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Safety and efficacy of L-threonine produced by fermentation using *Escherichia coli* CGMCC 7.232 for all animal species

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Abstract

The product subject of this assessment is L-threonine produced by fermentation with a genetically modified strain of *Escherichia coli* (CGMCC 7.232). It is intended to be used in feed and water for drinking for all animal species and categories. The production strain and its recombinant DNA were not detected in the additive. The product L-threonine, manufactured by fermentation with *E. coli* CGMCC 7.232, does not raise any safety concern with regard to the genetic modification of the production strain. L-Threonine produced using *E. coli* CGMCC 7.232 is considered safe for the target species. The FEEDAP Panel has concerns regarding the safety of the simultaneous administration of L-threonine via water for drinking and feed. L-Threonine produced using *E. coli* CGMCC 7.232 is safe for the consumer. In 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. L-Threonine produced using *E. coli* CGMCC 7.232 is safe for the environment. 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.

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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 7.232 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, to deliver the present output.

The production strain and its recombinant DNA were not detected in the additive. The product L-threonine, manufactured by fermentation with *E. coli* CGMCC 7.232, does not raise any safety concern with regard to the genetic modification of the production strain.

L-Threonine produced using *E. coli* CGMCC 7.232 is considered safe for the target species. The FEEDAP Panel has concerns regarding the safety of the simultaneous administration of L-threonine via water for drinking and feed.

L-Threonine produced using *E. coli* CGMCC 7.232 is safe for the consumer.

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.

L-Threonine produced using *E. coli* CGMCC 7.232 is safe for the environment.

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.

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1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003¹ establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from Welding GmbH & Co KG.² for authorisation of the product L-Threonine, 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 4 October 2017.

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 produced by fermentation using a genetically modified strain of *Escherichia coli* (CGMCC 7.232), when used under the proposed conditions of use (see Section 3.1.7).

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 *Escherichia 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 waving 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) (Ph. Eur., 2016). 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 10 opinions on the safety and efficacy of L-threonine produced by genetically modified strains of *E. coli* (EFSA FEEDAP Panel, 2013, 2014a,b,c,d, 2015a,b, 2016a,b, 2017).

The Joint FAO/WHO Expert Committee on Food Additives evaluated L-threonine as a 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 Welding GmbH & Co KG. Esplanase 39, 20354 Hamburg, Germany.

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, 10.09.1999, 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 dossier⁷ in support of the authorisation request for the use of L-threonine as a feed additive.

The FEEDAP Panel used the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA or other expert bodies, peer-reviewed scientific papers, 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.⁸

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, 2008), Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance (EFSA FEEDAP Panel, 2012c), Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use (EFSA GMO Panel, 2011) and Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2017).

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* (CGMCC 7.232). 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.

3.1. Characterisation

3.1.1. Characterisation of the active substance/additive

L-Threonine (International Union of Pure and Applied Chemistry name: (2S,3R)-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 weight of 119.12 Da. The molecular formula of L-threonine is C₄H₉NO₃. The structural formula is given in Figure 1.

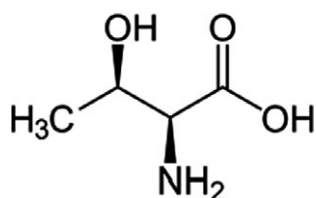


Figure 1: Molecular structure of L-threonine

The additive contains, by specification, $\geq 98.5\%$ L-threonine, $< 0.5\%$ moisture, and unspecified amount of other amino acids and $< 1\%$ other substances. The analysis of five batches of the additive (ISO 13903 method) showed an average of threonine of 100% 'as is' (range 97.6–101.9%).⁹ The loss

⁷ FEED dossier reference: FAD-2017-0037.

⁸ The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2017-0037_threonine.pdf

⁹ Technical dossier/Section II/Annex 2.1.3 and supplementary information April 2018/Annex Q5b. Analytical method ISO 13903

on drying was 0.2%. The amount of unidentified material was in average lower than 1% on a dry matter basis.

Analysis of five additional batches (VDLUFA, equivalent to the EU official method (Regulation (EC) No 152/2009)) showed an average of threonine of 98.7% (range 97.5–100.2%) on dry matter basis.¹⁰ The loss on drying was not specified.

The specific optical rotation was measured in three batches of the final product and was -27.9° in all three batches,¹¹ which is within the range (-29.0 to -27.6°) established for L-threonine in the Ph. Eur. (2016) and demonstrates the identity of the L-enantiomer.

3.1.2. Impurities

Three batches were analysed for heavy metals (cadmium, mercury and lead) and were found below the limit of detection (LOD). Arsenic (analysed in three batches) was up to 0.014 mg/kg.¹² None of these concentrations were considered of concern.

The microbiological quality of three batches of the product was tested by counting Enterobacteriaceae (negative), *E. coli* (negative), *Salmonella* spp. (negative in 25 g) and yeasts (negative).¹³ Regarding mycotoxins (ochratoxin A, aflatoxins (unspecified), zearalenone, fumonisin, deoxynivalenol and citrinin); all values were below the limit of quantification (LOQ)¹⁴ and are considered to be of no concern.

Dioxins (polychlorinated dibenzofurans (PCDF), polychlorinated dibenzo(p)dioxins (PCDD)) and dioxin-like polychlorinated biphenyls (PCBs) were measured in three batches of the final product.¹⁵ PCDF/D amounted < 0.14 ng WHO-TEQ/kg in each of the three batches. PCBs amounted 0.13 ng WHO-TEQ/kg in each of the three batches. The sum of PCDF/D and dioxin-like PCBs was 0.269 ng WHO-TEQ/kg in each of the three batches.

One batch of the additive was tested for inhibitory activity against the list of reference strains of the EFSA Guidance on microbial studies (EFSA, 2008).¹⁶ For all strains, the minimum inhibitory concentration (MIC) values were $> 1,280$ mg/L. The MICs were in all cases greater than 4 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 2.14 to 5.81 IU/mg.¹⁷

Viable cells of the production strain were not detected in 1 g of product of three batches of the additive, tested in triplicate.¹⁸ The samples were first incubated in non-selective broth at 37°C for 48 h, followed by culturing on non-selective plates at 37°C for 24 h. No colonies of the production strain were detected on the plates.

The absence of recombinant DNA of the production strain was confirmed in three samples of 1 g of the final product tested in triplicate by targeting a 969 bp DNA fragment (LOD: 10 ng/g additive).¹⁹

3.1.3. Physical characteristics

The additive is a white granulated product, practically odourless, with a solubility in water of 650 g/L (at 20°C) and a pH of 5.72 (10% solution in water at 20°C).²⁰ It has a density of 650 kg/m³.

The particle size distribution of the final product (three batches) was analysed by laser diffraction.²¹ The fractions of particles having a diameter <100 , <50 and <10 μm were in the range of 62–70%, 26–38% and 2–4% (v/v), respectively.

¹⁰ Technical dossier/Section II/Annex 2.1.4a and supplementary information April 2018/Annex answers to SIn and Q8 statement conformity analyses amino acids. Analytical method VDLUFA.

¹¹ Technical dossier/Supplementary information April 2018/Annex Q7.

¹² Technical dossier/Section II/Annex 2.1.4a. LOD (in mg/kg) was 0.01 for lead and 0.002 for cadmium and mercury.

¹³ Technical dossier/Section II/Annex 2.1.4a.

¹⁴ Technical dossier/Section II/Annex 2.1.4a. LOQ (in $\mu\text{g}/\text{kg}$) was 5 for ochratoxin A, 1.7 for aflatoxins, 17 for zearalenone, 25 for fumonisin, 134 for deoxynivalenol and 15 for citrinin.

¹⁵ Technical dossier/Section II/Annex 2.1.4f.

¹⁶ Technical dossier/Section II/Annex 2.2.2.2 and Supplementary information January 2017/Annex 9.

¹⁷ Technical dossier/Section II/Annex 2.1.4e.

¹⁸ Technical dossier/Supplementary information June 2018/Annex 2.

¹⁹ Technical dossier/Supplementary information April 2018/Q4.

²⁰ Technical dossier/Section II/Annex 2.5.2.

²¹ Technical dossier/Section II/Annexes 2.1.5a, 2.1.5b and 2.1.5c.

Analytical data on the dusting potential (three batches) of the final product (Stauber–Heubach method) ranged from 3 to 9.5 g/m³.²²

3.1.4. Characterisation of the production organism

The additive is produced by a genetically modified strain of *E. coli* K-12 (YPT_{Thr}001), which has been deposited in the China General Microbiological Culture Collection Center (CGMCC) with the accession number CGMCC 7.232.²³ The identity of the strain was confirmed as *E. coli* [REDACTED]

[REDACTED] The strain is a derivative of *E. coli* K-12 strain [REDACTED]
Bauer et al., 2007.

The susceptibility of the production strain to relevant antibiotics (ampicillin, gentamicin, kanamycin, streptomycin, tetracycline, ciprofloxacin, colistin and fosfomycin) was demonstrated by broth microdilution following the guidance on microorganisms used as feed additive or as production organisms (EFSA FEEDAP Panel, 2017) and including *E. coli* ATCC 25922 as a control strain.²⁷ No antimicrobial resistance genes were found in the genome of the production strain by bioinformatic analysis using CARD database.²⁸

3.1.4.1. Information relating to the genetically modified microorganism

Characteristics of the recipient or parental microorganism

The recipient strain [REDACTED] is well-characterised and its safety (non-pathogenicity) has been documented (Gorbach, 1978). The strain was shown to be ineffective in colonising the human gut (Smith, 1975). Its genome has been fully sequenced (Hayashi et al., 2006).

Characteristics of the donor organism [REDACTED]

Description of the genetic modification process

²² Technical dossier/Section II/Annexes 2.4.3a, 2.4.3b and 2.4.3c.

²³ Technical dossier/Section II/Annex 2.2.1.2a.

²⁷ Technical dossier/Supplementary information June 2018/Annex 1.

3.1.4.2. Information related to the production process

L-Threonine is produced by fermentation using the production strain *E. coli* GCMCC 7.232.

The applicant declared that no antibiotics are used in the production process.³²

3.1.5. Stability and homogeneity

The shelf-life of three batches of the product was tested at 25 and 40°C when stored in sealed plastic bags protected from light for 12 and 6 months, respectively.³³ No losses were detected.

The stability of the product (three batches) in a vitamin/mineral premixture (containing 16 g choline/kg) supplemented with 100 g L-threonine/kg was studied when stored in plastic bags at room temperature for 6 months.³⁴ No losses were observed.

The stability of the product (three batches) was tested in a compound feed for piglets (mash and pelleted feed) when supplemented at 0.5% L-threonine.³⁵ The basal diet consisted in barley, soybean meal and soybean and contained 0.56% threonine. Pelleting was performed at 55–60°C. The effect of pelleting represented a loss of 0–2%. Samples were stored in closed plastic bags protected from light. After three months storage at room temperature, no loss was observed in the meal or in the pelleted feed. As total threonine (protein-bound plus free threonine) was analysed instead of supplemental threonine, the results of this stability study might not accurately reflect the 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%.³⁶ The coefficient of variation was 3.5%. Total threonine (protein-bound plus free threonine) was analysed. Consequently, the result obtained might not accurately reflect the 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. No losses were observed.³⁷ 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.³⁸

3.1.6. Physico-chemical incompatibilities in feed

No physico-chemical 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 provided that it is not supplemented at the same time in feed. 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 recipient organism [REDACTED] is considered to be safe.

³² Technical dossier/Section II/Annex 2.3i.

³³ Technical dossier/Section 2.4.1 and Annex 2.1.4a; supplementary information April 2018/Annex answers to SIn.

³⁴ Technical dossier/Section 2.4.1 and Annexes 2.1.4a and 2.4.1c.

³⁵ Technical dossier/Section 2.4.1 and Annexes 2.1.4b and 2.4.1c.

³⁶ Technical dossier/Section 2.4.2 and Annex 2.4.1c.

³⁷ Technical dossier/Section 2.4.1 and Annex 2.1.4a.

³⁸ Guidance for the preparation of dossiers for nutritional additives. EFSA Journal 2012;10(1):2535.

The production strain confers increased L-threonine synthesis by [REDACTED]

[REDACTED] Those traits do not raise safety concern.

[REDACTED] analysis of the non-coding sequences in the inserted regions of the production strain showed the insertion of a partial sequence of [REDACTED] and therefore is of no concern. [REDACTED] No antimicrobial resistance genes were found in the genome of the production strain [REDACTED]

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 from 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 (2.14–5.81 IU/mg) is similar to or lower than that observed in other feedingstuffs and is therefore of no concern for the target species (Cort et al., 1990). The data submitted allows excluding the presence of recombinant DNA and antibiotic resistance genes in the final product (see Section 3.1.2). Therefore, the FEEDAP Panel concludes that L-threonine produced by *E. coli* CGMCC 7.232 is safe for the target species when used respecting animals' requirements (EFSA FEEDAP Panel 2014a).

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

3.2.2.1. Conclusions on safety for the target species

L-Threonine produced using *E. coli* CGMCC 7.232 is considered safe for the target species. 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 L-threonine > 99% on a dry matter basis 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. No viable cells of the production strain and/or no recombinant DNA were found in the final product. Therefore, the FEEDAP Panel concluded that L-threonine produced using *E. coli* CGMCC 7.232 is safe for the consumer.

3.2.4. Safety for the user

No studies to support the safety of the additive for the user were provided.

3.2.4.1. Effects on the respiratory system

Dusting potential was measured in three batches of the additive and ranged from 3 to 9.5 g/m³. The percentage of particles with diameters < 100, < 50 and < 10 µm were 62–70%, 26–38% and 2–4% (v/v, three batches of the final product), 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 2.14 to 5.81 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 30,664 IU per 8-h working day, indicating a risk by inhalation due to exposure to endotoxins for people handling the additive (see Appendix A).

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.

Viable cells of the production organism and its recombinant DNA were not detected in the final product. The final product does not pose any environmental safety concern associated with the genetic modification of the production strain. L-Threonine produced using *E. coli* CGMCC 7.232 is considered safe for the environment.

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 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 Regulation³⁹ and Good Manufacturing Practice.

4. Conclusions

The production strain and its recombinant DNA were not detected in the additive. The product L-threonine, manufactured by fermentation with *E. coli* CGMCC 7.232, does not raise any safety concern with regard to the genetic modification of the production strain.

L-Threonine produced using *E. coli* CGMCC 7.232 is considered safe for the target species. The FEEDAP Panel has concerns regarding the safety of the simultaneous administration of L-threonine via water for drinking and feed.

L-Threonine produced using *E. coli* CGMCC 7.232 is safe for the consumer.

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.

L-Threonine produced using *E. coli* CGMCC 7.232 is safe for the environment.

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

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.

5. Recommendations

The description of the additive should contain the statement 'produced by fermentation with *Escherichia coli* CGMCC 7.232'.

Documentation provided to EFSA

- 1) L-Threonine produced using *E. coli* CGMCC 7.232 for all animal species. July 2017. Submitted by Welding GmbH & Co KG
- 2) L-Threonine produced using *E. coli* CGMCC 7.232 for all animal species. Supplementary information. April 2018. Submitted by Welding GmbH & Co KG.
- 3) L-Threonine produced using *E. coli* CGMCC 7.232 for all animal species. Supplementary information. June 2018. Submitted by Welding GmbH & Co KG.
- 4) Evaluation report of the European Union Reference Laboratory for Feed Additives on the Methods(s) of Analysis for of L-threonine produced by *E. coli* CGMCC 7.232 for all animal species.
- 5) Comments from Member States.

Chronology

Date	Event
27/06/2017	Dossier received by EFSA
31/07/2017	Reception mandate from the European Commission
04/10/2017	Application validated by EFSA – Start of the scientific assessment
07/12/2017	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. <i>Issues: characterisation of the production strain and of the additive, stability and safety for the user.</i>
04/01/2018	Comments received from Member States
02/02/2018	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
23/04/2018	Reception of supplementary information from the applicant - Scientific assessment re-started
18/06/2018	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. <i>Issues: characterisation of the production strain.</i>
29/06/2018	Reception of supplementary information from the applicant - Scientific assessment re-started
02/10/2018	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

References

- Bauer AP, Dieckmann SM, Ludwig W and Schleifer KH, 2007. Rapid identification of *Escherichia coli* safety and laboratory strain lineages based on Multiplex-PCR. *FEMS Microbiology Letters*, 269, 36–40.
- 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, A-Physiology Pathology, Clinical Medicine*. 37, 130–137.
- EFSA (European Food Safety Authority), 2008. Technical Guidance: Microbial Studies. *The EFSA Journal*, 836, 1–3.
- 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, 2 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, 2 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 FEEDAP Panel (EFSA Panel on additives and products or substances used in animal feed), Rycken G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Borjes G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, Lopez-Alonso M, Lopez Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Anguita M, Galobart J and Innocenti ML, 2017. Guidance on the identity, characterisation and conditions of use of feed additives. EFSA Journal 2017;15(10):5023, 12 pp. <https://doi.org/10.2903/j.efsa.2017.5023>
- 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>
- Gorbach SL, 1978. Risk assessment of recombinant DNA experimentation with *Escherichia coli* K12. Proceedings from a workshop at Falomuth, Massachusetts. Journal of Infectious Diseases, 137, 613–714.
- Hayashi K, Morooka N, Yamamoto Y, Fujita K, Isono K, Choi S, Ohtsubo E, Baba T, Wanner BL, Mori H and Horiuchi T, 2006. Highly accurate genome sequences of *Escherichia coli* K-12 strains MG1655 and W3110. Molecular Systems Biology, 2, 2006.0007. <https://doi.org/10.1038/msb4100049>
- Health Council of the Netherlands, 2010. Endotoxins. Health-based recommended occupational exposure limit. Publication no 2010/04OSH, 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.
- Ph. Eur. (European Pharmacopoeia), 2016. 9th Edition. European Directorate for the Quality of Medicines and Health Care.

- Rylander R, 1999. Health effects among workers in sewage treatment plants. *Occupational and Environmental Medicine*, 56, 354–357.
- Smith HW, 1975. Survival of orally administered *E. coli* K-12 in alimentary tract of man. *Nature*, 255, 500–502.
- Thorn J, 2001. The inflammatory response in humans after inhalation of bacterial endotoxin: a review. *Inflammatory Response*, 50, 254–261.

Abbreviations

CGMCC	China general microbiological culture collection centre
EURL	European Union Reference Laboratory
FCC	Food Chemical Codex
FEEDAP	EFSA scientific Panel on additives and products or substances used in animal feed
GMO	EFSA scientific Panel on genetically modified microorganisms
HSE	British health safety Executive
IEC-VIS/FD	ion exchange chromatography coupled with post-column derivatisation and visible or fluorescence detection
IU	International unit of endotoxin activity. One IU corresponds to one endotoxin unit (EU).
LOD	limit of detection
LOQ	limit of quantification
MIC	Minimum inhibitory concentration
PCB	dioxin-like polychlorinated biphenyl
PCDD	polychlorinated dibenzo(<i>p</i>)dioxins
PCDF	polychlorinated dibenzofurans
RSDr	relative standard deviation for repeatability
RSDR	relative standard deviation for reproducibility
WHO	World Health Organization

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/day. With an uncertainty factor of 2, maximum inhalation exposure would occur for $2 \times 800 = 1,600$ s (0.444 h/day). Again, assuming a respiration volume of 1.25 m³/h, the inhalation volume providing exposure to potentially endotoxin-containing dust would be $0.444 \times 1.25 = 0.556$ m³/day. This volume should contain no more than 900 IU endotoxin, so the dust formed from the product should contain no more than $900/0.556 = 1,619$ IU/m³.

Calculation of endotoxin content of dust

Two key measurements are required to evaluate the potential respiratory hazard associated with endotoxin content of the product (the dusting potential of the product, expressed in g/m³; 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/m³, then the content of endotoxins of the dust, c IU/m³, is obtained by the simple multiplication $a \times b$. 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 7.232 including consideration of using filter half mask (FF P2 or FF P3)⁴⁰ as a preventative measure

Calculation	Identifier	Description	Amount	Source
	a	Endotoxin content IU/g product	5,810	Technical dossier
	b	Dusting potential (g/m ³)	9.5	Technical dossier
a × b	c	Endotoxin content in the air (IU/m ³)	55,195	
	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	30,664	
	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	

⁴⁰ 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.

Calculation	Identifier	Description	Amount	Source
I /10		Endotoxins inhaled (IU)/eight-hour working day reduced by filter half mask FF P2 (reduction factor 10)	3,066	
I /20		Endotoxins inhaled (IU)/eight-hour working day reduced by filter half mask FF P3 (reduction factor 20)	1,533	

References

- 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.2012.253>
- 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-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 2010/04OSH, 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 7.232 for all animal species

In the current application authorisation is sought under Articles 4(1) for L-threonine produced by *Escherichia coli* CGMCC 7.232, under the category/functional group 3(c) 'nutritional additives//amino acids, their salts and analogues' according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for all animal species. L-threonine is already authorised as a feed additive under Commission Directive 88/485/EEC.

For the quantification of L-threonine in the feed additive, the Applicant submitted the ring-trial validated method EN ISO 17180:2013 based on ion exchange chromatography coupled with post-column derivatisation and visible or fluorescence detection (IEC-VIS/FD). The following performance characteristics are reported: a relative standard deviation for repeatability (RSD_r) ranging from 0.7 to 1.4%; and a relative standard deviation for reproducibility (RSD_R) ranging from 1.9 to 2.3%. In addition, the EURL identified the 'L-threonine monograph' of the Food Chemical Codex (FCC) for the identification of L-threonine in the feed additive.

For the quantification of L-threonine in premixtures, feedingstuffs and water the Applicant submitted the ring-trial validated Community method (Commission Regulation (EC) No 152/2009) based on IEC coupled with photometric detection (IEC-VIS). This method, designed only for the analysis of premixtures and feedingstuffs, does not distinguish between the salts and the amino acid enantiomers. The following performance characteristics were reported for the quantification of total threonine: RSD_r ranging from 1.9% to 2.7%, and RSD_R ranging from 3.8% to 5.2%. In the frame of the stability studies, the Applicant presented experimental data obtained analysing threonine in water with the VDLUFA official method based on IEC-VIS/FD. The results presented are considered sufficient to demonstrate the suitability of the procedure for the analysis of the amino acid in water. Hence, the EURL recommends for official control this method to quantify threonine in water.

In the frame of this authorisation, the EURL recommends for official control (i) the 'L-threonine monograph' of the FCC based on infrared absorption for the identification of L-threonine in the feed additive; (ii) the ring-trial validated method EN ISO 17180:2013 based on IEC-VIS/FD to quantify free threonine in feed additive and premixtures (containing more than 10% threonine); (iii) the Community method based on IEC-VIS for the quantification of threonine in premixtures and feedingstuffs; and (iv) the analytical method described by VDLUFA (4.11.6) based on IEC-VIS/FD to quantify 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.