SCIENTIFIC OPINION

EFSA Journal

ADOPTED: 19 April 2016

doi: 10.2903/j.efsa.2016.4470

Safety of L-threonine, technically pure, produced by fermentation with *Escherichia coli* CGMCC 7.58 for all animal species based on a dossier submitted by Feedway Europe NV

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP)

Abstract

L-Threonine, technically pure, is a feed additive produced by fermentation using a genetically modified (GM) strain of *Escherichia coli* (CGMCC 7.58). The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP Panel) of EFSA issued an opinion on the safety and efficacy of the product, which concluded that the recipient strain and its genetic modification, including the presence or absence of an antibiotic resistance gene in the product under assessment, are insufficiently characterised. Consequently, the FEEDAP Panel could not make a conclusion on the safety of the product L-threonine produced by fermentation with this recombinant strain of *E. coli* for target animals, consumers, users and the environment. Furthermore, the level of endotoxins present in the product and its dusting potential represent a risk for the user by inhalation. The European Commission asked EFSA to deliver an opinion on the safety of L-threonine for all animal species based on additional data submitted by the applicant on the characterisation of the additive and of the genetic modification of the production strain. No recombinant antibiotic resistance genes are present in the production strain and therefore in the final product. L-Threonine technically pure, manufactured by fermentation using *E. coli* CGMCC 7.58, does not raise safety concerns for the target species, consumers, users and the environment with regard to the genetic modification of the production strain. A risk from exposure to endotoxins for people handling the additive is expected. Concerns remain about possible dermal sensitisation.

Keywords: nutritional additives, amino acids, L-threonine, safety, efficacy

Requestor: European Commission

Question number: EFSA-Q-2015-00555

Correspondence: feedap@efsa.europa.eu

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

Acknowledgements: The Panel wishes to thank the members of the Working Group on Amino Acids, including Lucio Costa, Noël Dierick and Lubomir Leng, and the members of the Working Group on Genetically Modified Microorganisms, including Boet Glandorf, Lieve Herman and Sirpa Kärenlampi, 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), 2016. Scientific opinion on the safety of L-threonine, technically pure, produced by fermentation with *Escherichia coli* CGMCC 7.58 for all animal species based on a dossier submitted by Feedway Europe NV. EFSA Journal 2016;14(5):4470, 11 pp. doi:10.2903/j.efsa.2016.4470

ISSN: 1831-4732

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

Summary

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on L-threonine, technically pure, produced by fermentation using the genetically modified (GM) strain *Escherichia coli* CGMCC 7.58 for all animal species.

In 2015, FEEDAP Panel of the European Food Safety Authority (EFSA) adopted an opinion on the safety and efficacy of L-threonine produced by fermentation using *E. coli* CGMCC 7.58. In that opinion, the FEEDAP Panel concluded that the recipient strain and its genetic modification, including the presence or absence of an antibiotic resistance gene in the product under assessment, are insufficiently characterised. Consequently, the FEEDAP Panel could not make a conclusion on the safety of the product L-threonine produced by fermentation with this recombinant strain of *E. coli* for target animals, consumers, users and the environment. Furthermore, the level of endotoxins present in the product and its dusting potential were found to have an inhalation risk for the user.

The applicant provided additional information in relation to the characterisation of the additive, the genetic modification process of the production strain and the production process. The FEEDAP Panel has performed the assessment of those new data following an approach in line with the principles laid down in Regulation (EC) No 429/2008 and the relevant guidance documents.

This product was characterised in a previous scientific opinion (EFSA FEEDAP Panel, 2015).

The production strain is a derivative of *E. coli* K-12, which is considered safe. No recombinant antibiotic resistance genes are present in the production strain and therefore in the final product. L-Threonine technically pure, manufactured by fermentation using *E. coli* CGMCC 7.58, does not raise safety concerns for the target species, consumers, users and the environment with regard to the genetic modification of the production strain.

Due to improvements at several steps of the manufacturing process, the level of endotoxins present in the product and its dusting potential have been reduced. Nevertheless, endotoxins represent still a health risk for the user upon inhalation. In the absence of any data on sensitisation, the product should be considered as a potential dermal sensitiser.

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

Regulation (EC) No 1831/2003 establishes rules governing the Community authorisation of additives for animal nutrition and, in particular, Article 9 defines the terms of the authorisation by the Commission.

The applicant, Feedway Europe NV, is seeking a Community authorisation of L-threonine, technically pure to be used as a nutritional additive for all animal species (Table 1).

Table 1: Description of the substances

Category of additive	Nutritional additive
Functional group of additive	Amino acids, their salts and analogues
Description	L-Threonine, technically pure
Target animal category	All animal species
Applicant	Feedway Europe NV
Type of request	New opinion

On 10 March 2015, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP Panel) of the European Food Safety Authority (EFSA), in its opinion concluded that the recipient strain and its genetic modification, including the presence or absence of an antibiotic resistance gene in the product under assessment, are insufficiently characterised. Consequently, the FEEDAP Panel could not make a conclusion on the safety of the product L-threonine produced by fermentation with this recombinant strain of *E. coli* for target animals, consumers, users and the environment. Regardless of the assessment of the genetic modification, the FEEDAP Panel had concerns with respect to the microbial contamination of the product and the safety of the simultaneous oral administration of L-threonine via water for drinking and feed. Finally, the level of endotoxins present in the product and its dusting potential were found to have an inhalation risk for the user.

The Commission gave the possibility to the applicant to submit complementary information in order to complete the assessment on the safety and to allow a revision of Authority's opinion.

The Commission has now received new data on the safety of L-threonine, technically pure produced by *Escherichia coli*.

In view of the above, the Commission asks the Authority to deliver a new opinion on the safety of L-threonine, technically pure as nutritional additive for all animal species based on the additional data submitted by the applicant.

1.2. Additional information

L-Threonine (minimum content of L-threonine 98%, 'as is' basis) was first authorised for use in animal nutrition by Directive 88/485/EEC. It is currently included in the European Union Register of Feed Additives pursuant to Regulation (EC) No 1831/2003 concerning additives in feedingstuffs.

The applicant has provided additional information on the physical properties of the additive, the genetic modification of the production strain and on the production process.

Although the additive was initially intended to be used in all animal species as a nutritional additive (functional group amino acids, their salts and analogues) to feed and to water for drinking, during the assessment, the applicant requested to withdraw the use of the additive in water for drinking.[1]

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier[2] following a previous application on the same product.[3]

[1] Technical dossier/Correspondence and mandate/FAD-2015-0029 withdrawal partial app to EC and to EFSA.
[2] FEED dossier reference: FAD-2015-0029.
[3] FEED dossier reference: FAD-2010-0291.

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety of L-threonine produced by *E. coli* CGMCC 7.58 is in line with the principles laid down in Regulation (EC) No 429/2008[4] and the relevant guidance documents: Guidance on nutritional additives (EFSA FEEDAP Panel, 2012a), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012b), and Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use (EFSA GMO Panel, 2011).

3. Assessment

The current application is for the authorisation of L-threonine produced by a genetically modified (GM) strain of *E. coli* (CGMCC 7.58). It is intended to be used in all animal species as a nutritional additive (functional group amino acids, their salts and analogues) to feed.

3.1. Characterisation

This product has been characterised in a previous opinion (EFSA FEEDAP Panel, 2015), with the exception of the production strain, which was not adequately identified, and the genetic modification, for which the absence of antibiotic resistance genes in the production strain (and therefore in the product), was insufficiently proven.

Additional data have been submitted on the genetic modification process of the production strain, the physicochemical properties of the additive and on its manufacturing process.

3.1.1. Impurities

The previously assessed product had an average endotoxin activity (two batches analysed) of 927 International units of endotoxin activity (IU)/mg.[5] An improvement has been introduced in the downstream process to reduce the amount of bacterial endotoxins in the final product. The filtration by ceramic membrane has been modified (from a former pore size of 300 kDa to the current smaller size of 20 kDa); reverse osmosis has also been applied to reduce the amount of endotoxins in the process water.[6] New data have been submitted on the bacterial endotoxin activity of the product, measured in three batches. The values ranged from < 0.50 to 1.44 IU/mg (European Pharmacopoeia 2.6.14 method).[7]

In March 2015 assessment, it was noted that one batch had > 10,000 total aerobic count colony-forming units (CFU)/g and it was recommended to review the production process to ensure that the microbial contamination of the product is low. The applicant has now provided microbiological analyses (three batches) of total aerobic counts showing that no colonies were found after aerobic incubation of 100 μL of a 5% solution on LB medium at 37°C for 48 h. The production strain, when used as positive control, performed as expected.[8] The previous opinion indicated that the numbers of yeasts (< 100 CFU/g) and filamentous fungi (≤ 150 CFU/g) were low, and that *Salmonella* spp. were absent in 25 g samples. No data on these were provided in the new application.

3.1.2. Physical properties

The applicant stated that the downstream process had been modified to increase the crystal size of the final product and consequently decrease its dustiness. The evaporation temperature has been lowered (75–72°C), the evaporation rate reduced (2.5–2.0 m^3/h), the evaporation time prolonged (12–15 h) and the cooling time extended from 3 to 5 h.[9]

[4] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.
[5] Technical dossier FAD-2010-0291/Supplementary information January 2013/Attachment 3.
[6] Technical dossier/Supplementary information March 2016/Attachment 1.
[7] Technical dossier/Technology improvement on production process, Supplementary information on endotoxin levels and Attachments 1–3.
[8] Technical dossier/Supplementary information March 2016/Attachment 10.
[9] Technical dossier/Technology improvement on production process.

New analytical data have been provided to characterise the particle size and dustiness of the new final product. The particle size distribution (three batches analysed by laser diffraction) indicated a fraction of 59–60% (v/v) particles < 100 μm, of 24–26% (v/v) of particles < 50 μm and 3% (v/v) of particles < 10 μm diameter (mean diameter of the particles of the additive was about 85 μm).[10] The former product (five batches analysed, method not specified) showed a range of 69–75% of particles having a diameter < 100 μm and a range of 12–15% of particles having a diameter < 50 μm; one additional batch analysed by sieving had about 16% (w/w) of the particles with a diameter < 50 μm.[11] The dusting potential (three batches analysed by the Stauber–Heubach method) ranged from 0.74 to 1.25 g/m^3.[12] The dusting potential of the product assessed in 2015 (one batch analysed) was 2.4 g/m^3.[13]

3.1.3. Characterisation of the production microorganism *E. coli* CGMCC 7.58[14]

In the former dossier submitted for the characterisation of the production strain (EFSA FEEDAP Panel, 2015), no data were provided to confirm the identity of the recipient strain. Moreover, no conclusive evidence was provided regarding the absence of an antibiotic resistance gene (which was transiently used in the genetic modification) in the genome of the production strain or in the product. Therefore, uncertainty remained regarding the identity and safety of the production strain and of the product.

New data were provided in the dossier submitted for the present evaluation, which confirmed the identity of the production strain as *E. coli* K-12 derivative.[15]

Furthermore, the applicant provided an analysis indicating that the antibiotic resistance gene is not present in the production strain.[16]

3.2. Safety

In its previous assessment (EFSA FEEDAP Panel, 2015), the FEEDAP Panel could not conclude on the safety of the product L-threonine produced by fermentation with this recombinant strain of *E. coli* for target animals, consumers, users and the environment. The reason was that the recipient strain and its genetic modification, including the presence or absence of an antibiotic resistance gene in the production strain (and therefore in the product), were insufficiently characterised.

The production strain has been adequately identified as an *E. coli* K-12 derivative. *E. coli* K-12 is considered safe. The applicant provided evidence that the production strain does not carry the antibiotic resistance genes transiently used during the genetic modification. Absence in the product of recombinant DNA corresponding to the inserted sequences was previously shown (EFSA FEEDAP Panel, 2015). Therefore, L-threonine, manufactured by fermentation with *E. coli* CGMCC 7.58, does not raise any safety concern for the target species, the consumer, the user and the environment with regard to the genetic modification of the production strain.

3.2.1. Safety for the user

In the former opinion (EFSA FEEDAP Panel, 2015), it was concluded that there were no concerns for users in respect of irritation to skin or eyes but that the product may have the potential to be a dermal sensitiser and should be handled accordingly. The level of endotoxins present in the product and its dusting potential indicated a risk for the user by inhalation.

No additional toxicity studies have been submitted on skin sensitisation potential of the product.

3.2.1.1. Effects on the respiratory system

Although a previous assessment was performed in 2015 (EFSA FEEDAP Panel, 2015), the technological improvement in the manufacturing process makes necessary to re-assess the relevance of the bacterial endotoxin activities found in the final product for the user when exposed by inhalation.

[10] Technical dossier/Supplementary information March 2016/Attachments 4 and 5.
[11] Technical dossier FAD-2010-0291/Section II.1.5 and Annex II.1.1.
[12] Technical dossier/Supplementary information March 2016/Attachment 4.
[13] Technical dossier FAD-2010-0291/Supplementary information January 2013/Attachment 4.
[14] This section has been amended following the applicable provisions on confidentiality.
[15] Technical dossier/Supplementary information March 2016/Attachment Conf 1 L-Threonine molecular identification.
[16] Technical dossier/Supplementary information March 2016/Attachment Conf 2 L-Threonine detection of antibiotic-resistant.

The bacterial endotoxin activity (the three new batches) was up to 1.44 IU/mg.[17] The dusting potential ranges from 0.74 to 1.25 g/m^3.[18]

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 (2012b) is described in Appendix A. The health-based recommended threshold for the quantity of inhaled endotoxins per working day is 900 IU, derived from provisional occupational exposure limits given by the Dutch Expert Committee on Occupational Safety (DECOS) (Health Council of the Netherlands, 2010) and the Health and Safety Executive (HSE, 2013). Based on the calculation of the potential endotoxin content in dust (Wallace et al., 2016), the inhalation exposure could be up to 1,000 endotoxin IU per 8-h working day, indicating a risk from the exposure to endotoxins for people handling the additive.

3.2.1.2. Conclusions on safety for the user

The product L-threonine produced by *E. coli* CGMCC 7.58 is not irritant but is a potential skin sensitiser. It is a risk for the users by inhalation.

4. Conclusions

The production strain is an *E. coli* K-12 derivative. No recombinant antibiotic resistance genes are present in the production strain and therefore in the final product. L-Threonine manufactured by fermentation with *E. coli* CGMCC 7.58 does not raise safety concerns for the target species, consumers, users and the environment with regard to the genetic modification of the production strain.

The level of endotoxins present in the product and its dusting potential indicate a health risk for the user upon inhalation. The product should be considered as a potential dermal sensitiser.

Documentation provided to EFSA

1) Dossier L-Threonine produced by *Escherichia coli* for all animal species. October 2015. Submitted by Feedway Europe NV.
2) Dossier L-Threonine produced by *Escherichia coli* for all animal species. Supplementary information. November 2015. Submitted by Feedway Europe NV.
3) Dossier L-Threonine produced by *Escherichia coli* for all animal species. Supplementary information. March 2016. Submitted by Feedway Europe NV.

References

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

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

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* for all animal species based on a dossier submitted by HELM AG on behalf of Meihua Holdings Group Co. Ltd. EFSA Journal 2015;13(3):4051, 17 pp. doi:10.2903/j.efsa.2015.4051A

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

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

HSE (Health and Safety Executive), 2013. Occupational hygiene implications of processing waste at materials recycling facilities (MRFs). RR977 Research Report, 41 pp.

Wallace RJ, Gropp J, Dierick N, Costa LG, Martelli G, Brantom PG, Bampidis V, Renshaw DW and Leng L, 2016. Risks associated with endotoxins in feed additives produced by fermentation. Environmental Health, 15, 1–7.

[17] Technical dossier/Supplementary information on endotoxin levels and Attachments 1–3.
[18] Technical dossier FAD-2010-0291/Supplementary information January 2013/Attachment 4 dusting potential.

Abbreviations

CGMCC	China General Microbiological Culture Collection Center
CFU	colony-forming unit
DECOS	Dutch Expert Committee on occupational safety
FEEDAP	EFSA Panel on Additives and Products or Substances Used in Animal Feed
GM	genetically modified
GMO	EFSA Panel on Genetically Modified Microorganisms
HSE	British Health Safety Executive
IU	International unit of endotoxin activity. One IU corresponds to one EU

Appendix A – Safety for the user

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

Calculation of maximum acceptable levels of exposure from feed additives

The likely exposure time according to EFSA guidance (EFSA FEEDAP Panel, 2012b) for additives added in premixtures assumes a maximum of 40 periods of exposure per day, each comprising 20 s, equal to 800 s per day. With an uncertainty factor of 2, maximum inhalation exposure would occur for $2 \times 800 = 1{,}600$ s (0.444 h per day). Again, assuming a respiration volume of 1.25 m^3/h, the inhalation volume providing exposure to potentially endotoxin-containing dust would be $0.444 \times 1.25 = 0.556$ m^3 per day. This volume should contain no more than 900 International unit of endotoxin activity (IU) endotoxin, so the dust formed from the product should contain no more than $900/0.556 = 1{,}619$ IU/m^3.

Calculation of endotoxin content of dust

Two key measurements are required to evaluate the potential respiratory hazard associated with endotoxin content of the product (the dusting potential of the product, expressed in g/m^3; the endotoxin activity of the dust, determined by the *Limulus* amoebocyte lysate assay (expressed in IU/g)).[19] If data for the dust are not available, the content of endotoxins of the product can be used instead. If the content of endotoxins of the relevant additive is *a* IU/g and the dusting potential is *b* g/m^3, then the content of endotoxins of the dust in the air, *c* IU/m^3, is obtained by the simple multiplication $a \times b$. This resulting value is further used for calculation of potential inhalatory exposure by users to endotoxin from the additive under assessment (Table A.1) (EFSA FEEDAP Panel, 2012b).

Table A.1: Estimation of user exposure to endotoxins from the additive L-threonine produced by *Escherichia coli* CGMCC 7.58, including consideration of using filter half mask (FF P2 or FF P3)[(a)] as a preventative measure

Calculation	Identifier	Description	Amount	Source
	a	Endotoxin content IU/g product	1,440	Technical dossier
	b	Dusting potential (g/m^3)	1.25	Technical dossier
$a \times b$	*c*	Endotoxin content in the air (IU/m^3)	1,800	
	d	No of premixture batches made/working day	40	EFSA FEEDAP Panel (2012b)
	e	Time of exposure (s)/production of one batch	20	EFSA FEEDAP Panel (2012b)
$d \times e$	*f*	Total duration of daily exposure/worker (s)	800	
	g	Uncertainty factor	2	EFSA FEEDAP Panel (2012b)
$f \times g$	*h*	Refined total duration of daily exposure (s)	1,600	
h/3 600	*i*	Refined total duration of daily exposure (h)	0.44	
	j	Inhaled air (m^3)/8-h working day	10	EFSA FEEDAP Panel, 2012b
$j/8 \times i$	*k*	Inhaled air during exposure (m^3)	0.556	
$c \times k$	*l*	Endotoxin inhaled (IU) during exposure/8-h working day	1,000	
	m	Health-based recommended exposure limit of endotoxin (IU/m^3)/8-h working day	90	Health Council of the Netherlands (2010)
$m \times j$	*n*	Health-based recommended exposure limit of total endotoxin exposure (IU)/ 8-h working day	900	

[19] The Limulus amebocyte lysate is an aqueous extract of circulating amebocytes from the horseshoe crab (*Limulus polyphemus*). This extract reacts with minute quantities of bacterial endotoxin (lipopolysaccharide from the walls of Gram-negative bacteria) and this reaction is the basis of the assay used for the detection and quantification of bacterial endotoxins.

Calculation	Identifier	Description	Amount	Source
//10		Endotoxins inhaled (IU)/8-h working day reduced by filter half mask FF P2 (reduction factor 10)	100	
//20		Endotoxins inhaled (IU)/8-h working day reduced by filter half mask FF P3 (reduction factor 20)	50	

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

Reference

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