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SCIENTIFIC OPINION

Scientific Opinion on the safety and efficacy of *Pediococcus pentosaceus* (NCIMB 30068) as a silage additive for all animal species[1]

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

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ABSTRACT

Pediococcus pentosaceus is a technological additive intended to improve the ensiling process at a minimum proposed dose of 1×10^8 colony-forming units (CFU)/kg fresh material. The *P. pentosaceus* strain was found to be resistant to tetracycline by an unidentified mechanism and thus may pose a risk for the spread of genes coding for resistance to an antibiotic of human and veterinary importance. Thus, the additive containing this strain is not considered safe for the target animals and consumers of products from animals fed the treated silage. The additive should be regarded as a skin and eye irritant and a potential skin and respiratory sensitiser, and treated accordingly. Since the *P. pentosaceus* strain carries an uncharacterised resistance to tetracycline, the FEEDAP Panel cannot conclude on its safety for the environment. A total of four studies with laboratory-scale silos were made 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. Although the additive showed a tendency to increase lactic acid production and reduce pH in the ensiled material, overall there was insufficient evidence of a beneficial effect on the preservation of nutrients.

KEY WORDS

technological additive, silage additive, *Pediococcus pentosaceus*, QPS, safety, efficacy

[1] On request from the European Commission, Question No EFSA-Q-2012-00081, adopted on 6 March 2014.

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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 the target animals, consumers, users and for the environment, and on the efficacy of a product based on a specific strain of *Pediococcus pentosaceus* when used as a technological additive intended to improve the ensiling process at a minimum proposed dose of 1×10^8 colony-forming units (CFU)/kg fresh material.

P. pentosaceus NCIMB 30068 is resistant to tetracycline by an unidentified mechanism and, consequently, the qualified presumption of safety approach to safety assessment cannot be applied. Taking into consideration that (i) the resistance to tetracycline is not intrinsic in the *P. pentosaceus* species, (ii) the genetic basis of the tetracycline resistance has not been established and (iii) a potential for horizontal gene transfer amongst bacteria cannot be excluded, the FEEDAP Panel concludes that the strain may pose a risk for the spread of genes coding for resistance to tetracycline, an antibiotic of human and veterinary importance. Therefore, the additive based on the *P. pentosaceus* strain is not considered safe for the target animals and consumers of products from animals fed the treated silage.

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

Since the strain carries an uncharacterised resistance to tetracycline, the FEEDAP Panel cannot conclude on its safety for the environment.

Studies with laboratory-scale silos, each lasting at least 90 days, were carried out using samples of forage of differing water-soluble carbohydrate content representing material considered 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. The FEEDAP Panel concluded that, although the additive showed a tendency to increase lactic acid production and reduce pH in the ensiled material, overall there was insufficient evidence of a beneficial effect on the preservation of nutrients.

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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 Microferm Limited[5] for re-evaluation of the product *Pediococcus pentosaceus* (NCIMB 30068), 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.[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 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 *Pediococcus pentosaceus* (NCIMB 30068), 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] Microferm Limited, Spring Lane North, Malvern Link, WR14 1BU, Worcester, United Kingdom.
[6] EFSA Dossier reference: FAD-2010-0272.

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

Additive	*Pediococcus pentosaceus* NCIMB 30068			
Registration number/EC No/No	-			
Category(ies) of additive	Technological additives			
Functional group(s) of additive	Silage additive			
Description				
Composition, description	Chemical formula	Purity criteria	Method of analysis	
Pediococcus pentosaceus (NCIMB 30068)		*E. coli* <100 CFU/g *Salmonella* nil in 25 g Yeast/Mould<100 CFU/g	BS EN 15786:2009	
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, *Pediococcus pentosaceus*, 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 species *P. pentosaceus* 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 *P. pentosaceus* of unknown origin is deposited with the National Collection of Industrial and Marine Bacteria (NCIMB, UK) with the accession number NCIMB 30068.[7] 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 GenBank enabled the strain to be unambiguously identified as *P. pentosaceus*. Multilocus sequence typing based on sequencing four specific genes (*rpoA, pheS, atpA* and *dnaK*) was proposed as a means of strain-specific detection.[8] Although the method is suitable for the discrimination of closely related strains, its effectiveness depends on the selection of sequences to be compared. No data were provided to illustrate that comparison of the four gene fragments chosen in this case is able to distinguish between NCIMB 30068 and other *P. pentosaceus* 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).[9] The minimum inhibitory concentration (MIC) values for the *P. pentosaceus* strain are below or equal to the EFSA cut-off values except for tetracycline and ampicillin. The MIC for ampicillin is within the normal variation around the mean (one dilution step above the cut-off) and, thus, does not give rise concerns for safety. The MIC value for tetracycline is unclear. A total of three experiments were performed with different results. In the first study submitted, an MIC of 4 µg/mL was recorded. However, this test was made with an inappropriate medium for lactic acid bacteria. The two subsequent studies were made in the same period by two independent laboratories using the same growth medium and test conditions (ISO sensitest-MRS, 30 °C and under aerobiosis) but reported different values (≥ 64 µg/mL and 25 µg/mL). Both values exceed the cut-off value for tetracycline and trigger the need for further investigation.

To determine the genetic nature of the tetracycline resistance the whole genome of *P. pentosaceus* NCIMB 30068 was sequenced and the reads were assembled both by using the *de novo* assembly approach and by assembling against the genome sequence of *P. pentosaceus* ATCC 25745.[10] Identified coding sequences were annotated using basic local alignment search tool. Only 15 proteins sequences, annotated with the term "resistance", and not the whole genome, were further checked against the Antibiotic Resistance Database (ARDB). Using this approach, no currently known tetracycline resistance genes were identified in the genome sequence and the genetic nature of tetracycline resistance was not established. EFSA guidance (EFSA FEEDAP Panel, 2012) states that "*The absence of known antimicrobial resistance genes (e.g. based on analysis utilising the ARBD) is*

[7] Technical dossier/Section II.
[8] Technical dossier/Supplementary information August 2012.
[9] Technical dossier/Supplementary information November 2013/Annexes II.2.2.2.2 and 3.
[10] Technical dossier/Supplementary information November 2013/Annexes II.2.2.2.

not sufficient to explain the nature of the detected resistance". Thus, in the absence of information on the genetic nature of a demonstrated tetracycline resistance, the FEEDAP Panel cannot conclude on the extent of the risk of horizontal gene transfer to other bacteria in the food chain and in the environment.

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 (not specified). Material safety datasheets are provided for all medium components and cryoprotectants but no purity criteria are included.

No minimum content of *P. pentosaceus* in the final product is specified. Analysis of five production batches gave a mean value of 7.8×10^{11} CFU/g additive (range 6.1×10^{11} to 1.0×10^{12} CFU/g additive, coefficient of variation (CV) = 18.2 %).[12]

The additive is routinely monitored for microbial contamination. Limits are set for *Escherichia coli* (< 100 CFU/g), yeasts/moulds (< 100 CFU/g) and *Salmonella* spp. (absence in 25 g of the additive). Data from three batches confirmed compliance with the set limits.[13]

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, consequently, these substances are not routinely monitored in batches. Three batches of one of the medium components and three batches of *P. pentosaceus* (excipient not given) were tested for heavy metals (lead, cadmium and mercury), arsenic and aflatoxins B_1, B_2, G_1 and G_2. Aflatoxins were not detected (limit of detection: 0.1 µg/kg). Contamination with heavy metals and arsenic was low and of no concern (lead < 0.4 mg/kg, cadmium ≤ 0.1 mg/kg, mercury < 0.02 mg/kg and arsenic < 0.2 mg/kg).[14]

A single batch of a powder formulation of the additive was examined for particle size distribution by laser diffraction.[15] The mean particle size was 95 µm with approximately 54 % by weight of the additive consisting of particles with a diameter below 100 µm, 29 % particles with a diameter below 50 µm and 5 % particles with a diameter below 10 µm. No data on dusting potential were provided.[16]

2.3. Stability

2.3.1. Shelf life

Three batches of the product were standardised to give a count of 1×10^{11} CFU/g using maltodextrin, and another three batches were standardised to a level of 2.5×10^{10} CFU/g using dextrose as carrier. The samples were stored in sealed aluminium foil bags at ambient temperature.[17] Viability losses were insignificant over six months but were approximately 10 % after 12/15 months in the case of the maltodextrin formulation. Insignificant losses were observed for the dextrose formulation during the entire experimental period.

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.[18] An experiment was designed to mirror practical conditions, in which, typically, 10 g of product would be dissolved in 2 L of water and applied to 1 tonne of forage to deliver 1×10^{9} CFU/kg. Three batches of the solution of the *P. pentosaceus* strain were

[11] This section has been edited following the confidentiality claims made by the applicant.
[12] Technical dossier/Section II_2.1.3.
[13] Technical dossier/Section II_2.1.4.
[14] Technical dossier/Section II/2.1.4.2.
[15] Technical dossier/Section II_2.1.5.
[16] Technical dossier/Section II.1.5.2 and Supplementary information August 2012.
[17] Technical dossier/Section II_2.4.1.1 and Supplementary information August 2012.
[18] Technical dossier/Section II_2.4.1.2.

stored at room temperature and samples removed over seven days. Viable counts indicated that the strain was fully stable for at least four days under these conditions. Some losses (up to 20 %) were observed at seven days.

The strain of *P. pentosaceus* is also intended for use in grow-up formulations in which numbers of bacteria are increased by incubation before application to forage.[19] Typically, a silage additive with 1.3×10^{10} CFU/g would be mixed with water at the rate of 1 000 g per 25 L, left overnight, then a further 25 L added, and applied to forage at 2 L per tonne. Since the growth of the strain is encouraged, the product is also formulated to contain glucose, nitrogen sources and buffer salts. The ability of the organism to grow under these conditions was monitored for a period of seven days in three replicate studies. Numbers of organisms essentially doubled after one to two days, but thereafter declined, falling below the initial count on day 7.

2.4. Conditions of use

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

The applicant also anticipates the use of silage premixtures which include the strain under application combined with other authorised (microbial) additives. In such cases, the *P. pentosaceus* strain could be used at a lower concentration than when used alone. The product may also be used in a grow-up formulation.

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.[20]

3. Safety

3.1. Safety for the target animals and consumers

Pediococcus pentosaceus NCIMB 30068 is resistant to tetracycline by an unidentified mechanism and consequently, the QPS approach to safety assessment cannot be applied. Taking into consideration that:

- the resistance to tetracycline is not intrinsic in the *P. pentosaceus* species;
- the genetic basis of the tetracycline resistance of *P. pentosaceus* NCIMB 30068 has not been established; and
- a potential for horizontal gene transfer amongst bacteria cannot be excluded;

the FEEDAP Panel concludes that *P. pentosaceus* NCIMB 30068 may pose a risk for the spread of genes coding for resistance to tetracycline, an antibiotic of human and veterinary importance. Therefore, the additive based on *P. pentosaceus* 30068 is not considered safe for the target animals and consumers of products from animals fed the treated silage.

3.2. Safety for the user[21]

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

[19] Technical dossier/Section II_2.4.1.2.
[20] The full report is available on the EURL website: http://irmm.jrc.ec.europa.eu/SiteCollectionDocuments/FinRep-FAD-uorg3.pdf
[21] This section has been edited following the confidentiality claims made by the applicant.

accordingly. A significant fraction of the only batch of the product tested contained a high proportion of fine particles that have the potential to reach the respiratory surface of the lungs when inhaled. 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 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. The examples of excipients listed by the applicant (dextrose, maltodextrin) to be used in the preparation of the final formulation(s) do not introduce additional risks.

3.3. Safety for the environment

P. pentosaceus can be commonly found in plant materials. The use of the species in animal nutrition is not expected to measurably increase numbers of the organism in the environment. However, due to the antibiotic resistance of this specific strain, the FEEDAP Panel cannot conclude on the extent of the risk of horizontal gene transfer to other bacteria in the food chain and in the environment.

4. Efficacy

In the original submission, five laboratory experiments were described made with different forages.[22] However, these were not further considered owing to deficiencies in the reporting of results and unreliable statistical analysis.

Upon request, the applicant submitted four experiments performed in house Forages used in the studies represented materials easy to ensile (two maize silage), moderately difficult to ensile and difficult to ensile (Table 2) as defined in Regulation (EC) No 429/2008. The duration of ensiling was not given but could be estimated from the dates of harvest and silo opening, which were specified. On this basis, the maize samples were ensiled for approximately 270 days in study 1 and for 147 days in study 2, and the grass–clover mix and the lucerne were ensiled for 92 days in the remaining two studies.

All of the studies used mini-silos (volume not indicated) capable of holding 1 kg chopped forage material with the capacity to vent gas. The ambient temperature during ensiling was controlled at 20-21 ± 2–3 °C. In each case, the contents of four replicate silos were sprayed with the additive at several concentrations (apparently not confirmed by analysis) suspended in water. Forage for the control silos were sprayed with an equal volume of water.

[22] Technical dossier/Section IV and Supplementary information August 2012/Annexes IV.1-16.

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

Study no	Test material	Dry matter content (% fresh material)	Water-soluble carbohydrate content (% fresh material)
1[23]	Maize	29.9	12.7
2[24]	Maize	33.0	6.8
3[25]	Grass–clover mix	24.8	3.0
4[26]	Lucerne	19.0	1.2

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 to determine silage dry matter content, pH, lactic and volatile fatty acids concentrations, ethanol, ammonia and total nitrogen.

Statistical evaluation of data was made by Kruskal–Wallis and Mann–Whitney tests comparing data of each treatment with the average value for the corresponding control silos. Significance was assumed at $P < 0.05$.

Table 3: Summary of the analysis of ensiled material recovered at the end of the ensiling period with *Pediococcus pentosaceus* NCIMB 30068

Study no	Application rate (CFU/kg forage)	Dry matter loss (%)	pH	Lactic acid (% fresh material)	Acetic acid (% fresh material)	Ammonia-N (% total N)
1	0	4.6	3.7	1.2	0.5	3.5
	1×10^6	3.3	3.6*	1.6*	0.3*	3.3
	1×10^7	3.5	3.6*	1.6*	0.3*	3.4
2	0	6.6	4.0	0.5	0.4	2.0
	1×10^8	4.3	3.5*	1.1*	0.2*	2.9*
	1×10^9	3.9*	3.5*	1.1*	0.2*	3.0*
3	0	4.7	4.5	1.4	0.5	10.2
	1×10^6	5.3	4.3*	1.7*	0.2*	9.4
	1×10^7	4.9	4.3	1.8*	0.3	9.6
	1×10^8	4.3	4.3*	2.0*	0.2*	8.8*
4	0	6.7	4.5	1.3	0.6	12.2
	1×10^6	4.9*	4.4	2.7*	0.4*	12.1
	1×10^7	3.9*	4.5	1.9*	0.3*	11.8
	1×10^8	4.6*	4.4	1.9*	0.4*	11.2

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

Lactic acid concentration was significantly increased in all studies and pH reduced in three of the studies. However, a significant reduction in dry matter loss was observed in only one study at the proposed minimum application rate and in another one at a higher concentration (1×10^9 CFU/kg forage). No consistent effects on ammonia-N were observed.

Overall, there was insufficient evidence of a beneficial effect of the additive on the preservation of nutrients.

[23] Technical dossier/Supplementary information November 2013/Maize B.
[24] Technical dossier/Supplementary information November 2013/Maize A.
[25] Technical dossier/Supplementary information November 2013/Grass/clover.
[26] Technical dossier/Supplementary information November 2013/Lucerne.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

Pediococcus pentosaceus NCIMB 30068 is resistant to tetracycline and may pose a risk for the spread of genes coding for resistance to an antibiotic of human and veterinary importance. Therefore, the additive based on this strain is not considered safe for the target animals and consumers of products from animals fed the treated silage.

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

Since the strain carries an uncharacterised resistance to tetracycline, the FEEDAP Panel cannot conclude on the safety of *P. pentosaceus* NCIMB 30068 for the environment.

Although use of the additive showed a tendency to increase lactic acid production and reduce pH in the ensiled material, overall there was insufficient evidence of a beneficial effect on the preservation of nutrients.

RECOMMENDATIONS

The applicant should specify a minimum declared content of *P. pentosaceus* NCIMB 30068 in any final product.

DOCUMENTATION PROVIDED TO EFSA

1. *Pediococcus pentosaceus* (NCIMB 30068). November 2010. Submitted by Microferm Limited.
2. *Pediococcus pentosaceus* (NCIMB 30068). Supplementary information. August 2012. Submitted by Microferm Limited.
3. *Pediococcus pentosaceus* (NCIMB 30068). Supplementary information. November 2013. Submitted by Microferm Limited.
4. *Pediococcus pentosaceus* (NCIMB 30068). Supplementary information. January 2014. Submitted by Microferm Limited.
5. Comments from Member States received through the ScienceNet.

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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