SCIENTIFIC OPINION

Scientific Opinion on the safety and efficacy of *Bacillus subtilis* KCCM 10673P and *Aspergillus oryzae* KCTC 10258BP as feed additives for all animal species[1]

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

European Food Safety Authority (EFSA), Parma, Italy

This scientific opinion has been amended following the adoption of the decision of the Commission on confidentiality claims submitted by the applicant, in accordance with Article 8(6) and Article 18 of Regulation (EC) No 1831/2003. The modified section is indicated in the text.

ABSTRACT

PepSoyGen-C is described as pure cultures of *Aspergillus oryzae* and *Bacillus subtilis* added simultaneously to feed materials to reduce antinutritional factors. The applicant is seeking its authorisation as a technological additive, under a newly proposed, currently non-existent functional group, "substances for the reduction of anti-nutritional factors". The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) considers that the two microbial cultures should be regarded as two independent feed additives that are the subject of the evaluation. The additives are poorly characterised. The inclusion level is likely to be 2 % by weight of the total substrate. The ratio of the two cultures is described as 50/50 on a weight basis. A dose expressed in colony-forming units per kilogram of feed is not proposed. No evidence of toxigenic potential or resistance to antibiotics of human and veterinary importance was found, according to the current guidance documents. Therefore, the *B. subtilis* additive is presumed safe for target animals, consumers of products fed the additive and the environment. The *B. subtilis* additive should be considered as having the potential to be a skin and eye irritant and a skin sensitiser and be treated accordingly. In the absence of data on production of toxic secondary metabolites in *A. oryzae*, the FEEDAP Panel cannot draw conclusions on its safety for the target species and consumers of products fed the additive. The *A. oryzae* additive should be considered as having the potential to be a skin and eye irritant and a skin and respiratory sensitiser and be treated accordingly. The use of the additive as a technological feed additive is not expected to pose a risk to the environment. The FEEDAP Panel cannot draw conclusions on the efficacy of the additives in reducing antinutritional factors in soybean and other feed materials.

KEY WORDS

technological additive, substances for the reduction of antinutritional factors, *Bacillus subtilis* KCCM 10673P, *Aspergillus oryzae* KCTC 10258BP, safety, efficacy, soybean

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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 and efficacy of the product PepSoyGen-C as a feed additive for all animal species. The additive is said to be composed of a mixture of pure cultures of *Aspergillus oryzae* and of *Bacillus subtilis*. PepSoyGen-C is intended to treat feed materials such as soybean meal for use in all animal species in order to reduce antinutritional factors. For this purpose the applicant is seeking an authorisation for PepSoyGen-C as a technological additive under a newly proposed, currently non-existent functional group, "substances for the reduction of antinutritional factors". The FEEDAP Panel considers that the two microbial cultures should be regarded as two independent feed additives that are the subject of the evaluation.

The additives are poorly characterised. They are said to be combined only at the point of application to the target feed. The applicant indicates that the inclusion level is likely to be 2 % by weight of the total substrate (20 g/kg feed material). The ratio of the two cultures is described as 50/50 on a weight basis. A dose expressed as colony-forming units per kilogram of feed is not proposed.

No evidence of toxigenic potential or resistance to antibiotics of human and veterinary importance was found, judging by the current guidance documents. Therefore, *B. subtilis* KCCM 10673P is presumed safe for target animals, consumers of products fed the additive and the environment. The additive should be considered as having the potential to be a skin and eye irritant and a skin sensitiser and be treated accordingly.

In the absence of data on production of toxic secondary metabolites, other than aflatoxins, in *A. oryzae* KCTC 10258BP, the FEEDAP Panel cannot draw conclusions on its safety for the target species and the consumer of products fed the additive. The additive should be considered as having the potential to be a skin and eye irritant and a skin and respiratory sensitiser and be treated accordingly. The use of the additive as a technological feed additive is not expected to pose a risk to the environment.

A single *in vitro* study was provided in which the concentrations of stachyose, raffinose and trypsin inhibitor were measured in multiple batches of soybean meal after treatment with the additives. However, as the study suffers from a number of deficiencies, the FEEDAP Panel is not in a position to draw conclusions on the efficacy of the additives in reducing antinutritional factors in soybean and other feed materials.

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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 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from the company Regal BV[5] for authorisation of PepSoyGen-C, *Bacillus subtilis* KCCM 10673P and *Aspergillus oryzae* KCTC 10258BP, to be used as a feed additive for piglets, chickens for fattening, calves for fattening and for rearing, fish (salmonidae and other fish), lambs and goats for rearing and fattening, and dogs (category: zootechnical additive; functional group: other zootechnical additives) under the conditions mentioned in Table 1. During the assessment, the applicant requested a change in the category from "zootechnical additive" to "technological additive", in the functional group from "other zootechnical additives" to a new functional group of "substances for the reduction of anti-nutritional factors" and in the target animals from the above list to "all animal species". Table 1 was modified accordingly.

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive). EFSA received directly from the applicant the technical 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 29 January 2010.

The additive PepSoyGen-C is a preparation of *Bacillus subtilis* KCCM 10673P *and Aspergillus oryzae* KCTC 10258BP. This product has not been previously authorised in the European Union.

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 *Bacillus subtilis* KCCM 10673P *and Aspergillus oryzae* KCTC 10258BP, 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] Regal BV on behalf of Nutraferma Co, Wilhelminalaan 90, 6042 EP Roermond, the Netherlands.
[6] EFSA Dossier reference: FAD-2009-0007.

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

Additive	*Bacillus subtilis* GR-101 and *Aspergillus oryzae* GB-107
Registration number/ EC No/No	
Category of additive	1. 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 SANCO reference: 3933683)

Description			
Composition, description	Chemical formula	Purity criteria	Method of analysis
Bacillus subtilis GR-101 and *Aspergillus oryzae* GB-107	-	-	NEN-EN15784 (2009) US/FDA CFSAN-BAM 2001

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 species	-	-	-	-

Other provisions and additional requirements for the labelling	
Specific conditions or restrictions for use	To be used to treat feed materials to diminish anti-nutritional factors. The additive as such will not be brought on the market, only treated feed materials
Specific conditions or restrictions for handling	-
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
-	-	-	-

ASSESSMENT

1. Introduction

This opinion is based on data provided by the applicant on a soybean meal (intended to be sold under the trade name PepSoyGen) fermented by the additive PepsSoyGen-C, composed of pure cultures of *Aspergillus oryzae* and *Bacillus subtilis.* The additive is intended to be used as a technological feed additive, under a newly proposed, currently non-existent, functional group, "substances for the reduction of anti-nutritional factors" in feedingstuffs for all animal species.

The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) for the purpose of this assessment considers that the feed material soybean should not be regarded as a feed additive and that the two microbial cultures should be regarded as two independent feed additives intended to be added to feed materials to reduce antinutritional factors. These two microbial cultures are the subject of the evaluation.

The bacterial species *B. subtilis* 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 requires evidence that the strain lacks toxigenic potential and does not show resistance to antibiotics of human and veterinary importance.

2. Characterisation

2.1. Characterisation of the active agents

2.1.1. Characterisation of *Bacillus subtilis*

The active agent is made of viable cells of *B. subtilis* of unknown origin. The strain is deposited in the Korean Culture Center of Microorganisms with the accession number KCCM 10673P.[7] It has not been genetically modified. Its identity was obtained by biochemical tests and by sequence analysis of the complete 16S rRNA gene.[8] Information on its genetic stability was not provided.

Antimicrobial susceptibility was tested by the disc agar technique and by broth dilution.[9] The tests included the battery of antibiotics listed in the Technical Guidance on the update of the criteria used in the assessment of bacterial resistance to antibiotics of human and veterinary importance (EFSA FEEDAP Panel, 2012). No minimum inhibitory concentration (MIC) value of the antimicrobials tested against the *B. subtilis* strain was identified above the cut-off values; therefore, *B. subtilis* KCCM 10673P is susceptible to the antibiotics tested.

The assessment of the toxigenic potential of *B. subtilis* KCCM 10673P was made in accordance with the Technical Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition (EFSA FEEDAP Panel, 2011). Polymerase chain reaction (PCR) testing for non-ribosomally synthesised peptides was carried out, and the primers for surfactin biosynthesis genes were those described by Tapi et al. (2010).[10] The results showed the strain to be positive for surfactin biosynthesis genes. Cytoxicity of the strain was assessed on Vero cells using the culture supernatants (Lindbäck and Granum, 2005).[11] No evidence of cytotoxicity was seen with the non-concentrated culture supernatants, as required by the revised Guidance (EFSA FEEDAP Panel, 2014). Consequently, this strain is considered to be non-toxigenic.

[7] Technical dossier/Supplementary information February 2015/Section II/Annexes 2.2.1.2.c.
[8] Technical dossier/ Supplementary information February 2015/Section II/Annexes 2.2.1.2.a,b.
[9] Technical dossier/Supplementary information February 2015/Section II/Annexes 2.2.2.2.a,b.
[10] Technical dossier/ Supplementary information February 2015/Section II/Annex 2.2.2.2.c.
[11] Technical dossier/ Supplementary information February 2015/Section II/Annex 2.2.2.2.m.

2.1.2. Characterisation of *Aspergillus oryzae*

The active agent is made of viable cells of *A. oryzae* of unknown origin. It is deposited in the Korean Culture Center of Microorganisms with the accession number KCTC 10258BP.[12] It has not been genetically modified. It was identified by sequence analysis of the DNA internal transcribed spacer and 5.8S rRNA gene.[13] Information on the genetic stability of the strain was not provided.

The absence of the metabolic pathways for aflatoxin production in *A. oryzae* KCTC 10258BP was investigated via PCR by searching for the following genes:

- *apa-2*, coding for the regulatory protein AflR, a transcriptional activator of aflatoxin biosynthesis (Chang et al., 1993);
- *ver-1* encoding versicolorin A dehydrogenase, which converts versicolorin A to sterigmatocystin (Chang et al., 1992; Skory et al., 1992);
- *omt-1* gene encodes sterigmatocystin-*O*-methyltransferase and is required for conversion of demethylsterigmatocystin and dehydrodemethylsterigmatocystin to sterigmatocystin and dihydrosterigmatocystin, respectively.

None of the genes of the biosynthetic pathways for aflatoxin production was detected in *A. oryzae* KCTC 10258BP, and this is considered an adequate approach to exclude aflatoxin synthesis (Kim et al., 2014).[14]

No information was provided on the potential for production of other secondary metabolites of concern, including antibiotics such as penicillin, whose biosynthetic pathways are known to occur in strains of *A. oryzae* (Inglis et al., 2013).

2.2. Characterisation of the additives[15]

The manufacturing process of *B. subtilis* is fully described in the dossier. The product is in liquid form. No minimum content of colony-forming units (CFU) per gram of additive was declared, and the only information available is based on an analysis of five batches of the pure culture, which shows a mean of 4.3×10^8 CFU/g (range: $3.7–5.3 \times 10^8$ CFU/g).[16]

The manufacturing process of *A. oryzae* is fully described in the dossier. The product is in solid form. is grown on a semi-solid medium containing wheat bran and the resulting biomass is spray dried. Similarly, no minimum content of colony-forming units per gram of additive was specified, and the only information available is based on an analysis of five batches of the pure culture, which shows a mean of 2.7×10^8 CFU/g (range: $2.2–3.0 \times 10^8$ CFU/g).[17]

No information on chemical and microbiological impurities was provided for the additives.

The particle size distribution and dusting potential of the *A. oryzae* additive were not provided.

Shelf-life data were provided in relation to the proposed use of the product (see section 2.3). Three batches of *B. subtilis* held under refrigeration were shown to be stable for five days.[18] No data for the *A. oryzae* culture were provided.

[12] Technical dossier/Supplementary information February 2015/Section II/Annexes 2.2.1.2.d.
[13] Technical dossier/Supplementary information February 2015/Section II/Annex 2.2.1.2.e.
[14] Technical dossier/Supplementary information February 2015/Section II/Annex 2.2.2.2.p.
[15] This section has been amended following the provisions of Article 8(6) and Article 18 of Regulation (EC) No 1831/2003.
[16] Technical dossier/Supplementary information February 2015/Annex 2.1.3.a.
[17] Technical dossier/Supplementary information February 2015/Annex 2.1.3.a.
[18] Technical dossier/Supplementary information February 2015/Section II.

The additives are foreseen for addition to feedingstuffs (e.g. soybean) as "substance for reduction of antinutritional factors". No further demonstration of the stability of the additives in feedingstuffs and of their homogeneous distribution is considered necessary, once the efficacy has been demonstrated.

2.3. Conditions of use

The applicant stated that the additives will not be marketed to feed manufacturers or farmers but used by the applicant to treat feed materials (e.g. soybean meal) for all animal species.[19]

The two additives are said to be combined only at the point of application to the target feed. The applicant indicates that the inclusion level is likely to be 2 % by weight of the total substrate (20 g/kg feed material). The ratio of the two cultures is described as 50/50 on a weight basis. A dose expressed in CFU/kg feed is not proposed.

2.4. Evaluation of the analytical methods by the European Union Reference Laboratory (EURL)

EFSA has verified the EURL report as it relates to the methods used for the control of the active agents in animal feed. The executive summary of the EURL report can be found in Annex A.

3. Safety

3.1. *Bacillus subtilis* KCCM 10673P

The species *B. subtilis* is considered by EFSA to be suitable for the QPS approach to safety assessment (EFSA, 2007; EFSA BIOHAZ Panel, 2013). In the view of the FEEDAP Panel, the identity of the production strain is established as *B. subtilis*. Moreover, the toxigenic potential and the antibiotic resistance qualifications have been met. Therefore, *B. subtilis* KCCM 10673P is presumed safe for the target species, the consumer and the environment.

No data are provided on the potential of the additive to induce skin/eye irritation or skin sensitisation. Therefore, the additive should be considered as having the potential to be a skin and eye irritant and a skin sensitiser and be treated accordingly.

3.2. *Aspergillus oryzae* KCTC 10258BP

A. oryzae is not considered by EFSA to be suitable for a QPS approach to safety assessment.

3.2.1. Safety for the target animals

No tolerance studies were submitted by the applicant. In the absence of these and of data on the potential for production of toxic secondary metabolites (other than aflatoxins) and antimicrobial activity in the *A. oryzae* additive, the FEEDAP Panel cannot draw conclusions on its safety for target animals given feed materials produced using the additive.

3.2.2. Safety for the consumer

The additive is intended to grow in the target feed. In the absence of information on the potential for production of toxic secondary metabolites (other than aflatoxins), the FEEDAP Panel is not in the position to assess whether hazardous residues derived from the use of *A. oryzae* KCTC 10258BP are present in animal tissues or products.

3.2.3. Safety for the user

No information was available on the particle size and dusting potential of the additive. No data were provided on the potential of the additive to induce skin/eye irritation or skin sensitisation. Therefore, the additive should be considered as having the potential to be a skin and eye irritant and a skin sensitiser

[19] Technical dossier/Supplementary information February 2015/Section II.

and be treated accordingly. The potential for users to be exposed to dust from the additive could not be excluded. In addition, given the proteinaceous nature of the active agent, the additive should also be considered to have the potential to be a respiratory sensitiser and treated accordingly.

3.2.4. Safety for the environment

A. oryzae is ubiquitous in nature. Its use as a technological feed additive is not expected to pose a risk to the environment.

4. Efficacy

The function of the additives is described as a treatment for feed materials (e.g. soybean) to reduce antinutritional factors such as raffinose, stachyose and trypsin inhibitor factor.

An outline description of a single *in vitro* study was provided in which the concentrations of stachyose, raffinose and trypsin inhibitor were measured in multiple batches of soybean meal after treatment with the additives.[20] However, the values of these antinutritional factors before treatment were not determined. Instead reference was made to published values of different soybeans used in animal nutrition (conventional and low-trypsin-inhibitor raw or heated soybean and soybean meal).[21] The study suffers from the following deficiencies: (i) the dose of the additives added to the feed material is not reported: (ii) the feed material used is not characterised (e.g. raw or heated soybean, whole grain or meal); (iii) the initial concentration of antinutritional factors was not measured; (iv) no demonstration of efficacy for feed materials other than soybean was provided.

Considering the above, the FEEDAP Panel is not in a position to draw conclusions on the efficacy of the additives in reducing antinutritional factors in soybean and other feed materials.

CONCLUSIONS

The additives *A. oryzae* KCTC 10258BP and *B. subtilis* KCCM 10673P were insufficiently characterised.

The liquid preparation *B. subtilis* KCCM 10673P is presumed safe for the target species, consumers and the environment. It should be considered to be a skin and eye irritant and a skin sensitiser.

Concerning the solid preparation of *A. oryzae* KCTC 10258BP, in the absence of data on toxic secondary metabolites (other than aflatoxins) and on the potential for antibiotic production, the FEEDAP Panel cannot draw conclusions on its safety for the target species and the consumer. The additive should be considered to be a skin and eye irritant and a skin and respiratory sensitiser. Its use is not expected to pose a risk to the environment.

The FEEDAP Panel cannot draw conclusions on the efficacy of the additives in reducing antinutritional factors in soybean and other feed materials.

REMARK

In the view of the FEEDAP Panel, the functional group "substances for the reduction of antinutritional factors" needs a definition. The endpoints for assessing the efficacy and the safe use of any additive assigned to this functional group should be defined.

[20] Technical dossier/Supplementary information February 2015/Section IV/Annexes 4.1a–d.
[21] Technical dossier/Supplementary information September 2013/Section IV/Annex 4.1d.

DOCUMENTATION PROVIDED TO EFSA

1. Dossier PepSoyGen-C. February 2009. Submitted by Regal BV on behalf of Nutraferma Co.
2. Dossier PepSoyGen-C. Supplementary information. September 2013. Submitted by Regal BV on behalf of Nutraferma Co.
3. Dossier PepSoyGen-C. Supplementary information. February 2015. Submitted by Regal BV on behalf of Nutraferma Co.
4. Evaluation report of the Community Reference Laboratory for Feed Additives on the methods of analysis for PepSoyGen-C.
5. Comments from Member States received through the ScienceNet.

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Skory CD, Chang P-K, Cary J and Linz JE, 1992. Isolation and characterization of a gene from *Aspergillus parasiticus* associated with the conversion of versicolorin A to sterigmatocystin in aflatoxin biosynthesis. Applied Environmental Microbiology, 58, 3527–3537.

Tapi A, Chollet-Imbert M, Scherens B and Jacques P, 2010. New approach for the detection of non-ribosomal peptide synthetase genes in *Bacillus* strains by polymerase chain reaction. Applied Microbiology and Biotechnology, 85, 1521–1531. doi: 10.1007/s00253-009-2176-4

ANNEX

Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Methods of Analysis for PepSoyGen-C[1]

In the current application authorisation is sought for the probiotic PepSoyGen-C, which consists of two active agents *Bacillus subtilis GR-101* and *Aspergillus oryzae GB-107*,[2] under the category 'zootechnical additives', functional group 'other zootechnical additives' according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorization is sought for the use of *Bacillus subtilis GR-101* and *Aspergillus oryzae GB-107* for piglets, chicken for fattening, calves for fattening and for rearing, fish (salmonidae and other fish), lambs and goats for rearing and fattening and for dogs.[3] The product is to be used to treat feed materials to diminish anti-nutritional factors and will enter animals in live form. The proposed conditions of use do not include minimum or maximum concentrations of the feed additive. According to the applicant the *product* as such will not be placed on the market, but feed materials previously treated with the product.

For the enumeration of *Bacillus subtilis GR-101* in *premixtures* and *feedingstuffs* the CEN method (EN 15784) has been validated at a range between 10^5 and 10^9 CFU/g. The performance characteristics of the CEN method reported after logarithmic transformation of measured values (CFU) are:

- for the *premixtures*: (1) a standard deviation for *repeatability* (s_r) of 0.09 $\log_{10}$ CFU/g and (2) a standard deviation for *reproducibility* (s_R) of 0.32 $\log_{10}$ CFU/g.
- for the *feedingstuffs*: (1) $s_r = 0.07$ $\log_{10}$ CFU/g and (2) $s_R = 0.35$ $\log_{10}$ CFU/g and
- a limit of detection (LOD) of 1×10^5 CFU/kg in *feedingstuffs*.

However, since this specific application does not include target levels of *Bacillus subtilis GR-101* in *feedingstuffs*, the EURL cannot evaluate the suitability of this CEN standard.

For the enumeration of *Aspergillus oryzae GB-107* the applicant proposes internationally recognised US FDA/CFSAN BAM spread plate method for enumeration of yeasts, moulds and mycotoxins. The applicant considers that further validation or verification is not necessary since this is an official US method. However another international standard exists for the enumeration of yeasts and moulds (ISO 21527–1). No performance characteristics of this spread plate method were provided except the LOD of 1 x 10^5 CFU/kg in feed. As this method was not tested on the product, the EURL cannot evaluate the suitability of this ISO method for official control. Molecular methods were used by the applicant for identification of active agents. The EURL recommends for official control for *Bacillus subtilis GR-101*, Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for bacterial identification. For *Aspergillus oryzae GB-107* the EURL recommends for official control Polymerase Chain Reaction (PCR), generally recognised standard methodology for identification of yeasts and moulds.

Further testing or validation is not considered necessary.

[1] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2009-0007.pdf
[2] GR-101 and GB-107 are in-house identifiers for *Bacillus subtilis* KCCM 10673P and *Aspergillus oryzae* KCTC 10258BP, respectively.
[3] In the course of the assessment, the applicant requested a change in the category from "zootechnical additive" to "technological additive", in the functional group from "other zootechnicals" to "substances for the reduction of anti-nutritional factors" and in the target animals from the above list to "all animal species".