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## Safety and efficacy of L-tryptophan produced by fermentation with *Escherichia coli* KCCM 80135 for all animal species

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

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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 using *Escherichia coli* KCCM 80135 when used as a nutritional additive in feed and water for drinking for all animal species. The production strain and its recombinant DNA were not detected in the additive. L-Tryptophan produced by fermentation with *E. coli* KCCM 80135 does not raise any safety concern with regard to the genetic modification of the production strain. The use of L-tryptophan produced using *E. coli* KCCM 80135 in supplementing feed to compensate for tryptophan deficiency in feedingstuffs is safe for non-ruminant target species. Using unprotected forms of tryptophan in ruminants can be a risk. The use of L-tryptophan produced by fermentation using *E. coli* KCCM 80135 in animal nutrition presents no safety concerns to consumers of animal products. L-Tryptophan produced by *E. coli* KCCM 80135 is not toxic by inhalation. The additive is not an irritant to skin and eyes, and it is not a skin sensitiser. The additive under assessment is considered safe for the environment. It is regarded as an effective source of the amino acid L-tryptophan for all non-ruminant species. If the additive L-tryptophan is intended for use in ruminants, it should be protected from ruminal degradation.

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## 1. Introduction

### 1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003<sup>1</sup> 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<sup>2</sup> for authorisation of the product L-Tryptophan produced using *Escherichia coli* KCCM 80135, 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 15 March 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 *E. coli* KCCM 80135, when used as a nutritional additive in feed and water for drinking for all animal species 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 as a nutritional feed additive for use in all animal species.<sup>3</sup> The product under assessment, L-tryptophan produced by *E. coli* KCCM 80135, has not been previously authorised as feed additive in the European Union (EU).

L-Tryptophan is authorised for use in food,<sup>4</sup> cosmetics<sup>5</sup> and as a veterinary medicinal product.<sup>6,7</sup> 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 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).

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 FEEDAP 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) and concluded that doses  $\geq 250$  mg/day may represent a risk of adverse health effects for children, adolescents and adults.

<sup>1</sup> 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.

<sup>2</sup> CJ Europe GmbH, Ober der Roeth 4, 65824 Schwalbach am Taunus, Germany.

<sup>3</sup> Commission implementing regulation (EU) 2017/873 of 22 May 2017 concerning the authorisation of L-tryptophan produced by *Escherichia coli* as feed additive for all animal species. OJ L 134, 23.5.2017, p. 14.

<sup>4</sup> 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.

<sup>5</sup> 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.

<sup>6</sup> 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.

<sup>7</sup> 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. OJ 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<sup>8</sup> in support of the authorisation request for the use of L-Tryptophan produced by fermentation using *E. coli* KCCM 80135 as an additive for feed and water for drinking in all animal species.

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 the L-Tryptophan produced by fermentation with *E. coli* K12 KCCM 80135 in animal feed. The Executive Summary of the EURL report can be found in Annex A.<sup>9</sup>

### 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 80135 is in line with the principles laid down in Regulation (EC) No 429/2008 and the relevant guidance documents: Guidance on nutritional additives (EFSA FEEDAP Panel, 2012a), Tolerance and efficacy studies in target animals (EFSA FEEDAP Panel, 2011), Technical Guidance for assessing the safety of feed additives for the environment (EFSA, 2008a), 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), Technical Guidance: Microbial Studies (EFSA, 2008b) and Guidance on microorganisms used as feed additive or as production organisms (EFSA FEEDAP Panel, 2018).

## 3. Assessment

L-Tryptophan minimum 98% produced by fermentation using *E. coli* KCCM 80135 is intended to be used as a nutritional feed additive under the functional group 'amino acids, their salts and analogues' in feed and water for drinking for all animal species.

### 3.1. Characterisation

#### 3.1.1. Characterisation of the production organism

[REDACTED]

The susceptibility of the production strain against the list of antibiotics proposed for *E. coli* by the FEEDAP Panel (EFSA FEEDAP Panel, 2012d) was tested using broth microdilution. [REDACTED]

##### 3.1.1.1. Information relating to the genetic modification

###### *Characterisation of the recipient or parental microorganism*

The recipient strain is *E. coli* KCCM 11132P, which was derived from *E. coli* K-12 W3110 by classical mutagenesis and genetic modification. This strain harbours an [REDACTED]. *E. coli* K-12 is well characterised and its safety (non-pathogenicity) has been documented (Gorbach, 1978). *E. coli* K-12 was shown to be ineffective in colonizing the human gut (Smith, 1975), and its genome has been fully sequenced (Hayashi et al., 2006). *E. coli* KCCM 11132P

<sup>8</sup> FEED dossier reference: FAD-2017-0033.

<sup>9</sup> The full report is available on the EURL website: <https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2017-0033-tryptophan.pdf>

was previously characterised by the FEEDAP Panel (EFSA FEEDAP Panel, 2015b), and did not raise safety concerns.

#### *Characteristics of the newly introduced DNA*

Different genetic elements were introduced in the production strain to improve the L-tryptophan production:

[REDACTED]

#### *Description of the genetic modification process*

[REDACTED]

### **3.1.2. Manufacturing process**

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

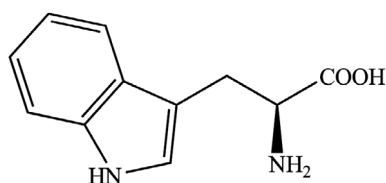
[REDACTED]

### **3.1.3. Characterisation of the active substance/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*)- $\alpha$ -amino-1-*H*-indole-3-propanoic acid, L- $\alpha$ - aminoindole-3-propionic acid, L- $\alpha$ -amino-3-indolepropionic acid, 2-amino-3-indolylpropanoic acid, L- $\beta$ -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<sub>11</sub>H<sub>12</sub>N<sub>2</sub>O<sub>2</sub>, the molecular weight is 204.23 g/mol. The structural formula is given in Figure 1.

<sup>14</sup> Technical dossier/Section II/Annex\_CONFID\_II\_2\_04.

[REDACTED]



**Figure 1:** Structural formula of L-tryptophan

According to the specification, the product contains  $\geq 98\%$  L-tryptophan 'as is', and  $\leq 1\%$  moisture.

The analysis of five batches of L-tryptophan showed an average content of L-tryptophan of 98.3% on as is basis (range 98.2–98.4%),<sup>19</sup> moisture ranged 0.35–0.50%, ammonium ranged 0.06–0.10%, glutamic acid ranged 0.03–0.04%, valine ranged 0.3–0.4% and ash ranged 0.13–0.18%.<sup>20</sup> On a dry matter basis, the amount of identified material is, on average, 99.1% (range 99.0–99.1%). The tryptophan content on dry matter basis was 98.8% (98.7–98.8%).

The specific optical rotation of three batches of the final product ranged from  $-30.8$  to  $-31.4^\circ$ ,<sup>21</sup> which is within the range described in the European Pharmacopoeia ( $-30$  to  $-33^\circ$ ) for this amino acid and confirms the identity of the L-enantiomer.<sup>22</sup>

### 3.1.3.1. Impurities

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

Analysis of microbial contamination of the final product (3 batches) indicated that *Salmonella* spp. (25-gram samples), *E. coli*, and coliforms were absent whereas total bacterial count, was  $< 10^3$  colony forming unit (CFU)/g; and yeasts and filamentous fungi were  $< 5 \times 10^1$  CFU/g.<sup>24</sup>

Mycotoxins were analysed in three batches of the final product. Aflatoxins (B1, B2, G1 and G2), ochratoxin A, zearalenone, deoxynivalenol and fumonisins (B1 and B2) had concentrations below the LOD.<sup>25</sup>

Dioxins (polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs)) and dioxin-like polychlorinated biphenyls (Co-planar PCBs) were analysed in three batches of the final product. Both parameters were found below the LOD.<sup>26</sup>

The endotoxin activity (3 batches analysed by *Limulus* amoebocyte lysate test) ranged from 0.33 to 0.42 IU/mg.<sup>27</sup>

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 analysed by high-performance liquid chromatography (HPLC) (EurPh 2.2.29) in three batches of the final product were  $< 3$  mg/kg in all cases.<sup>28</sup> No MTCA was detected in three batches of the final product.<sup>29</sup> As specified in the European Pharmacopoeia 9th edition (2017), the maximum permitted content of EBT (impurity A) and the sum of all other impurities (B-L, including MTCA) in L-tryptophan are 10 mg/kg and 390 mg/kg, respectively.

<sup>19</sup> Technical dossier/Section II/Annex II.6.1.

<sup>20</sup> Technical dossier/Section II/Annex II.1.3. Analysis of L-tryptophan performed by reverse phase HPLC and UV detection.

<sup>21</sup> Technical dossier/Section II/Annex II.1.4.

<sup>22</sup> European Pharmacopoeia monograph 1/2015:1272.

<sup>23</sup> Technical dossier/Section II/Annex II.1.4. LOD in  $\mu\text{g}/\text{kg}$  were 5 for mercury and 1 for cadmium, lead and arsenic.

<sup>24</sup> Technical dossier/Section II/Annex II.1.4 and supplementary information July 2018/ SIN CJ L-trp 80135/Response to question 3(ii).

<sup>25</sup> Technical dossier/Section II/Annex II.1.4. LOD (in  $\mu\text{g}/\text{kg}$ ) 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.

<sup>26</sup> Technical dossier/Section II/Annex II.1.4. LOD (in  $\mu\text{g}/\text{kg}$ ) was 0.05 for PCDD/F and 0.13 for Co-PCBs

<sup>27</sup> Technical dossier/Supplementary information July 2018/Annex SIN 2.

<sup>28</sup> Technical dossier/Section II/Annex II.1.4 and supplementary information July 2018/SIN CJ L-trp 80135/Response to question 3 (v).

<sup>29</sup> Technical dossier/Supplementary information July 2018/Annex SIN 3. LOD was 3 mg/kg.

### 3.1.3.2. Physical characteristics

The product under assessment is a solid pale brownish odourless powder. The hydrogen potential (pH) in 10% solution at 20°C ranges from 4.5 to 7. The bulk density ranges from 350 to 500 kg/m<sup>3</sup>.<sup>32</sup> Its water solubility is 10.6 g/L at 20°C.<sup>33</sup>

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 ranged 82–91, 62–69 and 18–26 µm (v/w), respectively.<sup>34</sup>

The dusting potential of the product under assessment (three batches analysed by Stauber–Heubach method) showed values ranging from 0.60 to 0.85 g/m<sup>3</sup>.<sup>35</sup>

### 3.1.3.3. Stability and homogeneity

The applicant submitted data of stability tests made with L-tryptophan produced using *E. coli* strains different of the *E. coli* production strain of the product under assessment to support its stability. In particular, the test shelf life at 25°C was performed with L-tryptophan produced using *E. coli* KCCM 11132P (EFSA FEEDAP Panel, 2015b), whereas the tests of shelf life at 40°C, the stability in water for drinking were performed with L-tryptophan produced using *E. coli* KCCM 80152 (EFSA FEEDAP Panel, 2019c) of the same producer. As the manufacturing process is the same and the product characteristics are very similar, the FEEDAP Panel considers that the stability and homogeneity studies performed testing L-tryptophan of the other two production strains are indicative of the stability of the product under assessment. No losses were observed in the shelf life studies (6 months or 2.5 years) and losses were < 1% in water for drinking (after 48 h).<sup>36</sup>

The following studies on stability in premixtures and feedingstuffs and on the capacity of the additive to distribute homogeneously in premixtures and in pelleted feed were performed with the product under assessment.<sup>37</sup>

The stability of three batches in a vitamin mineral premixture (containing 4% choline chloride) at a supplementation rate of 5% was tested at 25±2°C for 6 months. The premixture was packed in aluminium vacuum bags. The losses observed ranged from 0.4 to 2.2% after the 6-month period.<sup>38</sup>

The stability of three batches of the additive 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 for 3 months. The packaging was aluminium vacuum bags. After the 3-month period, the observed losses ranged from 0.5 to 4.6%.<sup>39</sup>

The stability of three batches of the additive in a pelleted feed (basal diet described only in nutritional components) at a supplementation rate of 0.2% was tested after storage at 25°C in paper/polyethylene bags for 3 months. Preconditioning temperature was ≤ 72°C and pellets were dried at 60–65°C. The losses ranged from 0% to 7% depending on the batch considered.<sup>40</sup>

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 5.7%.<sup>41</sup>

The capacity of the additive to distribute homogeneously in a pelleted complete feed for chickens for fattening when supplemented at 0.2% was studied analysing 10 subsamples. After mixing, the complete feed was preconditioned using water steam at ≤ 72°C, pelleted at 81°C, dried at 60–65°C

<sup>32</sup> Technical dossier/Section II/Annex II.1.1.

<sup>33</sup> Technical dossier/Section II.1.5.

<sup>34</sup> Technical dossier/Section II/Annex II.1.6.

<sup>35</sup> Technical dossier/Section II/Annex II.1.7.

<sup>36</sup> Technical dossier/Section II/Annexes II.4.2 and II.4.5.

<sup>37</sup> Technical dossier/Supplementary information July 2018/SIN CJ L-trp 80135/Response to question 1.

<sup>38</sup> Technical dossier/Section II/Annex II.4.3 and supplementary information July 2018/SIN CJ L-trp 80135/Response to question 4 (1).

<sup>39</sup> Technical dossier/Section II/Annex II.4.4 and supplementary information July 2018/SIN CJ L-trp 80135/Response to question 4 (1).

<sup>40</sup> Technical dossier/Supplementary information July 2018/Annex SIN 4.

<sup>41</sup> Technical dossier/Section II/Annex II.4.7.



and then cooled for 10 min to 23°C. The CV was 2.9%.<sup>42</sup> No data were provided on the stability of the additive during feed processing.

#### 3.1.3.4. Physicochemical incompatibilities

No physicochemical incompatibilities in feed are expected with other additives, medical 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/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 and the amino acid composition of the unsupplemented diet.<sup>43</sup>

### 3.2. Safety

#### 3.2.1. Safety of the genetic modification

The recipient organism *E. coli* KCCM 11132P is considered to be safe. The production strain KCCM 80135

The applicant provided sufficient information that neither the production strain nor its recombinant DNA is present in the final product. The product L-tryptophan, manufactured by fermentation with *E. coli* KCCM 80135, 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 on average 98.8% L-tryptophan and less than 1% unidentified material on a dry matter basis. The endotoxin activity ranged from 0.33 to 0.42 IU/mg. These values are negligible 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.

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* KCCM 80135 is safe for non-ruminant target species when used to supplement the diet in appropriate amounts. The EFSA FEEDAP Panel (2015a) 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. Consequently, using unprotected forms of tryptophan in ruminants can be a risk.

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).

##### 3.2.2.1. Conclusions on safety for the target species

The use of L-tryptophan produced using *E. coli* KCCM 80135 in supplementing feed to compensate for tryptophan deficiency in feedingstuffs is safe for non-ruminant target species. Using unprotected forms of tryptophan in ruminants can be a risk.

<sup>42</sup> Technical dossier/Section II/Annex II.4.6.

<sup>43</sup> Technical dossier/Section II.5.1.

The FEEDAP Panel has concerns on the safety of the simultaneous oral administration of L-tryptophan via water for drinking and feed.

### 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. 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* KCCM 80135 is highly purified (average of 98.8% L-tryptophan and < 1% unidentified material on a dry matter basis) and (2) the concentrations of EBT and MTCA are < 3 mg/kg additive, no additional toxicological data are required.

#### 3.2.3.1. Conclusions on safety for the consumer

The use of L-tryptophan produced by fermentation with *E. coli* KCCM 80135 in animal nutrition presents no concern to consumers of animal products.

### 3.2.4. 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.<sup>37</sup> Only the irritation tests (skin and eyes) were performed with the product under assessment. The acute inhalation and the dermal sensitisation tests were performed with L-tryptophan produced by a different production strain (*E. coli* KCCM 11132P).<sup>44</sup> As the purity and physical characteristics of the test item are very similar to the ones of the product under assessment, the production process is the same and the strains share a common lineage, the FEEDAP Panel considers that the results of the toxicological studies performed with L-tryptophan originating from *E. coli* KCCM 11132P can be used to support the safety for the user of L-tryptophan produced with *E. coli* KCCM 80135.

#### 3.2.4.1. Effects on the respiratory system

About 44% of particles have a diameter < 88 µm and the dusting potential is up 0.85 g/m<sup>3</sup> (see Section 3.1.3). Therefore, inhalation exposure of users is likely.

In an acute inhalation toxicity study in accordance with the Organisation for Economic Co-operation and Development (OECD) Guideline 403,<sup>45</sup> a group of 10 RccHan<sup>TM</sup>: WIST strain rats (5 males and 5 females) were exposed to a concentration of 5.1 mg L-tryptophan (98.5% purity)/l air for 4 h (nose only exposure system). The signs observed (decreased/increased respiratory rate, hunched posture, piloerection 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<sub>50</sub>) for acute inhalation toxicity after 4 h exposure is considered to be > 5.1 mg/l.

The endotoxin activity in the additive was up to 0.42 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) (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 198 endotoxin IU/working day, indicating thus no risk by inhalation exposure to endotoxins for persons handling the additive.

<sup>44</sup> FAD-2017-0052/Technical dossier/Section III/Annex III.3.7.

<sup>45</sup> Technical dossier/Section III/Annex III.3.2.

### 3.2.4.2. 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) in sodium chloride (0.9% solution) was applied to incubated adult cattle corneas for 4 h.<sup>46</sup> 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  $\leq 3$ , 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) was applied (triplicate tissues) topically on the epidermal surface for 15 min, rinsed and followed by a post-exposure incubation period of 42 h.<sup>47</sup> 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  $\mu$ L of Dulbecco's phosphate-buffered saline solution) or positive (10  $\mu$ L of sodium lauryl sulfate 5% w/v) controls. The relative mean viability of the test item treated tissues 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) in accordance with OECD Guideline 429, L-tryptophan (98.5% purity) caused no signs of toxicity, visual local skin irritation or irritation indicated by an  $\geq 25\%$  increase in mean ear thickness.<sup>48</sup> Consequently, the additive was classified as non skin sensitiser.

### 3.2.4.3. Conclusions on safety for the user

L-Tryptophan produced by *E. coli* KCCM 80135 is not toxic by inhalation. The endotoxin activity present in the final product does not represent a risk for the persons handling the additive when exposed by inhalation. The additive is not an irritant to skin and eyes, and it is not a skin sensitiser.

### 3.2.5. Safety for the environment

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. It is concluded that the use of the product under assessment, produced by *E. coli* KCCM 80135 as a feed additive does not represent a risk to the environment.

The production organism and its DNA were not detected in the final product. The additive does not pose any environmental safety concern associated with the genetic modification of the production strain.

## 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.

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

<sup>46</sup> Technical dossier/Section III/Annex III.3.3.

<sup>47</sup> Technical dossier/Section III/Annex III.3.4.

<sup>48</sup> Technical dossier/Section III/Annex III.3.5.

### 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<sup>49</sup> and Good Manufacturing Practice.

## 4. Conclusions

The production strain and its recombinant DNA were not detected in the additive. L-Tryptophan produced by fermentation with *E. coli* KCCM 80135 does not raise any safety concern with regard to the genetic modification of the production strain.

The use of L-tryptophan produced using *E. coli* KCCM 80135 in supplementing feed to compensate for tryptophan deficiency in feedingstuffs is safe for non-ruminant target species. Using unprotected forms of tryptophan in ruminants can be a risk.

The use of L-tryptophan produced by fermentation using *E. coli* KCCM 80135 in animal nutrition presents no safety concerns to consumers of animal products.

L-Tryptophan produced by *E. coli* KCCM 80135 is not toxic by inhalation. The additive is not an irritant to skin and eyes, and it is not a skin sensitiser.

The additive under assessment is considered safe for the environment.

The additive under assessment is regarded as an effective source of the amino acid L-tryptophan for all non-ruminant species. If the product L-tryptophan is intended for use in 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.

## Chronology

Date	Event
14/06/2017	Dossier received by EFSA. Feed grade L-tryptophan produced with <i>Escherichia coli</i> K12 KCCM80135. Submitted by CJ Europe GmbH.
30/06/2017	Reception mandate from the European Commission
15/03/2018	Application validated by EFSA – Start of the scientific assessment
07/05/2018	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, stability, genetic modification process.</i>
15/06/2018	Comments received from Member States
15/06/2018	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
04/07/2018	Reception of supplementary information from the applicant - Scientific assessment re-started
12/11/2018	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, characterisation of the production strain.</i>
19/12/2018	Request to the EURL to evaluate the method to measure tryptophan in the additive.
30/01/2019	Reception of the evaluation of the EURL of the method to measure tryptophan in the additive
06/03/2019	Reception of supplementary information from the applicant - Scientific assessment re-started
02/04/2019	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

<sup>49</sup> Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

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## Abbreviations

BCOP	bovine corneal opacity and permeability
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'-Ethyldiene-bis-L-tryptophan
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
FID	fluorescence detection
HPLC	high-performance liquid chromatography
HSE	UK Health and Safety Executive
IUPAC	International Union of Pure and Applied Chemistry
IVIS	<i>in vitro</i> irritancy score
LC <sub>50</sub>	lethal concentration, median
LOD	limit of detection
MTCA	1-methyl-1,2,3,4-tetrahydro-beta-carboline-3-carboxylic acid
MIC	minimum inhibitory concentration
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
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzodioxin
PCDF	polychlorinated dibenzofuran
RH	relative humidity
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, 2015a,b)

### Calculation of maximum acceptable levels of exposure from feed additives

The probable exposure time according to EFSA guidance (EFSA FEEDAP Panel, 2012b) for additives added in premixtures assumes a maximum of 40 periods of exposure per day, each comprising 20 s =  $40 \times 20 = 800$  seconds/day. With an uncertainty factor of 2, maximum inhalation exposure would occur for  $2 \times 800 = 1,600$  seconds = 0.444 hours/day. Again, assuming a respiration volume of 1.25 m<sup>3</sup>/hour, the inhalation volume providing exposure to potentially endotoxin-containing dust would be  $0.444 \times 1.25 = 0.556$  m<sup>3</sup>/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<sup>3</sup>.

### 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<sup>3</sup>, 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<sup>3</sup>, then the content of endotoxins of the dust,  $c$  IU/m<sup>3</sup>, 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, 2012b).

**Table A.1:** Estimation of user exposure to endotoxins from the additive L-tryptophan produced by *Escherichia coli* KCCM 80135, 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	420	Technical dossier
	$b$	Dusting potential (g/m <sup>3</sup> )	0.85	Technical dossier
$a \times b$	$c$	Endotoxin content in the air (IU/m <sup>3</sup> )	357	
	$d$	No of premixture batches made/working day	40	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012b)
	$e$	Time of exposure (s) per production of one batch	20	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012b)
$d \times e$	$f$	Total duration of daily exposure/worker (s)	800	
	$g$	Uncertainty factor	2	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012b)
$f \times g$	$h$	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 <sup>3</sup> ) per eight-hour working day	10	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012b)
$j/8 \times i$	$k$	Inhaled air during exposure (m <sup>3</sup> )	0.56	
$c \times k$	$l$	<b>Endotoxin inhaled (IU) during exposure per eight-hour working day</b>	<b>198.3</b>	
	$m$	Health-based recommended exposure limit of endotoxin (IU/m <sup>3</sup> ) per eight-hour working day	90	Health Council of the Netherlands, 2010
$m \times j$	$n$	<b>Health-based recommended exposure limit of total endotoxin exposure (IU) per eight-hour working day</b>	<b>900</b>	



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

## **Annex A – Executive Summary of the EURL report on the analytical methods submitted in connection with the application for authorisation of L-tryptophan produced by fermentation with *Escherichia coli* K12 KCCM 80135**

In the current application, authorisation is sought under Article 4(1) for L-tryptophan produced by fermentation with *Escherichia coli* K12 KCCM 80135, 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 Implementing Regulation (EU) 2017/873. 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, premixtures, feedingstuffs and water, the Applicant submitted a single-laboratory validated analytical method based on High-Performance Liquid Chromatography (HPLC) with photometric detection. However, based on the data provided, the EURL cannot recommend this method for official control.

The EURL previously evaluated and recommended (i) the ring-trial validated EN ISO 13904:2016 method based on HPLC with fluorescence detection (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 studies, the Applicant presented experimental data obtained analysing tryptophan in water with the VDLUFA official method based on HPLC-FLD 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.