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Safety and efficacy of the feed additive consisting of L-tryptophan produced by *Escherichia coli* KCCM 80210 for all animal species (Daesang Europe BV)

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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 the safety and efficacy of the feed additive consisting of L-tryptophan produced by fermentation with *Escherichia coli* KCCM 80210 when used as a nutritional additive in feed for all animal species and categories. The production strain *E. coli* KCCM 80210 is safe for the production of L-tryptophan and it was not detected in the final product. The Panel notes that two out of five batches of the additive do not comply with the minimum specification of 98% L-tryptophan on a dry matter basis proposed by the applicant. The use of L-tryptophan ($\geq 98\%$) produced by *E. coli* KCCM 80210 in supplementing feed to compensate for L-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 L-tryptophan is used in ruminants. The use of L-tryptophan produced by *E. coli* KCCM 80210 in animal nutrition raises no safety concerns to consumers of animal products and to the environment. The additive under assessment is considered a mild eye irritant. The endotoxin activity of the additive and its dusting potential indicate a risk by inhalation for the users. The additive is not a skin irritant and is not a skin sensitiser. The additive 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.

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Keywords: nutritional additive, amino acid, L-tryptophan, safety, efficacy, *Escherichia coli* KCCM 80210

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

Regulation (EC) No 1831/2003¹ 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 Daesang Europe BV² for authorisation of the feed additive consisting of L-tryptophan produced by fermentation using *Escherichia coli* KCCM 80210, 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 15 September 2020.

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 feed additive consisting of L-tryptophan ($\geq 98\%$) produced by fermentation with a genetically modified strain of *E. coli* (KCCM 80210), when used under the proposed conditions of use (see Section 3.1.4).

1.2. Additional information

The subject of the present assessment is the product consisting of L-tryptophan (minimum 98%) produced by a genetically modified strain of *E. coli* (KCCM 80210). L-Tryptophan produced by this bacterial strain had not been previously authorised as feed additive in the European Union.

L-Tryptophan ($\geq 98\%$) 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 (FEEDAP) 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–e, 2020a,b, c). The EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) 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, 9th Edition (2017), so that this citation matches the Reference List. Please confirm that this is correct. 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 Daesang Europe BV. Van Heuven Goedhartlaan 935, 1181 LD, Amsterdam (The Netherlands).

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 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. 35–56.

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 dossier⁷ in support of the authorisation request for the use of the product consisting of L-tryptophan (minimum 98.0%) produced by fermentation using *E. coli* KCCM 80210 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 the product consisting of L-tryptophan produced by fermentation using *E. coli* KCCM 80210 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 the additive under assessment is in line with the principles laid down in Regulation (EC) No 429/2008⁹ and the relevant guidance documents: Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012), 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 an additive consisting of L-tryptophan ($\geq 98\%$) produced by fermentation with a genetically modified strain of *Escherichia coli* (KCCM 80210). It is proposed to be used as nutritional additive (functional group: amino acids, their 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 derivative of *E. coli* K-12 which is deposited in the Korean Culture Collection of Microorganisms (KCCM) with accession number KCCM 80210.¹⁰

A bioinformatic analysis of the whole genome sequence (WGS) of the production strain confirmed its identity as an *E. coli* K-12 derivative.¹¹ This was based

E. coli K-12 is well characterised and its safety (non-pathogenicity) has been documented (Gorbach, 1978). The strain has been shown to be ineffective in colonising the human gut (Smith, 1975) and its genome (MG1655 and W3110) has been fully sequenced (Hayashi et al., 2006).

⁷ FEED dossier reference: FAD-2020-0038.

⁸ The full report is available on the EURL website: <https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2020-0038-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.

¹⁰ Technical dossier/Section II/Annexes Section II/Annex 2.2.1.2a CONFID.

¹¹ Technical dossier/Section II/Annexes Section II/Annex 2.2.1.2c CONFID, Annex 2.2.1.2d CONFID and Annex 2.2.1.2e CONFID.

[REDACTED]¹²
The production strain was tested for its susceptibility to all the antimicrobials listed for 'Enterobacteriaceae' in the Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018a,b).¹³ All minimum inhibitory concentration (MIC) values were below or equal to the cut-off values set in the Guidance and, therefore, the strain is considered susceptible to those antibiotics.

The WGS of the production strain was interrogated for the presence of antimicrobial resistance (AMR) genes [REDACTED]

[REDACTED]¹²
This, and the fact that the production strain was not phenotypically resistant to any of the antibiotics tested, suggests that the production strain does not carry antibiotic resistance genes of concern.

The WGS of the production strain was also interrogated for the presence of toxin and virulence factor genes [REDACTED]¹²

3.1.1.1. Information regarding to the genetically modified microorganism

Characterisation of the recipient microorganism

[REDACTED]¹⁴

Characteristics of the introduced sequences

[REDACTED]¹⁴
[REDACTED]¹⁴

Description of the genetic modification

[REDACTED]¹⁴
[REDACTED]¹⁴
[REDACTED]¹²
[REDACTED]

¹² Technical dossier/Section II/Annexes Section II/Annex 2.2.1.2c CONFID.

¹³ Technical dossier/Section II/Annexes Section II/Annex 2.2.2.2.

¹⁴ Technical dossier/Section II/Annexes Section II/Annex 2.2.1.2b CONFID.

3.1.2. Manufacturing process

L-Tryptophan is produced by fermentation using *E. coli* KCCM 80210.

¹⁵

¹⁶

3.1.3. Characterisation of the product/active substance

L-Tryptophan (International Union of Pure and Applied Chemistry (IUPAC) name: (2S)-2-amino-3-(1H-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 C₁₁H₁₂N₂O₂, the molecular weight is 204.23 g/mol. The structural formula is given in Figure 1.

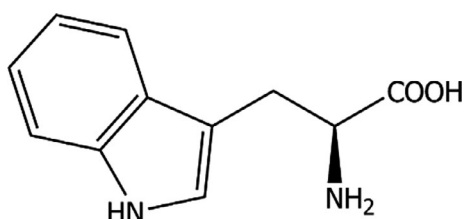


Figure 1: Structural formula of L-tryptophan

According to the specification, the product contains $\geq 98\%$ L-tryptophan on a DM basis and $\leq 1\%$ moisture.

The analysis of five batches of the additive showed an average content of tryptophan of 98.4% on 'as is' basis (range 97.3–99.7%),¹⁷ moisture was 0.1% and ash ranged 0.02–0.06%.¹⁸ On a dry matter basis, the tryptophan content was on average 98.5% (97.4–99.8%), corresponding to the amount of identified material. Two out of the five analysed batches did not reach the specification of minimum 98% tryptophan on a DM basis.

The specific optical rotation was measured in five batches of the additive and was on average -32.3° (range -32.0 to -32.4°),¹⁸ 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.¹⁹

3.1.3.1. Undesirable substances

Five batches of the additive were analysed for heavy metals (cadmium, lead and mercury) and arsenic. All values were below the respective limit of detection (LOD) except for lead which ranged from 0.03 to 0.06 mg/kg.²⁰ Polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and coplanar dioxin-like polychlorinated biphenyls (Co-planar PCBs) were analysed in three batches and found below the corresponding limit of quantification (LOQ).²¹ The calculated (upper bound) levels of dioxins and the sum of dioxins and dioxin-like-PCBs were calculated to be 0.137 ng WHO-PCDD/F-TEQ/kg and 0.269 ng WHO-PCDD/F-PCB-TEQ/kg, respectively (in all three batches). In relation to mycotoxins, aflatoxins (not specified), ochratoxin A, zearalenone, fumonisins (B1, B2 and B3), deoxynivalenol and citrinin were analysed in three batches of the additive. All analytical values

¹⁵ Technical dossier/Section II/Annex 2.3.1a Confid.

¹⁶ Technical dossier/Section II/Annex 2.3.1.b.5.

¹⁷ Technical dossier/Section 2.6.1 and Annex II.6.1. Spontaneous submission of additional information, Q2. Supplementary information November 2021/Annex 2 TRP feed grade. Tryptophan analysed by European Pharmacopoeia 9.0, 2.2.56, method I.

¹⁸ Technical dossier/Section II/Annexes 2.1.3a to e.

¹⁹ European Pharmacopoeia monograph 1/2017:1272.

²⁰ Technical dossier/Section II/Annex 2.1.4a. LOD in mg/kg were 0.01 for arsenic and lead, and 0.002 for cadmium and mercury.

²¹ Technical dossier/Section II/Annex 2.1.4b1 to d1. LOQ in ng/kg ranged 0.04–2 for PCDD/Fs; and ranged 1–20 for PCBs, depending on the substance considered.

were found below the LOD except for citrinin in one batch (15.6 µg/kg).²² The detected amounts of these undesirable substances do not raise safety concerns.

Microbial contamination was analysed in three batches. *Salmonella* spp. was absent in 25 g samples. In 1 g samples, *E. coli* and *Enterobacteriaceae* were not detected; and yeast and moulds ranged 510–700 colony-forming units (CFU)/g.²³

The concentration of 1,1'-ethylidene-bis-L-tryptophan (EBT) was < 10 mg/kg in all three batches analysed. The concentration of 1-methyl-1,2,3,4-tetrahydro-beta-carboline-3-carboxylic acid (MTCA) was measured in three batches and was 1 mg/kg in all cases.²⁴

Endotoxin activity was measured in three batches (*Limulus* amoebocyte lysate assay) and ranged from 1,720 to 5,180 EU/g.²⁵

The presence of viable cells of the production strain in the final product was tested in three batches of the additive.²⁶

[REDACTED] No growth was detected.

The presence of DNA from the production strain was tested in three batches of the additive in triplicate.²⁷

[REDACTED] No DNA of the production strain was detected.

3.1.3.2. Physical-chemical properties

The additive is described as a white to yellowish white odourless crystalline powder. It has a solubility in water of 10.6 g/L (20°C)²⁸ and a density of 550–650 kg/m³.

Dusting potential was analysed in three batches (Stauber-Heubach method) and the values ranged from 0.6 to 0.8 g/m³.²⁹ Particle size distribution of the final product was measured in three batches and the fractions of particles below 11, 52 and 105 µm diameter ranged 21–25, 64–74 and 76–86%, respectively.³⁰

3.1.3.3. Stability and homogeneity

The shelf-life of the additive (three batches) was tested at 25°C and 40°C when stored in bags protecting from light and air exchange for 6 months.³¹ Losses observed at both temperatures ranged from 0% to 2% depending on the batch considered.

The stability of the additive (three batches) in a vitamin mineral premixture containing choline chloride (30,000 mg/kg) was studied when supplemented at an inclusion rate of 4%.³² The samples were stored at room temperature in bags protecting from light and air exchange for 6 months. Losses observed ranged from 3% to 10%.

The stability of the additive (three batches) was studied in a complete feed for chickens for fattening, when supplemented at 0.2%. The basal diet consisted of wheat, maize and soybean meal.³³ Mash and pelleted feed were tested after storage at room temperature in bags protecting from light and air exchange for 3 months. Pelleting temperature was about 70°C. Pelleting the mash feeds produced losses up to 2%. After 3 months storage, only a loss of 2% was observed in one of the batches for each mash and pelleted feed.

²² Technical dossier/Section II/Annex 2.1.4a. LOD in µg/kg were 1.7 for aflatoxins, 5 for ochratoxin A, 17 for zearalenone, 25 for the sum of the three fumonisins, 134 for DON and 15 for citrinin.

²³ Technical dossier/Section II/Annexes 2.1.3a to e and annex 2.1.4a.

²⁴ Technical dossier/Section II/Annexes 2.1.4g1 to g3 for EBT and 2.1.4h1 to h3 for MTCA. EBT analysed according to European Pharmacopoeia 10th Edition, monograph 1272; MTCA analysed by HPLC with fluorescent detector.

²⁵ Technical dossier/Section II/Annex 2.1.4i.

²⁶ Technical dossier/Section II/Annex 2.1.4.e.

²⁷ Technical dossier/Section II/Annex 2.1.4f CONFID.

²⁸ Technical dossier/Section II/Annex 2.5.2.6.

²⁹ Technical dossier/Section II/Annexes 2.1.5a to c.

³⁰ Technical dossier/Section II/Annexes 2.1.4b2, c2 and d2.

³¹ Technical dossier/Section II.4.1 and Annex 2.1.4a.

³² Technical dossier/Section II.4.1, annex 2.1.4b received as spontaneous submission of supplementary information, and premixture composition in annex 2.4.1.

³³ Technical dossier/Section II.4.1, annex 2.1.4b, and feed composition in annex 2.4.1c.

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 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 80210 as a feed additive does not represent a risk to the environment.

3.2.2.1. Conclusions on the safety for the target species, consumers and the environment

The use of L-tryptophan produced using *E. coli* KCCM 80210 to supplement 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 use of L-tryptophan produced by fermentation using *E. coli* KCCM 80210 in animal nutrition is considered safe for the consumers and for the environment.

3.2.3. Safety for the user

The applicant provided an *in vivo* acute inhalation toxicity study, a dermal irritation study, an eye irritation study and a dermal sensitisation study testing the additive under assessment.

3.2.3.1. Effects in the respiratory system

The highest measured dusting potential of the additive under assessment was 0.8 g/m³ and the fraction of particles having a diameter < 52 µm ranged from 64% to 74% (see Section 3.1.3.2). Hence, the users can be exposed to the additive by inhalation.

In an acute inhalation toxicity study in rats performed in accordance with OECD Guideline 403 and consistent with good laboratory practices (GLP), the additive showed an inhalation median lethal concentration (LC₅₀) greater than 5.13 mg/L air and the test item required no classification.³⁵

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 1,720 to 5,180 IU/g. 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, 2012, 2014b) is described in 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 upon the calculation of the potential endotoxin content in dust, the inhalation exposure is calculated as 2,300 endotoxin IU per working day, indicating that inhalation exposure to endotoxins for persons handling the additive is above the recommended threshold.

3.2.3.2. Effects on skin and eyes

The skin irritation potential of the additive was tested in a valid study in rabbits performed according to OECD guideline 404 and consistent with GLP, which showed that it is not a skin irritant and has no corrosive effect on skin.³⁶

The eye irritation potential of the additive was tested in a valid study in rabbits performed according to OECD guideline 405 and consistent with GLP, which showed that according to the results obtained and under the assayed experimental conditions, the test item was classified as mild irritant to eyes.³⁷

In a valid skin sensitisation study following local lymph node assay (LLNA) performed in mice according to OECD guideline 429 and compliant with GLP, it was found that the additive does not have to be classified as a skin sensitiser.³⁸

3.2.3.3. Conclusions on safety for the user

L-Tryptophan produced using *E. coli* KCCM 80210 is not a skin irritant and it is not a skin sensitiser but is mild irritant to eyes. The endotoxin activity of the additive and its dusting potential indicate a risk by inhalation for the users.

³⁵ Technical dossier/Section III/Annex 3.3.1.1.

³⁶ Technical dossier/Section III/Annex 3.3.1.2a.

³⁷ Technical dossier/Section III/Annex 3.3.1.2b.

³⁸ Technical dossier/Section III/Annex 3.3.1.2c.

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 Regulation³⁹ and Good Manufacturing Practice.

4. Conclusions

The Panel notes that two out of five batches of the additive do not comply with the minimum specification of 98% L-tryptophan on a dry matter basis as proposed by the applicant.

The use of L-tryptophan ($\geq 98\%$) produced using *E. coli* KCCM 80210 to supplement feed to compensate for L-tryptophan deficiency in feedingstuffs is safe for non-ruminant species. There may be a risk for an increased production of toxic metabolites when unprotected L-tryptophan is used in ruminants.

The use of L-tryptophan produced by fermentation using *E. coli* KCCM 80210 in animal nutrition is considered safe for the consumers and for the environment.

L-Tryptophan produced using *E. coli* KCCM 80210 is not a skin irritant and it is not a skin sensitiser but is mild irritant to eyes. The endotoxin activity of the additive and its dusting potential indicate a risk by inhalation for the users.

The product L-tryptophan produced by fermentation using *E. coli* KCCM 80210 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. Recommendation(s)

The FEEDAP Panel recommends that the specification of the additive includes limits for L-tryptophan-related impurities in compliance with the European Pharmacopeia.

6. Documentation as provided to EFSA/Chronology

Date	Event
29/05/2020	Dossier received by EFSA. L-tryptophan produced by fermentation with <i>Escherichia coli</i> KCCM 80210 for all animal species. Submitted by Daesang Europe BV.
07/07/2020	Reception mandate from the European Commission
15/09/2020	Application validated by EFSA – Start of the scientific assessment
21/09/2020	Reception of spontaneous supplementary information from the applicant – <i>Issues: Characterisation of the additive</i>
26/11/2020	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. <i>Issues: Characterisation of the additive, manufacturing process</i>
27/11/2017	Reception of supplementary information from the applicant - Scientific assessment re-started
27/11/2020	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
15/12/2020	Comments received from Member States
27/01/2021	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

³⁹ Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 October 2003 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

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Abbreviations

CAS	Chemical Abstracts Service
CFU	colony-forming unit
CV	coefficient of variation
DM	dry matter
EBT	1,1'-ethylidene-bis-L-tryptophan
EURL	European Union Reference Laboratory
LOD	limit of detection
LOQ	limit of quantification
MIC	minimum inhibitory concentration
MTCA	1-methyl-1,2,3,4-tetrahydro-beta-carboline-3-carboxylic acid
RH	relative humidity
WHO	World Health Organization
VKM	Norwegian Scientific Committee for Food Safety
WGS	Whole genome sequence

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, 2015a,b).

Calculation of maximum acceptable levels of exposure from feed additives

The probable exposure time according to EFSA guidance (EFSA FEEDAP Panel, 2012) for additives added in premixtures assumes a maximum of 40 periods of exposure per day, each comprising 20 s = $40 \times 20 = 800$ s/day. With an uncertainty factor of 2, maximum inhalation exposure would occur for $2 \times 800 = 1,600$ s = 0.444 h/day. Again, assuming a respiration volume of 1.25 m³/h, the inhalation volume providing exposure to potentially endotoxin-containing dust would be $0.444 \times 1.25 = 0.556$ m³/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/m³.

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/m³, 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, $a \times b$. 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, 2012).

Table A.1: Estimation of user exposure to endotoxins from the additive L-tryptophan produced by *Escherichia coli* KCCM 80210, 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	5,180	Technical dossier
	b	Dusting potential (g/m ³)	0.8	Technical dossier
a × b	c	Endotoxin content in the air (IU/m ³)	4,144	
	d	No of premixture batches made/working day	40	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012)
	e	Time of exposure (s) per production of one batch	20	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012)
d × e	f	Total duration of daily exposure/worker (s)	800	
	g	Uncertainty factor	2	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012)
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 8-h working day	10	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012)
j/8 × i	k	Inhaled air during exposure (m ³)	0.56	
c × k	l	Endotoxin inhaled (IU) during exposure per 8-h working day	2,300	
	m	Health-based recommended exposure limit of endotoxin (IU/m ³) per 8-h working day	90	HCN, 2010
m × j	n	Health-based recommended exposure limit of total endotoxin exposure (IU) per 8-h working day	900	

Calculation	Identifier	Description	Amount	Source
I/10		Endotoxins inhaled (IU) per 8-h working day reduced by filter mask FF P2 (reduction factor 10)	230	
I/20		Endotoxins inhaled (IU) per 8-h working day reduced by filter mask FF P3 (reduction factor 20)	115	

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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 fermentation using *Escherichia coli* KCCM 80210

In the current application, an authorisation is sought under Article 4(1) for L-tryptophan produced by fermentation with *Escherichia coli* KCCM 80210 (W008), 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. The authorisation is sought for all animal species.

According to the Applicant, the feed additive contains L-tryptophan with a minimum purity of 98% (w/w). The feed additive is intended to be mixed either in premixtures or added directly to feedingstuffs. However, the Applicant did not propose a minimum or maximum L-tryptophan content in feedingstuffs.

For the determination of tryptophan in the feed additive and premixtures, the Applicant submitted the ring-trial validated EN ISO 13904:2016 method based on high-performance liquid chromatography with fluorescence detection (HPLC-FLD). The method is dedicated for the determination of free tryptophan in commercial products and premixtures (containing more than 2% (w/w) of tryptophan) and for the determination of free and total tryptophan in feedingstuffs.

For the determination of tryptophan in feedingstuffs, the Applicant submitted a ring-trial validated European Union (EU) method, where the procedure to determine tryptophan in feedingstuffs is identical to the one described in the above-mentioned EN ISO 13904:2016 method. The EU method is applicable for the determination of free (synthetic and natural) and total (peptide-bound and free) amino acid using an HPLC method with fluorescence detection (FLD). The method does not distinguish between the amino acid enantiomers.

Based on the performance characteristics available, the EURL recommends for official control the above-mentioned two ring-trial validated methods based on HPLC-FLD to determine tryptophan in the feed additive, premixtures and/or feedingstuffs.

In addition, for the identification of the feed additive, the EURL recommends the 'L-tryptophan monograph' of the Food Chemical Codex (FCC), where different tests (including one based on optical rotation) are described.

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.