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Safety and efficacy of *Lactobacillus casei* DSM 28872 as a silage additive for all animal species

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Abstract

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed was asked to deliver a scientific opinion on the safety and efficacy of a strain of *Lactobacillus casei* when used as a technological additive intended to improve ensiling at a proposed application rate of 1×10^8 CFU (when used alone) or 5×10^7 CFU (when used in combination) kg/fresh matter. The species *L. casei* 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 absence of resistance to antibiotics of human and veterinary significance. As the identity of the strain was clearly established and as no antibiotic resistance was detected, the use of the strain in the production of silage is presumed safe for livestock species, consumers of products from animals fed treated silage and the environment. In the absence of data, no conclusion can be drawn on the skin and eye irritancy of the additive. The additive should be considered to have the potential to be a respiratory sensitiser. Five studies with laboratory-scale silos were made using forage of differing water-soluble carbohydrate content. Replicate silos containing forages treated at the proposed application rate were compared to identical silos containing the same but untreated forage. The mini-silos were stored for 90 days at 20–24°C. At the end of the ensiling period, the content of the silos was analysed and dry matter losses determined. Results showed that the *L. casei* strain applied at a minimum dose of 5×10^7 CFU/kg has the potential to improve the production of silage from easy and moderately difficult to ensile forage species by reducing dry matter loss and enhancing protein preservation.

Keywords: technological additive, silage additive, *Lactobacillus casei*, 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 Microferm Limited[2] for authorisation of the product *Lactobacillus casei* DSM 28872, when used as a feed additive for all animal 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 dossiers in support of this application. The particulars and documents in support of the application were considered valid by EFSA as of 3 May 2016.

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 casei* DSM 28872, 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. casei* DSM 28872. It has not been previously authorised as a feed additive in the European Union (EU).

The species *L. casei* is considered by EFSA to be suitable for the Qualified Presumption of Safety (QPS) approach to safety assessment (EFSA, 2007; EFSA BIOHAZ Panel, 2013). This approach requires the identity of the strain to be conclusively established and evidence that the strain does not show acquired resistance to antibiotics of human and veterinary importance.

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier[3] in support of the authorisation request for the use of *Lactobacillus casei* DSM 28872 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 casei* DSM 28872 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: 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

[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] Microferm Limited, Spring Lane North, Malvern Link WR141BU Worcestershire United Kingdom.
[3] FEED dossier reference: FAD-2016-0016.
[4] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.
[5] The full report is available on the EURL website https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2016-0016.pdf

(EFSA FEEDAP Panel, 2012b) and Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance (EFSA FEEDAP Panel, 2012c).

3. Assessment

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, *L. casei*, and is intended to be added to forages to promote ensiling (technological additive, functional group: silage additive) with the eventual use of the silage in any animal species.

3.1. Characterisation

3.1.1. Characterisation of the active agent

The strain of *L. casei* was originally isolated from cut grass and is deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSZM) with the accession number DSM 28872.[6] It has not been genetically modified. Strain identity was established by its phenotypic properties and by the full 16S rRNA gene sequence (1,469 bp) and by multilocus sequence testing (MLST) based on four housekeeping genes (*rpoA, pheS, atpA and dnaK*) which by comparison with sequences recorded in databases gave an unambiguous identification.[7] It was recognised that MLST could also provide a strain-specific detection method but no direct evidence of this was provided.

Genetic stability was examined by comparison of the master culture with three consecutive working cultures using random amplification of polymorphic DNA-polymerase chain reaction (RAPD-PCR).[8] No differences in the resultant RAPD patterns were observed.

The strain was tested for antibiotic susceptibility using a serial dilution method.[9] The battery of antibiotics tested included those recommended by EFSA for *L. casei/paracasei* (EFSA FEEDAP Panel, 2012d). All of the minimum inhibitory concentration (MIC) values for the *L. casei* strain were equal to or fell below the cut-off values defined by the FEEDAP Panel. Consequently, no further investigation is considered necessary and the strain is considered to be susceptible to all relevant antibiotics.

3.1.2. Characterisation of the product[10]

The manufacturing process is detailed in the dossier. The final additive consists of approximately one-third freeze-dried cell mass, the remainder being carrier.

Each batch of *Lactobacillus casei* DSM 28872 received from the producer is routinely examined for total coliforms, *Escherichia coli*, *Salmonella* spp., total yeasts and filamentous fungi. No microbial contaminants were detected in five batches ($<$ 10 CFU/g or absence of *Salmonella* in 1 g).[11]

Five batches of the additive were examined for the presence of heavy metals (Cd, Pb and Hg), arsenic and aflatoxins.[12] Heavy metal and arsenic were found only in trace amounts ($<$ 0.1 mg/kg *L. casei* production batch). Aflatoxins were found at a mean value of 0.24 μg/kg. Based on these results, routine analyses to detect the presence of heavy metals, arsenic and aflatoxins are not applied to the batches provided by the producer.

No specific data were provided on the particle size distribution or dusting potential of the additive under assessment.

3.1.3. Stability

Three batches were standardised to a count of 1×10^{11} CFU/g using maltodextrin as a carrier and a further three batches were standardised to a count of 2.5×10^{10} CFU/g using glucose.[13] The

[6] Tehcnical dossier/Section II/Annex II.8.
[7] Tehcnical dossier/Section II and Supplementary information November 2016/Annex II.5.
[8] Tehcnical dossier/Section II/Annex II.2.
[9] Tehcnical dossier/Section II/Annex II.1.
[10] This section has been amended following the confidentiality claims made by the applicant.
[11] Technical dossier/Section II/Annex II.4.
[12] Technical dossier/Section II/Annex II.6.
[13] Technical dossier/Section II.

samples were stored in aluminium foil bags at ambient temperature. Losses were < 0.5 log after 18 months storage for both formulations.

Lactobacillus casei DSM 28872 was standardised to a count of 1×10^{11} CFU/g using glucose as a carrier and including diammonium phosphate (5%) and dipotassium phosphate (2.5%) as buffers. Three samples (each of 5 g) were suspended in 1 L water giving a count of 5×10^8 CFU/mL and stored for 7 days at room temperature. No loss of viability was detected after 3 days and even after 7 days counts were < 0.2 log of the initial value.

3.1.4. Conditions of use

The additive is intended for use with all forages and for all animal species at a proposed minimum dose of 1×10^8 CFU/kg forage if used alone, or 5×10^7 CFU/kg forage if used in combination with other authorised microorganisms. It can be applied dry or dispersed in water.

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. Consequently, *L. casei* DSM 28872 is considered 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.

3.2.2. 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. Given the proteinaceous nature of the active agent, the additive also 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 indicated that the additive could be incorporated into a number of different formulations 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 described do not introduce additional risks.

3.3. Efficacy

Five laboratory experiments were made with different forage samples selected to represent materials easy to ensile (studies 1,[14] 2[15] and 3[16]), moderately difficult to ensile (study 4[17]) and difficult to ensile (study 5[18]), as specified by Regulation (EC) No 429/2008 (Table 1).

In all of the studies, forage was ensiled in mini-silos with a capacity of 4.5 L. All of the silos were fitted with air-locks to vent gas. The additive was dissolved in water and sprayed on the forage at an intended concentration of 5×10^7 CFU/kg fresh matter (not confirmed by analysis). Forage for the control silos were sprayed with an equal volume of water, but without the additive. Four replicate silos were prepared for each experimental treatment (with or without the additive). The ambient temperature during ensiling was controlled at 20 ± 2°C and the duration of the experiments was 90–91 days.

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

Study	Test material	Dry matter content (%)	Water-soluble carbohydrate content (% fresh matter)
1	Grass/legume mixture (74:26)[(a)]	25.2	4.3
2	Grass/legume mixture (68:32)[(a)]	43.4	3.4

[14] Technical dossier/Section IV/Annexes IV.1 and IV.4.
[15] Technical dossier/Section IV/Annexes IV.5 and IV.6.
[16] Technical dossier/Section IV/Annexes IV.5 and IV.7.
[17] Technical dossier/Section IV/Annexes IV.1 and IV.3.
[18] Technical dossier/Section IV/Annexes IV.1 and IV.2.

Study	Test material	Dry matter content (%)	Water-soluble carbohydrate content (% fresh matter)
3	Grass/legume mixture (95:5)[(a)]	20.9	3.0
4	Grass/legume mixture (72:28)[(a)]	40.8	2.3
5	Grass/legume mixture (33:67)[(a)]	21.8	1.2

(a): Grass and legume percentages in the mixture, where the predominant legumes were red clover and lucerne, and the grasses were predominately timothy and meadow fescue.

Silos were opened at the end of the experiment and the contents were analysed to determine dry matter (DM) content, pH, lactic and volatile fatty acids concentrations, ethanol, 2,3 butanediol, ammonia and total nitrogen. DM loss during ensiling was calculated. Statistical evaluation of data was by a non-parametric test (Wilcoxon Kruskal–Wallis test), comparing treated versus control silos. Significance was declared at $p < 0.05$.

Table 2: Summary of the analysis of ensiled material recovered at the end of the ensiling period with *Lactobacillus casei* DSM 28872

Study	Application rate (CFU/kg forage)	Dry matter loss (%)	pH	Lactic acid (% dry matter)	Acetic acid (% dry matter)	Ammonia-N (% total N)
1	0	8.5	4.6	5.8	9.2	10.7
	5×10^7	2.5*	4.0*	11.2*	7.1*	3.6*
2	0	2.2	4.6	7.1	1.6	6.4
	5×10^7	1.9	4.5*	6.8	1.4	6.3
3	0	12.1	5.2	1.1	2.3	23.5
	5×10^7	2.5*	4.0*	10.6*	1.1*	3.5*
4	0	1.7	4.8	4.5	1.1	7.3
	5×10^7	1.5	4.3*	6.7*	0.7*	6.4*
5	0	3.9	4.6	7.8	3.6	8.8
	5×10^7	2.9	4.5*	8.5*	3.7	8.4

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

The addition of *Lactobacillus casei* DSM 28872 to the forages tested produced a generally consistent response (Table 2). The pH of the ensiled material was significantly reduced compared to the untreated silage as a result of increased lactic acid production (significant in four of the five trials). This appeared to lead to the better preservation of nutrients as indicated by the reduction in dry matter losses and the reduction in the ammonia nitrogen fraction. Although these changes were seen in all trials, the reduction in dry matter only reached significance in two studies with easily ensiled material, and reduction in the percentage of ammonia nitrogen in three studies (two with easy to ensile and one with moderately difficult to ensile material).

In study 3, there was evidence of substantial clostridial activity in the control silage as indicated by a high concentration of butyrate (1.2% fresh matter (FM)) and 2,3 butanediol (0.6% FM). This appeared to have been controlled by the addition of the additive since both of these parameters were below the limit of quantification (LOQ < 0.01% FM) in the treated forage.

However, benefits in terms of nutrient preservation could only be significantly demonstrated with easy and moderately difficult to ensile forages although the same trends were observed with the single example of difficult to ensile material.

4. Conclusions

As the identity of the strain *Lactobacillus casei* DSM 28872 has been established and no antibiotic resistance of concern detected, following the QPS approach to safety assessment, the use of this strain in the production of silage is presumed safe for target species, consumers of products from animals fed treated silage and for the environment.

In the absence of data, no conclusion can be drawn on the skin and eye irritancy of the additive. The additive should be considered to have the potential to be a respiratory sensitiser.

Lactobacillus casei DSM 28872 at a minimum dose of 5×10^7 CFU/kg has the potential to improve the production of silage from easy and moderately difficult to ensile forage species by reducing dry matter loss and enhancing protein preservation.

Documentation provided to EFSA

1) *Lactobacillus casei* DSM 28872. March 2016. Submitted by Microferm Limited.
2) *Lactobacillus casei* DSM 28872. Supplementary information November 2016. Submitted by Microferm Ltd.
3) Evaluation report of the European Union Reference Laboratory for Feed Additives on the Methods(s) of Analysis for *Lactobacillus casei* DSM 28872.

References

EFSA (European Food Safety Authority), 2007. Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. EFSA Journal 2007;5(12):587, 16 pp. doi:10.2903/j.efsa.2007.587

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2013. Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2013 update). EFSA Journal 2013;11(11):3449, 106 pp. doi:10.2903/j.efsa.2013.3449

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2011. Technical guidance: tolerance and efficacy studies in target animals. EFSA Journal 2011;9(5):2175, 15 pp. doi:10.2903/j.efsa.2011.2175

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012a. Guidance for the preparation of dossiers for technological additives. EFSA Journal 2012;10(1):2528, 23 pp. doi:10.2903/j.efsa.2012.2528

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012b. Guidance for establishing the safety of additives for the consumer. EFSA Journal 2012;10(1):2537, 12 pp. doi:10.2903/j.efsa.2012.2537

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012c. Guidance on studies concerning the safety of use of the additive for users/workers. EFSA Journal 2012;10(1):2539, 5 pp. doi:10.2903/j.efsa.2012.2539

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012d. 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

Abbreviations

CFU	colony-forming unit
DM	dry matter
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EURL	European Union Reference Laboratory
FM	fresh matter
LOQ	limit of quantification
MIC	minimum inhibitory concentration
MLS	multilocus sequence
PFGE	pulsed field gel electrophoresis
RAPD-PCR	randomly amplified polymorphic DNA - polymerase chain reaction amplification
QPS	Qualified Presumption of Safety

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for *Lactobacillus casei* DSM 28872

In the current application authorisation is sought under Article 4(1) for *Lactobacillus casei* DSM 28872 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* in silage for all animal species.

According to the Applicant, the *feed additive* contains as *active substance* viable cells of the non-genetically modified strain *Lactobacillus casei* DSM 28872. The *feed additive* is to be marketed as a powder containing a minimum *Lactobacillus casei* DSM 28872 content of 8×10^{10} Colony Forming Units (CFU)/g. The *feed additive* is intended to be added dry or sprayed onto silage at a minimum dose of 5×10^{7} CFU/kg fresh silage.

For the identification of *Lactobacillus casei* DSM 28872, the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for microbial identification.

For the enumeration of *Lactobacillus casei* DSM 28872 in *feed additive*, the Applicant submitted the ring-trial validated spread plate method EN 15787 specifically designed 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 casei* DSM 28872 in the *feed additive per se*.

The Applicant did not provide any experimental method or data for the determination of *Lactobacillus casei* DSM 28872 in silage. Since the unambiguous determination of the content of *Lactobacillus casei* DSM 28872 initially added to silage is not achievable by analysis, 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.