for consumers of products from animals fed treated silage and for the environment.

Although users at the farm level are exposed to silage additives for only a short period of time when preparing the aqueous suspension, the potential of the additives to be irritants and/or to act as a skin sensitisers cannot be excluded. Given the proteinaceous nature of the active agents and the high dusting potential of the products, the FEEDAP Panel considers it prudent to treat these additives as skin and respiratory sensitisers.

The efficacy of *L. brevis* DSM 23231 (strain A) to improve the preservation of nutritive value or increase the aerobic stability of silage was not demonstrated.

L. buchneri DSM 22501 (strain B) has the potential to improve the production of silage from easy, moderately difficult and difficult to ensile materials by reducing the pH and ammonia nitrogen and by increasing the preservation of dry matter.

L. buchneri NCIMB 40788—CNCM I-4323 (strain C) has the potential to improve the aerobic stability of easy, moderately difficult and difficult to ensile forage materials.

L. buchneri ATCC PTA 6138 (strain D) and ATCC PTA-2494 (strain E) also have the potential to improve the aerobic stability of easy to ensile materials.

DOCUMENTATION PROVIDED TO EFSA

1. *Lactobacillus brevis* (IFA 92−DSM 23231), *Lactobacillus buchneri* (CNCM 1819−DSM 22501), *Lactobacillus buchneri* (NCIMB 40788—CNCM I-4323), *Lactobacillus buchneri* (ATCC PTA-6138) and *Lactobacillus buchneri* (ATCC PTA-2494). September 2010. Submitted by SILAC-EEIG-Silage Additives.

2. *Lactobacillus brevis* (IFA 92−DSM 23231), *Lactobacillus buchneri* (CNCM 1819−DSM 22501), *Lactobacillus buchneri* (NCIMB 40788—CNCM I-4323), *Lactobacillus buchneri* (ATCC PTA-6138) and *Lactobacillus buchneri* (ATCC PTA-2494). Supplementary dossier. July 2012. Submitted by SILAC-EEIG-Silage Additives.

3. Evaluation report of the European Union Reference Laboratory for Feed Additives on the methods(s) of analysis for *Lactobacillus brevis* (DSM 23231), *Lactobacillus buchneri* (DSM 22501), *Lactobacillus buchneri* (NCIMB 40788—CNCM I-4323), *Lactobacillus buchneri (*PTA-6138) and *Lactobacillus buchneri* (PTA-2494) for all animal species.

4. Comments from Member States received through the ScienceNet.

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. The EFSA Journal, 587, 1–16.

EFSA Panel on Biological Hazards (BIOHAZ), 2012a. Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2012 update). EFSA Journal, 10(12):3020. 84 pp.

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2012b. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA Journal, 10(6):2740. 10 pp.

Honig H. 1985. Determination of aerobic deterioration—System Völkenrode. Landbauforsch. Völkenrode, SH 2, p. 3.

APPENDIX

Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Methods of Analysis for *Lactobacillus brevis* (DSM 23231), *Lactobacillus buchneri* (DSM 22501), *Lactobacillus buchneri* (NCIMB 40788−CNCM I-4323), *Lactobacillus buchneri* (PTA-6138) and *Lactobacillus buchneri* (PTA-2494) for all animal species[33]

This report is on the evaluation of feed additives "*micro-organisms used as silage agents"*, which is related to the application of (1) forty two *micro-organisms* for which authorisation is sought under Article 10(2) and (2) three additional *micro-organisms* for which authorisation is sought under Article 4(1). Authorisation is sought for all the above mentioned *micro-organisms* under category/functional group 1(k), technological additives/silage additives, according to Annex I of Regulation (EC) No 1831/2003. The list of *micro-organisms* of interest and the minimum activities in the *feed additives* and in *silage*, as sought in the authorisation, are presented in Table 1.[34] The intended use of the current applications is for all animal species, except for FAD-2011-0001, for which pigs, bovines, sheep, goats and horses are specified.

For identification and characterisation of *Saccharomyces cerevisiae* the EURL recommends for official control Polymerase Chain Reaction (PCR), a generally recognised standard methodology for identification of yeasts. For identification and characterisation of all the other *micro-organisms* of concern (i.e. *lactococci*, *lactobacilli*, *pediococci* and *bacilli*) the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for microbial identification.

The EURL recommends for enumeration in the *feed additives* the following ring trial validated methods:

- Pour plate method using MRS agar (ISO 15214) for *Lactococci;*
- Spread plate method using MRS agar (EN 15787) for *Lactobacilli*;
- Spread plate method using MRS agar (EN 15786) for *Pediococci;*
- Spread plate method using tryptone soya agar (EN 15784) for *Bacilli;* and
- Pour plate method using CGYE agar (EN 15789) for *Saccharomyces.*

None of the Applicants provide experimental data for the determination of micro-organisms in silage. Furthermore, the unambiguous determination of the content of micro-organisms added to silage is not achievable by analysis. Therefore the EURL cannot evaluate nor recommend any method for official control to determine any of the forty five micro-organisms of concern 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.

[33] The EURL produced a combined report for the *L. lactis, L. plantarum, L. buchneri, L. paracasei, L. rhamnosus, L. salivarius, L. casei, L. brevis, L. pentosus, P. acidilactici, P. pentosaceus, Bacillus, Saccharomyces cerevisiae* and *Lactococcus lactis.*

[34] Full list provided in EURL evaluation report, available from the EURL website: http://irmm.jrc.ec.europa.eu/SiteCollectionDocuments/FinRep-uorg-silage-group1.pdf