Safety and efficacy of a feed additive consisting of L-arginine (produced by Corynebacterium glutamicum CGMCC 20516) for all animal species (Eppen Europe SAS)

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Abstract

Following a request from the European Commission, the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the safety and efficacy of the feed additive consisting of L-arginine produced by fermentation using a non-genetically modified strain of Corynebacterium glutamicum (CGMCC 20516). The additive is intended to be used in feed and water for drinking for all animal species and categories. The production strain was considered suitable for the qualified presumption of safety (QPS) approach to safety assessment. No viable cells of C. glutamicum CGMCC 20516 were detected in the final product. L-Arginine produced using C. glutamicum CGMCC 20516 is safe for the target species when supplemented in appropriate amounts to the diet according to the nutritional needs of the target species, for the consumer and for the environment. In the absence of data, the FEEDAP Panel could not conclude on the safety of L-arginine produced by C. glutamicum CGMCC 20516 for the users. The additive L-arginine produced by fermentation using C. glutamicum CGMCC 20516 is regarded as an efficacious source of the essential amino acid L-arginine for non-ruminant species. For the supplemental L-arginine to be as efficacious in ruminants as in non-ruminant species, it requires protection against degradation in the rumen.

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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 \(\text{L-arginine produced by Corynebacterium glutamicum CGMCC 20516, 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). EFSA received directly from the applicant the technical dossier in support of this application. The particulars and documents in support of the application were considered valid by EFSA as of 12 November 2021.

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 \(\text{L-arginine produced by C. glutamicum CGMCC 20516, when used under the proposed conditions of use (see Section 3.1.6).}

1.2. Additional information

\(\text{L-Arginine produced by C. glutamicum CGMCC 20516 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 dossier\(^3\) in support of the authorisation request for the use of \(\text{L-arginine produced by C. glutamicum CGMCC 20516 as a feed additive.}

The FEEDAP Panel used the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA or other expert bodies, peer-reviewed scientific papers, and other scientific reports, to deliver the present output.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of \(\text{L-arginine in animal feed. The Executive Summary of the EURL report can be found in Annex A.}\(^4\)

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of \(\text{L-arginine produced by C. glutamicum CGMCC 20516 is in line with the principles laid down in Regulation (EC) No 429/2008}\(^5\) 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, 2017b), Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2017c), Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017d),}

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\(^1\) 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.

\(^2\) Eppen Europe SAS, 10 Rue de la Paix 75,002 Paris, France.

\(^3\) FEED dossier reference: FAD-2021-0038.

\(^4\) The full report is available on the EURL website: https://joint-research-centre.ec.europa.eu/publications/fad-2021-0038_en.

\(^5\) 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.
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) and Guidance on the assessment of the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019).

3. Assessment

The additive L-arginine produced by fermentation using *C. glutamicum* CGMCC 20516 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 is produced by a non-genetically modified strain of *C. glutamicum* which is deposited in the China General Microbiological Culture Collection Center (CGMCC) with accession number CGMCC 20516.6

The genome of the production strain was sequenced and used for identification purposes.7 The taxonomic identification of the production strain CGMCC 20156 as *C. glutamicum* was confirmed Therefore, the production strain was unambiguously identified as *C. glutamicum*.8

A high-yielding L-arginine strain was selected and named/deposited as CGMCC 20516.9

The susceptibility of the production strain to the battery of antibiotics recommended by the FEEDAP Panel (EFSA FEEDAP Panel, 2018b) was tested by broth microdilution following the method of the Clinical and Laboratory Standards Institute (CLSI).11 All the minimum inhibitory concentration (MIC) values were below the corresponding cut-off values for ‘*Corynebacterium* and other Gram-positive’ (EFSA FEEDAP Panel, 2018b). Therefore, the production strain is considered susceptible to all relevant antibiotics.

The WGS data of the production strain was interrogated for the presence of antimicrobial resistance (AMR) genes

10 No hits of concern were identified.

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6 Technical dossier/Section II/Annexes/Annex 2.2.1.2.a CONFID.
7 Technical dossier/Section II/Annexes/Annex 2.2.1.2.c CONFID, Annex 2.2.1.2.d CONFID and Annex 2.2.1.2.e CONFID.
8 Technical dossier/Supplementary Information March 2022/EFSA-Q-2021-00494_SIn_211210 Answers and Annex 1.d CONFID.
9 Technical dossier/Section II/Annexes/Annex 2.2.1.2.b CONFID.
10 Technical dossier/Section II/Annexes/Annex 2.2.1.2.c CONFID.
11 Technical dossier/Section II/Annexes/Annex 2.2.2.2.
3.1.2. Manufacturing process

L-Arginine is produced by fermentation using *C. glutamicum* CGMCC 20516.\(^\text{12}\) The applicant stated that no antimicrobials are used during the manufacturing process.\(^\text{13}\)

3.1.3. Characterisation of the active substance

L-Arginine (International Union of Pure and Applied Chemistry (IUPAC) name: (S)-2-amino-5-guanidinopentanoic acid; synonym 2-amino-5-guanidinova leric acid, a compound identified with the Chemical Abstracts Service (CAS) No 74–79-3, and the European Inventory of Existing Commercial chemical Substances (EINECS) No 200–811-1. It has a molecular mass of 174.2 Da. The molecular formula of L-arginine is C\(_6\)H\(_{14}\)N\(_4\)O\(_2\). The structural formula is given in Figure 1.

![Figure 1: Structural formula of L-arginine](image)

The applicant declared that the product contains by specification \(\geq 98.5\%\) L-arginine on a dry matter (DM) basis and \(\leq 1\%\) water. Batch-to-batch variation data were provided for five batches of the additive.\(^\text{14}\) The content of the active substance for the additive was on average 100.1\% (range: 99.7–100.4\%) on DM basis. Moisture ranged from 0.4\% to 0.8\%.

The specific optical rotation measured in three batches of the additive ranged from +26.9 to +27.1° which falls within the reference range (+25.5 to +28.5°) set in the European Pharmacopoeia (2020) and confirms the L-enantiomer of arginine in the additive.\(^\text{15}\)

Three batches of the product were analysed for chemical impurities.\(^\text{16}\) Lead, cadmium, mercury and arsenic were below their limits of quantification (LOQs). Polychlorinated dibeno-p-dioxins and dibenzofurans (PCDD/F) and dioxin-like polychlorinated biphenyls (PCBs) levels were below the corresponding LOQs in three batches of the additive.\(^\text{17}\) The levels of dioxins and the sum of dioxins and dioxin-like-PCBs (upper bond) were calculated to be 0.14 ng WHO-PCDD/F-TEQ/kg and 0.27 ng WHO-PCDD/F-PCB-TEQ/kg, respectively. Regarding the mycotoxin content, the analysis of those batches showed that the levels of aflatoxins (not specified), ochratoxin A and zearalenone were below their corresponding limit of detection (LOD). Fumonisin (B1 + B2 + B3), deoxynivalenol and citrinin ranged between \(<\) LOD = 44.1 \(\mu\)g/kg, 192–231.4 \(\mu\)g/kg and 43.4–223.7 \(\mu\)g/kg, respectively.\(^\text{18}\)

The same three batches of the product were analysed for microbiological contamination: *Salmonella* spp., yeasts and moulds, Enterobacteriaceae and *Escherichia coli* were not detected in 25 g.\(^\text{19}\)

The detected amounts of the above impurities do not raise safety concerns.

\(^\text{12}\) Technical dossier/Section II/Annexes/Annex 2.3.1.a and Supplementary information March 2022/Annex 3.a CONFID.

\(^\text{13}\) Technical dossier/Section II/Annexes/Annex 2.3.1.b.

\(^\text{14}\) Technical dossier/Section II/Annexes/Annex 2.1.3.a and Supplementary information March 2022/EFDAQ-2021-00494_SI n_211210 Answers. Analytical method to determine L-arginine in the additive was stated to be VDLUFA III.4.11.1.

\(^\text{15}\) Technical dossier/Section II/Annexes/Annex 2.1.5.g to I and Supplementary information March 2022/Annex 4.

\(^\text{16}\) Technical dossier/Supplementary information March 2022/Annex 5. Limit of quantification in \(\mu\)g/kg were 10 for lead and arsenic and 2 for mercury and cadmium.

\(^\text{17}\) Technical dossier/Section II/Annexes/Annex 2.1.4.a to c.

\(^\text{18}\) Technical dossier/Supplementary information March 2022/Annex 5. Limit of detection in \(\mu\)g/kg were 1.8 for aflatoxins, 134 for deoxynivalenol, 17 for zearalenone, 25 for fumonisin, 2.8 for ochratoxin and 15 for citrinin.

\(^\text{19}\) Technical dossier/Supplementary information March 2022/Annex 5.
The presence of viable cells of the production strain was tested in three batches of the final product. Samples of 10 g of each batch (triplicate samples) were dissolved in 80 mL of 0.9% NaCl, the pH was adjusted to 6.5–7.5 and the volume was adjusted to 90 mL. From the diluted sample, 10 mL (corresponding to 1 g of the original sample) were taken and passed through a 0.45-μm mixed cellulose esters (MCE) filter; the filter was then placed on selective medium (Corynebacterium agar containing 128 mg/L fosfomycin) and incubated at 30°C for 5 days. Positive controls with samples from each of the three batches and 0.9% NaCl spiked with the production strain were included. No growth was detected in any of the three batches tested.

3.1.4. Physical properties of the additive

The additive is a white crystalline powder with an approximate density of 1,400–1,500 kg/m³ and a solubility in water of 148.7 g/L (20°C). The pH (1 g/20 mL, measured in three batches) was on average 10.6 (range 10.5–10.8). The dusting potential was analysed (Stauber–Heubach method) in three batches of the final product. The values ranged from 2.8 to 3.1 g/m³. The particle size distribution of three batches of the final product was measured (laser diffraction). The fractions of particles with diameters < 10 μm, < 50 μm and < 100 μm ranged 1–2%, 5–7% and 12–16%, respectively.

3.1.5. Stability and homogeneity

The shelf-life of the additive (3 batches) was studied when stored at room temperature or at 40°C (packaging not described) for 6 months. No losses were observed at the end of the storage period at both conditions.

The stability of the additive (three batches) in vitamin-mineral premixture for poultry (containing choline chloride) was studied when supplemented at 4% and stored at room temperature in paper bags free from light for 6 months. Losses at the end of the storage period ranged from 11.2% to 22.4%.

The stability of the additive (three batches) in feed (mash and pellet form) for poultry was studied when supplemented at 0.2%, stored at room temperature in paper bags free from light for 3 months. The diet consisted of wheat, soybean meal, rapeseed meal, soybean oil and contained a background concentration of arginine of 1.27%. The pelleting process was performed at 78–79°C and resulted in a loss ranging from 1% to 4%. Losses at the end of the storage period ranged from 6.1% to 9.4% in the mash feed and were up to 3.5% in pelleted feed.

The stability of the additive (three batches) in water for drinking was studied when supplemented at 0.2%. Samples were stored at 20°C for 48 h. Losses at the end of the storage period were on average 6.7%.

The capacity for homogeneous distribution of the additive in feed was studied in 10 subsamples of the poultry pelleted feed described above. Total arginine was analysed and the background concentration of arginine in feed was subtracted. The resulting coefficient of variation was 10.3%.

3.1.6. Conditions of use

L-Arginine is intended to be used in feed and water to achieve an adequate amino acid profile and to meet the L-arginine requirements for all animal species. It can be added directly to complete feed, through complementary feed, premixtures and 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 unsupplemented diet.

20 Technical dossier/Section II/Annexes/Annex 2.1.4.d.
21 Technical dossier/Section II/Annexes/Annex 2.5.2.
22 Technical dossier/Section II/Annexes/Annex 2.1.5.g to f.
23 Technical dossier/Section II/Annexes/Annex 2.1.5.d to f.
24 Technical dossier/Section II/Annexes/Annex 2.1.5.a to c.
25 Technical dossier/Section II/Annexes/Annex 2.4.1.a to c and Supplementary information March 2022/Annex 7.
26 Technical dossier/Supplementary information March 2022/Annex 7.
3.2. Safety

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

Safety concerns from the additive may derive either from the amino acid or from the residues of the fermentation process/production strain remaining in the final product. The L-arginine under assessment is highly purified (less than 1% unidentified material). The production strain CGMCC 20516 belongs to a species, *C. glutamicum*, that qualifies for the qualified presumption of safety (QPS) approach to safety assessment (EFSA, 2007a) when used for production purposes (EFSA BIOHAZ Panel, 2020). The strain was unambiguously identified as *C. glutamicum* and was shown to be susceptible to all relevant antibiotics. There were also no viable cells in the final product. It can be concluded that no safety concerns for target animals, consumers and the environment would arise from the fermentation residues that may be present in the final additive.

The metabolism of L-arginine, the lysine-arginine antagonism, the arginine requirements for the different species/categories and the effects of supplementation of arginine in excess to the diets have been already reviewed in former FEEDAP opinions (EFSA, 2007b; EFSA FEEDAP Panel, 2016).

L-Arginine produced by *C. glutamicum* CGMCC 20516 is safe for the target species when supplemented in appropriate amounts to the diet according to the nutritional needs of the target species. Regarding the use in water, the FEEDAP Panel reiterates its concerns over the safety for the target species of L-arginine administered simultaneously via water for drinking and feed owing to the risk of nutritional imbalances and hygienic reasons (EFSA FEEDAP Panel, 2010).

The amino acid L-arginine 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 biomass or excreted as such. The absorbed L-arginine will be incorporated into body proteins or excreted as urea/uric acid and as carbon dioxide. The use of L-arginine produced by *C. glutamicum* CGMCC 20516 in animal nutrition is considered safe for the consumers and for the environment.

3.2.2. Safety for the user

No studies were submitted to support the safety of the additive for the user.

The additive under assessment is a powder with a dusting potential up to 3.1 g/m³ and containing a fraction of inhalable particles (< 100 μm diameter) up to 16%. Therefore, exposure of users by inhalation is possible.

In the absence of data, the FEEDAP Panel cannot conclude on the safety of L-arginine produced by *C. glutamicum* CGMCC 20516 for the users.

3.3. Efficacy

Efficacy studies are not required for amino acids that occur naturally in plant and animal proteins. The nutritional role of the amino acid L-arginine is well established in the scientific literature.

In beef or dairy cattle fed a variety of diets, L-arginine has not been identified to be limiting (Schwab et al., 2005). The rapid degradation of L-arginine by ruminal microorganisms has been described in a previous opinion (EFSA FEEDAP Panel, 2017a). Consequently, for the supplemental L-arginine to be as efficacious in ruminants as in non-ruminant species, it requires 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 and Good Manufacturing Practice.

4. Conclusions

L-Arginine produced using *C. glutamicum* CGMCC 20516 is safe for the target species when supplemented in appropriate amounts to the diet according to the nutritional needs of the target species.
species. The FEEDAP Panel has concerns on the use of amino acids in water for drinking for hygienic reasons, and due to the risk of imbalances when administered simultaneously via feed and water.

The use of L-arginine produced using *C. glutamicum* CGMCC 20516 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 safety of L-arginine produced by *C. glutamicum* CGMCC 20516 for the users.

The additive L-arginine produced by fermentation using *C. glutamicum* CGMCC 20516 is regarded as an efficacious source of the essential amino acid L-arginine for non-ruminant nutrition. For the supplemental L-arginine to be as efficacious in ruminants as in non-ruminant species, it requires protection against degradation in the rumen.

### 5. Documentation provided to EFSA/Chronology

| Date       | Event                                                                 |
|------------|----------------------------------------------------------------------|
| 19/03/2021 | Dossier received by EFSA. L-Arginine produced by *Corynebacterium glutamicum* CGMCC 20516 for all animal species. Submitted by Eppen Europe SAS. |
| 06/07/2021 | Reception mandate from the European Commission                        |
| 12/11/2021 | Application validated by EFSA – Start of the scientific assessment    |
| 10/12/2021 | Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterisation, user safety* |
| 18/01/2022 | Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives |
| 02/03/2022 | Comments received from Member States                                    |
| 08/03/2022 | Reception of supplementary information from the applicant - Scientific assessment re-started |
| 29/06/2022 | Opinion adopted by the FEEDAP Panel. End of the Scientific assessment |

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Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| AMR          | antimicrobial resistance          |
| ANI          | average nucleotide identity       |
| ATCC         | American Type Culture Collection  |
| CAS          | Chemical Abstracts Service        |
| CGMCC        | China General Microbiological Culture Collection Center |
| EINECS       | European Inventory of Existing Commercial chemical Substances |
| EURL         | European Union Reference Laboratory |
| FEEDAP       | EFSA 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  |
| QPS          | qualified presumption of safety   |
| PCB          | polychlorinated biphenyl          |
| PCDD/F       | polychlorinated dibenzo-p-dioxins and dibenzofurans |
| WGS          | whole genome sequence            |

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Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of the Analysis for L-arginine produced by fermentation with Corynebacterium glutamicum CGMCC 20516

In the current application an authorisation is sought under Article 4(1) for L-arginine produced by fermentation with Corynebacterium glutamicum CGMCC 20516, under the category/functional group 3 (c) ‘nutritional additives’/amino acids, their salts and analogues’ according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for all animal species [2].

According to the Applicant, the feed additive (L-arginine) has a minimum purity (mass fraction) of 98.5%. The feed additive is intended to be added directly into feedingstuffs or through premixtures and water for drinking with no proposed minimum or maximum limits of L-arginine in feedingstuffs.

For the identification of L-arginine in the feed additive the EURL recommends “L-arginine” monograph of the Food Chemical Codex (FCC).

For the determination of L-arginine in the feed additive, premixtures, feedingstuffs and water the Applicant proposed the ring-trial validated EN ISO 13903 method, which is equivalent to the European Union (EU) method for the determination of amino acids (Commission Regulation (EC) No 152/2009 (Annex III, F)).

The EU method is dedicated for the determination of free (synthetic and natural) and total (peptide-bound and free) amino acids (including arginine) in premixtures and feedingstuffs using an ion-exchange chromatography (IEC) coupled to post-column derivatisation and optical (visible -VIS) detection. The method does not distinguish between salts of amino acids and it cannot differentiate between enantiomers of the acids.

The EU method was further ring-trial validated for the determination of total arginine in feed resulting in the above-mentioned equivalent standard method EN ISO 13903. The following performance characteristics were reported for the determination of total arginine content in feed ranging from 4.0 to 43.5 g/kg: RSDr ranging from 2.3 to 3.3% and RSDR ranging from 7.2 to 9.7%.

In addition, the Applicant provided acceptable experimental data in the frame of the stability studies for the determination of arginine in the feed additive and water, thus demonstrating the applicability of the EU method for these two additional matrices.

Based on the performance characteristics available, the EURL recommends for official control the ring-trial validated European Union (EU) method based on ion-exchange chromatography coupled to post-column derivatisation and optical detection (IEC-VIS) for the determination of arginine in the feed additive, premixtures, feedingstuffs and water.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761) is not considered necessary.