European Food Safety Authority

EFSA Journal 2015;13(9):4236

SCIENTIFIC OPINION

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[1]

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

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

This opinion concerns L-threonine as a feed additive produced by four different strains derived from *Escherichia coli* K-12. Three strains are genetically modified (GM): NRRL B-30843, KCCM11133P and DSM 26131. L-Threonine produced by *E. coli* DSM 26131 could not be assessed because of the insufficient molecular characterisation of the genetic modification, and the lack of data on both the absence of the production strain and its recombinant DNA from the final product. No safety concerns were found in the products related to the genetic modification of the other GM strains or to antibiotic resistance of the producer strains. L-Threonine products made by fermentation using *E. coli* strains NRRL B-30843, KCCM11133P and DSM 25085 are free of the production strain and have a high purity (≥ 98.8 %). L-Threonine, technically pure, produced by *E. coli* strains NRRL B-30843, KCCM11133P and DSM 25085 is safe for the target animals when used in appropriate amounts to supplement threonine-deficient feeds, for the consumer of animal products and for the environment. The FEEDAP Panel considers that L-threonine produced by *E. coli* strains NRRL B-30843, KCCM11133P or DSM 25085 is not an irritant to eyes and skin, and is not a skin sensitiser. There is no risk from inhalation of L-threonine, but concerns may arise from the content of endotoxins in the products. These L-threonine products are 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. The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) has concerns regarding the safety of the simultaneous oral administration of L-threonine via water for drinking and feed.

KEY WORDS

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

[1] On request from the European Commission. Questions No EFSA-Q-2012-00113, adopted on 9 September 2015.

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[3] Acknowledgement: The Panel wishes to thank the members of the Working Group on Amino Acids, including Paul Brantom, Lucio Costa, Noël Dierick, Lubomir Leng and Giovanna Martelli, and the members of the Working Group on Genetically Modified Microorganisms of FEEDAP, including Pier Sandro Cocconcelli, Boet Glandorf, Lieve Herman Sirpa Kärenlampi and Christoph Tebbe, for the preparatory work on this scientific opinion.

Suggested citation: 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-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. doi:10.2903/j.efsa.2015.4236

Available online: www.efsa.europa.eu/efsajournal

SUMMARY

Following a request from the European Commission, the European Food Safety Authority (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP Panel) was asked to deliver a scientific opinion on L-threonine produced by fermentation with genetically modified (GM) strains of *Escherichia coli* K-12 (NRRL B-30843, DSM 26131, KCCM11133P) or with a non-GM *E. coli* strain (DSM 25085) for all animal species.

L-Threonine is an essential amino acid for all animal species. It is commonly considered the second and third limiting amino acid in cereal-based diets for pigs and poultry, respectively. It is widely used in the feed industry to optimise dietary protein.

Three of the strains are GM: *E. coli* NRRL B-30843, *E. coli* DSM 26131 and *E. coli* KCCM11133P. The final product obtained from *E. coli* strain NRRL B-30843 contains no cultivable production organisms or recombinant DNA. The product from *E. coli* strain KCCM11133P contains no cultivable production organisms, but the possible presence of fragments spanning the full length of recombinant genes cannot be ruled out. However, no genes of concern remain in the production strain KCCM11133P. No safety concerns were found in these two products related to the genetic modification. The genetic modification of *E. coli* DSM 26131 and its possible consequences for the product could not be assessed because of insufficient data.

L-Threonine products made by fermentation with *E. coli* strains NRRL B-30843, KCCM11133P or DSM 25085 are free of the production strain and have a high purity (≥ 98.8 %). No recombinant DNA could be detected in L-threonine produced by fermentation of the GM strains *E. coli* NRRL B-30843 and KCCM11133P.

L-Threonine, technically pure, produced by *E. coli* NRRL B-30843, KCCM11133P or DSM 25085 strains is safe for the target animals when used in appropriate amounts to supplement feed to compensate for threonine deficiency in feedingstuffs. However, the FEEDAP Panel has concerns regarding the safety of the simultaneous oral administration of L-threonine via water for drinking and feed.

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 products made by *E. coli* strains NRRL B-30843, KCCM11133P and DSM 25085, no risks are expected for the consumer from their use as a feed additive.

The FEEDAP Panel considers that L-threonine produced by *E. coli* strains NRRL B-30843, KCCM11133P or DSM 25085 is not an irritant to eyes and skin, and is not a skin sensitiser. There is no risk from inhalation of L-threonine but concerns may arise from the content of endotoxins in the products.

The use of the product L-threonine produced by *E. coli* strains NRRL B-30843, KCCM11133P or DSM 25085 in animal nutrition does not pose a risk to the environment.

The L-threonine products are 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.

L-Threonine produced by the genetically modified microorganism (GMM) *E. coli* DSM 26131 could not be assessed because of the insufficient molecular characterisation of the genetic modification, and the lack of data on both the absence of the production strain and its recombinant DNA from the final product. In particular, the EFSA FEEDAP Panel could not conclude on the safety of this product for target animals, and on the safety concerning consumers, users and the environment.

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BACKGROUND

Regulation (EC) No 1831/2003[4] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7. 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 a time limit or pursuant to Directive 82/471/EEC.

The European Commission received a request from the consortium AMAC/EEIG – Amino Acids Authorisation Consortium[5] for authorisation and re-evaluation of the product L-threonine as a feed additive for all animal species (category: nutritional additives; 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 4(1) (authorisation of a feed additive or new use of a feed additive), and under Article 10(2) (re-evaluation of an authorised feed additive). EFSA received directly from the applicant the technical dossier in support of this application.[6] According to Article 8 of that Regulation, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. The particulars and documents in support of the application were considered valid by EFSA as of 27 April 2012.[7]

The additive L-threonine is an amino acid produced by fermentation with genetically modified strains of *Escherichia coli* K-12 (NRRL B-30843, DSM 26131, KCCM11133P) or with a non-GM *E. coli* strain (DSM 25085). L-Threonine is permanently authorised as feed nutritional additive for use in all animal species.[8]

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.[9] 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.[10] 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 a pharmacologically active substance in veterinary medicinal products and is not a subject to maximum residue levels when used in food-producing animals.[11] L-Threonine is also registered as an

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

[5] On 19/12/2012, the rights of AMAC/EEIG were transferred to FEFANA asbl, Avenue Louise 130A, Box 1, 1050 Brussels, Belgium). Companies: ADM Specialty Ingredients (Europe) B.V.; Chr. Olesen Group representing Zhejiang Guoguang Biochemistry Co. Ltd.; CJ Europe GmbH.; and Evonik Degussa GmbH.

[6] EFSA Dossier reference: FAD-2010-0058.

[7] A new mandate was received in EFSA on 12/01/2012.

[8] 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.

[9] Commission Directive 2006/141/EC on infant formulae and follow-on formulae, OJ L 401, 30.12.2006, p. 1–33.

[10] 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. 19–25.

[11] Commission Regulation (EC) No 1931/1999, amending Annexes I, II and III of Council Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. OJ L 240, 10.09.1999, p. 3–10.

ingredient in cosmetic products as antistatic, hair conditioning, hair waving or straightening (Commission decision 2006/257/EEC).

The Scientific Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) issued five opinions on the safety and efficacy of L-threonine for all animal species, produced by different genetically modified strains of *Escherichia coli* (EFSA FEEDAP Panel, 2013, 2014a, b, c, d).

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, 2008a, 2010).

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 animal(s), consumer, user and the environment and the efficacy of the product L-threonine produced by fermentation using *Escherichia coli* (NRRL B-30843, DSM 26131, KCCM11133P or DSM 25085), 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
Registration number/EC No/No (if appropriate)	3.3.1.
Category of additive	3. Nutritional additives
Functional group(s) of additive	c. Amino Acids, their salts and analogues

Description			
Composition, description	Chemical formula	Purity criteria (if appropriate)	Method of analysis (if appropriate)
L-Threonine	$C_4H_9NO_3$	minimum 98%	Regulation (EC) No 152/2009

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

Conditions of use (*See appendix for details*)				
Species or category of animal	Maximum Age	Minimum content	Maximum content	Withdrawal period (if appropriate)
		mg/kg of complete feedingstuffs, supplementary feed (based on end feed) and in water		
All animal species and categories	-	-	-	-
Other provisions and additional requirements for the labelling				
Specific conditions or restrictions for use (if appropriate)	Not applicable			
Specific conditions or restrictions for handling (if appropriate)	Please refer to MSDS			
Post market monitoring (if appropriate)	Not applicable			
Specific conditions for use in complementary feedingstuffs or water (if appropriate)	Not applicable			

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

ASSESSMENT

1. Introduction[12]

L-Threonine is an essential amino acid for humans and animals. L-Threonine is currently authorised without a time limit under Council Directive 70/524/EEC for use in all species as a nutritional additive. No maximum levels of L-threonine in feeds are established in the European Union (EU). The applicant, a consortium of four companies, asks for the re-evaluation of the use of L-threonine, produced by a microbiological fermentation process using genetically modified (GM) or non-GM strains derived from *Escherichia coli* K-12, as a feed additive (nutritional additives, functional group amino acids and their salts and analogues) for all animal species and categories and a new use of this feed additive (use in water).

The application contains data from four sources of L-threonine obtained by fermentation using GM strains of *E. coli* (NRRL B-30843, DSM 26131, KCCM 11133P) or a non-GM *E. coli* strain (DSM 25085).[13]

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. Under EU conditions, L-threonine is usually the second limiting amino acid, after L-lysine, in the diet of pigs and the third, after sulphur amino acids and L-lysine, for poultry.

2. L-Threonine produced by *E. coli* DSM 25085

2.1. Characterisation

2.1.1. Characterisation of the active substance/additive

L-Threonine (International Union of Pure and Applied Chemistry (IUPAC) 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 (CAS) No 72-19-5, and the European Inventory of Existing Commercial chemical Substances (EINECS) No 200-774-1, has a molecular weight of 119.12 Da, and the molecular formula of L-threonine is $C_4H_9NO_3$. The structural formula is given in Figure 1.

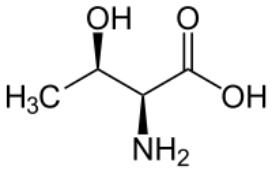

Figure 1: Molecular structure of L-threonine.

The specifications of the product allows a minimum of 98 % L-threonine, a maximum of 1.5 % water and a maximum of 0.5 % ash.[14]

The analysis of five batches of threonine (EU official method of Regulation 152/2009)[15] showed an average content of threonine of 99.9 % 'as is' (range 99.5 to 100.4 %), water 0.08 % (range from 0.05

[12] This section has been amended following the confidentiality claims made by the applicant.
[13] Technical dossier/Supplementary information July 2012 and supplementary information October 2013/Conf/Annex Qxxii.
[14] Technical dossier/Section II.1.3.
[15] Technical dossier/Supplementary information July 2012/Annexes June 2012/Annex Qii. Supplementary information October 2014/Annexes/Annex Qi.

to 0.1 %) and L-cystine 0.1 % (range from 0.05 to 0.15 %).[16] On a dry matter basis, the amount of unidentified material was < 1 %.

The specific optical rotation of five batches of the final product was analysed. In all cases the obtained values were within the range given by the European Pharmacopoeia (range from –27.6 to –29.0 °)[17] and confirms the identity of the L-enantiomer.[18]

2.1.2. Impurities[19]

Historical data (2009 to 2013, number of batches analysed not reported) were provided on heavy metals and arsenic, which were below the limit of detection (LOD).[20] Dioxin concentrations during 2006–2013 (number of batches analysed not reported) ranged from < 0.078 to <0.5 ng World Health Organization polychlorinated dibenzodioxin/dibenzofuran toxic equivalents (WHO-PCDD/F-TEQ/kg).[21]

The microbial contamination of three batches was analysed: *Salmonella* (in 25 g) and *E. coli* (in 1 g) were absent; anaerobic spore formers, Enterobacteriaceae, yeasts and filamentous fungi were <10 colony-forming units (CFU)/g; total viable count (30 °C) and aerobic spore formers reached values of up to 90 CFU/g in all three cases. These three batches were analysed for mycotoxins (aflatoxins B1, B2, G1, G2, ochratoxin A) and in all cases the concentrations were below the LOD.[22]

The detected amounts of the aforementioned contaminants/impurities were of no concern and often below the detection limits.

The antimicrobial activity of one batch of the final product was tested against a reference panel of bacteria (*E. coli* American Culture Type Collection (ATCC) 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 and *Bacillus subtilis* ATCC 6633 (EFSA, 2008)). The minimal inhibitory concentration was >8 g/L.[23] No evidence of antimicrobial activity was detected at concentrations up to eight times that recommended for supplementation of feeds.

No evidence was provided on the resistance of the producer strain to antibiotics used in human and veterinary medicine. Nevertheless, a report based on polymerase chain reaction (PCR) targeting a gene involved in threonine biosynthesis was provided that indicated the absence of DNA from the producer strain in the final product (three batches).[24]

Bacterial endotoxin activity was measured in one batch of the final product (European Pharmacopoeia EP 2.6.14 method) and was 0.34 international unit (IU)/mg.[25]

2.1.3. Physical properties

The dossier contained generic (non-strain-specific) information on the physical properties of L-threonine: it is a white crystalline powder with a bulk density of 585–715 kg/m^3 and a melting point

[16] Technical dossier/Supplementary information October 2014/Annexes/Annex Qi.
[17] European Pharmacopoeia monograph 1/2015:1272.
[18] Technical dossier/Supplementary information October 2014/Annexes/Annex Qii optical rotation.
[19] This section has been amended following the confidentiality claims made by the applicant.
[20] Technical dossier/Supplementary information October 2014/Annexes/Annex Qiii impurities. LOD of lead, cadmium and mercury 0.1 mg/kg; of arsenic 0.05 mg/kg. LOD of dioxins 0.01 ng WHO-PCDD/F-TEQ/kg.
[21] Technical dossier/Supplementary information October 2014/Annexes/Annex Qiii impurities heavy metals and Supplementary information July 2012/Annexes June 2012/Annex Qiii.
[22] Technical dossier/Supplementary information October 2014/Annexes/Annex Qiii impurities_microb. LOD of aflatoxins B1, B2, G1 and G2 0.08 µg/kg; LOD of ochratoxin A 0.1 µg/kg.
[23] Technical dossier/Supplementary information October 2014/Annexes/Annex Qv MIA.
[24] Technical dossier/Supplementary information October 2014/Conf 080814/Analysis Thre absence DNA.
[25] Technical dossier/Supplementary information March 2015/Annexes Qi.

of 256 °C. The isoelectric point of L-threonine is pH 6.2. It is readily soluble in water (86–90 g/L water at 20 °C).[26] The pK_a is 2.7–9.6.

Particle size distribution was carried out by sieve analysis. The particle fraction below 100 µm was 40 % and that below 50 µm 15 %. No information was provided regarding the respirable fraction (< 10 µm).[27]

No data were provided on dusting potential despite it having been requested.

2.1.4. Characterisation of the production organism *E. coli* DSM 25085[28]

The *E. coli* production strain has been deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ) with the accession number DSM 25085.[29]

The dossier contains information on the strain *E. coli* DSM 25085 (an *E. coli* K-12 derivative) that allows to conclude that it does not represent safety concerns when used as production strain of the product under assessment. [30]

Although the parent strain *E. coli* K-12 is not included on the European Food Safety Authority (EFSA) qualified presumption of safety (QPS) list (EFSA BIOHAZ Panel, 2013), it is not considered a human or animal pathogen, it has a long history of apparent safe use in industrial production and is scientifically recognised as a safe bacterial strain not producing toxic substances (US Environmental Protection Agency, 1997; http://epa.gov/biotech_rule/pubs/fra/fra004.htm; Gorbach, 1978). *E. coli* K-12 has been used widely as a model organism in research in microbial genetics and physiology, and has widespread use in industrial applications. *E. coli* K-12 is one of the most extensively studied of all microorganisms. Its genome sequence was published in 1997 (Blattner et al., 1997) and confirmed the absence of toxigenic potential. It does not appear in the most comprehensive review of pathogenic *E. coli* published by Nataro and Kaper (1998). Indeed, strain K-12 is commonly used as a 'base-model' (safety reference strain) against which the safety of other *E. coli* strains is assessed, (see Kaper et al. (2004)). In support of this, the applicant provided evidence of the absence of virulence factors.[31]

2.1.5. Manufacturing process[32]

General information was provided on the production process. L-Threonine is produced by fed-batch fermentation of the production strain using various sources of carbohydrates, of nitrogen, salts and vitamins. At the end of the fermentation process, the biomass is heat inactivated and separated from the broth. The L-threonine containing solution is evaporated and subsequently crystallisation takes place prior to solid–liquid separation of the product crystals and drying. The material safety data sheet (MSDS) of the product under application was available. [33]

No viable cells of the production strain were present in three batches of the final product.[34]

The producer declared that no antimicrobial substances (including antibiotics) are used in the production process.[35]

[26] Technical dossier/Section II.2.2.
[27] Technical dossier/Section II.1.5.
[28] This section has been amended following the confidentiality claims made by the applicant.
[29] Technical dossier/Supplementary information February 2012/Conf_030212/Safe Deposit.
[30] Technical dossier/Supplementary information October 2013/Conf/Annex Qxxii and Supplementary information May 2015/Annex Qi.
[31] Technical dossier/Supplementary information October 2014/Conf/Annex analysis Thre absence virulence and Annex analysis Thre absence DNA.
[32] This section has been amended following the confidentiality claims made by the applicant.
[33] Technical dossier/Section II/Section II.3 and Annex II.3.3.
[34] Technical dossier/Supplementary information October 2014/Annex Qiii impurities viable cells.
[35] Technical dossier/Supplementary information October 2014 /Annex Qi antimicrobial.

2.1.6. Stability and homogeneity

2.1.6.1. Shelf life

One batch of the additive was stored at 10–25 °C, 20–75 % relative humidity (RH) (packaging not described), for two years. No losses of threonine were detected. Similarly, no losses were observed after storing another batch at 85, 60 and 45 °C for three days, three weeks and three months, respectively.[36]

2.1.6.2. Stability in premixtures

No losses of threonine were detected when L-threonine was included at 5 % in a vitamin/mineral premixture (containing choline chloride) and stored at 10–25 °C, 20–75 % RH (packaging not described), for six months.[37]

2.1.6.3. Stability in feedingstuffs

A complete feed for chickens for fattening, mainly consisting of wheat, soybean meal and maize, was supplemented with 0.1 % L-threonine (one batch), conditioned (65 °C), pelleted (95 °C), packaged (not described) and stored for three months under ambient conditions. No losses of threonine due to feed processing or storage were observed. [38]

2.1.6.4. Stability in water

L-Threonine (three batches) was added at concentrations of 0.5, 1, 5 and 10 g/L water for drinking and stored for three days at 20–27 °C. No losses of threonine were detected; however, the solutions became turbid after two days.[39]

2.1.6.5. Homogeneity

The ability to homogeneously distribute in a premixture and a complete feed was tested in one batch of the product. Ten subsamples each of a vitamin/mineral premixture (inclusion rate 5 %) and a complete feed for chickens for fattening (inclusion rate 0.1 %) were analysed for threonine. The coefficients of variation (CV) were 2.2 and 2.7 %, respectively.[40]

2.1.7. Physico-chemical incompatibilities in feed

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

2.1.8. Conditions of use

L-Threonine, technically pure, is proposed to be used in feeds in order to achieve the adequate amino acid profile and meet the requirements on L-threonine for all animal species. It can be added directly to the feedingstuffs/complementary feedingstuffs or via premixture. No inclusion levels are proposed as the requirements in quantitative terms depend on the species, the physiological state of the animal, the performance level and the environmental conditions, as well as the amino acid composition of the unsupplemented diet.

The L-threonine produced by *E. coli* DSM 25085 is proposed to be used also in water for drinking.

[36] Technical dossier/Supplementary information July 2012/Annexes June 2012/Annexes Qv.
[37] Technical dossier/Supplementary information July 2012/Annexes June 2012/Annexes Qvi.
[38] Technical dossier/Supplementary information July 2012/Annexes June 2012/Annexes Qvii.
[39] Technical dossier/Supplementary information July 2012/Annexes June 2012/Annex Qx.
[40] Technical dossier/Supplementary information July 2012/Annexes June 2012/Annexes Qxi L Thr homogeneity mixing profile premix and Qxi L Thr homogeneity mixing profile feed A.

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

EFSA has verified the EURL report as it relates to the methods used for the control of the L-threonine in animal feed. The Executive Summary of the EURL report can be found in Appendix A.[41]

2.2. Safety

2.2.1. 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 product under authorisation, which contains ≥ 99.6 % threonine and < 1 % unidentified material on a dry matter basis. The level of endotoxins in this product (< 1 IU/mg) is negligible in comparison with that observed in other feedingstuffs (1 000 IU/mg; Cort et al., 1990) and is therefore of no concern for the target species. Therefore, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP Panel) considers that the safety concerns for the target species are highly unlikely to arise from this product.

The tolerance of the target species to L-threonine and their requirements for this amino acid have already been described in a previous FEEDAP Panel opinion (EFSA FEEDAP Panel, 2014a). In this previous opinion, the FEEDAP Panel concluded that the use of the amino acid L-threonine in supplementing feed to compensate for threonine deficiency in feedingstuffs is safe for the target animals. The margin of safety is higher in pigs than in poultry. However, excess doses would create amino acid imbalances, with negative consequences on animal performance and increased nitrogen excretion. The consequences of using L-threonine as a feed additive in ruminants were revised in a previous opinion (EFSA FEEDAP Panel, 2014c). The FEEDAP Panel, in line with its previous statement (EFSA FEEDAP Panel, 2010), recommends that amino acids, their salts and analogues should generally not be used in water for drinking because of the risk of imbalances and for hygiene reasons.

2.2.1.1. Conclusions on the safety for the target species

The use of L-threonine produced by *E. coli* DSM 25085 in supplementing feed to compensate for threonine deficiency in feedingstuffs is safe for the target animals. However, excess doses would create amino acid imbalances with negative consequences on animal performance. The FEEDAP Panel has concerns on the safety of the simultaneous oral administration of L-threonine via water for drinking and feed.

2.2.2. Safety for the consumer

Absorption, distribution, metabolism and excretion of the amino acid L-threonine are described in a previous opinion (EFSA FEEDAP Panel, 2013).

As a general principle, conventional toxicology studies are considered to be inappropriate for amino acids. Dietary intakes of amino acids that lead to amounts significantly lower or greater than that which is optimum for health and performance will inevitably cause a physiological imbalance and, consequently, adverse effects.

The product under assessment is produced by fermentation. Concerns for the consumer would derive not from the amino acid itself, which will be incorporated into protein, but from possible residues from the fermentation. In this case, the product originating from *E. coli* DSM 25085 is highly purified (≥ 99.6 % threonine and < 1 % unidentified material on a dry matter basis). Therefore, the FEEDAP Panel concludes that this product is considered of no safety concern for consumers.

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

2.2.2.1. Conclusions on safety for the consumer

The FEEDAP Panel concludes that the use of L-threonine produced by *E. coli* DSM 25085 in animal nutrition is safe for consumers.

2.2.3. Safety for the user

2.2.3.1. Effects on eyes, skin and the respiratory tract

The applicant provided the results of an acute inhalation toxicity study, skin and eye irritation test and a dermal sensitisation test with two L-threonine products manufactured using two strains of *E. coli*, which were different from the strain used for the product under application, but also derived from *E. coli* K-12.[42] Those tests were already assessed in a previous opinion (EFSA FEEDAP Panel, 2014a) and showed no evidence of specific concerns for user safety.

Since the product under application is similar to and has higher purity than the products tested, and that all strains share *E. coli* K-12 as a common lineage, the FEEDAP Panel considers that these data are relevant also to the product under assessment. The Panel concludes that there is no evidence of specific concerns for user safety (inhalation toxicity, skin and eyes irritation and dermal sensitisation) for the product produced by *E. coli* DSM 25085.

2.2.3.2. Inhalation exposure to endotoxin

The production species, *E. coli*, is a Gram-negative bacterium. Although the K-12 strain and its derivatives are safe from the point of view of enterotoxins and other virulence factors (Gorbach, 1978; EPA, 1997; Bauer et al., 2007), *E. coli* K-12 retains lipopolysaccharide in its cell envelope (Luchi and Morrison, 2000; Svensson et al., 2005; Gao et al., 2006), which potentially may result in endotoxin activity in the final product. The user can suffer from occupational respiratory disease depending on the level of endotoxins in the air and dust (Rylander, 1999; Thorn, 2001). The bacterial endotoxin activity (one batch analysed) was 0.34 IU/mg. No dusting potential is available for this specific product. The applicant instead provided the dusting potential of another product (produced by *E. coli* DMS 26131; section 5.1.3), considered the worse-case scenario for the L-threonine products of this application, and the measured value was 7.4 g/m^3.[43]

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, 2012), is described in Appendix B. The threshold for the quantity of inhaled endotoxins per working day is 900 IU derived from provisional occupational exposure limits given by the Dutch Expert Committee on Occupational Safety (DECOS) (Health Council of the Netherlands, 2010) and the UK Health and Safety Executive (HSE, 2013). Based upon the calculation of the content of endotoxin in dust and the dusting potential of the product from *E. coli* DMS 26131, exposure would be 1 409 IU per eight-hour working day, indicating a risk of inhalation exposure to endotoxins for people handling the additive.

2.2.3.3. Conclusions on safety for the user

The FEEDAP Panel considers that L-threonine produced by *E. coli* DSM 25085 is not an irritant to eyes and skin, and is not a skin sensitiser. There is no risk from inhalation of L-threonine but concerns may arise from the content of endotoxins in the product.

2.2.4. Safety for the environment

The amino acid L-threonine is a physiological and natural component of proteins of living organisms. When consumed, most will be absorbed, and any non-absorbed fraction will be incorporated in the intestinal microbial mass and excreted as such. The excess of absorbed L-threonine will be excreted as

[42] Technical dossier/Supplementary information August 2014/Conf/Response letter conf info.
[43] Technical dossier/Supplementary information June 2012/Answer to Qiv and Annex Qiv L-threonine PSD and dusting potential.

urea/uric acid and as carbon dioxide. The use of the additive in water for drinking, when given in addition to complete diets with a well-balanced amino acid profile, would disturb the nitrogen balance and increase nitrogen excretion via urine.

The amino acid L-threonine itself does not pose any risk for the environment. It is concluded that the use of the product L-threonine, technically pure, produced by *E. coli* DSM 25085, as a feed additive does not represent a risk to the environment.

2.3. Efficacy

Efficacy studies are not required for amino acids naturally occurring in proteins of plants and animals. 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 is regarded as an effective source of threonine.

The efficacy of the amino acid L-threonine is briefly described in a previous opinion (EFSA FEEDAP Panel, 2014a). L-Threonine is efficacious as a supplemented amino acid to maintain or restore the adequate balance of dietary amino acids for all animal species. For the supplemental L-threonine to be as efficacious in ruminants as in non-ruminant species, it requires protection against degradation in the rumen.

2.4. Post-marketing 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.

2.5. Conclusions and recommendations on L-threonine produced by *E. coli* DSM 25085

2.5.1. Conclusions

L-Threonine, technically pure, produced by *E. coli* DSM 25085 is safe for the target animals when used in appropriate amounts to supplement feed to compensate for threonine deficiency in feedingstuffs. The FEEDAP Panel has concerns on the safety of the simultaneous oral administration of L-threonine via water for drinking and feed.

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 made by *E. coli* DSM 25085, no risks are expected for the consumer from its use as a feed additive.

The FEEDAP Panel considers that L-threonine produced by *E. coli* DSM 25085 is not an irritant to eyes and skin, and is not a skin sensitiser. There is no risk from inhalation of L-threonine but concerns may arise from the content of endotoxins in the product.

The use of the product L-threonine produced by *E. coli* DSM 25085 in animal nutrition does not pose a risk to the environment.

The product, L-threonine, 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.

2.5.2. Recommendations

The description of the additive should contain the statement ‘produced by fermentation with *Escherichia coli* DSM 25085’.

[44] OJ L 35, 8.2.2005, p. 1.

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 additives under application be set to 98.5 %.

3. L-Threonine produced by *E. coli* NRRL B-30843

3.1. Characterisation

3.1.1. Characterisation of the active substance/additive

The characterisation of the active substance/additive has been described previously (see section 2.1.1).

The specifications of the product allows a minimum of 98 % L-threonine, a maximum of 1.5 % water and a maximum of 0.5 % ash.[45]

Five batches were analysed (method ISO 13903) and the average threonine content was 99.7 % on a 'as is' basis (ranging from 98.4 to 100.5 %). Water ranged from 0.2 to 0.4 % and ash ranged from 0.04 to 0.08 %.[46] The amount of unidentified material was < 1 % on a dry matter basis.

The specific optical rotation of five batches of the final product was analysed. In all cases the obtained values were within the range given by the European Pharmacopoeia (range –27.6 to –29.0 °)[47] and confirmed the identity of the L-enantiomer.[48]

3.1.2. Impurities

Heavy metals (cadmium, lead and mercury), as well as arsenic, were analysed in three batches and these elements were undetectable. One batch was analysed for microbiological impurities: *Salmonella* was not detected in 100 g of the product, *E. coli* was < 3 CFU/g and *Enterobacteriaceae*, yeasts and filamentous fungi were < 10 CFU/g. Aflatoxin B1 and ochratoxin A were below the LOD in another batch of the final product.[49]

The detected amounts of the aforementioned contaminants/impurities were of no concern and, in fact, levels of contaminants/impurities were often below the detection limits.

The antimicrobial activity of the product (one batch) was tested against a reference panel of bacteria (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212 and *B. subtilis* ATCC 6633 (EFSA, 2008b). The minimal inhibitory concentration was > 8 g/L.[50] No evidence of antimicrobial activity was detected at concentrations up to eight times that recommended for supplementation of feeds.

No evidence was provided on the resistance of the producer strains to antibiotics used in human and veterinary medicine. However, the demonstrated absence of producer bacterial DNA in the final product (see section 3.1.5) provides assurance that any transmissible resistance genes would not be transferred intact to the product.

[45] Technical dossier/Section II.1.3.
[46] Technical dossier/Supplementary information October 2014/Annexes/Annex Qi.
[47] European Pharmacopoeia monograph 1/2015:1272.
[48] Technical dossier/Supplementary information October 2014/Annexes/Annex Qii optical rotation.
[49] Technical dossier/Supplementary information October2014/Annexes/Annexes Qiii Impurities heavy metals, microb, mycotox. LOD of (in mg/kg) of arsenic and cadmium was 0.01, of lead 0.1, and of mercury 0.001. LOD of aflatoxin B1 and ochratoxin A was 1 µg/kg.
[50] Technical dossier/Supplementary information October 2014/Annexes/Annexes Qv MIA.

Bacterial endotoxin activities were measured in three batches of the final product by the United States Pharmacopeia (USP) method and the detected bacterial endotoxin activity ranged from 1 to 54 IU/mg.[51]

3.1.3. Physical properties

General information on physical properties has been described above (section 2.1.3).

Sieve analysis indicated that the particle fraction below 100 µm was 1.2 % and that below 50 µm was 0.4 %. No information was provided on the respirable fraction (< 10 µm).[52]

No data were provided on dusting potential.

3.1.4. Characterisation of the production organism *E. coli* NRRL B-30843[53]

The *E. coli* production strain has been deposited in the NRRL culture collection of the United States Department of Agriculture (USDA) with the accession number NRRL B-30843.[54]

The recipient strain is a derivative of *E. coli* K-12. *E. coli* K-12 is a well characterised Gram-negative bacterium and its safety (non-pathogenicity) has been reviewed extensively (section 2.1.4). The technical dossier contains detailed and sufficient information on the recipient microorganisms including safety aspects, the origin and function of the different genetic elements introduced in the production strain, the genetic modification process and the genetic and phenotypic traits introduced.[55]

3.1.5. Manufacturing process[56]

General information on the manufacturing process has been described above (section 2.1.5).

Material safety data sheets of the substances used in the fermentation media were provided.[57]

The applicant declared that no antimicrobial substances (including antibiotics) were used in the production process.[58]

Neither the production strain nor its recombinant DNA was detected in the final product (three and six batches analysed, respectively). [59]

3.1.6. Stability and homogeneity

No specific data on the shelf life of this product, or its stability in premixtures, feedingstuffs or water, or its mixing properties were available despite the information having been requested.

3.1.7. Physico-chemical incompatibilities in feed

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

3.1.8. Conditions of use

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

[51] Technical dossier/Supplementary information March 2015/Annexes Qi.
[52] Technical dossier/Section II.1.5.
[53] This section has been amended following the confidentiality claims made by the applicant.
[54] Technical dossier/Supplementary information February 2012/Conf_030212/Annex II.
[55] Technical dossier/Supplementary information October 2013/Conf_071013; Supplementary information October 2013/Conf_071013 and Supplementary information October 2014/Conf 080814.
[56] This section has been amended following the confidentiality claims made by the applicant.
[57] Technical dossier/Supplementary information January 2015/Conf 120115.
[58] Technical dossier/Supplementary information October 2014/Annex Qi antimicrobial.
[59] Technical dossier/Supplementary information October 2014/Conf 080814 and Conf_071013.

The product L-threonine produced with *E. coli* NRRL B-30843 is proposed to be used also in water for drinking.

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

EFSA has verified the EURL report as it relates to the methods used for the control of the L-threonine product in animal feed. The Executive Summary of the EURL report can be found in Appendix A.[60]

3.2. Safety

3.2.1. Safety aspects of the genetic modification[61]

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

3.2.2. 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 product under authorisation, which contains ≥ 98.8 % threonine and less than 1 % unidentified material on a dry matter basis. The level of endotoxins in this product (< 1 to 54 IU/mg) is negligible in comparison to that observed in other feedingstuffs (1 000 IU/mg; Cort et al., 1990) and is therefore of no concern for the target species. No safety concerns arose from the genetic modification. Therefore, the FEEDAP Panel considers that safety concerns for the target species are highly unlikely to arise from L-threonine produced by *E. coli* NRRL B-30843.

The tolerance of the target species to L-threonine and their requirements for this amino acid have already been described (section 2.2.1; EFSA FEEDAP Panel, 2014a). The FEEDAP Panel, in line with its previous statement (EFSA FEEDAP Panel, 2010), recommends that amino acids, their salts and analogues should generally not be used in water for drinking because of the risk of imbalances and for hygiene reasons.

3.2.3. Safety for the consumer

Considerations for consumer safety are the same as described above (section 2.2.2), except that the producer strain is a GMM. As the genetic modification is of no concern, the FEEDAP Panel concludes that the use of the product in animal nutrition is safe for consumers.

3.2.4. Safety for the user

3.2.4.1. Effects on eyes, skin and the respiratory tract

Data on user safety were not provided for this product. Results using other L-threonine products were provided as described above (section 2.2.3.1). Similarly, it can be concluded that there is no evidence of specific concerns for user safety (inhalation toxicity, skin and eyes irritation and dermal sensitisation) arising from the product produced by fermentation with *E. coli* NRRL B-30843.

3.2.4.2. Inhalation exposure to endotoxin

As described above (section 2.2.3.2), endotoxin is a potential hazard in products from *E. coli* K-12. The endotoxin activity (analysed in three batches) ranged from 1 to 54 IU/mg. No dusting potential is available for this specific product, so calculations were again based on the dusting potential from the product formed by *E. coli* DSM 26131 (section 5.1.3). Based upon the calculation of the content of

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

[61] This section has been amended following the confidentiality claims made by the applicant.

endotoxin in dust and the dusting potential of 7.4 g/m^3, exposure would be 222 000 IU per eight-hour working day, indicating a risk of inhalation exposure to endotoxins for people handling the additive.

3.2.4.3. Conclusions on safety for the user

The FEEDAP Panel considers that L-threonine produced by *E. coli* NRRL B-30843 is not an irritant to eyes and skin, and is not a skin sensitiser. There is no risk from inhalation of L-threonine but concerns may arise from the content of endotoxins.

3.2.5. Safety for the environment

As L-threonine itself presents no environmental concerns (section 2.2.4), the only potential risk from the product would arise from the genetic modification of the production strain. Neither *E. coli* NRRL B-30843 nor its recombinant DNA was detected in the final product. The final product does not trigger any environmental safety concern associated with the genetic modification.

3.3. Efficacy

Efficacy considerations are the same as described above (section 2.3).

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[62] and Good Manufacturing Practice.

3.5. Conclusions and recommendations on L-threonine produced with *E. coli* NRRL B-30843

3.5.1. Conclusions

L-Threonine manufactured by fermentation with *E. coli* NRRL B-30843 does not give rise to any safety concern with regard to its genetic modification.

L-Threonine, technically pure, produced by *E. coli* NRRL B-30843 is safe for the target animals when used in appropriate amounts to supplement feed to compensate for threonine deficiency in feedingstuffs. Regardless of the assessment of the genetic modification, the FEEDAP Panel has concerns on the safety of the simultaneous oral administration of L-threonine via water for drinking and feed.

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 products made by *E. coli* NRRL B-30843, no risks are expected for the consumer from their use as a feed additive.

L-Threonine produced by *E. coli* NRRL B-30843 is not an irritant to eyes and skin, and is not a skin sensitiser. There is no risk from inhalation of L-threonine but concerns may arise from the content of endotoxins.

The use of the product L-threonine produced by *E. coli* NRRL B-30843 in animal nutrition does not pose a risk to the environment.

The additive 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.

[62] OJ L 35, 8.2.2005, p. 1.

3.5.2. Recommendations

The description of the additive should contain the statement 'produced by fermentation with *Escherichia coli* NRRL B-30843'.

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 additives under application be set to 98.5 %.

4. L-Threonine produced by *E. coli* KCCM11133P

4.1. Characterisation

4.1.1. Characterisation of the active substance/additive

The characterisation of the active substance/additive has been described previously (see section 2.1.1).

The specification of the product allows a minimum of 98 % L-threonine, a maximum of 1.5 % water and a maximum of 0.5 % ash.[63]

Five batches were analysed (method VDLUFA 4.11.6) and the average threonine content was 99.4 % on a 'as is' basis (ranging from 99.3 to 99.6 %). Water was ≤0.1 %.[64] The amount of unidentified material was < 1 % on a dry matter basis.

The specific optical rotation of five batches of the final product was analysed. In all cases the obtained values were within the range given by the European Pharmacopoeia (range –27.6 to –29.0 °)[65] and confirmed the identity of the L-enantiomer. [66]

4.1.2. Impurities

The levels of arsenic, lead, cadmium, chromium and nickel were analysed in three batches and were below detection limits. Copper was 0.8–3.9 mg/kg, while zinc was 5.9–65.1 mg/kg and mercury 0.02–0.09 mg/kg.[67]

Another batch of the final product was analysed for microbial contamination and mycotoxins. *Salmonella* was absent, *E. coli* and clostridia were $<10^2$ CFU/g, and the total aerobic bacterial count was 1.2×10^3 CFU/g. The levels of aflatoxin (unspecified), ochratoxin, deoxynivalenol, zearalenone and fumonisins B1 + B2 were below the LOD.[68]

The detected amounts of the aforementioned contaminants/impurities were of no concern and often below the detection limits.

The antimicrobial activity of the product (one batch) was tested against a reference panel of bacteria (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212 and *B. subtilis* ATCC 6633; EFSA, 2008b). The minimal inhibitory concentration was >8 g/L.[69] No evidence of antimicrobial activity was detected at concentrations up to eight times that recommended for supplementation of feeds.

[63] Technical dossier/Section II.1.3.
[64] Technical dossier/Supplementary information July 2012/Annexes June 2012/Annex Qii. Supplementary information October 2014/Annexes/Annex Qi.
[65] European Pharmacopoeia monograph 1/2015:1272.
[66] Technical dossier/Supplementary information October 2014/Annexes/Annex Qii optical rotation.
[67] Technical dossier/Supplementary information July 2012/Annexes June 2012/Annex Qiii heavy metals. LOD (in mg/kg) of arsenic and lead was 1, of cadmium was 0.1 and of nickel and chromium was 0.2.
[68] Technical dossier/Supplementary information October 2014/Annexes/Annex Qiii impurities. LOD (in µg/kg) of aflatoxin was 1.7, of ochraoxin <5, of deoxynivalenol < 134, of zearalenone <17 and of fumonisins B1+B2 <25.
[69] Technical dossier/Supplementary information October 2014/Annexes/Annexes Qv MIA.

No evidence was provided on the resistance of the producer strains to antibiotics used in human and veterinary medicine. However, the demonstrated absence of producer bacterial DNA in the final product (section 4.1.4) provides assurance that any transmissible resistance genes would not be transferred intact to the product.

Bacterial endotoxin activities were measured (EP 2.6.14 method) in three batches of the final product. The bacterial endotoxin activity ranged from 18 to 30 IU/mg.[70]

4.1.3. Physical properties

General information on physical properties has been described above (section 2.1.3).

Sieve analysis indicated that the particle fraction below 100 µm was 68 % and that below 50 µm was 25 %. No information was provided regarding the respirable fraction (<10 µm).[71]

No data were provided on dusting potential.

4.1.4. Characterisation of the production organism *E. coli* KCCM11133P[72]

The *E. coli* production strain was deposited in the Korean Culture Centre for Microorganisms under accession number KCCM11133P.[73]

The recipient strain is a derivative of *E. coli* K-12. *E. coli* K-12 is a well-characterised Gram-negative bacterium and its safety (non-pathogenicity) has been reviewed extensively (section 2.1.4). The technical dossier contains detailed and sufficient information on the recipient microorganism including safety aspects, the origin and function of the different genetic elements introduced in the production strain, the genetic modification process and the genetic and phenotypic traits introduced. [74]

4.1.5. Manufacturing process[75]

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

Material safety data sheets of the substances used in the fermentation media were provided.[76]

Neither the production strain nor its recombinant DNA was detected in three batches of the final product. [77]

The applicant declared that no antimicrobial substances (including antibiotics) were used in the production process.[78]

4.1.6. Stability and homogeneity

No specific data on the shelf life of this product, or its stability in premixtures, feedingstuffs or water, or its mixing properties were available despite the information having been requested.

4.1.7. Physico-chemical incompatibilities in feed

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

[70] Technical dossier/Supplementary information March 2015/Annexes Qi.
[71] Technical dossier/Section II.1.5.
[72] This section has been amended following the confidentiality claims made by the applicant.
[73] Technical dossier/Supplementary information October 2013/Conf_071013/Annex_CONFID_Qxvi_L-Thr.
[74] Technical dossier/Supplementary information October 2013/Conf_071013.
[75] This section has been amended following the confidentiality claims made by the applicant.
[76] Technical dossier/Supplementary information January 2015/ Conf 120115.
[77] Technical dossier/Supplementary information October 2013/Conf_071013 and Annex_CONFID_Qxxi_L-thr.
[78] Technical dossier/Supplementary information October 2014/Annex Qi antimicrobial.

4.1.8. Conditions of use

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

The L-threonine produced with *E. coli* KCCM11133P is proposed to be used also in water for drinking.

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

EFSA has verified the EURL report as it relates to the methods used for the control of the L-threonine in animal feed. The Executive Summary of the EURL report can be found in Appendix A.[79]

4.2. Safety

4.2.1. Safety aspects of the genetic modification[80]

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

4.2.2. 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 L-threonine produced by *E. coli* KCCM11133P, which contains ≥99.4 % threonine and less than 1 % unidentified material on a dry matter basis. The level of endotoxins in the product (18 to 30 IU/mg) is negligible in comparison to that observed in other feedingstuffs (1 000 IU/mg; Cort et al., 1990) and is therefore of no concern for the target species. No safety concerns arose from the genetic modification. The FEEDAP Panel considers that safety concerns for the target species are highly unlikely to arise from L-threonine produced by *E. coli* KCCM11133P.

The tolerance of the target species to L-threonine and their requirements for this amino acid have already been described (section 2.2.1; EFSA FEEDAP Panel, 2014a). The FEEDAP Panel, in line with its previous statement (EFSA FEEDAP Panel, 2010), recommends that amino acids, their salts and analogues should generally not be used in water for drinking because of the risk of imbalances and for hygiene reasons.

4.2.3. Safety for the consumer

Considerations for consumer safety are the same as described above (section 2.2.2), except that the producer strain is a GMM. As the genetic modification is of no concern, the FEEDAP Panel concludes that the use of the product in animal nutrition is safe for consumers.

4.2.4. Safety for the user

4.2.4.1. Effects on eyes, skin and the respiratory tract

Data on user safety were not provided for this product. Results using other L-threonine products were provided as described above (section 2.2.3.1). Similar conclusions can be drawn that there is no evidence of specific concerns for user safety (inhalation toxicity, skin and eyes irritation and dermal sensitisation) for the product produced by fermentation with *E. coli* KCCM11133P.

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

[80] This section has been amended following the confidentiality claims made by the applicant.

4.2.4.2. Inhalation exposure to endotoxin

As described above (section 2.2.3.2), endotoxin is a potential hazard in products from *E. coli* K-12. The bacterial endotoxin activity (analysed in three batches) ranged from 18 to 30 IU/mg. No dusting potential is available for this specific product, so calculations were again based on the dusting potential from the product formed by *E. coli* DSM 26131 (section 5.1.3). Based upon the calculation of the content of endotoxin in dust and the dusting potential of 7.4 g/m^3, exposure would be 123 000 IU per eight-hour working day indicating a risk of inhalation exposure to endotoxins for people handling the additive.

4.2.4.3. Conclusions on the safety for the user

The FEEDAP Panel considers that L-threonine produced by *E. coli* KCCM11133P is not an irritant to eyes and skin, and is not a skin sensitiser. There is no risk from inhalation of L-threonine but concerns may arise from the content of endotoxins.

4.2.5. Safety for the environment

As L-threonine itself presents no environmental concerns (section 2.2.4), the only potential risk from the product would arise from the genetic modification of the production strain. Neither *E. coli* KCCM11133P nor its recombinant DNA was detected in the final product. The final product does not trigger any environmental safety concern associated with the genetic modification.

4.3. Efficacy

Efficacy considerations are the same as described above (section 2.3).

4.4. Post-market monitoring

The FEEDAP Panel considers that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation[81] and Good Manufacturing Practice.

4.5. Conclusions and recommendations on L-threonine produced by *E. coli* KCCM11133P

4.5.1. Conclusions

L-Threonine, manufactured by fermentation with *E. coli* KCCM11133P, does not give rise to any safety concern with regard to its genetic modification.

L-Threonine, technically pure, produced by *E. coli* KCCM11133P is safe for the target animals when used in appropriate amounts to supplement feed to compensate for threonine deficiency in feedingstuffs. Regardless of the assessment of the genetic modification, the FEEDAP Panel has concerns on the safety of the simultaneous oral administration of L-threonine via water for drinking and feed.

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 made by *E. coli* KCCM11133P, no risks are expected for the consumer from its use as a feed additive.

The FEEDAP Panel considers that L-threonine produced by *E. coli* KCCM11133P is not an irritant to eyes and skin, and is not a skin sensitiser. There is no risk from inhalation of L-threonine but concerns may arise from the content of endotoxins.

The use of the product, L-threonine, produced by *E. coli* KCCM11133P in animal nutrition does not pose a risk to the environment.

[81] OJ L 35, 8.2.2005, p. 1.

The additive 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.

4.5.2. Recommendations

The description of the additive should contain the statement 'produced by fermentation with *Escherichia coli* KCCM11133P.'

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 additives under application be set to 98.5 %.

5. L-Threonine produced by *E. coli* DSM 26131

5.1. Characterisation

5.1.1. Characterisation of the active substance/additive

The characterisation of the active substance/additive has been described previously (see section 2.1.1).

The specification of the product allows a minimum of 98 % L-threonine, a maximum of 1.5 % water and a maximum of 0.5 % ash.[82]

Five batches were analysed (method not described) and the average threonine content was 98.9 % on a 'as is' basis (ranging from 98.8 to 99.0 %). Water was 0.1 % and ash ranged from 0.03 to 0.04 %.[83] As no information was available on the method of analysis used, the purity of the product should be taken with some caution. It is not possible to conclude on the amount of unidentified material.

The specific optical rotation of five batches of the final product was analysed. In all cases the obtained values were within the range given by the European Pharmacopoeia (range –27.6 to –29.0°).[84]

5.1.2. Impurities

The purity data (unspecified number of batches) showed levels of lead ranging from < 0.05 to < 10 mg/kg, arsenic ranging from 0.052 to < 0.5 mg/kg, nickel 0.84 mg/kg, mercury < 0.1 mg/kg, cadmium < 0.5 mg/kg and chromium < 5.0 mg/kg. Mycotoxins (unspecified) were not detected. *Salmonella* was not detected in 25 g. These data should be regarded with caution because of the uncertainty regarding the identity of the product and the quality of some analytical reports submitted.[85]

The detected amounts of the aforementioned contaminants/impurities were of no concern and often below the detection limits.

No information was provided on the antimicrobial activity of the product, on the resistance of the production strain to antibiotics used in human and veterinary medicine or on the bacterial endotoxin activity of the product.

5.1.3. Physical properties

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

[82] Technical dossier/Section II.1.3.
[83] Technical dossier/Supplementary information July 2012/Annexes June 2012/Annex Qii .
[84] Technical dossier/Supplementary information July 2012/Annexes June 2012/Annex Qii .
[85] Technical dossier/Supplementary information July 2012/Annexes June 2012/Annex Qiii heavy metals. LOD (in mg/kg) of the second certificate for cadmium was 0.001, for mercury 0.0025 and for *Salmonella* is not specified.

Regarding the particle size distribution (sieve analysis) of the L-threonine produced by *E. coli* DSM 26131 (number of batches not specified), the particle fraction below 100 µm was 90 % and that below 50 µm was 55%. No information was provided on the respirable fraction (< 10 µm).[86]

Dusting potential (by the Stauber–Heubach method) was submitted only for one batch of the additive. The value was high, at 7.4 g/m^3.[87]

5.1.4. Characterisation of the production organism *E. coli* DSM 26131[88]

The *E. coli* production strain was deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ) under accession number DSM 26131.[89]

The information provided in the dossier on the identity of the production strain, the identity of the recipient strain, on the origin and function of the genetic elements introduced in the production strain, on the genetic modificiation process and on the genetic and phenotypic traits introduced is insufficient and does not allow to conclude on the safety of the production strain.

5.1.5. Manufacturing process

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

Material safety data sheets of the substances used in the fermentation media were not provided. No information was provided regarding the use of any antimicrobial compound (including antibiotics) in the production process.[90]

No information was provided on whether the production strain or the recombinant DNA genes are present in the final product.

5.1.6. Stability and homogeneity

No specific data on the shelf life of this product, or its stability in premixtures, feedingstuffs or water, or its mixing properties were available despite the information having been requested.

5.1.7. Physico-chemical incompatibilities in feed

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

5.1.8. Conditions of use

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

The L-threonine produced with *E. coli* DSM 26131 is proposed to be used also in water for drinking.

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

EFSA has verified the EURL report as it relates to the methods used for the control of the L-threonine in animal feed. The Executive Summary of the EURL report can be found in Appendix.[91]

[86] Technical dossier/Section II.1.5.
[87] Technical dossier/Supplementary information July 2012/Annexes June 2012/Annex Qiv.
[88] This section has been amended following the confidentiality claims made by the applicant.
[89] Technical dossier/Supplementary information July 2012/AMAC Conf 200712.
[90] Technical dossier/Supplementary information October 2014/2014-10-13 AMAC Threonine answer to EFSA.
[91] The full report is available on the EURL website: http://irmm.jrc.ec.europa.eu/SiteCollectionDocuments/FinRep-FAD-2010-0058+0081.pdf. This report was amended in June 2012: http://irmm.jrc.ec.europa.eu/SiteCollectionDocuments/amend-FinRep-FAD-2010-0058+0081.pdf

5.2. Safety

5.2.1. Safety aspects of the genetic modification

Owing to lack of information, the genetic modification could not be assessed sufficiently. The applicant provided no information that neither the production strain nor the recombinant genes are present in the final product. Therefore, the presence of cells of the production strain or its DNA in the final product cannot be excluded. This is considered a concern because the genetic modification is not well characterised. In particular, it is not known if the production strain carries genes conferring resistance to antibiotics. Therefore, the EFSA FEEDAP Panel cannot conclude on the safety of the product L-threonine from *E. coli* DSM 26131 with regard to the genetic modification.

5.2.2. Safety for the target species

Regarding L-threonine produced by *E. coli* DSM 26131, the data submitted do not allow the purity or the amount of unidentified material to be established or exclusion of the possible presence of recombinant DNA or antibiotic resistance genes in the final product. Therefore, the FEEDAP Panel cannot conclude on the safety of the L-threonine produced by *E. coli* DSM 26131 for the target species.

5.2.3. Safety for the consumer

The FEEDAP Panel cannot conclude on the product L-threonine produced by *E. coli* DSM 26131 because the data submitted do not allow conclusions on the amount of identified material to be made, and also do not exclude the possible presence of recombinant DNA, including antibiotic resistance genes used in the genetic modification process, in the final product.

5.2.4. Safety for the user

The data submitted do not allow the purity or the amount of unidentified material to be established or exclusion of the possible presence of recombinant DNA or antibiotic resistance genes in the final product. Therefore, the FEEDAP Panel cannot conclude on the safety of the L-threonine produced by *E. coli* DSM 26131 for the user.

5.2.5. Safety for the environment

The presence of viable cells of the production strain *E. coli* DSM 26131 or its recombinant DNA, which is not well characterised, cannot be excluded in the final product. The possible presence is a concern because such DNA, which may carry antibiotic resistance genes that could be present on plasmids, might be transferred to pathogenic bacteria present in the environment, including the animal gut. Moreover, if viable production strain cells or its recombinant genes were present in the product, its environmental impact should be assessed according to the 'Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use' (EFSA GMO Panel, 2011). Therefore, the EFSA FEEDAP Panel cannot conclude on the environmental safety of the product L-Threonine from *E. coli* DSM 26131, associated with the genetic modification.

5.3. Efficacy

Efficacy considerations are the same as described above (section 2.3).

5.4. 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[92] and Good Manufacturing Practice.

[92] OJ L 35, 8.2.2005, p. 1.

5.5. Conclusions and recommendations on L-threonine produced with *E. coli* DSM 26131

5.5.1. Conclusions

L-Threonine produced by the GMM *E. coli* DSM 26131 could not be assessed because of the insufficient molecular characterisation of the genetic modification, and the lack of data on both the absence of the production strain and its recombinant DNA from the final product. In particular, the EFSA FEEDAP Panel could not conclude on the safety of this product for target animals, and on the safety concerning consumers, users or the environment.

The additive 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. L-Threonine for all animal species. August 2011. Submitted by AMAC EEIG.
2. L-Threonine for all animal species. Supplementary information July 2012. Submitted by AMAC EEIG.
3. L-Threonine for all animal species. Supplementary information October 2013. Submitted by FEFANA Asbl.
4. L-Threonine for all animal species. Supplementary information October 2014. Submitted by FEFANA Asbl.
5. L-Threonine for all animal species. Supplementary information May 2015. Submitted by FEFANA Asbl.
6. Evaluation report of the European Union Reference Laboratory for Feed Additives on the Methods(s) of Analysis for L-threonine.
7. Comments from Member States received through the ScienceNet.

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Appendix A. Safety for the user

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

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

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

Calculation of maximum acceptable levels of exposure from feed additives. The likely exposure time, according to EFSA guidance (EFSA FEEDAP Panel, 2012), for additives added in premixtures assumes a maximum of 40 periods of exposure per day, each comprising 20 s = 40 × 20 = 800 seconds per day. With an uncertainty factor of 2, maximum inhalation exposure would occur for 2 × 800 = 1 600 s = 0.444 h/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/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) and the endotoxin activity of the dust, determined by the *Limulus* amoebocyte lysate assay (expressed in IU/g)). If data for the dust are not available, the content of endotoxins of the product can be taken instead. If the content of endotoxins of the relevant additive is *a* IU/g and the dusting potential is *b* g/m^3, then the content of endotoxins of the dust *c* IU/m^3 is obtained by simple multiplication *a* × *b*. This resulting value is further used for calculation of potential inhalatory exposure by users to endotoxin from the additives under assessment (Tables A1 to A3) (EFSA FEEDAP Panel, 2012).

Table A1 Estimation of user exposure to endotoxins from the additive L-threonine produced by *E. coli* DSM 25085, including consideration of using filter mask FF P2 or FF P3 as a preventative measure

Calculation	Identifier	Description	Amount	Source
	a	Endotoxin content (IU/g product)	340	Technical dossier
	b	Dusting potential (g/m^3) [(a)]	7.4	Technical dossier
a × *b*	*c*	Endotoxin content in the air (IU/m^3)	2 516	
	d	No of premixture batches made/working day	40	EFSA Guidance on User Safety (EFSA FEEDAP Panel, 2012)
	e	Time of exposure (seconds) per production of one batch	20	EFSA Guidance on User Safety (EFSA FEEDAP Panel, 2012)
d × *e*	*f*	Total duration of daily exposure/worker (seconds)	800	
	g	Uncertainty factor	2	EFSA Guidance on User Safety (EFSA FEEDAP Panel, 2012)
f × *g*	*h*	Refined total duration of daily exposure/worker (seconds)	1 600	
h/3 600	*i*	Refined total duration of daily exposure (hours)	0.44	
	j	Inhaled air (m^3) per eight-hour working day	10	EFSA Guidance on User Safety (EFSA FEEDAP Panel, 2012)
j/8 × *i*	*k*	Inhaled air during exposure (m^3)	0.56	
c × *k*	*l*	**Endotoxin (IU) inhaled during exposure per eight-hour working day** [(a)]	**1 409**	
	m	Health-based recommended exposure limit of endotoxin (IU/m^3) per eight-hour working day	90	Health Council of the Netherlands (2010)
m × *j*	*n*	**Health-based recommended exposure limit of total endotoxin (IU) exposure per eight-hour working day**	**900**	
l/10		Endotoxins inhaled (IU) per eight-hour working day reduced by filter mask FF P2 (reduction factor 10)	141	
l/20		Endotoxins inhaled (IU) per eight-hour working day reduced by filter mask FF P3 (reduction factor 20)	70	

(a): Based on the dusting potential measured in L-threonine produced by E. coli DSM 26131.

Table A2 Estimation of user exposure to endotoxins from the additive L-threonine produced by *E. coli* NRRL B-30843, including consideration of using filter mask FF P2 or FF P3 as a preventative measure

Calculation	Identifier	Description	Amount	Source
	a	Endotoxin content (IU/g product)	54 000	Technical dossier
	b	Dusting potential (g/m^3) [(a)]	7.4	Technical dossier
a × *b*	*c*	Endotoxin content in the air (IU/m^3)	399 600	
	d	No of premixture batches made/working day	40	EFSA Guidance on User Safety (EFSA FEEDAP Panel, 2012)
	e	Time of exposure (seconds) per production of one batch	20	EFSA Guidance on User Safety (EFSA FEEDAP Panel, 2012)
d × *e*	*f*	Total duration of daily exposure/worker (seconds)	800	
	g	Uncertainty factor	2	EFSA Guidance on User Safety (EFSA FEEDAP Panel, 2012)
f × *g*	*h*	Refined total duration of daily exposure/worker (seconds)	1 600	
h/3 600	*i*	Refined total duration of daily exposure (hours)	0.44	
	j	Inhaled air (m^3) per eight-hour working day	10	EFSA Guidance on User Safety (EFSA FEEDAP Panel, 2012)
j/8 × i	*k*	Inhaled air during exposure (m^3)	0.56	
c × *k*	*l*	**Endotoxin (IU) inhaled during exposure per eight-hour working day** [(a)]	**222 000**	
	m	Health-based recommended exposure limit of endotoxin (IU/m^3) per eight-hour working day	90	Health Council of the Netherlands (2010)
m × *j*	*n*	**Health-based recommended exposure limit of total endotoxin (IU) exposure per eight-hour working day**	**900**	
l/10		Endotoxins inhaled (IU) per eight-hour working day reduced by filter mask FF P2 (reduction factor 10)	22 200	
l/20		Endotoxins inhaled (IU) per eight-hour working day reduced by filter mask FF P3 (reduction factor 20)	11 100	

(a): Based on the dusting potential measured in L-threonine produced by E. coli DSM 26131.

Table A3 Estimation of user exposure to endotoxins from the additive L-threonine produced by *E. coli* KCCM11133P, including consideration of using filter mask FF P2 or FF P3 as a preventative measure

Calculation	Identifier	Description	Amount	Source
	a	Endotoxin content (IU/g product)	30 000	Technical dossier
	b	Dusting potential (g/m^3) [(a)]	7.4	Technical dossier
a × *b*	*c*	Endotoxin content in the air (IU/m^3)	222 000	
	d	No of premixture batches made/working day	40	EFSA Guidance on User Safety (EFSA FEEDAP Panel, 2012)
	e	Time of exposure (seconds) per production of one batch	20	EFSA Guidance on User Safety (EFSA FEEDAP Panel, 2012)
d × *e*	*f*	Total duration of daily exposure/worker (seconds)	800	
	g	Uncertainty factor	2	EFSA Guidance on User Safety (EFSA FEEDAP Panel, 2012)
f × *g*	*h*	Refined total duration of daily exposure/worker (seconds)	1 600	
h/3 600	*i*	Refined total duration of daily exposure (hours)	0.44	
	j	Inhaled air (m^3) per eight-hour working day	10	EFSA Guidance on User Safety (EFSA FEEDAP Panel, 2012)
j/8 × i	*k*	Inhaled air during exposure (m^3)	0.56	
c × *k*	*l*	**Endotoxin (IU) inhaled during exposure per eight-hour working day** [(a)]	**123 333**	
	m	Health-based recommended exposure limit of endotoxin (IU/m^3) per eight-hour working day	90	Health Council of the Netherlands (2010)
m × *j*	*n*	**Health-based recommended exposure limit of total endotoxin (IU) exposure per eight-hour working day**	**900**	
l/10		Endotoxins inhaled (IU) per eight-hour working day reduced by filter mask FF P2 (reduction factor 10)	12 333	
l/20		Endotoxins inhaled (IU) per eight-hour working day reduced by filter mask FF P3 (reduction factor 20)	6 167	

(a): Based on the dusting potential measured in L-threonine produced by E. coli DSM 26131.

Annex - Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for L-threonine[93]

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* (RSDr) ranging from 1.9 to 4.1%;
- a relative standard deviation for *reproducibility* (RSDR) 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 Feed Grade 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:

- RSDr ranging from 0.5 to 2.4%; and
- RSDR 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*.

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*.

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

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