SCIENTIFIC OPINION

EFSA Journal

ADOPTED: 18 April 2018

doi: 10.2903/j.efsa.2018.5275

Safety and efficacy of *Bacillus subtilis* KCCM 10673P and *Aspergillus oryzae* KCTC 10258BP when used as a technological feed additive for all animal species

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), Guido Rychen, Gabriele Aquilina, Giovanna Azimonti, Vasileios Bampidis, Maria de Lourdes Bastos, Georges Bories, Andrew Chesson, Pier Sandro Cocconcelli, Gerhard Flachowsky, Boris Kolar, Maryline Kouba, Marta López-Alonso, Secundino López Puente, Alberto Mantovani, Baltasar Mayo, Fernando Ramos, Maria Saarela, Roberto Edoardo Villa, Robert John Wallace, Pieter Wester, Anne-Katrine Lundebye, Carlo Nebbia, Derek Renshaw, Matteo Lorenzo Innocenti and Jürgen Gropp

Abstract

The additive consists of single strains of *Bacillus subtilis* and *Aspergillus oryzae*, to be used in combination as a technological additive (proposed functional group: Substances for the reduction of anti-nutritional factors) in feed materials for all animal species. An opinion on the two microorganisms was published previously. At this time, the safety of the *B. subtilis* strain for target animals, consumers, users and the environment was established, but no conclusions could be draw on the safety of the strain of *A. oryzae* for the target species and consumers or on the efficacy of the additive in reducing anti-nutritional factors. This opinion considers new information intended to address the identified deficiencies in the previous opinion. An extensive analytical search for secondary metabolites was made of the *A. oryzae* component of the additive and 15 metabolites were detected and quantified. Of the 15 metabolites, 13 occurred in ng/g additive concentrations and were considered not to be of concern. The remaining two compounds, cyclic dipeptides (2,5-diketopiperazines), were detected at concentrations up to 1.5 mg/kg. Both have been identified in a wide range of foods and beverages and there appears to be no reports of adverse reactions to such cyclic dipeptides in processed food. Consequently, none of the metabolites detected were considered likely to cause adverse effects in target animals fed treated feed material or in consumers of products derived from such animals. Data from the analysis of 18 batches of soybean before and after treatment with the two strains were presented in support of the efficacy of the additive. Two classes of anti-nutritional factors were considered; oligosaccharides of the raffinose series and trypsin inhibitor. The microbial strains in combination were able to substantially reduce the concentration of oligosaccharides and trypsin inhibitor naturally present in soybean. Other feed materials were not considered.

Keywords: *Bacillus subtilis*, *Aspergillus oryzae*, safety, secondary metabolites, trypsin inhibitor, oligosaccharides, soybean

Requestor: European Commission

Question number: EFSA-Q-2016-00220

Correspondence: feedap@efsa.europa.eu

Panel members: Gabriele Aquilina, Giovanna Azimonti, Vasileios Bampidis, Maria de Lourdes Bastos, Georges Bories, Andrew Chesson, Pier Sandro Cocconcelli, Gerhard Flachowsky, Jürgen Gropp, Boris Kolar, Maryline Kouba, Secundino López Puente, Marta López-Alonso, Alberto Mantovani, Baltasar Mayo, Fernando Ramos, Guido Rychen, Maria Saarela, Roberto Edoardo Villa, Robert John Wallace and Pieter Wester.

Acknowledgements: The Panel wishes to thank EFSA staff member(s): Jaume Galobart and Orsolya Holczknecht for the support provided to this scientific output.

Amendment: An editorial correction was carried out that does not materially affect the contents or outcome of this scientific output. It was related to the name of the applicant. To avoid confusion, the older version has been removed from the EFSA Journal, but is available on request, as is a version showing all the changes made.

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, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Lundebye A-K, Nebbia C, Renshaw D, Innocenti ML and Gropp J, 2018. Scientific Opinion on the safety and efficacy of *Bacillus subtilis* KCCM 10673P and *Aspergillus oryzae* KCTC 10258BP when used as a technological feed additive for all animal species. EFSA Journal 2018;16(5):5275, 8 pp. https://doi.org/10.2903/j.efsa.2018.5275

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 as provided by the requestor

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

The applicant, Regal BV on behalf of Nutraferma Co., is seeking a Community authorisation of *Bacillus subtilis* GR-101, *Aspergillus oryzae* GB-107 to be used as feed additives. (Table 1)

Table 1: Description of the substances

Category of additive	Technological additives
Functional group of additive	Substances for the reduction of anti-nutritional factors: substances or, where applicable microorganisms which reduce or remove anti-nutritional factors from feed (in accordance with SANTE reference: 3933683)
Description	*Bacillus subtilis* GR-101 and *Aspergillus oryzae* GB-107
Target animal category	All animal species
Applicant	Regal BV on behalf of Nutraferma Co.
Type of request	New opinion

On 8 September 2015, the Panel on Additives and Products or Substances used in Animal Feed of the European Food Safety Authority ('Authority'), in its opinion on the safety and efficacy of the product concluded that the additive *Aspergillus oryzae* KCTC 10258BP and *Bacillus subtilis* KCCM 10673P were insufficiently characterised. So, in the absence of data on production of toxic secondary metabolites in *A. oryzae*, the FEEDAP Panel couldn't draw conclusions on its safety for the target species and consumers of products and tissues derived from animals fed the additive. In addition, the FEEDAP Panel couldn't draw conclusions on the efficacy of the additive.

The Commission, after the consultation with the Member States at the Standing Committee on Plants, Animals, Food and Feed, decided to give the possibility to the applicant to submit complementary information in order to complete the assessment on the safety and efficacy and to allow a revision of Authority's opinion.

The Commission has now received new data on the safety and efficacy of *Bacillus subtilis* GR-101 and *Aspergillus oryzae* GB-107.

In view of the above, the Commission asks the Authority to deliver a new opinion on the safety and efficacy of *Bacillus subtilis* GR-101 and *Aspergillus oryzae* GB-107 as a feed additive based on the additional data submitted by the applicant.

1.2. Additional information

In 2009, the European Food Safety Authority (EFSA) was requested by the European Commission to evaluate a product consisting of two microbial strains (*B. subtilis* and *A. oryzae*) intended to be used to treat soybean and other feed materials to reduce the concentration of anti-nutritional factors. An opinion on the assessment of the two organisms as a technological additive in a proposed new functional group of 'substances for the reduction of anti-nutritional factors' for all animal species was published in 2015 (EFSA FEEDAP Panel, 2015). The safety of the *B. subtilis* strain for target animals, consumers, users and the environment was established, but no conclusions could be draw on the safety of the strain of *A. oryzae* for the target species and the consumer. The FEEDAP Panel also was unable to establish the efficacy of the additive in reducing anti-nutritional factors in soybean and other feed materials.

2. Data and Methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of additional information[1] to a previous application on the same product.[2]

[1] FEED dossier reference: FAD-2016-0014.
[2] FEED dossier reference: FAD-2009-0007.

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of *B. subtilis* and *A. oryzae* 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), Guidance for establishing the safety of additives for the consumer (EFSA FEEDAP Panel, 2012b) and Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012c).

3. Assessment

The additive consists of single strains of *B. subtilis* and *A. oryzae*, to be used in combination as a technological additive (proposed new functional group: Substances for the reduction of anti-nutritional factors) in feed materials for all animal species.

3.1. Characterisation

3.1.1. Characterisation of the active agents

Bacillus subtilis

The strain is deposited in the Korean Culture Center of Microorganisms with the accession number KCCM 10673P. The identity of the strain, its susceptibility to antibiotics and its toxigenic potential were considered in the previous opinion and no concerns were identified.

Aspergillus oryzae

The strain is deposited in the Korean Culture Center of Microorganisms with the accession number KCTC 10258BP. Data relating to strain identity was provided in the context of the previous opinion and considered satisfactory. Evidence of the absence of genes involved in the production of aflatoxin was also provided at that time. However, there was no information on the presence of other secondary metabolites of concern known to occur in some strains of *A. oryzae*. This issue has now been addressed by the applicant.

It was confirmed that the strain was isolated from a Korean traditional food (Meju, fermented soy paste). In an initial screen, the *A. oryzae* preparation derived from the solid substrate fermentation and used to treat the feed material (described as the seed culture) was examined for the presence of selected compounds of recognised concern, as described by Blumenthal (2004).[3] Although the species specific mycotoxins kojic acid, 3-nitropropionic acid and cyclopiazonic acid or the antibiotic penicillin G (benzylpenicillin) were not detected, the high-performance liquid chromatography (HPLC) fingerprint method employed had limited sensitivity. Subsequently, a far more extensive analysis of secondary metabolites of possible toxicological relevance was made of three batches using liquid chromatography with tandem mass spectrometry (LC–MS/MS). A battery of nearly 200 metabolites was considered.[4] The limit of detection (LOD) for each metabolite, individually determined using stock multianaylate solutions, were generally < 10 μg/kg. Using this method a total of 15 metabolites was detected and quantified (Table 2).

Table 2: Mean concentrations (μg/kg sample) of metabolites identified in the *A. oryzae* preparation derived from the solid substrate fermentation

Metabolite	Concentration (μg/kg sample)
Zearalenone	0.7
Enniatin A	0.2
Enniatin A1	1.1
Enniatin B	0.6
Enniatin B1	1.7
Enniatin B2	0.1
Beauvericin	1.0
Culmorin	9.5

[3] Technical dossier/ Annex A CoA Secondary metabolites Aspergillus.
[4] Technical dossier/Supplementary Information October 2016 and Supplementary Information November 2016.

Metabolite	Concentration (μg/kg sample)
3-Nitropropionic acid	1.8
Alternariaiol-monomethyl-ether	0.2
Emodin	1.9
Asperglaucide	5.6
Neoechinulin A	14.7
Cyclo (L-pro-L-tyr)	1,533
Cyclo (L-pro-L-val)	881

Several of the metabolites detected, such as zeralenone, members of the enniatin family and culmorin are associated with other fungal species, particularly *Fusarium* spp., and may have derived from the contamination of the soybean used in production.

3.1.2. Characterisation of the additive

The methods used for the production of each of the two cultures were described in the previous application and counts provided for five batches of each culture. The applicant has now defined a minimum specification for each culture allowing the conditions of use to be more precisely defined:

B. subtilis culture: minimum content 1.2×10^8 CFU/g compared to a mean for the five batches of 4.3×10^8 CFU/g (range 3.7–5.3 $\times 10^8$ CFU/g).

A. oryzae culture: minimum content 2.0×10^8 CFU/g compared to a mean for the five batches of 2.7×10^8 CFU/g (range 2.2–3.0 $\times 10^8$ CFU/g).

No data has been provided on microbiological or chemical impurities of the additive other than the new data provided for *A. oryzae*.

3.1.3. Conditions of use

In the original application, it is stated that there is no intention to make the two microbial cultures available to feed manufacturers or to farmers. They will be used only by the applicant to treat feed materials for eventual sale.

The two cultures are combined at the point of application with a total inclusion rate of 20 g/kg feed material on a 50:50 basis by weight delivering a minimum of 1.2×10^6 CFU *B. subtilis* and 2.0×10^6 CFU *A. oryzae*/kg feed material.

3.2. Safety

3.2.1. Safety for the target species, the consumer and the environment

In the previous opinion (EFSA FEEDAP Panel, 2015), it was concluded that the strain of *B. subtilis* met the criteria required for the Qualified Presumption of Safety (QPS) approach to safety assessment (EFSA BIOHAZ Panel, 2017) to be applied. On this basis, the strain was presumed safe for target species, the consumers of products derived from animals exposed to the bacterium and for the environment. The safety of *A. oryzae* for the environment was also already established in the previous opinion.

Strains of *A. oryzae* have a long and extensive history of use in the production of foods from soybean and the strain involved in this application was isolated from one such food item. An extensive search for potentially toxic metabolites derived directly from the metabolism of the *A. oryzae* strain or from the soybean on which it was grown identified a total of 15 metabolites/mycotoxins (Table 2). Of the 15 compounds, 13 occurred at concentrations below 15 μg/kg. Although most are known to be biologically active and have been tested for various pharmacological properties, toxicological studies generally are absent. However, application of the Threshold of Toxicological Concern (TTC) approach to all 13 compounds indicates a lack of concern for either target animals or consumers regardless of the Cramer class applied.

The remaining two compounds (Cyclo (L-Pro-L-Tyr) and Cyclo (L-Pro-L-Val)) both cyclic dipeptides (2,5-diketopiperazines), were detected at concentrations up to 1.5 mg/kg. Cyclic dipeptides are widely distributed in nature and are naturally produced by both microorganisms and by mammals. They are also found in many protein-rich processed foods, particularly when heat-processed, often contributing to the sensory properties. They are also recognised as common by-products of fermentation by yeasts and bacteria (see Review by Borthwick and Da Costa, 2017 and references therein). The two peptides

in Table 2 have been identified in a wide range of foods and beverages including bread, beer, cheese and Parma ham. There appear to be no reports of adverse reactions to cyclic dipeptides found in processed food.

The FEEDAP Panel considers that the detected metabolites occur at concentrations considered unlikely to cause adverse effects in target animals fed the treated feed material or in consumers of products derived from such animals.

3.2.2. Safety for the user

In the previous opinion, the Panel concluded that the additive should be considered to be a skin and eye irritant and a skin and respiratory sensitiser. No new data have been submitted, and therefore the previous conclusions still apply.

3.3. Efficacy

The additive is intended to be used reduce anti-nutritional factors concentration in feedingstuffs for all animal species. In particular, the additive is applied to soybean to reduce the concentration of anti-nutritional factors, through a solid state fermentation with *Bacillus subtilis* GR-101 and *Aspergillus oryzae* GB-107

Data from the analysis of 18 batches of soybean before and after treatment with the two microbial strains were presented in support of the efficacy of the additive.[5] The soybean samples were inoculated according to the proposed conditions of use: 20 g additive/kg feed, corresponding to a minimum of 1.2×10^6 CFU of *B. subtilis*/kg feed) and a minimum of 2×10^6 CFU of *A. oryzae*/kg feed). After inoculation, the soybean samples were deposited onto solid state fermentation beds with controlled air-flow and temperature. At the end of the fermentation period, the samples were dried and analysed. Two classes of anti-nutritional factors were considered: oligosaccharides of the raffinose series (raffinose and stachyose) and trypsin inhibitor.[6]

The mean content of raffinose in the 18 samples of untreated soybean was 1.47% dry matter (DM) (range 0.51–2.51% DM) and the mean content of stachyose was 6.11% DM (range 5.3–7.2% DM). Treatment of the soybean left only residual amounts of the two oligosaccharides in all cases. The content of raffinose fell to a mean of 0.02% DM (range 0–0.08% DM) and stachyose to 0.05% DM (range 0–0.1% DM). Similar result was seen with the trypsin inhibitor with the mean initial value of 2.9 trypsin inhibitor unit (TIU)/g (range 2.5–3.8 TIU/g) reducing by approximately 85% to a mean of 0.43 TIU/g (range 0.3–0.5 TIU/g).

No data was provided on other anti-nutritional factors or with feed materials other than soybean.

4. Conclusions

In the previous opinion, it was concluded that the strain of *B. subtilis* met the criteria required for the QPS approach to safety assessment to be applied. On this basis, the strain was presumed safe for target species and consumers of products derived from animals exposed to the bacterium.

The strain of *A. oryzae* has been exhaustively tested for the presence of secondary metabolites of potential concern. Those metabolites detected occur at concentrations considered unlikely to cause adverse effects in target animals fed the treated feed material or in consumers of products derived.

No new data have been submitted that modify the previous conclusions that the additive should be considered to be a skin and eye irritant and a skin and respiratory sensitiser.

Both organisms are ubiquitous in nature. Their use as a technological additive is not expected to pose a risk for the environment.

The two strains in combination, applied according to the conditions of use (minimum 1.2×10^6 CFU *of B. subtilis*/kg feed and minimum 2×10^6 CFU of *A. oryzae*/kg feed) are able to substantially reduce the concentration of oligosaccharides of the raffinose series and trypsin inhibitor naturally present in soybean. Other feed materials have not been considered.

Documentation provided to EFSA

1) *Bacillus subtilis* KCCM 10673P and *Aspergillus oryzae* KCTC 10258BP for all animal species. February 2016. Submitted by Nutraferma Co.

[5] Technical dossier/Annexes 1–18.
[6] Analysed according to AOCS Official Method Ba 12-75.

2) *Bacillus subtilis* KCCM 10673P and *Aspergillus oryzae* KCTC 10258BP for all animal species. Supplementary Information October 2016. Submitted by Nutraferma Co.
3) *Bacillus subtilis* KCCM 10673P and *Aspergillus oryzae* KCTC 10258BP for all animal species. Supplementary Information November 2016. Submitted by Nutraferma Co.
4) *Bacillus subtilis* KCCM 10673P and *Aspergillus oryzae* KCTC 10258BP for all animal species. Supplementary Information July 2017. Submitted by Nutraferma Co.

References

Blumenthal CZ, 2004. Production of toxic metabolites in *Aspergillus niger, Aspergillus oryzae*, and *Trichoderma reesei*: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. Regulatory Toxicology and Pharmacology, 39, 214–228.

Borthwick AD and Da Costa NC, 2017. 2,5-Diketopiperazines in food and beverages: taste and bioactivity. Critical Reviews in Food Science and Nutrition, 57, 718–742.

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2017. Ricci A, Allende A, Bolton D, Chemaly M, Davies R, Girones R, Herman L, Koutsoumanis K, Lindqvist R, Nørrung B, Robertson L, Ru G, Sanaa M, Simmons M, Skandamis P, Snary E, Speybroeck N, Ter Kuile B, Threlfall J, Wahlström H, Cocconcelli PS, Klein G (deceased), Prieto Maradona M, Querol A, Peixe L, Suarez JE, Sundh I, Vlak JM, Aguillera-Gomez M, Barizzone F, Brozzi R, Correia S, Heng L, Istace F, Lythgo C and Fernández Escámez PS, 2017. Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA. EFSA Journal 2017;15(3):4664, 177 pp. https://doi.org/10.2903/j.efsa.2018.4664

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. https://doi.org/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. https://doi.org/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. https://doi.org/10.2903/j.efsa.2012.2539

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2015. Scientific Opinion on the safety and efficacy of *Bacillus subtilis* KCCM 10673P and *Aspergillus oryzae* KCTC 10258BP as feed additives for all animal species. EFSA Journal 2015;13(9):4230, 11 pp. https://doi.org/10.2903/j.efsa.2015.4230

Abbreviations

CFU	colony forming unit
DM	dry matter
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
HPLC	high-performance liquid chromatography
LC–MS/MS	liquid chromatography with tandem mass spectrometry
LOD	limit of detection
QPS	Qualified Presumption of Safety
TIU	trypsin inhibitor unit
TTC	Threshold of Toxicological Concern