SCIENTIFIC OPINION

EFSA Journal

ADOPTED: 24 January 2017

doi: 10.2903/j.efsa.2017.4704

Efficacy of *Saccharomyces cerevisiae* (NBRC 0203), *Lactobacillus plantarum* (NBRC 3070) and *Lactobacillus casei* (NBRC 3425) as a silage additive for all species

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Abstract

The product under assessment is a preparation containing single strains of *Saccharomyces cerevisiae*, *Lactobacillus plantarum* and *Lactobacillus casei* to be used as a technological additive to improve the ensiling process. EFSA has been previously requested by the European Commission to evaluate this product. The safety of the additive for consumers, users, the environment and target animals was established at that time. Results from a number of *in vitro* ensiling studies made using a variety of grass forage and maize samples were also presented. These efficacy studies showed no evidence that the additive had the potential to conserve nutrients and so improve the production of silage. There was, however, evidence for improved aerobic stability once the ensiled material was exposed to air, but because of the apparent variability in the additive mix, an effective dose could not be established. The applicant has now provided a number of additional ensiling studies confirming that the additive when applied at 80 mL/tonne significantly increases the aerobic stability of ensiled material after exposure to air. This was shown in forage materials with dry matter contents varying between 30% and 70%. Fluorescence *in situ* hybridisation methods were also introduced to better characterise the additive. Although this method allows individual strains to be separately enumerated, bacterial and yeast numbers are expressed as cell numbers/mL and include counts of both viable and non-viable cells. As a result values cannot be equated to colony-forming units, the basis of any authorisation and the functional properties of the additive. Consequently, the Panel remains unable to identify a minimum specification for the product or a minimum effective dose.

Keywords: *Saccharomyces cerevisiae*, *Lactobacillus plantarum*, *Lactobacillus casei*, silage, efficacy, FISH

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Question number: EFSA-Q-2016-00130

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Suggested citation: EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Villa RE, Wallace RJ, Wester P, Brozzi R and Saarela M, 2017. Scientific Opinion on the efficacy of *Saccharomyces cerevisiae* (NBRC 0203), *Lactobacillus plantarum* (NBRC 3070) and *Lactobacillus casei* (NBRC 3425) as a silage additive for all species. EFSA Journal 2017;15(2):4704, 8 pp. doi: 10.2903/j.efsa.2017.4704

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition and in particular, Article 9 defines the terms of the authorisation by the Community.

The applicant, EM Agriton BV,[2] is seeking a Community authorisation of *Saccharomyces cerevisiae* (NBRC 0203), *Lactobacillus plantarum* (NBRC 3070) and *Lactobacillus casei* (NBRC 3425) to be used as a silage additive for all animal species (Table 1).

Table 1: Description of the substances

Category of additive	Technological
Functional group of additive	Silage additive
Description	*Saccharomyces cerevisiae* (NBRC 0203), *Lactobacillus plantarum* (NBRC 3070) and *Lactobacillus casei* (NBRC 3425)
Target animal category	All animal species
Applicant	EM Agriton BV
Type of request	New opinion

On 10 September 2013, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) of the European Food Safety Authority ("Authority"), in its opinion on the safety and efficacy of the product, conclude that the characterisation and the efficacy of *Saccharomyces cerevisiae* NBRC 0203 (IFO0203), *Lactobacillus plantarum* NBRC 3070 (ATCC 8014) and *Lactobacillus casei* (NBRC 3425) as silage additive was not established from the data provided by the applicant.

The Commission gave the possibility to the applicant to submit complementary information to complete the assessment on the characterisation and the efficacy to allow a revision of Authority's opinion. The data generated by the applicant and compiled in the above-mentioned supplementary information have been sent directly to the Authority by the applicant.

The Commission has now received new data on the characterisation and the efficacy of *Saccharomyces cerevisiae* (NBRC 0203), *Lactobacillus plantarum* (NBRC 3070) and *Lactobacillus casei* (NBRC 3425).

1.2. Terms of Reference

In view of the above, the Commission asks the Authority to deliver a new opinion on the characterisation and the efficacy of the product composed of *Saccharomyces cerevisiae* (NBRC 0203), *Lactobacillus plantarum* (NBRC 3070) and *Lactobacillus casei* (NBRC 3425) based on the additional data submitted by the applicant.

1.3. Additional information

In 2011, the European Food Safety Authority (EFSA) was requested by the European Commission to evaluate the product when used as a technological additive (functional group: silage additive) in forage for all animal species to improve the ensiling process. The safety of the additive for consumers, users, the environment and target animals was established at that time (EFSA FEEDAP Panel, 2013). However, the efficacy studies provided at that time showed no evidence that the additive had the potential to conserve nutrients and so improve the production of silage. There was some evidence for an effect on aerobic stability, but because of the inherent variability in the additive mix, a minimum effective dose could not be established.

[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] EM Agriton B.V.Molenstraat 10-1, 8391 AJ Noordwolde. The Netherlands.

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of additional information[3] to a previous application on the same product.[4]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the efficacy of the preparation of *Saccharomyces cerevisiae* (NBRC 0203), *Lactobacillus plantarum* (NBRC 3070) and *Lactobacillus casei* (NBRC 3425) 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, 2012).

3. Assessment

The additive is a preparation containing *Saccharomyces cerevisiae* (NBRC 0203), *Lactobacillus plantarum* (NBRC 3070) and *Lactobacillus casei* (NBRC 3425) to be used as a technological additive (functional group: silage additive) in forage for all animal species.

3.1. Characterisation

3.1.1. Product specification

From the very limited description provided, the Panel had difficulty in understanding the production process and how an additive of consistent composition could be guaranteed since it would appear that the fermentation medium is simultaneously inoculated with all three organisms. The final additive appears to consist simply of the fermentation broth, possibly with the volume adjusted, and is stored and sold as a liquid product. No new details about the production process have been provided.

Initially, the Applicant gave only combined counts for the two strains of lactobacilli and, because of the apparent variability in their numbers, it was not possible to establish a minimum specification for the additive. Subsequently, the Applicant applied fluorescence *in situ* hybridisation (FISH) methods using 16S rRNA gene-based probes for the separate enumeration of the two *Lactobacillus* species[5] and a peptide nucleic acid (PNA)-FISH method for *S. cerevisiae*.[6] The minimum specification for the additive is now given as:

L. casei $\geq 1.25 \times 10^7$ 'visible' bacteria/mL,
L. plantarum $\geq 10^5$ 'visible' bacteria/mL and
S. cerevisiae $\geq 10^4$ CFU/mL.

Data on eight batches of the additive derived using the FISH methods showed compliance with this minimum specification.[7] The mean count and range for each component strain was 4.2×10^5 ($0.5–9.5 \times 10^5$) for *S. cerevisiae,* 7.9×10^7 ($4.5–12.7 \times 10^7$) for *L. casei* and 3.9×10^5 ($1.2–8.0 \times 10^5$) for *L. plantarum* per mL of the liquid preparation of the additive.

However, any specification provided for the additive must be based on viability since this is the basis of the authorisation and relates to the functional properties of the additive. It is well recognised that most DNA hybridisation-based methods, including the methods applied here, are not dependent on viability and will stain dead or inert cells. A comparison of results obtained by FISH and by conventional plating for the two bacterial strains from five batches of product gave a mean count of total lactobacilli of 4.3×10^6 CFU/mL ($2.0–8.7 \times 10^6$ CFU/mL) by plate culture, while FISH gave a mean count of 1.8×10^8 cells/mL ($1.4–2.1 \times 10^8$ cells/mL).[8] The Applicant suggests that this difference might be explained by the propensity of the bacterial cells to clump and not form separate colonies on plating. In the view of the Panel a more likely explanation is that the FISH probes are also reacting with non-viable organisms.

[3] FEED dossier reference: FAD-2016-0001.
[4] FEED dossier reference: FAD-2010-0240.
[5] Technical dossier/Annexes 3.a and 3.b.
[6] Technical dossier/Annex 3.c.
[7] Technical dossier/Annex 1.a.
[8] Technical dossier/Annex 1.b.

3.1.2. Shelf life

From studies described in the initial application, the Panel was able to conclude that the product appears to have a shelf life of about 1 year at temperatures not greater than 25°C, but to be very sensitive to higher temperatures. However, in the absence of strain specific detection and because of the variable nature of the product, it was not possible to comment on the individual stability of the two lactobacilli strains. This study was followed by one in which the individual counts of the constituent strains was measured using FISH in four batches at monthly intervals for 6 months.[9] Although the storage conditions were not provided, on the basis of the previous study, it is assumed that the storage temperature was around 25°C. The results showed that counts for each individual strain was little changed after 6 months storage and, as a result, the ratio between strains remained fairly constant. However, as indicated previously, the FISH methods used are not specific for viable cells and so the results obtained are of questionable value.

3.1.3. Conditions of use

The recommended rate of addition is 80 mL additive per tonne fresh material. This would result in a minimum concentration of 1×10^6 cells of *L. casei*, 8×10^3 cells of *L. plantarum* and 8×10^2 CFU of *S. cerevisiae* per kg fresh forage material.

3.2. Efficacy

In the initial application, the results from nine ensiling experiments were described, made with a variety of forage materials. Significant improvement in aerobic stability was shown in three of the nine studies but a minimum effective dose could not be identified. The applicant has now submitted a further seven studies, again made with a variety of materials for ensiling (Table 2).

The first four studies followed a common design in which the test material was packed into 2.75 L minisilos each closed with a fermentation lock. Before packing, the forage was sprayed either with the additive at the recommended rate of (80 mL/tonne forage) or an equivalent amount of water. The additive batch used was, in each case, analysed and showed to conform to specification as established by FISH. Four replicate silos per treatment were then stored at unspecified temperature for 99–102 days depending on the experiment. At day 84, all silos were exposed to the air for a period of 24 h to provide an aerobic challenge. At the end of the experiments, silos were emptied and samples taken for analysis (e.g. dry matter content, volatile fatty acids, ethanol, nitrogen). No measure was reported for corrected dry matter loss. The fifth study also involved two treatments using six replicate mini silos per treatment, each holding approximately 1.5 kg of wilted grass. The duration of this study was 100 days.

Table 2: Characteristics of the forage samples used in the ensiling experiments

Study No	Test material	Dry matter content (% fresh material)	Water-soluble carbohydrate content (% fresh matter)
1[(a)]	Maize cob	66.8	2.94
2[(b)]	Maize cob	59.0	2.71
3[(c)]	*Lolium perenne* 1st cut	32.7	6.27
4[(d)]	*Lolium perenne* 2nd cut	38.9	5.68
5[(e)]	*Lolium perenne*	37.1	2.70
6[(f)]	Wheat bran	61.1	3.59
7[(f)]	Spelt bran	63.6	3.22

(a): Technical dossier/Annex 5.1.
(b): Technical dossier/Annex 5.2.
(c): Technical dossier/Annex 5.4.
(d): Technical dossier/Annex 5.5.
(e): Technical dossier/Annex 5.6.
(f): Technical dossier/Annex 5.3.

Studies six and seven made with wheat bran and spelt bran differed from the above. The number of replicate silos was increased to 12 per treatment but density of packing and exposure to air were

[9] Technical dossier/Annex 4.b.

introduced as additional variables in both control and test groups. Thus, five silos were packed at 'normal' density (311 kg/m^3 for wheat bran and 323 kg/m^3 for spelt bran), four at normal density but exposed to the air for 24 h at day 10 and the remaining three packed at a lower density (223 kg/m^3 for wheat bran and 233 kg/m^3 for spelt bran) without aerobic stress. All silos were opened after 28 days.

Subsamples were then from all replicate in the seven studies to assess aerobic stability and placed into an insulated box fitted with a probe to measure temperature rise over time. An increase of 3°C was taken as evidence of a loss of aerobic stability.

Normality of data was tested with Kolmogorov–Smirnov and equality of variances was tested by Levene's test. Normally distributed variables were subjected to analysis of variance with Tukey as *post hoc* test. If normality and equality were not fulfilled, a non-parametric Wilcoxon test was performed. Significance was declared at $p < 0.05$. Results from the seven studies are summarised in Table 3.

Table 3: Summary of the analysis of ensiled material recovered at the end of ensiling and the aerobic stability of the ensiled materials

Study number	Treatment	pH	Lactic acid (% DM)	Acetic acid (% DM)	NH_3-N as % total N	Aerobic stability (days)
Study 1	Control	3.9	2.67	0.62	3.7	2.7
	Treatment	4.1*	1.87*	0.90*	2.7*	6.4*
Study 2	Control	4.0	2.37	0.90	4.2	2.6
	Treatment	3.8*	2.39	1.59*	4.1	7.0*
Study 3	Control	4.4	3.61	0.65	10.2	5.8
	Treatment	4.6	6.21	0.95	8.4	6.9*
Study 4	Control	4.4	4.74	2.05	6.5	13.1
	Treatment	4.3*	6.30	2.87	6.3	14.7*
Study 5	Control	4.2	3.5	0.88	1.7	6.8
	Treatment	4.1	3.0*	1.21*	1.8	13.8*
Study 6	Norm Control	4.1	5.36	0.76	2.8	4.9
	Norm treated	4.3*	3.76*	2.12*	1.9*	7.3*
	Aerob control	4.3	4.46	0.75	2.8	1.9
	Aerob treated	4.2	3.84*	2.40*	1.9*	7.3*
	Low control	4.3	4.08	0.77	3.0	2.6
	Low treated	4.3	2.87*	1.53	1.8*	7.3*
Study 7	Norm Control	4.7	0.88	0.14	1.4	3.4
	Norm treated	4.5*	1.50*	0.47*	1.4	7.3*
	Aerob control	4.9	1.07	0.22	1.1	1.7
	Aerob treated	4.4*	2.13	0.70*	1.2	7.3*
	Low control	4.7	2.98	0.49	1.7	4.5
	Low treated	4.5*	3.08	0.96*	1.3	7.3*

*: Significantly different from the control value at a minimum of $p < 0.05$.
DM: dry matter.

The additional studies confirm the previous observation that 'overall there is no evidence of a consistent beneficial effect on the quality of silage produced which might arise from the presence of the additive'. However, as previously indicated, there was consistent evidence that the additive when applied at 80 mL/tonne significantly increased the period before deterioration occurred after the ensiled material was exposed to air. This was shown in all seven studies and covered material with dry matter contents varying between 30% and 70%.

The Panel notes that two of the studies were made with wheat and spelt bran. Bran can be added to wet forage to increase the dry matter content before ensiling and/or to reduce effluent production during silage making. The applicant also makes reference to a growing market for the inclusion of 'fermented bran' in feed for horses. However, ensiling bran alone is not representative of current

commercial practice. In addition, the application rate for these two studies appears to be substantially higher than applied to the other studies (5 L product solution to 11.25 kg bran). Even if these studies are discounted as not fully representative, there is sufficient evidence from the first five studies to conclude on the ability of the additive to improve aerobic stability.

In the first five studies, the FISH methods were used to demonstrate that the batch of additive used in the individual studies conformed to the minimum specification established using the same methodology. Although the FISH method showed that there is a greater consistency of product that previously demonstrated and allowed individual strains to be separately enumerated, bacterial and yeast numbers are expressed as cell numbers/mL or 'visible' bacteria/mL. As these values cannot be equated to CFU, it remains not possible to establish a minimum specification for the product or minimum effective dose based on viable numbers.

4. Conclusions

The additional studies confirm the previous observation that there is evidence that the additive when applied at 80 mL/tonne significantly increases aerobic stability of ensiled material after exposure to air. This was shown in forage material with dry matter contents varying between 30% and 70%.

Although the FISH method allows individual strains to be separately enumerated, bacterial and yeast numbers are expressed as cell numbers/mL or 'visible' bacteria/mL. As these values cannot be equated to CFU, it remains impossible to establish a minimum specification for the product or a minimum effective dose based on viable numbers.

Documentation provided to EFSA

1) Additional information on *Saccharomyces cerevisiae* (NBRC 0203), *Lactobacillus plantarum* (NBRC 3070) and *Lactobacillus casei* (NBRC 3425). December 2015. Submitted by EM Agriton B.V.
2) Additional information on *Saccharomyces cerevisiae* (NBRC 0203), *Lactobacillus plantarum* (NBRC 3070) and *Lactobacillus casei* (NBRC 3425). Supplementary information. December 2016. Submitted by EM Agriton B.V.

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Abbreviations

CFU	colony-forming unit
DM	dry matter
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
FISH	fluorescence *in situ* hybridization