DOI: 10.2903/j.efsa.2024.8708

SCIENTIFIC OPINION



Safety and efficacy of a feed additive consisting of L-threonine (produced with *Escherichia coli* CGMCC 7.455) for all animal species (Kempex Holland B.V.)

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Abstract

Following a request from the European Commission, EFSA was asked to deliver a scientific opinion on the safety and efficacy of the feed additive consisting of Lthreonine produced by fermentation with Escherichia coli CGMCC 7.455 when used as a nutritional additive in feed and water for drinking for all animal species and categories. The production strain is genetically modified. None of the introduced genetic modifications raised a safety concern. Viable cells of the production strain and its DNA were not detected in the final additive. Therefore, the final product does not give raise to any safety concern regarding the genetic modification of the production strain. The use of ∟-threonine (≥ 98.5%) produced with *E. coli* CGMCC 7.455 to supplement feed is safe for the target species. The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) has concerns on the safety of the simultaneous oral administration of L-threonine via water for drinking and feed due to possible amino acid imbalances and hygienic reasons. The use of Lthreonine produced with E. coli CGMCC 7.455 in animal nutrition raises no safety concerns to consumers of animal products and to the environment. In the absence of data, the FEEDAP Panel cannot conclude on the potential of the additive to be irritant to skin or eyes, or on its potential to be a dermal sensitiser. The endotoxin activity in the additive does not pose a risk for the user via inhalation. The additive L-threonine is regarded as an effective source of the amino acid L-threonine for all non-ruminant species. In order to be as efficacious in ruminants as in nonruminants, it should be protected from ruminal degradation.

KEYWORDS

amino acid, efficacy, Escherichia coli CGMCC 7.455, L-threonine, nutritional additive, safety

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EFSA Journal. 2024;22:e8708. https://doi.org/10.2903/j.efsa.2024.8708

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1 | INTRODUCTION

1.1 | Background and Terms of Reference

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

The European Commission received a request from Kempex Holland B.V.² for the authorisation of the additive consisting of L-threonine (produced with *Escherichia coli* CGMCC 7.455), when used as a feed additive for all animal species (category: nutritional additives; functional group: amino acids, their salts and analogues).

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive). The dossier was received on 27 January 2023 and the general information and supporting documentation are available at https://open.efsa.europa.eu/questions/EFSA-Q-2023-00049. The particulars and documents in support of the application were considered valid by EFSA as of 31 July 2023.

According to Article 8 of Regulation (EC) No 1831/2003, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment and on the efficacy of the feed additive consisting of L-threonine (produced with *E. coli* CGMCC 7.455), when used under the proposed conditions of use (see **Section 3.1.6**).

1.2 | Additional information

L-Threonine produced with *Escherichia coli* CGMCC 7.455 has not been previously authorised as a feed additive in the European Union.

2 | DATA AND METHODOLOGIES

2.1 | Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier³ in support of the authorisation request for the use of l-threonine (produced with *E. coli* CGMCC 7.455) as a feed additive.

The confidential version of the technical dossier was subject to a target consultation of the interested Member States from 17 August 2023 to 17 November 2023 for which the received comments were considered for the assessment.

In accordance with Article 38 of the Regulation (EC) No 178/2002⁴ and taking into account the protection of confidential information and of personal data in accordance with Articles 39 to 39e of the same Regulation, and of the Decision of EFSA's Executive Director laying down practical arrangements concerning transparency and confidentiality,⁵ a non-confidential version of the dossier has been published on Open.EFSA.

According to Article 32c(2) of Regulation (EC) No 178/2002 and to the Decision of EFSA's Executive Director laying down the practical arrangements on pre-submission phase and public consultations, EFSA carried out a public consultation on the non-confidential version of the technical dossier from 22 January to 12 February 2024 for which no comments were received.

The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) used the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA or other expert bodies, peerreviewed scientific papers, other scientific reports, to deliver the present output.

EFSA has verified the European Union Reference Laboratory report as it relates to the methods used for the control of the active substance in animal feed.⁶

⁵Decision https://www.efsa.europa.eu/en/corporate-pubs/transparency-regulation-practical-arrangements

⁶Evaluation report received on 06/10/2023 and available on the EU Science Hub https://joint-research-centre.ec.europa.eu/eurl-fa-eurl-feed-additives/eurl-fa-authorisat ion/eurl-fa-evaluation-reports_en

¹Regulation (EC) No 1831/2003 of the European Parliament and of the council of 22 September 2003 on the additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29. ²Kempex Holland B.V., Zeelandsedijk 15, 5408 SL, Volkel, The Netherlands.

³Dossier reference: FEED-2022-12513.

⁴Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, p. 1–48.

2.2 | Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of L-threonine (produced with *E. coli* CGMCC 7.455) is in line with the principles laid down in Regulation (EC) No 429/2008⁷ and the relevant guidance documents: Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012), Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017a), Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2017b), Guidance on the assessment of the safety of feed additives (EFSA FEEDAP Panel, 2017c), Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018a), Guidance on the characterisation of microorganisms used as feed additives for the environment (EFSA FEEDAP Panel, 2019), EFSA statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain (EFSA, 2021), and Guidance on the assessment of the safety of feed additives for the users (EFSA FEEDAP Panel, 2019).

3 | ASSESSMENT

The L-threonine under assessment is produced by fermentation with a genetically modified strain of *E. coli* (CGMCC 7.455) and it is intended to be used as a nutritional additive (functional group: amino acids, their salts and analogues) in feed and water for drinking⁸ for all animal species.

3.1 | Characterisation

3.1.1 | Characterisation of the production organism

The production strain is a genetically modified derivative of *E. coli* K12 **Example**, deposited in the China General Microbiological Culture Collection Center with accession number CGMCC 7.455.⁹

of the production strain confirmed its identity as an E.

coli K-12 derivative.¹⁰

and its ineffectiveness in colonising the human gut is reported (Smith, 1975).

The production strain was tested for its susceptibility to all the antimicrobials listed for '*Enterobacteriaceae*' in the Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018b).¹² All minimum inhibitory concentration (MIC) values were below or equal to the cut-off values set in the Guidance and the strain is considered susceptible to all relevant antimicrobials.

The WGS data of the production strain were interrogated for the presence of antimicrobial resistance

.¹³ The search evidenced

above the thresholds set by EFSA (EFSA, 2021),

Additional data demonstrated that the **Exercise** gene is intrinsic in *E. coli* (EFSA BIOHAZ Panel, 2023), and therefore, it can be concluded that no hits of concern were identified.

The WGS data of the production strain were also interrogated for the presence of toxin and virulence determinant genes.¹⁴

hits of concern were identified.

⁷Commission 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. ⁸RFI 240129 Q4 Statement conditions of use.

⁹2.2.1.2.a Certificate of deposition CGMCC 7.455.

¹⁰2.2.1.2.c Bioinformatin report T1445R1434.1_2022 and 2.2.1.2.c Bioinformatic report T1445R1434.2_2023_rev.

¹¹2.2.1.2.c Bioinformatin report T1445R1434.1_2022 and 2.2.1.2.c Bioinformatic report T1445R1434.2_2023_rev.

¹²2.2.2.2 MIC T1445R1386_2022_esigned.

¹³2.2.1.2.c Bioinformatin report T1445R1434.1_2022, 2.2.1.2.c Bioinformatic report T1445R1434.2_2023_rev, 2.2.1.2.c1 T1445_Annex1_Table_A1 and 2.2.1.2.c2 T1445_Annex1_Table_A2.

¹⁴2.2.1.2.c Bioinformatin report T1445R1434.1_2022 and 2.2.1.2.c Bioinformatic report T1445R1434.2_2023_rev.

3.1.1.1 | Information related to the genetically modified microorganism¹⁵

Description of the genetic modification



3.1.2 | Manufacturing process

L-Threonine is produced by fermentation with <i>E. coli</i> CGMCC 7.455. ¹⁶	

The applicant declared that no antimicrobials are used in the manufacturing process.¹⁷

3.1.3 | Characterisation of the additive

L-Threonine (International Union of Pure and Applied Chemistry (IUPAC) name: (2S,3R)-2-amino-3- hydroxybutanoic acid; synonyms: 2-amino-3-hydroxybutyric acid, α-amino-β-hydroxybutyric acid), a compound identified with the Chemical Abstracts Service (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 g/mol. The molecular formula of L-threonine is C₄H₉NO₃. The structural formula is given in Figure 1.

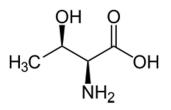


FIGURE 1 Structural formula of L-threonine.

¹⁵2.2.1.2.c Bioinformatin report T1445R1434.1_2022, 2.2.1.2.c Bioinformatic report T1445R1434.2_2023_rev and 2.2.1.2.b Modification process of Threonine producing strain.

¹⁶2.3.1.a and 2.3.1.c Amount of each used in the main fermentation Thr.

¹⁷2.3.1.b Statement letter antibiotic use.

According to the specification, the product contains \ge 98.5% L-threonine on a dry matter (DM) basis and \le 1% water. The analysis of five batches of the additive showed an average content of L-threonine of 100.7% on 'as is' basis (range 99.7%–101.2%).¹⁸ Moisture was 0.2% and residue on ignition 0.2%. On a DM basis, the L-threonine content was on average 100.9% (99.9%–101.4%), corresponding to the amount of identified material.

The specific optical rotation was measured in three batches of the additive and ranged from -28.2 to -28.3° .¹⁹ This range is within the reference values established for ∟-threonine in the European Pharmacopoeia (-27.6 to -29.0°) and confirmed the L-enantiomer of threonine (European Pharmacopoeia, 2023).

Three batches of the additive were analysed for cadmium, lead, mercury and arsenic. Arsenic, lead and mercury were below the limit of quantification (LOQ) of the corresponding methods.²⁰ Cadmium was < LOQ, except for one batch which was 0.003 mg/kg. Microbiological contamination was analysed in three batches and included Enterobacteriaceae, Escherichia *coli, Salmonella* spp., yeasts and filamentous fungi and none were detected in 25-g samples.²¹

Polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (PCBs) were analysed in three batches and found below the corresponding LOQ.²² The calculated upper bound concentration was 0.121 ng WHO-PCDD/F-TEQ/kg for the sum of dioxins and 0.237 ng WHO-PCDD/F-PCB-TEQ/kg for the sum of dioxins and dioxin-like-PCBs, respectively (all expressed in 88% dry matter).

The same three batches were analysed for mycotoxins.²³ Aflatoxins (not specified) ranged from 1.9 to 2.6 µg/kg; ochratoxin A was below the limit of detection (LOD) of the method in any batch; deoxynivalenol ranged between 149.2 and 386.8 µg/kg; fumonisins (B1 + B2 + B3) were below the LOD of the method, except for one batch which showed a value of 28.3 µg/kg; zearalenone and citrinin were below the LOD of the corresponding methods.

The FEEDAP Panel considers that the microbial contamination and the amounts of the above detected impurities do not raise safety concerns.

Endotoxin activity was analysed in three batches of the final product and in all cases the result was < 300 IU/g.²⁴

The presence of viable cells of the production strain was investigated

of the samples.

. No viable cells of the production strain were detected in any

The presence of DNA from the production strain was tested

. No DNA of the production strain was detected.

Physical properties of the additive 3.1.4

The additive appears as a white to beige crystalline powder.²⁷ The reported bulk density was 700 kg/m^{3.} The additive is soluble in water (> 33.3 g/L).²⁸

The dusting potential of three batches of the additive was determined using the Stauber-Heubach method and showed values < 100 mg/m³, the LOQ of the analytical method.²⁹

Stability and homogeneity 3.1.5

The shelf-life of the additive (three batches) was tested at room temperature when stored in bags, protected from light or moisture, for 6 months.³⁰ Losses < 3% were observed.

fumonisins; 1.8 μ g/kg for aflatoxins; 2.8 μ g/kg for ochratoxin A; 15 μ g/kg for citrinin.

²⁸Sect_2.1-2.2.

³⁰2.1.3 BtB purity stability 132446.

¹⁸2.1.3 BtB purity stability 132,446. Method of analysis in accordance with Commission Regulation 152/2009 Appendix III, method G and F.

¹⁹Other supporting document – Optical rotation threonine.

²⁰2.1.3 BtB purity stability 132446. Limit of detection were, respectively, 0.003 mg/kg for lead and arsenic; 0.0006 mg/kg for mercury and cadmium.

²¹2.1.3 BtB purity stability 132446.

²²2.1.4.a Dioxins. Upper bound concentrations are calculated on the assumption that all values of the different congeners below the limit of quantification are equal to the limit of quantification. TEQ = toxic equivalency factors for dioxins, furans and dioxin-like PCBs established by the WHO in 2005 (van den Berg et al., 2006). ²³2.1.3 BtB purity stability 132446 and RFI 240129 Q1 Statement LOD ochratoxin. LOD were, respectively, 134 µg/kg for deoxynivalenol; 17 for zearalenone; 25 µg/ for

²⁴2.1.4.b Endotoxins. Gel clot limit test. Ph Eur 2.6.14, method A. ²⁵2.1.4.c Absence viable cells T1445R1394.1_2022_esigned.

²⁶2.1.4.d Absence DNA T1445R1440_2022_confidential_esigned.

²⁷2.1.5.b Optical rotation L-Threonine (COA-5 Batches).

²⁹2.1.5.a Dust and RFI 240129 Q2 Statement Dusting potential. LOQ in g/m³ was 0.1.

The stability of the additive (three batches) in a premixture for piglets was studied when supplemented at an inclusion rate of 4%.³¹ The samples were stored at room temperature in paper bags protecting from light for 6 months. No losses were observed.

The stability of the additive (three batches) was studied in a complete feed, when supplemented at 0.2%. The basal diet consisted of **additive** (three batches) was studied in a complete feed, when supplemented at 0.2%. The basal diet consisted of **additive** (three batches) was and pelleted feed were tested after storage at room temperature in paper bags protecting from light for 3 months. Feed conditioning was performed at 60°C and the pelleting temperature was 73–75°C. Stability was maintained after pelleting the mash feeds. After 3 months of storage, no losses were observed neither in mash nor in pelleted feed.

The stability of the additive (three batches) in water for drinking was studied when supplemented at an inclusion rate of 0.2%.³³ The samples were stored at 20°C for 48 h. Negligible losses were observed.

One of the pelleted feeds described above was used to study the homogeneous distribution of the additive in feed.³⁴ Total threonine (protein-bound threonine + free threonine) was analysed in 10 subsamples. The coefficient of variation (CV) of total threonine was 3%. When the background content of threonine in the compound feed (protein bound threonine, 0.64%) was subtracted from the total threonine amount of each individual subsample, the resulting CV was 13%.

3.1.6 | Conditions of use

L-Threonine is intended to be used in complete feed for all animal species, directly or through complementary feed, premixtures or water. No inclusion levels have been proposed as the requirements, in quantitative terms, depend on the species, the physiological state of the animal, the performance level, the environmental conditions and the amino acid composition of the un-supplemented diet.

3.2 | Safety

3.2.1 | Safety of the production microorganism

The parental strain is *E. coli* K-12 which is considered to be safe. The genetic modifications performed to obtain the production strain *E. coli* CGMCC 7.455 have the purpose to increase the production of L-threonine. None of the introduced modifications raises a safety concern. The production strain does not carry acquired antimicrobial resistance genes. The production strain and its DNA were not detected in the final additive. Therefore, the final product does not raise any safety concern regarding the genetic modification of the production strain.

3.2.2 | Safety for the target species, consumers and the environment

The L-threonine requirements of the target animal species and the safety of this essential amino acid in non-ruminant and ruminant nutrition were summarised in previous opinions of the EFSA FEEDAP Panel (2013, 2015).

Safety concerns on the use of the additive may derive from the amino acid itself, L-threonine, and/or on the residues/ metabolites derived from the fermentation process.

The additive is produced by fermentation with a genetically modified *E. coli* K12 derivative and no safety concerns were identified for the production strain (See Section 3.2.1). Moreover, the additive is highly purified (\geq 98.5% L-threonine and the unidentified material is < 1% on a DM basis). The endotoxin activity was below 300 IU/g. These values are very low when compared with ca. 1,000,000 IU/g commonly found in feedingstuffs (Cort et al., 1990). The FEEDAP Panel considers that the use of the additive is safe for the target species when added to supplement the diets with appropriate amounts to satisfy animal requirements. Finally, the FEEDAP Panel reiterates its previous statement 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 (EFSA FEEDAP Panel, 2010). Moreover, it may result in an increased nitrogen excretion via urine. Therefore, the FEEDAP Panel has concerns on the safety of the simultaneous oral administration of threonine-containing additives via feed and water for drinking.

The absorption and metabolic fate of L-threonine in the organism were described in a previous opinion (EFSA FEEDAP Panel, 2013). The amino acid L-threonine, supplemented to feed, will be incorporated into proteins of tissues and/or products of animal origin and any of its potential excess will be metabolised and excreted. Therefore, the composition of tissues and products of animal origin will not be affected by the use of L-threonine in animal nutrition, which is thus considered safe for the consumer.

The amino acid L-threonine is a physiological and natural component of animal and plant proteins. When consumed, it will be absorbed, and the non-absorbed fraction will be incorporated into the intestinal microbial mass and excreted

³¹2.4.1.a Stability feed premix homogeneity 132,563 and RFI 240129 Q3 Statement conditions and pelleting.

³²2.4.1.a Stability feed premix homogeneity 132563, 2.4.1.c CONFID Feed composition RDS_5260 and RFI 240129 Q3 Statement conditions and pelleting.

³³2.1.3 BtB purity stability 132446.

³⁴2.4.1.a Stability feed premix homogeneity 132563.

as such. The use of amino acids 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 use of the additive L-threonine in animal nutrition would not lead to any localised increase in its concentration in the environment. The use of L-threonine produced with *E. coli* CGMCC 7.455 as a feed additive does not pose a risk to the environment.

3.2.2.1 | Conclusions on the safety for the target species, consumers and the environment

The use of L-threonine produced with *E. coli* CGMCC 7.455 to supplement feed is safe for the target species. The FEEDAP Panel has concerns on the safety of the simultaneous use of L-threonine via water for drinking and feed due to possible amino acid imbalances and hygienic reasons.

The use of L-threonine produced by fermentation with *E. coli* CGMCC 7.455 in animal nutrition is considered safe for the consumers and for the environment.

3.2.3 | Safety for the user

No specific data on inhalation toxicity, skin/eye irritation or dermal sensitisation were submitted to support the safety of the additive under assessment for the user. Although the dusting potential of the additive was < 100 mg/m³ (see Section 3.1.4), exposure of the users via inhalation cannot be excluded.

The bacterial endotoxin activity (analysed in three batches) was < 300 IU/g in any batch tested. The scenario used to estimate the exposure of persons handling the additive to endotoxins in the dust was based on the EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012) and considered a worst-case scenario in which the endotoxin activity was set at 300 IU/g and the dusting potential at 100 mg/m³. 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 is calculated as 17 endotoxin IU per working day, which compared with the threshold of 900 IU per working day indicates no risk resulting from exposure to endotoxins for the users.

In the absence of data, the FEEDAP Panel cannot conclude on the potential of the additive to be irritant to skin or eyes, or on its potential to be a dermal sensitiser. The endotoxin activity in the additive does not pose a risk for the user via inhalation.

3.3 | Efficacy

Efficacy studies are not required for amino acids naturally occurring in the proteins of plants and animals. The nutritional role of the amino acid L-threonine is well established in the scientific literature. The product L-threonine, technically pure, is regarded as an effective source of the amino acid L-threonine.

The efficacy of ∟-threonine for both non-ruminant and ruminant species was described in previous opinions (EFSA FEEDAP Panel, 2013, 2014). Supplemental ∟-threonine is degraded by ruminal microbiota if not given in a protected form.

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 Regulation³⁵ and good manufacturing practice.

4 | CONCLUSIONS

The production strain is genetically modified, and its viable cells and DNA were not detected in the final additive. Therefore, the final product does not give raise to any safety concern regarding the genetically modified production strain.

The use of L-threonine produced with *E. coli* CGMCC 7.455 in feed is safe for the target species. The FEEDAP Panel has concerns on the safety for the target species resulting from the simultaneous administration of L-threonine via water for drinking and feed due to possible amino acid imbalances and hygienic reasons.

The use of ∟-threonine produced by fermentation with *E. coli* CGMCC 7.455 in animal nutrition is considered safe for the consumers and for the environment.

In the absence of data, the FEEDAP Panel cannot conclude on the potential of the additive to be irritant to skin or eyes, or on its potential to be a dermal sensitiser. The endotoxin activity in the additive does not pose a risk for the user via inhalation.

The feed additive consisting of L-threonine produced by fermentation with *E. coli* CGMCC 7.455 is regarded as an effective source of the amino acid L-threonine for all non-ruminant species. In order to be as efficacious in ruminants as in non-ruminants, it should be protected from ruminal degradation.

ABBREVIATIONS

CAS	Chemical Abstracts Service
CGMCC	China General Microbiological Culture Collection
CV	coefficient of variation
DECOS	Dutch Expert Committee on Occupational Safety
DM	dry matter
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
IUPAC	International Union of Pure and Applied Chemistry
LOD	Limit of detection
LOQ	limit of quantification
MIC	minimum inhibitory concentration
PCBs	polychlorinated biphenyls
PCDDs	Polychlorinated dibenzo-p-dioxins
PCDFs	polychlorinated dibenzofurans
WGS	whole genome sequence

ACKNOWLEDGEMENTS

The Panel wishes to thank the following for the support provided to this scientific output (in alphabetical order of the last name): FEEDAP Working Group of Microbiology and Jaume Galobart.

CONFLICT OF INTEREST

If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

REQUESTOR

European Commission

QUESTION NUMBER

EFSA-Q-2023-00049

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How to cite this article: EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Bampidis, V., Azimonti, G., Bastos, M. L., Christensen, H., Durjava, M., Dusemund, B., Kouba, M., López-Alonso, M., López Puente, S., Marcon, F., Mayo, B., Pechová, A., Petkova, M., Ramos, F., Villa, R. E., Woutersen, R., Glandorf, B., Anguita, M., ... Pettenati, E. (2024). Safety and efficacy of a feed additive consisting of L-threonine (produced with *Escherichia coli* CGMCC 7.455) for all animal species (Kempex Holland B.V.). *EFSA Journal*, *22*(4), e8708. <u>https://doi.org/10.2903/j.efsa.2024.8708</u>



