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SCIENTIFIC OPINION

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Safety and efficacy of a feed additive consisting of L-isoleucine produced with *Corynebacterium glutamicum* CGMCC 20437 for all animal species (Eppen Europe SAS)

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) | **Vasileios Bampidis** | **Giovanna Azimonti** | **Maria de Lourdes Bastos** | **Henrik Christensen** | **Mojca Durjava** | **Birgit Dusemund** | **Maryline Kouba** | **Marta López-Alonso** | **Secundino López Puente** | **Francesca Marcon** | **Baltasar Mayo** | **Alena Pechová** | **Mariana Petkova** | **Fernando Ramos** | **Roberto Edoardo Villa** | **Ruud Woutersen** | **Anna Dioni** | **Joana Firmino** | **Matteo Innocenti** | **Jordi Tarrés-Call**

Correspondence: feedap@efsa.europa.eu

Abstract

Following a request from the European Commission, EFSA was asked to deliver a scientific opinion on the safety and efficacy of L-isoleucine produced by fermentation with *Corynebacterium glutamicum* CGMCC 20437 as a nutritional feed additive for use in feed and in water for drinking for all animal species. The production strain is non-genetically modified, qualifies for the QPS approach to safety assessment when used for production purposes, is susceptible to the relevant antibiotics and contains no antimicrobial resistance genes of concern. No viable cells of the production strain were detected in the final product. The additive does not give rise to any safety concern regarding the production strain. L-Isoleucine produced by fermentation with *Corynebacterium glutamicum* CGMCC 20437 is considered safe for the target species, the consumer and the environment. Regarding the use in water, the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) reiterates its concerns over the safety for the target species of L-isoleucine administered simultaneously via water for drinking and feed owing to the risk of nutritional imbalances and hygienic reasons. In the absence of data, the FEEDAP Panel is not in a position to conclude on the potential of L-isoleucine produced by fermentation with *Corynebacterium glutamicum* CGMCC 20437 to be irritant to skin and/or eyes, or as a dermal sensitiser. Due to the high dusting potential, exposure by inhalation is likely. L-Isoleucine produced by fermentation with *Corynebacterium glutamicum* CGMCC 20437 is considered as an efficacious source of the essential amino acid L-isoleucine for non-ruminant animal species. For the supplemental L-isoleucine to be as efficacious in ruminants as in non-ruminant species, it would require protection against degradation in the rumen.

KEYWORDS

amino acid, *Corynebacterium glutamicum* CGMCC 20437, efficacy, L-isoleucine, nutritional additive, safety

CONTENTS

1 | INTRODUCTION

1.1 | Background and Terms of Reference

Regulation (EC) No 1831/2003[1] 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 Eppen Europe SAS[2] for the authorisation of the additive consisting of L-isoleucine produced by fermentation with *Corynebacterium glutamicum* CGMCC 20437, 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 20 March 2023 and the general information and supporting documentation are available at https://open.efsa.europa.eu/questions/EFSA-Q-2023-00207. The particulars and documents in support of the application were considered valid by EFSA as of 11 October 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-isoleucine produced by fermentation with *Corynebacterium glutamicum* CGMCC 20437, when used under the proposed conditions of use (see Section 3.1.6).

1.2 | Additional information

The subject of the assessment is the feed additive consisting of L-isoleucine produced by fermentation with *Corynebacterium glutamicum* CGMCC 20437, intended for use as a nutritional additive in feed or water for drinking, for all animal species. This additive is not authorised in the European Union.

2 | DATA AND METHODOLOGIES

2.1 | Data

The present assessment is based on the data submitted by the applicant in the form of a technical dossier[3] in support of the authorisation request for the use of L-isoleucine as a feed additive.

The confidential version of the technical dossier was subject to a target consultation of the interested Member States from 10 October 2023 to 11 January 2024; the comments received were considered for the assessment.

In accordance with Article 38 of the Regulation (EC) No 178/2002[4] 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 13 February to 5 March 2024, for which no comments were received.

The EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) Panel used the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA, 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 L-isoleucine in animal feed.[6]

[1]Regulation (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.

[2]10 Rue de la Paix, 75,002, Paris (represented in the EU by Gang MENG).

[3]Dossier reference: FEED-2023-013997.

[4]Regulation (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.

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

[6]Evaluation report received on 05/12/2023 and available on the EU Science Hub https://joint-research-centre.ec.europa.eu/eurl-fa-eurl-feed-additives/eurl-fa-authorisation/eurl-fa-evaluation-reports_en

2.2 | Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of L-isoleucine produced by fermentation with *Corynebacterium glutamicum* CGMCC 20437 is in line with the principles laid down in Regulation (EC) No 429/2008[7] and the relevant guidance documents: 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 FEEEDAP Panel, 2017b), Guidance on the assessment of the safety of feed additives for the target species (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 or as production organisms (EFSA FEEDAP Panel, 2018b), the Guidance on the assessment of the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019) and Guidance on the assessment of the safety of feed additives for users (EFSA FEEDAP Panel, 2023).

3 | ASSESSMENT

The additive under assessment, L-isoleucine produced by fermentation with *Corynebacterium glutamicum* CGMCC 20437, is intended to be used as a nutritional additive (functional group: amino acids, their salts and analogues) in feed and water for drinking for all animal species.

3.1 | Characterisation

3.1.1 | Characterisation of the production organism

The additive L-isoleucine is produced by fermentation with a non-genetically modified strain of *C. glutamicum* which is deposited at the China General Microbiological Culture Collection Center (CGMCC) with the accession number 20437.[8] The strain was obtained [redacted],[9] and it has not been genetically modified.

The taxonomic identification of the production strain CGMCC 20437 as *C. glutamicum* was confirmed [redacted].[10] [redacted].

The susceptibility of the production strain to relevant antibiotics was tested against the list of antimicrobials described for '*Corynebacterium* and other Gram-positive' in the Guidance on characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018b).[11] All minimum inhibitory concentration (MIC) values were equal to or lower than the cut-off values specified in this guidance. Therefore, the strain is considered susceptible to the relevant antibiotics.

The WGS data of the production strain were searched for the presence of antimicrobial resistance (AMR) genes [redacted].[12] No hits of concern [redacted] were identified.

3.1.2 | Manufacturing process

L-Isoleucine is produced by fermentation with *Corynebacterium glutamicum* CGMCC 20437.[13] [redacted].[14]

[7]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.

[8]2.2.1.2a Strain deposition CGMCC20437.

[9]Annex 2.2.1.2b Strain development.

[10]Annex 2.2.1.2c Bioinformatic T1441R1398 2022 e signed.

[11]Annex 2.2.2.2 MIC antimicrobial susceptibility T144R1379_2022.

[12]Annex 2.2.1.2c Bioinformatic T1441R1398 2022 e signed.

[13]2.3.1.a Manufacturing.

[14]2.31.b Statement of no antibiotics-CGMCC20437.

3.1.3 | Characterisation of the active substance/additive

The active substance of the additive is L-isoleucine (International Union of Pure and Applied Chemistry name: (2S,3S)-2-amino-3-methylpentanoic acid, a compound identified with the Chemical Abstracts Service No 73–32-5, the European Inventory of Existing Commercial Chemical Substances No 200–798-2) and has a molecular mass of 131.17 g/mol. The chemical formula of L-isoleucine is $C_6H_{13}NO_2$ and the structural formula is presented in Figure 1.

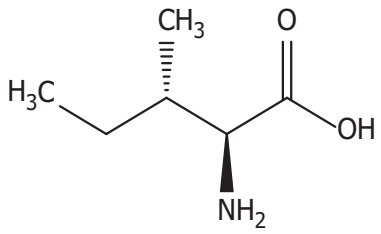

FIGURE 1 Structural formula of L-isoleucine.

The applicant declared that the additive contains by specification ≥ 93.5% L-isoleucine (on a dry matter (DM) basis), ≤ 2% moisture. Batch-to-batch variation data were provided for five batches of the additive.[15] The content of the active substance was on average 98.4% (range: 98.0%–98.8%) on a DM basis. The loss on drying ranged 0.2%–0.3%.[16]

The specific optical rotation was measured in three batches of the final product and ranged between +39.5° and +40.1°,[17] which is according to the specifications set by the applicant (+38.5° to +41.5°). A deviation is noted for one batch with respect to the European Pharmacopoeia range (+40° to +43°) of the specific optical rotation for this substance (PhEur, 2023).

Three batches of the additive were analysed for impurities.[18] Arsenic was below the limit of quantification (< LOQ)[19] of the analytical method; cadmium was 0.0021 mg/kg in all three batches; lead ranged 0.01–0.012 mg/kg except for one batch that was below the LOQ of the analytical method; and mercury ranged 0.016–0.017 mg/kg.[20] The analysis of the mycotoxins zearalenone, fumonisins B1 + B2 + B3, deoxynivalenol and citrinin showed values found below the limit of detection (LOD) of the respective analytical methods, while aflatoxins (not specified) and ochratoxin A were below their LOQs.[21]

Polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (DL-PCBs) were analysed in three batches and found below the corresponding LOQ except for one batch in which the concentration of PCB no. 105 was 8 ng/kg. The calculated upper bound (UB) concentration in all three batches was 0.137 ng WHO-TEQ/kg for the sum of PCDD/Fs, and 0.269 ng WHO-TEQ/kg for the sum of PCDD/Fs and DL-PCBs. The UB for the sum of non DL-PCBs was 0.003 mg/kg (in all three batches).[22]

Microbiological contamination was analysed by the determination of *Enterobacteriaceae*, *Escherichia coli*, *Salmonella* spp., yeasts and filamentous fungi and the results were below the LOD in all cases.[23]

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

The presence of viable cells of the production strain was investigated in three batches of the final product,[24] each batch tested in triplicate.[25] [redacted]. No colonies were detected in the plates inoculated with the samples of the batches tested. [redacted]

3.1.4 | Physical properties of the additive

The additive is an odourless white solid.[26] Its bulk density is 480–700 kg/m^3 and its solubility in water is reported to be > 33.3 g/L.[27]

[15] 2.1.3 BtB Impurity stability 129720. Analytical method for L-isoleucine stated to be according to EC Regulation 152/2009, Appendix III, G and F method.
[16] 2.1.3 BtB Impurity stability 129720.
[17] 2.1.5b Optical rotation.
[18] 2.1.3 BtB Impurity stability 129720.
[19] Other supporting document –2.1.3.a Statement Q2 RFI 240212.
[20] 2.1.3 BtB Impurity stability 129720 LOQ in mg/kg was 0.01 for arsenic and for lead.
[21] 2.1.3 BtB Impurity stability 129720 LODs in µg/kg were: 17–41 for zearalenone, 25 for fumonisine B1 + B2 + B3, 134 for desoxynivalenol and 15 for citrinin. LOQs in µg/kg were 5.8 for aflatoxins and 8.3 for ochratoxin A.
[22] 2.1.4.a Dioxins Isoleucine 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 PCDD/Fs and DL-PCBs established by WHO in 2005 (van den Berg et al., 2006).
[23] 2.1.3 BtB Impurity stability 129,720 Sect_2.6 LOD was 1 colony per 25 g sample in all cases.
[24] Other supporting document – 2.1.4.b1 Statement Q1 RFI 240212.
[25] 2.1.4b viable cells T1441R399_2022_esigned.
[26] 2.1.3a Statement Q2 RF1 240,212.
[27] Sect_2.1–2.2.

The dusting potential of three batches of the additive was determined using the Stauber–Heubach method and showed values ranging from 1.3 to 2.5 g/m^3.[28]

3.1.5 | Stability and homogeneity

The shelf life of the additive (three batches) was studied when stored at room temperature in commercial packaging bags (protected from light and moisture) for 6 months. No losses at the end of storage period were detected.[29]

The stability of the additive (three batches) in a premixture was studied when supplemented at 4% and stored at room temperature in paper bags for 6 months. No losses were detected.[30]

The stability of the additive (three batches) in a mash feed consisting of wheat, soybean meal and maize was studied when supplemented at 0.2%, stored in paper bags at room temperature for 3 months. No losses at the end of the storage period were observed. The stability of the additive in pelleted feed was studied in the mash feed described above (pelleting at 81°C),[31] under the same packaging and storage conditions, and no loss was detected. The pelleting process did not result in a loss of L-isoleucine.[32]

The stability of three batches of the additive in water for drinking was studied at an intended concentration of 0.2% for 48 h. No losses were observed at the end of the storage period.[33]

The homogeneous distribution of the additive (one batch) was studied in 10 subsamples of the pelleted feed described above which contained a background concentration of isoleucine (protein-bound isoleucine) of 0.85%. When total isoleucine (protein-bound plus free isoleucine) was analysed, the coefficient of variation (CV) was 4.1%. When the background concentration of isoleucine was subtracted from each subsample, the CV was 23.0%.[34]

3.1.6 | Conditions of use

L-Isoleucine is intended to be used directly in feedingstuffs/complementary feedingstuffs or via premixture and in water for drinking for all animal species. No inclusion levels are proposed, as the optimal daily allowance in quantitative terms depends on the species, the physiological state of the animal, the performance level and the environmental conditions, in particular on the amino acid composition of the unsupplemented diet.

3.2 | Safety

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

L-Isoleucine is one of the three branched-chain amino acid (BCAA) together with leucine and valine and one of the nine essential amino acids. The interaction of BCAAs fed at excessive levels has already been described in former FEEDAP opinions (EFSA, 2008; EFSA FEEDAP Panel, 2013, 2020).

Safety concerns from the additive may derive either from the amino acid itself (excessive levels referred above) and/or on the residues/metabolites derived from the fermentation process. The L-isoleucine under assessment is highly purified (≥ 93.5% L-isoleucine on a DM basis). The production strain was developed to increase the production of L-isoleucine and belongs to a species, *C. glutamicum*, that qualifies for the QPS approach to safety assessment when used for production purposes (EFSA BIOHAZ Panel, 2023). It is susceptible to the relevant antibiotics, [redacted], and no viable cells of the production strain were detected in the final product. It can be concluded that no safety concerns for target animals, consumers and the environment would rise from the fermentation material present in the final additive.

The use of amino acids in water for drinking, in addition to complete diets with a well-balanced amino acid profile, may represent a risk for the target species due to nutritional imbalances and hygienic 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 isoleucine-containing additives via feed and water for drinking.

Regarding the safety of the amino acid for consumers, L-isoleucine supplemented to feed will be incorporated into proteins of tissues and/or products of animal origin and any potential excess will be metabolised and excreted as urea/uric acid and as carbon dioxide. Therefore, the composition of tissues and products of animal origin will not be modified by the use of L-isoleucine in animal nutrition.

[28] 2.1.5.a Dust Isoleucine.
[29] Sect_2.4, 2.4.1.a Stability feed premix homogeneity 129957.
[30] 2.4.1.a Stability feed premix homogeneity 129957.
[31] Other supporting document – 2.4.1.a1 Statement Q3 RFI 240212.
[32] 2.4.1.a Stability feed premix homogeneity 129957.
[33] 2.4.1.a Stability feed premix homogeneity 129957.
[34] 2.4.1.a Stability feed premix homogeneity 129957.

The amino acid L-isoleucine is a physiological and natural component of the proteins of living organisms. When consumed, it will be absorbed, and the non-absorbed fraction will be incorporated into the intestinal microbial mass and excreted as such. Its use in animal nutrition would not lead to any localised increase of its concentration in the environment.

The FEEDAP Panel concludes that the use of L-isoleucine produced by fermentation with *Corynebacterium glutamicum* CGMCC 20437 to cover nutritional needs of animals is safe for the target species, for the consumer and for the environment.

3.2.2 | Safety for the user

No information was provided on the safety of the additive under assessment for users/workers.[35] The dusting potential of the additive (highest measured value 2.5 g/m^3, see Section 3.1.4) indicates that the user may be exposed by inhalation. In the absence of data, the FEEDAP Panel is not in the position to conclude on the potential of the additive to be irritant to skin and/or eyes, or as a dermal sensitiser.

3.3 | Efficacy

Efficacy studies are not required for amino acids naturally occurring in proteins of plants and animals. The nutritional role of the amino acid L-isoleucine is well established in the scientific literature. The additive L-isoleucine is regarded as an effective source of isoleucine for non-ruminant animal species. For the supplemental L-isoleucine to be as efficacious in ruminants as in non-ruminant species, it would require protection against degradation in the rumen.

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[36] and Good Manufacturing Practice.

4 | CONCLUSIONS

L-Isoleucine produced by fermentation with *Corynebacterium glutamicum* CGMCC 20437 is considered to be safe for the target species, for the consumer and for the environment. Regarding the use in water, the FEEDAP Panel reiterates its concerns over the safety for the target species of L-isoleucine administered simultaneously via water for drinking and feed owing to the risk of nutritional imbalances and hygienic reasons.

In the absence of data, the FEEDAP Panel is not in a position to conclude on the potential of L-isoleucine produced by fermentation with *Corynebacterium glutamicum* CGMCC 20437 to be irritant to skin and/or eyes, or as a dermal sensitiser. Due to the high dusting potential, exposure by inhalation is likely.

L-Isoleucine produced by fermentation with *Corynebacterium glutamicum* CGMCC 20437 is considered as an efficacious source of the essential amino acid L-isoleucine for non-ruminant animal species. For the supplemental L-isoleucine to be as efficacious in ruminants as in non-ruminant species, it would require protection against degradation in the rumen.

ABBREVIATIONS

CV	coefficient of variation
DM	dry matter
EINECS	European Inventory of Existing Commercial Chemical Substances
FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
LOD	limit of detection
LOQ	limit of quantification
MIC	minimum inhibitory concentration

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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.

[35]Other supporting document – 3.3.a Statement Q4 RF1 240212.

[36]Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

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PANEL MEMBERS
Vasileios Bampidis, Giovanna Azimonti, Maria de Lourdes Bastos, Henrik Christensen, Mojca Durjava, Birgit Dusemund, Maryline Kouba, Marta López-Alonso, Secundino López Puente, Francesca Marcon, Baltasar Mayo, Alena Pechová, Mariana Petkova, Fernando Ramos, Roberto Edoardo Villa and Ruud Woutersen.

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