SCIENTIFIC OPINION

EFSA Journal

ADOPTED: 5 July 2017

doi: 10.2903/j.efsa.2017.4939

# Safety and efficacy of L-threonine produced by fermentation with *Escherichia coli* CGMCC 11473 for all animal species

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), Guido Rychen, Gabriele Aquilina, Giovanna Azimonti, Vasileios Bampidis, Maria de Lourdes Bastos, Georges Bories, Andrew Chesson, Pier Sandro Cocconcelli, Gerhard Flachowsky, Jürgen Gropp, Boris Kolar, Maryline Kouba, Marta López-Alonso, Secundino López Puente, Alberto Mantovani, Baltasar Mayo, Fernando Ramos, Maria Saarela, Roberto Edoardo Villa, Pieter Wester, Lucio Costa, Noël Dierick, Lubomir Leng, Boet Glandorf, Lieve Herman, Sirpa Kärenlampi, Jaime Aguilera, Jordi Tarrés-Call and Robert John Wallace

## Abstract

The product subject of this assessment is L-threonine produced by fermentation with a genetically modified strain of *Escherichia coli* (CGMCC 11473). It is intended to be used in feed and water for drinking for all animal species and categories. It was not possible to characterise the genetic modification with the information provided. Uncertainty remained on the possible presence of cells from the production strain and their recombinant DNA in the product. Therefore, the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) could not conclude on the safety of the product L-threonine, produced by fermentation with *Escherichia coli* CGMCC 11473 for the target species, consumers and the environment. The FEEDAP Panel has concerns on the safety of the simultaneous oral administration of threonine containing additives via water for drinking and feed. In the absence of data, the FEEDAP Panel cannot conclude on the potential of the additive to be an irritant for skin and eyes or to be a skin sensitiser. There is a risk from the exposure by inhalation to endotoxins for persons handling the additive. The product under assessment is considered an efficacious source of the amino acid L-threonine for all animal species. For L-threonine to be as efficacious in ruminants as in non-ruminant species, it requires protection against degradation in the rumen.



**Keywords:** nutritional additives, amino acids and their salts and analogues, L-threonine, safety, efficacy, genetically modified microorganisms

**Requestor:** European Commission

**Question number:** EFSA-Q-2016-00074

**Correspondence:** feedap@efsa.europa.eu

**Panel members:** Gabriele Aquilina, Giovanna Azimonti, Vasileios Bampidis, Maria de Lourdes Bastos, Georges Bories, Andrew Chesson, Pier Sandro Cocconcelli, Gerhard Flachowsky, Jürgen Gropp, Boris Kolar, Maryline Kouba, Marta López-Alonso, Secundino López Puente, Alberto Mantovani, Baltasar Mayo, Fernando Ramos, Guido Rychen, Maria Saarela, Roberto Edoardo Villa, Robert John Wallace and Pieter Wester.

**Suggested citation:** EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wester P, Costa L, Dierick N, Leng L, Glandorf B, Herman L, Kärenlampi S, Aguilera J, Tarrés-Call J and Wallace RJ, 2017. Scientific Opinion on the safety and efficacy of L-threonine produced by fermentation with *Escherichia coli* CGMCC 11473 for all animal species. EFSA Journal 2017;15(7):4939, 16 pp. https://doi.org/10.2903/j.efsa.2017.4939

**ISSN:** 1831-4732



The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

## Summary

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-threonine produced by fermentation using the genetically modified strain *Escherichia coli* CGMCC 11473 for all animal species.

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of L-threonine was in line with the principles laid down in Regulation (EC) No 429/2008 and the relevant EFSA guidance documents. The FEEDAP Panel used the data provided by the applicant together with data from other sources, such as previous risk assessments by the European Food Safety Authority (EFSA) or other expert bodies, peer-reviewed scientific papers, other scientific reports and experts' knowledge, to deliver the present output.

The characterisation of the genetic modification could not be drawn from the information provided. Uncertainty remained on the possible presence of cells from the production strain and their recombinant DNA in the product. Therefore, the FEEDAP Panel could not conclude on the safety of the product L-Threonine, produced by fermentation with *Escherichia coli* CGMCC 11473 for the target species, consumers and the environment.

The FEEDAP Panel had concerns on the safety of the simultaneous oral administration of threonine containing additives via water for drinking and feed.

In the absence of data, the FEEDAP Panel could not conclude on the potential of the additive to be an irritant for skin and eyes or to be a skin sensitiser. There was a risk from the exposure to endotoxins by inhalation for persons handling the additive.

The product under assessment was considered an efficacious source of the amino acid L-threonine for all animal species. For L-threonine to be as efficacious in ruminants as in non-ruminant species, it requires protection against degradation in the rumen.

# Table of contents

# 1. Introduction

## 1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from Agri Nutrition BV[2] for authorisation of the product L-threonine produced by microbial fermentation with *Escherichia coli*, 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 April 2016.

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-threonine, when used under the proposed conditions of use (see Section 3.1.3).

## 1.2. Additional information

L-Threonine produced by eight different strains of *E. coli* (minimum content of 98% on dry matter basis) is currently authorised as a nutritional feed additive for use in all animal species (Commission Implementing Regulation (EU) 2016/1220).[3] The current application refers to L-threonine produced using a genetically modified strain of *E. coli*.

L-Threonine like other amino acids and other nitrogen compounds is authorised according to Commission Directive 2006/141/EC for infant formulae and follow-on formulae.[4] According to Commission Directive 2001/15/EC, amino acids such as L-threonine may be added in all dietary foods for particular nutritional uses including foods for particular nutritional uses intended for special medical purposes.[5] L-Threonine is also registered as an ingredient in cosmetic products as antistatic, hair conditioning, hair waiving or straightening (Commission decision 2006/257/EEC). L-Threonine is registered as pharmaceutical grade (for total parenteral nutrition) in many European countries and is described in a monograph of the European Pharmacopoeia (MG 01/2008:1049). According to Commission Regulation (EEC) 2377/90, L-threonine is also listed as pharmacologically active substance in veterinary medicinal products and is not subjected to maximum residue levels when used in food producing animals.[6]

The Scientific Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) issued nine opinions on the safety and efficacy of L-threonine produced by genetically modified strains of *E. coli* (EFSA FEEDAP Panel, 2013, 2014a–d, 2015a,b, 2016a,b).

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in contact with Food (AFC) issued two opinions related to threonine in the frame of the Flavouring Group Evaluation 26: amino acids from chemical group 34 (EFSA AFC Panel, 2006; EFSA, 2008a).

The Joint FAO/WHO Expert Committee on Food Additives evaluated L-threonine as food flavouring agent (JECFA, 2012).

[1] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.
[2] Agri Nutrition BV, Hanzestraat 1, 7006 RH, Doetinchem, The Netherlands.
[3] Commission implementing regulation (EU) 2016/1220 of 26 July 2016 concerning the authorisation of L-threonine produced by *Escherichia coli* as feed additive for all animal species. OJ L 201, 27.7.2016, p. 5.
[4] Commission Directive 2006/141/EC on infant formulae and follow-on formulae, OJ L 401, 30.12.2006, p 1.
[5] Commission Directive 2001/15/EC of 15 February 2001 on substances that may be added for specific nutritional purposes in foods for particular nutritional uses. OJ L/52, 22.2.2001, p. 19.
[6] 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, 10.9.1999, p. 3.

# 2. Data and methodologies

## 2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier[7] in support of the authorisation request for the use of L-threonine as a feed additive. The technical dossier was prepared following the provisions of Article 7 of Regulation (EC) No 1831/2003, Regulation (EC) No 429/2008[8] and the applicable EFSA guidance documents.

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-threonine in animal feed. The Executive Summary of the EURL report can be found in Annex A.[9]

## 2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of L-threonine 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), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012b), Technical Guidance: Microbial Studies (EFSA, 2008b), Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance (EFSA FEEDAP Panel, 2012c), and Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use (EFSA GMO Panel, 2011).

# 3. Assessment

The product which is the subject of this application is L-threonine produced by fermentation with a genetically modified strain of *E. coli*. L-Threonine is currently authorised for use as a nutritional additive, under the functional group 'amino acids, their salts and analogues'. The product under application is intended to be used in feed and water for drinking for all animal species and categories.

The objective of feed supplementation with essential amino acids is to complete the amino acid profile of the diet in order to closely meet individual amino acid requirements of animals or to compensate for potential imbalances. The supplementation of feedingstuffs with amino acids is a conventional measure for improving protein quality and utilisation. This supplementation became even more important when protein-reduced diets were introduced in animal husbandry for economic and environmental reasons. L-Threonine is well recognised as an essential amino acid in animal nutrition. Under European Union (EU) conditions, L-threonine seems to be the second most limiting amino acid, after L-lysine, in pigs and the third most limiting, after the sulfur amino acids and L-lysine, in poultry.

## 3.1. Characterisation

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

L-Threonine (International Union of Pure and Applied Chemistry name: (2*S*,3*R*)-2-amino-3-hydroxybutanoic acid; synonyms: 2-amino-3-hydroxybutyric acid, α-amino-β-hydroxybutyric acid), a compound identified with the Chemical Abstracts Service No 72-19-5 and the European Inventory of Existing Commercial Chemical Substances No 200-774-1, has a molecular mass of 119.12 Da. The molecular formula of L-threonine is $C_4H_9NO_3$. The structural formula is given in Figure 1.

[7] FEED dossier reference: FAD-2016-0003.

[8] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.

[9] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad2016_0003_l_threonine.pdf

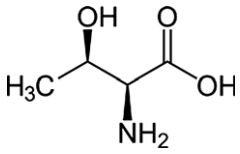


**Figure 1:** Molecular structure of L-threonine

The additive contains, by specification, ≥ 98.5% L-threonine, < 1% moisture and < 1.4% other substances.[10] The analysis of five batches of the additive showed an average of L-threonine of 99.1% 'as is' (range 98.3–99.5%).[11] The loss on drying was not provided. The amount of unidentified material was in average < 1% on a dry matter basis.

The specific optical rotation was measured in three batches of the final product and the average was −28.1° (range −28.3 to −27.7°),[12] which is within the range established for L-threonine in the European Pharmacopoeia (−29.0 to −27.6°) and demonstrates the identity of the L-enantiomer.

### 3.1.2. Impurities

Five batches were analysed for heavy metals (cadmium, mercury and lead) and were found under the limit of detection (LOD).[13] Arsenic was analysed in other five batches (average 1.1 mg/kg, range 0.1–4.6 mg/kg).[14] None of these amounts were considered of concern.

The microbiological quality of three batches of the product was tested by counting Enterobacteriaceae (negative), *E. coli* (negative) and *Salmonella* spp. (negative in 25 g);[15] filamentous fungi and yeasts (< 10 colony forming units (CFU)/g).[16] Regarding mycotoxins (ochratoxin A, aflatoxins (unspecified), zearalenone, fumonisin, deoxynivalenol and citrinin),[17] all values were below the limit of quantification (LOQ) (except deoxynivalenol which was ≤ 178 μg/kg and citrinin, which was ≤ 31 μg/kg) and are considered to be of no concern.

Dioxins and dioxin-like polychlorinated biphenyls (PCBs) were measured in three batches of the final product.[18] The sum of polychlorinated dibenzofurans (PCDF), polychlorinated dibenzo(p)dioxins (PCDD) and dioxin-like PCB ranged from 0.13 to 0.14 WHO-TEQ (ng/kg DM).

One batch of the additive was tested for inhibitory activity against the list of reference strains of the EFSA Guidance on microbial studies (EFSA, 2008a,b).[19] For all strains, the minimum inhibitory concentration values (MIC) were > 2,500 mg/L. The MICs were in all cases greater than four times the normal use level of the additive in feed, and therefore, the additive is considered not to have antimicrobial activity at feed use level.

Bacterial endotoxin activities were measured (*Limulus* Amoebocyte Lysate assay) in three batches of the final product and ranged from 288 to 319 IU/mg.[20]

No data were provided on the possible presence of viable cells of the production strain in the product. The applicant states that *E. coli* was not found in three batches of product (absence in 25 g); however, the methodology is not described. Test reports were provided in which DNA of the *E. coli* 16S rRNA gene was not found by PCR in one batch.[21] However, no information was provided on the amount of samples tested, and no details on the sensitivity of the method were provided. Therefore, uncertainty remains on the possible presence of the production strain and its DNA in the product.

[10] Technical dossier/Section II.2.1.3.
[11] Technical dossier/Section II/Annex 2.1.4a and Supplementary information January 2017/Annex 3. Analytical method in compliance with EU official method (EC) 152/2009.
[12] Technical dossier/Section II/Annex 2.1.3a and supplementary information January 2017/Answers to SIn 270516 revised version 161210.
[13] Technical dossier/Supplementary information January 2017/Annex 6. LOD (in mg/kg) was 5 for lead, 0.5 for cadmium and 0.01 for mercury.
[14] Technical dossier/Section II/Annex 2.1.3a.
[15] Technical dossier/Section II/Annex 2.1.4a.
[16] Technical dossier/Section II/Annex 2.1.3a and supplementary information January 2017/Annex 7. LOQ was < 10 CFU/g.
[17] Technical dossier/Section II/Annex 2.1.4a. LOQ (in μg/kg) was 5 for ochratoxin A, 1.7 for aflatoxins, 17 for zearalenone, 25 for fumonisin, 134 for deoxynivalenol and 15 for citrinin.
[18] Technical dossier/Supplementary information January 2017/Annexes 8a to 8c.
[19] Technical dossier/Section II/Annex 2.2.2.2 and Supplementary information January 2017/Annex 9.
[20] Technical dossier/Supplementary information January 2017/Annexes 10a and 10b.
[21] Technical dossier/Section II/Supplementary information January 2017/Annexes 12.4 to 12.6.

### 3.1.3. Physical characteristics

The particle size distribution of the final product (three batches) was analysed by laser diffraction.[22] The fractions of particles having a diameter < 100, < 50 and < 10 μm were 85%, 52% and 5% (v/v), respectively.

Analytical data on the dusting potential (three batches) of the final product (Stauber–Heubach method) ranged from 0.1 to 0.2 g/m$^3$.[23]

### 3.1.4. Characterisation of the production organism[24]

The additive under assessment is produced by a genetically modified strain of *E. coli* K-12, which has been deposited in the China General Microbiological Culture Collection Center (CGMCC) with the accession number CGMCC 11473.[25] The strain was identified as an *E. coli* K-12 derivative by whole genome sequence (WGS) analysis.[26]

A non-identified strain of *E. coli* K-12 was tested for antimicrobial susceptibility using agar diffusion against a battery of 12 antimicrobials. Out of the 10 clinically relevant antimicrobials recommended by EFSA (EFSA FEEDAP Panel, 2012c),[27] only five (gentamycin, tetracycline, ampicillin, kanamycin and chloramphenicol) were included in the battery. Of those, the MIC values were equal or below the corresponding cut-off values defined by the FEEDAP Panel, except for kanamycin, which was 256 mg/L. No information was provided on the genetic basis of this kanamycin resistance. The relationship between the tested strain and the production strain was not clarified. The genome of the production strain was not searched for genes conferring resistance to kanamycin or other antimicrobials. Therefore, uncertainty remains on the possible presence of antimicrobial resistance genes in the production strain.

#### 3.1.4.1. Information relating to the genetically modified microorganism

No information on the parental or recipient strain was submitted.

#### 3.1.4.2. Description of the genetic modification

No information was provided on the changes introduced in the production strain. The WGS of the production strain was compared to the genome of one of the publicly available genomes of *E. coli* K-12, which was used as a reference genome.[28] However, the reported analysis of the sequence and its interpretation were unclear, difficult to interpret and contained some contradictory statements. No characterisation of the genetic modification can be drawn from the report provided.

#### 3.1.4.3. Information related to the production process

L-Threonine is produced by fermentation using *E. coli* CGMCC 11473. After fermentation, the fermentation broth is inactivated and separated. L-Threonine is concentrated, purified, and crystallised.

According to the applicant, no antimicrobial substances are used during the production process.[29]

### 3.1.5. Stability and homogeneity

The shelf life of three batches of the product was tested at 25 and 40°C for 12 and 6 months, respectively.[30] The samples were kept in sealed bags protected from light during the storage period. Losses around 2% were observed at 25°C and around 1% at 40°C.

The stability of the product (three batches) in a vitamin/mineral premixture (containing 18.4 g choline chloride/kg) supplemented with 10% threonine was studied when stored in closed plastic bags at room temperature for 6 months.[31] Losses observed ranged from 0% (two batches) to 6%.

[22] Technical dossier/Section II/Annexes 2.1.5a, 2.1.5b and 2.1.5c.
[23] Technical dossier/Section II/Annexes 2.4.3a, 2.4.3b and 2.4.3c.
[24] This section has been amended following the provisions of Article 8(6) and Article 18 of Regulation (EC) No 1831/2003.
[25] Technical dossier/Section II/Annexes 2.2.1.2a and 2.2.1.2b.
[26] Technical dossier/Section II/ Supplementary information January 2017/Annex 13.4.
[27] Technical dossier/Supplementary information January 2017/Annex 12.1.
[28] Technical dossier/Section II/ Supplementary information January 2017/Annex 13.4 and annex I.11.
[29] Technical dossier/Supplementary information January 2017/Annex 2.
[30] Technical dossier/Section II.2.4.1 and Annex 2.1.4a and Supplementary information January 2017/Annexes 3 and 16.
[31] Technical dossier/Section II.2.4.1 and Annexes 2.1.4a and 2.4.1; Supplementary information January 2017/Response to Q16 and Annexes 3 and 16.

The stability of the product (three batches) was tested in a compound feed for piglets (mash and pelleted feed) when supplemented at 0.5% threonine.[32] The basal diet consisted in barley, wheat and soybean. Pelleting was performed at 65°C. The effect of pelleting represents a loss of 1–4%. The packaging and temperature conditions during storage were not described. After 3 months storage, a loss of 6% was observed in only one batch of meal and losses in pelleted feed ranged from 0% to 1%. As total threonine (protein-bound plus free threonine) was analysed instead of supplemental threonine, the results of this stability study might not reflect the real stability of the additive in feedingstuffs.

The capacity of the additive to distribute homogeneously in feed was tested in 10 samples of a pelleted feed supplemented at 0.5%.[30] The coefficient of variation was 1.5%. Total threonine (protein-bound plus free threonine) was analysed. Consequently, the result obtained might not reflect the real capacity of the additive to distribute homogeneously in feedingstuffs.

The stability of the product (three batches) in water was tested at a concentration of 5 g/L at room temperature for 24 h. The losses observed ranged from 0.4% to 2%.[33] The FEEDAP Panel notes that the concentration tested does not represent the practical use (concentrations about one-third of the usual supplemental level in feed are recommended for additives to be used in water for drinking) and that the storage period was shorter than the 48 h required in the corresponding guidance.[34]

#### 3.1.6. Physicochemical incompatibilities in feed

No physicochemical incompatibilities in feed are expected with other additives, medicinal products or feed materials.

#### 3.1.7. Conditions of use

It is proposed that L-threonine will be used in feeds to achieve an adequate amino acid profile and to meet the L-threonine requirements for all animal species. It can be added directly to feedingstuffs or complementary feedingstuffs, or via a premixture. It is also proposed to use the additive 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.

### 3.2. Safety

#### 3.2.1. Safety aspects of the genetic modification

The production strain is resistant to kanamycin, but the genetic basis for this resistance remains unknown. Potential resistance to five out of the 10 clinically relevant antimicrobials of those recommended by EFSA for *E. coli* is unknown. No characterisation of the genetic modification can be drawn from the information provided. Uncertainty remains on the genetic elements introduced and deleted, including possible genes conferring resistance to antimicrobials.

Uncertainty remains on the possible presence of cells from the production strain and their recombinant DNA in the product. Therefore, the FEEDAP Panel cannot conclude on the safety of the product L-threonine, produced by fermentation with *E. coli* CGMCC 11473, with regard to the genetic modification of the production strain.

#### 3.2.2. Safety for the target species

Tolerance studies with essential amino acids, such as L-threonine, cannot be designed in accordance with the protocols of conventional toxicity experiments because high dietary concentrations of a certain amino acid will result in amino acid imbalances and depression of feed intake and, hence, impaired performance. Nevertheless, in the case of nutritional additives produced by fermentation, the risks associated with the residues of the fermentation process in the final product need to be assessed. In this specific product, the active substance represents > 99% of the additive on a dry matter basis. The level of endotoxins in the product (288–319 IU/mg) is similar to that observed in other feedingstuffs and is therefore of no concern for the target species (Cort et al., 1990). The data

[32] Technical dossier/Section II.2.4.1 and Annexes 2.1.4a and 2.4.1; Supplementary information January 2017/Annexes 3 and 16.
[33] Technical dossier/Section II.2.4.1 and Annex 2.1.4a.
[34] Guidance for the preparation of dossiers for nutritional additives. EFSA Journal 2012;10(1):2535.

submitted cannot exclude the presence of recombinant DNA and antibiotic resistance genes in the final product. Therefore, the FEEDAP Panel cannot conclude on the safety of L-threonine produced by *E. coli* CGMCC 11473 for the target species.

The requirements and the effects of excess L-threonine in the diet have already been described in a previous opinion (EFSA FEEDAP Panel, 2014a). In that opinion, the FEEDAP Panel concluded that feed supplementation with the amino acid L-threonine, to compensate for threonine deficiency in feedingstuffs, is safe for the target animals. The margin of safety is higher in pigs than in poultry. Excess doses, however, would create amino acid imbalances with negative consequences on animal performance. Correct dosing when formulating diets requires knowledge of the amino acid content in feed materials and the requirement of animals. The FEEDAP Panel considers that the same conclusions would apply to the active substance L-threonine.

The FEEDAP Panel, in its previous statement (EFSA FEEDAP Panel, 2010), identified risks of nutritional imbalances and hygienic concerns in amino acids when administered in water for drinking.

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

The possible presence in the final product of the viable production strain and its recombinant DNA and/or antibiotic resistance genes cannot be excluded. Therefore, the FEEDAP Panel cannot conclude on the safety of L-threonine produced by *E. coli* CGMCC 11473 for the target species.

In addition, the FEEDAP Panel has concerns regarding the safety of the simultaneous administration of L-threonine via water for drinking and feed.

### 3.2.3. Safety for the consumer

The absorption, distribution, metabolism and excretion of L-threonine were described in a previous scientific opinion of the FEEDAP Panel (EFSA FEEDAP Panel, 2013). The use of 99% pure amino acid L-threonine in animal nutrition, to meet the animal requirements, does not give rise to concerns for the safety of the consumer.

The product under assessment, however, is produced by fermentation. The concerns for the consumer would not derive from the amino acid itself, which will be incorporated into the proteins of the animal tissues/products, but from the possible residues from the fermentation process. The possible presence in the final product of the viable production strain and its recombinant DNA and/or antibiotic resistance genes cannot be excluded. Therefore, the FEEDAP Panel cannot conclude on the safety of L-threonine produced by *E. coli* CGMCC 11473 for the consumer.

### 3.2.4. Safety for the user

No studies were submitted to support the safety for the user.

#### 3.2.4.1. Effects on the respiratory system

Dusting potential was measured in three batches and ranged 0.1–0.2 g/m$^3$. The percentage of particles with diameters $< 10$, $< 50$ and $<100$ μm were 5%, 52% and 85% (v/v, three batches), respectively. Therefore, workers may be exposed by inhalation.

Users can suffer from occupational respiratory disease depending on the level of endotoxins in air and dust (Rylander, 1999; Thorn, 2001). The bacterial endotoxin activity (analysed in three batches) ranged from 288 to 319 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 Appendix A. The threshold for the quantity of inhaled endotoxins per working day is 900 IU, derived from the provisional occupational exposure limits given by the Dutch Expert Committee on Occupational Safety (Health Council of the Netherlands, 2010) and the UK Health and Safety Executive (HSE, 2013). Based upon calculations of the content of endotoxins in dust, exposure would be 35,444 IU per eight-hour working day, indicating a risk by inhalation due to exposure to endotoxins for people handling the additive.

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

In the absence of data, the FEEDAP Panel cannot conclude on the potential of the additive to be irritant to skin and eyes or to be a skin sensitiser. There is a risk from the inhalation exposure to endotoxins for persons handling the additive.

### 3.2.5. Safety for the environment

The amino acid L-threonine is a physiological and natural component of the proteins of living organisms. When consumed, it will be absorbed, and the non-absorbed fraction will be incorporated into the intestinal microbial mass and excreted as such. The absorbed L-threonine will be incorporated into body protein or excreted as urea/uric acid and as carbon dioxide.

Uncertainty remains, however, on the possible presence of genes conferring antimicrobial resistance in the genome of the production strain, and on the possible presence of cells from the production strain and their DNA in the product. Therefore, the FEEDAP Panel cannot conclude on the environmental safety of the product L-threonine, produced by fermentation with *E. coli* CGMCC 11473, with regard to the genetic modification of the production strain.

## 3.3. Efficacy

Efficacy studies are not required for amino acids which naturally occur in the proteins of plants and animals. The nutritional role of L-threonine is well established in the scientific literature. Since most of the studies have been performed with supplemental L-threonine, the product L-threonine, technically pure, is regarded as an effective source of the amino acid L-threonine.

The efficacy of L-threonine for both non-ruminant and ruminant species was described in previous opinions (EFSA FEEDAP Panel, 2013, 2014a). Supplemental L-threonine is degraded by ruminal microbiota if not given in a protected form.

The product is considered an efficacious source of the amino acid L-threonine for all animal species. For the supplemental L-threonine to be as efficacious in ruminants as in non-ruminant species, it must be protected 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[35] and Good Manufacturing Practice.

# 4. Conclusions

The genetic modification cannot be assessed. Uncertainty remains on the possible presence of cells from the production strain and/or their recombinant DNA in the product, including possible genes conferring antimicrobial resistance. Therefore, the FEEDAP Panel cannot conclude on the safety of the product L-threonine, produced by fermentation with *E. coli* CGMCC 11473 for the target species, consumers and the environment.

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

In the absence of data, the FEEDAP Panel cannot conclude on the potential of the additive to be irritant to skin and eyes or to be a skin sensitiser. There is a risk from the exposure to endotoxins for persons handling the additive.

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

# Documentation provided to EFSA

1) Dossier L-threonine produced by *Escherichia coli* CGMCC 11473. January 2016. Submitted by Agri Nutrition B.V.
2) Dossier L-threonine produced by *Escherichia coli* CGMCC 11473. Supplementary information. January 2017. Submitted by Agri Nutrition B.V.
3) Evaluation report of the European Union Reference Laboratory for Feed Additives on the Methods of Analysis for L-threonine produced by *Escherichia coli* CGMCC 11473.
4) Comments from Member States.

[35] Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

## References


Cort N, Fredriksson G, Kindahl H, Edqvist LE and Rylander R. 1990. A clinical and endocrine study on the effect of orally administered bacterial endotoxin in adult pigs and goats. Journal of Veterinary Medicine, Series A 37, 130–137.

EFSA (European Food Safety Authority), 2008a. Scientific Opinion of the Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) on flavouring group evaluation 26, revision 1: amino acids from chemical group 34. EFSA Journal 2008;6(11):790, 51 pp. https://doi.org/10.2903/j.efsa.2008.790

EFSA (European Food Safety Authority), 2008b. Technical Guidance: Microbial Studies. EFSA Journal 2008,6 (10):836, 3 pp. https://doi.org/10.2903/j.efsa.2008.836

EFSA AFC Panel (EFSA Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food), 2006. Flavouring group evaluation 26: amino acids from chemical group 34. EFSA Journal 2006;4(5):373, 48 pp. https://doi.org/10.2903/j.efsa.2006.373

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances Used in Animal Feed), 2010. Scientific Opinion on the use of feed additives authorised/applied for use in feed when supplied via water. EFSA Journal 2010;8(12):1956, 9 pp. https://doi.org/10.2903/j.efsa.2010.1956

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012a. Guidance for the preparation of dossiers for nutritional additives. EFSA Journal 2012;10(1):2535, 14 pp. https://doi.org/10.2903/j.efsa.2012.2535

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012b. Guidance on studies concerning the safety of use of the additive for users/workers. EFSA Journal 2012;10(1):2539, 5 pp. https://doi.org/10.2903/j.efsa.2012.2539

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012c. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA Journal 2012;10(6):2740, 10 pp. https://doi.org/10.2903/j.efsa.2012.2740

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2013. Scientific opinion on the safety and efficacy of L-threonine produced by Escherichia coli (FERM BP-10942) for all animal species based on a dossier submitted by Ajinomoto Eurolysine SAS. EFSA Journal 2013;11(7):3319, 19 pp. https://doi.org/10.2903/j.efsa.2013.3319

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2014a. Scientific Opinion on the safety and efficacy of L-threonine (ThreAMINO®) produced by Escherichia coli K-12 (DSM 25086) for all animal species and categories based on a dossier by Evonik Industries A.G. EFSA Journal 2014;12(2):3564, 2 pp. https://doi.org/10.2903/j.efsa.2014.3564

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2014b. Scientific Opinion on the safety and efficacy of L-threonine produced by Escherichia coli for all animal species, based on a dossier submitted by HELM AG on behalf of Global Bio-Chem Technology. EFSA Journal 2014;12(5):3674, 3 pp. https://doi.org/10.2903/j.efsa.2014.3674

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2014c. Scientific Opinion on the safety and efficacy of L-threonine produced by Escherichia coli for all animal species, based on a dossier submitted by HELM AG on behalf of Star Lake Bioscience Co. EFSA Journal 2014;12(6):3726, 14 pp. https://doi.org/10.2903/j.efsa.2014.3726

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2014d. Scientific Opinion on the safety and efficacy of L-threonine produced by Escherichia coli (FERM BP-11383) for all animal species, based on a dossier submitted by Ajinomoto Eurolysine S.A.S. EFSA Journal 2014;12(10):3825, 14 pp. https://doi.org/10.2903/j.efsa.2014.3825

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2015a. Scientific Opinion on the safety and efficacy of L-threonine produced by *Escherichia coli* for all animal species based on a dossier submitted by HELM AG on behalf of Meihua Holdings Group Co. Ltd. EFSA Journal 2015;13(3):4051, 17 pp. https://doi.org/10.2903/j.efsa.2015.4051

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2015b. Scientific Opinion on the safety and efficacy of L-threonine produced by *Escherichia coli* strains NRRL B-30843, DSM 26131, KCCM11133P or DSM 25085 for all animal species based on a dossier submitted by AMAC EEIG. EFSA Journal 2015;13(9):4236, 34 pp. https://doi.org/10.2903/j.efsa.2015.4236

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2016a. Scientific opinion on the safety of L-threonine produced by fermentation using *Escherichia coli* CGMCC 3703, for all animal species based on a dossier submitted by GBT Europe GmbH. EFSA Journal 2016;14(1):4344, 13 pp. https://doi.org/10.2903/j.efsa.2016.4344

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2016b. Scientific opinion on the safety of L-threonine, technically pure, produced by fermentation with *Escherichia coli* CGMCC 7.58 for all animal species based on a dossier submitted by Feedway Europe NV. EFSA Journal 2016;14 (5):4470, 11 pp. https://doi.org/10.2903/j.efsa.2016.4470

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2011. Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use. EFSA Journal 2011;9 (6):2193, 54 pp. https://doi.org/10.2903/j.efsa.2011.2193

Health Council of the Netherlands, 2010. Endotoxins. Health-based recommended occupational exposure limit. Publication no 2010/04 OSH, 100 pp.

HSE (Health and Safety Executive), 2013. Occupational hygiene implications of processing waste at Materials Recycling Facilities (MRFs). RR977, 41 pp.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 2012. Evaluation of certain food additives: seventy-sixth report of the Joint FAO/WHO Expert Committee on Food Additives. WHO technical report series No 974. ISBN 978 92 4 120974 8.

Rylander R, 1999. Health effects among workers in sewage treatment plants. Occupational and Environmental Medicine, 56, 354–357.

Thorn J, 2001. The inflammatory response in humans after inhalation of bacterial endotoxin: a review. Inflammatory Response, 50, 254–261.

## Abbreviations

| | |
|---|---|
| AFC | Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in contact with Food |
| CFU | colony forming units |
| CGMCC | China general microbiological culture collection centre |
| EURL | European Union Reference Laboratory |
| FEEDAP | EFSA scientific Panel on additives and products or substances used in animal feed |
| GM | genetically modified |
| GMO | EFSA scientific Panel on genetically modified microorganisms |
| HSE | British health safety Executive |
| IU | International unit of endotoxin activity. One IU corresponds to one endotoxin unit (EU) |
| JECFA | Joint FAO/WHO Expert Committee on Food Additives |
| LOD | limit of detection |
| LOQ | limit of quantification |
| MIC | minimum inhibitory concentration |
| PCB | polychlorinated biphenyl |
| PCDD | polychlorinated dibenzo(p)dioxins |
| PCDF | polychlorinated dibenzofurans |
| PCR | polymerase chain reaction |
| WGS | whole genome sequence |

## Appendix A – Safety for the user

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

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

The likely 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, equal to = 800 s per day. With an uncertainty factor of 2, maximum inhalation exposure would occur for 2 × 800 = 1,600 s (0.444 h per day). Again, assuming a respiration volume of 1.25 $m^3$/h, the inhalation volume providing exposure to potentially endotoxin-containing dust would be 0.444 × 1.25 = 0.556 $m^3$ per day. This volume should contain no more than 900 IU endotoxin, so the dust formed from the product should contain no more than 900/0.556 = 1,619 IU/$m^3$.

### Calculation of endotoxin content of dust

Two key measurements are required to evaluate the potential respiratory hazard associated with endotoxin content of the product [the dusting potential of the product, expressed in g/$m^3$; 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 used 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 the simple multiplication a × b. This resulting value is further used for calculation of potential inhalatory exposure by users to endotoxin 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-threonine produced by *Escherichia coli* CGMCC 11473, including consideration of using filter half mask (FF P2 or FF P3)[(a)] as a preventative measure

| Calculation | Identifier | Description | Amount | Source |
|---|---|---|---|---|
| | *a* | Endotoxin content IU/g product | 319,000 | Technical dossier |
| | *b* | Dusting potential (g/$m^3$) | 0.2 | Technical dossier |
| *a* × *b* | *c* | Endotoxin content in the air (IU/$m^3$) | 63,800 | |
| | *d* | No of premixture batches made/working day | 40 | EFSA FEEDAP Panel (2012b) |
| | *e* | Time of exposure (s)/production of one batch | 20 | EFSA FEEDAP Panel (2012b) |
| *d* × *e* | *f* | Total duration of daily exposure/worker (s) | 800 | |
| | *g* | Uncertainty factor | 2 | EFSA FEEDAP Panel (2012b) |
| *f* × *g* | *h* | Refined total duration of daily exposure (s) | 1,600 | |
| *h*/3 600 | *i* | Refined total duration of daily exposure (h) | 0.44 | |
| | *j* | Inhaled air ($m^3$)/eight-hour working day | 10 | EFSA FEEDAP Panel (2012b) |
| *j*/8 × *i* | *k* | Inhaled air during exposure ($m^3$) | 0.56 | |
| *c* × *k* | *l* | **Endotoxin inhaled (IU) during exposure/ eight-hour working day** | **35,444** | |
| | *m* | Health-based recommended exposure limit of endotoxin (IU/$m^3$)/eight-hour working day | 90 | Health Council of the Netherlands (2010) |
| *m* × *j* | *n* | **Health-based recommended exposure limit of total endotoxin exposure (IU)/ eight-hour working day** | **900** | |
| *l* /10 | | Endotoxins inhaled (IU)/eight-hour working day reduced by filter half mask FF P2 (reduction factor 10) | 3,544 | |

| Calculation | Identifier | Description | Amount | Source |
|---|---|---|---|---|
| / /20 | | Endotoxins inhaled (IU)/eight-hour working day reduced by filter half mask FF P3 (reduction factor 20) | 1,772 | |

(a) Filtering face piece or filtering half mask according to European standard EN 149. They are graded from 1 to 3 depending on their filtering capacity.

## References

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012 Guidance on studies concerning the safety of use of the additive for users/workers. EFSA Journal 2012;10(1):2539, 5 pp. https://doi.org/10.2903/j.efsa.2012.253

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2015. Scientific Opinion on the safety and efficacy of L-lysine monohydrochloride produced by fermentation with *Escherichia coli* for all animal species based on a dossier submitted by HELM AG on behalf of Meihua Holdings Group Co. Ltd. EFSA Journal 2015;13(3):4052, 16 pp. https://doi.org/10.2903/j.efsa.2015.4052

Health Council of the Netherlands, 2010. Endotoxins. Health-based recommended occupational exposure limit. Publication no 2010/04 *OSH*, 100 pp.

# Annex A – European Union Reference Laboratory (EURL) evaluation report on the analytical methods submitted in connection with the application for authorisation of L-threonine produced by *E. coli* CGMCC 11473 for all animal species.

In the current application authorisation is sought under Articles 4(1) for L-threonine, 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. 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-threonine concentrations in premixtures and feedingstuffs.

For the quantification of threonine in premixtures and feedingstuffs, the Applicant submitted the ring-trial validated Community method for amino acids using an amino acid analyser or high-performance liquid chromatography (HPLC) equipped with ion exchange column (IEC). The method applies for the determination of free (synthetic and natural) and total (peptide-bound and free) amino acids. Only performance characteristics for the determination of total threonine are reported: – a relative standard deviation for repeatability (RSDr) ranging from 1.9% to 4.1%; and – a relative standard deviation for reproducibility (RSDR) ranging from 3.8% to 11.7%. Based on the performance characteristics available, the EURL recommends for official control this Community method to quantify threonine in premixtures and feedingstuffs.

For the quantification of L-threonine in the feed additive and water, the Applicant suggested to apply the above-mentioned Community method specifically designed for the analysis of premixtures and feedingstuffs. No experimental data were provided to demonstrate the applicability of this method to the quantification of L-threonine in these two matrices. However, the EURL recommends for official control, based on previous 'L-threonine' reports (e.g. FAD-2013-0028), (i) the 'L-threonine' monograph of the Food Chemical Codex (FCC) for the identification of L-threonine in the feed additive, using infrared absorption together with optical rotation and (ii) the ring-trial validated method (EN ISO 17180:2013) based on IEC coupled with post-column derivatisation and ultraviolet (UV) or fluorescence detection (FD) for the determination of L-threonine in the feed additive and concentrated premixtures (more than 10%). Precisions of the order of 1–2% were reported for a commercial product with threonine content of 96%.

The EURL could not evaluate nor recommend any method for the official control to quantify L-threonine 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.