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Safety of L-threonine produced by fermentation using *Escherichia coli* CGMCC 3703, for all animal species based on a dossier submitted by GBT Europe GmbH

EFSA 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 strain of *Escherichia coli*. The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) of the European Food Safety Authority (EFSA) issued an opinion on the safety and efficacy of the product, in which it could not conclude on the safety of this additive for target animals, consumer, user and the environment, due to insufficient characterisation of the genetic modification. The European Commission asked EFSA to deliver an opinion on the safety of L-threonine, technically pure, as a nutritional additive for all animal species based on additional data submitted by the applicant. Neither the production strain nor its recombinant genes are present in the final product. Therefore, the FEEDAP Panel concludes that L-threonine produced using *E. coli* CGMCC 3703 does not raise any safety concern for the target species, consumer, user and the environment with regard to the genetic modification of the production strain. The FEEDAP Panel reiterated their concerns on the safety of amino acids, including L-threonine, for target species when administered simultaneously via water for drinking due to amino acid imbalances. As the estimated maximum exposure to endotoxins by inhalation was below the provisional occupational exposure limit, no risk from the exposure to endotoxins for people handling the additive is expected.

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

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Summary

Following a request from the European Commission (EC), the European Food Safety Authority (EFSA) was asked to deliver a scientific opinion on the safety of L-threonine, technically pure, produced by fermentation using the genetically modified strain *Escherichia coli* CGMCC 3703 for all animal species.

In 2014, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) of EFSA adopted an opinion on the safety and efficacy of L-threonine produced by fermentation with *E. coli* CGMCC 3703. The FEEDAP Panel could not conclude on the safety of that product for target animals, consumer, user and the environment because the genetic modification, including the presence/absence of recombinant DNA and of antibiotic resistance genes in the product, was insufficiently characterised.

The applicant provided additional information in relation to the characterisation of the production microorganism, the characterisation of the additive and its stability. 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.

Neither the production strain nor its recombinant genes were present in the final product. Therefore, the FEEDAP Panel concluded that L-threonine, manufactured by fermentation with *E. coli* CGMCC 3703, did not raise safety concerns for the target species, consumers, users and the environment with regard to the genetic modification of the production strain.

The FEEDAP Panel reiterated their concerns on the safety of amino acids, including L-threonine, for target species when administered simultaneously via water for drinking due to amino acid imbalances.

As the estimated maximum exposure to endotoxins by inhalation was below the provisional occupational exposure limit, no risk from the exposure to endotoxins for people handling the additive is expected.

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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, GBT Europe GmbH, is seeking a Community authorisation of L-threonine, technically pure, produced by fermentation using *Escherichia coli* 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, produced by <i>Escherichia coli</i>
Target animal category	All animal species
Applicant	GBT Europe GmbH
Type of request	New opinion

On 10 April 2014, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) of the European Food Safety Authority ('Authority'), in its opinion on the safety and efficacy of the product could not conclude on the safety of the L-threonine produced by fermentation with this recombinant strain of *E. coli* for target animals, consumer, user and the environment. Regardless of the assessment of the genetic modification, the FEEDAP Panel had concerns about the safety of amino acids, including L-threonine, for target species when administered simultaneously via water for drinking due to amino acids imbalances.

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

The Commission has now received new data on the safety of L-threonine, technically pure, produced by fermentation using *E. coli* CGMCC 3703.

In view of the above, the Commission asks the Authority to deliver a new opinion on the safety of L-threonine, technically pure, produced by fermentation using *E. coli* CGMCC 3703 as a nutritional additive for all animal species based on the additional data submitted by the applicant.

1.2. Additional information

In 2012, the European Food Safety Authority (EFSA) was requested by the European Commission (EC) to re-evaluate the product L-threonine produced by *E. coli* CGMCC 3703 when used as a nutritional additive (functional group: functional group amino acids, their salts and analogues) for all animal species without time limit and without maximum content in feed (EFSA FEEDAP Panel, 2014). The amino acid L-threonine itself was considered safe for target animals, consumers and the environment. However, the genetic modification, including the possible presence of recombinant DNA and of antibiotic resistance genes in the product, was insufficiently characterised. Consequently, the FEEDAP Panel could not conclude on the safety of the L-threonine produced by fermentation using *E. coli* CGMCC 3703 for target animals, consumer, user and the environment.

The applicant has provided additional information on the characterisation of the production microorganism, the characterisation of the additive and its stability.

2. Data and Methodologies

2.1. Data

The present assessment is based on the data submitted by the applicant in the form of additional information¹ following a previous application on the same product.²

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the characterisation and safety of L-threonine produced by *E. coli* CGMCC 3703 is consistent with the principles laid down in Regulation (EC) No 429/2008³ and the relevant guidance documents: Guidance on nutritional additives (EFSA FEEDAP Panel, 2012a), Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use (EFSA GMO Panel, 2011), Technical guidance - Update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance (EFSA, 2008, revised in 2012b), Technical Guidance: Microbial Studies (EFSA, 2008) and Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012c).

3. Assessment

L-Threonine, technically pure, (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 current application is for the authorisation of L-threonine, technically pure, produced by a genetically modified (GM) strain of *E. coli* (CGMCC 3703), a derivative of *E. coli* K-12. It is intended to be used in all animal species as a nutritional additive in feed and in water for drinking.

3.1. Characterisation

This product has been characterised in a previous scientific opinion (EFSA FEEDAP Panel, 2014). Additional data have been submitted on the characterisation of the production strain, on the physical properties of the additive and its stability in complete feed, and on the manufacturing process.

3.1.1. Characterisation of the production organism⁴

The technical dossier contains information on the susceptibility of the production strain against the list of antibiotics proposed for *E. coli* in the technical guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance (EFSA FEEDAP Panel, 2012).⁵

Although the parent strain *E. coli* K-12 is not included in EFSA's 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 (EPA, 1997; 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

¹ Dossier reference: FAD-2015-0012.

² Dossier reference: FAD-2010-0289.

³ 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.

⁴ This section of the scientific output is temporarily unavailable to the public pending a final decision on the confidentiality request submitted by the applicant. A revised version of the opinion will be made available as soon as this decision-making process is concluded.

⁵ Technical dossier/Supplementary information April 2015/Annex 2.1.3.

strain) against which the safety of other *E. coli* strains is assessed, (see Kaper et al., 2004). It can be therefore assumed that there is no antibiotic or enterotoxin production from *E. coli* K-12.

Characterisation of the genetic modification⁶

In the former assessment of the production strain (EFSA FEEDAP Panel, 2014), the genetic modification, including the presence/absence of recombinant DNA and of antibiotic resistance genes in the product, was insufficiently characterised.

The applicant provided additional information⁷ which contains sufficient details 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.

3.1.2. Physical properties

The applicant submitted data on dusting potential measured in three batches of the final product (Stauber–Heubach). The values ranged between 0.10 and 0.13 g/m³.⁸

3.1.3. Manufacturing process⁹

The applicant declared that no antimicrobial compounds (including antibiotics) were used in the production process.¹⁰ It has been demonstrated that the final product does not contain antimicrobial compounds.¹¹

Neither viable cells of the production strain,¹² nor its recombinant DNA was detected in three batches of the final product.¹³

3.1.4. Stability and homogeneity

In the previous opinion, the stability of the additive in complete feed and the capacity of the additive to distribute homogeneously in feed were evaluated by measuring total threonine (protein-bound plus free L-threonine) instead of free L-threonine. The FEEDAP Panel noted that the results obtained might not fully represent the stability of the added free amino acid or its ability to distribute homogeneously in compound feedingstuffs.

A new stability study in complete feed for piglets was provided. L-Threonine (three different batches) was used to supplement (0.15%) a mash and pelleted complete feed for piglets (three batches; total L-threonine content: 0.85%). Samples were collected after mixing and after pelleting at 80°C. The feedingstuffs were stored in paper bags for 3 months at 5–30°C and 30–75% relative humidity. No losses were detected as a result of processing or storage for three months.¹⁴

Free threonine was analysed in 10 subsamples of the above pelleted complete feed for piglets to determine the capacity of the additive to distribute homogeneously in feed. The coefficient of variation was 4.4%.¹⁵

⁶ This section of the scientific output is temporarily unavailable to the public pending a final decision on the confidentiality request submitted by the applicant. A revised version of the opinion will be made available as soon as this decision-making process is concluded.

⁷ Technical dossier/Section II/Annex 2.1.1.

⁸ Technical dossier/Supplementary information April 2015/Annex 2.1.9.

⁹ This section of the scientific output is temporarily unavailable to the public pending a final decision on the confidentiality request submitted by the applicant. A revised version of the opinion will be made available as soon as this decision-making process is concluded.

¹⁰ Technical dossier/Supplementary information April 2015/Annexes 2.2.1 and 2.2.2.

¹¹ Technical dossier/Supplementary information April 2015/Annexes 2.1.5 to 2.1.7 and Annex 2.1.4.

¹² Technical dossier/Supplementary information April 2015/Annex 2.2.4 and Supplementary information September 2015/Annex II.

¹³ Technical dossier/ Section II/Annex 2.2.5–2.2.10 and Supplementary information September 2015/Annex I.

¹⁴ Technical dossier/Supplementary information April 2015/Annex 2.1.8.

¹⁵ Technical dossier/Supplementary information April 2015/Annex 2.1.8.

3.2. Safety¹⁶

In the previous scientific opinion (EFSA FEEDAP Panel, 2014), the genetic modification of the production strain was insufficiently characterised and the safety of the strain could not be assessed. The applicant provided sufficient information that neither the production strain nor its recombinant genes are present in the final product, given the limits of detection provided. Therefore, L-threonine, manufactured by fermentation with *E. coli* CGMCC 3703, 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.

The applicant did not provide new data on the safety of the product for target species when administered in the diet and in addition via water for drinking. The FEEDAP Panel reiterates its concerns on the safety of the amino acid L-threonine for target species when administered via water for drinking.

The applicant has submitted new data on the dusting potential of the additive that allows estimating the risk posed by the bacterial endotoxin present in the final product for the user when exposed by inhalation. 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 was up to 0.55 IU/mg.¹⁷ Dusting potential was measured in three batches and ranged between 0.10 and 0.13 g/m³. Summary data provided for three batches indicate that 7% (w/w) of particles have a diameter below 100 µm and 1.7% < 50 µm.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 (2012c) 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 UK Health and Safety Executive (HSE, 2013). Based upon the calculation of the potential endotoxin content in dust, the inhalation exposure could be up to 40 endotoxin IU per eight-hour working day, indicating no risk from the exposure to endotoxins for people handling the additive.

4. Conclusions

Neither the production strain nor its recombinant genes are present in the final product. Therefore, the FEEDAP Panel concludes that L-threonine, manufactured by fermentation using *E. coli* CGMCC 3703, 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 FEEDAP Panel reiterates their concerns on the safety of amino acids, including L-threonine, for target species when administered simultaneously via water for drinking.

As the estimated maximum exposure to endotoxins by inhalation is below the provisional occupational exposure limit, no risk from the exposure to endotoxins for people handling the additive is expected.

5. Recommendations

The description of the additive should contain the statement 'produced by fermentation with *Escherichia coli* CGMCC 3703'.

¹⁶ This section of the scientific output is temporarily unavailable to the public pending a final decision on the confidentiality request submitted by the applicant. A revised version of the opinion will be made available as soon as this decision-making process is concluded.

¹⁷ Technical dossier FAD-2010-0289/Supplementary information November 2013/Annex 1.

¹⁸ Technical dossier FAD-2010-0289/Supplementary information January 2013/Attachment 2.

Documentation provided to EFSA

1. L-Threonine technically pure produced by fermentation with *E. coli*. Supplementary information. April 2015. Submitted by Global Bio-Chem (GBT Europe GmbH).
2. L-Threonine technically pure produced by fermentation with *E. coli*. Supplementary information. September 2015. Submitted by Global Bio-Chem (GBT Europe GmbH).

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Abbreviations

CGMCC	China General Microbiological Culture Collection Centre
DECOS	Dutch Expert Committee on Occupational Safety
DNA	deoxyribonucleic acid
EC	European Commission
EPA	Environmental Protection Agency of US
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
GM	genetically modified
GMO	EFSA Panel on Genetically Modified Organisms
HBROEL	Health-based recommended exposure limit
HSE	British Health and Safety Executive
IU	International unit of endotoxin activity. One IU corresponds to one EU (endotoxin unit).
LPS	lipopolysaccharide
PCR	polymerase chain reaction

Appendix – Safety for the user

The effects of 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, 2012c) for additives added in premixtures assumes a maximum of 40 periods of exposure per day, each comprising 20 s = $40 \times 20 = 800$ 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 m³/h, the inhalation volume providing exposure to potentially endotoxin-containing dust would be $0.444 \times 1.25 = 0.556$ m³ per day. This volume should contain no more than 900 IU endotoxin, so the dust formed from the product should contain no more than $900/0.556 = 1\,619$ IU/m³.

Calculation of endotoxin content of dust

Two key measurements are required to evaluate the potential respiratory hazard associated with endotoxin content of the product (the dusting potential of the product, expressed in g/m³; the endotoxin activity of the dust, determined by the *Limulus* amoebocyte lysate assay (expressed in IU/g)). If data for the dust are not available, the content of endotoxins of the product can be used instead. If the content of endotoxins of the relevant additive is a IU/g and the dusting potential is b g/m³, then the content of endotoxins of the dust, c IU/m³, is obtained by the simple multiplication $a \times b$. This resulting value is further used for calculation of potential inhalatory exposure by users to endotoxin from the additive under assessment (Table 2) (EFSA FEEDAP Panel, 2012c).

Table 2: Estimation of user exposure to endotoxins from the additive L-threonine produced by *Escherichia coli* CGMCC 3703, including consideration of using filter mask FF P2 or FF P3 as preventative measure

Calculation	Identifier	Description	Amount	Source
	a	Endotoxin content (IU/g product)	550	Technical dossier
	b	Dusting potential (g/m ³)	0.13	Technical dossier
a × b	c	Endotoxin content in the air (IU/m ³)	71.5	
	d	No of premixture batches made per working day	40	EFSA FEEDAP Panel (2012c)
	e	Time of exposure (s) per production of one batch	20	EFSA FEEDAP Panel (2012c)
d × e	f	Total duration of daily exposure/worker (s)	800	
	g	Uncertainty factor	2	EFSA FEEDAP Panel (2012c)
f × g	h	Refined total duration of daily exposure/worker (s)	1 600	
h/3 600	i	Refined total duration of daily exposure (h)	0.44	
	j	Inhaled air (m ³) per 8-hour working day	10	EFSA FEEDAP Panel (2012c)
j/8 × i	k	Inhaled air during exposure (m ³)	0.56	
c × k	l	Endotoxin (IU) inhaled during exposure per 8-hour working day	40	
	m	Health-based recommended exposure limit of endotoxin (IU/m ³) per 8-hour working day	90	Health Council of the Netherlands (2010)
m × j	n	Health-based recommended exposure limit of total endotoxin exposure (IU) per 8-hour working day	900	
l/10		Endotoxins inhaled (IU) per 8-hour working day reduced by filter mask FF P2 (reduction factor 10)	4.0	
l/20		Endotoxins inhaled (IU) per 8-hour working day reduced by filter mask FF P3 (reduction factor 20)	2.0	