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Safety and efficacy of L-lysine monohydrochloride and L-lysine sulfate produced using *Corynebacterium glutamicum* CCTCC M 2015595 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 L-lysine sulfate produced using *Corynebacterium glutamicum* CCTCC M 2015595 when used as nutritional additive in feed and water for drinking for all animal species. The active substance is L-lysine and it is produced in two different forms (monohydrochloride or sulfate). Owing to the uncertainties regarding the possible genetic modification of the strain used to obtain the production strain *C. glutamicum* CCTCC M 2015595 and on the possible presence of viable cells and DNA of the production strain in the final product, the FEEDAP Panel cannot conclude on the safety of the additives L-lysine HCl and L-lysine sulfate produced with *C. glutamicum* CCTCC M 2015595 for the target species, the consumers, the users and the environment. For both products, the FEEDAP Panel has concerns regarding the safety for the target species when the additives are administered via water for drinking. In the absence of data, the FEEDAP Panel cannot conclude on the safety of the additive for the user. The products under assessment are considered efficacious sources of the amino acid L-lysine for all animal species. For these products to be as efficacious in ruminants as in non-ruminant species, they require protection against degradation in the rumen.

Keywords: nutritional additive, amino acid, lysine monohydrochloride, lysine sulfate, 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 Kempex Holland B.V.[2] for authorisation of the products L-lysine monohydrochloride and L-lysine sulfate, 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 30 November 2016.

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 L-lysine sulfate produced by fermentation with *Corynebacterium glutamicum* CCTCC M 2015595, when used under the proposed conditions of use (see Section 3.3).

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 European Union (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 *C. glutamicum* for all animal species (EFSA, 2007a; EFSA FEEDAP Panel, 2015a, 2016, 2019a,b); and three opinions on the safety and efficacy of concentrated liquid L-lysine (base), concentrated liquid L-lysine monohydrochloride and/or L-lysine monohydrochloride produced by *Escherichia coli* for all animal species (EFSA FEEDAP Panel, 2013, 2014, 2015a,b).

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 and L-lysine sulfate produced by fermentation with *C. glutamicum* CCTCC M 2015595 as a feed additive.

[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] Kempex Holland B.V. Zeelandsedijk 15, 5408 SL, Volkel, The Netherlands.
[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, 30.8.88, pp. 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, pp. 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-2016-0052.

The FEEDAP Panel used the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA, peer-reviewed scientific papers, other scientific reports and experts' knowledge, 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 the L-lysine monohydrochloride and L-lysine sulfate produced by fermentation with *C. glutamicum* CCTCC M 2015595 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 monohydrochloride and L-lysine sulfate produced by fermentation with *C. glutamicum* CCTCC M 2015595 is in line with the principles laid down in Regulation (EC) No 429/2008 and the relevant guidance documents: Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2017a), 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, 2017b), Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017c) and Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012).

3. Assessment

The current application is for the authorisation of L-lysine HCl (minimum 78.8% lysine on dry matter (DM) basis) and L-lysine sulfate (minimum 55% lysine on DM basis) produced by fermentation by a strain of *C. glutamicum* (CCTCC M 2015595). These products are intended to be used as a nutritional additive (functional group: amino acids, their salts and analogues) in feed and/or in water for drinking for all animal species. The active substance of both forms of the additive is L-lysine.

3.1. Characterisation of the production organism

The applicant purchased a strain of *C. glutamicum* (1.0563) from the China Center for Type Culture collection (CCTCC). No information was provided on the origin and history of modifications of that strain, including whether or not it has been genetically modified.[10] That strain was chemically and physically mutated (3 g/L nitroguanidene during 40 min and ultraviolet radiation for 90 s). The mutants were screened for high lysine production capacity, the production strain was selected and deposited in the CCTCC with accession number CCTCC M 2015595.[11]

[redacted]

As no information has been provided on the original strain, it is not possible to assess whether it has been genetically modified and sequences of concern eventually introduced that would remain in the production strain.

3.2. Manufacturing process

[redacted]

[9] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2016-0052_lysine.pdf
[10] Technical dossier/Supplementary information November 2018/Answers to Sin and Annexes 1.a and 1.b.
[11] Technical dossier/Section II/Annex 2.2.1.2b.

[redacted]

[redacted]

3.3. Conditions of use

According to the applicant, both forms of the additive can be added directly in compound feed, through complementary feed or through premixtures and they are aimed for all animal species.[15] No proposed inclusion levels are provided, 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, as well as the amino acid composition of the unsupplemented diet.

The applicant states that the additive (both forms) can be used in water but should not be simultaneously administered via water for drinking and feed.

3.4. L-Lysine monohydrochloride

3.4.1. Characterisation

3.4.1.1. Characterisation of the active substance/additive

L-Lysine HCl (IUPAC name: (2*S*)-2,6-diaminohexanoic acid monohydrochloride, synonym L-lysine hydrochloride, a compound identified with the CAS No 657-27-2 and the 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.

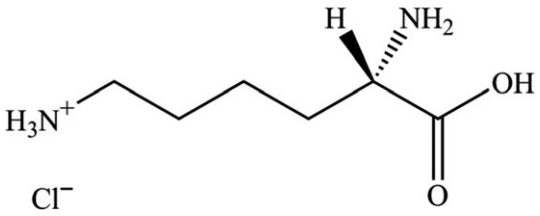

Figure 1: Molecular structure of L-lysine HCl

The product contains by specification ≥ 78.8% L-lysine on DM basis; ≤ 1% moisture; ≤ 0.5% other amino acids; ≤ 0.5% total sugars; ≤ 0.3% ash and ≤ 0.05% ammonium salt.[16]

The average lysine content analysed in six batches was 77.9% (range 76.9–78.7%) on as is basis.[17] The content of chloride was calculated to be 20%. The water content was in the range 0.4–0.6%. On a DM basis, the average lysine content was 78.3% (range 77.4–79.0%) and five of the six analysed batches did not meet the specification. The sum of quantified components was on average 98.3%.[18]

The specific optical rotation was measured in three batches ranged from +20.7° to +20.9° and confirm that the additive is the L-stereoisomer of lysine.[19]

3.4.1.2. Impurities

Analytical data (three batches) on the content of heavy metals (lead, cadmium and mercury) and arsenic were below the limit of detection (LOD) except one that showed mercury levels of 0.015 mg/kg.[20] Dioxins and the sum of dioxin plus dioxin-like PCBs in three batches of the product were < 0.135 ng WHO-PCDD/F TEQ/kg and < 0.269 ng WHO-PCDD/F-PCB TEQ/kg.[21] The concentrations of these undesirable substances do not raise safety concerns.

[redacted]

[15] Technical dossier/Section 2.5.1 and supplementary information January 2018/Answers FAD-2016-0052/Q7a-f.
[16] Technical dossier/Section II/Table 2.1.3a.
[17] Technical dossier/Supplementary information January 2018/Annexes Q7a to f. HPLC method accredited by the Dutch Accreditation Council (L172).
[18] Technical dossier/Section II/2.1.3.c CoA 5 batches Lys HCl.pdf.
[19] Technical dossier/Supplementary information January 2018/Annex Q8. National standard of the People's Republic of China for feed grade L-lysine HCl. Reference values of the Chinese standard for L-lysine HCl range +18.0 to +21.5°.
[20] Technical dossier/Annex 2.1.4a and supplementary information January 2018/Q11. LOD (in mg/kg) was 0.01 for mercury and cadmium, 0.04 for arsenic and 0.05 for lead.
[21] Technical dossier/Supplementary information January 2018/Annexes Q21.

Microbiological contamination (analysed in three batches) showed that *Salmonella* spp. was absent in 25 g. *E. coli*, and coliforms most probable number (MPN) was < 30/g, respectively. Yeasts and filamentous fungi were not detected.[22] Regarding the mycotoxin content the same batches showed aflatoxins (B1, B2, G1, G2), zearalenone, fumonisins (B1, B2), ochratoxin A and deoxynivalenol (DON) below the LOD. The amounts of the afore mentioned contaminants do not raise safety concerns.

The applicant provided information to support the absence of viable cells of the production strain in three batches of L-lysine HCl.[23] 1-g sample was diluted in 9 mL of distilled water (three replicates per batch) and after 2 h at 22°C shaking the sample every hour, 1 mL of the suspension was spread on LB plates (2 × 0.5 mL on two different plates). Plates incubated at 30°C for 2 days and 6 days before inspection of growth. Identification of the strains was performed using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometer (MALDI-TOF) analyses of clones for each batch and of colonies from the positive control. Every sample presented colonies after 2 days that morphologically corresponded to *C. glutamicum*. On day 6, the number of colonies had increased (calculated to range between 987 and 1,067 colony forming unit (CFU)/g depending on the batch considered) and had evolved differently by comparison with day 2. Colonies of control plate were identified as *C. glutamicum* as expected. 1–3 colonies of each phenotype were recovered for each replicate. These colonies were grouped into seven different morphotypes. MALDI-TOF results classified correctly the positive control as *C. glutamicum*, yielded a fully reliable and reproducible result for morphotypes 1 and 2 (*Acinetobacter radioresistens* and *Pseudomonas fulva*, respectively), a reproducible result (genus level) for morphotypes 3–5 (*Lactobacillus*, *Micrococcus* and *Rhodococcus fascians*), and an unreliable result for morphotype 6 (bacteria from desferrioxamine B (DFB) group). Morphotype 7 and seven additional ungrouped colonies could not be identified. Morphotypes 3–7 represented < 40 CFU/g of sample.

Although the available data do not indicate the presence of viable cells of the production strain, uncertainty remains due to the following weaknesses:

- The limited amount of product tested (0.1 g)
- There were viable cells that could not be identified. The high bacterial growth hampered the identification of all colonies

Therefore, the presence of viable cells of the production strain in the three batches of L-lysine HCl tested cannot be excluded.

The applicant did not provide conclusive data on the absence of DNA of the production strain in the final product.[24] This would be of relevance in the case the original strain was genetically modified.

3.4.1.3. Physical properties

L-Lysine HCl is a white or light brown powder, with a bulk density of 550–650 kg/m^3 at 25°C,[25] pH 5.4–5.6 (10% solution at 25°C)[26] and a water solubility of about 600 g/L at 20°C.[27]

The dusting potential (Stauber–Heubach) was analysed in three batches and was 0.2 g/m^3 in all cases.[28]

Concerning the particle size, three batches were analysed by laser diffraction. The fractions below 100, 50 and 10 μm diameter (v/v) were 3.6–5.9%, 1.6–2.4% and < 1%, respectively.[29]

3.4.1.4. Stability and homogeneity

The shelf life of the additive was studied in three batches of the additive kept in closed bags either at room temperature for 1 year or at 40°C for 8 h. No losses were observed.[30]

The stability of the additive (three batches) in a vitamin/mineral premixture (containing 16 g/kg choline chloride) was studied when added at 10% and stored in sealed plastic bags at ambient temperature for 6 months.[31] No losses were observed.

[22] Technical dossier/Annex 2.1.4a and supplementary information January 2018/Q9. LOD (in μg/kg) was 2 for aflatoxins B1, B2, G1 and G2; 10 for zearalenone; 50 for fumonisins B1 and B2; 5 for ochratoxin A and 134 for deoxynivalenol.
[23] Technical dossier/Supplementary information September 2018/Annex 1; supplementary information November 2018/Annex 5.
[24] Technical dossier/Section 2.1.4 and Annexes 2.1.4c to e.
[25] Technical dossier/Section 2.1.5.
[26] Technical dossier/Section II/Annex 2.1.3c.
[27] Technical dossier/Section II/Annex 2.5.2a.
[28] Technical dossier/Section II/Annex 2.4.3a.
[29] Technical dossier/Section II/Annex 2.1.5 and supplementary information January 2018/Q12.
[30] Technical dossier/Section II/Annex 2.4.1a and supplementary information January 2018/Q13.
[31] Technical dossier/Section II/Annex 2.4.1c and 2.4.1d; supplementary information January 2018/Q14a.

The stability of the additive (thee batches) was studied in a piglet compound feed (basal diet consisted on barley and soybean meal, containing tabulated values/calculated nutrients 7.99 g lysine/kg DM and 187 mg choline/kg DM)[32] when supplemented at 0.5%. The stability was studied in meal and pelleted feed. Pelleting conditions, stability during feed processing and storage conditions (packaging and temperature) were not reported. No losses were observed.[33]

The stability of the additive (three batches) was studied at a concentration of 0.5% in water for drinking (equivalent to a concentration of 4 mg lysine/mL) and stored at room temperature for 24 h.[34] No losses were detected

The capacity of one batch of L-lysine HCl (one batch) to distribute homogeneously in the piglet compound feed described above supplemented with 0.5% free lysine was studied in 10 subsamples. The coefficient of variation (CV) was 2.2%.[35]

3.4.1.5. Physico-chemical incompatibilities in feed

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

3.4.2. Safety of L-lysine HCl

L-Lysine requirements of different non-ruminant species and animal categories, absorption and metabolic fate of L-lysine, tolerance to L-lysine excess and the lysine to arginine antagonism have been described in detail in previous opinions. No safety concerns for ruminants would arise from ruminal lysine metabolism (EFSA FEEDAP Panel, 2013, 2014). The use of the amino acid 'per se' will not raise safety concerns for the target animals provided it is supplemented in appropriate amounts to the diets. However, 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 use via water for drinking.

Absorption, distribution, metabolism and excretion of L-lysine were described in a previous scientific opinion of the EFSA FEEDAP Panel (2013). The use of the amino acid L-lysine itself in animal nutrition is considered safe for consumers.

The amino acid L-lysine is a physiological and natural component of animals and plants. It is not excreted as such (but as urea/uric acid and carbon dioxide). The use of L-lysine in animal nutrition would not lead to any localised increase in the concentration of L-lysine or its metabolites in the environment.

Potential concerns would arise from the fermentation process. *C. glutamicum* is regarded by EFSA to be suitable for the Qualified Presumption of Safety (QPS) approach to safety assessment only when used as production organism (EFSA, 2007b; EFSA BIOHAZ Panel, 2019). The identity of the production strain has been established as *C. glutamicum* and the absence of antimicrobial resistance has been proven. However, uncertainty remains regarding the possible genetic modification of the original strain and the introduction of sequences of concern. In addition, the available data do not allow to conclude on the presence/absence of viable cells and of DNA of the production strain in the final product. Therefore, the FEEDAP Panel cannot conclude on the safety of the additive L-lysine HCl produced by *C. glutamicum* CCTCC M 2015595 for the target species, consumer and the environment.

3.4.2.1. Safety for the user

The fractions below 100, 50 and 10 μm diameter (v/v) were 3.6–5.9%, 1.6-2.4% and < 1%, respectively. The dusting potential was 0.2 g/m^3 in all three batches (see Section 3.4.1.3)

No specific studies to support the assessment of the safety for the user performed with L-lysine monohydrochloride were available. In the absence of data, the FEEDAP Panel cannot conclude on the potential of the L-lysine HCl to be toxic by inhalation, irritant to skin or eyes, or on its potential to be a dermal sensitiser. Furthermore, owing to the uncertainties regarding the possible genetic modification of the original production strain and the presence of viable cells of the production strain in the final product, the FEEDAP Panel cannot conclude on the safety of the additive L-lysine HCl produced using *C. glutamicum* CCTCC M 2015595 for the users.

[32] Technical dossier/Section II/Annex 2.4.1f.
[33] Technical dossier/Section II/Annex 2.4.1d and 2.4.1f.
[34] Technical dossier/Section II/Annex 2.4.1g.
[35] Technical dossier/Section II/Annex 2.4.1d and 2.4.1f and supplementary information January 2018/Q16.

3.5. L-Lysine sulfate

3.5.1. Characterisation

3.5.1.1. Characterisation of the active substance/additive

L-Lysine sulfate (CAS No 60343-69-3) has a molecular weight of 390.38 g/mol. The molecular formula is $[NH_2–(CH_2)_4–CH(NH_2)–COOH]_2\ SO_4$ and the molecular structure is given in Figure 2. The theoretical content of lysine in the lysine sulfate is 75%.

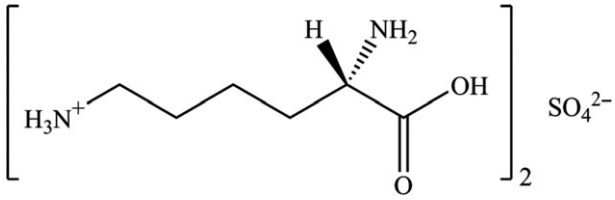

Figure 2: Molecular structure of L-lysine sulfate

L-Lysine sulfate solid contains by specification ≥ 73% lysine sulfate (≥ 55% lysine on DM basis), ≤ 5% other amino acids, ≤ 7% total sugars, ≤ 4% loss on drying, ≤ 4% residues on ignition, ≤ 3% ammonium salt and ≥ 6% protein.[36]

The compositional data of six batches showed an average total lysine concentration of 53.6% on as is basis (range 51.9–55.8%),[37] corresponding to an average 55.4% lysine on a DM basis. Three out of the six batches analysed did not meet the specification. The average sulfate content (calculated from analysed sulfur) was 19.8% (range 18–21.3%) as is, corresponding to 20.4% on a DM basis. The calculated proportion of sulfate not associated with lysine in relation to total lysine was about 8.5%. Water content was on average 3.2% (range 2.4–3.5%). Crude ash was on average 2.2% (range 1.5–2.7%). Ammonium (measured in three batches) represented 0.6%. The sum of total other amino acids was 7.5% on a DM basis, total sugars were 0.3% and protein not related to free amino acids (measured in three batches) averaged 8%.[38]

As regards the amount of identified material, three batches were analysed and about 97% of the material on a DM basis was identified.

3.5.1.2. Impurities

The content of heavy metals (lead, cadmium and mercury) and arsenic in the additive (three batches analysed) showed cadmium and lead values below the limit of detection.[39] Arsenic ranged from < 0.04 to 0.14 mg/kg and mercury from < 0.01 to 0.05 mg/kg. Dioxins and the sum of dioxin plus dioxin-like PCBs in three batches of the product were < 0.137 to 0.247 ng WHO-PCDD/F TEQ/kg and < 0.269 to 0.367 ng WHO-PCDD/F-PCB TEQ/kg.

Microbiological contamination (analysed in three batches) showed that *Salmonella* spp. was absent in 25 g samples. *E. coli*, and coliforms MPN was < 30/g, respectively. Yeasts and filamentous fungi were not detected.[40] Regarding the mycotoxin content, the same batches showed aflatoxins (B1, B2, G1, G2), ochratoxin A below the LOD (except one batch which had a concentration of ochratoxin A of 13.8 μg/kg), DON ranged from 457 to 2,917 μg/kg.[41] Zearalenone ranged from 73 to 140 μg/kg and fumonisins (B1, B2) from 240 to 950 μg/kg.

The amounts of the aforementioned contaminants/impurities do not raise safety concerns.

The applicant provided supplementary information to support the absence of viable cells of the production strain in three batches of L-lysine sulfate.[42] A 1-g sample was diluted in 9 mL of distilled water (three replicates per batch) and after 2 h at 22°C shaking the sample every hour, 1 mL of the suspension was spread on LB plates (2 × 0.5 mL on two different plates). Plates incubated at 30°C for 2 days and

[36] Technical dossier/Section II/Table 2.1.3b.
[37] Method HPLC accredited by the Dutch Accreditation Council (L172).
[38] Technical dossier/Supplementary information January 2018/Annexes Q18a to l.
[39] Technical dossier/Section II/Annex 2.1.4b.
[40] Technical dossier/Annex 2.1.4b and supplementary information January 2018/Q9.
[41] Technical dossier/Annex 2.1.4b and supplementary information January 2018/Q9. LOD (in μg/kg) was 2 for aflatoxins B1, B2, G1 and G2; 5 for ochratoxin A and 134 for deoxynivalenol.
[42] Technical dossier/Supplementary information September 2018/Annex 1 and supplementary information November 2018/Annex 5.

6 days before inspection of growth. Identification of the strains was performed using MALDI-TOF analyses of clones for each batch and of colonies from the positive control. Every sample presented colonies after 2 days that morphologically corresponded to *C. glutamicum*. On day 6, the number of colonies had increased (calculated to range between 1,597 and 2,190 CFU/g depending on the batch considered) and had evolved differently by comparison with day 2. One to three colonies of each morphotype were recovered for each replicate. These colonies were grouped into six different morphotypes. MALDI-TOF results identified correctly the positive control as *C. glutamicum*, yielded a fully reliable and reproducible result for morphotypes 1 and 2 (*Acinetobacter radioresistens* and *Pseudomonas fulva*, respectively), a reproducible result (genus level) for morphotypes 3 and 4 (*Lactobacillus* and *Rhodococcus fascians*), and an unreliable result for morphotype 5 (bacteria from DFB group). Morphotype 6 and three additional ungrouped colonies could not be identified. Morphotypes 2 to 6 represented < 10 CFU/g of sample.

Although the available data do not indicate the presence of viable cells of the production strain, uncertainty remains due to the following weaknesses:

- The limited amount of product tested (0.1 g).
- There were viable cells that could not be identified. The high bacterial growth hampered the identification of all colonies.

Therefore, the presence of viable cells of the production strain in the three batches of L-lysine SO4 tested cannot be excluded.

The applicant did not provide conclusive data on the absence of DNA of the production strain in the final product.[24] This would be of relevance in the case the original strain was genetically modified.

3.5.1.3. Physical properties

The product under assessment is a brown to light brown granulate. It has a bulk density of 600–650 g/cm^3.[25] Its solubility in water is 200 g/L at 20°C.[43]

Concerning the particle size, three batches were analysed by laser diffraction. The fractions below 100, 50 and 10 μm diameter (v/v) were 0–6.4%, 0–2.7% and ≤ 0.1%, respectively.[29]

The dusting potential (three batches, Stauber–Heubach method) ranged from < 0.1 to 1 g/m^3.[44]

3.5.1.4. Stability and homogeneity

The shelf life of the additive was studied in three batches of the additive kept in closed bags either at room temperature for 1 year or at 40°C for 8 h. No losses were observed.[45]

The stability of the additive (three batches) in a vitamin/mineral premixture (containing 16 g/kg choline chloride, and apparently 1.5 g/kg lysine) was studied when added at 10% and stored in sealed plastic bags at room temperature for 3 months.[46] Losses ranged from 2.8% to 3.8%.

The stability of the additive (three batches) was studied in a piglet compound feed (basal diet consisted on barley and soybean meal, containing 0.8% lysine and 187 mg/kg choline)[32] when supplemented at 0.5%. The stability was studied in meal and pelleted feeds, but the pelleting conditions were not described. Storage conditions were not reported. No losses were observed in the content of lysine.[47]

The stability of L-lysine sulfate (three batches) was studied when solved at a concentration of 0.5% in water for drinking (equivalent to a concentration of 3.74 mg lysine/mL) and stored at room temperature for 24 h.[48] Losses up to 4% were detected.

The capacity of one batch of L-lysine sulfate to distribute homogeneously in the piglet compound feed (pelleted) described above supplemented with 0.5% free lysine was studied in 10 subsamples. The CV was 3.5%.[47]

3.5.1.5. Physico-chemical incompatibilities in feed

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

[43] Technical dossier/Supplementary information January 2018/Answer to Q24.
[44] Technical dossier/Section II/Annex 2.4.3b.
[45] Technical dossier/Section II/Annex 2.4.1b.
[46] Technical dossier/Section II/Annex 2.4.1c and 2.4.1^e and supplementary information January 2018/Q26.
[47] Technical dossier/Section II/Annex 2.4.1e and 2.4.1f.
[48] Technical dossier/Section II/Annex 2.4.1h.

3.5.2. Safety of L-lysine sulfate

General considerations regarding the safety of L-lysine additives have been discussed in Section 3.4.2.

There is a high inherent content of sulfate in L-lysine sulfate which could be a safety concern for the target species, depending on the supplementation level and the tolerance of the target species (EFSA FEEDAP Panel, 2015b).

With regard to the high content of sulfate in L-lysine sulfate, the EFSA FEEDAP Panel (2019b) already concluded that the formulation of the complete feed should carefully take into account the maximum tolerable level of total S, as established by NRC (2005) and set in ruminant diets at 3 g S/kg DM (diet rich in concentrate) and at 5 g S/kg DM (diet rich in roughage) and in non-ruminant diets at 4 g S/kg DM. Also, the contribution of S/sulfate present in water for drinking to the total S intake should be considered, especially when the content is high.[49]

The studies, already published in the scientific literature and provided also by the applicant (Drewnoski et al. (2014) in feedlot cattle; Kerr et al. (2014), Bobeck et al. (2013) in growing pigs; Kim et al. (2014) in growing/finishing pigs and Spears et al. (2011) in steers), confirm the statement by NRC (2005), as specified above. Consequently, no negative effects are to be expected at normal use levels for the target species provided that the total S intake complies with the recommendations of established scientific bodies.

Potential concerns would arise from the fermentation process. *C. glutamicum* is regarded by EFSA to be suitable for the Qualified Presumption of Safety (QPS) approach to safety assessment only when used as production organism (EFSA, 2007b; EFSA BIOHAZ Panel, 2019). The identity of the production strain has been established as *C. glutamicum* and the absence of antimicrobial resistance has been proven. However, uncertainty remains regarding the possible genetic modification of the original strain and the introduction of sequences of concern. In addition, the available data do not allow to conclude on the presence/absence of viable cells and of DNA of the production strain in the final product. Therefore, the FEEDAP Panel cannot conclude on the safety of the additive L-lysine SO_4 produced by *C. glutamicum* CCTCC M 2015595 for the target species, consumer and the environment.

3.5.2.1. Safety for the user

In relation to the particle size distribution, the fractions below 100, 50 and 10 μm diameter (v/v) were 0–6.4%, 0–2.7% and ≤ 0.1%, respectively. The dusting potential ranged from < 1 to 1 g/m^3 (see Section 3.5.1.3).

No specific toxicity studies for user safety performed with the L-lysine sulfate under assessment were available. In the absence of data, the FEEDAP Panel cannot conclude on the potential of this product to be toxic by inhalation, or irritant to skin or eyes, or on its potential to be a dermal sensitiser. Furthermore, owing to the uncertainties regarding the possible genetic modification of the original production strain and the presence of viable cells of the production strain in the final product, the FEEDAP Panel cannot conclude on the safety of the additive L-lysine sulfate produced with *C. glutamicum* CCTCC M 2015595 for the user.

3.6. 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-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 EFSA opinions (EFSA FEEDAP Panel, 2013, 2014). In general, the products L-lysine HCl and L-lysine sulfate 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, would require protection against degradation in the rumen.

3.7. Post-marketing 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[50] and Good Manufacturing Practice.

[49] See appendix A of EFSA EFSA FEEDAP Panel et al., 2019a,b,2019c.

[50] 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.

4. Conclusions

Owing to the uncertainties regarding the possible genetic modification of the strain used to obtain the production strain and on the possible presence of viable cells and DNA of the production strain in the final product, the FEEDAP Panel cannot conclude on the safety of the additives L-lysine HCl and L-lysine sulfate produced with *C. glutamicum* CCTCC M 2015595 for the target species, the consumers, the users and the environment.

For both products, the FEEDAP Panel has concerns regarding the safety for the target species when the additives are administered via water for drinking.

In the absence of data, the FEEDAP Panel cannot conclude on the safety of the additive for the user.

The products under assessment are considered efficacious sources of the amino acid L-lysine for all animal species. For these products to be as efficacious in ruminants as in non-ruminant species, they require protection against degradation in the rumen.

Documentation provided to EFSA

1) Dossier L-lysine monohydrochloride and L-lysine sulfate from *Corynebacterium glutamicum*. September 2016. Submitted by Kempex Holland B.V.
2) Dossier L-lysine monohydrochloride and L-lysine sulfate from *Corynebacterium glutamicum*. Supplementary information. January 2018. Submitted by Kempex Holland B.V.
3) Dossier L-lysine monohydrochloride and L-lysine sulfate produced by fermentation with *Corynebacterium glutamicum*. Supplementary information. April 2018. Submitted by Kempex Holland B.V.
4) Dossier L-lysine monohydrochloride and L-lysine sulfate produced by fermentation with *Corynebacterium glutamicum*. Supplementary information. September 2018. Submitted by Kempex Holland B.V.
5) Dossier L-lysine monohydrochloride and L-lysine sulfate produced by fermentation with *Corynebacterium glutamicum*. Supplementary information. November 2018. Submitted by Kempex Holland B.V.
6) Evaluation report of the European Union Reference Laboratory for Feed Additives on the Methods(s) of Analysis of L-lysine monohydrochloride and L-lysine sulfate from *Corynebacterium glutamicum* CCTCC M 2015595.
7) Comments from Member States.

Chronology

Date	Event
16/08/2016	Dossier received by EFSA
23/09/2016	Reception mandate from the European Commission
30/11/2016	Application validated by EFSA – Start of the scientific assessment
07/02/2017	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, conditions of use, characterisation of the production strain, characterisation of both forms of the additive, safety for the target species and safety for the user.*
28/02/2017	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
01/03/2017	Comments received from Member States
22/01/2018	Reception of supplementary information from the applicant - Scientific assessment re-started
01/03/2018	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 the production strain and characterisation of both forms of the additive.*
23/04/2018	Reception of supplementary information from the applicant - Scientific assessment re-started
14/05/2018	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 both forms of the additive under assessment.*
11/09/2018	Reception of supplementary information from the applicant - Scientific assessment re-started

Date	Event
16/10/2018	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterisation of the production strain and characterisation of both forms of the additive under assessment.*
12/11/2018	Reception of supplementary information from the applicant - Scientific assessment re-started
26/02/2019	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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Abbreviations

CCTCC	China Center for Type Culture collection
CAS	Chemical Abstracts Service
CFU	colony forming unit
CV	coefficient of variation
DFB	desferrioxamine B
DM	dry matter
DON	deoxynivalenol
EINECS	European Inventory of Existing Commercial Chemical Substances
EURL	European Union Reference Laboratory
FEEDAP	EFSA Scientific Panel on additives and products or substances used in animal feed
IEC-UV/FD	Ion Exchange Chromatography coupled with post-column derivatisation and Ultraviolet or Fluorescence Detection

IUPAC	International Union of Pure and Applied Chemistry
LOD	limit of detection
MALDI-TOF	matrix-assisted laser desorption/ionisation time-of-flight mass spectrometer
MIC	minimum inhibitory concentration
MPN	most probable number
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo(*p*)dioxins
PCDF	polychlorinated dibenzofurans
QPS	Qualified Presumption of Safety (QPS
RSDr	Relative standard deviation for repeatability
RSDR	Relative standard deviation for reproducibility
TEQ	Toxin equivalent
WHO	World Health Organization

Annex A – Evaluation report of the analytical methods submitted in connection with the application for authorisation of L-lysine monohydrochloride and L-lysine sulfate from *Corynebacterium glutamicum* CCTCC M 2015595

In the current application, authorisation is sought under Article 4(1) for *L-lysine monohydrochloride and L-lysine sulfate from Corynebacterium glutamicum CCTCC M 2015595,* 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. *L-lysine* is already authorised as a *feed additive* under Commission Directive 88/485/EEC.

For the quantification of *L-lysine monohydrochloride* and *L-lysine sulfate* in *feed additive*, the Applicant submitted the ring-trial validated ISO method EN ISO 17180:2013 based on Ion Exchange Chromatography coupled with post-column derivatisation and Ultraviolet or Fluorescence Detection (IEC-UV/FD). The following performance characteristics are reported: a relative standard deviation for repeatability (RSDr) ranging from 0.7 to 1.7%; a relative standard deviation for reproducibility (RSDR) ranging from 1.5 to 2.5%; and a recovery rate (RRec) ranging from 97.8 to 100%. In addition, the EURL identified the "L-lysine monohydrochloride monograph" of the Food Chemical Codex (FCC) for the characterisation of *L-lysine monohydrochloride* in the *feed additive* and the generic European Pharmacopoeia monograph on sulfates (Ph. Eur. 20301) for the identification of *sulfates* in *L-lysine sulfate.*

For the quantification of *L-lysine monohydrochloride* and *L-lysine sulfate* in *premixtures, feedingstuffs* and *water* the Applicant submitted the ring-trial validated Community method (Commission Regulation (EC) No 152/2009) based on IEC coupled with post-column derivatisation using an amino acid analyser or high performance liquid chromatography equipped with ion exchange column and photometric detection (UV). This method, designed only for the analysis of *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 3.5% and RSDR ranging from 3.0 to 13.1%. Since the Applicant provided no experimental data to determine *L-lysine* in *water,* the EURL cannot evaluate nor recommend a method for the official control to determine *L-lysine* in *water.* Based on the performance characteristics presented, the EURL recommends for official control the four standard methods mentioned above for the identification or quantification of *lysine* in the *feed additive, premixture* and/or *feedingstuffs.*

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