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SCIENTIFIC OPINION

Scientific Opinion on the safety and efficacy of *Lactobacillus brevis* (DSMZ 16680) as a silage additive 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

This scientific output, published on 31 March 2014, replaces the earlier version published on 15 January 2014.[4]

ABSTRACT

Lactobacillus brevis is a technological additive intended to improve the ensiling process at a minimum proposed dose of 1.0×10^8 colony-forming units (CFU)/kg fresh material. The bacterial species *L. brevis* is considered by the European Food Safety Authority 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 in the production of silage is considered safe for livestock species, for consumers of products from animals fed the treated silage and for the environment. The additive should be regarded as a skin and eye irritant and a potential skin and respiratory sensitiser, and treated accordingly. The FEEDAP Panel concluded that *L. brevis* has the potential to increase aerobic stability of the treated silage at a minimum proposed dose of 1.0×10^8 CFU/kg fresh material. This was demonstrated in forage materials with a dry matter content of 29–46 %.

KEY WORDS

technological additive, silage additive, *Lactobacillus brevis*, QPS, safety, efficacy

[1] On request from the European Commission, Question No EFSA-Q-2012-00086, adopted on 5 December 2013.

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

[4] Revision 1: This scientific opinion has been edited following the confidentiality claims made by the applicant. The modified sections are indicated in the text.

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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 target animals, consumers, users and the environment, and on the efficacy of a product based on a specific strain of *Lactobacillus brevis* when used as a technological additive intended to improve the ensiling process at a minimum proposed dose of 1.0×10^8 CFU/kg fresh material.

The bacterial species *L. brevis* is considered by EFSA to be suitable for the qualified presumption of safety approach and not to require any specific demonstration of safety other than confirming the absence of resistance to antibiotics of human and veterinary clinical significance. As the identity of the strain has been clearly established and as no antibiotic resistance of concern was detected, the use of the strain in the production of silage is presumed safe for livestock species, for consumers of products from animals fed the treated silage and for the environment.

The additive should be regarded as a skin and eye irritant and a potential skin and respiratory sensitiser, and treated accordingly.

Studies with laboratory-scale silos, each lasting at least 250 days, were carried out using samples of forage of differing water-soluble carbohydrate content. In each case, replicate silos containing treated forage were compared with identical silos containing the same but untreated forage. At the end of fermentation, silos were opened, contents were analysed and a sub-sample was monitored for aerobic stability. A rise of 3 °C was taken as indicative of spoilage. The FEEDAP Panel concluded that *L. brevis* has the potential to increase aerobic stability of the treated silage at the minimum recommended dose of 1.0×10^8 CFU/kg fresh material. This was demonstrated in forage materials with a dry matter content of 29–46 %.

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BACKGROUND

Regulation (EC) No 1831/2003[5] 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 Microferm Limited[6] for re-evaluation of the product *Lactobacillus brevis* (DSMZ 16680) to be used as a feed additive 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.[7] 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 14 May 2012.

This product was 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 product *Lactobacillus brevis* (DSMZ 16680), when used under the conditions described in Table 1.

[5] 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.
[6] Microferm Limited, Spring Lane North, Malvern Link, Worcester WR14 1BU, United Kingdom.
[7] EFSA Dossier reference: FAD-2010-0277.

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

Additive	*Lactobacillus brevis* DSMZ 16680			
Registration number/EC No/No	-			
Category of additive	Technological			
Functional group of additive	Silage additive			
Description				
Composition, description	Chemical formula	Purity criteria	Method of analysis	
Lactobacillus brevis (DSMZ 16680)		*E. coli* <100 CFU/g *Salmonella* nil in 25 g Yeast/mould<100 CFU/g	BS EN 15787:2009	
Trade name				
Name of the holder of authorisation				
Conditions of use				
Species or category of animal	Maximum Age	Minimum content	Maximum content	Withdrawal period
		CFU/kg of complete feedingstuffs		
All animal species				
Other provisions and additional requirements for the labelling				
Specific conditions or restrictions for use				
Specific conditions or restrictions for handling	Respiratory sensitiser, wear appropriate PPE including dust masks and gloves, wash hands after use.			
Post-market monitoring				
Specific conditions for use in complementary feedingstuffs				
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. The present additive is based on a preparation of a single strain of one of those six genera, *Lactobacillus brevis*, and is intended to be added to forages to promote ensiling (technological additive, functional group: silage additive) for the eventual use of the silage in all animal species. The heterofermentative 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 resistance to antibiotics of human and veterinary importance.

2. Characterisation

2.1. Identity and properties of the active agent

The strain of *L. brevis* of unknown origin is deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DEU) with the accession number DSMZ 16680.[8] It has not been genetically modified. Strain identity was established by its phenotypic properties and by the partial 16S rRNA gene sequence which by comparison with sequences recorded in databases enabled the strain to be unambiguously identified as *L. brevis*. Multi locus sequence typing based on sequencing four specific genes (*rpoA, pheS, atpA* and *dnaK*) was proposed as a means of strain-specific detection.[9] Although this method is considered appropriate, no data were provided to illustrate that comparison of the four gene fragments chosen in this case is able to distinguish between DSMZ 16680 and other *L. brevis* strains. No evidence of genetic stability has been provided.

The strain was tested for antibiotic susceptibility using a broth microdilution method. The battery of antibiotics tested included the ones recommended by EFSA (EFSA FEEDAP Panel, 2012).[10] The minimum inhibitory concentration values for the *L. brevis* strain are below or equal to the EFSA cut-off values except for tetracycline which is exceeded by a single dilution. This is within the normal variation around the mean and, thus, does not raise concerns for safety.

2.2. Production and characteristics of the additive[11]

The manufacturing process is detailed in the dossier. The resultant additive consists of approximately 38 % cells, 2 % spent medium and 60 % excipients. Material safety datasheets are provided for all medium components and cryoprotectants but no purity criteria are included.

No minimum content of *L. brevis* in the final product is specified. Analysis of five production batches gave a mean value of 5.5×10^{11} colony-forming units (CFU)/g additive (range $4.6–6.4 \times 10^{11}$ CFU/g additive, coefficient of variation (CV) 13 %).

The additive is routinely monitored for microbial contamination. Limits are set for *Escherichia coli* (< 100 CFU/g), filamentous fungi (< 100 CFU/g) and *Salmonella* spp. (absence in 25 g of the additive). Data from three batches confirmed compliance with the set limits.

Given the nature of the fermentation medium and the excipients, the probability of contamination with heavy metals or mycotoxins is considered to be low and is consequently not included in routine monitoring of batches. Three batches of one of the medium components and three batches of *L. fermentum* (excipient not given) were tested for heavy metals (lead, cadmium and mercury), arsenic

[8] Technical dossier/Section II/Annex II.2–1.
[9] Technical Dossier/Supplementary information August 2012.
[10] Technical Dossier/Section II_2.2.2 and supplementary information August 2012 and September 2013.
[11] This section has been edited following the confidentiality claims made by the applicant.

and aflatoxins B_1, B_2, G_1 and G_2. Aflatoxins were not detected (< 0.1 mg/kg). Contamination with heavy metals and arsenic was low and of no concern (cadmium ≤ 0.1 mg/kg, mercury< 0.02 mg/kg and arsenic < 0.2 mg/kg). Lead values (0.4–3.9 mg/kg) were higher than normally encountered in microbial preparations. However, considering the extent of dilution in ensiled material and the levels normally considered acceptable in feedstuffs (< 10 mg/kg), this is not considered a hazard.[12]

Three batches of the additive were examined for particle size distribution by laser diffraction.[13] The average particle size was 88 µm, with 57 % by weight of the additive consisting of particles with diameters below 100 µm, 30 % below 50 µm and 4 % below 10 µm. No data on dusting potential were provided.[14]

2.3. Stability

2.3.1. Shelf life

Three batches *L. brevis* were standardised with maltodextrin to give a count of 1×10^{11} CFU/g or to a level of 2.5×10^{10} CFU/g using dextrose.[15] The samples were stored in sealed aluminium foil bags at ambient temperature. Viability losses were small over six months but were 10–14 % after nine months and 17–24 % after 15 months in the case of the maltodextrin formulation and up to 7–10 % after nine months and 16–21 % after 15 months for the for the dextrose formulation.

2.3.2. Stability in water

A batch of product was standardised to give a count of 1×10^{11} CFU/g using dextrose and ammonium and potassium phosphates as buffer salts. An experiment was designed to mirror practical conditions where, typically, 10 g of product would be dissolved in 2 L of water and applied to one tonne of forage to deliver 1×10^9 CFU/kg. Three batches of the solution of the *L. brevis* were stored at room temperature and samples removed over seven days. Viable counts remained essentially constant for four days with small losses thereafter.

2.4. Conditions of use

The additive is intended for direct use with all forages for all animal species at a minimum proposed dose of 1.0×10^8 CFU/kg fresh material as an aqueous suspension.

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

The EURL considered that the conclusions and recommendations reached in the previous assessment are valid and applicable for the current application.[16]

3. Safety[17]

In the view of the FEEDAP Panel, the antibiotic resistance qualification has been met and the identity of the strain established. Consequently, *L. brevis* DSMZ 16680 is considered by EFSA to be suitable for the QPS approach to safety assessment and is presumed safe for the target species, consumers of products from animals fed treated silage and the environment.

No data are available on skin/eye irritation or skin sensitisation. Therefore, the additive should be considered to have the potential to be a skin and eye irritant and a skin sensitiser and should be treated accordingly.

[12] Technical dossier/Section II/2.1.4.2.
[13] Technical Dossier /Section II.
[14] Technical Dossier /Section II_2.1.5.2 and supplementary information August 2012.
[15] Technical dossier/Section II.1.5.2 and supplementary information August 2012.
[16] The full report is available on the EURL website: http://irmm.jrc.ec.europa.eu/SiteCollectionDocuments/FinRep-FAD-uorg3.pdf
[17] This section has been edited following the confidentiality claims made by the applicant.

A significant fraction of the product is potentially inhalable and exposure via a respiratory route is possible. Although users at the farm level are exposed to the additive for only a short period of time when preparing the aqueous suspension or when applying the additive to forage, given the proteinaceous nature of the active agent, the additive should be considered to have the potential to be a respiratory sensitiser and should be treated accordingly.

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 does not provide an exhaustive list of cryoprotectants and carriers since the product is "generic". But it can be reasonably assumed that multiple formulations of the additive exist, which cannot be all directly tested for user safety. Excipients (dextrose, maltodextrin) used by the applicant in the preparation of the final formulation(s) do not introduce additional risks.

4. Efficacy

A total of four laboratory experiments are described made with different types of forage samples. The duration of the experiments differed considerably, ranging from 252 to 430 days. All of the studies used mini-silos capable of holding 1 kg of chopped forage material with the capacity to vent gas (volume not indicated). In each case, the contents of four replicate silos were sprayed with the additive (different concentrations, apparently not confirmed by analysis) suspended in water. Forage for the control silos were sprayed with an equal volume of water. Ambient temperature was not described. The forage samples used (Table 2) represent material easy to ensile (study 3) and difficult to ensile (studies 2 and 4). The water-soluble carbohydrate content was not measured in the whole crop maize silage used in study 1.

Replicate silos were opened at the end of the experiment and the contents were analysed by near infrared reflectance spectroscopy for proximate composition and by other methods for the remaining parameters (dry matter content, pH, lactic and volatile fatty acids concentrations, ethanol, ammonia and total nitrogen). A sub-sample was taken for continuous measurement of temperature, a 3 °C rise being taken as indicative of spoilage.

Statistical evaluation of data was made by Kruskal–Wallis and Mann–Whitney tests comparing single datapoints on each parameter with those from the corresponding control silos. Significance was assumed at $P < 0.05$.

Table 2: Characteristics of the forage materials used in the ensiling studies

Study	Test material	Dry matter content (% fresh material)	Water-soluble carbohydrate content (% fresh material)
1[18]	Whole crop maize	29.4	n.d
2[19]	Whole crop wheat	38.7	1.2
3[20]	Grass (wilted)	46.3	7.2
4[21]	Grass/clover	19.0	0.6

n.d: not determined.

The results of the four studies with application rates of 1×10^8 or 1×10^9 CFU/kg are summarised in Table 3.

[18] Technical dossier/Section IV and supplementary information September 2013/Annex IV.1–IV.3.
[19] Technical dossier/Section IV and supplementary information September 2013/Annex IV.10–IV.12.
[20] Technical dossier/Section IV and supplementary information September 2013/Annex IV.4–IV. 6.
[21] Technical dossier/Section IV and supplementary information September 2013/Annex IV.7–IV.9.

Table 3: Summary of the analysis of ensiled material recovered at the end of the ensiling studies

Study (duration in days)	Application rate (CFU/kg forage)	Dry matter loss (%)	pH	Lactic acid (% ensiled material	Acetic acid (% ensiled material	Ammonia-N (% total N)	Aerobic stability (hours)
1 (252)	0	4.8	3.6	1.4	0.4	3.8	25.5
	1×10^8	5.5	3.7	1.6 *	0.5 *	4.3	129.5 *
	1×10^9	4.7	3.6	1.2	0.6 *	3.9	133.5 *
2 (359)	0	4.3	4.2	1.3	0.6	11.3	78.5
	1×10^8	3.1	4.1	1.7	0.9 *	12.4	> 240 *
	1×10^9	3.5	4.1	1.6	1.0 *	12.1	> 240 *
3 (430)	0	6.8	4.4	2.8	0.7	3.6	98
	1×10^8	5.2	4.5	3.2	1.4 *	4.1 *	> 240 *
	1×10^9	5.5	4.5	3.8	1.5 *	3.5	> 240 *
4 (252)	0	7.4	4.6	0.7	0.8	16.7	> 168
	1×10^8	6.1	4.4	1.5 *	1.0	12.5 *	> 168
	1×10^9	6.4	4.4	0.9	1.4 *	12.6 *	> 168

*Significantly different from control value at $P < 0.05$.

As would be expected of a heterofermentative strain, no effect on the preservation of nutrients was observed. However, positive results were seen at both application rates in three out of four studies when forage samples were examined for aerobic stability (measuring a rise of 3 °C as indicative of spoilage). This was related to a significant increase in acetic acid concentration. In study 4 there was no loss in aerobic stability of the control sample over the experimental period and consequently, no improvement in stability on addition of *L. brevis* was seen.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

As the identity of *L. brevis* DSMZ 16680 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 considered safe for the target species, for consumers of products from animals fed treated silage and for the environment.

The additive should be regarded as a skin and eye irritant and a potential skin and respiratory sensitiser, and treated accordingly.

L. brevis DSMZ 16680 has the potential to increase aerobic stability of the treated silage at the minimum recommended dose of 1.0×10^8 CFU/kg fresh material. This was demonstrated in forage materials with a dry matter content of 29–46 %.

RECOMMENDATIONS

The applicant should specify a minimum declared content of *L. brevis* DSMZ 16680 in any final product.

DOCUMENTATION PROVIDED TO EFSA

1. *Lactobacillus brevis* (DSMZ 16680). November 2010. Submitted by Microferm Limited.

2. *Lactobacillus brevis* (DSMZ 16680). Supplementary information, August 2012. Submitted by Microferm Limited.

3. *Lactobacillus brevis* (DSMZ 16680). Supplementary information, September 2013. Submitted by Microferm Limited.

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 2007, 587, 1–16.

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA Journal 2012;10(6):2740, 10 pp. doi:10.2903/j.efsa.2012.2740

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