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Safety and efficacy of L-tryptophan produced by fermentation with *Escherichia coli* CGMCC 7.248 for all animal species

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Abstract

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 L-tryptophan produced by fermentation with a genetically modified strain of *Escherichia coli* CGMCC 7.248 when used as a nutritional additive in feed and water for drinking for all animal species and categories. The production strain *E. coli* CGMCC 7.248 and its recombinant DNA were not detected in the final product. The product L-tryptophan, manufactured by fermentation with *E. coli* CGMCC 7.248, does not give rise to any safety concern with regard to the genetic modification of the production strain. L-Tryptophan produced by *E. coli* CGMCC 7.248 is safe for non-ruminant target species. The use of unprotected L-tryptophan in ruminant feed should be avoided. L-Tryptophan produced by fermentation by *E. coli* CGMCC 7.248 is safe for the consumer. The level of endotoxins present in the product and its dusting potential indicate an inhalation risk for the user. L-Tryptophan produced by *E. coli* CGMCC 7.248 is not a skin or eye irritant but it is a dermal sensitiser. The use of L-tryptophan produced by *E. coli* CGMCC 7.248 in animal nutrition does not pose a risk to the environment. The product under assessment is regarded as an effective source of the amino acid L-tryptophan for all non-ruminant species. For the supplemental L-tryptophan to be as efficacious in ruminants as in non-ruminant species, it requires protection against degradation in the rumen.

Keywords: nutritional additive, amino acid, L-tryptophan safety, efficacy, genetically modified microorganism

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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 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 Andrés Pintaluba, S.A.[2] for authorisation of the product Feed Grade L-tryptophan (L-tryptophan), produced by fermentation with *Escherichia coli* CGMCC 7.248 when used as a feed additive for all animal species (category: nutritional additives; functional group: amino acids, salts and analogues).

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). The particulars and documents in support of the application were considered valid by EFSA as of 19 July 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 Feed Grade L-tryptophan (L-tryptophan) produced by a strain of *E. coli* CGMCC 7.248, when used under the proposed conditions of use (see Section 3.1.4).

1.2. Additional information

L-Tryptophan (minimum content of 98% on dry matter basis) produced by fermentation with six strains of *E. coli* (KCCM 11132P, DSM 25084, FERM BP-11200, FERM BP-11354, CGMCC 7.59 or CGMCC 3667) is currently authorised for use as a nutritional additive for all animal species.[3] The product under assessment, L-tryptophan (minimum 98%) produced by a genetically modified strain of *E. coli* CGMCC 7.248, has not been previously authorised as a feed additive in the European Union (EU).

L-Tryptophan is authorised for use in food,[4] cosmetics[5] and as a veterinary medicinal product.[6,7]

L-Tryptophan is described in the European Pharmacopoeia (2017), monograph 01/2017:1272.

The EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) published nine opinions on the safety and efficacy of L-tryptophan produced by different strains of *E. coli* for all animal species (EFSA FEEDAP Panel, 2013, 2014a,b, 2015a,b, 2016a,b, 2017a,b).

The Panel on Dietetic Products, Nutrition and Allergies (NDA) of EFSA issued a scientific opinion on the substantiation of health claims related to L-tryptophan (EFSA NDA Panel, 2011). The Panel on Nutrition, Dietetic Products, Novel Food and Allergy of the Norwegian Scientific Committee for Food Safety (VKM) published an opinion on histidine, methionine, S-adenosylmethionine and tryptophan added to foods and drinks and in food supplements (VKM, 2013) and another on L-tryptophan in food supplements and energy drinks (VKM, 2016).

[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] Andrés Pintaluba S.A., Prudenci Bertrana 5, 43206 Reus, Spain. Representing Henan Julong Biological Engineering Co., Ltd.
[3] Commission Implementing Regulation (EU) 2017/873 of 22 May 2017 concerning the authorisation of L-tryptophan produced by *Escherichia coli* as a feed additive for all animal species, OJ L 134, 22.5.2017, p. 14.
[4] Regulation (EU) No 609/2013 of the European Parliament and of the Council of 12 June 2013 on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control and repealing Council Directive 92/52/EEC, Commission Directives 96/8/EC, 1999/21/EC, 2006/125/EC and 2006/141/EC, Directive 2009/39/EC of the European Parliament and of the Council and Commission Regulations (EC) No 41/2009 and (EC) No 953/2009, OJ L 181, 29.6.2013, p. 35.
[5] Commission Decision of 9 February 2006 amending Decision 96/335/EC establishing an inventory and a common nomenclature of ingredients employed in cosmetic products. OJ L 97, 5.4.2006, pp. 1–528.
[6] Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. OJ L 15, 20.1.2010, p. 1.
[7] Regulation (EC) No 470/2009 of the European Parliament and of the Council of 6 May 2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin, repealing Council Regulation (EEC) No 2377/90 and amending Directive 2001/82/EC of the European Parliament and of the Council and Regulation (EC) No 726/2004 of the European Parliament and of the Council. OL L 152, 16.6.2009, p. 11.

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[8] in support of the authorisation request for the use of L-tryptophan produced by fermentation with *E. coli* CGMCC 7.248 as a feed additive.

The FEEDAP Panel used the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA or other expert bodies, peer-reviewed scientific papers, and other scientific reports, to deliver the present output.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of L-tryptophan produced by *E. coli* CGMCC 7.248 in animal feed. The Executive Summary of the EURL report can be found in Annex A.[9]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of Feed Grade L-tryptophan is in line with the principles laid down in Regulation (EC) No 429/2008[10] and the relevant guidance documents: Guidance on nutritional additives (EFSA FEEDAP Panel, 2012a), Technical guidance: Tolerance and efficacy studies in target animals (EFSA FEEDAP Panel, 2011), Technical Guidance for assessing the safety of feed additives for the environment (EFSA, 2008), Guidance for establishing the safety of additives for the consumer (EFSA FEEDAP Panel, 2012b), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012c), Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance (EFSA FEEDAP Panel, 2012d) and Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use (EFSA GMO Panel, 2011).

3. Assessment

The subject of the present assessment is L-tryptophan (minimum 98%) produced by fermentation with a genetically modified strain of *E. coli* (CGMCC 7.248). It is intended to be used as nutritional additive (functional group amino acids, salts and analogues) in feed for all animal species and categories.

3.1. Characterisation

3.1.1. Characterisation of the production organism

The additive is produced by a genetically modified strain of *E. coli,* which is deposited in the China General Microbiological Culture Collection Center (CGMCC) with deposition number CGMCC 7.248.[11] A bioinformatic analysis of the whole genome sequence (WGS) of the production strain confirmed the identity of the production strain as an *E. coli* K-12 (MG1655) derivative [redacted]

E. coli CGMCC 7.248 was tested for antibiotic susceptibility using broth microdilution using the battery of antibiotics tested that EFSA recommended (EFSA FEEDAP Panel, 2012d) for *E. coli*. All minimum inhibitory concentration values were below the corresponding cut-off values defined by the FEEDAP Panel.[13] [redacted]

[8] FEED dossier reference: FAD-2017-0019.
[9] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2017-0019_tryptophan.pdf
[10] 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.
[11] Technical dossier/Section II/Annex_II_2_1a.
[redacted]
[13] Technical dossier/Section II/Annex_II_2_1c.

3.1.1.1. Characteristics of the recipient or parental microorganism

The recipient strain is *E. coli* K-12 MG1655. *E. coli* K-12 MG1655 is well-characterised and its safety (non-pathogenicity) has been documented (Gorbach, 1978). The strain was shown to be ineffective in colonising the human gut (Smith, 1975). Its genome has been fully sequenced (Blattner et al., 1997; Hayashi et al., 2006).

3.1.1.2. Characteristics of the donor organism

[redacted]

3.1.1.3. Description of the genetic modification process

[redacted]

The production strain CGMCC 7.248 was subsequently obtained by [redacted] No added antimicrobial resistance genes were found in the genome of the production strain.

3.1.2. Manufacturing process

[redacted]

3.1.3. Characterisation of the additive

L-Tryptophan (International Union of Pure and Applied Chemistry (IUPAC) name: (2*S*)-2-amino-3-(1*H*-indol-3-yl) propanoic acid; synonyms: (*S*)-α-amino-1-*H*-indole-3-propanoic acid, l-α-aminoindole-3-propionic acid,-l-α-amino-3-indolepropionic acid, 2-amino-3-indolylpropanoic acid, l-β-3-indolylalanine) has the Chemical Abstracts Service (CAS) No 73-22-3 and European Inventory of Existing Commercial Chemical Substances (EINECS) No 200-795-6. The chemical formula is $C_{11}H_{12}N_2O_2$, the molecular weight is 204.23 g/mol. The structural formula is given in Figure 1.

[redacted]

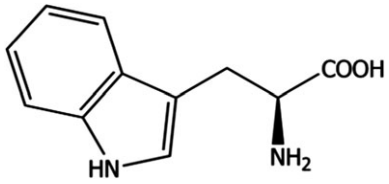

Figure 1: Structural formula of L-tryptophan

The L-tryptophan content of the product is specified as ≥ 98% on 'as is' basis, the other components being water (≤ 0.5%), crude ash (≤ 0.5%) and other amino acids (e.g. phenylalanine or leucine, ≤ 1%).[16] The analysis of five batches of L-tryptophan showed an average content of tryptophan of 98.8% on dry matter basis (range 98.3–99.7%), water 0.1%, ash 0.2%[17] and other amino acids as tyrosine or phenylalanine were below the limit of detection (LOD) or up to 0.031% phenylalanine (in only one batch).[18] On a dry matter basis, the amount of identified material is on average 99.0% (98.5–99.9%).

The specific optical rotation of five batches of the final product was on average –30.5° (range –29.1 to –31.7°),[19] which is within the range described in the European Pharmacopoeia (–30 to –33°) for this amino acid and confirms the identity of the L-enantiomer.[20]

3.1.3.1. Impurities

Three batches of the final product were analysed for heavy metals (lead, cadmium and mercury) and arsenic. Lead, cadmium and arsenic were below the LOD, and mercury ranged from LOD to 0.013 mg/kg.[21] Fluorine measured in three batches was < 10 mg/kg in all of them.[22]

Analysis of microbial contamination of the final product (three batches) indicated that *Salmonella* spp. (in 25-g samples) was absent; total aerobic bacterial count ranged from 5.5×10^2 to 2.2×10^3 CFU/g; aerobic spore formers ranged from 7.2×10^2 to 1.9×10^3 CFU/g and anaerobic spore formers from < 4 to 1.4×10^2 CFU/g; *E. coli*, *Staphylococcus* coagulase positive, *Clostridium perfringens*, sulfite-reducing bacteria, thermotolerant coliforms, total coliforms, faecal streptococci and Enterobacteriaceae were < 10 CFU/g; and yeast and filamentous fungi were < 10^2 CFU/g.[23]

Aflatoxins B1, B2, G1 and G2, ochratoxin A, zearalenone, T2 and HT-2 toxins, fumonisins B1 and B2 and deoxynivalenol had concentrations below the limit of quantification (LOQ) in three batches.[24]

Dioxins (polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs)) and dioxin-like polychlorinated biphenyls (DL-PCBs) were analysed in three batches of the final product. The sum of dioxins and DL-PCBs (WHO-PCDD/F-PCB-TEQ) ranged from 0.15 to 0.34 ng/kg L-tryptophan on dry matter basis.[25] Non-DL-PCBs were analysed in two batches and total of the six PCBs were 1,200 ng/kg on dry matter basis in both batches.

The endotoxin activity (three batches analysed by Limulus amoebocyte lysate test) ranged from 0.82 to 2.03 IU/mg.[26]

1,1′-Ethylidene-bis-L-tryptophan (EBT) and 1-methyl-1,2,3,4-tetrahydro-beta-carboline-3-carboxylic acid (MTCA), present in a specific brand of L-tryptophan produced by fermentation, were implicated in the eosinophilia–myalgia syndrome outbreak that occurred in humans in New Mexico in 1989 (Hertzman et al., 1990). The concentrations of EBT and MTCA were analysed in three batches of the final product. EBT concentrations were < 10 mg/kg in all cases and MTCA was not detected.[27] The

[16] Technical dossier/Section II/Annex II.1.3a.
[17] Technical dossier/Section II/Annex II.1.3b and Supplementary information November 2018/Annex II.1.3d. L-tryptophan analysed by method ISO 13904:2016.
[18] Technical dossier/Section II/Annex II.1.3c. Analysis performed by HPLC and LOD in % was 0.02.
[19] Technical dossier/Section II/Annex II.1.3b.
[20] European Pharmacopoeia 9th edition (2019), monograph 1/2015:1272.
[21] Technical dossier/Section II/Annex II.1.4e and supplementary information November 2018/Annex II.1.4g. LOD in mg/kg were 0.01 for mercury and arsenic, 0.02 for lead and 0.5 for cadmium.
[22] Technical dossier/Section II/Annex II.1.4c.
[23] Technical dossier/Section II/Annex II.1.4a.
[24] Technical dossier/Section II/Annex II.1.4a. LOQ (in μg/kg) was 2 for aflatoxins, ochratoxin A and T-2 toxin; 10 for zearalenone and fumonisins B1 and B2; and 20 for HT-2 toxin and deoxynivalenol.
[25] Technical dossier/Section II/Annex II.1.4c and II.1.4d.
[26] Technical dossier/Section II/Annex II.1.4b.
[27] Technical dossier/Section II/Annexes II.1.4a and II.1.4b and supplementary information November 2018/Annex II.1.4f. EBT and MTC were analysed by HPLC DAD.

maximum permitted content of EBT in L-tryptophan, as specified by the European Pharmacopoeia (2017), is 10 mg/kg.

No viable cells of the production strain were found in three batches of the concentrate of the final product (each tested in triplicate). Tests were done by incubating a 1% solution of 0.2 g of product in non-selective medium for 24 and 48 h at 37°C and plating 0.2 mL of these cultures on selective solid medium and incubating the plates at 30°C for 2 days.[28]

The absence of recombinant DNA of the production strain was confirmed in three samples of 1 g of the final product tested in triplicate [redacted]

Overall, the data on undesirable contaminants (chemical and microbiological) as well as on tryptophan impurities do not give rise to safety concerns.

3.1.3.2. Physical characteristics

The product under assessment is a white or slightly yellow crystalline powder with slight odour. Tapped density ranged from 380 to 390 kg/m^3 and bulk density from 200 to 210 kg/m^3.[23] It is sparingly soluble in water. The pH measured in five batches ranged from 5.9 to 6.3.[19]

The particle size distribution (three batches analysed by laser diffraction) showed that the percentages of particles having a diameter below 100, 50 and 10 μm were 98%, 69% and 15% (v/v), respectively.[30] The dusting potential (three batches analysed by the Stauber–Heubach method) ranged from 0.77 to 0.96 g/m^3.[31]

3.1.3.3. Stability and homogeneity

The shelf-life of the additive (three batches, commercial packaging consisting in polyethylene bags, protected from light) was tested at 25°C and at 40°C for 29 and 6 months, respectively.[32] No losses were observed at 25°C after 29 months and up to 1% at 40°C after 6 months.

The stability of three batches in three different vitamin–mineral premixtures: one for piglets, another for chickens for fattening (both without choline chloride) and a third one for pregnant sows (containing 50,000 mg choline chloride/kg) at a supplementation rate of 0.51% were tested at 25°C for 6 months. The premixtures were packed as described above. The losses observed after the 6-month period were 2.6%, 0% and 9% in premixtures for piglets, chickens for fattening and sows, respectively.[33]

Stability in complete feeds was tested in three batches of complete feeds for piglets (based on barley, soybean meal, whey, maize and fat), for chicken for fattening (based on maize and soybean meal) or for pregnant sows (based on maize, sunflower and beet pulp) at a supplementation rate of 0.06% tryptophan, after storage at 25°C for 3 months. Mash and pelleted feed were tested. The packaging was the one described above. The pelleting temperatures were ~ 60°C, ~ 50°C and ~ 60°C and the pelleting process induced a loss of 11%, 4% or 1% of the supplemented additive, respectively. For piglets feed, after the 3-month period, the observed losses were of 12% and 7% in mash feed and in pelleted feed, respectively.[34] For chicken for fattening feed, the observed loss in mash feed was 11% and there was no loss in pelleted feed. For pregnant sows feed, no losses were observed in mash or in pelleted feed.

The capacity of the additive to distribute homogeneously in the premixture described above was studied by analysing 10 subsamples. The coefficient of variation (CV) was 4.1%.[35]

The capacity of the additive to distribute homogeneously in the complete feed for piglets described above was studied analysing 10 subsamples, both of mash and pelleted feed. The CVs were 1.0% and 1.4%, respectively.[36]

[28] Technical dossier/Section II/Annex II.2.1c.

[redacted]

[30] Technical dossier/Section II/Annex II.1.5c.

[31] Technical dossier/Section II/Annex II.1.5a.

[32] Technical dossier/Section II/Annexes II.4.1a and II.4.1b.

[33] Technical dossier/Section II/Table II.23 and Annexes II.4.1d and II.4.1[e] and supplementary information November 2018/Annexes II.4.1k, II.4.1m and II.4.1o.

[34] Technical dossier/Section II/Annexes II.4.1f and II.4.1g and supplementary information November 2018/Annexes II.4.1q and II.4.1s.

[35] Technical dossier/Section II/Annex II.4.1d and II.4.2b.

[36] Technical dossier/Section II/Annex II.4.1f and II.4.2c.

3.1.3.4. Physicochemical incompatibilities

No physico-chemical incompatibilities in feed are expected with other additives, medicinal products or feed materials.

3.1.4. Conditions of use

It is proposed that L-tryptophan will be used in feeds to achieve an adequate amino acid profile and to meet the L-tryptophan requirements for all animal species. It can be added directly to feedingstuffs. No inclusion levels have been proposed, as the requirements, in quantitative terms, depend on the species, the physiological state of the animal, the performance level, the environmental conditions and the amino acid composition of the unsupplemented diet.

3.2. Safety

3.2.1. Safety aspects of the genetic modification

The recipient organism *E. coli* K-12 MG1655 is considered to be safe. The production strain CGMCC 7.248 [redacted] Those traits do not raise any safety concern. No added antimicrobial resistance genes were found in the genome of the production strain.

The applicant provided sufficient information that neither the production strain nor its recombinant DNA are present in the final product. The product L-tryptophan, manufactured by fermentation with *E. coli* CGMCC 7.248, does not give rise to any safety concern with regard to the genetic modification of the production strain.

3.2.2. Safety for the target species

Tolerance studies are not normally required for highly purified amino acids. Such tolerance studies with a certain indispensable amino acid will inevitably result in amino acid imbalances, with depression of feed intake and hence impaired performance and increased nitrogen excretion. This is also the case for the product under application which contains 98.8% tryptophan and $\leq$ 1% unidentified material on a dry matter basis. The endotoxin activity ranged from 0.82 to 2.03 IU/mg. These values are compared with ca 1,000 IU/mg commonly found in feedingstuffs (Cort et al., 1990). Therefore, at the usual conditions of use of the additive in feed, the endotoxins added by the additive would be insignificant compared to the background in feed. Therefore, the FEEDAP Panel considers that safety concerns for target species are unlikely to arise from this product. Since no particular safety concerns arose from the *E. coli* CGMCC 7.248, the FEEDAP Panel confirms the safety for the target species of L-tryptophan produced by this strain.

The L-tryptophan requirements of the target animal species and the safety of the use of this essential amino acid in non-ruminant and ruminant nutrition were summarised in previous opinions of the EFSA FEEDAP Panel (2013, 2015a).

Given the high purity of the product, the FEEDAP Panel considers that the use of L-tryptophan produced with *E. coli* CGMCC 7.248 is safe for non-ruminant target species when used to supplement the diet in appropriate amounts. The FEEDAP Panel reiterates that ruminal metabolism of unprotected L-tryptophan may result in the production of toxic quantities of 3-methylindole (skatole), which causes pulmonary disease (fog fever; emphysema) in cattle and goats (Hammond et al., 1978). Consequently, only a protected form of L-tryptophan should be used in ruminants.

3.2.2.1. Conclusions on safety for the target species

The use of L-tryptophan produced using *E. coli* CGMCC 7.248 in supplementing feed to compensate for tryptophan deficiency in feedingstuffs is safe for non-ruminant target species. The use of unprotected L-tryptophan in ruminant feed poses safety concerns.

3.2.3. Safety for the consumer

The absorption and metabolic fate of L-tryptophan were described in a previous opinion (EFSA FEEDAP Panel, 2013).

The amino acid L-tryptophan, supplemented to feed, will be incorporated into proteins of tissues and/or products of animal origin and any of their potential excess will be metabolised and excreted as

urea/uric acid and carbon dioxide. Therefore, the composition of tissues and products of animal origin will not be affected by the use of L-tryptophan in animal nutrition.

The product under assessment is produced by fermentation. Concerns for the consumer would derive not from the amino acid itself, which will be incorporated into proteins, but from possible residues from fermentation. Considering that (1) the product originating from *E. coli* CGMCC 7.248 is highly purified (98.8% L-tryptophan and < 1% unidentified material on a dry matter basis) and (2) the concentrations of EBT and MTCA are < 10 mg/kg additive or below LOD, respectively, no additional toxicological data are required.

3.2.3.1. Conclusions on safety for the consumer

L-Tryptophan product produced by fermentation with *E. coli* CGMCC 7.248 presents no concern to consumers of animal products.

3.2.4. Safety for the user

The applicant provided an acute inhalation toxicity study, dermal and eye irritation studies and a skin sensitisation study performed with the L-tryptophan produced by *E. coli* CGMCC 7.248.[37]

3.2.4.1. Effects on the respiratory system:

`Practically all particles have a diameter < 100 μm and the dusting potential is up to 1 g/m^3 (see Section 3.1.3).

In an acute inhalation toxicity study performed in accordance with the Organisation for Economic Co-operation and Development (OECD) Guideline 436,[38] the mass median aerodynamic diameter (MMAD) of the test item (98.1% on dry matter by potentiometric method) was found too large (approx. 28.4 μm with 14% of particles < 4 μm) for the generation trials. As the proper atmosphere to conduct the test could not be established, the FEEDAP Panel cannot conclude on the potential acute inhalation toxicity of the additive under assessment.

The effects of endotoxin inhalation and the exposure limits have been described in a previous opinion (EFSA FEEDAP Panel, 2015a). The scenario used to estimate the exposure of persons handling the additive to endotoxins in the dust, based on the EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012c) is described in the Appendix A. The health-based recommended threshold for the quantity of inhaled endotoxins per working day is 900 IU, derived from provisional occupational exposure limits given by the Dutch Expert Committee on Occupational Safety (DECOS) (Health Council of the Netherlands, 2010) and the UK Health and Safety Executive (HSE, 2013). Based upon the calculation of the potential endotoxin content in dust, the inhalation exposure could be up to 1,083 endotoxin IU per working day, indicating thus an inhalation exposure to endotoxins for persons handling the additive.

3.2.4.2. Effects on skin and eyes

In an acute dermal irritation study in accordance with OECD Guideline 404, 0.5 g of the product under assessment was applied on the skin of three rabbits for 4 h.[39] No lesions were observed and the additive is considered not irritant for the skin.

In an eye irritation study in accordance with OECD 405, 0.1 g of the test item was introduced in the conjunctival sac of the eye of three female New Zealand white rabbits and surveyed up to 72 h post-instillation.[40] No ocular changes were observed at 48 and 72 h. The product is considered not irritant for the eye.

In a skin sensitisation study (Magnusson and Kligman maximisation test) in accordance with OECD Guideline 406, the test item caused sensitisation reaction (discrete or patchy erythema) in 30% of the animals (allergenicity grade III). Consequently, the additive should be considered as a skin sensitiser.[41]

3.2.4.3. Conclusions on safety for the user

The level of endotoxins present in the product and its dusting potential indicate an inhalation risk for the user. The additive is not a skin and eye irritant but it is a skin sensitiser.

[37] Technical dossier/Supplementary information November 2018/2 tryptophan reply/Reply to question 12.
[38] Technical dossier/Seciton III/Annex III.3a.
[39] Technical dossier/Section III/Annex III.3c.
[40] Technical dossier/Section III/Annex III.3b.
[41] Technical dossier/Section III/Annex III.3d.

3.2.5. Safety for the environment

The production strain *E. coli* CGMCC 7.248 and its recombinant DNA were not detected in the final product. The product does not pose any environmental safety concern associated with the genetic modification of the production strain. L-Tryptophan produced using *E. coli* CGMCC 7.248 is safe for the environment.

The amino acid L-tryptophan is a physiological and natural component of the proteins of living organisms. When given to animals, it is not excreted as such, but as urea/uric acid, indole-related compounds and carbon dioxide. The use of the product L-tryptophan in animal nutrition would not lead to any localised increase in the concentration in the environment.

The use of L-tryptophan produced by *E. coli* CGMCC 7.248 in animal nutrition does not pose a risk to the environment.

3.3. Efficacy

Efficacy studies are not required for amino acids naturally occurring in the proteins of plants and animals. The nutritional role of L-tryptophan is well established in the scientific literature. The additive feed grade L-tryptophan is regarded as an effective source of the amino acid L-tryptophan.

Overdosing of supplemental L-tryptophan may increase skatole and indole in the hind gut resulting in boar taint of pork (Zamaratskaia and Squires, 2008).

The efficacy of this essential amino acid in non-ruminant and ruminant nutrition was summarised in a previous opinion of the EFSA FEEDAP Panel (2014b). Supplemental L-tryptophan is degraded by ruminal microbiota if not given in a protected form.

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[42] and Good Manufacturing Practice.

4. Conclusions

The production strain *E. coli* CGMCC 7.248 and its recombinant DNA were not detected in the final product. The product L-tryptophan, manufactured by fermentation with *E. coli* CGMCC 7.248, does not give rise to any safety concern with regard to the genetic modification of the production strain.

The use of L-tryptophan produced by *E. coli* CGMCC 7.248 is safe for non-ruminant target species. The use of unprotected L-tryptophan in ruminant feed poses safety concerns.

As L-tryptophan produced by fermentation by *E. coli* CGMCC 7.248 is highly pure (≥ 98.8% on dry matter basis) and because neither the amino acid L-tryptophan nor its metabolites accumulate in animal tissues/products, and the concentrations of EBT and MTCA are low (< 10 mg/kg additive each), this product presents no concern to consumers of animal products.

The level of endotoxins present in the product and its dusting potential indicate an inhalation risk for the user. L-Tryptophan produced by *E. coli* CGMCC 7.248 is not a skin or eye irritant but it is a dermal sensitiser.

The use of L-tryptophan produced by *E. coli* CGMCC 7.248 in animal nutrition does not pose a risk to the environment.

The product under assessment is regarded as an effective source of the amino acid L-tryptophan for all non-ruminant species. For the supplemental L-tryptophan to be as efficacious in ruminants as in non-ruminant species, it requires protection against degradation in the rumen.

5. Recommendations

It is recommended that specification of the additive complies with the European Pharmacopeia with regards L-tryptophan related impurities such as 1,1′-ethylidene-bis- L-tryptophan (EBT) < 10 mg/kg (European Pharmacopoeia, 2017).

[42] 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.

Documentation provided to EFSA

1) Feed grade L-tryptophan produced with Escherichia coli CGMCC 7.248. 2017. Submitted by Andrés Pintaluba S.A.
2) Feed grade L-tryptophan produced with Escherichia coli CGMCC 7.248. Supplementary information. November 2018. Submitted by Andrés Pintaluba S.A.
3) Evaluation report of the European Union Reference Laboratory for Feed Additives on the Methods of Analysis for L-tryptophan produced by *Escherichia coli* CGMCC 7.248.
4) Comments from Member States.

Chronology

Date	Event
31/3/2017	Dossier received by EFSA
7/6/2017	Reception mandate from the European Commission
19/7/2017	Application validated by EFSA – Start of the scientific assessment
19/10/2017	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: manufacturing process, characterisation of the additive and of the production microorganism, stability and safety for the user*
19/10/2017	Comments received from Member States
17/11/2017	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
26/11/2018	Reception of supplementary information from the applicant - Scientific assessment re-started
22/1/2019	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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Abbreviations

CAS	Chemical Abstracts Service
CFU	colony forming unit
CGMCC	China General Microbiological Culture Collection Center
CV	coefficient of variation
DL-PCB	dioxin-like polychlorinated biphenyls
DECOS	Dutch Expert Committee on Occupational Safety
DM	dry matter
EBT	1,1′-ethylidene-bis-L-tryptophan
EINECS	European Inventory of Existing Commercial Chemical Substances
EURL	European Union Reference Laboratory
FCC	Food Chemical Codex
FD	fluorescence detection
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
GMO	EFSA Panel on Genetically Modified Organisms
HPLC-DAD	high-performance liquid chromatography with diode array detection
HSE	Health and Safety Executive
IUPAC	International Union of Pure and Applied Chemistry
LOD	limit of detection
LOQ	limit of quantification
MMAD	mass median aerodynamic diameter
MTCA	1-methyl-1,2,3,4-tetrahydro-beta-carboline-3-carboxylic acid
NDA	EFSA Panel on Dietetic Products, Nutrition and Allergies
OECD	Organisation for Economic Co-operation and Development
PCDD	polychlorinated dibenzodioxin
PCDF	polychlorinated dibenzofuran
VKM	Norwegian Scientific Committee for Food Safety
TEQ	toxic equivalent
WHO	World Health Organization
WGS	whole genome sequence

Appendix A – Calculation of exposure to endotoxins

Calculation of maximum acceptable levels of exposure from feed additives

The probable exposure time according to EFSA guidance (EFSA FEEDAP Panel, 2012c) for additives added in premixtures assumes a maximum of 40 periods of exposure per day, each comprising 20 s = 40 × 20 = 800 s/day. With an uncertainty factor of 2, maximum inhalation exposure would occur for 2 × 800 = 1,600 s = 0.444 h/day. Again, assuming a respiration volume of 1.25 m^3/h, the inhalation volume providing exposure to potentially endotoxin-containing dust would be 0.444 × 1.25 = 0.556 m^3/day. This volume should contain no more than 900 IU endotoxin, so the dust formed from the product should contain no more than 900/0.556 = 1,619 IU/m^3.

Calculation of endotoxin content of dust

Two key measurements are required to evaluate the potential respiratory hazard associated with the endotoxin content of the product (the dusting potential of the product, expressed in g/m^3, and the endotoxin activity of the dust, determined by the Limulus amoebocyte lysate assay (expressed in IU/g)). If data for the dust are not available, the content of endotoxins of the product can be taken instead. If the content of endotoxins of the relevant additive is a IU/g and the dusting potential is b g/m^3, then the content of endotoxins of the dust, c IU/m^3, is obtained by simple multiplication, a × b. This resulting value is further used for calculation of the potential inhalatory exposure of users to endotoxins from the additive under assessment (Table A.1) (EFSA FEEDAP Panel, 2012c).

Table A.1: Estimation of user exposure to endotoxins from the additive L-tryptophan produced by *Escherichia coli* CGMCC 7.248, including consideration of using a filter mask FF P2 or FF P3 as a preventative measure

Calculation	Identifier	Description	Amount	Source
	a	Endotoxin content IU/g product	2,030	Technical dossier
	b	Dusting potential (g/m^3)	0.96	Technical dossier
a × *b*	*c*	Endotoxin content in the air (IU/m^3)	1,949	
	d	No of premixture batches made/working day	40	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012c)
	e	Time of exposure (s) per production of one batch	20	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012c)
d × *e*	*f*	Total duration of daily exposure/worker (s)	800	
	g	Uncertainty factor	2	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012c)
f × *g*	*h*	Refined total duration of daily exposure/worker (s)	1,600	
h/3,600	*i*	Refined total duration of daily exposure (h)	0.44	
	j	Inhaled air (m^3) per eight-hour working day	10	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012c)
j/8 × *i*	*k*	Inhaled air during exposure (m^3)	0.56	
c × *k*	*l*	**Endotoxin inhaled (IU) during exposure per eight-hour working day**	**1,083**	
	m	Health-based recommended exposure limit of endotoxin (IU/m^3) per eight-hour working day	90	Health Council of the Netherlands (2010)
m × *j*	*n*	**Health-based recommended exposure limit of total endotoxin exposure (IU) per eight-hour working day**	**900**	
l/10		Endotoxins inhaled (IU) per eight-hour working day reduced by filter mask FF P2 (reduction factor 10)	108	
l/20		Endotoxins inhaled (IU) per eight-hour working day reduced by filter mask FF P3 (reduction factor 20)	54	

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for L-tryptophan produced by *Escherichia coli* CGMCC 7.248

In the current application, authorisation is sought under Article 4(1) for L-tryptophan produced by *Escherichia coli* CGMCC 7.248, under the category/functional group 3(c) 'nutritional additives'/'amino acids, their salts and analogues', according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for all animal species. L-Tryptophan is already authorised as a feed additive under Commission Directive 88/485/EEC.

For the quantification of L-tryptophan in the feed additive, the Applicant validated and further verified the method based on titration described in the European Pharmacopoeia monograph 01/2017:1272. For the quantification of L-tryptophan in premixtures and feedingstuffs, the Applicant submitted two single-laboratory validated and further verified analytical methods based on high-performance liquid chromatography with diode array detection (HPLC-DAD).

However, the EURL previously evaluated (i) the ring-trial validated Community method based on HPLC coupled with fluorescence detection (FD) for the quantification of L-tryptophan in feedingstuffs; and (ii) the ring-trial validated EN ISO 13904:2016 method 'Animal feeding stuffs – Determination of tryptophan content' for the quantification of L-tryptophan in feed additive and premixtures (containing more than 2% of tryptophan). Based on the performance characteristics available, the EURL recommends for official control these two ring-trial validated methods to quantify tryptophan in the feed additive, premixtures and/or feedingstuffs. In addition, the EURL identified the 'L-tryptophan monograph' of the Food Chemical Codex (FCC) for the identification of the feed additive.

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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.