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Safety and efficacy of L-lysine monohydrochloride and concentrated liquid L-lysine (base) produced by fermentation using *Corynebacterium glutamicum* strain KCCM 10227 for all animal species

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Abstract

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on L-lysine monohydrochloride and concentrated liquid L-lysine (base) produced using *Corynebacterium glutamicum* KCCM 10227 when used as nutritional additives in feed and water for drinking for all animal species. The active substance is L-lysine. L-lysine HCl and concentrated liquid L-lysine (base) produced by the strain *C. glutamicum* KCCM 10227 do not represent a risk for the target species, the consumer and the environment. L-lysine HCl produced by *C. glutamicum* KCCM 10227 is hazardous by inhalation, it is not irritant to skin but mildly irritant to eyes and it is not a skin sensitiser. Concentrated liquid L-lysine (base) produced by *C. glutamicum* KCCM 10227 is hazardous by inhalation, not irritant to skin and eyes and it is not a skin sensitiser. L-lysine HCl and concentrated liquid L-lysine (base) are considered as efficacious sources of the essential amino acid L-lysine for non-ruminant animal species. For the supplemental L-lysine to be as efficacious in ruminants as in non-ruminant species, it would require protection against degradation in the rumen.

Keywords: nutritional additive, amino acid, lysine monohydrochloride, lysine base, safety, efficacy

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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 a feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from CJ Europe GmbH[2] for authorisation of the product L-lysine monohydrochloride and concentrated liquid L-lysine (base), 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 particulars and documents in support of the application were considered valid by EFSA as of 8 May 2018.

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 product L-lysine monohydrochloride and concentrated liquid L-lysine (base), when used under the proposed conditions of use (see Section 3.1.6).

1.2. Additional information

L-lysine is currently authorised for its use in all animal species as a nutritional additive.[3] No maximum content in feedingstuffs is established in the EU.

L-lysine is authorised for use in food,[4] cosmetics[5] and as a veterinary medicinal product.[6,7]

L-lysine hydrochloride is described in a monograph of the European Pharmacopoeia (PhEur 9th edition, 2017) monograph 01/2008:0930.

The scientific Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) has published several opinions on the safety and efficacy of L-lysine and/or its salts produced by fermentation using different strains of *C. glutamicum* for all animal species (EFSA, 2007; EFSA FEEDAP Panel, 2015b, 2016b, 2017a, 2019a,b), and others on the safety and efficacy of L-lysine and/or its salts produced by fermentation using different strains of *Escherichia coli* (EFSA FEEDAP Panel, 2013, 2014, 2015a,b,c, 2016a).

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[8] in support of the authorisation request for the use of L-lysine monohydrochloride (HCl) and concentrated liquid L-lysine (base) as additive in feed and water for drinking.

[1] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.
[2] CJ Europe GmbH. Ober der Roeth 4, 65824 Schwallbach am Taunus, Germany.
[3] Commission Directive 88/485/EEC of 26 July 1988 amending the Annex to Council Directive 82/471/EEC concerning certain products used in animal nutrition. OJ L 239, 30.8.1988, p. 36–39.
[4] Regulation (EU) No 609/2013 of the European Parliament and of the Council of 12 June 2013 on food intended for infants and young children, food for special medical purposes and total diet replacement for weight control and repealing Council Directive 92/52/EEC, Commission Directives 96/8/EC, 1999/21/EC, 2006/125/EC and 2006/141/EC, Directive 2009/39/EC of the European Parliament and of the Council and Commission Regulations (EC) No 41/2009 and (EC) No 953/2009, OJ L 181, 29.6.2013, p. 35.
[5] Commission Decision of 9 February 2006 amending Decision 96/335/EC establishing an inventory and a common nomenclature of ingredients employed in cosmetic products. OJ L 97, 5.4.2006, p. 1–528.
[6] Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. OJ L 15, 20.1.2010, p. 1.
[7] Regulation (EC) No 470/2009 of the European Parliament and of the Council of 6 May 2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin, repealing Council Regulation (EEC) No 2377/90 and amending Directive 2001/82/EC of the European Parliament and of the Council and Regulation (EC) No 726/2004 of the European Parliament and of the Council. OL L 152, 16.6.2009, p. 11.
[8] FEED dossier reference: FAD-2018-0028.

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, 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 L-lysine HCl and concentrated liquid L-lysine (base) produced by *C. glutamicum* KCCM 10227 in animal feed. The Executive Summary of the EURL report can be found in Annex A.[9]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of L-lysine HCl and concentrated liquid L-lysine (base) is in line with the principles laid down in Regulation (EC) No 429/2008[10] and the relevant guidance documents: Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2017b), Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018), Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017c), Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017d), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012) and Guidance for assessing the safety of feed additives for the environment (EFSA, 2008).

3. Assessment

The product subject of this application is L-lysine in the forms of monohydrochloride (HCl) or concentrated liquid L-lysine (base) produced by fermentation with a chemically mutated strain *C. glutamicum*. The applicant is requesting the authorisation of these products as nutritional additive, under the functional group 'amino acids, their salts and analogues'. The product under application is intended to be used in feed and water for drinking for all animal species and categories.

3.1. Characterisation

3.1.1. Characterisation of the production microorganism

The additive is produced by a strain of *C. glutamicum,* which is deposited in [redacted] as *C. glutamicum* KCCM 10227.[redacted] *C. glutamicum* is a Gram-positive, non-pathogenic bacterium, which has been recommended for qualified presumption of safety (QPS) when used for the production of amino acids (EFSA BIOHAZ Panel, 2019). The production strain was obtained by chemical mutagenesis from the type strain *C. glutamicum* ATCC 13032 and it is not genetically modified.[12]

The identity of the production strain has been confirmed to belong to the species *C. glutamicum* [redacted] It shares 100% identity with the type strain *C. glutamicum* ATCC 13032.

The susceptibility of the production strain to relevant antibiotics was tested against the list of antimicrobials described for '*Corynebacterium* and other Gram+' in the Guidance on characterisation of microorganisms (EFSA FEEDAP Panel, 2018). All measured minimum inhibitory concentration (MIC) values were lower than the cut-off values specified in such guidance.[redacted]

3.1.2. Manufacturing process

[redacted]

[9] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2018-0028_l-lysine.pdf.

[10] 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.

[redacted]

[12] Technical dosssier/Supplementary information November 2018/02 SIN CJ, Section 2.

[redacted]

[redacted]

3.1.3. Characterisation of the L-lysine monohydrochloride

L-lysine HCl (International Union of Pure and Applied Chemistry (IUPAC) name: (2S)-2,6-diaminohexanoic acid monohydrochloride, synonym L-(+)-2,6-Diamino-N-Caproic Acid Monohydrochloride, a compound identified with the Chemical Abstracts Service (CAS) No 657-27-2 and the European Inventory of Existing Commercial chemical Substances (EINECS) No 211-519-9), has a molecular weight of 182.65 g/mol. The theoretical content of lysine in lysine monohydrochloride is 80%. The molecular formula is NH_2-$(CH_2)_4$-$CH(NH_2)$-COOH-HCl and the molecular structure is given in Figure 1.

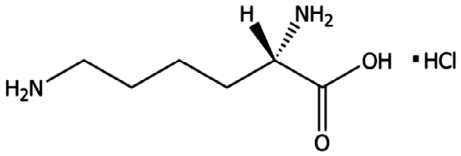

Figure 1: Molecular structure of L-lysine HCl

The specification is for an additive containing ≥ 78% L-lysine on 'as is' basis, ≤ 1% water, and ≤ 0.3% ash.[18]

The applicant provided data of five batches of the additive.[19] L-lysine was 79.6% (range 79.5–79.8%) on 'as is' basis.[20] Water was 0.1% (range 0.1–0.2%).

Analytical data of five additional bathes showed an average of chloride of 19.1% (range 19.1–19.2%), loss on drying was 0.1% (range 0.1–0.2%), ash was on average 0.04% (range 0.01–0.07%), sulfate was 0.02% and sodium, potassium and ammonium 0.01% each. Lysine was not analysed.[21]

The specific optical rotation was measured in three batches (European Pharmacopoeia method 2.2.7) and ranged from +21.9° to +22.3°.[22] This is within the range of the reference values established in the European Pharmacopoeia (range between +21.0° and +22.5°) and confirms the L enantiomer of lysine in the additive.

3.1.3.1. Impurities of L-lysine HCl

Five batches were analysed for undesirable substances. Levels of heavy metals (cadmium, lead, mercury, chromium, copper, nickel and zinc) and arsenic were reported. All of them were below the limit of detection (LOD) except for mercury, ranging from 0.014 to 0.023 mg/kg.[23]

Five batches were analysed for polychlorinated dibenzodioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (PCB).[24] PCDD/F ranged from 0.16 to 0.33 ng TEQ-WHO/kg. PCBs ranged from 0.14 to 0.27 ng TEQ-WHO/kg.

In reference to the microbiological contamination, analytical data (five batches) were submitted. *Salmonella* spp. (25 g per sample) was absent; *E. coli*, filamentous fungi and yeasts were below the LOD.[25] Regarding the mycotoxin content, analytical data of the same batches showed levels of ochratoxin, zearalenone and deoxynivalenol (DON) below the LOD.[26] Aflatoxins ranged from < LOD to 0.4 μg/kg.

[redacted]

[18] Technical dossier/Section II.1.3.
[19] Technical dossier/Section II/Annex II.1.4, Annex II.6.1 and supplementary information November 2018/Annex SIN 01.
[20] Technical dossier/Section II.1.4.
[21] Technical dossier/Section II/Annex II.1.6.
[22] Technical dossier/Section II/Annex II.1.3 and supplementary information November 2018/Annex SIN 3.
[23] Technical dossier/Section II/Annex II.1.8. LOD (in mg/kg) were 1 for lead and arsenic; 0.1 for cadmium and 0.2 for chrome, copper, nickel and zinc.
[24] Technical dossier/Section II/Annex II.1.10.
[25] Technical dossier/Section II/Annex II.1.10 and supplementary information November 2018/02 SIN CJ, Section 3.3. LOD was < 10^3 colony forming units (CFU)/g
[26] Technical dossier/Section II/Annex II.1.8. LOD (in μg/kg) were 0.1 for aflatoxins, 5 for ochratoxin, 17 for zearalenone and 134 for deoxynivalenol.

The absence of viable cells of the production strain in the final product was studied [redacted] No colonies were detected.

3.1.3.2. Physical characteristics of L-lysine monohydrochloride

The additive is a pale brownish free flowing crystalline powder with an approximate density of 550–750 kg/m^3, pH 5.0–6.5 (at 10% solution) and with a water solubility of about 642 g/L at 30°C.[28]

Concerning the particle size, three batches were analysed by laser diffraction.[29] There were no particles with a diameter < 10 μm. The fraction of particles having a diameter < 50 and < 100 μm ranged 0–0.2% and 0.49–1.9%, respectively.

The dusting potential was analysed (Stauber–Heubach method) in the same three batches and ranged from 1.27 to 1.69 g/m^3.[30]

3.1.3.3. Stability and homogeneity

The shelf-life of L-lysine HCl (three batches) was studied when stored in sealed brown glass bottles at 25°C and 40°C for 6 months. Losses ranged from 0 to 0.4% and from 0 to 0.1%, respectively.[31]

The stability of the additive (three batches) in a vitamin/mineral premixture containing choline chloride (40,000 mg/kg) was studied when supplemented at 5% with the product under assessment.[32] Three samples per batch were collected in sealed containers and stored at 25°C for 6 months. No losses were observed.[32]

The stability of three batches of the additive in a mash feed for chickens for fattening (with a basal diet containing maize, soy bean extracted oil meal and wheat) when supplemented at 0.4% lysine HCl was examined. Three samples per batch were collected in sealed containers and stored at 25°C for 3 months. Losses in mash feed ranged from 0 to 3%.[33]

The stability of three batches of L-lysine HCl in water for drinking was studied at nominal concentration of 0.5 g/L, at 25°C and 40°C for 48 h. No losses were detected.[34] The FEEDAP Panel notes that the concentration of the additive tested is too high to be used under practical conditions in water for drinking.

The capacity of the additive (one batch) to distribute homogeneously was studied in the premixture and in the mash feed described above and a pelleted feed for chicken for fattening (inclusion rate 0.4% via premixture). Analyses of 10 subsamples yielded a coefficient of variation (CV) of 6% for the premixture,[35] 8% for the mash feed[36] and 2% for the pelleted feed for chicken for fattening.[37]

3.1.4. Characterisation of concentrated liquid L-lysine (base)

L-lysine (IUPAC name (2S)-2,6 diaminohexanoic acid; synonym (S)-2,6-Diaminocaproic acid), a compound identified with the CAS No 56-87-1 and the EC-No 201-300-6, has a molecular weight of 146.2 g/mol. The molecular formula is $H_2N(CH_2)CH(NH_2)CO_2H$. The molecular structure is given in Figure 2.

[redacted]

[28] Technical dossier/Section II/Annex II_1_01.
[29] Technical dossier/Section II/Annex II.1.12.
[30] Technical dossier/Section II/Annex II.1.13.
[31] Technical dossier/Section II/Annex II.4.1.
[32] Technical dossier/Section II/Annex II.4.5.
[33] Technical dossier/Section II/Annex II.4.7.
[34] Technical dossier/Section II/Annex II.4.3.
[35] Technical dossier/Section II/Annex II.4.13.
[36] Technical dossier/Section II/Annex II.4.9.
[37] Technical dossier/Section II/Annex II.4.11.

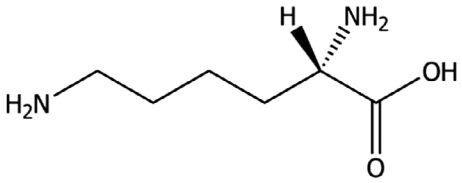

Figure 2: Molecular structure of L-lysine

The product is specified to contain ≥ 50% lysine and ≤ 50% water.[38] Compliance with the specification was shown in five batches in which L-lysine was on average 50.4% on 'as is' basis (range 50.0–51.0%). Water content was 47.8% (range 45.5–49.3%).[39]

Five additional batches where analysed and showed an average content of lysine of 51.1% (range 50.9–51.2%), 46.2% water (range 45.9–46.6%), 0.1% of free amino acids other than lysine (glutamic acid, alanine, isoleucine and arginine), 0.03% ammonium, 0.06% sodium, 0.14% potassium, 0.9% chloride and 0.15% sulfate. Ash was on average 0.3% (range 0.28–0.32%).[40] Total identified material on 'as is' was 98.5% (98.3–98.9%).

3.1.4.1. Impurities of concentrated liquid L-lysine (base)

Levels of heavy metals (cadmium, lead, mercury, chromium, copper, nickel and zinc) and arsenic (analysed in five batches) were reported. All values were below the LOD except for mercury (ranged from < LOD to 0.06 mg/kg); zinc (< LOD to 8.63 μg/kg) and copper (< LOD to 2.4 μg/kg).[41] The same batches were analysed for dioxins, furans and dioxine-like PCBs. PCDD/F ranged from 0.16 to 0.18 ng WHO-TEQ/kg and the sum of PCDD/F and PCBs ranged from 0.30 to 0.31 ng WHO-TEQ/kg.[42]

In reference to the microbiological contamination, analytical data (five batches) showed that *Salmonella* spp. (25 g sample) was not detected; *E. coli*, filamentous fungi and yeasts were below the LOD; and total bacterial count ranged from below the LOD to 1.5 x 10^3 CFU/g.[43] Regarding the mycotoxin content, analytical data of three batches showed levels of aflatoxins (B1, B2, G1, G2), ochratoxin A, zearalenone and DON below the limit of quantification (LOQ).[44]

The absence of viable cells of the production strain in the final product was studied [redacted] No colonies could be detected.

3.1.4.2. Physical characteristics of concentrated liquid L-lysine (base)

The concentrated liquid L-lysine is a dark brown liquid with an approximate density of 1,120–1,170 kg/m^3 and a pH 9–11 (at 20°C). It is very easily soluble in water and has a boiling point ranging from 110 to 120°C.[45] The viscosity ranged from 75 to 86 cp.[46] The surface tension ranged from 49 to 50 mN/m.[47]

3.1.4.3. Stability and homogeneity of concentrated liquid L-lysine (base)

The shelf-life of concentrated liquid L-lysine (three batches) was studied when stored in sealed brown glass bottles at 25°C and 40°C for 6 months. No losses were observed.[48]

The stability of the additive (three batches) in a vitamin/mineral premixture containing choline chloride (40,000 mg/kg) when supplemented at 11% (representing an addition of 5.5% lysine) was

[38] Technical dossier/Section II.1.3 and annexes II.1.2, II.1.5 and II.6.1.
[39] Technical dossier/Section II/Annex II.1.5 and Supplementary information November 2018/Annex SIN 02.
[40] Technical dossier/Section II/Annex II.1.7.
[41] Technical dossier/Section II/Annex II.1.9. LOD (in mg/kg) was 1 for arsenic and lead; 0.1 for cadmium; 0.2 for coper, chromium and nickel; and 0.01 for mercury.
[42] Technical dossier/Section II/Annex II.1.11.
[43] Technical dossier/Section II/Annex II.1.11 and supplementary information November 2018/02 SIN CJ, section 3.3. LOD (in CFU/g) was 10^3 for *E. coli*, filamentous fungi, yeast and total bacterial count.
[44] Technical dossier/Section II/Annex II.1.9. LOQ (in μg/kg) was 0.4 for aflatoxins (B1, B2, G1, G2), 0.2 for ochratoxin A, 10 for zearalenone and 20 for deoxynivalenol.
[45] Technical dossier/Section II/Annex II.1.2.
[46] Technical dossier/Section II/Annex II.1.14.
[47] Technical dossier/Section II/Annex II.1.15.
[48] Technical dossier/Section II/Annex II.4.2.

studied. Three samples per batch were collected and stored in aluminium bags at 25°C for 6 months. Losses ranged from 0 to 4.2%, depending on the batch considered.[49]

The stability of three batches of the concentrated liquid L-lysine was studied in a mash feed for chickens for fattening (basal diet containing maize, soy bean extracted oil meal and wheat) when supplemented with the additive at 0.8% (corresponding to 0.4% lysine). Three samples per batch were collected and stored in aluminium bags at 25°C, RH 60% for 3 months. Losses (6%) were observed in only one batch.[50]

The stability of three batches of liquid L-lysine in water for drinking was studied at 0.5 g/L nominal concentration (0.25 g lysine/L), at 25°C and 40°C for 48 h. Losses were ranged from 0 to 4% and from 0 to 5%, respectively.[51]

The capacity of the additive (three batches) to distribute homogeneously was studied in the premixture and in the mash feed described above and in a pelleted feed for chicken for fattening (supplemented with 0.8% (corresponding to 0.4% lysine)). Analysis of 10 subsamples yielded a CV of 3% for the premixture,[35] 13% for the mash feed[52] and 4% for the pelleted feed for chicken for fattening.[53]

3.1.5. Physico-chemical incompatibilities in feed

No physico-chemical incompatibilities in feed are expected with other additives, medicinal products or other feed materials.

3.1.6. Conditions of use

L-lysine is proposed to be used in feeds in order to achieve the adequate amino acid profile and meet the requirements on L-lysine for all animal species. It can be added directly to the feedingstuffs, complementary feedingstuffs or via premixture.[54] The use of both forms of L-lysine in water for drinking is also proposed.[55] No inclusion levels are proposed as the requirements in quantitative terms depend on the species, the physiological state of the animal, the performance level and the environmental conditions, as well as the amino acid content of the unsupplemented diet.

3.2. Safety of concentrated liquid L-lysine (base) and L-lysine HCl

3.2.1. Safety for the target species, consumer and environment

Both forms of the additive are highly purified and are produced by fermentation using a strain that belongs to a species that qualifies for the QPS approach for safety assessment. Concerns from the use of the additive would not derive from L-lysine, which is considered safe, but may arise from residues of the fermentation process/production strain remaining in the final product. Since the identity of the production strain has been established as *C. glutamicum*, it is susceptible to relevant antimicrobials used in human and veterinary medicine and no viable cells of the production strain are in the final products, both forms of L-lysine produced by *C. glutamicum* KCCM 10227 are considered safe for the target species provided that it is supplemented in appropriate amounts to the diets. Due to the risk of nutritional imbalances and hygienic reasons, associated to the use of amino acids via water for drinking (EFSA FEEDAP Panel, 2010), the FEEDAP Panel has concerns on the safety of the simultaneous oral administration of lysine-containing additives via feed and water for drinking.

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

The amino acid L-lysine 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. The absorbed L-lysine will be incorporated into body

[49] Technical dossier/Section II/Annex II.4.6.
[50] Technical dossier/Section II/Annex II.4.8.
[51] Technical dossier/Section II/Annex II.4.4.
[52] Technical dossier/Section II/Annex II.4.10.
[53] Technical dossier/Section II/Annex II.4.12.
[54] Technical dossier/Section II.5.1.
[55] Technical dossier/Section II.4.1.3 and supplementary information November 2018/02 SIN CJ, section 3.6.

protein or excreted as urea/uric acid and as carbon dioxide. 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 FEEDAP Panel concludes that both forms of L-lysine produced by *C. glutamicum* KCCM 10227 are safe for the target species, for the consumer and for the environment.

3.2.2. Safety for the user

L-lysine HCl

The applicant submitted an acute inhalation toxicity study, and *in vitro* eye irritation study, an *in vitro* skin irritation study and a skin sensitisation study performed with the product under assessment.

- Effects on the respiratory system

The dusting potential of the additive ranged from 1.27 to 1.69 g/m^3.

The applicant submitted an acute inhalation toxicity study, in accordance with OECD Guideline 403, performed with the additive under assessment.[56] Ten RccHan™: WIST strain rats (five males and five females) were exposed to a dust atmosphere (5 mg additive/L air, all particles had a diameter < 10 μm) for 4 h using a nose only exposure system, followed by a 14-day observation period. The relative humidity during exposure was very low (23–27%). Common abnormalities observed during the first day after exposure included decreased respiratory rate, hunched posture, piloerection and wet fur. One male rat died the second day post-exposure; it was considered a treatment-related effect. The remaining animals showed no signs on day 2 post-exposure. At necropsy on day 14 post-exposure, six of nine rats showed abnormal red dark patches in lungs and another one had lungs abnormally red. The rest recovered normality on day 2 post-exposure. The acute inhalation median lethal concentration (4-h LC_{50}) of L-lysine monohydrochloride in the Wistar strain rat was greater than 5 mg/L. Persistent alterations were observed in almost all exposed animals at the end of the 14-day post-treatment observation period, indicating a potential for inhalation toxicity.

The FEEDAP Panel concludes that the additive is hazardous by inhalation.

- Effects on skin and eyes

In an *in vitro* skin irritation test (Human Skin Model Test) according to OECD Guideline 439, reconstructed human epidermis membranes were topically exposed to the additive under assessment for 15 min. Viability of the epidermal cells was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test 42 h after exposure. The positive and negative controls performed as expected. The mean viability of the skin membranes was 92% compared to the negative control group.[57] The additive was considered not irritant for the skin.

In an *in vitro* eye irritation test (Bovine Corneal Opacity and Permeability Assay) according to OECD Guideline 437, the product under assessment was applied to bovine corneas at a concentration of 20% w/v in sodium chloride 0.9% w/v for 240 min.[58] Negative and positive control items were tested concurrently. The two endpoints, decreased light transmission through the cornea (opacity) and increased passage of sodium fluorescein dye through the cornea (permeability), were combined in an empirically derived formula to generate an in vitro irritancy score (IVIS). The positive and negative controls performed as expected. As the IVIS of the additive (5.3) was > 3 and ≤ 55, no prediction on eye irritation could be made. According to the irritant classification using the classification scheme adopted by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, 2006), the IVIS of 5.3 correspond to mild irritation for the eyes.[59]

In a skin sensitisation study using local lymph node assay in the mouse, in accordance with OECD Guideline 429, the product under assessment was tested topically at 5, 10 or 25% w/w in 1% Plurionic in distilled water (25 μL/ear).[60] A control group was treated with 1% Pluronic in distilled water alone. The stimulation index expressed as the mean radioactive incorporation for each treatment group divided by the mean radioactive incorporation of the vehicle control group were close to 1 in all

[56] Technical dossier/Section II/Annex III.3.4.
[57] Technical dossier/Section II/Annex III.3.8.
[58] Technical dossier/Section II/Annex III.3.6.
[59] Technical dossier/Supplementary information November 2018/02 SIN CJ, section 4.2.
[60] Technical dossier/Section II/Annex III.3.10.

concentrations tested (1, 1.15 and 1.03 for the concentrations of 5, 10 and 25% w/w, respectively). As all IVIS were < 3, the additive was not considered to be a skin sensitiser.

Liquid L-lysine (base)

The applicant submitted an acute inhalation toxicity study, and *in vitro* eye irritation study, an *in vitro* skin irritation study and a skin sensitisation study performed with the product under assessment.

- Effects on the respiratory system

In an acute inhalation toxicity study performed in accordance with OECD Guideline 403, a group of 10 RccHan™: WIST strain rats (five males and five females) was exposed to an aerosol atmosphere of 5.4 mg additive/l air for 4 h and surveyed during 14 day thereafter.[61] The particle size distribution of the aerosol showed that the inhalable fraction (< 4 μm diameter) was 81% (geometric SD 2.5%). All animals exhibited hunched posture one day after exposure but recovered to normality on day 3 post-exposure. At postmortem examination, the 14th day after exposure, dark patches in the lungs were observed in 4 of 10 rats. The acute inhalation median lethal concentration (4 h LC_{50}) of the additive in the Wistar strain rat was greater than 5.35 mg/L. The FEEDAP Panel considers that concentrated liquid L-lysine (base) is hazardous by inhalation.

- Effects on skin and eyes

In an *in vitro* skin irritation test (Human Skin Model Test), according to OECD Guideline 439, reconstructed human epidermis membranes were topically exposed to the additive under assessment for 15 min.[62] Viability of the epidermal cells was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test 42 h after exposure. The positive and negative controls performed as expected. The mean viability of the skin membranes was 78% compared to the negative control group. The additive was classified as non-irritant for the skin.

In an *in vitro* eye irritation test (Bovine Corneal Opacity and Permeability Assay) according to OECD Guideline 437, the product under assessment was applied to bovine corneas at a concentration of 20% w/v in sodium chloride 0.9% w/v for 10 min followed by an incubation period of 120 min.[63] Negative and positive control items were tested concurrently. The two endpoints, decreased light transmission through the cornea (opacity) and increased passage of sodium fluorescein dye through the cornea (permeability), were combined in an empirically derived formula to generate an IVIS. The positive and negative controls performed as expected. As the IVIS of the additive was ≤ 3, the additive does not require classification and is considered non irritant to eyes.

In a skin sensitisation study using local lymph node assay in the mouse, in accordance with OECD Guideline 429, the product under assessment was tested topically at 25, 50 or 100% v/v in ethanol/distilled water (25 μL/ear).[64] A control group was treated with ethanol/distilled water alone. The stimulation index expressed as the mean radioactive incorporation for each treatment group divided by the mean radioactive incorporation of the vehicle control group were 1.3, 1.3 and 1.7 for the concentrations of 25, 50 and 100% v/v, respectively. As all IVIS were < 3, the additive was not considered to be a skin sensitiser.

3.2.2.1. Conclusions on the safety for the user

L-Lysine HCl produced by *C. glutamicum* KCCM 10227 is hazardous by inhalation, it is not irritant to skin but mildly irritant to eyes and it is not a skin sensitiser.

Concentrated liquid L-lysine (base) produced by *C. glutamicum* KCCM 10227 is hazardous by inhalation, not irritant to skin and eyes and it is not a skin sensitiser.

3.3. Efficacy of L-lysine HCl and concentrated liquid L-lysine (base)

Efficacy studies are not required for amino acids naturally occurring in proteins of plants and animals. The nutritional role of the amino acid L-lysine is well established in the scientific literature. The efficacy of L-lysine for both non-ruminant and ruminant species was described in two previous opinions (EFSA FEEDAP Panel, 2013, 2014). In general, products concentrated liquid L-lysine (base) and L-lysine

[61] Technical dossier/Section III/Annex III.3.5.
[62] Technical dossier/Section III/Annex III.3.9.
[63] Technical dossier/Section III/Annex III.3.7.
[64] Technical dossier/Section II/Annex III.3.11.

HCl are considered as efficacious sources of the essential amino acid L-lysine for non-ruminant animal species. For the supplemental L-lysine 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[65] and Good Manufacturing Practice.

4. Conclusions

L-Lysine HCl and concentrated liquid L-lysine (base) produced by the strain *C. glutamicum* KCCM 10227 do not represent a risk for the target species, for the consumer and for the environment.

L-Lysine HCl produced by *C. glutamicum* KCCM 10227 is hazardous by inhalation, it is not irritant to skin but mildly irritant to eyes and it is not a skin sensitiser. Concentrated liquid L-lysine (base) produced by *C. glutamicum* KCCM 10227 is hazardous by inhalation, not irritant to skin and eyes and it is not a skin sensitiser.

L-lysine HCl and concentrated liquid L-lysine (base) are considered as efficacious sources of the essential amino acid L-lysine for non-ruminant animal species. For the supplemental L-lysine to be as efficacious in ruminants as in non-ruminant species, it would require protection against degradation in the rumen.

Chronology

Date	Event
4/5/2018	Dossier received by EFSA. L-lysine HCl and concentrated liquid L-lysine (base) produced using *Corynebacterium glutamicum* for all animal species. Submitted by CJ Europe GmbH
29/5/2018	Reception mandate from the European Commission
10/7/2018	Application validated by EFSA – Start of the scientific assessment
9/10/2018	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: manufacturing process, characterisation of the production strain and of the additive, stability of the additive, conditions of use and safety for the user*
10/10/2018	Comments received from Member States
10/10/2018	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
22/11/2018	Reception of supplementary information from the applicant – Scientific assessment re-started
19/12/2018	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 of L-lysine HCl and concentrated liquid L-lysine base*
21/12/2017	Reception of supplementary information from the applicant – Scientific assessment re-started
3/4/2019	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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Abbreviations

CAS	Chemical Abstracts Service
CFU	colony-forming unit
CV	coefficient of variation
EURL	European Union Reference Laboratory
FCC	Food Chemical Codex
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
ICCVAM	The Interagency Coordinating Committee on the Validation of Alternative Methods
IEC-VIS	Ion exchange chromatography coupled with photometric detection
IVIS	In vitro irritancy score
LOD	limit of detection
LOQ	limit of quantification
PCB	polychlorinated biphenyls
PCDDs	polychlorinated dibenzodioxins
RSDr	relative standard deviation for repeatability

Annex A – Evaluation Report on the Analytical Methods submitted in connection with the Application for Authorisation of L-lysine monohydrochloride and concentrated liquid L-lysine (base) produced by fermentation using *Corynebacterium glutamicum* strain KCCM 10227

In the current application, authorisation is sought under Article 4(1) for L-lysine monohydrochloride and concentrated liquid L-lysine produced by *Corynebacterium glutamicum* KCCM10227, 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. Authorisation is sought for all animal species.

According to the Applicant, the dry crystalline powdered L-lysine monohydrochloride has a minimum purity (mass fraction) of 98% (minimum of 78% of L-lysine) and concentrated liquid L-lysine contains a minimum of 50% of L-lysine.

For the quantification of lysine in the feed additive, the Applicant submitted a slightly modified protocol of the Community method dedicated for the determination of amino acids in feed. However, the European Union Reference Laboratory (EURL) previously evaluated lysine dossiers and recommended for the quantification of lysine in the feed additives and premixtures (containing more than 10% lysine) the ring-trial validated method EN ISO 17180:2013 based on ion exchange chromatography coupled to visible or fluorescence detection (IEC-VIS/FLD). This standard method does not distinguish between the salts of amino acids and cannot differentiate between enantiomers. It applies for products containing more than 10% of amino acid. The following performance characteristics are reported: a relative standard deviation for repeatability (RSDr) ranging from 0.7 to 1.7% and a relative standard deviation for reproducibility (RSDR) ranging from 1.5 to 2.5%. In addition, the EURL identified the "L-lysine monohydrochloride monograph" of the Food Chemical Codex (FCC) for the identification of L-lysine monohydrochloride in the feed additive.

For the quantification of L-lysine in premixtures and feedingstuffs, the Applicant submitted the ring-trial validated Community method (Commission Regulation (EC) No 152/2009) based on IEC-VIS. This method, designed only for the analysis of amino acids in premixtures and feedingstuffs, does not distinguish between the salts and the amino acid enantiomers. The following performance characteristics were reported for the quantification of total lysine: RSDr ranging from 2.1 to 2.8% and RSDR ranging from 3 to 6.7%.

In the frame of the stability studies, the Applicant presented experimental data obtained analysing lysine in water with a slightly modified protocol of the VDLUFA 4.11.6 method based on IEC-VIS/FLD. The results presented are considered sufficient to demonstrate the suitability of the procedure for the analysis of the amino acid in water.

In the frame of this authorisation, the EURL recommends for official control (i) the "L-lysine monohydrochloride monograph" of the FCC based on infrared absorption for the identification of L-lysine monohydrochloride in the feed additive; (ii) the ring-trial validated method EN ISO 17180:2013 based on IEC-VIS/FLD to quantify free lysine in the feed additive and premixtures (containing more than 10% lysine); (iii) the Community method based on IEC-VIS for the quantification of lysine in premixtures and feedingstuffs; and (iv) a slightly modified VDLUFA 4.11.6 method based on IEC-VIS/FLD to quantify lysine in 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) is not considered necessary.