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SCIENTIFIC OPINION

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[1]

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

European Food Safety Authority (EFSA), Parma, Italy

This scientific output, published on 19 August 2014, replaces the earlier version published on 29 July 2013.[4]

ABSTRACT

The product L-threonine, technically pure, is an essential amino acid that contains by specification ≥ 98 % L-threonine. It is produced by a genetically modified *Escherichia coli* strain. Neither the production strain nor its recombinant DNA was detectable in the final product. The final product does not raise safety concerns with regard to the genetic modification. L-Threonine, technically pure, is considered safe for the target animals when used to meet the requirements. The toxicological studies, including mutagenicity tests, performed with the additive or other sources of L-threonine did not reveal any toxic effect. Since the composition of tissues and products of animal origin will not be changed by the use of L-threonine in animal nutrition, and considering the high purity of the product under assessment, no risks are expected for the consumer from the use of L-threonine, technically pure, as a feed additive. The product is not irritating to the skin or eyes and is not likely to be a skin sensitiser. Although there is a potential for user exposure by inhalation, there is no evidence of acute toxicity by the inhalation route. The use of the product as a feed additive does not represent a risk to the environment. L-Threonine, technically pure, is considered as an efficacious source of L-threonine for all animal species. Response in ruminants requires some degree of protection of threonine from ruminal degradation.

KEY WORDS

nutritional additives, amino acids and their salts and analogues, L-threonine, safety, efficacy, genetically modified micro-organisms

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[1] On request from the European Commission Question No EFSA-Q-2012-00114 adopted on 9 July 2013.

[2] Panel members: Gabriele Aquilina, Vasileios Bampidis, Maria De Lourdes Bastos, Lucio Guido Costa, Gerhard Flachowsky, Mikolaj Antoni Gralak, Christer Hogstrand, Lubomir Leng, Secundino López-Puente, Giovanna Martelli, Baltasar Mayo, Fernando Ramos, Derek Renshaw, Guido Rychen, Maria Saarela, Kristen Sejrsen, Patrick Van Beelen, Robert John Wallace and Johannes Westendorf. Correspondence: FEEDAP@efsa.europa.eu

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[4] Revision 1: This scientific opinion has been edited following the adoption of the decision of the Commission on confidentiality claims submitted by the applicant, in accordance with Article 8(6) and Article 18 of Regulation (EC) No 1831/2003. The modified sections are indicated in the text.

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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 the safety and efficacy of the product L-threonine, technically pure, for all animal species.

Threonine is an essential amino acid, considered as the second limiting amino acid in pig feed and probably as the third limiting in poultry feed. Supplementing feedingstuffs with free threonine would ensure that the animal requirements for that amino acid are met.

The product L-threonine, technically pure, contains by specification ≥ 98 % L-threonine. It is produced by a genetically modified *Escherichia coli* strain.

Neither the production strain nor its recombinant DNA was detectable in the final product. The final product does not raise safety concerns with regard to the genetic modification.

L-Threonine, technically pure, is considered safe for the target animals when used to meet the requirements.

The toxicological studies, including mutagenicity tests, performed with the additive or other sources of L-threonine did not reveal any toxic effect. Since the composition of tissues and products of animal origin will not be changed by the use of L-threonine in animal nutrition, and considering the high purity of the product under assessment, no risks are expected for the consumer from the use of L-threonine, technically pure, as a feed additive.

The product is not irritating to the skin or eyes and is not likely to be a skin sensitiser. Although there is a potential for user exposure by inhalation, there is no evidence of acute toxicity by the inhalation route.

The use of the product as a feed additive does not represent a risk to the environment.

L-Threonine, technically pure, is considered as an efficacious source of L-threonine for all animal species. Response in ruminants requires some degree of protection of threonine from ruminal degradation.

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BACKGROUND

Regulation (EC) No 1831/2003[5] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular Article 10(2) of that Regulation also specifies that for existing products within the meaning of Article 10(1), an application shall be submitted in accordance with Article 7, at the latest one year before the expiry date of the authorisation given pursuant to Directive 70/524/EEC for additives with a limited authorisation period, and within a maximum of seven years after the entry into force of this Regulation for additives authorised without time limit or pursuant to Directive 82/471/EEC.

The European Commission received a request from the company Ajinomoto Eurolysine S.A.S.[6] for re-evaluation of the product L-threonine, when used as a feed additive for all animal species (category: nutritional additive; functional group: amino acids, their salts and analogues) under the conditions mentioned in Table 1.

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 10(2) (re-evaluation of an authorised feed additive). EFSA received directly from the applicant the technical dossier in support of this application.7 According to Article 8 of that Regulation, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. The particulars and documents in support of the application were considered valid by EFSA as of 27 April 2012.[8]

L-threonine, technically pure, (minimum content of 98 % as is basis) is currently authorised as a nutritional feed additive for use in all animal species.[9]

L-threonine like other amino acids and other nitrogen compounds is authorized according to Commission Directive 2006/141/EC for infant formulae and follow-on formulae.[10] 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.[11] L-threonine is also registered as an ingredient in cosmetic products as antistatic, hair conditioning, hair waiving or straightening (Commission decision 2006/257/EEC). 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.[12]

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in contact with Food (AFC) on a request from the Commission, issued and opinion (EFSA, 2008) related to Flavouring Group Evaluation 26, revision 1: amino acids from chemical group 34 (EFSA-Q-2003-169B).

[5] 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.
[6] Ajinomoto Eurolysine S.A.S. Rue de Courcelles, 153, 75817, Paris 17ème - FRANCE.
[7] EFSA Dossier reference: FAD-2010-0081.
[8] A new mandate was received in EFSA on 12/01/2012.
[9] Commission Directive 88/485/EEC of 26 July 1988 amending the Annex to Council Directive 82/471/EEC concerning certain products used in animal nutrition. OJ L 239, 30.08.1988, p. 36.
[10] Commission Directive 2006/141/EC on infant formulae and follow-on formulae, OJ L 401, 30.12.2006, p. 1.
[11] 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.
[12] 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.

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in contact with Food (AFC) issued and opinion on L-threonine (EFSA, 2010) related to Flavouring Group Evaluation 26: amino acids from chemical group 34.

TERMS OF REFERENCE

According to Article 8 of Regulation (EC) No 1831/2003, EFSA shall determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment and the efficacy of the product L-threonine, when used under the conditions described in Table 1.

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

Additive	L-threonine, technically pure
Registration number/EC No/No (if appropriate)	
Category(-ies) of additive	(c): Nutritional additive
Functional group(s) of additive	3 (c): Amino acids, their salts and analogues

Description			
Composition, description	Chemical formula	Purity criteria (if appropriate)	Method of analysis (if appropriate)
White to pale yellow crystalline powder	C4H9NO3 or CH3-CH(OH)-CH(NH2)-COOH	not less than 98 % L-threonine (product as is)	High Pressure Liquid Chromatography, 1) foor feedingstuffs: Regulation (EC) No 152/2009 of 27 January 2009, Annex III, Part F (or equivalent: ISO 13903:2005) 2) pure products/premixtures: Regulation(EC) 152/2009, Annex III, Part F in adapting dilutions or AOACMethod 999.13

Trade name (if appropriate)	L-threonine, feed grade (Ajinomoto Eurolysine):
Name of the holder of authorisation (if appropriate)	

Conditions of use				
Species or category of animal	Maximum Age	Minimum content	Maximum content	Withdrawal period (if appropriate)
		mg/kg of complete feedingstuffs		
All species	Not applicable	Not applicable	Not applicable	None (essential amino acid)

Other provisions and additional requirements for the labelling	
Specific conditions or restrictions for use (if appropriate)	None
Specific conditions or restrictions for handling (if appropriate)	Like for any product which may generate dust, wear a mask and safety goggles
Post-market monitoring (if appropriate)	Not considered necessary in view of adequate monitoring and operating conditions during the manufacturing process to ensure quality and safety of the additive. HACCP and Traceability systems are in place. Ajinomoto Eurolysine has been certified ISO 9001 and FAMI-QS.
Specific conditions for use in complementary feedingstuffs (if appropriate)	None

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

ASSESSMENT

This opinion is based on data provided by a single company involved in the production/distribution of L-threonine produced by a genetically modified microorganism. The FEEDAP Panel has sought to use the data provided together with data from other sources to deliver an opinion.

1. Introduction[13]

Threonine, technically pure (minimum content of L-threonine 98 %, on an 'as is' basis), was first authorised by Commission Directive 88/485/EEC of 26 July 1988 amending the Annex to Council Directive 82/471/EEC concerning certain products used in animal nutrition.

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).

The application is for L-threonine, technically pure, a purified fermentation product produced by a genetically modified (GM) *Escherichia coli* (FERM BP-10942). It is intended to be used in all animal species. The objective of feed supplementation with essential amino acids is to complete the amino acid profile of the diet in order to closely meet the requirement of individual amino acids of the animals or to compensate for potential imbalances. The supplementation of feedingstuffs with amino acids is a conventional measure in improving the protein quality and utilisation. This supplementation became even more important since 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 (pigs) limiting amino acid after L-lysine and the third after sulphur amino acids and L-lysine (poultry).

2. Characterisation

2.1. Characterisation of the active substance/additive

L-Threonine (International Union of Pure and Applied Chemistry (IUPAC) name (2*S*,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 (CAS) No 72-19-5 and the European Inventory of Existing Commercial chemical Substances (EINECS) No 200-774-1, is an essential amino acid. It has a molecular weight of 119.12 Da and a nitrogen content of 11.76 %. The molecular formula of L-threonine is $C_4H_9NO_3$; the structural formula is given in Figure 1.

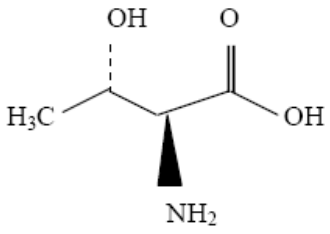

Figure 1: Structural formula of L-threonine

The product L-threonine, technically pure, is a white to pale yellow crystalline powder, with no particular odour. It contains by specification not less than 98 % L-threonine (on as is basis) and not more than 1.5 % impurities (e.g. moisture and accompanying substances/impurities). No evidence of the potential occurrence of D-threonine in L-threonine, technically pure, was given in the dossier.

Batch-to-batch variation on five samples showed a mean L-threonine content of 99.5 % (range 99.03–99.91 %), and a water content < 0.4 %. Analyses performed on 809 samples during a 2.5-year period

[13] This section has been edited following the provisions of Article 8(6) and Article 18 of Regulation (EC) No 1831/2003.

showed an average L-threonine content of 99.7 % (range 98.5–100 %). The sum of quantifiable N compounds (NH_3, NO_2, NO_3, betaine) in five samples was < 0.008 % and that of quantifiable biogenic amines was < 0.027 %. No quantifiable sugars or organic acids were present. The sum of inorganic anions and cations in five samples amounted to < 0.2 %.

2.2. Impurities[14]

The mycotoxin content (aflatoxin B1, B2, G1, G2, deoxynivalenol and ochratoxin A (< 1 μg/kg); zearalenone (< 5 μg/kg); T2-toxin (< 10 μg/kg); HT2-toxin (< 50 μg/kg); fumosins B1 and B2 (< 100 μg/kg)) analysed in three batches of L-threonine, technically pure, was very low.[15] The content of arsenic and cadmium (< 0.01 mg/kg), chromium and copper (< 0.05 mg/kg), iron (< 0.1 mg/kg), lead (< 0.02 mg/kg), mercury (< 0.002 mg/kg) and nickel (< 0.05 mg/kg) analysed in five batches of the additive was very low. Residues of organochlorine and organophosphorus pesticides, and of dioxins and dioxin-like polychlorinated biphenyls (PCBs), analysed in three batches of the additive were all below the maximum levels fixed by Directive 2002/32/EC. Polycyclic aromatic hydrocarbons (PAHs) were all below 0.5 μg/kg. Microbiological controls performed on five lots show very low levels of aerobic microbiota (total bacterial count (< 100 colony-forming units (CFU)/g), moulds (< 10 CFU/g), yeasts (< 10 CFU/g), *Salmonella* (0 in 50 g), *Clostridium perfringens* (< 10 CFU/g), *Staphylococcus aureus* (< 10 CFU/g)), and those indicative of contamination such as *Streptococcus* (< 20 CFU/g), Enterobacteriaceae (< 10 CFU/g) or coliforms (< 10 CFU/g).

The absence of antimicrobial activities was investigated on three batches of the additive.[16] The absence of antibiotic resistance of the L-threonine-producing strain to most of (but not restricted to) the antibiotics described in the guidance on the assessment of bacterial antimicrobial susceptibility of the FEEDAP Panel (with the exception of ampicillin, sulphonamide and apramycin that were not tested) was confirmed by a minimum inhibitory concentration analysis.[17]

2.3. Physical properties

The product has a bulk density of 0.5–0.6 kg/L, the melting point is 256 °C and the product shows a solubility in water of 9.76 g/100 mL.

Particle size distribution was analysed in four samples and showed an average particle size (v/v) of 133 μm, with 10 % of particles < 36.8 μm, 50 % < 103.2 μm and 90 % < 268.1 μm. The dustiness test (according to EN 15051) showed an inhalable fraction of 12 347 mg/kg, a thoracic fraction of 4 217 mg/kg and a respirable fraction of 664 mg/kg (fractions according to EN 481). The applicant therefore classified L-threonine, technically pure, as a very fine powder with a high dusting potential.[18]

2.4. Characterisation of the production organism[19]

L-Threonine is produced by a genetically modified strain of *E. coli*, deposited in the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology culture collection with deposition number FERM BP-10942. The identity of the strain was confirmed by means of ribotyping and serotyping.[20]

The recipient strain is a derivative of *Escherichia coli* K-12. *E. coli* K-12 is a well-characterised Gram-negative bacterium and its safety (non-pathogenicity) has been reviewed extensively (Gorbach, 1978). The technical dossier contains detailed and sufficient information on the recipient microorganism including safety aspects, the origin and function of the different genetic elements

[14] This section has been edited following the provisions of Article 8(6) and Article 18 of Regulation (EC) No 1831/2003.
[15] Technical dossier/Section II.2.1.3.3.2.
[16] Technical dossier/Section II.2.2.2.1 and Annex II.57.1.
[17] Supplementary information (July 2012)/Section II.BA.1.4.1.2 and Annex II.46.
[18] Technical dossier/Section II.2.1.5.1 and II.2.1.5.2/Annexes II.44 to II.46.
[19] This section has been edited following the provisions of Article 8(6) and Article 18 of Regulation (EC) No 1831/2003.
[20] Supplementary information (July 2012)/Section II.BA.1.4.1.1.

introduced in the production strain, the genetic modification process and the genetic and phenotypic traits introduced.

2.4.1. Information relating to the production process

L-Threonine is produced by fermentation of the production strain. After the fermentation step, the fermentation broth is inactivated and the bacterial cells are removed. The L-threonine is concentrated, purified and crystallised.

Neither the production strain nor its recombinant DNA was detected in three samples of the final product.[21]

2.5. Stability

2.5.1. Shelf-life

The stability of the product L-threonine, technically pure, packed in punctured nylon polyethylene bags, has been tested in one batch at different environmental conditions (5 °C/60 % relative humidity (RH), 25 °C/30 % RH, 25 °C/60 % RH, 40 °C/30 % RH and 40 °C/60 % RH) for up to 24 months. There were no indications of significant changes in the concentrations of L-threonine during the 24 months of storage between samples from different time points and stored at any of the five environmental conditions applied.[22] The applicant submitted data on the stability of the L-threonine, technically pure, from two additional batches.[23] The shelf-life was measured throughout a nine-month period at 5, 25 and 40 °C and a fixed relative humidity of 60 %. There were no indications of significant changes in the concentrations of L-threonine during the nine months of storage between samples from different time points and stored at any of the three environmental conditions applied. The applicant proposes an expected shelf-life of the product L-threonine, technically pure, of three years for the 25-kg packs and of one year for big bags and for storage in silos.[24]

2.5.2. Stability in premixtures

Three batches of the additive were incorporated in a premixture for piglets containing minerals, vitamins (including choline chloride), enzymes and other amino acids and were stored in punctured polyethylene bags for six months (two batches) and nine months (one batch).[25] Storage conditions were 5, 25 and 40 °C and 60 % RH. No losses were observed at 5 and 25 °C; two batches showed losses at 40 °C (7 % after nine months in one batch and 8 % after six months in another batch).

2.5.3. Stability in compound feed

Three batches of the additive were incorporated in a grower pig feed. Pelleting resulted in a loss ≤ 5 % threonine. The pelleted samples were stored in punctured polyethylene bags for three months (two batches) and six months (one batch).[26] Storage conditions were 5, 25 and 40 °C and 60 % RH. No losses were observed at 5 and 25 °C, two batches showed a loss at 40 °C of 10 % after three months.

2.6. Homogeneity

Homogeneity of L-threonine, technically pure, was tested in 10 subsamples, each of a piglet premixture, a mash pig feed and a pellet pig feed. The coefficients of variation were 1.3, 2.8 and 3.1 %, respectively.[27]

[21] Supplementary information (July 2012)/Confidential information.
[22] Technical dossier/Section II/2.4.1.1.2.
[23] Supplementary information (July 2012)/Section II/Point III.2.
[24] Technical dossier/Section II/2.4.1.1.3.
[25] Technical dossier/Section II/2.4.1.2.1; Supplementary information (July 2012)/Section II/Point III.3.
[26] Technical dossier/Section II/2.4.1.2.2 ; Supplementary information (July 2012)/Section II/Point III.4.
[27] Technical dossier/Section II/2.4.2.1; Technical dossier/Section II/2.4.2.2.

2.7. Conditions of use

According to the applicant, L-threonine, technically pure, is intended to be used in all animal species without limitations in dose or time. It is proposed to be supplemented to feedingstuffs which are deficient in the essential amino acid L-threonine. The inclusion in compound feedingstuffs can be achieved by direct incorporation or via a premixture.

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

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

3. Safety

3.1. Safety aspects of the genetic modification[28]

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

3.2. Safety for the target species

Tolerance studies are not normally required for highly purified amino acids independent of the manufacturing process. This is the case for the additive under authorisation, which contains > 99 % threonine. Therefore, the FEEDAP Panel considers that safety concerns for target species are highly unlikely to arise from this fermentation additive.

Depending on the species, genetics, sex and physiological state of the non-ruminant animal, the requirements according to NRC (1994, 1998) for total threonine range between 0.35 and 1.0 % in the feed. Common feed materials contain 0.3–1.8 % threonine. Supplementation levels of compound feeds with threonine are in the range 0.05–0.15 %. Under EU conditions, L-threonine seems to be the second limiting amino acid after L-lysine (pigs) and third after sulphur amino acids and L-lysine (poultry).

A literature review of studies with overdoses of amino acids, including L-threonine, mostly in the rat, was provided.[29]

In the weanling rat, excessive intake of threonine reduced weight gain and food intake, depending on the protein content of the diet (Muramatsu et al., 1971; Sawar et al., 1995). In adult rats, excess dietary threonine has also been shown to slightly decrease growth and feed intake. High threonine intake (four times the normal level) from conception until the offspring were 90 days of age markedly decreased body weight at all ages (Castagné et al., 1995). The body weight of pups of the high-threonine group was reduced and did not reach the final body weight of other groups with lower threonine supply. In growing pigs fed a 20 % protein-containing diet with up to 4 % L-threonine (Edmonds and Baker, 1987a; Edmonds et al., 1987), body weight gain and feed intake were weakly reduced. In chickens for fattening fed a 23.1 % protein-containing diet with up to 4 % L-threonine, body weight gain and feed intake were reduced (Edmonds and Baker, 1987b). Edmonds and Baker (1987a, b) concluded from their studies with rats, pigs and chickens that rats are equally as sensitive to excess threonine as chickens are, with both species being more sensitive than the pig.

It is generally known that imbalances in amino acid intake will result in various biochemical adaptations, including reduced protein synthesis and eventually reduced body weight gain. The available data on excess threonine suggest that the effects on body weight derive from an imbalance and not from specific toxicity.

[28] This section has been edited following the provisions of Article 8(6) and Article 18 of Regulation (EC) No 1831/2003.
[29] Technical dossier/Secction III.3.1.1.2.

Correct dosing in formulating diets requires knowledge on the amino acid content in feed materials and the requirements of animals. These data are all freely accessible to feed compounders.

3.2.1. Conclusions on the safety for the target species

The FEEDAP Panel concludes that L-threonine technically pure, produced by *E. coli* (FERM BP 10942), is safe for all animal species. However, excess doses would create amino acid imbalances with negative consequences on animal performance.

3.3. Safety for the consumer

The product under assessment is produced by fermentation. The concerns for the consumer would not derive from the amino acid itself, which will be incorporated into protein, but for possible residues from the fermentation. In this case, the additive is highly purified and, therefore, following the provisions of the guidance on nutritional additives, it is considered of no concern for consumers.

In the particular case of L- threonine, this is confirmed by the lack of effects in the studies provided by the applicant with the additive under application:[30] (1) two bacterial reverse mutation tests (OECD 471) with *Salmonella* Typhimurium strains TA 1535, TA 1537, TA 98 and TA 100 and a tryptophan-requiring *Escherichia coli* strain WP2;[31] two gene mutation tests (in mouse lymphoma test) with L-threonine, technically pure, (TK locus (OECD 476),[32] Chinese hamster ovary cell assay test, chromosome aberration (mutation frequency of TK locus) (OECD 473)[33]); and a bone marrow *in vivo* micro-nucleus test (OECD 474)[34] in rats by oral administration with L-threonine, technically pure, all showing the lack of genotoxicity including mutagenicity; and (2) three acute oral studies (OECD 401)[35] and two sub-chronic toxicity studies (OECD 408)[36] with L-threonine, technically pure, in rats.

Further studies were performed with a different source of L-threonine—a reproduction and developmental study (Matsueda and Niiyama, 1982)[37] and a behavioural developmental study in the rat (Castagné et al., 1995)—which also showed no safety concerns.[38]

Absorption, distribution, metabolism and excretion are briefly described in Appendix B.

3.3.1. Conclusions regarding the safety for the consumer

L-Threonine will be incorporated in the protein of the body of the target animal and any potential excess will be metabolised. Since the composition of tissues and products of animal origin will not be changed by the use of L-threonine in animal nutrition, and considering the high purity of the product under assessment, no risks are expected for the consumer from the use of L-threonine, technically pure, as a feed additive.

3.4. Safety for the user

3.4.1. Effects on the respiratory system

The additive under assessment is a fine powder with a high dusting potential. Therefore, inhalation exposure of users is likely.

[30] Technical dossier/Section III.3.2.2.2.
[31] Technical dossier/Section III/Annexes III.27 and III.28.
[32] Technical dossier/Section III/Annexes III.29 and III.30.
[33] Technical dossier/Section III/Annex III.31.
[34] Technical dossier/Section III/Annex III.32.
[35] Technical dossier/Section III/Annexes III. 24 to III.26.
[36] Technical dossier/Section III/Annexes III.33 and III.34.
[37] Technical dossier/Section III/Annex III.35.
[38] Technical dossier/Section III/Annex III.10.

An acute inhalation test with the additive under application in rats, carried out according to OECD Guideline 403, showed no mortality and no adverse effects at the dose of 5.22 g/m^3 for male and female rats.[39]

3.4.2. Effects on the skin and eyes

An acute dermal irritation/corrosion study carried out in albino rabbits according to OECD Guideline 404 gave results which indicate that L-threonine, technically pure, will not be irritating to human skin.[40]

An acute eye irritation/corrosion study in albino rabbits, carried out according to OECD Guideline 405, gave results which indicate that L-threonine, technically pure, will not be irritating to human eyes.[41]

A maximisation study for sensitisation in guinea pigs, conducted according to OECD Guideline 406, provided no evidence that L-threonine, technically pure, is a skin sensitiser.[42]

3.4.3. Conclusions on the safety for the user

The product is not irritating to the skin or eyes and is not likely to be a skin sensitiser. Although there is a potential for user exposure by inhalation, there is no evidence of acute toxicity by the inhalation route.

3.5. Safety for the environment

Neither the production strain nor its recombinant DNA was detectable in the final product. The final product does not trigger an environmental safety concern associated with the genetic modification.

L-Threonine is a natural component of animals and plants. Its use in animal nutrition would not lead to any localised increase in concentration in the environment. The final product does not trigger an environmental safety concern associated with the genetic modification. It is concluded that the use of the product as a feed additive does not represent a risk to the environment.

4. Efficacy

Efficacy studies are not required for amino acids naturally occurring in plant or animal protein. The applicant provided technical bulletins on the use of threonine in pig and poultry nutrition, based on peer-reviewed articles.[43]

The nutritional role of threonine is well established in the scientific literature. Since most of the studies have been performed with supplemental L-threonine, the additive L-threonine, technically pure, is regarded as an effective source of threonine. Response in ruminants requires some degree of protection of threonine from ruminal degradation.

Data on requirements, allowances and recommendations for threonine in the different animal species and categories, and consequently for feed supplementation, are easily accessible in the standard literature for animal nutrition experts.

5. Post-market monitoring

The FEEDAP Panel considers that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation[44] and Good Manufacturing Practice.

[39] Technical dossier/Section III/Annex III.36.
[40] Technical dossier/Section III/Annex III.38.
[41] Technical dossier/Section III/Annex III.37.
[42] Technical dossier/Section III/Annex III.39.
[43] Technical dossier/Section IV/Annexes IV.1, IV.2, IV.3 and IV.4.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

Neither the production strain nor its recombinant DNA was detectable in the final product. The final product does not raise safety concerns with regard to the genetic modification.

L-Threonine, technically pure, is considered safe for the target animals when used to meet the requirements.

The toxicological studies, including mutagenicity tests, performed with the additive or other sources of L-threonine, did not reveal any toxic effect. Since the composition of tissues and products of animal origin will not be changed by the use of L-threonine in animal nutrition, and considering the high purity of the product under assessment, no risks are expected for the consumer from the use of L-threonine, technically pure, as a feed additive.

The product is not irritating to the skin or eyes and is not likely to be a skin sensitiser. Although there is a potential for user exposure by inhalation, there is no evidence of acute toxicity by the inhalation route.

The use of the product as a feed additive does not represent a risk to the environment.

L-Threonine, technically pure, is considered as an efficacious source of L-threonine for all animal species. Response in ruminants requires some degree of protection of threonine from ruminal degradation.

RECOMMENDATIONS

The description of the additive should contain the statement "produced by fermentation with *Escherichia coli* (FERM BP-10942)".

Considering the analytical data and to better standardise product quality, the FEEDAP Panel recommends that the specification for the minimum L-threonine content in the additive be set to 98.5 %.

DOCUMENTATION PROVIDED TO EFSA

1. Application for the renewal of authorisation of L-threonine, technically pure as nutritional additive. January 2012. Submitted by Ajinomoto Eurolysine SAS.

2. Application for the renewal of authorisation of L-threonine, technically pure as nutritional additive. Supplementary information. July 2012. Submitted by Ajinomoto Eurolysine SAS.

3. Application for the renewal of authorisation of L-threonine, technically pure as nutritional additive. Supplementary information. April 2013. Submitted by Ajinomoto Eurolysine SAS.

4. Evaluation report of the Community Reference Laboratory for Feed Additives on the methods(s) of analysis for L-threonine.

5. Comments from Member States received through the ScienceNet.

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APPENDICES

Appendix A. Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Methods of Analysis for L-threonine[45]

In the current application authorisation is sought for *L-Threonine* under Articles 4(1) and 10(2), category 'nutritional additives' and functional group 3(c) 'amino acids, their salts and analogues' according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of *L-Threonine* for all animal species and categories. The *feed additive* is intended to be mixed either in *premixtures* or added directly to complete *feedingstuffs* or *water*. The two Applicants (FAD-2010-0058 and FAD-2010-0081) suggested no minimum or maximum *L-Threonine* concentrations in *premixtures*, *feedingstuffs* and *water*.

For the determination of *L-Threonine* in *premixtures* and *feedingstuffs* the Applicants submitted the ring-trial validated Community method for amino acids. The method applies for the determination of *free* (synthetic and natural) and *total* (peptide-bound and free) amino acids, using an amino acid analyzer or High Performance Liquid Chromatography (HPLC) equipment. However, only performance characteristics for the determination of total *L-Threonine* are reported:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 1.9 to 4.1%;
- a relative standard deviation for *reproducibility* (RSD_R) ranging from 3.8 to 11.7%.

Based on the performance characteristics presented, the EURL recommends for official control, the ring-trial validated Community method based on ion exchange chromatography coupled with post-column derivatisation to determine *L-Threonine* in *premixtures* and *feedingstuffs*.

For the determination of the *active substance* in the *feed additive*, both Applicants submitted the abovementioned ring trial validated Community method designed for the analysis of *premixtures* and *feedingstuffs*. One Applicant (FAD-2010-0081) identified an alternative ring-trial validated method developed by the "Association of Official Agricultural Chemists" "Lysine, Methionine and *Threonine* in Technically pure Amino Acids and Premixes" (AOAC 999:13 - 2004). Additionally the EURL identified a ring-trial validated method developed by the "Association of German Agricultural Analytical and Research Institutes" (VDLUFA, Germany – Method 4.11.6). Both the methods are based on the same principle of the Community method (i.e. extraction of the sample with diluted hydrochloric acid and measuring the target analyte with HPLC coupled with post-column derivatisation system) and are explicitly designed to determine free *L-Threonine* in *feed additive* and *premixtures* at amino acid contents higher than 100 g/kg. The following performance characteristics covering both methods were reported:

- RSD_r ranging from 0.5 to 2.4%; and
- RSD_R ranging from 0.9 to 4.7%

Based on the performance characteristics presented, the EURL recommends for official control, the ring trial validated AOAC 999:13 and VDLUFA 4.11.6 methods based on ion exchange chromatography coupled with post-column derivatisation to determine the free *L-Threonine* in the *feed additive.*

[45] The full report is available on the EURL website: http://irmm.jrc.ec.europa.eu/SiteCollectionDocuments/FinRep-FAD-2010-0058+0081.pdf. The report was amended in June 2012: http://irmm.jrc.ec.europa.eu/SiteCollectionDocuments/amend-FinRep-FAD-2010-0058+0081.pdf

The Applicants provided no experimental data for the identification of *L-Threonine* in *water*. Therefore, the EURL cannot evaluate nor recommend a method for the official control to determine *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.

Appendix B. The metabolic fate of L-threonine[46]

Threonine released during enzymatic digestion is rapidly absorbed via different sodium-dependent and -independent transport systems in the brush border membrane of the small intestine (Munck and Munck, 1994; Massey et al., 1998). In pigs, the apparent ileal digestibility of threonine in feed ingredients varies: about 63 % for cottonseed and linseed meal, 59–72 % for cereals and 77–90 % for soybean meal/soy isolate, milk/whey and fish meals (NRC, 1998). Apparent digestibility of crystalline L-threonine is about 100 %, as for most crystalline amino acids (e.g. Izquierdo et al., 1988; Chung and Baker, 1992). However, microbial degradation in the small intestine may negatively affect its bioavailability (Dierick et al., 1986; Van der Meulen et al., 1998; Bikker et al., 2006; Dai et al., 2012). Once absorbed, L-threonine is used in the body for tissue protein synthesis; mucin production in the gut; collagen, elastin and tooth enamel formation; and as a precursor for glycine and for propionic acid. L-Threonine is both glucogenic and ketogenic: it has two catabolic pathways and one is closely linked to that of glycine and L-serine (Rodwell, 1990; Voet and Voet, 1995). L-Threonine may be catabolised by threonine dehydratase (TDH) in the cytosol to NH_4^+ and 2-ketobutyrate, which is rapidly and irreversibly converted to CO_2, or it may be metabolised by threonine dehydrogenase (TDG) in the mitochondria to form 2-amino-3-ketobutyrate, which is then cleaved by 2-amino-ketobutyrate CoA ligase to form glycine and acetyl-CoA (Dale, 1978; Bird and Nunn, 1983). The TDG pathway has been shown to account for 80 % of threonine disposal in growing pigs (Ballevre et al., 1990; le Floc'h et al., 1994) and rats (Bird and Nunn 1983; Moundras et al., 1992), making it the major degradative pathway, but it accounts for only 7–10 % of threonine disposal in humans (Darling et al., 2000). L-Threonine is among the amino acids most rapidly degraded by ruminal microorganisms, with an estimated half-life in the rumen of 1.1 hours (Chalupa, 1976). Therefore, little if any dietary L-threonine provided to ruminants would be expected to reach the abomasum intact and be absorbed. For a detailed scheme of threonine metabolic pathway see Bird and Nunn (1983) and Darling et al. (1999).

Threonine is catabolised in the cytosol by threonine dehydratase to yield 2-ketobutyric acid. Ketobutyric acid can be transaminated to 2-aminobutyric acid by alanine aminotransferase or further degraded by branched-chain α-keto acid dehydrogenase (BCKDH) or pyruvate dehydrogenase (PDH) to propionyl coenzyme A (CoA) in the mitochondria. Threonine is also catabolised in the mitochondria by threonine dehydrogenase to 2-amino-3-ketobutyric acid which can be further degraded mainly to glycine and acetyl CoA in vivo. Cofactors include pyridoxal (vitamin B6, or nicotinamide adenine dinucleontide) and co-enzyme A. * Denotes the fate of the labelled carbon of L-(1–^{13}C)threonine through the degradative pathways (Bird and Nunn, 1983; Darling et al., 1999).

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