European Food Safety Authority

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## SCIENTIFIC OPINION

# Scientific Opinion on the safety and efficacy of L-tryptophan, technically pure, produced by *Escherichia coli* strains DSM 25084, KCCM 11132P or SARI12091203 for all animal species based on a dossier submitted by AMAC EEIG[1]

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

European Food Safety Authority (EFSA), Parma, Italy

## ABSTRACT

The product L-tryptophan is a nutritional feed additive produced by fermentation using one of three strains of *Escherichia coli*. L-Tryptophan produced by *E. coli* SARI12091203 could not be assessed because the data submitted did not permit the identity and safety of the strain, and the purity of the additive, to be determined. The EFSA Panel on Additives and Products or Substance used in Animal Feed (FEEDAP) could not conclude on the safety of this product for target animals, for consumers, users and the environment. Strain KCCM 11132P is genetically modified, but its product gives no cause for concern on GM grounds because no recombinant DNA or live bacteria were detected. L-Tryptophan produced by *E. coli* KCCM 11132P or *E. coli* DSM 25084 is safe for non-ruminant target species when supplemented to diets in appropriate amounts, but should not be given to ruminants in an unprotected form because of the formation of skatole (3-methylindole) during ruminal fermentation. L-Tryptophan produced by *E. coli* KCCM 11132P or *E. coli* DSM 25084 contains low concentrations of 1,1′-ethylidene-bis-L-tryptophan and 1-methyl-1,2,3,4-tetrahydro-beta-carboline-3-carboxylic acid and is safe for the consumer of animal products. The level of endotoxins present in the products of *E. coli* KCCM 11132P or *E. coli* DSM 25084 and its possible dusting potential indicate an inhalation risk for the user. In the absence of data on the potential for dermal sensitisation it is concluded that such potential may exist. No other concerns for user safety (inhalation toxicity, skin and eyes irritation) regarding the L-tryptophan produced by *E. coli* DSM 25084 were identified. The use of the product L-tryptophan produced by *E. coli* KCCM 11132P or *E. coli* DSM 25084 in animal nutrition does not pose a risk to the environment. The products under application are efficacious sources of the amino acid L-tryptophan for animal nutrition.



## KEY WORDS

nutritional additive, amino acids, L-tryptophan, safety, efficacy, genetically modified microorganisms

[1] On request from the European Commission. Questions No EFSA-Q-2011-00946, adopted on 10 September 2015.

[2] Panel members: Gabriele Aquilina, Vasileios Bampidis, Maria de Lourdes Bastos, Georges Bories, Andrew Chesson, Pier Sandro Cocconcelli, Maria Luisa Fernandez-Cruz, Gerhard Flachowsky, Jürgen Gropp, Boris Kolar, Maryline Kouba, Secundino Lopez Puente, Marta Lopez-Alonso, Alberto Mantovani, Baltasar Mayo, Fernando Ramos, Guido Rychen, Maria Saarela, Roberto Edoardo Villa, Robert John Wallace and Pieter Wester. Correspondence: feedap@efsa.europa.eu

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## SUMMARY

Following a request from European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP Panel) was asked to deliver a scientific opinion on L-tryptophan, technically pure, produced using microbial fermentation by *Escherichia coli* strains DSM 25084, KCCM 11132P or SARI12091203and to be used as a nutritional feed additive for all animal species.

L-Tryptophan products made by fermentation with *E. coli* KCCM 11132P and *E. coli* DSM 25084 are free of the production strain. No recombinant DNA was detected in the final product from *E. coli* KCCM 11132P, indicating no safety concern with regard to its genetic modification.

The use of L-tryptophan produced by *E. coli* KCCM 11132P or *E. coli* DSM 25084 in supplementing feed to compensate for tryptophan deficiency in feedingstuffs is safe for non-ruminant target species. However, excess doses would create amino acid imbalances with negative consequences on animal performance. The use of unprotected L-tryptophan in ruminant feed should be avoided. The FEEDAP Panel has concerns on the safety of L-tryptophan for target species when administered via water for drinking.

As the L-tryptophan products produced by fermentation with *E. coli* KCCM 11132P or *E. coli* DSM 25084 are highly pure and because neither the amino acid L-tryptophan nor its metabolites accumulate in animal tissues, and the concentrations of 1,1′-ethylidene-bis-L-tryptophan (EBT) and 1-methyl-1,2,3,4-tetrahydro-beta-carboline-3-carboxylic acid (MTCA) are low (< 10 mg/kg additive each), these products present no concern to consumers of animal products.

The level of endotoxins present in the products of *E. coli* KCCM 11132P or *E. coli* DSM 25084 and its possible dusting potential indicate an inhalation risk for the user. In the absence of data on the potential for dermal sensitisation it is concluded that such potential may exist. No other concerns for user safety (inhalation toxicity, skin and eyes irritation) regarding the L-tryptophan produced by *E. coli* KCCM 11132P or *E. coli* DSM 25084 were identified.

The use of the product L-tryptophan produced by *E. coli* KCCM 11132P or *E. coli* DSM 25084 in animal nutrition does not pose a risk to the environment.

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

L-Tryptophan produced by *E. coli* SARI12091203 could not be assessed because the data submitted did not permit the identity and safety of the strain, and the purity of the additive, to be determined. The European Food Safety Authority (EFSA) FEEDAP Panel could not conclude on the safety of this product for target animals, and on the safety concerning consumers, users and the environment.

The FEEDAP Panel made some recommendations on labelling.

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## BACKGROUND

Regulation (EC) No 1831/2003[4] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7. In particular Article 10(2) of that Regulation also specifies that for existing products within the meaning of Article 10(1), an application shall be submitted in accordance with Article 7, at the latest one year before the expiry date of the authorisation given pursuant to Directive 70/524/EEC for additives with a limited authorisation period, and within a maximum of seven years after the entry into force of this Regulation for additives authorised without time limit or pursuant to Directive 82/471/EEC.

The European Commission received a request from the consortium AMAC/EEIG - Amino Acids Authorisation Consortium[5], for authorisation of a new use (i.e. use in water for drinking), and/or re-evaluation of authorisation of the product L-tryptophan technically pure when used as a feed additive for all animal species (category: nutritional additive; functional group: amino acids, their salts and analogues) under the conditions described in Table 1.

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), and under Article 10(2) (re-evaluation of an authorised feed additive). EFSA received directly from the applicant the technical dossier in support of this application.[6] According to Article 8 of that Regulation, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. The particulars and documents in support of the application were considered valid by EFSA as of 24 November 2011.

L-Tryptophan technically pure, was first authorised for use in animal nutrition by Directive 88/485/EEC.[7] It is currently authorised as a nutritional additive (functional group amino acids, their salts and analogues) for use in all animal species without time limit and without maximum content in feed.

Amino acids such as L-tryptophan may be used in the manufacture of infant formulae and follow-on formulae in order to satisfy the requirements on amino acids and other nitrogen compounds (Commission Directive 2006/141/EC).[8] L-Tryptophan is described in the European Pharmacopoeia (2010), monograph 01/2009:1272. L-Tryptophan is also listed as a pharmacologically active substance in veterinary medicinal products and is not subject to maximum residue levels when used in food producing animals.[9] Tryptophan is registered as an ingredient for use in cosmetics as antistatic, for hair conditioning and masking (Commission Decision 2006/257/EC).[10]

The EFSA Panel on Additives and Products or Substances used in Animal Feed published four opinions on the safety and efficacy of L-tryptophan produced by different strains of *Escherichia coli*

[4] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition OJ L 268, 18.10.2003, p. 29.

[5] On 19/12/2012, the rights of AMAC/ EEIG were transferred to FEFANA asbl, Avenue Louise 130A, Box 1, 1050 Brussels, Belgium). Companies: Chr. Olesen Group representing Dream Garden Biotechnology, CJ Europe GmbH and Evonik Degussa GmbH.

[6] EFSA Dossier references: FAD-2010-0056.

[7] Commission Directive 88/485/EEC of 326 July 1988 amending the Annex to Council Directive 82/471/EEC concerning certain products used in animal nutrition. OJ L 239/36, 30.8.1988, p. 4.

[8] Commission Directive 2006/141/EC on infant formulae and follow-on formulae, OJ L 401, 30.12.2006, p. 1.

[9] Commission Regulation (EC) No 1931/1999, amending Annexes I, II and III of Council Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. OJ L 240, 10.09.1999, p. 3.

[10] Commission decision 2006/257/EC of 9 February 2006 amending Decision 96/335/EC establishing an inventory and a common nomenclature to ingredients employed in cosmetic products. OJ L 97, 5.4.2006, p. 1.

for all animal species (EFSA FEEDAP Panel, 2013, 2014a, b, 2015). 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, 2011).

## TERMS OF REFERENCE

According to Article 8 of Regulation (EC) No 1831/2003, EFSA shall determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment and the efficacy of the product L-tryptophan technically pure, produced by fermentation with *Escherichia coli* strains DSM 25084, KCCM 11132P or, SARI12091203 when used under the conditions described in Table 1.

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

| | |
|---|---|
| **Additive** | L-Tryptophan, technically pure |
| **Registration number/EC No/No** (if appropriate) | 3.4.1. |
| **Category of additive** | 3. Nutritional additives |
| **Functional group(s) of additive** | c. Amino Acids, their salts and analogues |

| **Description** | | | |
|---|---|---|---|
| Composition, description | Chemical formula | Purity criteria (if appropriate) | Method of analysis (if appropriate) |
| L-Tryptophan | $C_{11}H_{12}N_2O_2$ | minimum 98% | Regulation (EC) No 152/2009 and Commission Directive (EC) No 2000/45 |

| | |
|---|---|
| **Trade name** (if appropriate) | Not appropriate |
| **Name of the holder of authorisation** (if appropriate) | Not appropriate |

| **Conditions of use** | | | | |
|---|---|---|---|---|
| Species or category of animal | Maximum Age | Minimum content | Maximum content | Withdrawal period (if appropriate) |
| | | mg/kg of complete feedingstuffs, supplementary feed (based on end feed) and in water | | |
| All animal species and categories | - | - | - | - |

| **Other provisions and additional requirements for the labelling** | |
|---|---|
| Specific conditions or restrictions for use (if appropriate) | Not applicable |
| Specific conditions or restrictions for handling (if appropriate) | Please refer to MSDS |
| Post market monitoring (if appropriate) | Not applicable |
| Specific conditions for use in complementary feedingstuffs or water (if appropriate) | Not applicable |

| **Maximum Residue Limit (MRL)** (if appropriate) | | | |
|---|---|---|---|
| Marker residue | Species or category of animal | Target tissue(s) or food products | Maximum content in tissues |
| - | - | - | - |

## ASSESSMENT

### 1. Introduction[11]

L-Tryptophan is an essential amino acid for all animal species. L-Tryptophan, technically pure (minimum content of L-tryptophan 98 %, 'as is' basis), was first authorised for use in animal nutrition by Directive 88/485/EEC. It is currently included in the European Union Register of Feed Additives.

The current application is for the re-evaluation of L-tryptophan, technically pure, produced either by genetically modified (GM) strains or by non-GM strains of *Escherichia coli*. It is intended to be used in all animal species as a nutritional additive in feed and in water for drinking.

The objective of feed supplementation with essential amino acids is to complete the amino acid profile of the diet in order to closely meet the individual amino acid requirements of the animals or to compensate for potential imbalances. The supplementation of feedingstuffs with amino acids is a conventional measure in improving the protein quality and utilisation. L-Tryptophan is well recognised as an essential amino acid in animal nutrition. Under European feeding practices, after L-lysine and L-threonine, L-tryptophan is one of the next most limiting amino acid in pig diets. As well as serving as a building block for proteins, tryptophan is a critical precursor for the functions of nervous and immune systems. Emerging evidence from recent studies shows that tryptophan and its metabolites (e.g. serotonin (5-hydroxytryptamine (5-HT)) and melatonin) can regulate feed intake, reproduction, immunity, neurological function and anti-stress responses (Le Floc'h and Sève, 2007; Yao et al., 2011).

### 2. L-Tryptophan produced by *E. coli* DSM 25084

#### 2.1. Characterisation

##### 2.1.1. 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*)-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 $C_{11}H_{12}N_2O_2$, and the molecular weight is 204.23 g/mol. The structural formula is given in Figure 1.

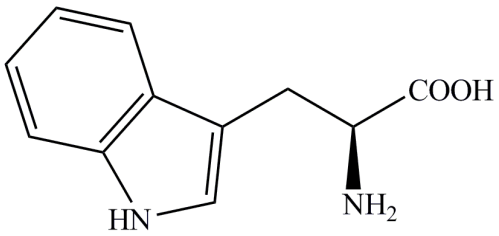


**Figure 1:** Structural formula of L-tryptophan.

The L-tryptophan content of the product is specified as ≥98 %, the other components being water (≤ 1 %) and ash (≤ 1 %).

The analysis of five batches of L-tryptophan (official European Union (EU) method) showed an average content of L-tryptophan of 99.1 %, on a 'as is' basis (range from 98.3 to 99.9 %),[12] water 0.0–0.1 % and ash 0.0–0.1 %. Other constituents detected in three batches were phenylalanine

[11] This section has been amended following the confidentiality claims made by the applicant.
[12] Technical dossier/Supplementary information January 2014/Annex Qxi.

(≤ 0.38 %) and tyrosine (≤ 0.29 %).[13] On a dry matter basis, the amount of identified material is, on average, 99.2 % (98.4–99.9 %).

The specific optical rotation of three batches of the final product was, on average, –31.1 ° (range from –31.1 to –31.3 °),[14] 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.[15]

### 2.1.2. Impurities

L-Tryptophan produced by *E. coli* DSM 25084 contained concentrations of arsenic < 2 mg/kg, lead < 5 mg/kg, mercury < 0.1 mg/kg and cadmium < 0.5 mg/kg.[16] The detected amounts of these impurities were negligible and often below the limits of detection (LODs)

Aflatoxins B1, B2, G1 and G2, as well as ochratoxin A, concentrations were below the LOD in three batches.[17]

Analysis of microbial contamination of the final product (three batches) indicated that *Salmonella* (in 25 g) was absent; the total plate count was ≤ $2 \times 10^2$ colony-forming units (CFU)/g, while anaerobic spore formers were <$2 \times 10^2$ CFU/g, yeast, filamentous fungi, *E. coli* and aerobic spore formers were < $10^2$ CFU/g and Enterobacteriaceae were < 10 CFU/g.[18]

Historical data (2006–2010) on dioxins and dioxin-like polychlorinated biphenyls (PCBs) showed that the amount detected was negligible (< 0.5 ng World Health Organization polychlorinated dibenzodioxin/dibenzofuran toxic equivalents (WHO-PCDD/F-TEQ/kg)). [19]

The endotoxin activity (three batches by European Pharmacopoeia method 2.6.14) ranged from 23.9 to 31.6 international unit (IU)/mg.[20]

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). Therefore, the applicant was requested to provide relevant data for the present application. The concentrations of EBT and MTCA analysed in three batches were < 10 mg/kg in all cases.[21] The maximum permitted content of EBT in L-tryptophan, as specified by the European Pharmacopoeia (2010), is 10 mg/kg. Higher amounts of MTCA have been found in some alcoholic beverages (Adachi et al., 2000).

### 2.1.3. Physical properties

The dossier contained only generic information on the physical properties of L-tryptophan: L-tryptophan feed grade is a white, yellow or light-grey crystalline powder. Its density is 1.34 g/cm$^3$ and its solubility in water is 11.4 g/L at 25 °C.[22]

[13] Technical dossier/Supplementary information January 2014/Annexes/Annex Qxi.
[14] Technical dossier/Supplementary information January 2014/Annexes Qv.
[15] European Pharmacopoeia monograph 1/2015:1272.
[16] Technical dossier/Supplementary information December 2012/Annex Qv and xi dioxins, heavy metals and arsenic.
[17] Technical dossier/Supplementary information December 2012/Annex Qv and xi. Supplementary information October 2014/Annex Qvi contam, LOD (in µg/kg) was 0.08 for aflatoxins and 0.1 for ochratoxin A.
[18] Technical dossier/Supplementary information December 2012/Annex Qvi and v and Supplementary information May 2015/Annex plate count.
[19] Technical dossier/Supplementary information December 2012/Annex Qv and xi.
[20] Technical dossier/Supplementary information May 2015/Annexes Qi.
[21] Technical dossier/Supplementary information December 2012/Annexes Qvi.
[22] Technical dossier/Annex II.3.1.

The particle size distribution of L-tryptophan produced by *E. coli* DSM 25084 (one batch) was measured by laser diffraction (v/v). The percentages below 100, 50 and 10 µm were 99, 80 and 18 %, respectively.[23]

The dusting potential (Stauber–Heubach method) of L-tryptophan produced by *E. coli* DSM 25084 was measured in three batches of the final product and ranged from 2.3 to 2.9 g/m$^3$.[24]

### 2.1.4. Characterisation of the production organism *E. coli* DSM 25084[25]

The *E. coli* production strain has been deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ) with the accession number DSM 25084.[26]

The dossier contains information on the strain *E. coli* DSM 25084 (an *E. coli* K-12 derivative) that allows to conclude that it does not represent safety concerns when used as production strain of the product under assessment. [27]

The production strain for L-tryptophan production is a derivative of *E. coli* K-12. Although *E. coli* K-12 is not included on the European Food Safety Authority (EFSA) qualified presumption of safety (QPS) list (EFSA FEEDAP Panel, 2013), it is not considered a human or animal pathogen, it has a long history of apparent safe use in industrial production and is scientifically recognised as a safe bacterial strain not producing toxic substances (US Environmental Protection Agency, 1997; http://epa.gov/biotech_rule/pubs/fra/fra004.htm; Gorbach, 1978). *E. coli* K-12 has been a widely used model organism in microbial genetics and physiology research, and has widespread use in industrial applications. *E. coli* K-12 is one of the most extensively studied of all microorganisms. Its genome sequence was published in 1997 (Blattner et al., 1997), which confirmed the absence of toxigenic potential. It does not appear in the most comprehensive review of pathogenic *E. coli* published by Nataro and Kaper (1998). Indeed, strain K-12 is commonly used as a 'base-model' (safety reference strain) against which the safety of other *E. coli* strains is assessed, (see Kaper et al., 2004).

Phenotypical analyses of antibiotic resistance were not provided. However, when targeting a gene involved in tryptophan biosynthesis in the production strain, no amplification was detected in three batches of the final product by polymerase chain reaction (PCR),[28] which is considered acceptable proof that the final product cannot contain entire genes coding for antibiotic resistance.

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### 2.1.5. Manufacturing process[29]

The dossier contains general information on the production process. Carbon and nitrogen sources, mineral salts and vitamins are used in fermentation media. After the fermentation, the biomass is inactivated by acidification and heating and separated. The fermentation broth is subsequently concentrated and L-tryptophan crystallised.[30] The material safety data sheet (MSDS) of the product under assessment is available.[31]

The qualitative composition of the fermentation media and the corresponding MSDS were provided.[32]

[23] Technical dossier/Supplementary information October 2014/Annexes Qi Particle size distribution.
[24] Technical dossier/Supplementary information October 2014/Annexes Qi Dusting Potential.
[25] This section has been amended following the confidentiality claims made by the applicant.
[26] Technical dossier/Supplementary information June 2012/Certificate of deposition.
[27] Technical dossier/Supplementary information May 2015/Annex Q.
[28] Technical dossier/Supplementary information October 2014/Confidential/Annex 2.
[29] This section has been amended following the confidentiality claims made by the applicant.
[30] Technical dossier/Section II.3.1.
[31] Technical dossier/Section II/Annexes/Annex II.3.1.
[32] Technical dossier/Supplementary information January 2015/Confidential/Annexes.

No viable cells of the production strain *E. coli* DSM 25084 were detected in three batches of the final product.[33]

The applicant stated that no antimicrobial compounds (including antibiotics) were used in the production process.[34]

### 2.1.6. Stability and homogeneity

#### 2.1.6.1. Shelf life

The shelf life of one batch of the additive (packaging unknown) was tested at 10 to 30 °C and 20 to 70 % relative humidity.[35] No losses were detected after 36 months.

#### 2.1.6.2. Stability in premixtures

The stability of one batch in a vitamin–mineral premixture (containing choline chloride), for turkeys at a supplementation rate of 4.9 %, was tested at 10–25 °C for six months. The premixture was packed in sealed bags consisting of a double paper layer and an inner plastic layer. The loss was 20 % after the six-month period.[36]

#### 2.1.6.3. Stability in feedingstuffs

The stability of one batch in a complete feed for chickens for fattening (based on maize, soybean meal and wheat), at a supplementation rate of 0.1 %, was tested after storage at ambient temperature (10–25 °C) for three months. The compound feed was stored packed in sealed bags consisting of a double paper layer and an inner plastic layer. The loss was of 4 % after the three-month period.[37]

#### 2.1.6.4. Stability in water

The stability of one batch at four concentrations (0.1, 0.5, 1.0 and 5.0 g/L) was tested in water at 25–28 °C for three days under laboratory conditions. No losses were detected.[38]

#### 2.1.6.5. Homogeneity

The homogeneous mixing of L-tryptophan in feed for chickens for fattening and premixtures at two inclusion rates (0.1 % and 4.9 %) was studied from 10 subsamples. The mean analysed values were 0.097 and 4.71 %, respectively, and the coefficients of variation 1.4 and 2.6 %.[39]

### 2.1.7. Physico-chemical incompatibilities in feed

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

### 2.1.8. Conditions of use

According to the applicant, the product is intended as a supplement to the feed for all animal species and categories without maximum content in feed or time of administration. It can be administered directly to the compound feed or via premixtures. It is proposed to be used also in water for drinking. No proposed inclusion levels are provided, as the optimal daily allowance in quantitative terms

[33] Technical dossier/Supplementary information October 2014/AMAC conf/Annex Qvi viable cells.
[34] Technical dossier/Supplementary information October 2014/Annex Qvii antimicrobial.
[35] Technical dossier/Supplementary information December 2012/Annex Qvii; Supplementary information October 2014/Annex Qx.
[36] Supplementary information December 2012/Annexes Qviii; Supplementary information January 2014/Annex Qvii; and Supplementary information October 2014/Annex Qx.
[37] Technical dossier/Supplementary information December 2012/Annexes Qix; Supplementary information January 2014/Annex Qviii; and Supplementary information October 2014/Annex Qx.
[38] Technical dossier/Supplementary information January 2014/Annex Qix; Supplementary information October 2014/Annex Qx.
[39] Technical dossier/Section II.4.2/Tables II.4.2a and b and supplementary information December 2012/Annexes Qx.

depends on the species, the physiological state of the animal, the performance level and the environmental conditions, as well as the amino acid composition of the unsupplemented diet.

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

EFSA has verified the EURL report as it relates to the methods used for the control of the product, L-tryptophan, in animal feed. The Executive Summary of the EURL report can be found in the Annex.

## 2.2. Safety

### 2.2.1. Safety for the target species

Tolerance studies are not normally required for highly purified amino acids. Such tolerance studies with a certain indispensable amino acid will inevitably result in amino acid imbalances, with depression of feed intake and hence impaired performance and increased nitrogen excretion. This is also the case for the product under application, which contains > 98.4 % tryptophan and less than 1 % unidentified material on a dry matter basis. The endotoxin activity ranged from 23.9 to 31.6 IU/mg. These values are compared with ca. 1 000 IU/mg commonly found in feedingstuffs (Cort et al., 1990). Therefore, at the usual conditions of use of the additive in feed, the endotoxins added by the additive would be insignificant compared with the background in feed. Therefore, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP Panel) considers that safety concerns for target species are unlikely to arise from this product. Since no particular safety concerns arose from the *E. coli* DSM 25084, the FEEDAP Panel confirms the safety for the target species of L-tryptophan produced by this strain.

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

Given the high purity of the product, the FEEDAP Panel considers that the use of L-tryptophan produced by the non-GM *E. coli* DSM 25084 is safe for non-ruminant target species when used to supplement the diet in appropriate amounts. The FEEDAP Panel reiterates that ruminal metabolism of unprotected L-tryptophan may result in the production of toxic quantities of 3-methylindole (skatole), which causes pulmonary disease (fog fever; emphysema) in cattle and goats (Hammond et al., 1978). Consequently, only a protected form of L-tryptophan should be used in ruminants.

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).

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

The use of L-tryptophan produced by *E. coli* DSM 25084 in supplementing feed to compensate for tryptophan deficiency in feedingstuffs is safe for non-ruminant target species. However, excess doses would create amino acid imbalances with negative consequences on animal performance. The use of unprotected L-tryptophan in ruminant feed should be avoided.

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

### 2.2.2. Safety for the consumer

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

As a general principle, conventional toxicology studies are considered to be inappropriate for proteogenic amino acids. Dietary intakes of amino acids that lead to amounts significantly below or above the optimum amount for health and performance will inevitably cause a physiological imbalance and, consequently, adverse effects.

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 the non-GM *E. coli* DSM 25084 is highly purified (> 98.4 % L-tryptophan and < 1 % unidentified material on a dry matter basis) and (2) the concentrations of EBT and MTCA are < 10 mg/kg additive, no additional toxicological data are required.

Amino acids 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. Therefore, the FEEDAP Panel concludes that the product, L-tryptophan, produced by fermentation with *E. coli* DSM 25084 presents no concern to consumers of animal products.

### 2.2.3. Safety for the user

The applicant provided toxicity studies on acute inhalation toxicity, dermal and eye irritation performed with the L-tryptophan produced by *E. coli* DSM 25084.[40]

#### 2.2.3.1. Effects on the respiratory system

In an acute inhalation toxicity study carried out in accordance with the Organisation for Economic Co-operation and Development (OECD) Guideline 403, six male and six female Crl:CD(SD)IGS BR Sprague–Dawley rats were exposed to L-tryptophan dust at a concentration of 5.17 g/m$^3$ (median aerodynamic diameter 3.1 µm) for four hours and monitored for a further 14 days.[41] There was no mortality or effect on clinical signs, body weight, body weight gain or gross pathology in the exposed animals.

The production species, *E. coli*, is a Gram-negative bacterium. Although the K-12 strain and its derivatives are safe from the point of view of enterotoxins and other virulence factors (Gorbach, 1978; EPA, 1997; Bauer et al., 2007), *E. coli* K-12 retains lipopolysaccharide in its cell envelope (Luchi and Morrison, 2000; Svensson et al., 2005; Gao et al., 2006), which potentially may result in endotoxin activity in the final product. The user can suffer from occupational respiratory disease depending on the level of endotoxins in the air and dust (Rylander, 1999; Thorn, 2001). The inhalation of endotoxins may cause the following acute symptoms: dry cough, dyspnoea accompanied by diminished lung function, fever and general malaise (Appendix A). The bacterial endotoxin activity was analysed in three batches and ranged from 24 to 32 IU/mg. The dusting potential measured in three batches ranged from 2.3 to 2.9 g/m$^3$. About 70 % (w/w) of the particles had a diameter smaller than 50 µm and about 17 % lower than 10 µm.

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 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 51 556 endotoxin IU per working day, thus indicating an inhalation exposure to endotoxins for persons handling the additive.

[40] Technical dossier/Supplementary information October 2014/Conf/Response letter Sin 01042014.
[41] Technical dossier/Section III/Reference III.3.1.

2.2.3.2. Effects on skin and eyes

In an acute dermal irritation study (in accordance with OECD Guideline 404), the shaved dorsal skin of three New Zealand White (NZW) rabbits was exposed to 0.5 g of L-tryptophan in 0.1 mL sterile water (gauze patch) for four hours. At the end of exposure the treated skin was cleaned and then examined at 1, 24, 48 and 72 hours after exposure for signs of irritancy. No skin irritancy or systemic effects were observed.[42]

In an eye irritation test (in accordance with OECD Guideline 405), 100 mg of L-tryptophan was applied to the conjunctival sack of one of the eyes of three NZW rabbits, without rinsing. Observations were performed at 1, 24, 48 and 72 hours after application. The exposure induced a transient slight eye irritation visible after one hour in all of the rabbits; the effects were no longer present at the examination 24 hours after treatment.[43]

2.2.3.3. Conclusions on safety for the user

The level of endotoxins present in the product and its possible dusting potential indicate an inhalation risk for the user. In the absence of data on the potential for dermal sensitisation it is concluded that such potential may exist. No other concerns for user safety (inhalation toxicity, skin and eyes irritation) regarding the L-tryptophan produced by *E. coli* DSM 25084 were identified.

### 2.2.4. 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 excreted not 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 the additive 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. It is concluded that the use of the product L-tryptophan, technically pure, produced by *E. coli* DSM 25084 as a feed additive does not represent a risk to the environment.

## 2.3. 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 L-tryptophan, technically pure, 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 used in ruminants, it should be protected from ruminal degradation.

## 2.4. 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[44] and Good Manufacturing Practice.

[42] Technical dossier/Section III/Reference III.3.2.
[43] Technical dossier/Section III/Reference III.3.3.
[44] OJ L 35, 8.2.2005, p. 1.

## 2.5. Conclusions and recommendations on L-tryptophan produced by *E. coli* DSM 25084

### 2.5.1. Conclusions

The use of L-tryptophan produced by *E. coli* DSM 25084 as a feed supplement is safe for non-ruminant target species. However, excess doses would create amino acid imbalances with negative consequences on animal performance. The use of unprotected L-tryptophan in ruminant feed should be avoided. The FEEDAP Panel has concerns on the safety of L-tryptophan for target species when administered via water for drinking.

As L-tryptophan produced by fermentation by *E. coli* DSM 25084 is highly pure (≥98.4 % on a dry matter basis) and because neither the amino acid L-tryptophan nor its metabolites accumulate in animal tissues, and the concentrations of EBT and MTCA are low (< 10 mg/kg additive each), this product presents no concern to consumers of animal products.

The level of endotoxins present in the product and its possible dusting potential indicate an inhalation risk for the user. In the absence of data on the potential for dermal sensitisation it is concluded that such potential may exist. No other concerns for user safety (inhalation toxicity, skin and eyes irritation) regarding the L-tryptophan produced by *E. coli* DSM 25084 were identified.

The use of L-tryptophan produced by *E. coli* DSM 25084 in animal nutrition does not pose a risk to the environment.

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

### 2.5.2. Recommendations

The description of the additive should contain the statement 'produced by fermentation with *Escherichia coli* DSM 25084.'

The specification of the additive should be amended indicating a 1,1′-ethylidene-bis-L-tryptophan (EBT) content <10 mg/kg.

## 3. L-Tryptophan produced by *E. coli* KCCM 11132P

## 3.1. Characterisation

### 3.1.1. Characterisation of the active substance/additive

The characterisation of the active substance and the specification of the additive have been described above (section 2.1.1).

Five batches were analysed (VDLUFA 4.11.2) and the average tryptophan content was 99.6 % on a 'as is' basis (ranging from 99.3 to 99.8 %).[45] Water ranged from 0.1 to 0.6 % and ash from < 0.1 to 0.3 %.

Analytical data on specific optical rotation (five batches) showed an average of –31.2 ° (range –31.1 to –31.3 °).[46] This is within the range described in the European Pharmacopoeia (–30 to –33 °) for this amino acid and confirms the identity of the L-enantiomer.[47]

[45] Technical dossier/Supplementary information January 2015/Annex Qiii CoAs new.
[46] Technical dossier/Supplementary information January 2014/Annexes Qv.
[47] European Pharmacopoeia monograph 1/2015:1272.

### 3.1.2. Impurities

L-Tryptophan produced by *E. coli* KCCM 11132P had concentrations of arsenic <1.0 mg/kg, lead < 1.0 mg/kg, mercury ranging from < 0.01 to 0.12 mg/kg and cadmium <0.1 mg/kg (three batches analysed).[48] The detected amounts of these impurities were negligible and often below the LODs. Other impurities declared were zinc (ranging from 14.7 to 21.2 mg/kg), copper (ranging from 1.9 to 3.6 mg/kg) and chromium (ranging from 1.9 to 2.7 mg/kg).

Regarding the content of mycotoxins in the final product, one batch of the additive was analysed to determine the levels of aflatoxins (3.3 µg/kg) and ochratoxin A (< 5 µg/kg).

Analysis of microbial contamination of the final product indicated that *Salmonella* (in 25 g) was absent in one batch. Sulphate-reducing clostridia were also absent in the same batch.[49]

The content of dioxins and dioxin-like PCBs was analysed in one batch of the additive.[50] The amount detected was negligible (0.085 ng TE-PCDD/F-WHO 1998 (upper bound) and PCBs under the LOD).

The endotoxin activity was determined in three batches (EP 2.6.14 method). Two batches contained > 0.5 IU/mg (but were not further quantified) and the third 2 900 IU/mg.[51]

The concentrations of EBT and MTCA were analysed in three batches, for reasons described above (section 2.1.2). EBT averaged 3 mg/kg (range from 0.8 to 4.4 mg/kg) and MTCA 6 mg/kg (range from 4.2 to 8.0 mg/kg).

### 3.1.3. Physical properties

General information provided in the dossier has been described above (see section 2.1.3).

The particle size distribution of L-tryptophan produced by *E. coli* KCCM 11132P (one batch analysed) was measured by laser diffraction (v/v). The percentages below 100, 50 and 10 µm were about 90, 67 and 17 %, respectively.[52]

The dusting potential (Stauber–Heubach) measured in one batch was 2.5 g/m$^3$.[53]

### 3.1.4. Characterisation of the production organism *E. coli* KCCM 11132P[54]

The product, L-tryptophan, is produced by a GM strain of *E. coli*, deposited at the Korean Culture Center of Microorganisms (KCCM) with the accession number KCCM 11132P.[55] The identity of the production strain was confirmed by 16S rRNA gene sequence analysis.[56] Information is provided in the dossier on the susceptibility of the production strain to antimicrobials of human and veterinary importance.[57]

The recipient strain is a derivative of *E. coli* K-12.[58] *E. coli* K-12 is a well-characterised Gram-negative bacterium which safety (non-pathogenicity) has been reviewed extensively (see section 2.1.4). The technical dossier contains detailed and sufficient information on the recipient

[48] Technical dossier/Supplementary information December 2012/Annex Qv heavy metals and arsenic.
[49] Technical dossier/Supplementary information December 2012/Annex Qiv, v and xi.
[50] Technical dossier/Supplementary information December 2012/Annex Qiv, v and xi.
[51] Technical dossier/Supplementary information May 2015/Annex Q1 Analysis of Microbial Endotoxins.
[52] Technical dossier/Supplementary information October 2014/Annexes Qi Particle size distribution.
[53] Technical dossier/Supplementary information October 2014/Annexes Qi Dusting Potential.
[54] This section has been amended following the confidentiality claims made by the applicant.
[55] Technical dossier/Supplementary information December 2012/ Annex Qi_L-tryptophan certificate of deposition.
[56] Technical dossier/Supplementary information June 2012/ Annex C_II_2_01.
[57] Technical dossier/Supplementary information January 2015/Conf/Annex Trp Qv 04-2014; Supplementary information November 2013/Attachment 1; Supplementary information June 2012/ Annex C_II_2_02, and Supplementary information January 2015/Conf_220115/Trp Annex CONFID_Q3_11-2014.
[58] Technical dossier/Supplementary information June 2012/Confid Sect II Identity.

microorganism including safety aspects, the origin and function of the different genetic elements introduced in the production strain, the genetic modification process and the genetic and phenotypic traits introduced. [59]

### 3.1.5. Manufacturing process[60]

The generic information contained in the dossier has been described above (see section 2.1.5).

The qualitative composition of the fermentation media and the corresponding MSDS were provided.[61]

Neither viable cells of the production strain nor its recombinant DNA was detected in three batches of the final product. [62]

Information was provided in the dossier on whether antimicrobial compounds (including antibiotics) were used during the manufacturing process.[63]

### 3.1.6. Stability and homogeneity

#### 3.1.6.1. Shelf life

The shelf life of one batch of the additive was studied when packed in a 10 kg three-ply paper bag with one-ply polyethylene inner lining and stored at ambient temperature shielded from sunlight for 2.5 years. No losses were detected.[64]

#### 3.1.6.2. Stability in premixtures

The stability of one batch was tested in a vitamin–mineral premixture (containing choline chloride),[65] at a supplementation rate of 3.4 % at 25 and 40 °C, stored in sealed brown glass for six months. Losses of 3 % were observed only at 25 °C.[66]

#### 3.1.6.3. Stability in feedingstuffs

The stability of one batch was tested in a mash compound feed for pigs for fattening (based on wheat, barley, soybean meal and maize) at a supplementation rate of 0.05 %, packed in sealed brown glass and stored at 25 and 40 °C for three months. The mash was conditioned at 60 °C for eight seconds and 2.25 bar.[67] Losses of 12 and 25 % were observed at 25 and 40 °C, respectively. The influence of the processing was not reported. [68]

#### 3.1.6.4. Stability in water for drinking

The stability of another batch in water at 0.1 g/L concentration was tested at 25 and 40 °C following 48 hours of storage. No losses were detected. [69]

[59] Technical dossier/Supplementary information June 2012/ Annex C_II_2_02.
[60] This section has been amended following the confidentiality claims made by the applicant.
[61] Technical dossier/Supplementary information January 2015/Confidential/add Info L-trp.
[62] Technical dossier/Supplementary information January 2015/Conf_220115/Trp Annex CONFID_Q5_11-2014 and Supplementary information April 2014/Annex_CONFID_Qi_L-Trp_Abs recomb DNA_26032014.
[63] Technical dossier/Supplementary information October 2014/ Supplementary information October 2014/Conf/Annex confide Qiv.
[64] Supplementary information January 2014/Annex Qiv.
[65] Supplementary information October 2014/AMAC conf/Annex Qix and Supplementary information January 2015/AMAC conf/Annex Qix composition.
[66] Technical dossier/Supplementary information January 2014/Annex Qvii.
[67] Technical dossier/Supplementary information October 2014/AMAC conf/Annex Qix.
[68] Technical dossier/Supplementary information January 2014/Annex Qviii.
[69] Technical dossier/Supplementary information December 2012/Annex Qxi

3.1.6.5. Homogeneity

The capacity of one batch of L-tryptophan to homogeneously distribute in mash feed (described above) at an inclusion rate of 0.05 % was studied in 10 subsamples. The average value was 0.044 % and the coefficient of variation 1.6 %.[70]

### 3.1.7. Physico-chemical incompatibilities in feed

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

### 3.1.8. Conditions of use

The general conditions of use provided in the dossier have been described above (see section 2.1.8).

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

EFSA has verified the EURL report as it relates to the methods used for the control of L-tryptophan in animal feed. The Executive Summary of the EURL report can be found in Annex.

## 3.2. Safety

### 3.2.1. Safety aspects of the genetic modification[71]

The recipient organism is considered to be safe. The molecular characterisation of the genetic modifications does not raise safety concerns regarding the final product.

### 3.2.2. Safety for the target species

Safety considerations follow closely those described above (section 2.2.1).

The endotoxin activity of this L-tryptophan ranged from > 0.5 to 2 900 IU/mg. These values are compared with circa 1 000 IU/mg commonly found in feedingstuffs (Cort et al., 1990). Therefore, at the usual conditions of use of the additive, the endotoxins added by the additive would not add significantly to the background in feed. Therefore, the FEEDAP Panel considers that safety concerns for target species are highly unlikely to arise from this additive. Since no particular safety concerns arose from the modification of the GM *E. coli* KCCM 11132, the FEEDAP Panel confirms the safety for the target species of L-tryptophan produced by this strain, with qualifications concerning its use in ruminants (section 2.2.1).

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), and general considerations for consumer safety have been discussed above (section 2.2.2). Considering that the product originating from the GM *E. coli* KCCM 11132P is also highly purified (> 99.8 % L-tryptophan and < 1 % unidentified material on a dry matter basis) and contains low concentrations of EBT and MTCA (< 10 mg/kg additive), no additional toxicological data are required. This product presents no concern to consumers of animal products.

### 3.2.4. Safety for the user

In all the toxicity studies submitted, the test item was the product of *E. coli* DSM 25084 (see section 2.2.4).[72] As the production strain *E. coli* KCCM 11132P shares a common ancestor (*E. coli* K-12) with

[70] Technical dossier/Supplementary information January 2014/Annex Qx.
[71] This section has been amended following the confidentiality claims made by the applicant.

*E. coli* DSM 25084, the production process is similar, as is product composition (amount of unidentified material < 1 % on a dry matter basis), the FEEDAP Panel considers that the outcomes of the toxicity studies described above (see sections 2.2.3.1 and 2.2.3.2) can also apply to the product of *E. coli* KCCM 11132P.

#### 3.2.4.1. Effects in the respiratory system

The possible problems regarding *E. coli* endotoxins have been discussed above (section 2.2.3). The bacterial endotoxin activity was analysed in three batches and ranged from > 0.5 to 2 900 IU/mg. The dusting potential measured in one batch was 2.5 g/m$^3$. About 70 % (w/w) of the particles had a diameter smaller than 50 µm and about 17 % lower than 10 µm.

Based upon the calculation of the potential endotoxin content in dust (section 2.2.3; Appendix A), the inhalation exposure could be up to 4 027 778 endotoxin IU per eight-hour working day, indicating a potentially high inhalation exposure to endotoxins for persons handling the additive.

#### 3.2.4.2. Conclusions on safety for the user

The level of endotoxins present in the product and its possible dusting potential indicate a severe inhalation risk for the user. In the absence of data on the potential for dermal sensitisation it is concluded that such potential may exist. No other concerns for user safety (inhalation toxicity, skin and eyes irritation) regarding the L-tryptophan produced by *E. coli* KCCM 11132P were identified.

### 3.2.5. Safety for the environment

Neither the production strain *E. coli* KCCM 11132P nor its recombinant DNA was detected in the final product. The final product does not pose any environmental safety concern associated with the genetic modification. No environmental issues arise from the metabolism and excretion of metabolic products (section 2.2.4).

The amino acid L-tryptophan is a physiological and natural component of animals and plants. 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 the additive 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. It is concluded that the use of the product, L-tryptophan, technically pure, produced by *E. coli* KCCM 11132P as a feed additive does not represent a risk to the environment.

## 3.3. Efficacy

Efficacy considerations for supplemental L-tryptophan have been described above (section 2.3).

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

## 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[73] and Good Manufacturing Practice.

[72] Supplementary information October 2014/Conf/Response letter Sin 01042014.
[73] OJ L 35, 8.2.2005, p. 1.

### 3.5. Conclusions and recommendations on L-tryptophan produced by fermentation with *E. coli* KCCM 11132P

#### 3.5.1.1. Conclusions

L-Tryptophan made by fermentation using *E. coli* KCCM 11132P is free of the production strain and has a high purity (≥ 99.8 % on a dry matter basis). No recombinant DNA was detected in the product.

The use of L-tryptophan produced by *E. coli* KCCM 11132P in supplementing feed to compensate for tryptophan deficiency in feedingstuffs is safe for non-ruminant target species. However, excess doses would create amino acid imbalances with negative consequences on animal performance. The use of unprotected L-tryptophan in ruminant feed should be avoided. The FEEDAP Panel has concerns on the safety of L-tryptophan for target species when administered via water for drinking.

As the L-tryptophan product is highly pure and because neither the amino acid L-tryptophan nor its metabolites accumulate in animal tissues, and the concentrations of EBT and MTCA are low, this product presents no concern to consumers of animal products.

The level of endotoxins present in the product and its possible dusting potential indicate a severe inhalation risk for the user. In the absence of data on the potential for dermal sensitisation it is concluded that such potential may exist. No other concerns for user safety (inhalation toxicity, skin and eyes irritation) regarding the L-tryptophan produced by *E. coli* KCCM 11132P were identified

The use of L-tryptophan produced by *E. coli* KCCM 11132P in animal nutrition does not pose a risk to the environment.

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

#### 3.5.1.2. Recommendations

The description of the additive should contain the statement 'produced by fermentation with *Escherichia coli* KCCM 11132P.'

The specification of the additive should be amended indicating a 1,1'-ethylidene-bis-L-tryptophan (EBT) content < 10 mg/kg.

## 4. L-Tryptophan produced by *E. coli* SARI12091203

### 4.1. Characterisation

#### 4.1.1. Characterisation of the active substance/additive

Characterisation of the active substance and the specification of the additive have been described previously (section 2.1.1).

Five batches were analysed (method not described), providing an average tryptophan content of 98.9 % on an a 'as is' basis (ranging from 98.6 to 99.3 %).[74] Water ranged from < 0.1 to 0.3 % and ash from 0.3 to 0.5 %. As no information was available on the method of analysis used, the purity of the product should be taken with some caution. It is not possible to conclude on the amount of unidentified material.

[74] Technical dossier/Section II.1.3/Table II.1.3c. and Supplementary information October 2014/AMAC conf, response to Qii.

Analytical data on specific optical rotation (three batches) showed an average of –30.3 ° (range from –29.5 to –31.3 °).[75] This is within the range described in the European Pharmacopoeia (from –30 to –33 °) for this amino acid and confirms the identity of the L-enantiomer.[76]

### 4.1.2. Impurities

L-Tryptophan produced by *E. coli* SARI12091203 (three batches) contained concentrations of arsenic ranging from < LOD to 0.012 mg/kg, lead ranging from 0.016 to 0.2 mg/kg, mercury < LOD and cadmium 0.03 mg/kg (mercury and cadmium were analysed in only one batch).[77] The detected amounts of these impurities were negligible and often below the LOD.

Aflatoxin B1 was analysed in three batches of the final product, and in all cases was <0.3 μg/kg (LOD).

Analysis of microbial contamination of the final product indicated that *Salmonella* (in 25 g) was absent in one batch.

No data were available on dioxins and dioxin-like PCBs levels or on the endotoxin activity of the final product.

The concentrations of EBT and MTCA were analysed in three batches, for reasons described above (section 2.1.2). The contents of EBT and MTCA analysed in one batch of the final product were < 10 mg/kg (LOD) for EBT and < 10 mg/kg for MTCA.

### 4.1.3. Physical properties

General information provided in the dossier has been described above (see section 2.1.3).

No specific data for the L-tryptophan produced with *E. coli* SARI12091203 are available regarding its particle size distribution or dusting potential.

### 4.1.4. Characterisation of the production organism

The applicant stated that the production strain is *E. coli*,[78] deposited in the Shanghai Advanced Research Institute, Chinese Academy of Sciences culture collection with deposition number SARI12091203.[79] However, no evidence was provided regarding the identity and characterisation of the strain, whether it is genetically modified or the relation of the production strain with the strain deposited in the culture collection.

Therefore, in the absence of adequate information, the FEEDAP Panel cannot assess the safety of the production strain.

### 4.1.5. Manufacturing process[80]

The generic information contained in the dossier has been described above (see section 2.1.5).

The qualitative composition of the fermentation media and the corresponding MSDS were not provided.[81]

No information was available on whether antimicrobial compounds (including antibiotics) were used during the manufacturing process.[82]

[75] Technical dossier/Supplementary information January 2014/Annexes Qv.
[76] European Pharmacopoeia monograph 1/2015:1272.
[77] Supplementary information December 2012/Annex Qiv and v. LOD (in mg/kg) of arsenic was 0.005 and of mercury 0.001.
[78] Technical dossier/Supplementary information June 2012/Tryptophan strain description.
[79] Technical dossier/Supplementary information December 2012/Annex Qi L-tryptophan non GMO statement and certificate dep.
[80] This section has been amended following the confidentiality claims made by the applicant.
[81] Technical dossier/Supplementary information January 2015.

### 4.1.6. Stability and homogeneity

#### 4.1.6.1. Shelf life

The shelf life was tested for 24 months at room temperature or for 12 months at 40 °C when the product was stored in transparent polyethylene bags. No losses were observed.[83]

#### 4.1.6.2. Stability in premixtures, feedingstuffs and water for drinking

The dossier contains no specific information on the stability of L-tryptophan produced by *E. coli* SARI12091203 in premixtures, feedingstuffs or water for drinking despite it having been requested.

#### 4.1.6.3. Homogeneity

No specific information on the capacity of L-tryptophan produced by *E. coli* SARI12091203 to distribute homogeneously in premixtures, feedingstuffs or water was provided despite it having been requested.

### 4.1.7. Physico-chemical incompatibilities in feed

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

### 4.1.8. Conditions of use

The general conditions of use provided in the dossier have been described above (see section 2.1.8).

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

EFSA has verified the EURL report as it relates to the methods used for the control of the L-tryptophan in animal feed. The Executive Summary of the EURL report can be found in Appendix A.

## 4.2. Safety

Regarding L-tryptophan produced by *E. coli* SARI12091203, the data submitted did not permit an assessment of the identity and the safety of the strain and of the purity of the additive.

## 4.3. Efficacy

Efficacy considerations for supplemental L-tryptophan have been described above (section 2.3).

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

[82] Technical dossier/Supplementary information October 2014.
[83] Technical dossier/Supplementary information December 2012/Annex Qvii new.

### 4.4. 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[84] and Good Manufacturing Practice.

### 4.5. Conclusions and recommendations on L-tryptophan produced by fermentation with *E. coli* SARI12091203

#### 4.5.1. Conclusions

The data submitted did not permit an assessment of the identity and the safety of the strain and of the purity of the additive L-tryptophan produced by *E. coli* SARI12091203 The EFSA FEEDAP Panel could not conclude on the safety of this product for target animals, and on the safety concerning consumers, users and the environment.

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

## DOCUMENTATION PROVIDED TO EFSA

1. L-Tryptophan. August 2011. Submitted by AMAC EEIG (Amino Acids Authorisation Consortium European Economic Interest Grouping).
2. L-Tryptophan. Supplementary information. December 2012. Submitted by AMAC EEIG.
3. L-Tryptophan. Supplementary information. January 2014. Submitted by FEFANA Asbl.
4. L-Tryptophan. Supplementary information. October 2014. Submitted by FEFANA Asbl.
5. L-Tryptophan. Supplementary information. January 2015. Submitted by FEFANA Asbl.
6. L-Tryptophan. Supplementary information. May 2015. Submitted by FEFANA Asbl.
7. Evaluation report of the European Union Reference Laboratory for Feed Additives on the Methods(s) of Analysis for L-tryptophan.
8. Comments from Member States received through the ScienceNet.

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## Appendix A. Safety for the user

*Effects of endotoxin inhalation.* There is abundant evidence in the literature that workers exposed to high endotoxin levels by inhalation suffer impaired lung function. The Dutch Expert Committee on Occupational Safety (Health Council of the Netherlands, 2010) summarised the evidence as follows. The inhalation of endotoxins may cause the following acute symptoms: dry cough, dyspnoea accompanied by diminished lung function, fever and general malaise. After several hours, the following symptoms may develop: bronchoconstriction, headache and aching joints. The acute effects have been observed in the context of research with volunteers and reported in the context of epidemiological research among occupationally exposed people. It has been demonstrated that, in asthma sufferers and people with inflammations of the nasal mucosa, exposure to lipopolysaccharides (LPS) can lead to bronchial obstruction, accompanied by increased reactivity. Epidemiological research has produced evidence to suggest that prolonged exposure to endotoxins may lead to chronic bronchitis and diminished lung function.

Workers in sewage plants, poultry sheds, sawmills and materials recycling facilities (Health Council of the Netherlands, 2010; HSE, 2013) are particularly exposed to high levels of respirable endotoxins, which leads to chronic bronchitis and diminished lung function (Health Council of the Netherlands, 2010). Thorn (2001) concluded that inhalation of 30 to 40 µg LPS was a threshold dose for inducing clinical symptoms and lung function changes in healthy subjects. The threshold dose for inducing changes in blood neutrophils may be less than 0.5 µg LPS.

*Exposure limits.* The Health Council of the Netherlands (2010) proposed a health-based recommended exposure limit (HBROEL) of 90 IU/$m^3$ (eight-hour time-weighted average) for endotoxins in the workplace. The statutory maximum exposure permitted by the UK Health and Safety Executive (HSE, 2013) is the same. As respiration in humans may reach 1.25 $m^3$/hour (EFSA FEEDAP Panel, 2012b), inhalation volume over an eight-hour working day would be 8 × 1.25 = 10 $m^3$. Thus, the maximum permissible total daily exposure by the user, without protection, would be 10 × 90 = <u>900 IU</u>.

*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 seconds = 40 × 20 = 800 seconds/day. With an uncertainty factor of 2, maximum inhalation exposure would occur for 2 × 800 = 1 600 seconds = 0.444 hours/day. Again, assuming a respiration volume of 1.25 $m^3$/hour, 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 = <u>1 619 IU/$m^3$</u>.

*Calculation of endotoxin content of dust.* Two key measurements are required to evaluate the potential respiratory hazard associated with the endotoxin content of the product (the dusting potential of the product, expressed in g/$m^3$, and the endotoxin activity of the dust, determined by the *Limulus* amoebocyte lysate assay (expressed in IU/g)). If data for the dust are not available, the content of endotoxins of the product can be taken instead. If the content of endotoxins of the relevant additive is *a* IU/g and the dusting potential is *b* g/$m^3$, then the content of endotoxins of the dust, *c* IU/$m^3$, is obtained by simple multiplication, *a* × *b*. This resulting value is further used for calculation of the potential inhalatory exposure of users to endotoxins from the additives under assessment (Tables B1 and B2) (EFSA FEEDAP Panel, 2012b).

**Table A1**. Estimation of user exposure to endotoxins from the additive L-tryptophan produced by *E. coli* DSM 25084, 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) | 32 000 | Technical dossier |
| | *b* | Dusting potential (g/m$^3$) | 2.9 | Technical dossier |
| *a* × *b* | *c* | Endotoxin content in the air (IU/m$^3$) | 92 800 | |
| | *d* | No of premixture batches made/working day | 40 | EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012b) |
| | *e* | Time of exposure (seconds) per production of one batch | 20 | EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012b) |
| *d* × *e* | *f* | Total duration of daily exposure/worker (seconds) | 800 | |
| | *g* | Uncertainty factor | 2 | EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012b) |
| *f* × *g* | *h* | Refined total duration of daily exposure/worker (seconds) | 1600 | |
| *h*/3 600 | *i* | Refined total duration of daily exposure (hours) | 0.44 | |
| | *j* | Inhaled air (m$^3$) per eight-hour working day | 10 | EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012b) |
| *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** | **51 556** | |
| | *m* | Health-based recommended exposure limit of endotoxin (IU/m$^3$) per eight-hour working day | 90 | Health Council of the Netherlands, 2010 |
| *m* × *j* | *n* | **Health-based recommended exposure limit of total endotoxin exposure (IU) per eight-hour working day** | **900** | |
| l/10 | | Endotoxins inhaled (IU) per eight-hour working day reduced by filter mask FF P2 (reduction factor 10) | 5 156 | |
| l/20 | | Endotoxins inhaled (IU) per eight-hour working day reduced by filter mask FF P3 (reduction factor 20) | 2 578 | |

**Table A2**. Estimation of user exposure to endotoxins from the additive L-tryptophan produced by *E. coli* KCCM11132P, including consideration of using a filter mask FF P2 or FF P3 as a preventative measure

| Calculation | Identifier | Description | Amount | Source |
|---|---|---|---|---|
| | *a* | Endotoxin content (IU/g product) | 2 900 000 | Technical dossier |
| | *b* | Dusting potential ($g/m^3$) | 2.5 | Technical dossier |
| *a* × *b* | *c* | Endotoxin content in the air ($IU/m^3$) | 7 250 000 | |
| | *d* | No of premixture batches made/working day | 40 | EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012b) |
| | *e* | Time of exposure (seconds) per production of one batch | 20 | EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012b) |
| *d* × *e* | *f* | Total duration of daily exposure/worker (seconds) | 800 | |
| | *g* | Uncertainty factor | 2 | EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012b) |
| *f* × *g* | *h* | Refined total duration of daily exposure/worker (seconds) | 1 600 | |
| *h*/3 600 | *i* | Refined total duration of daily exposure (hours) | 0.44 | |
| | *j* | Inhaled air ($m^3$) per eight-hour working day | 10 | EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012b) |
| *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** | **4 027 778** | |
| | *m* | Health-based recommended exposure limit of endotoxin ($IU/m^3$) per eight-hour working day | 90 | Health Council of the Netherlands (2010) |
| *m* × *j* | *n* | **Health-based recommended exposure limit of total endotoxin exposure (IU) per eight-hour working day** | **900** | |
| l/10 | | Endotoxins inhaled (IU) per eight-hour working day reduced by filter mask FF P2 (reduction factor 10) | 402 778 | |
| l/20 | | Endotoxins inhaled (IU) per eight-hour working day reduced by filter mask FF P3 (reduction factor 20) | 201 389 | |

## 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[1]

In the current application authorisation is sought for *L-Tryptophan* under Articles 4(1) and 10(2), category 'nutritional additives' and functional group 3(c) 'amino acids, their salts and analogues' according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of *L-Tryptophan* for all animal species and categories. The *feed additive* is intended to be mixed either in *premixtures* or added directly to complete *feedingstuffs* or *water*. The Applicant suggested no minimum or maximum *L-Tryptophan* concentrations in *premixtures*, *feedingstuffs* and *water*.

For the determination of *L-Tryptophan* in *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated Community method (Commission Regulation (EC) No 152/2009). The method applies for the determination of *free* (synthetic and natural) and *total* (peptide-bound and free) amino acid, using High Performance Liquid Chromatography (HPLC) equipment. The following performance characteristics are reported:

* For free *L-Tryptophan:*

- a relative standard deviation for *repeatability* ($RSD_r$) of 1.3%;
- a relative standard deviation for *reproducibility* ($RSD_R$) ranging from 4.7 to 5.1%.

* For total *L-Tryptophan:*

- a relative standard deviation for *repeatability* ($RSD_r$) ranging from 0.8 to 1.9%;
- a relative standard deviation for *reproducibility* ($RSD_R$) ranging from 1.5 to 6.3%.

Based on the performance characteristics presented, the EURL recommends for official control the ring-trial validated Community method, based on reversed phase HPLC with fluorescence detection, to determine *L-Tryptophan* in *premixtures* and *feedingstuffs*.

For the determination of the *active substance* in the *feed additive* the Applicant submitted the above-mentioned ring trial validated Community method designed for the analysis of *premixtures* and *feedingstuffs*. Nevertheless the EURL identified, for the determination of the amino acid in the *feed additive*, a titrimetric method among the internationally recognised European Pharmacopoeia methods. No performance characteristics of this method are provided. However, the EURL considers this method suitable to be used within the frame of official control.

The Applicant provided neither experimental data nor experimental method for the determination of *L-Tryptophan* in *water*. Therefore, the EURL cannot evaluate nor recommend a method for the official control to determine *L-Tryptophan* in *water*.

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) is not considered necessary.

[1] The full report is available on the EURL website: http://irmm.jrc.ec.europa.eu/SiteCollectionDocuments/FinRep-FAD-2010-0056.pdf