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Safety and efficacy of L-tryptophan produced by fermentation with *Escherichia coli* KCCM 10534 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 non-genetically modified strain of *Escherichia coli* KCCM 10534 when used as a nutritional additive in feed and water for drinking for all animal species and categories. The production strain *E. coli* KCCM 10534 is safe for the production of L-tryptophan and it was not detected in the final product. The use of L-tryptophan produced using *E. coli* KCCM 10534 in supplementing feed to compensate for tryptophan deficiency in feedingstuffs is safe for non-ruminant target species. There may be a risk for an increased production of toxic metabolites when unprotected tryptophan is used in ruminants. The FEEDAP Panel has concerns on the safety of the simultaneous oral administration of L-tryptophan via water for drinking and feed due to possible amino acid imbalances. The use of L-Tryptophan produced by *E. coli* KCCM 10534 in animal nutrition raises no safety concerns to consumers of animal products and to the environment. The additive under assessment is considered not toxic by inhalation, it is not a skin or eye irritant and is not a skin sensitiser. The endotoxin activity of the additive and its dusting potential indicate a risk by inhalation for the users. The product L-tryptophan is regarded as an effective source of the amino acid L-tryptophan for all non-ruminant species. In order to be as efficacious in ruminants as in non-ruminants, it should be protected from ruminal degradation.

Keywords: nutritional additive, amino acid, L-tryptophan, safety, efficacy, *Escherichia coli*

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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 CJ Europe GmbH[2] for authorisation of the product L-tryptophan, feed grade, when used as a feed additive for all animal species (category: nutritional additives; functional group: amino acids, their 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 18 September 2018.

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 L-tryptophan produced by fermentation with *Escherichia coli* KCCM 10534, when used under the proposed conditions of use (see Section 3.1.5).

1.2. Additional information

The subject of the present assessment is L-tryptophan (minimum 98%) produced by a non-genetically modified strain of *E. coli* (KCCM 10534). L-Tryptophan produced by this bacterial strain had not been previously authorised as a feed additive in the European Union.

L-Tryptophan (≥ 98%) produced by fermentation with specific strains of *E. coli* is currently authorised for use as a nutritional additive in the European Union, under the functional group 'amino acids, their salts and analogues'.[3]

L-Tryptophan is authorised for use in food for nutritional purposes,[4] and for use in cosmetics.[5] It is authorised for use as a veterinary medical product without maximum residue limits.[6]

The EFSA Panel on Additives and Products or Substances used in Animal Feed published several 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, 2019a,b,c,d,e). 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).

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

The Norwegian Scientific Committee for Food Safety assessed the safety of L-tryptophan in food (VKM, 2013) 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] CJ Europe GmbH, Ober der Roeth 4, 65824 Schwalbach am Taunus, Germany.
[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, 23.5.2017, p. 14–17.
[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 2006141/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. 35–56.
[5] Commission Decision 2006/257/EC of 9 February 2006 amending Decision 96/335/EC establishing an inventory and a common nomenclature of ingredients employed in cosmetic products. OJ L 97/1, 5.4.2006, p. 598.
[6] Commission Regulation (EC) No 37/2010 of 22 December 2009, on pharmacologically active substances and their classification regarding maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. OJ L 15, 20.1.2010, pp. 1.

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[7] in support of the authorisation request for the use of L-tryptophan produced [redacted] with *E. coli* KCCM 10534 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, other scientific reports and experts' knowledge, 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 fermentation with *E. coli* KCCM 10534 in animal feed. The Executive Summary of the EURL report can be found in Annex A.[8]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of L-tryptophan produced by fermentation with *E. coli* KCCM 10534 is in line with the principles laid down in Regulation (EC) No 429/2008[9] and the relevant guidance documents: Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012a–d), Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2017c), Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018a), Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017d), Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017e), Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018b) and Guidance for assessing the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019f).

3. Assessment

The product subject of this application is L-tryptophan (≥ 98%) produced [redacted] with a non-genetically modified strain of *E. coli* (KCCM 10534). It is proposed to be used as a nutritional additive (functional group: amino acids, their salts and analogues) to feed and water for drinking in all animal species and categories.

3.1. Characterisation

3.1.1. Characterisation of the production organism

The production strain was deposited in the Korean Culture Collection of Microorganisms (KCCM) with accession number KCCM 10534.[redacted]

The production strain was identified as *E. coli* [redacted]

[7] FEED dossier reference: FAD-2018-0038.

[8] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2018-0038_tryptophan.pdf

[9] 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.

[redacted]

[13] Technical dossier/Section II.2.1.2.1.

[redacted] the strain is considered susceptible to those antibiotics.

The whole genome sequencing (WGS) of the production strain was interrogated for the presence of antimicrobial resistance (AMR) genes [redacted] No hits of concern were identified.

3.1.2. Manufacturing process

The dossier contains information on the production process. [redacted]

3.1.3. Characterisation of the product/active substance

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.

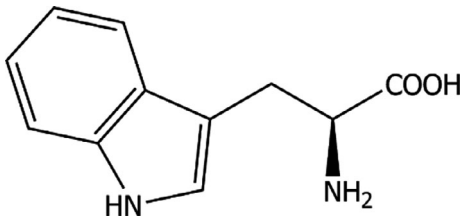

Figure 1: Structural formula of L-tryptophan

According to the specification, the product contains ≥ 98% L-tryptophan 'as is' and ≤ 1% moisture. The minimum L-tryptophan content on a dry matter (DM) basis is 99%.[18]

The analysis of five batches of the additive showed an average content of tryptophan of 98.4% on 'as is' basis (range 98.34–98.41%),[19] moisture ranged 0.39–0.43%, ammonium ranged 0.05–0.08%, glutamic acid ranged 0.04–0.05%, valine ranged 0.03–0.05%, lysine was 0.01% and ash ranged 0.13–0.16%.[20] On a dry matter basis, the tryptophan content was on average 98.8% (98.7–98.8%) and the amount of identified material was 99.1% in all batches. Although the specification was reached on 'as is' basis, none of the batches achieved the specification of minimum 99% L-tryptophan on DM basis.

The specific optical rotation was measured in three batches of the additive and was on average –31.2° (range –31.1 to –31.3°),[21] 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.[22]

[redacted]

[15] Technical dossier/Supplementary information September 2019/03_SIN_CJ_L-TRP.
[16] Technical dossier/Section II.2.2.2.
[redacted]
[18] Technical dossier/Section II/Annex II.1.1.
[19] Technical dossier/Section 2.6.1 and Annex II.6.1. Analytical method: HPLC with C18 column using potassium phosphate buffer as mobile phase.
[20] Technical dossier/Section II/Annex II.1.2. Analysis of L-tryptophan performed by HPLC, other amino acids by aa amino acid analyser (detection at 570 nm after post-column derivatisation with ninhydrin).
[21] Technical dossier/Section II/Annex II.1.3.
[22] European Pharmacopoeia monograph 1/2015:1272.

3.1.3.1. Undesirable substances

Three batches of the final product were analysed for heavy metals (lead, cadmium and mercury) and arsenic. All analytical values were below the limit of detection (LOD).[23]

In three batches analysed, nitrofurans (furazolidone, furaltadone, nitrofurazone and nitrofurantoin) and nitrofuran metabolites were below the corresponding LODs.[24] A multiresidue pesticide analysis showed that none of the 368 pesticides was detected.[25]

Analysis of microbial contamination of the final product (three batches) indicated that *Salmonella* spp. (25 g samples), *E. coli*, and coliforms were absent whereas total bacterial count, was $< 10^3$ colony forming unit (CFU)/g; yeasts and filamentous fungi were < 50 CFU/g.[26]

Mycotoxins were analysed in three batches of the final product. Aflatoxins (B1, B2, G1 and G2), ochratoxin A, zearalenone, deoxinivalenol and fumonisins (B1 and B2) were in concentrations below the corresponding LOD.[27]

The endotoxin activity of three batches of the final product was evaluated by the *Limulus* amoebocyte lysate assay (European Pharmacopoeia 2.6.14 method D) and the values ranged from 310 to 360 IU/g.[28]

The concentrations of 1,1′-ethylidene-bis-L-tryptophan (EBT) and of 1-methyl-1,2,3,4-tetrahydro-beta-carboline-3-carboxylic acid (MTCA) were analysed in three batches of the final product and were < 3 mg/kg in all cases.[29]

The presence of viable cells of the production strain in the final product was investigated [redacted] No colonies were detected.

3.1.3.2. Physic-chemical properties

The additive under assessment is a solid granulated pale brownish odourless crystalline product. The pH in 1% solution at 20°C ranges from 4.5 to 7. The bulk density ranges from 350 to 500 kg/m^3. The water solubility is 10.6 g/L at 20°C.[18] The pKa values are 2.38 (carboxyl function) and 9.39 (amino group) and the octanol/water partition coefficient (Log K_{ow}) is −1.46 (at pH 5).[31]

Analytical data (three batches analysed by Stauber–Heubach method) were submitted on the dusting potential of the product under assessment. The values ranged from 1.98 to 4.74 g/m^3.[32]

The particle size distribution (three batches analysed by sieving) showed that the fraction of particles < 125 μm and < 88 μm ranged from 64% to 68% and from 38% to 45%, respectively.[33]

3.1.3.3. Stability and homogeneity

No information on the shelf life, stability (in premixtures, feedingstuffs and water for drinking) and capacity of the additive under assessment to distribute homogeneously in feed was provided. The applicant provided information on the shelf life, stability in premixtures, feedingstuffs and water for drinking, and on the capacity of L-tryptophan to distribute homogeneously in feed performed with an L-tryptophan originating from different strains (*E. coli* KCCM 80135 or KCCM 80152) of the same producer. Such stability studies were described in previous opinions (EFSA FEEDAP Panel, 2017a, 2019). As the production process is the same and the product characteristics are very similar, the FEEDAP Panel considers that the results of those studies can be applicable to the product under assessment.

[23] Technical dossier/Section II/Annex II.1.3. LOD in μg/kg were 5 for mercury and 1 for cadmium, lead and arsenic.
[24] Technical dossier/Section II/Annex II.1.3. LOD (in μg/kg) was 0.09-0.15 for nitrofurans and 0.11-0.16 for nitrofuran metabolites.
[25] Technical dossier/Section II/Annex II.1.4. LOD (in μg/kg) was 0.5–8.
[26] Technical dossier/Section II/Annex II.1.3 and supplementary information November 2018/02 SIn CJ, response to question 2i.
[27] Technical dossier/Section II/Annex II.1.3. LO (in μg/kg) was 0.1 for aflatoxins (B1, B2, G1, G2) and ochratoxin A; 1.5 for zearalenone; 0.5 for deoxynivalenol and 5 for fumonisins B1 and B2.
[redacted]
[29] Technical dossier/Section II/Annex II.1.3, and supplementary information November 2018/Annex SIN 2, LOD (mg/kg) was 3 for EBT and MTCA.
[redacted]
[31] Technical dossier/Section II.2.2.
[32] Technical dossier/Section II/Annex II.1.6 and supplementary information November 2018/Annex SIN 4.
[33] Technical dossier/Section II/Annex II.1.5.

The shelf-life of three batches of L-tryptophan produced by *E. coli* strain KCCM 80152 was tested at 25 ± 2°C and at 40 ± 2°C (packaging not described) for 6 months.[34] No losses were observed.

The stability of three batches of L-tryptophan produced by *E. coli* KCCM 80135 in a vitamin–mineral premixture (containing 4% choline chloride) at a supplementation rate of 0.5% was tested at 25 ± 2°C for 6 months. The premixture was packed in aluminium bags. The losses observed ranged from 0.4% to 2.2% after the 6-month period.[35]

The stability of three batches of L-tryptophan produced by *E. coli* KCCM 80135 in a complete feed for chickens for fattening (mash feed based on maize, soybean meal and wheat), at a supplementation rate of 0.4%, was tested after storage at 25 ± 2°C packed in aluminium bags for 3 months. After the 3-month period, the observed losses ranged from 0.5% to 4.6%.[36]

The stability of three batches of L-tryptophan produced by *E. coli* KCCM 80135 in pelleted feed for chicken for fattening (crude protein 16.5%, crude fat 6.2% and crude fibre 3.1%) supplemented at 0.2% was studied when stored at 25°C in plastic bags for 3 months. Preconditioning was performed at 72°C and drying at 60–65°C. Pelleting represented a loss ranging from 0% to 1% depending on the batch considered. The observed losses of L-tryptophan ranged from 0% to 7%, depending on the batch considered.[37]

The stability of the additive in water for drinking (three batches of L-tryptophan produced by *E. coli* KCCM 80152) was measured at a concentration of 0.5 g/L when stored at 25 or 40°C for 48 h. Losses were < 1% in both cases.[38]

The capacity of the additive (L-tryptophan produced by *E. coli* KCCM 80135) to distribute homogeneously in the premixture used in the stability test was studied by analysing 10 subsamples. The coefficient of variation (CV) was 5.7%.[39]

The capacity of the additive (L-tryptophan produced by *E. coli* KCCM 80135) to distribute homogeneously in a pelleted complete feed for chickens for fattening when supplemented at 0.2% was studied analysing 10 subsamples. The CV was 2.9%.[40]

3.1.4. Physico-chemical incompatibilities

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

3.1.5. 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 complete/complementary feedingstuffs or via premixtures. The additive is also proposed for use in water for drinking. 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, the water intake and the amino acid composition of the unsupplemented diet.[41]

3.2. Safety

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

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

The additive is highly purified (> 98% tryptophan and less than 1% unidentified material on a dry matter basis) and is produced [redacted] using a strain that is considered safe. Concerns on the use of the additive would not derive from the L-tryptophan, which is considered safe but may arise from residues of the fermentation process/production strain remaining in the final product.

[34] Technical dossier/Section II/Annex II.4.02.
[35] Technical dossier/Section II/Annex II.4.03.
[36] Technical dossier/Section II/Annex II.4.04.
[37] Technical dossier/Section II/Annex II.4.09 and supplementary information November 2018/02 SIN CJ, reply to question 4.
[38] Tehcnical dossier/Section II/Annex II.4.05.
[39] Technical dossier/Section II/Annex II.4.07.
[40] Technical dossier/Section II/Annex II.4.06.
[41] Technical dossier/Section II.5.1.

The endotoxin activity was up to 360 IU/g. These values are very low when 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 with the background in feed. Since the production strain was identified as an *E. coli* K12 derivative, was not genetically modified, it was susceptible to antimicrobials of clinical human and veterinary relevance, and no viable cells of the production strain were found in the final product, L-tryptophan produced with *E. coli* KCCM 10534 is safe for non-ruminant target species when used to supplement the diet in appropriate amounts to satisfy the animal requirements.

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., 1979). Consequently, only a protected form of L-tryptophan should be used in ruminants.

The FEEDAP Panel recommended in a previous statement that amino acids, their salts and analogues should generally not be used in water for drinking because of the risk of imbalances and for hygiene reasons (EFSA FEEDAP Panel, 2010).

The absorption and metabolic fate of L-tryptophan in the organism 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 its potential excess will be metabolised and excreted. Therefore, the composition of tissues and products of animal origin will not be affected by the use of L-tryptophan in animal nutrition. EBT and 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 < 3 mg/kg additive in both cases and do not represent a safety concern according to the European Pharmacopoeia 9th edition (2017) that established a maximum permitted content of EBT (impurity A) and the sum of all other impurities (B–L, including MTCA) in L-tryptophan as 10 and 390 mg/kg, respectively.

The amino acid L-tryptophan is a physiological and natural component of animals and plants. When given to animals, it is not excreted as such, but as urea/uric acid, indole-related compounds and carbon dioxide. The use of amino acids in water for drinking, when given in addition to complete diets with a well-balanced amino acid profile, would disturb the nitrogen balance and increase nitrogen excretion via urine. 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* KCCM 10534 as a feed additive does not represent a risk to the environment.

3.2.1.1. Conclusions on the safety for the target species, consumer and the environment

The use of L-tryptophan produced using *E. coli* KCCM 10534 in supplementing feed to compensate for tryptophan deficiency in feedingstuffs is safe for non-ruminant species. There may be a risk for an increased production of toxic metabolites when unprotected tryptophan is used in ruminants. The FEEDAP Panel has concerns on the safety of the simultaneous oral administration of L-tryptophan via water for drinking and feed due to possible amino acid imbalances.

The use of L-tryptophan produced by fermentation using *E. coli* KCCM 10534 in animal nutrition is considered safe for the consumers and for the environment.

3.2.2. Safety for the user

The applicant did not submit studies performed with L-tryptophan produced by fermentation with *E. coli* KCCM 10534 to assess the safety for the user. The applicant provided an acute inhalation toxicity test, an eye irritation test, a skin irritation test and a dermal sensitisation test performed with L-tryptophan produced by different production strains (*E. coli* KCCM 11132P in the case of the acute inhalation and the dermal sensitisation tests; and *E. coli* KCCM 80135 in the case of the eye and skin irritation tests).[42] Data obtained in such tests were assessed in previous opinions (EFSA FEEDAP Panel, 2017a, 2019). As the purity and physical characteristics of the test item are very similar to the ones of the product under assessment and the production process is the same (*E. coli* KCCM 80135) or very similar (*E. coli* KCCM 11132P),[43] the FEEDAP Panel considers that the results of the toxicological

[42] Technical dossier/Section III.3 and Table III.3.1d.
[43] Technical dossier/Section III/Annex III.3.07.

studies performed with L-tryptophan originating from *E. coli* KCCM 11132P or *E. coli* KCCM 80135 can be used to support the safety for the user of L-tryptophan produced with *E. coli* KCCM 10534.

3.2.2.1. Effects on the respiratory system

The dusting potential of the additive under assessment can be up to 4.7 g/m^3 and the fraction of particles having a diameter < 88 μm ranged from 38% to 45% (see Section 3.1.3.2).

In an acute inhalation toxicity study performed in accordance with OECD Guideline 403,[44] a group of 10 RccHan™:WIST strain rats (5 males and 5 females) were exposed to a concentration of 5.1 mg L-tryptophan (98.5% purity, produced using strain *E. coli* KCCM 11132P)/L air for 4 h (nose only exposure system). The signs observed (decreased/increased respiratory rate, hunched posture, pilo-erection and wet fur) disappeared on day 4 after exposure. No mortality occurred and no macroscopic lesions were observed at the necropsy. The lethal concentration that would kill 50% of the rat population (LC_{50}) for acute inhalation toxicity after 4 h exposure is considered to be > 5.1 mg/L.

Users can suffer from occupational respiratory disease depending on the level of endotoxins in air and dust (Rylander, 1999; Thorn, 2001). The bacterial endotoxin activity (analysed in three batches) ranged from 0.31 to 0.36 IU/mg.

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, 2012b) 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) (HCN, 2010) and the UK Health and Safety Executive (HSE, 2013). Based on the calculation of the potential endotoxin content in dust, the inhalation exposure is calculated as 948 endotoxin IU per working day, indicating that inhalation exposure to endotoxins for persons handling the additive is above the recommended threshold.

3.2.2.2. Effects on skin and eyes

In an *in vitro* bovine corneal opacity and permeability (BCOP) assay (method B.47 of Commission Regulation (EC) No 440/2008), 0.75 mL of a concentration of 20% L-tryptophan (w/v, 98.7% purity, produced by strain *E. coli* KCCM 80135) in sodium chloride (0.9% solution) was applied to incubated adult cattle corneas for 4 h.[45] Negative (sodium chloride solution 0.9%) and positive control (imidazole 20% solution in sodium chloride 0.9%) items were tested concurrently. The two endpoints measured, decreased light transmission through the cornea (opacity) and increased passage of sodium fluorescein dye through the cornea (permeability) were combined in an empirically derived formula to obtain an *in vitro* irritancy score (IVIS). As the IVIS score for the test item was ≤ 3, no classification is required. The controls performed as expected.

In an *in vitro* skin irritation study using reconstructed human epidermis model (EPISKIN™) in accordance with OECD Guideline 439, 10 mg of L-tryptophan (98.7% purity, produced by strain *E. coli* KCCM 80135) was applied (triplicate tissues) topically on the epidermal surface for 15 min, rinsed and followed by a post-exposure incubation period of 42 h.[46] Potential cytotoxicity of the test item was measured by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay and compared with negative (10 μL of Dulbecco's phosphate-buffered saline solution) or positive (10 μL of sodium lauryl sulphate 5% w/v) controls. The relative mean viability of the exposed tissues to the test item was 110% after 42 h post-exposure incubation period. The controls performed as expected. The test item was considered not irritant for the skin.

In a *in vivo* skin sensitisation study (local lymph node assay in mouse) performed in accordance with OECD Guideline 429, L-tryptophan (98.5% purity, produced using strain *E. coli* KCCM 11132P) caused no signs of toxicity, visual local skin irritation or irritation indicated by an ≥ 25% increase in mean ear thickness.[47] Consequently, the additive was classified as non-skin sensitiser.

3.2.2.3. Conclusions on safety for the user

The additive is not considered toxic by inhalation, it is not a skin or eye irritant and is not a skin sensitiser. The endotoxin activity of the additive and its dusting potential indicate a risk by inhalation for the users.

[44] Technical dossier/Section III/Annex III.3.02.
[45] Technical dossier/Section III/Annex III.3.03.
[46] Technical dossier/Section III/Annex III.3.04.
[47] Technical dossier/Section III/Annex III.3.05.

3.3. Efficacy

Efficacy studies are not required for amino acids naturally occurring in the proteins of plants and animals. The nutritional role of the amino acid 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.

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). The FEEDAP Panel reiterates that, if the product L-tryptophan is used in ruminants, it should be protected from ruminal degradation.

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

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[48] and Good Manufacturing Practice.

4. Conclusions

The use of L-tryptophan produced using *E. coli* KCCM 10534 in supplementing feed to compensate for tryptophan deficiency in feedingstuffs is safe for non-ruminant target species. There may be a risk for an increased production of toxic metabolites when unprotected tryptophan is used in ruminants.

The FEEDAP Panel has concerns on the safety of the simultaneous oral administration of L-tryptophan via water for drinking and feed due to possible amino acid imbalances.

The use of L-Tryptophan produced by *E. coli* KCCM 10534 in animal nutrition raises no safety concerns to consumers of animal products and to the environment.

The additive under assessment is considered not toxic by inhalation, it is not a skin or eye irritant and is not a skin sensitiser. The endotoxin activity of the additive and its dusting potential indicate a risk by inhalation for the users.

The product L-tryptophan is regarded as an effective source of the amino acid L-tryptophan for all non-ruminant species. In order to be as efficacious in ruminants as in non-ruminants, it should be protected from ruminal degradation.

5. Recommendations

It is recommended that the specification of the additive complies with the European Pharmacopeia with regard to L-tryptophan impurities.

The specifications on dry matter basis should reflect the actual content of L-tryptophan 98.7%.

Chronology

Date	Event
15/06/2018	Dossier received by EFSA. Feed grade L-tryptophan produced with *Escherichia coli* KCCM 10534. Submitted by CJ Europe GmbH
06/07/2018	Reception mandate from the European Commission
18/09/2018	Application validated by EFSA – Start of the scientific assessment
22/10/2018	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 of the additive and stability*
13/12/2018	Reception of supplementary information from the applicant - Scientific assessment re-started
18/12/2018	Comments received from Member States
18/12/2018	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives

[48] 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
07/05/2019	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 of the production strain and of the additive*
30/09/2019	Reception of supplementary information from the applicant - Scientific assessment re-started
21/10/2019	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 of the additive*
04/11/2019	Reception of supplementary information from the applicant - Scientific assessment re-started
18/03/2020	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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Abbreviations

CAS	Chemical Abstracts Service
CFU	colony forming unit
CV	coefficient of variation
DECOS	Dutch Expert Committee on Occupational Safety
DM	dry matter
EBT	1,1′-ethylidene-bis-L-tryptophan (EBT)
EINECS	European Inventory of Existing Commercial Chemical Substances
EURL	European Union Reference Laboratory
FCC	Food Chemical Codex
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
FLD	fluorescence detection
HPLC	high-performance liquid chromatography
HSE	UK Health and Safety Executive
IUPAC	International Union of Pure and Applied Chemistry
IVIS	*in vitro* irritancy score
KCCM	Korean Culture Collection of Microorganisms
LC_{50}	lethal concentration, median

LOD	limit of detection
MIC	minimum inhibitory concentration
MTCA	1-methyl-1,2,3,4-tetrahydro-beta-carboline-3-carboxylic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NDA	EFSA Panel on Dietetic Products, Nutrition and Allergies
OECD	Organisation for Economic Co-operation and Development
RH	relative humidity
WGS	whole genome sequencing
WHO	World Health Organization
VKM	Norwegian Scientific Committee for Food Safety

Appendix A – Safety for the user

The effects of endotoxin inhalation and the exposure limits have been described in a previous opinion (EFSA FEEDAP Panel, 2015).

Calculation of maximum acceptable levels of exposure from feed additives

The probable exposure time according to EFSA guidance (EFSA FEEDAP Panel, 2012a–d) 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/m3, then the content of endotoxins of the dust, *c* IU/m3, 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, 2012a–d).

Table A.1: Estimation of user exposure to endotoxins from the additive L-tryptophan produced by *Escherichia coli* KCCM 10534, 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	360	Technical dossier
	b	Dusting potential (g/m^3)	4.74	Technical dossier
a × *b*	*c*	Endotoxin content in the air (IU/m^3)	1,706	
	d	No of premixture batches made/ working day	40	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012a–d)
	e	Time of exposure (s) per production of one batch	20	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012a–d)
d × *e*	*f*	Total duration of daily exposure/worker (s)	800	
	g	Uncertainty factor	2	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012a–d)
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, 2012a–d)
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**	**948**	
	m	Health-based recommended exposure limit of endotoxin (IU/m^3) per eight-hour working day	90	HCN (2010)

Calculation	Identifier	Description	Amount	Source
$m \times j$	n	**Health-based recommended exposure limit of total endotoxin exposure (IU) per eight-hour working day**	**900**	
//10		Endotoxins inhaled (IU) per eight-hour working day reduced by filter mask FF P2 (reduction factor 10)	95	
//20		Endotoxins inhaled (IU) per eight-hour working day reduced by filter mask FF P3 (reduction factor 20)	47	

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Annex A – Executive summary of the evaluation report of the European Union Reference Laboratory for feed additives on the methods of analysis for L-tryptophan produced by fermentation using *Escherichia coli* KCCM 10534

In the current application authorisation is sought under Article 4(1) for L-tryptophan produced by fermentation with *Escherichia coli* KCCM 10534, 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. According to the Applicant, the product has a minimum purity of 98% and it is intended to be mixed either in premixtures or added directly to feedingstuffs or water for drinking. However, the Applicant did not propose a minimum or maximum L-tryptophan content in feedingstuffs.

For the quantification of L-tryptophan in the feed additive the Applicant submitted a single-laboratory validated analytical method based on High Performance Liquid Chromatography (HPLC) and photometric detection. Furthermore, for the quantification of L-tryptophan in premixtures and feedingstuffs the Applicant submitted the VDLUFA 4.11.2 method, based on HPLC coupled with fluorescence detection (FLD).

However, the EURL previously evaluated and recommended (i) the ring-trial validated EN ISO 13904:2016 method based on HPLC-FLD for the quantification of L-tryptophan in feed additive and premixtures (containing more than 2% of tryptophan); and (ii) the ring-trial validated Community method based on HPLC-FLD for the quantification of L-tryptophan in feedingstuffs. 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.

Furthermore, in the frame of the stability and homogeneity studies, the Applicant presented experimental data obtained analysing tryptophan in water with a slightly modified version of the VDLUFA method 4.11.2 based on HPLC-FLD and dedicated for the determination of tryptophan in feed. The results presented are considered sufficient to demonstrate the suitability of the method for the analysis of the amino acid in water. Hence, the EURL recommends for official control this method to quantify tryptophan in water.

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.