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## Safety of concentrated L-lysine (base), L-lysine monohydrochloride and L-lysine sulfate produced using different strains of *Corynebacterium glutamicum* for all animal species based on a dossier submitted by FEFANA asbl

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### Abstract

The EFSA FEEDAP Panel previously (2016) could not conclude on the safety of certain concentrated liquid L-lysine (base), L-lysine monohydrochloride (HCl) and L-lysine sulfate products manufactured using different strains of *Corynebacterium glutamicum*. New information on the safety of these products was provided by the applicant. The recipient strain *C. glutamicum* KCTC 12307BP qualifies for qualified presumption of safety (QPS) approach for safety assessment, the genetic modification does not introduce any safety concern and no introduced antibiotic resistance genes remain in its genome. Even if uncertainty remains concerning the absence/presence of the production strain and/or its recombinant DNA in the final products, these would not raise safety concerns. The liquid L-lysine (base) and L-lysine HCl produced by *C. glutamicum* KCTC 12307BP or *C. glutamicum* KCCM 11117P; and L-lysine HCl produced by *C. glutamicum* NRRL B-50547 are considered safe for the target species, consumers and the environment. Regarding the safety for the user, concentrated liquid L-lysine (base) and L-lysine HCl produced by *C. glutamicum* KCTC 12307BP, *C. glutamicum* NRRL B-50547 or *C. glutamicum* KCCM 11117P are not irritant to skin or eyes and they are not skin sensitisers. L-Lysine HCl is not hazardous by inhalation. The use of *C. glutamicum* DSM 24990 in the production of L-lysine sulfate is considered safe for the target species, consumers, users or the environment. No negative effects are to be expected for the target species within the proposed inclusion levels of 0.5–30 g lysine sulfate/kg complete feed provided that the total S intake complies with the recommendations of established scientific bodies. The use of *C. glutamicum* KCCM 10227 in the production of L-lysine sulfate is considered safe for the target species, consumers, users and the environment with regard to antimicrobial resistance. No negative effects are to be expected for the target species within common inclusion levels provided that the total S intake complies with the recommendations of established scientific bodies.

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## Summary

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 the safety of concentrated liquid L-lysine (base), L-lysine monohydrochloride and L-lysine sulfate produced using different strains of *C. glutamicum* as a nutritional additive for all animal species based on the additional data submitted by the applicant.

The approach followed by the FEEDAP Panel to assess the safety of concentrated liquid L-lysine (base), L-lysine monohydrochloride and L-lysine sulfate (solid or liquid) is in line with the principles laid down in Regulation (EC) No 429/2008 and the relevant guidance documents.

Uncertainty remains concerning the absence/presence of the production strain *C. glutamicum* KCTC 12307BP and/or its recombinant DNA in the final products liquid L-lysine (base) and L-lysine HCl. The recipient strain qualifies for qualified presumption of safety (QPS) approach for safety assessment, the genetic modification does not introduce any safety concern and no introduced antibiotic resistance genes remain in the genome of the production strain. Therefore, the presence of viable cells and/or its recombinant DNA in the products would not raise safety concerns. The liquid L-lysine (base) and L-lysine HCl produced by *C. glutamicum* KCTC 12307BP are considered safe for the target species, consumers and the environment.

The production strain *C. glutamicum* NRRL B-50547 carries a full gene of [REDACTED]. Since no cells and recombinant DNA from the production strain were found in the product L-lysine HCl, the product does not raise safety concerns with respect to the genetic modification of the production strain provided that the downstream process is performed ensuring that no recombinant DNA of the production strain is present in the final product. L-Lysine HCl produced by *C. glutamicum* NRRL B-50547 does not raise safety concerns and it is considered safe for the target species, consumers and the environment.

The products concentrated liquid L-lysine (base) and L-lysine monohydrochloride, produced using *C. glutamicum* KCCM 11117P, do not raise safety concerns with respect to the potential presence of viable cells of the production strain or its recombinant DNA. Consequently, the liquid L-lysine (base) and L-lysine HCl produced by *C. glutamicum* KCCM 11117P are considered safe for the target species, consumers and the environment.

Regarding the safety for the user, concentrated liquid L-lysine (base) and L-lysine HCl produced by *C. glutamicum* KCTC 12307BP, *C. glutamicum* NRRL B-50547, *C. glutamicum* KCCM 11117P are not irritant to skin or eyes and they are not skin sensitisers. L-Lysine HCl is not hazardous by inhalation.

The use of *C. glutamicum* DSM 24990 in the production of L-lysine sulfate is considered safe for the target species, consumers, users or the environment. No negative effects are to be expected for the target species within the proposed inclusion levels of 0.5–30 g lysine sulfate/kg complete feed provided that the total S intake complies with the recommendations of established scientific bodies.

The use of *C. glutamicum* KCCM 10227 in the production of L-lysine sulfate is considered safe for the target species, consumers, users and the environment with regard to antimicrobial resistance. No negative effects are to be expected for the target species within common inclusion levels provided that the total S intake complies with the recommendations of established scientific bodies.

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## 1. Introduction

### 1.1. Background and Terms of Reference as provided by the requestor

Regulation (EC) No 1831/2003 establishes rules governing the Community authorisation of additives for use in animal nutrition and, in particular, Article 9 defines the terms of the authorisation by the Commission.

The applicant, FEFANA asbl,<sup>1</sup> is seeking a Community authorisation of concentrated liquid L-lysine (base), L-lysine monohydrochloride and L-lysine sulfate produced using different strains of *Corynebacterium glutamicum* to be used as a nutritional additive for all animal species. (Table 1).

**Table 1:** Description of the substances

<b>Category of additive</b>	Nutritional additive
<b>Functional group of additive</b>	Amino acids, their salts and analogues
<b>Description</b>	Concentrated liquid L-lysine (base), L-lysine monohydrochloride and L-lysine sulfate produced using different strains of <i>Corynebacterium glutamicum</i>
<b>Target animal category</b>	All animal species
<b>Applicant</b>	FEFANA asbl
<b>Type of request</b>	New opinion

On 02 December 2015, the Panel on Additives and Products or Substances used in Animal Feed of the European Food Safety Authority ("Authority"), in its opinion on the safety and efficacy of the product could not conclude on the safety of concentrated liquid L-lysine (base), L-lysine monohydrochloride and L-lysine sulfate produced using different strains of *Corynebacterium glutamicum*.

The Commission has now received new data on the safety of concentrated liquid L-lysine (base), L-lysine monohydrochloride and L-lysine sulfate produced using different strains of *C. glutamicum*.

In view of the above, the Commission asks the Authority to deliver a new opinion on the safety of concentrated liquid L-lysine (base), L-lysine monohydrochloride and L-lysine sulfate produced using different strains of *C. glutamicum* as a nutritional additive for all animal species based on the additional data submitted by the applicant.

### 1.2. Additional information

The active substance of the three products under application, L-lysine, is produced either by genetically modified strains of *C. glutamicum* (KCTC 12307BP, NRRL B-50547 or KCCM 11117P) in the case of concentrated liquid L-lysine (base) and L-lysine monohydrochloride technically pure, or by chemically mutated strains of *C. glutamicum* (DSM 24990 or KCCM10227) in the case of L-lysine sulfate solid. The applicant withdrew the application for L-lysine sulfate (solid and liquid forms) produced by *C. glutamicum* DSM 16615 during the assessment. L-Lysine is currently authorised for its use in all animal species as a nutritional additive (functional group amino acids, their salts and analogues).<sup>2</sup> No maximum content in feedingstuffs is established in the EU.

The applicant has provided additional information on the characterisation of the additives, the characterisation of the production strains and on their safety.

The initially proposed use in water for drinking of concentrated liquid L-lysine base and L-lysine monohydrochloride produced by *C. glutamicum* KCTC 12307BP was withdrawn by the applicant.<sup>3</sup>

Product from *C. glutamicum* rich in protein (inactivated fermentation by-product from the production of amino acids by culture of *Corynebacterium glutamicum* on substrates of vegetable or chemical origin, ammonia or mineral salts, it may be hydrolysed) with up to 0.3% antifoaming agents, 1.5% filtration/clarifying agents and 2.9% propionic acid and with a declared content of crude protein and propionic acid if > 0.5%; are listed in the Catalogue of feed materials (Commission Regulation

<sup>1</sup> FEFANA asbl, Avenue Louise 130a, 1050 Brussels, Belgium. The consortium includes 5 companies (ADM Specialty Ingredients (Europe) BV, Daesang Corp., CJ Europe GmbH, Evonik Degusa GmbH, and VitaLys).

<sup>2</sup> 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.

<sup>3</sup> Technical dossier/ [REDACTED] Attachment 4.

(EU) 2017/1017).<sup>4</sup> *C. glutamicum* is regarded as qualified presumption of safety (QPS) only when used as a production organism for amino acids (EFSA BIOHAZ Panel, 2013, 2017).

## 2. Data and methodologies

### 2.1. Data

The present assessment is based on data submitted by the applicant in the form of additional information<sup>5</sup> to a previous application of the same product.<sup>6</sup>

### 2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety of concentrated liquid L-lysine (base), L-lysine monohydrochloride and L-lysine sulfate (solid or liquid) is in line with the principles laid down in Regulation (EC) No 429/2008<sup>7</sup> and the relevant guidance documents: Guidance on nutritional additives (EFSA FEEDAP Panel, 2012a), Guidance for establishing the safety of additives for the consumer (EFSA FEEDAP Panel, 2012b), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012c), Guidance on the assessment of bacterial resistance to antibiotics of human or veterinary importance (EFSA FEEDAP Panel, 2012d), Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018), Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use (EFSA GMO Panel, 2011) and Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (EFSA BIOHAZ Panel, 2013, 2017 update).

## 3. Assessment

The products under application are concentrated liquid L-lysine (base), L-lysine HCl, L-lysine sulfate solid and L-lysine sulfate liquid. L-Lysine is currently authorised for use in feeds for all animal species. The products under application are produced by fermentation with different *C. glutamicum* strains. They are intended to be used in all animal species as nutritional additives (functional group amino acids, their salts and analogues) in feed. L-Lysine HCl and the solid form of L-lysine sulfate are also proposed for their use in water for drinking.

Concentrated liquid L-lysine base and L-lysine HCl are produced by microbial fermentation by genetically modified (GM) strains of *C. glutamicum* (KCTC 12307BP, NRRL B-50547 or KCCM 11117P).

L-Lysine sulfate solid and liquid are produced by microbial fermentation by chemically mutated strains of *C. glutamicum* (DSM 24990 or KCCM10227).

*C. glutamicum* is considered QPS approach for safety assessment for amino acid production when specific requirements are met (species identity confirmation and the absence of transmissible antibiotic resistance) (EFSA BIOHAZ Panel, 2013, 2017). When the production strain is a GM *C. glutamicum*, the genetic modification should not raise safety concerns.

In the previous assessment, the FEEDAP Panel concluded that the use of L-lysine HCl technically pure produced using *C. glutamicum* NRRL B-50547 in animal nutrition is considered a hazard for the target species, consumer, user and environment due to the presence of a recombinant antimicrobial resistance gene in most batches of the product.

The FEEDAP Panel could not conclude on:

- The safety of the products concentrated liquid L-lysine (base) and L-lysine HCl, technically pure, produced using *C. glutamicum* KCCM 11117P (the presence of the production strain and/or its recombinant DNA in concentrated liquid L-lysine (base) and L-lysine HCl technically pure could not be excluded; and the genetic basis of the resistance of the production strain to clindamycin was not investigated) or KCTC 12307BP (incomplete data on its identity, safety of

<sup>4</sup> Commission Regulation (EU) 2017/1017 of 15 June 2017 amending Regulation (EU) No 68/2013 on the Catalogue of feed materials. OJ L 159/48, 21.6.2017, pp. 48–119.

<sup>5</sup> FEED dossier reference: FAD-2016-0029.

<sup>6</sup> FEED dossier reference: FAD-2010-0067.

<sup>7</sup> 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.

genetic modification, potential for antimicrobial resistance and the possible presence of the production strain and its recombinant DNA in the final products concentrated liquid L-lysine (base) and L-lysine HCl technically pure) for the target animal species, consumer, the user and the environment.

- The safety of L-lysine sulfate solid and/or liquid originating from *C. glutamicum* KCCM 10227 or DSM 24990. The genetic base of resistance to at least one of the antimicrobials used in medical and veterinary practice was not sufficiently elucidated. The intrinsic high sulfate content in the product might have the potential to cause adverse effects in the target species.
- The dermal sensitisation and on the irritancy of L-lysine HCl to skin or eyes. The concerns regarding the safety of the genetic modification and potential presence of transmissible resistance to antimicrobials might also have implications for the safety of the user.

Regardless of the assessment of the genetic modifications, potential transmissible antimicrobial resistance or the absence of studies on safety for the user, the FEEDAP Panel had concerns on the safety of the administration of the amino acids, including L-lysine, via water for drinking for the target species because of the risk of imbalances and for hygiene reasons.

Additional data have been submitted on the characterisation of the production strains and/or the products under assessment.

These products had been characterised in a previous scientific opinion (EFSA FEEDAP Panel, 2016). The specifications of the products under assessment are:

- Concentrated liquid L-lysine (base): minimum 50% lysine, maximum 48% water;
- L-Lysine monohydrochloride: minimum 78% lysine, maximum 1.5% water; and
- L-Lysine sulfate solid: minimum 40% lysine.

### 3.1. Concentrated liquid L-lysine (base) and L-lysine HCl produced by *C. glutamicum* KCTC 12307BP

In its previous opinion (EFSA FEEDAP Panel, 2016), the FEEDAP Panel could not conclude on the safety of concentrated liquid L-lysine (base) and L-lysine HCl produced by the GM *C. glutamicum* KCTC 12307BP for the target animals, consumers and environment due to the lack of adequate information on the identity of the production strain, its genetic modification, its potential to harbour transferable antimicrobial resistance and lack of evidence of absence of the production strain or its recombinant DNA in the products.

The applicant provided new information on the characterisation of the strain, the absence of the production strain or its recombinant DNA in the final product, characterisation of the products and studies on the safety of both products for the user. The new information provided is assessed below.

#### 3.1.1. Characterisation of the *C. glutamicum* KCTC 12307BP and the absence of viable cells and DNA in the final products

In the former assessment (EFSA FEEDAP Panel, 2016), evidence for the identity of the production strain was not submitted. In addition, no information on the process of genetic modification was provided.

#### Antimicrobial susceptibility of the genetically modified microorganism

*C. glutamicum* KCTC 12307BP was tested for antimicrobial susceptibility using [REDACTED] method. The battery of antimicrobials tested was that recommended by EFSA (EFSA FEEDAP Panel, 2012c). All minimum inhibitory concentration (MIC) values were below the corresponding cut-off for 'Other Gram +', except for clindamycin. The MIC is 2 mg/L and the cut-off 0.25 mg/L.<sup>8</sup> No convincing explanation on the genetic basis of this resistance was provided. In the meantime, based in new scientific evidence, the FEEDAP Panel set the cut-off value of clindamycin from 0.25 to 4 mg/L for *Corynebacterium*, as reflected in the guidance on characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018). Therefore, the strain is considered susceptible to all relevant antimicrobials, and of no concern for the target animals, users, consumers or the environment.

<sup>8</sup> [REDACTED]

### Information on the parental/recipient organism

The recipient organism was

[REDACTED]

analysis.<sup>10</sup>

Information regarding the donor organism

All sequences introduced in the production strain derive from

[REDACTED]

### Information regarding the genetic modification

[REDACTED]

### Absence of viable cells and DNA in the final product

In the former assessment (EFSA FEEDAP Panel, 2016), no information on the absence of viable cells and DNA in the final products was provided.

New data were provided in which the production strain was not found in three batches of each final product (L-lysine base and L-lysine HCl), by incubating in non-selective liquid medium at 30°C for 4 h, followed by plating on selective medium at 30°C for 10 days. However, the amount of sample analysed

<sup>9</sup> [REDACTED]  
<sup>10</sup> [REDACTED]



was not reported. Therefore, uncertainty remains on the absence of viable production cells in the final products.<sup>11</sup>

No recombinant DNA was found in three batches of each final product (L-lysine base and L-lysine\_HCl), each tested in triplicate, by [REDACTED]. However, no control DNA was added to the samples before DNA extraction as requested. Therefore, a limit of detection could not be established and uncertainty remains on the absence of recombinant DNA in the final products.

### 3.1.2. Characterisation of the additives

New analytical data on impurities of concentrated liquid L-lysine (base) and L-lysine monohydrochloride (three batches each) have been submitted. All six batches yielded similar analytical results. As regards [REDACTED]. Regarding the microbiological analyses, all batches were [REDACTED].

<sup>12</sup>

New certificates of analysis have been submitted showing aerobic plate count at 30°C < 10<sup>2</sup> CFU/g and the absence of vegetative anaerobic mesophilic bacteria in three batches of L-lysine HCl and concentrated liquid L-lysine base (50%).<sup>13</sup>

Regarding the physical properties, new data on particle size distribution (laser diffraction) of three batches of L-lysine HCl showed a fraction of particles < 10 µm ranging from 0 to 0.43% (v/v), a fraction of particles < 50 µm ranging from 0 to 2.2% (v/v) and a fraction of particles < 100 µm ranging from 0 to 5.3% (v/v).<sup>14</sup>

### 3.1.3. Safety of concentrated liquid L-lysine (base) and L-lysine monohydrochloride produced using *C. glutamicum* KCTC 12307BP

#### Safety of the genetic modification

Uncertainty remains concerning the absence/presence of the production strain and/or its recombinant DNA in the final products. Nevertheless, as the recipient strain *C. glutamicum* KCTC 12307BP qualifies for QPS approach for safety assessment, the genetic modification does not introduce any safety concern and no introduced antibiotic resistance genes remain in the genome of the production strain, the presence of viable cells and/or its recombinant DNA in the products do not raise safety concern.

#### Safety for the target species, consumers and the environment

The safety of L-lysine in animal nutrition when used in appropriate amounts is well established in the scientific literature. For nutritional additives produced by fermentation, the risk associated with the residues of the fermentation process in the final product needs to be assessed.

The production strain *C. glutamicum* KCTC 12307BP has been unambiguously identified and it is susceptible to antimicrobials relevant for human or veterinary medicine. The recipient strain qualifies for QPS approach for safety assessment; the genetic modification does not introduce any safety concern and no introduced antibiotic resistance genes remain in the genome of the production strain. Although uncertainty remains concerning the possible presence of the production strain and/or its recombinant DNA in the final products, this does not raise safety concerns considering the above reasons.

Consequently, the concentrated liquid L-lysine (base) and L-lysine HCl produced by *C. glutamicum* KCTC 12307BP are considered safe for the target species, consumers and the environment.

#### Safety for the user

In the former assessment (EFSA FEEDAP Panel, 2016), no specific studies concerning the user safety performed with either of the two products were available. Concentrated liquid L-lysine (base)

<sup>11</sup> Technical dossier/Supplementary information June 2017/Annexes Q3.

<sup>12</sup> [REDACTED]

<sup>13</sup> Technical dossier/Supplementary information August 2016/[REDACTED]/Attachments 2–7.

<sup>14</sup> Technical dossier/Supplementary information August 2016/[REDACTED]/Attachments 8–10.

was considered as corrosive due to its high pH. In the absence of data, the FEEDAP Panel could not conclude on the dermal sensitisation and on the irritancy of L-lysine HCl to skin or eyes.

The applicant has provided new studies on the safety for the user performed with L-lysine HCl and concentrated liquid L-lysine produced by *C. glutamicum* KCTC 12307BP.

#### *Concentrated liquid L-lysine (base)*

##### **Effects on skin and eyes**

An acute dermal irritation study was performed with concentrated liquid L-lysine (50%) in rabbits in accordance with OECD Guideline 404.<sup>15</sup> None of the rabbits showed any skin reaction. Consequently, the product is classified as not irritating to human skin.

An acute eye irritation study was conducted with concentrated liquid L-lysine (50%, pH 9.95) in rabbits, in accordance with OECD Guideline 405.<sup>16</sup> Some redness of conjunctiva and chemosis was observed within 24 h post-application in different grades in all three rabbits. As the recorded scores were lower than the threshold value for classifying a product as an eye irritant (0.7 vs 2), the test material is considered not irritant for human eye.

The skin sensitisation potential of the product concentrated liquid L-lysine (50%) was studied in guinea pigs in accordance with OECD Guideline 406 (Guinea pig maximisation test).<sup>17</sup> No dermal reactions were observed at the 24- or 48-h post-challenge. Consequently, the test item has no sensitising properties.

#### *L-lysine monohydrochloride*

##### **Effects on the respiratory system**

Although the additive has very low fraction of particles below 100 µm (up to 5.3%), its dusting potential is high (7.1 g/m<sup>3</sup>, EFSA FEEDAP Panel, 2016), indicating that exposure of the user by inhalation is possible.

An acute inhalation toxicity study in accordance with OECD Guideline 436 was performed with L-lysine HCl (99.4% pure).<sup>18</sup> Six CRL (WI) BR rats (3 males and 3 females) were exposed by inhalation ('nose only') to a concentration of 5.1 mg L-lysine HCl/L air for 4 h. No mortality was observed. Dyspnoea during or shortly after exposure (all rats, 4 of them up to day 1) or weak grip and limb tone (one female rat, the first days of the observation period) were observed. No signs were observed from day 6 to the end of the study. Only one rat showed a slight body weight loss (2 g) during days 1–3 of the observation period. No macroscopic lesions were observed at the necropsy. The acute inhalation median lethal dose of the product is > 5.1 mg/L.

##### **Effects on skin and eyes**

An acute dermal irritation/corrosion study according to OECD Guideline 404 was carried out with L-lysine HCl (99.5% pure).<sup>19</sup> None of the three female albino rabbits used showed dermal irritation/corrosion at any observation time (1, 24, 48 and 72 h post-administration). Thus, the product L-lysine HCl is classified as not irritating to human skin.

An acute eye irritation/corrosion test in accordance with OECD Guideline 405 was performed using L-lysine HCl (99.5% pure).<sup>20</sup> No irritation/corrosion was observed in the eyes of the tested rabbits during the observation period. Thus, the product L-lysine HCl is classified as not irritating to human eyes.

The skin sensitisation potential of the product L-lysine HCl (99.5%) was studied in guinea pigs in accordance with OECD Guideline 406 (Guinea pig maximisation test).<sup>21</sup> No dermal reactions were observed at the 24- or 48-h post-challenge. Consequently, the test item has no sensitising properties.

#### *Conclusions on the safety for the user*

Neither the concentrated liquid L-lysine (base) nor the L-lysine HCl produced by *C. glutamicum* KCTC 12307BP are irritant to skin or eyes and they are not skin sensitisers. L-Lysine HCl is not hazardous by inhalation.

<sup>15</sup> Technical dossier/Supplementary information August 2016/[REDACTED]/Attachment 11.

<sup>16</sup> Technical dossier/Supplementary information August 2016/[REDACTED]/Attachment 12.

<sup>17</sup> Technical dossier/Supplementary information August 2016/[REDACTED]/Attachment 13.

<sup>18</sup> Technical dossier/Supplementary information September 2016/Daesang L-lysine HCl acute inhalation study.

<sup>19</sup> Technical dossier/Supplementary information August 2016/AMAC OECD 404 L-lysine HCl.

<sup>20</sup> Technical dossier/Supplementary information August 2016/AMAC OECD 405 L-lysine HCl.

<sup>21</sup> Technical dossier/Supplementary information August 2016/AMAC OECD 406 L-lysine HCl.

### 3.1.4. Conclusions on concentrated liquid L-lysine (base) and L-lysine monohydrochloride produced using *C. glutamicum* KCTC 12307BP

Uncertainty remains concerning the absence/presence of the production strain and/or its recombinant DNA in the final products. Nevertheless, as the recipient strain *C. glutamicum* KCTC 12307BP qualifies for QPS approach for safety assessment, the genetic modification does not introduce any safety concern and no introduced antibiotic resistance genes remain in the genome of the production strain, the presence of viable cells and/or its recombinant DNA in the products do not raise safety concern.

The liquid L-lysine (base) and L-lysine HCl produced by *C. glutamicum* KCTC 12307BP are considered safe for the target species, consumers and the environment.

Neither the concentrated liquid L-lysine (base) nor the L-lysine HCl produced by *C. glutamicum* KCTC 12307BP are irritant to skin or eyes and they are not skin sensitisers. L-Lysine HCl is not hazardous by inhalation for the user.

## 3.2. L-Lysine monohydrochloride produced using *C. glutamicum* NRRL B-50547

### 3.2.1. Absence of DNA of the production strain in the final product of L-lysine HCl

The production strain carries a gene conferring resistance to an antimicrobial and in the previous assessment it was found in the additive. The FEEDAP Panel, in its opinion of 2016 on the safety and efficacy of the product, concluded that L-lysine HCl produced by the GM *C. glutamicum* NRRL B-50547, raised safety concerns for the target species, consumers, users and the environment due to the presence of a [REDACTED] gene in the additive. The applicant stated that this resulted from a failure of the downstream process that has been resolved. New analyses have now been provided demonstrating that no full-length [REDACTED] gene was found [REDACTED] in [REDACTED] three new batches of the product.<sup>22</sup> Re-analysis of one of the original batches [REDACTED] tested in the original dossier confirmed that it contains the full-length [REDACTED] gene.

### 3.2.2. Characterisation of the L-lysine HCl

New data have been provided on the particle size distribution of the L-lysine HCl (three batches) measured in by sieving. No particles with a diameter < 50 µm were found and the fraction of particles with a diameter of < 105 µm was 0.1% (w/w). 88% of the particles had diameters ranging from 420 to 1,190 µm.<sup>23</sup>

### 3.2.3. Safety of L-lysine monohydrochloride produced using *C. glutamicum* NRRL B-50547

#### Safety of the genetic modification

The production strain contains a [REDACTED] gene in its genome as a result of the genetic modification. This resistance gene was present in three earlier batches of the L-lysine HCl product. The absence of viable cells of the production strain was previously shown (EFSA FEEDAP Panel, 2016). The analyses of three newly produced batches did not reveal the presence of recombinant DNA from the production strain.<sup>24</sup> Therefore, the product does not raise safety concern with respect to the genetic modification of the production strain.

#### *Safety for the target species, consumer and environment*

The safety of the L-lysine HCl was already considered in the previous assessment and the only concern was the presence of recombinant DNA in the product. As described above, no recombinant DNA was detected in three batches of the additive and consequently L-lysine HCl produced by *C. glutamicum* NRRL B-50547 does not raise safety concerns and it is considered safe for the target species, consumers and the environment.

<sup>22</sup> [REDACTED]

<sup>23</sup> Technical dossier/Supplementary information August 2016/ [REDACTED] particle size distribution.

<sup>24</sup> [REDACTED]

### Safety for the user

In the former opinion, no specific studies for user safety performed with either of these two products were available. Concentrated liquid L-lysine (base) was considered as corrosive due to its high pH. In the absence of data, the FEEDAP Panel could not conclude on the dermal sensitisation and on the irritancy of L-lysine HCl to skin or eyes. In addition, the concerns regarding the presence of transmissible antibiotic resistance in the L-lysine HCl would also have implications for the safety of the user.

The new data on particle size distribution indicate that there are practically no particles of < 100 µm diameter. The dusting potential of one analysed batch (Stauber–Heubach) was 0.3 g/m<sup>3</sup> (EFSA FEEDAP Panel, 2016). The dusting potential and the particle size distribution indicate low potential for exposure by inhalation; therefore, the FEEDAP Panel concludes that there are no concerns for the user by inhalation.

No new toxicological studies performed with concentrated liquid L-lysine (base) or L-lysine HCl produced by *C. glutamicum* NRRL B-50547 have been submitted. The results of studies on user safety (acute dermal and ocular irritation/corrosion studies, skin sensitisation studies and an acute inhalation study) performed with concentrated liquid L-lysine (base) and/or L-lysine HCl of *C. glutamicum* KCTC 12307BP (see Section 3.1.3) indicated that these products were neither irritant to skin and eyes and nor skin sensitisers. As the product from *C. glutamicum* NRRL B-50547 has similar physical characteristics (particle size distribution), the dusting potential is lower, the production strains share a common lineage, the production process and the composition of the growth medium are similar, the FEEDAP Panel considers that the outcome of these toxicological studies can be extended to the product under assessment.

### Conclusions on the safety for the user

The FEEDAP Panel considers that the conclusions for the safety for the user of the concentrated liquid L-lysine (base) and L-lysine HCl produced by *C. glutamicum* KCTC 12307BP can be extended to the additives under assessment. Therefore, concentrated liquid L-lysine (base) and L-lysine HCl produced by *C. glutamicum* NRRL B-50547 are not irritant to skin or eyes and they are not skin sensitisers. L-lysine HCl is not hazardous by inhalation.

#### 3.2.4. Conclusions L-lysine monohydrochloride produced using *C. glutamicum* NRRL B-50547

The production strain carries a full gene of [REDACTED]. Since no cells and recombinant DNA from the production strain were found in the product, the product does not raise safety concerns with respect to the genetic modification of the production strain provided that the downstream process is performed ensuring that no recombinant DNA of the production strain is present in the final product.

L-lysine HCl produced by *C. glutamicum* NRRL B-50547 does not raise safety concerns and it is considered safe for the target species, consumers and the environment.

Concentrated liquid L-lysine (base) and L-lysine HCl produced by *C. glutamicum* NRRL B-50547 are not irritant to skin or eyes and they are not skin sensitisers. L-lysine HCl is not hazardous by inhalation for the user.

#### 3.3. Concentrated liquid L-lysine (base) and L-lysine monohydrochloride produced by *C. glutamicum* KCCM 11117P

The FEEDAP Panel, in its opinion on the safety and efficacy of the product (EFSA FEEDAP Panel, 2016), could not conclude on the safety of concentrated liquid L-lysine (base) and L-lysine HCl produced by the GM *C. glutamicum* KCCM 11117P for the target species, consumer and environment due to the uncertainty regarding the presence/absence of the production strain and/or its recombinant DNA in both products, and the resistance of the production strain to clindamycin. Information on particle size of L-lysine HCl was also inadequate, and implications for user safety were unclear.

The applicant has provided new information on characterisation of the production strain and of the additives.

### 3.3.1. Characterisation of *C. glutamicum* KCCM 11117P and the absence of viable cells and DNA in the final products

#### Antimicrobial resistance of the genetically modified microorganism

In the former assessment (EFSA FEEDAP Panel, 2016), the production strain was reported to be susceptible to the antibiotics listed in the technical guidance for the assessment of bacterial antimicrobial susceptibility (EFSA FEEDAP Panel, 2012d) for 'Other Gram +', except for clindamycin. The MIC is 2 mg/L and the cut-off 0.25 mg/L.<sup>25</sup> In the meantime, based in new scientific evidence, the FEEDAP Panel set the cut-off value of clindamycin from 0.25 to 4 mg/L for *Corynebacterium*, as reflected in the guidance on characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018). Therefore, the strain is considered susceptible to all relevant antimicrobials, and of no concern for the target animals, users, consumers or the environment.

#### Absence of viable cells and DNA in the final product

In the former assessment (EFSA FEEDAP Panel, 2016), the absence of viable cells of the production strain KCCM11117P was tested in three batches of the solid product only. No viable cells were detected; however, no controls were included. No recombinant DNA was detected in five batches of L-lysine HCl by [REDACTED] in *C. glutamicum* KCCM11117P. However, the DNA extraction methodology was not reported to include a cell lysis step suitable for *C. glutamicum*. No information was provided for the concentrated liquid L-lysine (base) product.

New analyses were provided, in which no cells of the production strain were detected in three samples of 0.1 g (solid product) or 1 mL (liquid product), all tested in triplicate, by plating on non-restrictive media and cultivating at 37°C for 2 days. Adequate controls were used.

Furthermore, no recombinant DNA was found in [REDACTED] three independent batches of each product tested in triplicate by [REDACTED].

### 3.3.2. Physical characterisation of L-lysine HCl

New data have been provided on the particle size distribution of the product L-lysine HCl (three batches) measured by laser diffraction.<sup>26</sup> About 80% of the particles have a size ranging from 260 to 1,170 µm diameter. The fraction of particles < 100 µm diameter ranged from 0.47 to 0.87% (v/v) and practically no particles were found < 50 µm (0–0.14% v/v).

### 3.3.3. Safety of concentrated liquid L-lysine (base) and L-lysine monohydrochloride produced using *C. glutamicum* KCCM 11117P

#### Safety for the target species, consumer and the environment

The safety of L-lysine in animal nutrition when used in appropriate amounts is well established in the scientific literature. For nutritional additives produced by fermentation, the risk associated with the residues of the fermentation process in the final product needs to be assessed. The production strain has been identified as *C. glutamicum*, the GM does not raise safety concerns and is sensitive to antimicrobials relevant for human or veterinary medicine and no safety concerns arise from the production process. Neither viable cells of the production strain nor recombinant DNA were found in the final products. Consequently, the liquid L-lysine (base) and L-lysine HCl produced by *C. glutamicum* KCCM 11117P are considered safe for the target species, consumers and the environment.

#### Safety for the user

In the former opinion (EFSA FEEDAP Panel, 2016), no specific studies for user safety performed with either of these two products were available. Concentrated liquid L-lysine (base) was considered as corrosive due to its high pH. In the absence of data, the FEEDAP Panel could not conclude on the dermal sensitisation and on the irritancy of L-lysine HCl to skin or eyes. In addition, the concerns regarding the presence of transmissible antibiotic resistance in the L-lysine HCl would also have implications for the safety of the user.

<sup>25</sup> Technical dossier/[REDACTED]/Annexes/Annex II.1.2.

<sup>26</sup> Technical dossier/Supplementary information August 2016/[REDACTED]/ Particle size distribution.

Regarding the L-lysine HCl, the new data on particle size distribution shows no respirable fraction.<sup>27</sup> The dusting potential of one analysed batch (Stauber–Heubach) was 3.1 g/m<sup>3</sup> (EFSA FEEDAP Panel, 2016). Although the dusting potential indicates that the additive could reach the upper respiratory mucosa, the absence of respirable particles indicates no concerns for exposure by inhalation for the user.

No new toxicological studies performed with concentrated liquid L-lysine (base) or L-lysine HCl produced using *C. glutamicum* KCCM11117P have been submitted. The results of studies concerning user safety (acute dermal and ocular irritation/corrosion studies, skin sensitisation studies and an acute inhalation study) performed with concentrated liquid L-lysine (base) and/or L-lysine HCl of *C. glutamicum* KCTC 12307BP (see Section 3.2.3), indicated that the products were not irritant to skin and eyes and not skin sensitisers. As the product from *C. glutamicum* KCCM11117P has similar physical characteristics (particle size distribution), the dusting potential is lower, the production strains share a common lineage, the production process and the composition of the growth medium are similar, the FEEDAP Panel considers that the outcome of these toxicological studies can be extended to the product under assessment.

#### Conclusions on the safety for the user

The FEEDAP Panel considers that the conclusions for the safety for the user of the concentrated liquid L-lysine (base) and L-lysine HCl produced by *C. glutamicum* KCTC 12307BP can be extended to the additives under assessment. Therefore, concentrated liquid L-lysine (base) and L-lysine HCl produced by *C. glutamicum* KCCM 11117P are not irritant to skin or eyes and they are not skin sensitisers. L-Lysine HCl is not hazardous by inhalation.

#### 3.3.4. Conclusions on concentrated liquid L-lysine (base) and L-lysine monohydrochloride produced using *C. glutamicum* KCCM 11117P

The products concentrated liquid L-lysine (base) and L-lysine monohydrochloride, produced using *C. glutamicum* KCCM 11117P, do not raise safety concerns with respect to the potential presence of viable cells of the production strain or its recombinant DNA. Consequently, the liquid L-lysine (base) and L-lysine HCl produced by *C. glutamicum* KCCM 11117P are considered safe for the target species, consumers and the environment.

Concentrated liquid L-lysine (base) and L-lysine HCl produced by *C. glutamicum* KCCM 11117P are not irritant to skin or eyes and they are not skin sensitisers. L-Lysine HCl is not hazardous by inhalation for the user.

#### 3.4. L-Lysine sulfate (solid) produced using *C. glutamicum* DSM 24990

The FEEDAP Panel, in its opinion of 2016, concluded that 'For L-lysine sulfate originating from non-genetically modified strain DSM 24990, the FEEDAP Panel cannot conclude on the safety for the target species, the consumer and the environment due to uncertainties that this product may contain antibiotic resistance genes. The high sulfate content in the product also has the potential to cause adverse effects in the target species. The FEEDAP Panel reiterates its concerns on the safety of the administration of amino acids, including L-lysine, via water for drinking because of the risk of imbalances and for hygiene reasons'.

The applicant has provided new data on the sensitivity of the production strain to antimicrobials (clindamycin and chloramphenicol); on the tolerance of target species to sulfur; and a subchronic 90-day oral repeated dose toxicity study in rat.

##### 3.4.1. Characterisation of *C. glutamicum* DSM 24990 and the absence of viable cells and DNA in the final product

###### Antimicrobial resistance of *C. glutamicum* DSM 24990

In the former assessment (EFSA FEEDAP Panel, 2016), the production strain was found susceptible to the antimicrobials listed in the technical guidance of the assessment of antimicrobial susceptibility (EFSA FEEDAP Panel, 2012d) for 'Other Gram positive' except for streptomycin, ampicillin, clindamycin and chloramphenicol. Resistance to streptomycin and ampicillin were not associated with mobile genetic elements. Clindamycin had a MIC of 4 mg/L (cut-off 0.25 mg/L) and chloramphenicol a MIC of 8 mg/L (cut-off 2 mg/L) and information about the genetic basis of the resistance to clindamycin and chloramphenicol was not provided.

<sup>27</sup> Technical dossier/Supplementary information August 2016/ [REDACTED] Particle size distribution.

In the meantime, based in new scientific evidence, the FEEDAP Panel set the cut-off values of clindamycin and chloramphenicol to 4 mg/L for *Corynebacterium* (EFSA FEEDAP Panel, 2018). Therefore, the MICs identified do not raise safety concerns in relation to the antimicrobial resistance.

Therefore, the production strain is considered susceptible to all relevant antimicrobials, and of no concern for the target animals, users, consumers or the environment.

### **Absence of viable cells and DNA in the final product**

In the former assessment (EFSA FEEDAP Panel, 2016), the absence of viable cells of the production strain was considered demonstrated but no data were provided on the absence of DNA in the product. The reassessment of clindamycin and chloramphenicol sensitivity indicates no concern relating to transmissible antibiotic resistances, thus the possible presence of DNA in the product is no longer of concern.

## **3.4.2. Safety of L-lysine sulfate (solid) produced using *C. glutamicum* DSM 24990**

### **Toxicological studies**

The company provided spontaneously a 90-day study in CD<sup>®</sup> rats in accordance with OECD 408 testing guideline using the L-lysine sulfate under assessment as teste item (Biolys<sup>®</sup> containing 56.5% lysine and approx. 20% sulfate by specification).<sup>28</sup> Ten animals/sex/group were treated with dietary concentrations adjusted, based on feed consumption, in order to obtain a constant exposure to 0 (control group), 177, 531 and 1,770 mg additive/kg body weight (bw), respectively (corresponding to an actual intake of 103 mg lysine and 35 mg sulfate; 314 mg lysine and 106 mg sulfate; 1,026 mg lysine and 350 mg sulfate per kg bw, respectively). In addition, the control and top dose level included also a recovery group of 5 animals/sex/group, which were killed after 28 additional days of treatment withdrawal. In compliance with the OECD guideline, tested parameters included haematology and coagulation, clinical biochemistry, urinalysis, ophthalmological and auditory examinations, gross pathology and histopathology. No treatment-related effects were detected on behaviour, survival, weight gain, feed intake, as well as ophthalmological and auditory examinations.

The main treatment-related adverse effect was epithelial degeneration of proximal and distal renal tubules in both male and female rats at the top dose (10/10 in males and 8/10 in females, vs 0/10 in controls of both sexes). Urinary pH was significantly decreased at all dose levels in females with an apparent dose-related trend; in males only a numerical decrease in the intermediate and high-dose groups was seen. The FEEDAP panel considers that a decreased urinary pH may reflect an effect of supplementation with high doses of lysine sulfate; however such effect should be considered as adverse only in conjunction with other alterations, such histopathological changes in renal tubules. The tubular lesions appeared to be reversible, as they were not observed after the recovery period. In top dose males of the recovery group, urinary pH and blood leucocyte count were significantly decreased.

Based on widespread, although apparently reversible, tubular damage at the high dose level, the FEEDAP Panel identifies the dose level of 531 mg additive/kg bw (corresponding to an intake of 314 mg lysine and 106 mg sulfate) as a no adverse effect level (NOAEL). The FEEDAP also notes that proliferative lesions in bladder, induced in rats by another additive based on L-lysine sulfate (EFSA, 2007), were not detected in this more recent study.

### **Safety for the target species**

In the former assessment (EFSA FEEDAP Panel, 2016), *C. glutamicum* DSM 24990 was found to be resistant to clindamycin and chloramphenicol and the FEEDAP Panel considered that the presence of potentially transmissible antibiotic resistances is of concern for the safety to the target species. Furthermore, the high inherent content of sulfate in this product could be a safety concern for the target species (EFSA FEEDAP Panel, 2015b) depending on the supplementation level and the tolerance of the different target species.

As the production strain has been shown to be susceptible to all relevant antibiotics, there is no longer any concern for safety of the target species with regard to antimicrobial resistance.

In relation to the concentrations of sulfate/sulfur (S) present in the product, the applicant reviewed the literature and provided new data on studies performed in pigs (Kerr et al., 2011; Kim et al., 2014) showing that no negative effects are to be expected within the proposed inclusion levels of 0.5–27 g

<sup>28</sup> Technical dossier/Supplementary information November 2018/Annex 1.

additive/kg feed (corresponding to 0.4 to 18.9 g lysine sulfate/kg complete feed), which would add about 0.03–1.6 g additional S/kg complete feed.<sup>29</sup>

However, consideration should be given to (i) some feed materials may already contain, besides S-amino acids, a high background amount of sulfate and/or other S species (e.g. dried distillers' grains with solubles, biomasses, rapeseed meal, sugarbeet pulp and molasses/vinasses, grass, alfalfa), (ii) adverse effects of excess of sulfate/S in animal nutrition are well described (e.g. reduction of feed intake, diarrhoea, polioencephalomalacia), and (iii) it is impractical to analyse all S species individually in feed. Therefore, the formulation of the 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 dry matter (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 (see Appendix A).

Also the contribution of sulfate present in water for drinking to the total S intake should be considered.

#### *Conclusion on the safety for the target species*

As the production strain has been shown to be susceptible to all relevant antibiotics, there is no longer any concern for safety of the target species regarding antimicrobial resistance. No negative effects are to be expected within the proposed inclusion levels of 0.5–27 g additive/kg complete feed for the target species provided that the total S intake complies with the recommendations of established scientific bodies.

#### **Safety for the consumer**

In the former assessment (EFSA FEEDAP Panel, 2016), the FEEDAP Panel considered that the presence of potentially transmissible antibiotic resistances was of concern for the safety for the consumer. Furthermore, there were indications of possible adverse effects (proliferative lesions in the urogenital tract of rats) from a 90-day toxicity study reported in a previous FEEDAP opinion on a similar L-lysine sulfate product (EFSA, 2007).

As the production strain has been shown to be susceptible to all relevant antibiotics, there is no longer any concern for safety of the consumer with regard to antimicrobial resistance.

With regard to the indications of possible adverse effects found in a former 90-day oral subchronic toxicity study (EFSA, 2007), the applicant observed that the additive composition and production process are different.<sup>30</sup> The additive assessed in 2007 was produced using syrup, molasses, fermented grass juice from the green crop drying industry, grain, starch products and hydrolases thereof as carbon sources, vs [REDACTED] used as carbon sources in the product under assessment. Furthermore, the amount of active substance in the previous additive (EFSA, 2007) was lower (47.5% vs 51.8% lysine) and the sulfate higher (23% vs 17.5% sulfate) than the additive under assessment. Therefore, the results of the previous 90-day oral subchronic toxicity study cannot be referred to the additive under assessment.

The applicant provided the new 90-day subchronic oral toxicity study described above. Considering that consumer exposure to the additive resulting to the use of the additive in animal feed would be very low (EFSA BIOHAZ Panel, 2013), based on the NOAEL derived from the rat 90-day study, the FEEDAP Panel considers that no safety concerns for the consumer are expected.

The production strain qualifies for the QPS approach of safety assessment for the production of amino acids (EFSA BIOHAZ Panel, 2017). No safety concerns are expected to originate from the production strain or its metabolites. The product is therefore considered safe for consumers of products from animals receiving L-lysine sulfate produced using *C. glutamicum* DSM 24990.

#### *Conclusion for the consumer*

The use of L-lysine sulfate produced by *C. glutamicum* DSM 24990 in animal nutrition is considered safe for the consumer.

#### **Safety for the user**

In the former assessment (EFSA FEEDAP Panel, 2016), it was considered that the uncertainties related with the potential to transfer antibiotic resistance may have implications on the safety for the user.

<sup>29</sup> Technical dossier/Evonik/Sulfate content.

<sup>30</sup> Technical dossier/Evonik/90d-tox-statement.



As the production strain has been shown to be susceptible to all relevant antibiotics, there is no longer any concern for safety of the user with regard to antimicrobial resistance.

### Safety for the environment

In the former assessment (EFSA FEEDAP Panel, 2016), the FEEDAP Panel could not conclude on the environmental safety of this L-lysine sulfate product due to uncertainties about the potential presence of transmissible antibiotic resistance genes.

As the production strain has been shown to be susceptible to all relevant antibiotics, there is no longer any concern for safety of the environment with regard to antimicrobial resistance.

#### 3.4.3. Conclusions on L-lysine sulfate (solid) produced using *C. glutamicum* DSM 24990

As the production strain has been shown to be susceptible to all relevant antibiotics, the use of *C. glutamicum* DSM 24990 in the production of L-lysine sulfate is considered safe for the target species, consumers, users or the environment.

As regards the safety of the product for the target species, no negative effects are to be expected within the proposed inclusion levels of 0.5–30 g lysine sulfate/kg complete feed provided that the total S intake complies with the recommendations of established scientific bodies.

### 3.5. L-Lysine sulfate (solid) produced using *C. glutamicum* KCCM-10227

The FEEDAP Panel, in its opinion of 2016, concluded that 'For L-lysine sulfate originating from non-genetically modified strain *C. glutamicum* KCCM-10227, the FEEDAP Panel cannot conclude on the safety for the target species, the consumer and the environment due to uncertainties that this product may contain antibiotic resistance genes. The high sulfate content in the product also has the potential to cause adverse effects in the target species. The FEEDAP Panel reiterates its concerns on the safety of the administration of amino acids, including L-lysine, via water for drinking because of the risk of imbalances and for hygiene reasons'.

#### 3.5.1. Characterisation of *C. glutamicum* KCCM-10227 and the absence of viable cells and DNA in the final product

In the former assessment (EFSA FEEDAP Panel, 2016), the production strain was found to be susceptible to antibiotics listed in the technical guidance for the assessment of bacterial antimicrobial susceptibility (EFSA Feedap Panel, 2012d) for 'Other Gram +', except for clindamycin (MIC 2 mg/L and cut-off 0.25 mg/L). In the meantime, based in new scientific evidence, the FEEDAP Panel set the cut-off value of clindamycin from 0.25 to 4 mg/L for *Corynebacterium*, as reflected in the guidance on characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018). Therefore, the strain is considered susceptible to all relevant antimicrobials, and of no concern for the target animals, users, consumers or the environment.

#### Absence of viable cells in the final product

In the former assessment (EFSA FEEDAP Panel, 2016), the absence of viable cells of the production microorganism in one batch of the final product was considered demonstrated although the presence of DNA of the production strain in the final product could not be excluded.

The applicant provided new data on the absence of viable cells of the production strain in three batches of the former product. Total plate count (complex agar plate) identified  $< 3 \times 10^2$  CFU/g of final product and no *C. glutamicum* were identified by PCR.<sup>31</sup>

As the production strain has been shown to be susceptible to all relevant antibiotics, the possible presence of DNA in the product is no longer of concern.

<sup>31</sup> Technical dossier/CJ Europe/Annex 6.

### 3.5.2. Safety of L-lysine sulfate (solid) produced using *C. glutamicum* KCCM 10227

#### Safety for the target species

In the former assessment (EFSA FEEDAP Panel, 2016), it was considered that in the absence of information on the genetic basis of the resistance to clindamycin the product could not be considered safe for the target species. In addition, it was noted that there is a high inherent content of sulfate in this product. These levels of sulfate could be a safety concern for the target species (EFSA FEEDAP Panel, 2015b) depending on the supplementation level and the tolerance of the target species. The FEEDAP Panel reiterated its previous statement that amino acids, their salts and analogues should generally not be used in water for drinking because of the risk of imbalances and for hygiene reasons (EFSA FEEDAP Panel, 2010).

As the production strain has been shown to be susceptible to all relevant antibiotics, the use of *C. glutamicum* KCCM 10227 in the production of L-lysine sulfate is considered safe for the target species, with regard to antimicrobial resistance.

In relation to the concentrations of sulfate/sulfur (S) present in the product, the applicant reviewed the literature

[REDACTED]

<sup>32</sup> As described above (see Section 3.4.2), these sulfur levels would not represent a safety concern for the target species.

#### Safety for the consumer

In the former assessment (EFSA FEEDAP Panel, 2016), it was considered that in the absence of information on the genetic basis of the resistance to clindamycin the product could not be considered safe for the consumer. Furthermore, there were indications of possible adverse effects (proliferative lesions in the urogenital tract of rats) from a 90-day toxicity study reported in a previous FEEDAP opinion on a similar L-lysine sulfate product (EFSA, 2007).

As the strain has been shown to be susceptible to all relevant antibiotics, the use of *C. glutamicum* KCCM 10227 in the production of L-lysine sulfate is considered safe for the target species, with regard to antimicrobial resistance.

With regard to the indications of possible adverse effects found in a former 90-day oral subchronic toxicity study (EFSA, 2007), the applicant did not provide additional data. The FEEDAP Panel observed that composition of the additive and the production process are different with respect to the product assessed in 2007. The additive assessed in 2007 was produced using syrup, molasses, fermented grass juice from the green crop drying industry, grain, starch products and hydrolases thereof as carbon sources; vs [REDACTED] used in the product under assessment. Furthermore, the amount of active substance in the previous additive (EFSA, 2007) was lower (47.5% vs 59.9% lysine) and the sulfate higher (23% vs 20.1% sulfate) than in the additive under assessment. Therefore, the results of the 90-day oral subchronic toxicity study assessed in 2007 cannot be referred as relevant for the additive under assessment.

The use of L-lysine produced by *C. glutamicum* KCCM 10227 in animal nutrition is considered safe for the consumer.

#### Safety for the user and the environment

In the former assessment (EFSA FEEDAP Panel, 2016), it was considered that the uncertainties related with the potential to transfer resistance to clindamycin may have implications on the safety for the user and the environment.

As the strain has been shown to be susceptible to all relevant antibiotics, there is no longer any concern for safety of the user and the environment with regard to antimicrobial resistance.

<sup>32</sup> [REDACTED]

### 3.5.3. Conclusions on L-lysine sulfate (solid) produced using *C. glutamicum* KCCM 10227

As the strain has been shown to be susceptible to all relevant antibiotics, the use of *C. glutamicum* KCCM 10227 in the production of L-lysine sulfate is considered safe for the target species, with regard to antimicrobial resistance.

Regarding the safety of the product for the target species, no negative effects are to be expected within common inclusion levels provided that the total S intake complies with the recommendations of established scientific bodies.

## 4. Conclusions

Uncertainty remains concerning the absence/presence of the production strain *C. glutamicum* KCTC 12307BP and/or its recombinant DNA in the final products liquid L-lysine (base) and L-lysine HCl. The recipient strain qualifies for QPS approach for safety assessment, the genetic modification does not introduce any safety concern and no introduced antibiotic resistance genes remain in the genome of the production strain. Therefore, the presence of viable cells and/or its recombinant DNA in the products would not raise safety concerns. The liquid L-lysine (base) and L-lysine HCl produced by *C. glutamicum* KCTC 12307BP are considered safe for the target species, consumers and the environment.

The production strain *C. glutamicum* NRRL B-50547 carries a full gene of [REDACTED]. Since no cells and recombinant DNA from the production strain were found in the product L-lysine HCl, the product does not raise safety concerns with respect to the genetic modification of the production strain provided that the downstream process is performed ensuring that no recombinant DNA of the production strain is present in the final product. L-Lysine HCl produced by *C. glutamicum* NRRL B-50547 does not raise safety concerns and it is considered safe for the target species, consumers and the environment.

The products concentrated liquid L-lysine (base) and L-lysine monohydrochloride, produced using *C. glutamicum* KCCM 11117P, do not raise safety concerns with respect to the potential presence of viable cells of the production strain or its recombinant DNA. Consequently, the liquid L-lysine (base) and L-lysine HCl produced by *C. glutamicum* KCCM 11117P are considered safe for the target species, consumers and the environment.

Regarding the safety for the user, concentrated liquid L-lysine (base) and L-lysine HCl produced by *C. glutamicum* KCTC 12307BP, *C. glutamicum* NRRL B-50547, *C. glutamicum* KCCM 11117P are not irritant to skin or eyes and they are not skin sensitizers. L-Lysine HCl is not hazardous by inhalation.

The use of *C. glutamicum* DSM 24990 in the production of L-lysine sulfate is considered safe for the target species, consumers, users or the environment. No negative effects are to be expected for the target species within the proposed inclusion levels of 0.5–30 g lysine sulfate/kg complete feed provided that the total S intake complies with the recommendations of established scientific bodies.

The use of *C. glutamicum* KCCM 10227 in the production of L-lysine sulfate is considered safe for the target species, consumers, users and the environment with regard to antimicrobial resistance. No negative effects are to be expected for the target species within common inclusion levels provided that the total S intake complies with the recommendations of established scientific bodies.

## Documentation provided to EFSA

- 1) Dossier L-lysine and related compounds produced by fermentation with *Corynebacterium glutamicum*. May 2016. Submitted by FEFANA absl.
- 2) Dossier L-lysine and related compounds produced by fermentation with *Corynebacterium glutamicum*. Spontaneous supplementary information. August 2016. Submitted by FEFANA absl.
- 3) Dossier L-lysine and related compounds produced by fermentation with *Corynebacterium glutamicum*. Spontaneous supplementary information. September 2018. Submitted by FEFANA absl.
- 4) Dossier L-lysine and related compounds produced by fermentation with *Corynebacterium glutamicum*. Supplementary information. January 2017. Submitted by FEFANA absl.
- 5) Dossier L-lysine and related compounds produced by fermentation with *Corynebacterium glutamicum*. Spontaneous supplementary information. November 2018. Submitted by FEFANA absl.

## Chronology

Date	Event
30/05/2016	Dossier received by EFSA
30/05/2016	Reception mandate from the European Commission
17/06/2016	Application validated by EFSA – Start of the scientific assessment
20/08/2016	Reception of spontaneous supplementary information from the applicant
26/09/2016	Reception of spontaneous supplementary information from the applicant
26/10/2016	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. <i>Issues: Characterisation of four production strains and safety for the user.</i>
30/01/2017	Reception of supplementary information from the applicant - Scientific assessment re-started
12/11/2018	Reception of spontaneous supplementary information from the applicant
28/11/2018	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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## Abbreviations

AIV	Artturi Ilmari Virtanen
Asbl	<i>Association sans but lucratif</i>
CFU	colony forming unit
DDGS	dried distillers grains and solubles
DM	dry matter
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
GM	genetically modified
ICP	inductively coupled plasma
MIC	minimum inhibitory concentration
MTL	maximum tolerable level
PCR	polymerase chain reaction
QPS	qualified presumption of safety
TMR	total mixed ration
WHO	World Health Organization

## Appendix A – Considerations on sulfate/sulfur in animal nutrition

### A.1. Total S content and S species in feed materials and feed additives

Sulfur is an essential nutrient in animal nutrition. In feed materials, it is present in organic (e.g. methionine, cystine with 21.5% and 26.5% S; glucosinolates (isothiocyanates) with about 16% S) and inorganic forms (e.g. sulfates with 33% S). Normally S-amino acids are the main S species in feed materials. However, fertilising intensities and/or technological treatments during processing of feed materials (forages, dried distillers grains and solubles (DDGS), sugar beet pulp and molasses/vinasses) may contribute to higher S-contents and also other S species may become important. Mineral containing feed materials may also contain S (e.g. sulfates of Ca, Mg, Na) while  $MgSO_4$  (Epsom salt) and  $Na_2SO_4$  (Glauber's salt) are also known as laxatives for short term application in various animal species (Kamphues et al. (2014).

Kerr et al. (2008) report total S content, measured by thermal combustion (CNS) and inductively coupled plasma (ICP), in 464 samples originating from 17 plant-based and 13 animal-based feed materials. Only for a small number of feedstuffs, differences between both methods were apparent. The mean S content was high in corn gluten meal (10.6 g/kg DM), corn gluten feed (7.4 g/kg DM), DDGS (6.9 g/kg DM) and in soybean meal (4.7 g/kg DM); intermediate in wheat flour/middlings (2.2 g/kg DM) and low in barley, corn, oats, sorghum and wheat (< 2g/kg DM). In animal-based feed materials, the S content ranged between 4.2 and 18.4 g/kg DM. These figures are in line with more recent data of NRC (2012) and Kamphues et al. (2014).

For some specific feed materials, Kamphues et al. (2014) provided some data on S species. In sugar beet pulp, molasses and vinasses, the total S content ranges between 1.6 and 4.4, 7.7 and 9.8, and 5.0 and 10.0 g/kg DM, respectively, mainly originating from sulfate (50–95%). In DDGS, the total S content ranges between 3.2 and 11.8 g/kg DM, with 2.8–65.0% originating from sulfate. In rapeseed meal, the total S content ranges between 6.6 and 7.6 g/kg DM with important contribution amounting to 20–40% from sulfate and to about 10% from glucosinolates. Grass, grass silage, hay, lucerne and maize silage contain between 1.2 and 8.9, 1.9 and 4.8, 1.2 and 4.2, 1.4 and 4.2 and 0.8 and 1.5 g total S/kg DM, respectively, with about 50% originating from sulfate.

More complete data on S contents (organic/inorganic) of currently used feed materials in the EU are reported in the databases of Dutch Central Feedstuff Bureau (CVB, 2011), French National Institute for Agricultural Research (INRA, 2004) and Spanish Foundation for the Development of Animal Nutrition (FEDNA, 2015). A lot of trace element feed additives (e.g. Zn, Fe, Mn, Co, Cu) and lysine are also marketed in the sulfate form.

### A.2. Total S content and S species in compound feeds/total mixed ration

Compound feeds normally contain feed materials and feed additives bearing also S (e.g. sulfate-based trace elements; lignosulfonates), while total mixed ration (TMR) includes forages/silages/AIV (Artturi Ilmari Virtanen) silages/roughages and concentrates/complementary feeds.

Recent data (Kamphues et al., 2014) show that pig compound feeds contain between 2.19 and 3.97 g S/kg DM and between 0.98 and 3.41 g sulfate/kg DM, poultry feeds between 2.37 and 4.57 g S/kg DM and between 0.98 and 6.60 g sulfate/kg DM and TMR for dairy cows between 2.37 and 6.32 g S/kg DM and between 1.83 and 8.06 g sulfate/kg DM.

### A.3. Tolerance to S and sulfate in animals

S and several S-species may have multiple effects on animal health/yields and it is difficult/impossible to show clear dose-response effects. Organic sulfur-containing compounds are absorbed across the intestinal wall by specific transport processes. The sulfur-containing amino acids are incorporated into proteins or they are used to produce other organic sulfur-containing compounds. Eventually, all these organic sulfur-containing compounds will be catabolised to inorganic sulfate. Sulfates fed at physiological doses to animals are relatively well absorbed. Urinary excretion is the main route. However differences in metabolism of S and of S species exist between ruminants and non-ruminants, the former being more sensible to S-excess than the latter. Therefore, the aetiology of toxicosis is very different in the two groups of animals. Specific effects of S and of various S species are reviewed by some authors (e.g. McDowell, 2003; Daenicke and Schenkel, 2009; Suttle, 2010; Kamphues et al., 2014).

### A.3.1. Tolerance to S and sulfate in ruminants

In ruminants, most dietary sulfur, whether ingested as amino acids or inorganic sulfur, is reduced to sulfide ( $H_2S$ ) by the ruminal flora that use sulfur as an electron acceptor. Sulfide can be incorporated into microbial protein by certain types of microbiota or the sulfide can be absorbed into the portal circulation where it is quickly and efficiently (in most cases) oxidised to sulfate in the liver and excreted principally via the urine. In general, ruminants are more sensitive to S-excess than non-ruminants and the toxicity is related to the form of S ingested. The margin between the desirable and harmful concentration of S in the ruminant diet is very small (only 2- to 3-fold). Reduction in feed intake and growth rate in cattle and sheep are observed with 3–4 g S/kg DM (Kandyliis, 1984; Zinn et al., 1997). The main reason for this are processes in the rumen leading to high sulfide formation. Absorbed  $H_2S$  may have harmful effects on the central nervous system and may contribute to the development of brain lesions (polioencephalomalacia). In bulls, during a 20-day balance trial, no influence on faeces quality could be detected while feeding 14% beet vinasse in DM, corresponding to 7.1 g S from sulfate/kg DM (Stemme et al., 2005). In addition, many interactions between high S/ $SO_4$ -intake and metabolic processes/disturbances are described, such as: the well-known Cu x S x Mo interactions, the potential negative effects of S on trace element absorption (e.g. Cu, Se, Zn) and interactions with some vitamins (vitamin A, thiamine, biotine, etc.). More details of effects of high S amounts in ruminant nutrition are reviewed by Kandyliis (1984), Daenicke and Schenkel (2009), Suttle (2010) and Kamphues et al. (2014). NRC (2005) recommended < 3 g S/kg DM in ruminant diets rich in concentrate and < 5 g S/kg DM in diets rich in roughage.

For water for drinking for ruminants, NRC (2005) sets maximum tolerable level (MTL) for sulfate at 600 mg/L, fed concentrate rich diets and at 2,500 mg/L fed high forage diets, while Kamphues et al. (2014) in his review proposes a maximum content between 500 and 800 mg/L. Surface and ground water supplies can contain as much as 3.5 g sulfate/L, particularly in the USA and Canada (Suttle, 2010). However, normal sulfate levels in the EU range between 25 and 75 mg/L, corresponding to 8–25 mg S/L. (Oude Elferink and Meijer, 2001). Tap water contains < 15 mg sulfate/L. If TMR intake by dairy cows would amount to 20 kg/day, the S intake from water (surface and ground water) would add only 0.02–0.125 g/kg TMR, which is a negligible amount.

### A.3.2. Tolerance to S and sulfate in non-ruminants

The NRC (2005) set MTL of S in non-ruminant feeds (pigs and poultry) at 4 g S/kg DM. More recent data are still in line with this figure. In a pig trial (14–34 kg body weight; duration 24 days), 6.25, 12.5, 25 and 50 g  $CaSO_4$  were added per kg of a control diet. The analysed total S-content amounted to 2.5 g/kg for the control diet and for the treatments 3.6, 5.0, 8.2 and 12.1 g S/kg feed. No negative effects up to 5.0 g S/kg feed were observed regarding performance, gut microbiota or immune functions (Kerr et al., 2011). Inclusion of up to 3.8 g S/kg in pig feed containing 30% DDGS (84-day trial; start at 34 kg body weight) has no negative effect on carcass parameters of growing–finishing pigs (██████████ 2014). In a 14-day trial for chickens for fattening, ██████████ (2013) compared S in DDGS (control) with S from lysine sulfate (different levels); diets contained 2.7 g total S/kg (control diet) and 2.9–3.4 g total S/kg (lysine sulfate diets); no differences in growth performance were noted. A high sulfur diet or water source may induce a cathartic effect in pigs and poultry, though wetter faeces/droppings do not seem to affect animal performance in and of themselves. In pigs, in a 12-day balance trial, increasing levels of sulfate in the diet up to 65 g sulfate (21 g S/kg feed) by inclusion of beet vinasses from 16% to 43% reduced the dry matter of faeces in a linear way from about 35% (control) to about 5% dry matter (Stemme et al., 2005).

For sulfate in water for drinking, NRC (2005) sets MTL's at 3,000 mg/L for pigs and at 1,000 mg/L for poultry, while Kamphues et al. (2014) in his review proposes a maximum content between 1,600 and 1,800 mg/L for pigs and < 500 mg/L for poultry. The German recommendations of water quality for food producing animals (Kamphues et al. 2007) do not distinguish between ruminants and non-ruminants (< 500 mg sulfate/L water). The limit for water for drinking according human regulations in Germany is given with 240 mg  $SO_4$ /L.

## A.4. Conclusions on the addition of lysine sulfate to animal feeds

Lysine sulfate additives contain between 50 and 60% lysine and between 17.5 and 20.2% sulfate, resulting in 5.8–6.7% S in the additive. Normal use levels of lysine in feeds ranges between 0.5 and 5 g/kg

feed, corresponding to 1.0–10.0 g lysine sulfate/kg feed; depending on the lysine background of the feed, the age, sex and physiological status of the animal, supplementation level of lysine may be as high as 12.5 g/kg feed, corresponding to about 25 g lysine sulfate/kg or 1.5 g additional S/kg feed.

When formulating compound feeds/TMR, consideration should be given to (i) some feed materials may already contain, besides S-amino acids, a high background amount of sulfate and/or other S species (e.g. DDGS, biomasses, rapeseed meal, sugarbeet pulp and molasses/vinasses, grass, alfalfa), (ii) adverse effects of excess of sulfate/S in animal nutrition are well described (e.g. reduction of feed intake, diarrhoea, disturbances in the well-known Cu x S x Mo interactions in ruminants, negative effects on the absorption of some trace element (e.g. Cu, Se, Zn), polioencephalomalacia), and (iii) it is impractical to analyse all S species individually in feed. Therefore, the formulation of the 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.

Kamphues et al. (2014) proposed to consider S-compounds (mainly SO<sub>4</sub>) as an undesirable feed ingredient and MTL's should be fixed in the feed law.

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