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## Safety and efficacy of L-isoleucine produced by fermentation with *Corynebacterium glutamicum* KCCM 80189 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 the safety and efficacy of L-isoleucine produced by fermentation using *Corynebacterium glutamicum* KCCM 80189 when used as a nutritional additive in feed and water for drinking for all animal species. The production strain is not genetically modified. Viable cells of the production strain were not detected in the final additive. The additive does not give rise to any safety concern regarding the production strain. L-Isoleucine produced using *C. glutamicum* KCCM 80189 is considered safe for the target species, for the consumer and for the environment. L-Isoleucine produced by *C. glutamicum* KCCM 80189 is considered not toxic by inhalation, not irritant to skin or eyes and not a dermal sensitiser. Regarding the use in water, the FEEDAP Panel reiterates its concerns over the safety of L-isoleucine administered simultaneously via water for drinking and feed owing to the risk of nutritional imbalances and hygienic reasons. L-Isoleucine produced by *C. glutamicum* KCCM 80189 is considered as an efficacious source of the essential amino acid L-isoleucine for non-ruminant animal species. For the supplemental L-isoleucine to be as efficacious in ruminants as in non-ruminant species, it would require protection against degradation in the rumen.

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## 1. Introduction

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

Regulation (EC) No 1831/2003<sup>1</sup> establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from CJ Europe GmbH<sup>2</sup> for authorisation of the product L-iso-leucine produced by fermentation using *Corynebacterium glutamicum* KCCM 80189, 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 5 June 2019.

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-iso-leucine produced by fermentation using *C. glutamicum* KCCM 80189, when used under the proposed conditions of use (see Section 3.1.6).

### 1.2. Additional information

The product under assessment is based on L-iso-leucine produced by fermentation using *C. glutamicum* KCCM 80189. This product is not authorised in the European Union.

L-Isoleucine produced by fermentation using *Escherichia coli* FERM ABP-10641 is currently authorised as a nutritional additive for use in all animal species in accordance with Regulation (EU) No 348/2010<sup>3</sup>.

D,L-Isoleucine produced by chemical synthesis or protein hydrolysis is currently authorised as flavouring substance in feed (EU Flavour Information System (FLAVIS) Number [17.010]).<sup>4</sup>

L-Isoleucine is authorised for use in food as a nutritional<sup>5</sup> and flavourings<sup>6</sup> substance, for use in cosmetics<sup>7</sup> and as a veterinary medicinal product.<sup>8</sup>

EFSA has issued an opinion on the safety and efficacy of L-iso-leucine for all animal species (EFSA FEEDAP Panel and EFSA GMO Panel, 2010) and another one on the safety and efficacy of the use of amino acids (chemical group 34) when used as flavourings for all animal species, which included L-iso-leucine (EFSA FEEDAP Panel, 2014).

The EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) adopted two opinions (EFSA NDA Panel, 2010, 2014) on various health claims related to L-iso-leucine.

The European Pharmacopoeia (2017) has a monograph dedicated to isoleucine (01/2017:0770).

<sup>1</sup> 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.

<sup>2</sup> CJ Europe GmbH, Ober der Roeth 4, 65824, Schwalbach/Taunus, Germany.

<sup>3</sup> Commission Regulation (EU) No 348/2010 of 23 April 2010 concerning the authorization of L-iso-leucine as a feed additive for all animal species. OJ L 104, 24.4.2010, p. 29.

<sup>4</sup> Commission Implementing Regulation (EU) 2018/249 of 15 February 2018 concerning the authorisation of taurine, beta-alanine, L-alanine, L-arginine, L-aspartic acid, L-histidine, D,L-isoleucine, L-leucine, L-phenylalanine, L-proline, D,L-serine, L-tyrosine, L-methionine, L-valine, L-cysteine, glycine, monosodium glutamate and L-glutamic acid as feed additives for all animal species and L-cysteine hydrochloride monohydrate for all species except cats and dogs. OJ L 53, 23.2.2018, p. 134.

<sup>5</sup> 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 and amendments, OJ L 181, 29.6.2013, p. 35.

<sup>6</sup> Commission Implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC. OJ L 267, 2.10.2012, p. 1.

<sup>7</sup> Commission Decision of 9 February 2006 amending Decision 96/335/EC establishing an inventory and a common nomenclature of ingredients employed in cosmetic products (2006/257/EC). OJ L 97, 5.4.2006, p. 1.

<sup>8</sup> 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.

## 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<sup>9</sup> in support of the authorisation request for the use of L-isoleucine as an additive in feed and water for drinking.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the analytical determination of the L-isoleucine in animal feed. The Executive Summary of the EURL report can be found in Annex A.<sup>10</sup>

### 2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of L-isoleucine is in line with the principles laid down in Regulation (EC) No 429/2008<sup>11</sup> and the relevant guidance documents: Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012), Guidance on the 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, 2018a), 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), Guidance for assessing the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019) and Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018b).

## 3. Assessment

The additive under assessment, L-isoleucine produced by fermentation using *C. glutamicum* KCCM 80189, is intended to be used as a nutritional additive (functional group: amino acids, their salts and analogues) for all animal species.

### 3.1. Characterisation

#### 3.1.1. Characterisation of the production organism

The additive L-isoleucine is produced by [REDACTED] *C. glutamicum* which is deposited at the Korean Culture Centre of Microorganisms (KCCM) with the accession number KCCM 80189. [REDACTED]

The taxonomic identification of the production strain was confirmed [REDACTED] KCCM 80189, [REDACTED]

[REDACTED]

The susceptibility of the production strain KCCM 80189 was tested [REDACTED] Guidance on characterisation of

<sup>9</sup> FEED dossier reference: FAD-2019-0022.

<sup>10</sup> The full report is available on the EURL website: [https://ec.europa.eu/jrc/sites/jrcsh/files/finrep\\_fad-2019-0022-isoleucine.pdf](https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2019-0022-isoleucine.pdf)

<sup>11</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.

microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018a).

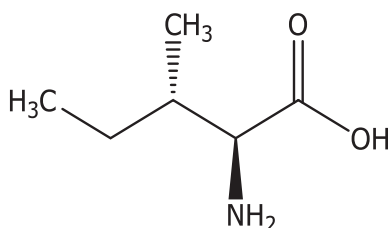
The WGS of the production strain was interrogated for the presence of antimicrobial resistance (AMR) genes

### 3.1.2. Manufacturing process

L-Isoleucine is produced by fermentation with *C. glutamicum* KCCM 80189.

### 3.1.3. Characterisation of the additive

L-Isoleucine<sup>19</sup> (International Union of Pure and Applied Chemistry (IUPAC) name: (2S,3S)-2-amino-3-methylpentanoic acid, a compound identified with the Chemical Abstracts Service (CAS) No 73-32-5, the European Inventory of Existing Commercial Chemical Substances (EINECS) No 200-798-2) is the active substance of the additive and has a molecular mass of 131.17 g/mol. The chemical formula of L-isoleucine is C<sub>6</sub>H<sub>13</sub>NO<sub>2</sub> and the structural formula is given in Figure 1.



**Figure 1:** Structural formula of L-isoleucine

The applicant declared that the product contains by specification  $\geq 90\%$  L-isoleucine (on an 'as is' basis),  $\leq 2\%$  moisture and  $\leq 1\%$  ash. Batch-to-batch variation data were provided for five batches of the additive.<sup>20</sup> The content of the active substance for the additive was 91.8% (range: 91.7–91.8%) on an 'as is' basis. The moisture content was in the range of 0.20–0.23%. Other analysed components were (average values of five batches) 0.24% ammonium, 0.03% sodium, 0.12% sulfate, 0.09% ash, 5.77%  $\alpha$ -amino-butyrac acid and 1.02% phenylalanine. L-Isoleucine produced by fermentation with *C. glutamicum* KCCM 80189 is a product with less than 1% unidentified impurities.

The specific optical rotation was measured in five batches of the final product and the average was  $+39.04^\circ$  (range  $+38.9^\circ$  to  $+39.2^\circ$ ),<sup>21</sup> which is according to the specifications set by the applicant ( $+38.5^\circ$  to  $+41.5^\circ$ ). The deviation in relation to the European Pharmacopoeia range ( $+40^\circ$  to  $+43^\circ$ ) for specific optical rotation might be due to the fact that the additive under assessment has a purity of 92%.

Three batches of the product were analysed for chemical impurities: lead (range 0.044–0.090 mg/kg), cadmium (range 0.02–0.03 mg/kg), mercury ( $< \text{LOD}$ ), arsenic ( $< \text{LOD}$ ), nickel (range 2.91–3.88 mg/kg), zinc (range 1.48–1.71 mg/kg), chromium ( $< \text{LOD}$ ), sum aflatoxins ( $< \text{LOD}$ ), ochratoxin A ( $< \text{LOD}$ ), zearalenone ( $< \text{LOD}$ ), deoxynivalenol ( $< \text{LOD}$ ), dioxins (polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/F)) 0.07 ng WHO-PCDD/F-TEQ/kg, sum of dioxins and dioxin-like polychlorinated

<sup>19</sup> Technical dossier/Section II/Annex/Annex \_II\_1\_01.

<sup>20</sup> Technical dossier/Section II/Annex/Annex \_II\_1\_02.

<sup>21</sup> Technical dossier/Supplementary information October 2019/Annex/Annex \_SIN\_01.



biphenyls (DL-PCBs) 0.12 ng WHO-PCDD/F-DL-PCB-TEQ/kg) and non-dioxin-like PCBs 0.60 µg/kg.<sup>22</sup> The same three batches of the product were analysed for microbiological contamination: *Salmonella* spp. (absent in 25 g), yeasts and moulds (< 100 colony forming units (CFU)/g), Enterobacteriaceae and *E. coli* (< 10 CFU/g).<sup>23</sup>

The absence of cells of the production strain was investigated in three batches of the final product, each batch tested in triplicate.

### 3.1.3.1. Physical characteristics of the additive

The additive is a white to yellowish crystalline powder. Its solubility in water is 3.0–3.5 g/100 g water. The bulk density of the additive is 600–850 kg/m<sup>3</sup> and its melting point of 270–290°C.

Three batches of the additive were analysed for particle size distribution by sieving method. The fraction of particles below 62 µm was 21%.<sup>25</sup> In three batches, the dusting potential measured according to Stauber–Heubach ranged between 1.8 and 2.7 g/m<sup>3</sup>.<sup>26</sup>

### 3.1.4. Stability and homogeneity

The shelf-life of L-isoleucine was evaluated in three batches of the additive when stored in bags corresponding to the commercial packaging at 30°C/65% relative humidity (RH) for 24 months.<sup>27</sup> No losses were observed in the content of L-isoleucine.

The stability of L-isoleucine (three batches) in a vitamin and mineral premixture for broilers (without choline chloride) was studied when added at 5% and stored at 25°C for 6 months (in closed bags). No losses were observed in the content of L-isoleucine in the premixture at the end of that period.<sup>28</sup>

The stability of L-isoleucine (three batches) was evaluated when added at 0.4% to a mash and pelleted feed (pelleting at 72°C) for broilers (without choline chloride) after storage at 25°C for 3 months (in closed bags). No losses were observed in the content of L-isoleucine in feed (both mash and pelleted) at the end of this period.<sup>29</sup> No losses were observed during feed processing.

L-Isoleucine (three batches) was suspended in water at 30°C and stored at 25°C or 40°C for up to 48 h.<sup>30</sup> No losses were observed in L-isoleucine.

The capacity of one batch of L-isoleucine to distribute homogeneously was studied in ten sub-samples of the pelleted and mash feeds and premixture used in the stability studies.<sup>31</sup> The homogeneity in mashed and pelleted feeds showed a coefficient of variation (CV) of 5%. Samples of the premixture showed a CV of 0.5%.

### 3.1.5. Physico-chemical incompatibilities in feed

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

### 3.1.6. Conditions of use

L-Isoleucine is intended to be used in feedingstuffs/complementary feedingstuffs or via premixture or water for drinking in all animal species. 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, in particular on the amino acid composition of the unsupplemented diet.

<sup>22</sup> Technical dossier/Section II/Annex/Annex\_II\_1\_03. LOD (in mg/kg) were 0.015 for lead, 0.01 for cadmium and mercury, 0.04 for arsenic, 0.20 for nickel and chromium, 0.50 for zinc. LOD (in µg/kg) were 0.2 for sum of aflatoxins, 0.5 for ochratoxin A, 50 for deoxynivalenol, 10 for zearalenone.

<sup>23</sup> Technical dossier/Section II/Annex/Annex\_II\_1\_03. The values reported represents LODs.

<sup>25</sup> Technical dossier/Section II/Annex/Annex\_II\_1\_04.

<sup>26</sup> Technical dossier/Section II/Annex/Annex\_II\_1\_05.

<sup>27</sup> Technical dossier/Section II/Annex/Annex\_II\_4\_01.

<sup>28</sup> Technical dossier/Section II/Annex/Annex\_II\_4\_03.

<sup>29</sup> Technical dossier/Section II/Annex/Annex\_II\_4\_04 and Annex\_II\_4\_05.

<sup>30</sup> Technical dossier/Section II/Annex/Annex\_II\_4\_02.

<sup>31</sup> Technical dossier/Section II/Annex/Annex\_II\_4\_02, Annex\_II\_4\_03, Annex\_II\_4\_04 and Annex\_II\_4\_05.

## 3.2. Safety

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

Safety concerns from the additive may derive either from the amino acid or from the residues of the fermentation process/production strain remaining in the final product. The L-isoleucine under assessment is highly purified (less than 1% unidentified material). The production strain KCCM 80189 belongs to a species, *Corynebacterium glutamicum*, that qualifies for the qualified presumption of safety (QPS) approach to safety assessment (EFSA, 2007) when used for production purposes (EFSA BIOHAZ Panel, 2019). The strain was unambiguously identified as *C. glutamicum*, was shown to be susceptible to the relevant antibiotics. There were also no viable cells in the final product. It can be concluded that no safety concerns for target animal, consumers and the environment would rise from the fermentation residues that may be present in the final additive.

Generally, it is well known that L-isoleucine is one of the three branched chain amino acid (BCAA) together with leucine and valine and one of the nine essential amino acids. As essential amino acid used as a nutritional additive, it is necessary to know the requirement for all animal species as well as to have information for the isoleucine content of the diet used. It is known (Harper et al., 1984) that isoleucine as a branched chain amino acid exerts a strong antagonism on the other two BCAAs, resulting in an alteration of the plasma and brain amino acid concentrations (imbalance) which is responsible for a reduced feed intake with impaired weight gain and feed efficiency. Maintenance requirements of animals for BCAAs may be influenced by these antagonisms. Excessive doses of amino acids lead to nutritional imbalances, which may provoke interactions and can eventually result in adverse effects (growth reduction, reduced feed consumption, changes in plasma amino acid pattern, mortality). Antagonisms occur widely in livestock nutrition due to adverse ratios of lysine and arginine and of the BCAAs in some common feedstuffs. Relative excesses of isoleucine or valine are better tolerated by almost all animal species than dietary overdoses of leucine. The interaction of BCAAs fed at excessive levels has already been described in the former FEEDAP opinion on L-valine (EFSA, 2008).

Regarding the safety of the amino acid for consumer, L-isoleucine supplemented to feed will be incorporated into proteins of tissues and/or products of animal origin but any of their potential excess will be metabolised, and therefore, the composition of tissues and products of animal origin will not be affected by the use of L-isoleucine in animal nutrition.

The amino acid L-isoleucine is a physiological and natural component of the proteins of living organisms. When consumed, it will be absorbed, and incorporated into body protein or excreted as urea/uric acid and as carbon dioxide.

The use of amino acids in water for drinking, in addition to complete diets with a well-balanced amino acid profile may represent a risk for the target species due to nutritional imbalances and hygienic reasons (EFSA FEEDAP Panel, 2010). Moreover, it may result in an increased nitrogen excretion via urine. Therefore, the FEEDAP Panel has concerns on the safety of the simultaneous oral administration of isoleucine-containing additives via feed and water for drinking.

The FEEDAP Panel concludes that L-isoleucine produced by *C. glutamicum* KCCM 80189 is safe for the target species, for the consumer and for the environment.

### 3.2.2. Safety for user

#### 3.2.2.1. Effects on the respiratory system

A valid acute inhalation test in laboratory animals, performed according to the Organisation for Economic Co-operation and Development (OECD) guideline 403, showed an LC<sub>50</sub> greater than 5.07 mg/L in male and female rats.<sup>32,33</sup>

#### 3.2.2.2. Effects on skin and eyes

The skin irritation potential of the additive was tested in a study<sup>34</sup> performed according to OECD guideline 439. The results of the study indicate that the additive should not be considered as a skin irritant.

<sup>32</sup> Technical dossier/Section III/Annex/Annex\_III\_3\_02.

<sup>33</sup> Technical dossier/Supplementary information October 2019/Annex/Annex\_SIN\_02.

<sup>34</sup> Technical dossier/Section III/Annex/Annex\_III\_3\_04.



The eye irritation potential of the additive was tested in a valid study performed according to OECD guideline 437. The results of the study indicate that the additive should not be considered as an eye irritant.<sup>35</sup>

In a valid dermal sensitisation study following OECD guideline 429 (local lymph-node assay) and Method B42 Skin Sensitization (local lymph node assay) of Commission Regulation (EC) No 440/2008, the additive did not show any skin sensitisation potential.<sup>36</sup>

### 3.2.2.3. Conclusions on safety for the user

L-Isoleucine produced by *C. glutamicum* KCCM 80189 is considered not toxic by inhalation, not irritant to skin or eyes and not a dermal sensitizer.

## 3.3. Efficacy

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

## 3.4. Post-market monitoring

The FEEDAP Panel considers that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation<sup>37</sup> and Good Manufacturing Practice.

## 4. Conclusions

The additive is produced by a non-genetically modified strain of *C. glutamicum* (*C. glutamicum* KCCM 80189). No viable cells of the production strain were detected in the final additive. The additive does not give rise to any safety concern regarding the production strain.

L-Isoleucine produced by *C. glutamicum* KCCM 80189 is considered to be safe for the target species, for the consumer and for the environment. Regarding the use in water, the FEEDAP Panel reiterates its concerns over the safety for the target species of L-isoleucine administered simultaneously via water for drinking and feed owing to the risk of nutritional imbalances and hygienic reasons.

L-Isoleucine produced by *C. glutamicum* KCCM 80189 is considered not toxic by inhalation, not irritant to skin or eyes and not a dermal sensitizer.

L-Isoleucine produced by *C. glutamicum* KCCM 80189 is considered as an efficacious source of the essential amino acid L-isoleucine for non-ruminant animal species. For the supplemental L-isoleucine to be as efficacious in ruminants as in non-ruminant species, it would require protection against degradation in the rumen.

## Documentation as provided to EFSA/Chronology

Date	Event
28/03/2019	Dossier received by EFSA. L-isoleucine produced by fermentation with <i>Corynebacterium glutamicum</i> KCCM 80189 for all animal species. Submitted by CJ Europe GmbH
15/04/2019	Reception mandate from the European Commission
05/06/2019	Application validated by EFSA – Start of the scientific assessment
22/07/2019	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 the production strain, characterisation of the additive, user safety</i>
05/09/2019	Comments received from Member States

<sup>35</sup> Technical dossier/Section III/Annex/Annex\_III\_3\_03.

<sup>36</sup> Technical dossier/Section III/Annex/Annex\_III\_3\_05.

<sup>37</sup> Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 January 2003 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

Date	Event
04/10/2019	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
14/10/2019	Reception of supplementary information from the applicant - Scientific assessment re-started
17/12/2019	Spontaneous Supplementary information from the applicant
28/01/2020	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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## Abbreviations

AMR	antimicrobial resistance
BCAA	branched chain amino acid
CAS	Chemical Abstracts Service
CFU	colony forming unit
CV	coefficient of variation
DL-PCB	dioxin-like polychlorinated biphenyl
EINECS	European Inventory of Existing Commercial Chemical Substances
EURL	European Union Reference Laboratory
FCC	Food Chemical Codex
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
FLAVIS	EU Flavour Information System
HPLC-FLD	high performance liquid chromatography coupled to fluorescence detection
IEC	ion exchange chromatography
IEC-VIS	ion exchange chromatography coupled to photometric detection
IUPAC	International Union of Pure and Applied Chemistry
LC <sub>50</sub>	lethal concentration, median
LOD	limit of detection
NDA	EFSA Panel on Dietetic Products, Nutrition and Allergies
OECD	Organisation for Economic Co-operation and Development
PCDD/F	polychlorinated dibenzo- <i>p</i> -dioxins and dibenzofurans
QPS	qualified presumption of safety
RH	relative humidity
RSD <sub>r</sub>	relative standard deviation for repeatability
RSD <sub>R</sub>	relative standard deviation for reproducibility
TEQ	toxic equivalents
WGS	whole genome sequence
WHO	World Health Organization

## Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Methods of Analysis for L-isoleucine produced by fermentation with *Corynebacterium glutamicum* KCCM 80189 for all animal species

In the current application authorisation is sought under Article 4(1) for *L-isoleucine produced by fermentation with Corynebacterium glutamicum* KCCM 80189, under the category/functional group 3(c) 'nutritional additives'/amino acids, their salts and analogues', according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for all animal species.

According to the Applicant *L-isoleucine* has a minimum purity (mass fraction) of 90%. The *feed additive* is intended to be added directly into *feedingstuffs* (or through *premixtures*) and *water* for drinking. However, the Applicant did not propose any minimum or maximum content of *L-isoleucine* in *feedingstuffs*.

For the quantification of L-isoleucine in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted an in-house validated analytical method based on high performance liquid chromatography coupled to fluorescence detection (HPLC-FLD). The Applicant did not present a verification study and therefore, the EURL cannot recommend the method for official control purposes.

For the quantification of *isoleucine* in feed a ring-trial validated European Union (EU) method (Commission Regulation (EC) No 152/2009) based on ion exchange chromatography (IEC) coupled to photometric detection exists (IEC-VIS). This method, designed for the analysis of amino acids in *premixtures* and *feedingstuffs*, does not distinguish between the salts and the amino acid enantiomers. This method was further ring-trial validated resulting in the EN ISO 13903:2005 method. The following performance characteristics were reported for the quantification of total *isoleucine*: relative standard deviation for repeatability (RSD<sub>r</sub>) ranging from 2.0 to 5.4% and relative standard deviation for reproducibility (RSD<sub>R</sub>) ranging from 6.8 to 14.3%.

The Applicant did not provide experimental data to determine *isoleucine* in *water*. Nevertheless, as concluded in a previous EURL report, the EURL recommends the EU method for official control for the quantification of *isoleucine* in the *feed additive* and *water*.

In addition, the EURL found the "L-isoleucine monograph" of the Food Chemical Codex (FCC) for the identification of *L-isoleucine* in the *feed additive*.

In the frame of this authorisation the EURL recommends for official control (i) the "L-isoleucine monograph" of the Food Chemical Codex (FCC) based on infrared absorption for the identification of *L-isoleucine* in the *feed additive*; and (ii) the European Union (EU) method based on IEC-VIS for the quantification of *isoleucine* in the *feed additive*, *premixtures*, *feedingstuffs* and *water*.

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