SCIENTIFIC OPINION

EFSA Journal

ADOPTED: 28 January 2020

doi: 10.2903/j.efsa.2020.6011

Assessment of the application for renewal of the authorisation of Amaferm® (fermentation product of *Aspergillus oryzae* NRRL 458) as a feed additive for dairy cows

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Abstract

AMAFERM® is a fermentation product produced by *Aspergillus oryzae* NRRL 458, containing alpha-amylase and cellulase enzyme activities, authorised for use as a feed additive for cows. This scientific opinion concerns the renewal of the authorisation of this additive for its use in dairy cows. In its former opinion, the EFSA Panel on Additives and Product or Substances used in Animal Feed (FEEDAP), based on the data and knowledge available at that time, concluded that the additive is safe for cows, for the consumers and the environment. In that assessment, the Panel also concluded that the additive is non-irritant or a dermal sensitiser but should be considered a potential respiratory sensitiser. The applicant provided new information regarding the characterisation of the additive in terms of enzyme activities as well as information concerning the production strain. Regarding the enzyme activities in the fermentation product, weaknesses and limitations in the methods of analysis were noted. The information regarding the production strain did not permit to confirm its taxonomic classification, moreover uncertainty remains regarding the presence of viable cells/spores in the final product. Therefore, the FEEDAP Panel could not confirm the previously drawn conclusions regarding the safety of the production strain and consequently could not confirm the safety for the target species and consumers. There was no need for assessing the efficacy of the additive in the context of the renewal of the authorisation.

Keywords: Zootechnical additives, digestibility enhancers, renewal, dairy cows

Requestor: European Commission

Question number: EFSA-Q-2016-00833

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Acknowledgements: The Panel wishes to acknowledge the contribution of Jaume Galobart, Orsolya Holczknecht and Konstantinos Sofianidis to this opinion.

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Suggested citation: EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Bampidis V, Azimonti G, Bastos ML, Christensen H, Dusemund B, Kos Durjava M, Kouba M, López-Alonso M, López Puente S, Marcon F, Mayo B, Pechová A, Petkova M, Ramos F, Sanz Y, Villa RE, Woutersen R, Cocconcelli PS, Dierick NA, Glandorf B, Herman L, Maradona MP, Rychen G, Saarela M and Anguita M, 2020. Scientific Opinion on the assessment of the application for renewal of the authorisation of Amaferm® (fermentation product of *Aspergillus oryzae* NRRL 458) as a feed additive for dairy cows. EFSA Journal 2020;18(2):6011, 9 pp. https://doi.org/10.2903/j.efsa.2020.6011

ISSN: 1831-4732

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

Table of contents

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 14(1) of that Regulation lays down that an application for renewal shall be sent to the Commission at the latest one year before the expiry date of the authorisation.

The European Commission received a request from Biozyme Incorporated[2] for renewal of the authorisation of the product Amaferm® (fermentation product of *Aspergillus oryzae* NRRL 458), when used as a feed additive for dairy cows (category: zootechnical additives; functional group: digestibility enhancers).

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 14(1) (renewal of the authorisation). EFSA received directly from the applicant the technical dossier in support of this application. The particulars and documents in support of the application were considered valid by EFSA as of 5 May 2017.

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 Amaferm® (fermentation product of *Aspergillus oryzae* NRRL 458), when used under the proposed conditions of use (see Section 3.1.1).

1.2. Additional information

The European Food Safety Authority (EFSA) adopted a scientific opinion on the safety and efficacy of the product for dairy cows and cattle for fattening (EFSA, 2006), which included the safety and efficacy of Amaferm for the target species, safety for the consumer, user and the environment.

The additive is authorised as a zootechnical additive (digestibility enhancers) for dairy cows.[3]

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[4] in support of the authorisation request for the use of Amaferm® (fermentation product of *Aspergillus oryzae* NRRL 458) as a feed additive.

The FEEDAP Panel used the data provided by the applicant together with data from other sources, as in previous risk assessments by EFSA.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the active substances 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 Amaferm (fermentation product of *Aspergillus oryzae* NRRL 458) is in line with the principles laid down in Regulation (EC) No 429/2008[6] and the relevant guidance documents: Guidance on the renewal of the authorisation of feed additives (EFSA FEEDAP Panel, 2013).

[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] Biozyme Incorporated, represented by J.E. van Eys, 24 Avenue de la Guillemotte, 78112 Fourqueux France.
[3] Commission Regulation (EC) No 537/2007 of 15 May 2007 concerning the authorisation of the fermentation product of *Aspergillus oryzae* (NRRL 458) (Amaferm) as a feed additive. OJ L 128, 16.5.2007, p. 13.
[4] FEED dossier reference: FAD-2016-0055.
[5] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2016-0055-amaferm.pdf
[6] 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.

3. Assessment

The additive Amaferm® (fermentation product of *Aspergillus oryzae* NRRL 458) is authorised as a zootechnical additive (functional group: digestibility enhancers) for dairy cows and this opinion deals with the request for the renewal of the authorisation.

3.1. Characterisation

The additive is authorised as a mixture of 4–5% of the fermentation product of *Aspergillus oryzae* NRRL 458, 94–95% wheat bran and 1% stainless steel grit (containing 5% cobalt carbonate) as microtracer. The authorisation specifies that the activities of the active substance are 3 IU[7] endo-1,4-beta-glucanase (cellulase; EC 3.2.1.4) per gram and 40 IU[8] alpha-amylase (amylase; EC 3.2.1.1).

According to the applicant, some minor changes have been applied to the manufacturing intended to improve the fermentation process. Antimicrobial substances are not used in the manufacturing[9]. The applicant reported that cobalt is not used anymore in the formulation of the additive, but no further details were provided at this regard.

The Panel noted that the enzyme activities given in the authorisation are not in line with the enzyme activities reported in the previous EFSA opinion (EFSA, 2006) and in the previous dossier submitted by the applicant. Following a request for clarification, the applicant confirmed that the new minimum specifications for Amaferm® are 0.3 mIU[10] cellulase/g and 20 mIU[11] amylase/g.[12] The applicant provided analytical data in four recent batches that demonstrated compliance with these new minimum specifications, average for cellulase 0.78 mIU/g and for amylase 33 mIU/g. Data from other batches analysed for the assessment of the methods by the EURL also showed compliance with the minimum specifications for enzyme activity.[13] The FEEDAP Panel notes that the performance of the method of analysis for cellulase does not allow to reliably quantify the enzyme activity in the feed additive at the minimum specifications. Moreover, no methods for the detection of the cellulase and amylase in premixtures and feed were made available by the applicant. Finally, no comparison is possible with the enzyme activities present in the additive now and those established at the time the first assessment, and therefore, a comparison of the composition of the additive is not possible.

The applicant provided analysis of three recent batches in which the content of arsenic, lead and *Salmonella* spp. were measured and not detected.[14] The applicant analysed three batches of the additive for the presence and quantification of mycotoxins and secondary metabolites from *A. oryzae* using a Liquid chromatography-mass spectrometry (LC-MS/MS) method.[15] This method allows to evaluate more than 200 substances and the applicant reported for each substance the limit of detection (LOD). Most of the compounds were below the LOD and for those that were detected the concentration was given. Among those detected the most relevant ones were: deoxynivalenol (0.6–1.0 mg/kg), don-3-glucoside (130–178 μg/kg), HT2 toxin (6.4–11.6 μg/kg), zearalenone (< 18.1–24.0 μg/kg), kojic acid (198 μg/kg in one batch, the other two < 4 μg/kg [LOD]), 3-nitropropionic acid (26.6–84.2 μg/kg) and tryptophol (415–699 μg/kg); and their concentrations were of no concern. Mycotoxins that could be produced by *Aspergillus* (ochratoxin A, aflatoxin and fumonisin) were not detected.

No antimicrobial activity was found in three batches of the fermentation product when tested up to 25 g/L against the following five reference strains: *S. aureus* DSM 1104, *E. faecalis* ATCC 29212, *B. subtilis* ATCC 6633, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853.[16]

The authorisation is given to a non-genetically modified strain of *A. oryzae* referred as NRRL 458. This strain (NRRL 458) is deposited at the American Type Culture Collection under the deposit number ATCC 9376.[17,18] [redacted]

[7] IU cellulose that liberates 1 micromole of glucose per minute from carboxymethylcellulose at pH 6.5 and at 39°C.
[8] IU amylase that liberates 1 micromole of glucose per minute from potato starch at pH 6.5 and at 39°C.
[9] Technical dossier/Supplementary information June 2018.
[10] one unit of endo-1,4-beta-glucanase activity (IU) 'refers to the cellulase that liberates 1 micromole of glucose per minute from carboxymethyl cellulose at pH 6.5 and at 38°C.' 'm' stands for milli.
[11] one unit of alpha-amylase activity (IU) 'refers to the amylase that liberates 1 micromole of glucose per minute from potato starch at pH 6.9 and at 38°C.' 'm' stands for milli.
[12] Technical dossier/Supplementary information February 2019.
[13] Technical dossier/Supplementary information February 2019/Annexes 10 to 15.
[14] Technical dossier/Section II/Annex II.8/Limits of detection not provided.
[15] Technical dossier/Supplementary information June 2018/Annex Q3a.
[16] Technical dossier/Supplementary information June 2018/Annexes Q6a and Q6b.
[17] Technical dossier/Section II and clarification note from the Applicant/Supplementary information June 2018/Annex Q2.
[18] ATCC on-line: http://www.lgcstandards-atcc.org/Products/All/9376.aspx?geo_country=it#history

[redacted].[19] Moreover, in order to taxonomically identify the production strain, the applicant sent, upon request, an analysis [redacted] but the comparison of the sequence did not permit to distinguish between *A. oryzae* and *A. flavus*.[redacted] Therefore, the taxonomic identification of the production strain as *A. oryzae* has not been confirmed.

The applicant checked microscopically the presence of spores in recent batches. The results showed a reduction in the number of spores in the final product compared to results obtained in the previous assessment (from 10^6 to 10^3 per gram). This discrepancy was justified by the applicant due to the minor changes in the manufacturing which would allow for a better germination of the spores.[12] The Panel notes that no confirmation was provided on whether the spores were from the production strain. The presence of viable cells/spores was investigated by plating samples of the additive on Potato Dextrose Agar (PDA) and the results showed no growth.[21] The presence of viable cells/spores was not detected when samples of the product were cultured probably due to a short time of incubation. Therefore, uncertainty remains on the presence of viable cells/spores of the production strain in the final product.

3.1.1. Conditions of use

The additive is currently authorised for use in dairy cows from 85 to 300 mg of additive/kg complete feed (12% moisture content, similar to 2–6 g/cow day). The authorisation includes Under 'Other provisions: Recommended dose: the quantity of the additive in daily ration should be 3 to 5 g/cow per day, and for user safety: shall be used breathing protection during handling and safety glasses shall be worn.' The applicant indicates in the renewal dossier that the dosage to be used is 2–5 g Amaferm per cow and day.

3.2. Safety

In the previous assessment, based on the data and knowledge available at that time, the FEEDAP Panel concluded that the additive is safe for dairy cows, for the consumers and the environment. It was also concluded that the additive is non-irritant or a dermal sensitiser but should be considered a potential respiratory sensitiser.

The data newly provided to confirm the identification of the production strain do not allow to distinguish between *A. oryzae* and *A. flavus.* The Panel notes that *A. flavus* is a human and animal opportunistic pathogen. Uncertainty remains on the presence of viable cells/spores of the production organism in the additive.

The applicant provided the outcome of a literature search on the safety of the additive from 2005 up to 2018.[22] The search was conducted in a library from a University allowing searching in 62 worldwide collections including Agricola, Agris, CAB abstracts, ECLAS, Medline, Pubnet, Scopus and Toxnet. The applicant did the following searches (hits retrieved indicated within brackets): *A. oryzae* and dairy cows (3 hits, 3 relevant), *A. oryzae* and health (79 hits, 2 relevant), *A. oryzae* and toxicity (24 hits, 0 relevant), *A. oryzae* and toxin (21 hits, 2 relevant), and *A. oryzae* and environment (210 hits, 0 relevant). Those found to be relevant in the search and that regarded the use in the target species did not indicate any safety concern, however, were not designed to establish the safety of the fermentation product. The other hits identified as relevant dealt with the toxigenic potential of the genus *Aspergillus but* were not specific to the production strain under assessment. The Panel notes that although the literature search was very limited, no papers were identified which would indicate a safety concern with regard to the use of the fermentation product from *A. oryzae* under assessment. However, considering that there is uncertainty regarding the identity of the strain as *A. oryzae* or *A. flavus*, the Panel considers that the suitability of the literature search performed is open to question until a definitive identification of the strain is provided.

In addition, the uncertainty regarding the presence of viable cells/spores of the production strain, should this be an *A. flavus* strain, in the final product may raise a concern for the target species, consumers and users. Therefore, taking into consideration all the above, the FEEDAP Panel considers

[19] Technical dossier/Section II/ Annex II.1.
[20] Technical dossier/Supplementary information June 2018/Annex Q1 and [redacted]
[21] Technical dossier/Supplementary information June 2018/Annex 7.
[22] Technical dossier/Supplementary information February 2019/Annex Q18a.

that the information provided by the applicant does not fulfil the minimum requirements to support that the additive remains safe under the approved conditions for target species, consumers and users.

With regard to the safety for the environment, the Panel notes that even if the production strain is *A. flavus*, this is a common fungus found in the environment, and its use as a feed additive will not significantly contribute to an increase in its presence in the environment. Therefore, the Panel considers that the use of Amaferm® remains safe for the environment.

3.3. Efficacy

The additive is authorised for use in dairy cows from 85 to 300 mg of additive/kg complete feed (12% moisture content, similar to 2–6 g/cow day). The present application for renewal of the authorisation does not include a proposal for amending or supplementing the conditions of the original authorisation that would have an impact on the efficacy of the additive. Therefore, there is no need for assessing the efficacy of the additive in the context of the renewal of the authorisation. However, due to the modification in the enzyme activities and the lack of data regarding the relationship with the activities previously defined, uncertainty remains on the levels of additive to be added to the diet that would correspond to the ones currently authorised.

3.4. Post-market monitoring

The FEEDAP Panel considers that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation[23] and Good Manufacturing Practice.

4. Conclusions

The data provided by the applicant does not allow the Panel to conclude that Amaferm® complies with the conditions of the authorisation. Uncertainty remains on the identification of the production strain as *A. oryzae* or *A. flavus*, an opportunistic pathogen. The Panel considers that the information provided by the applicant does not fulfil the minimum requirements to support that Amaferm® remains safe under the approved conditions for target species, consumers and the users. The Panel considers that the use of Amaferm® remains safe for the environment.

There is no need for assessing the efficacy of the additive in the context of the renewal of the authorisation.

Recommendations

The specifications of the product in terms of enzyme activity should be modified to reflect the current specifications and conditions of the method of analysis as described for the new batches analysed. Appropriate analytical methods addressing the limitations identified by the EURL on the methods of analysis should be developed.

Documentation as provided to EFSA/Chronology

Date	Event
02/09/2016	Dossier received by EFSA - Amaferm for dairy cows submitted by Biozyme Incorporated
07/06/2016	Reception mandate from the European Commission
05/05/2017	Application validated by EFSA – Start of the scientific assessment
05/08/2017	Comments received from Member States
22/09/2017	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: methods of analysis*
07/11/2017	Additional request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterisation and safety*

[23] Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

Date	Event
21/12/2018	Additional request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterisation and safety*
25/02/2019	Reception of supplementary information from the applicant (characterisation and safety)
04/06/2019	Reception of supplementary information from the applicant (methods of analysis) – Scientific assessment re-started
16/07/2019	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
28/01/2020	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

References

EFSA (European Food Safety Authority), 2006. Opinion of the Scientific Panel on Additives and Products or Substances used in Animal Feed on the safety and efficacy of the product "Amaferm" as a feed additive for dairy cows and cattle for fattening in accordance with Regulation (EC) No 1831/2003. EFSA Journal 2006;4 (3):337, 17 pp. https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2006.337

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2013. Guidance on the renewal of the authorisation of feed additives. EFSA Journal 2013;11(10):3431, 8 pp. https://doi.org/10.2903/j.efsa.2013.3431

Abbreviations

CMC	carboxymethyl cellulose
EURL	European Union Reference Laboratory
FEEDAP	Additives and Product or Substances used in Animal Feed
LC-MS/MS	Liquid chromatography-mass spectrometry
LOD	Limit of detection
PDA	Potato Dextrose Agar

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additive on the Method(s) of Analysis for fermentation product of *Aspergillus oryzae* NRRL 458

In the current application, authorisation is sought under Article 14 (renewal of authorisation) for fermentation product of Aspergillus oryzae NRRL 458 (Amaferm®), under the category/functional group (4 a) 'zootechnical additive'/'digestibility enhancers', according to the classification system of Annex 1 of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the feed additive for dairy cows.

Amaferm® is a preparation containing as active substances endo-1,4-beta-glucanase (EC 3.2.1.4) and alpha-amylase (EC 3.2.1.1) with a guaranteed minimum enzymatic activity content of 0.3 mIU/g and 4 mIU/g, respectively, where:

- one unit of endo-1,4-beta-glucanase activity (IU) 'refers to the cellulase that liberates 1 micromole of glucose per minute from carboxymethyl cellulose (CMC) at pH 6.5 and at 39 °C'; and
- one unit of alpha-amylase activity (IU) 'refers to the amylase that liberates 1 micromole of glucose per minute from potato starch at pH 6.5 and at 39 °C'

The feed additive is intended to be incorporated through premixtures or directly into feedingstuffs with a content ranging from 85 to 300 mg/kg complete feedingstuffs.

For the quantification of endo-1,4-beta-glucanase in the feed additive, the Applicant submitted a single-laboratory validated and further verified colorimetric method. The enzyme (cellulase) activity is quantified as the generation of reducing sugars from CMC. The reducing sugars are measured by colorimetry using tetrazolium blue chloride while glucose is used as standard. Based on the insufficient performance of the method presented in the validation and verification studies for the quantification of endo-1,4-beta-glucanase in the feed additive, the EURL does not consider this method suitable for the intended purpose.

The EURL is therefore unable to recommend a method for the official control of endo-1,4-beta-glucanase in the feed additive.

For the quantification of alpha-amylase in the feed additive, the Applicant submitted a single-laboratory validated and further verified colorimetric method. The enzyme activity is quantified as the generation of reducing sugars from potato starch. The reducing sugars are measured by colorimetry using 3,5-dinitrosalicylic acid colour reagent (DNS) while glucose is used as standard. The following performance characteristics were reported for the quantification of alpha-amylase in the feed additive: a recovery rate from 84.7% to 87.1% and precision expressed in terms of relative standard deviation for repeatability and intermediate precision from 6.5% to 7.6%.

Based on the performance characteristics available, the EURL recommends for official control the validated and further verified colorimetric method presented for the quantification of alpha-amylase in the feed additive.

Furthermore, the Applicant stated that an accurate quantification of the enzymes when added to premixtures and feeds is not achievable experimentally. The EURL is therefore unable to recommend methods for the official control of endo-1,4-beta-glucanase and alpha-amylase in premixtures and feedingstuffs.

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.