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Assessment of the application for renewal of authorisation of L-isoleucine produced by *Escherichia coli* FERM ABP-10641 as a nutritional additive, its extension of use in water for drinking and a new use as flavouring additive for all animal species

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Abstract

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on L-isoleucine produced by *Escherichia coli* FERM ABP-10641 (i) to renew the authorisation and to extend the use of the additive in water for drinking when used as a nutritional additive for all animal species and (ii) to evaluate a new use as a flavouring additive for all animal species. The applicant has provided evidence that the additive currently in the market complies with the existing conditions of authorisation. L-Isoleucine does not give rise to any safety concern regarding the production strain and its genetic modification. Considering that the production process has not been substantially modified and that no adverse effects have been reported in the literature search, the FEEDAP Panel considers that there is no evidence to reconsider the conclusions reached in previous assessments. Therefore, the Panel considers that the additive L-isoleucine produced by *E. coli* FERM ABP-10641 remains safe for the target species, consumer and for the environment when used as a nutritional additive in feed. The Panel extends these conclusions to the use of the additive as a flavouring compound. 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 is not irritant to skin and eyes and is not a dermal sensitiser but is hazardous by inhalation. The Panel confirms the previous conclusions that the additive is considered as a source of available isoleucine for non-ruminant animal species when used as a nutritional additive. It requires protection against degradation in the rumen to be as efficacious in ruminants as in non-ruminant species. The Panel considers the use of the additive in water for drinking to be equally effective than the use in feed when used as a nutritional additive. Since L-isoleucine is used in food as a flavouring compound, and its function in feed is essentially the same as that in food no further demonstration of efficacy is necessary.

Keywords: Nutritional, flavouring, additive, amino acid, L-isoleucine, feed additive, safety, efficacy

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1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7. In addition, Article 13(3) of that Regulation lays down that if the holder of an authorisation proposes changing the terms of the authorisation by submitting an application to the Commission, accompanied by the relevant data supporting the request for the change, the Authority shall transmit its opinion on the proposal to the Commission and the Member States. In addition, Article 14(1) of that Regulation lays down that an application for renewal shall be sent to the Commission at the latest one year before the expiry date of the authorisation.

The European Commission received a request from Ajinomoto Animal Nutrition[2] for (i) renewal of the authorisation of the product L-isoleucine (minimum 93.4% of L-isoleucine expressed on dry matter), (ii) the extension of use in water for all animal species (category: nutritional additives; functional group: amino acids, their salts and analogues) and for (iii) the authorisation of the product for all animal species under the category 'sensory additives' and the functional group 'flavouring compounds'.

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), under Article 13(3) (modification of the authorisation of a feed additive) and under Article 14(1) (renewal of the authorisation). The particulars and documents in support of the application were considered valid by EFSA as of 9 July 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-isoleucine produced by *Escherichia coli* FERM ABP-10641, when used under the proposed conditions of use (see Section 3.2).

1.2. Additional information

L-Isoleucine for feed use as a nutritional additive has been assessed by the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) and the EFSA Panel on Genetically Modified Organisms (GMO) (EFSA, 2010).

L-Isoleucine produced by *E. coli* FERM ABP-10641 is currently authorised as a nutritional additive, in the functional group amino acids their salts and analogous, for all animal species.[3]

L-Isoleucine for feed use as a flavouring additive has been assessed by the FEEDAP Panel (2014).

L-Isoleucine produced by chemical synthesis or protein hydrolysis is authorised as a sensory additive.[4] It is listed in the European Union Register of Feed Additives as feed flavourings (CAS No. 443-79-8/(d-, l-) Isoleucine/EU Flavour Information System (FLAVIS) Number [17.010].

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food adopted one opinion on consideration of amino acids and related substances evaluated by JECFA (63rd meeting) structurally related to amino acids from chemical group 34 evaluated by EFSA in FGE.26Rev1 (EFSA, 2008).

D,L-Isoleucine (FLAVIS No. 17.010) is listed in the European Union (EU) database of flavouring substances.[5]

[1] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.

[2] Ajinomoto Animal Nutrition, 32 rue Guersant, 75017, Paris, France.

[3] Commission Regulation (EU) No 348/2010 of 23 April 2010 concerning the authorisation of L-isoleucine as a feed additive for all animal species OJ L 104, 24.4.2010, p. 29–30.

[4] 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. 134–165.

[5] 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.

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

L-Isoleucine is authorised for use in nutritional supplements in infant formulae,[6,7] food,[8,9] cosmetics[10] and as a veterinary medicinal product.[11]

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[12] in support of the authorisation request for the use of L-isoleucine (minimum 93.4% of L-isoleucine expressed on dry matter) for all animal species 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 control of the L-isoleucine (minimum 93.4% of L-isoleucine expressed on dry matter) in animal feed. The Executive Summary of the EURL report can be found in Annex A.[13]

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[14] and the relevant guidance documents: Guidance on the renewal of the authorisation of feed additives (EFSA FEEDAP Panel, 2013) 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

L-Isoleucine (minimum 93.4% of L-isoleucine expressed on dry matter) is produced by fermentation with a genetically modified (GM) *E. coli* strain. The applicant seeks:

i) the renewal of the use as a nutritional additive in feed (functional group: amino acids, their salts and analogues),
ii) the extension of use in water, when used as a nutritional additive and
iii) a new use as sensory additive as a flavouring compound.

In all the three cases above described, the application is for all animal species.

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[6] Commission delegated Regulation (EU) 2016/127 of 25 September 2015 supplementing Regulation (EU) No 609/2013 of the European Parliament and of the Council as regards the specific compositional and information requirements for infant formula and follow-on formula and as regards requirements on information relating to infant and young child feeding.

[7] Commission Directive 2006/141/EC of 22 December 2006 on infant formulae and follow-on formulae and amending Directive 1999/21/EC.

[8] 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.

[9] Commission Directive 2006/125/EC of 5 December 2006 on processed cereal-based foods and baby foods for infants and young children (https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32006L0125&from=EN).

[10] Commission Decision of 9 February 2006 amending Decision 96/335/EC establishing an inventory and a common nomenclature of ingredients employed in cosmetic products (http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32006D0257&qid=1522920186832&from=EN).

[11] 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. 1–72. (*http://data.europa.eu/eli/reg/2010/37(1)/oj*)*

[12] FEED dossier reference: FAD-2019-0035.

[13] The full report is available on the EURL website: https://ec.europa.eu/jrc/en/eurl/feed-additives/evaluation-reports/fad-2019-0035?search&form-return

[14] 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.

3.1. Characterisation

3.1.1. Characterisation of the production organism

The additive L-isoleucine is produced by a GM strain of *E. coli* deposited at the International Patent Organism Depositary, National Institute of Technology and Evaluation, Japan with the accession number FERM ABP-10641.

The genomes of the parental [redacted] and the production FERM ABP-10641 strains were sequenced and the data were used to confirm the identification of the production strain as an *E. coli* K-12 derivative by molecular serotyping and bioinformatic analysis, [redacted]

A test for antibiotic susceptibility using a culture broth microdilution method to nine antibiotics confirmed the information already provided for the first authorisation. The minimum inhibitory concentration (MIC) values for all the clinically relevant antibiotics tested were equal or below to the cut-off values (EFSA FEEDAP Panel, 2018b). The whole genome sequence (WGS) analysis of the production strain was interrogated [redacted] The search did not identify any [redacted] antibiotic resistance gene. [redacted]

3.1.1.1. Information relating to the genetically modified microorganism

The production strain was assessed by EFSA in 2010 (EFSA FEEDAP Panel/EFSA GMO Panel, 2010) and it was concluded that the genetic modification does not raise safety concerns. [redacted] The Applicant states that the production strain has not been subject to any further modification.

3.1.2. Manufacturing process

L-Isoleucine is produced by fermentation (fed-batch fermentation) using the GM production strain *E. coli* FERM ABP-10641,[19] the cells in the fermentation broth are inactivated [redacted]. Filtration, [redacted] crystallisation, drying and packaging. The applicant stated that no antibiotics are used during fermentation process. The information submitted regarding the manufacturing process confirms that it is the same as the one described in the first assessment.

3.1.3. Characterisation of active substance/additive

L-Isoleucine (International Union of Pure and Applied Chemistry (IUPAC) name: (2~,3~)-2-amino-3-methylpentanoic acid), is a compound identified with the Chemical Abstract Service (CAS) No 73-32-5, and the European Inventory of Existing Commercial Chemical Substances (EINECS) No 200-798-2. It has a molecular weight of 131.17 Da. The chemical formula of L-isoleucine is $C_6H_{13}NO_2$. The structural formula is given in Figure 1.

[redacted]

[19] Technical dossier/Section II/Annex_Supp_II_1_Deposit certificate_ILE_FERM ABP-10641_ENG_JP.

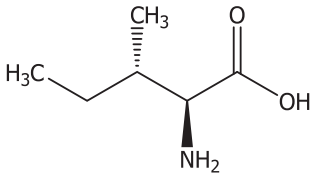

Figure 1: Structural formula of L-isoleucine

The additive is currently authorised with a minimum content of 93.4% L-isoleucine on a dry matter (DM) basis and less than 1% of unidentified material.

The content of L-isoleucine measured in five recent batches of the additive, with an average DM content of 99.92% and moisture 0.08%, averaged 95.7% (range 95.3–96.0%).[20]

Analytical data on the specific optical rotation of five batches was on average 40.4°[20] (range +40.3° to +40.4°), which is within the range in the European Pharmacopoeia (+40.0° to +43.0°) (PhEur, 2017) and confirms the identity of the L-enantiomer.[21]

The content of other free amino acids in the additive *as is* was 3.5% in three batches.[21] These accounted mostly of glutamic acid (0.035%), valine (2.02%), phenylalanine (0.12%), tyrosine (0.12%), serine (0.02%), alanine (0.32%), tryptophan (0.001%) and aminobutyric acid (alpha, beta and gamma) (0.95%).

Other components of the additive included ammonium nitrogen (0.026%), nitrites (ranged from < 5.0 to 6 mg/kg), nitrates (< 3.6 mg/kg) measured in three batches and reported on an 'as is' basis.[21,22]

Carbohydrates were not present in three batches. The sum of organic acids tested (acetate, succinate, butyrate, citrate, lactate, formate, oxalic acid, propionate, pyruvate) was calculated to be 20 mg/kg. Crude ash average content was 0.03%.[25]

3.1.4. Impurities

Five recent batches were analysed for the possible presence of arsenic, cadmium, chromium, copper, iron, lead, mercury, nickel, phosphorus, fluorine, melamine and (hydro)-cyanic acid. The levels found were below the limits of quantification (LOQs) of the analytical methods.[22,23]

In the same five recent batches, the content of dioxins (polychlorinated dibenzo-*p*-dioxins and furan (PCDD/F)) ranged between 0.053 and 0.055 ng WHO-PCDD/F-PCB TEQ/kg. The sum of dioxins and dioxin-like polychlorinated biphenyls (PCBs) ranged between 0.082 and 0.086 ng WHO-PCDD/F-PCB TEQ/kg.[25]

Residues of organochlorine (including pyrethroids) and organophosphorus pesticides, analysed in three batches of the additive, were all below the LOQs.[24,25]

The average content of biogenic amines in the additive 'as is' was 5 mg/kg (0.0005%) in three batches.[21] Their content in three recent batches ranged up to 3.44 mg/kg for cadaverine, 2.24 mg/kg for tyramine, < 5 mg/kg (LOQ) for tryptamine mg/kg, 3.22 mg/kg for putrescine and 2.03 mg/kg for spermidine.[25]

The microbial analyses of 10 batches of the product showed the absence of *Salmonella* spp. in 25 g of the additive, coliforms and *Staphylococcus* coagulase positive. Counts for yeasts and filamentous fungi were < 10 colony forming units (CFU)/g and counts of *Bacillus cereus* were < 100 CFU/g.[26]

Analysis of three recent batches showed values of aflatoxins (B1, B2, G1, G2), zearalenone, deoxynivalenol, ochratoxin A, T-2 and HT-2 toxins, fumonisins (B1, B2 and B3) below the LOQs.[22,27]

[20] Technical dossier/Section II/Annex_II_5_Mauclair_2019_ILE_Batches_7267_7274.
[21] Technical dossier/Section II/Annex_II_2_EurPharma_2017_9th_ed_ILE.
[22] Technical dossier/Section II/Annex_II_6_Eurofins_2018_ILE_Batches_7267_7271.
[23] Technical dossier/Section II/ Annex_II_20_Eurofins_Methods_2018_. LOQ: arsenic 0.05 mg/kg; cadmium 0.005 mg/kg; chromium 0.10 mg/kg; copper 0.1 mg/kg; iron 1 mg/kg; lead 0.02 mg/kg; mercury 0.005 mg/kg; nickel 0.10 mg/kg; phosphorus 3.0 mg/kg; fluorine 20 mg/kg; melamine 0.05 mg/kg; (hydro)cyanic acid 1.5 mg/kg.
[24] Technical dossier/Section II/ Annex_II_20_Eurofins_Methods_2018. LOQ: Organophosphorus Pesticides 0.01–0.03 mg/kg; Organochlorine Pesticides 0.002 to 0.1 mg/kg.
[25] Technical dossier/Section II/Annex_II_6_Eurofins_2018_ILE_Batches_7267_7271: < 1 (LOQ) for cadaverine, < 1 (LOQ) mg/kg for tyramine, < 5 (LOQ) mg/kg for tryptamine, < 1 (LOQ) mg/kg for putrescine and < 1 (LOQ) mg/kg for spermidine.
[26] Technical dossier/Section II/ Annex_II_10_Legrand_2018_ILE_8142_8144.
[27] Technical dossier/Section II/Annex II_20. LOQ: aflatoxins 0.1–0.2 µg/kg, zearalenone 20 µg/kg; deoxynivalenol 50 µg/kg; ochratoxin µg/kg; toxin T-2, HT-2 20 µg/kg; fumonisins: 20 µg/kg.

The amount of the impurities above described in the product does not raise a safety concern.

[redacted]

3.1.4.1. Presence of viable cells and recombinant DNA of the production organism

The presence of viable cells of the production strain was tested [redacted]

The presence of the production strain DNA was tested [redacted] No DNA was detected in any of the analysed batches.

Neither the production strain nor its recombinant DNA is present in the final product

3.1.5. Physical characteristics

The additive is a white powder. Its solubility in water at 23.7°C is 33.85 g/L, it has a pH ranging from 5 to 6.[30] The packed bulk density measured in three batches ranged from 698 to 706 kg/m^3.

Particle size distribution was analysed in five batches by laser light scattering. Most of the particles ranged from 100 to 1,000 μm diameter. The fraction of particles with a diameter below 10, 50 and 100 μm was 1.5, 4.2 and 6.8%, respectively.[31]

[redacted]

3.1.6. Stability and homogeneity

The shelf life of the additive (three batches produced in 2015) was measured when stored at 25°C and 40°C in closed polyethylene (PE) bottles with perforated caps for 36 months. Losses up to 0.5% of L-isoleucine were detected at 25°C in one batch. No losses were observed in any of the batches stored at 40°C.[32]

The stability of the additive (three batches produced in 2018) was measured in three types of vitamin/mineral premixtures (for piglets, pigs and chickens for fattening, one batch each) at 25°C or at 40°C in paper bags with plastic in nylon–PE bags for 6 months.[33] The concentration of choline chloride was 1.3% in piglets, 0.4% in pigs and 2% in chickens for fattening premixtures. For the piglet premixture, a maximum loss of 2.5 and 1.9% was observed at 25°C and 40°C, respectively. For the premixture for pigs for fattening, a maximum loss of 2.8 and 1.8% was observed at 25 and 40°C, respectively. For chickens for fattening, a maximum loss of 0.8 and 0.9% was observed at 25°C and 40°C, respectively.

The stability of the additive (three batches produced in 2018) in three types of pelleted feed (piglets, pigs and chickens for fattening, one batch each) was studied when stored at 25°C and 40°C in sealed plastic bags for 3 months.[34] Complete feeds for piglets, pigs and chickens for fattening were supplemented with L-isoleucine at 0.041, 0.032 and 0.072%, respectively. At the end of the storage period, the concentration of isoleucine was stable in all the feeds stored at the different temperatures.

[redacted]

[30] Technical dossier/Section II/ Annex _II_34_MSDS_2018_ILE_AANE.
[31] Technical dossier/Section II/Annex_II_7_Malvern_Mastersizer_ILE_Batches_7267_7274.
[32] Technical dossier/Section II/Annex _II_58_Auxenfans_et_al_2019.
[33] Technical dossier/Section II/Annex _II_67_Jurgens_et_al_2018_WUR1851_ILE_Stab_in_premix.
[34] Technical dossier/Section II/Annex_II _ 69_Jurgens_et_al_2018_WUR_1852_Stab_Feed_ILE.

The stability of the additive (three batches produced in 2017) in water for drinking was measured at 0.5, 2.5 and 5 g/L when stored at 25 and 40°C for 50 h.[35] No losses of L-isoleucine were observed.

The capacity of the additive to distribute homogeneously in premixtures (for piglets, pigs and chickens for fattening) and in feedingstuffs was studied.[33,34] Ten subsamples of each premixture and feedingstuff were analysed and considered to mix homogeneously.

3.1.7. Physico-chemical incompatibilities in feed

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

3.2. Conditions of use

The additive is currently authorised for use in feed for all animal species as a nutritional additive without a minimum or maximum content complete feed. The applicant proposes to maintain the same conditions of use as authorised in feed. The applicant also requests the use in water for drinking when used as a nutritional additive.

The applicant further requests a new use of the additive as a flavouring additive in feedingstuffs and premixtures for all animal species. As a feed flavouring in feed, the level of inclusion proposed is 25 mg/kg.

As a nutritional additive, 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, the environmental conditions and the amino acid composition of the unsupplemented diet.

The applicant does not propose to modify the conditions of use as authorised.

The authorisation for use as a nutritional additive for all animal species includes under other provisions an indication that breathing protection for the user shall be used during handling.

3.3. Safety

3.3.1. Safety aspects of the production organism

The genetic modifications performed to obtain the production strain have the purpose to increase the metabolic flow towards L-isoleucine [redacted]

The recipient organism *E. coli* K-12 is considered to be safe. None of the introduced modifications raise a safety concern. The applicant provided sufficient information that neither the production strain nor its recombinant DNA is present in the final product. The final product does not give rise to any safety concern with regards to the genetic modification of the production strain.

3.3.2. Safety for the target species, consumer and environment

The L-isoleucine subject of the current assessment was previously evaluated by the FEEDAP Panel (EFSA FEEDAP Panel, 2010). In that assessment, the Panel concluded that the additive was safe for the target species, the consumer and the environment when used as a nutritional additive.

In the present application, the applicant conducted three literature searches to confirm the safety of the product. The time span covered was from 2008 to November 2018.[36] The first search was performed using different databases (e.g. LILIVO, OVID, PubChem, PubMed, Web of Science, etc.). The search terms used were divided in different sections related to the laboratory animals, to humans, to pets and farm animals.[37] The second search in Google Scholar search[38] used terms referring to the use (e.g. water, feed), target species and their combination with terms referring to the safety. The third search used the additive CAS number in several Tox-net databases (ACToR, CCRIS, ChemiDplus, ECHA, GESTIS, HazMap, HPD, HSDB, PubChem, Reaxys).

[35] Technical dossier/Section II/Annex_II_ 72_Voudouris_and_Verkleij_2019_WUR_1865_Stab_in_water.

[36] Technical dossier/Section III/Annex_III_6-1_ScDB_Search_Report_111118.

[37] Laboratory animals: mouse, mice, rats, hamsters, guinea pigs, rabbits, monkeys, laboratory animals. Key words: 90-day toxicity study, adverse effect, gavage, NOEL, oral, toxicity, TTC; humans: humans, woman, men, children, adults, elderly, infants, adolescents, subjects, volunteers, patients, consumers. Key words: food supplement, MSDI, safety, Nutrition/ Dietetic Products, Novel Food and allergy, Pharmacovigilance, adverse/side-effect*, contraindicat*/contra-indicat*/interact*; pet, farm animals: pre-ruminants, protected ruminants, pigs, piglets, sow, poultry, broiler, layer, chicken, turkey, fish, salmonids, pets, cats, felines, dogs, canines, cattle, horse, sheep. Key words: Tolerance, nutrition/ feed/ food, dietetic products, safety, drinking water.

[38] Technical dossier/Section III/Annex_III_7-2_Google_Sch_Results_181112.

The Panel notes that none of the searches conducted included specific keywords for the production strain.

The applicant screened the titles against exclusion and inclusion criteria for their relevancy to assess the safety and efficacy of the additive[39]

As a result of the literature review the applicant submitted 21 full papers. Most of the papers concerned the effects of different supplementation levels on animal performance (e.g. performance, immunomodulation, resistance to stressors) but in none of these publications adverse events or safety issues concerning L-isoleucine were reported.

The additive is highly purified and is produced by fermentation using a GM strain that is considered safe. Concerns from the use of the additive would not derive from L-isoleucine, which is considered safe, but may arise from residues of the fermentation process/production strain remaining in the final product. Since the identity of the production strain has been established, it is susceptible to relevant antimicrobials used in human and veterinary medicine and no viable cells of the production strain or its DNA are in the final product; L-Isoleucine produced by *E. coli* FERM ABP-10641 is considered safe for the target species provided that it is supplemented in appropriate amounts to the diets.

Considering the above and the fact that the composition and the production process have not been modified, that the literature review has not identified any publication that would indicate a safety concern for the target animals, consumers and the environment, and the fact that the proposed conditions of use of the additive as a nutritional additive have not been modified (other than the request for use in water), the FEEDAP Panel considers that the use of L-isoleucine as a nutritional feed additive remains safe for the target species, consumers and the environment. These conclusions are extended to the additive used as flavouring compound considering the level of inclusion (25 mg/kg feed) that fall well below normal intake levels in the target animals (e.g. soya bean meal, a common feed material, contains approximately 2% isoleucine).[40]

Due to the risk of nutritional imbalances and hygienic reasons, associated to the use of amino acids via water for drinking (EFSA FEEDAP Panel, 2010), the FEEDAP Panel has concerns on the safety for the target species of the simultaneous oral administration of isoleucine-containing additives via feed and water for drinking.

3.3.3. Safety for the user

In this application, the applicant did not provide new studies to support the safety for the skin/eye irritation/skin sensitisation.

In the previous opinion (EFSA FEEDAP Panel, 2010), the Panel concluded that 'The product L-isoleucine is considered to be non-irritant to skin and eyes and not to be a dermal sensitiser. The submitted published literature gave no indications of skin/eye irritation/skin sensitisation'.

As concerns the effects of the respiratory system, the Panel concluded that 'the only detectable risk for the user could be derived from the dustiness of the product, but on the basis of an acute inhalation toxicity study, this is expected to be minor'.

In the current application dossier, additional data to support the assessment on the effects of the additive on the respiratory system were submitted.

3.3.3.1. Effects on the respiratory system

Dusting potential (measured in three batches) was up to 2.01 g/m^3 and the dust contained about 1.5% of particles having a diameter < 10 μm diameter.[31]

Users can suffer from occupational respiratory disease depending on the level of endotoxins in air and dust (Rylander, 1999; Thorn, 2001). The scenario used to estimate the exposure of persons handling the additive to endotoxins in the dust, based on the EFSA guidance on user safety (EFSA FEEDAP Panel, 2012), is described in Appendix A. The threshold for the quantity of inhaled endotoxins per working day is 900 IU, derived from the provisional occupational exposure limits given by the Dutch Expert Committee on Occupational Safety (Health Council of the Netherlands, 2010) and the UK Health and Safety Executive (HSE, 2013). Based on calculations of the content of endotoxins in dust (up to 911 EU/g), exposure by inhalation would be 1,017.28 IU per eight-hour working day, indicating a risk of exposure to endotoxins for people handling the additive (see Appendix A).

[39] Technical dossier/Section III/Annex_III_8_Literature_Search_Summary.
[40] https://www.feedipedia.org

The use of the additive as a flavouring and its use in water for drinking as a nutritional additive will not pose additional risks to the users than those indicated above.

3.3.3.2. Conclusions on safety for the user

The Panel, after taking into consideration the information provided in the dossier and considering that the manufacturing process did not change, confirms the previous conclusions also when its use is extended to water for drinking and when used as feed flavouring. L-Isoleucine is not irritant to skin and eyes, is not a dermal sensitiser. As concerns the effects on the respiratory system, the Panel concludes that there is a risk for people handling the additive from the exposure to endotoxins by inhalation.

3.4. Efficacy

The Panel confirms the previous conclusions that the product L-isoleucine is considered as a source of available isoleucine for non-ruminant animal species when used as a nutritional additive. To be as efficacious in ruminants as in non-ruminant species, it requires protection against degradation in the rumen. The Panel considers the use in water for drinking to be equally effective than the use in feed. Since L-isoleucine [17.010] is used in food as a flavouring compound, and its function in feed is essentially the same as that in food no further demonstration of efficacy is necessary.

3.5. 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[41] and Good Manufacturing Practice.

3.6. Conclusions

The applicant has provided evidence that the additive currently in the market complies with the existing conditions of authorisation.

L-Isoleucine does not give rise to any safety concern regarding the production strain and its genetic modification.

Considering that the production process has not been substantially modified and that no adverse effects have been reported in the literature search, the FEEDAP Panel considers that there is no evidence to reconsider the conclusions reached in previous assessments. Therefore, the Panel considers that the additive L-isoleucine produced by *E. coli* FERM ABP-10641 remains safe for the target species, consumer and for the environment when used as a nutritional additive in feed. The Panel extends these conclusions to the use of the additive as a flavouring compound. 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 is not irritant to skin and eyes and is not a dermal sensitiser but is hazardous by inhalation.

The Panel confirms the previous conclusions that the additive is considered as a source of available isoleucine for non-ruminant animal species when used as a nutritional additive. It requires protection against degradation in the rumen to be as efficacious in ruminants as in non-ruminant species. The Panel considers the use of the additive in water for drinking to be equally effective than the use in feed when used as a nutritional additive. Since L-isoleucine is used in food as a flavouring compound, and its function in feed is essentially the same as that in food no further demonstration of efficacy is necessary.

[41] Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

Documentation as provided to EFSA/Chronology

Date	Event
10/05/2019	Dossier received by EFSA. L-isoleucine. Submitted by Ajinomoto Animal Nutrition Europe
23/05/2019	Reception mandate from the European Commission
09/07/2019	Application validated by EFSA – Start of the scientific assessment
04/10/2019	Comments received from Member States
08/11/2019	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
02/04/2019	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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Abbreviations

AFC	EFSA Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food
CAS	Chemical Abstracts Service
CD	Commission Decision
CG	chemical group
CV	coefficient of variation
DM	dry matter
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
GM	genetically modified
GMO	EFSA Panel on Genetically Modified Organisms
HSE	UK Health and Safety Executive
IEC-VIS/FLD	ion-exchange chromatography coupled to visible or fluorescence detection
IUPAC	International Union of Pure and Applied Chemistry
LOQ	limit of quantification
MIC	minimum inhibitory concentration
MW	molecular weight

NDA	EFSA Panel on Dietetic Products, Nutrition and Allergies
PCB	polychlorinated biphenyl
PCDD/F	polychlorinated dibenzo-*p*-dioxins and furan
RH	relative humidity
RSDip	relative standard deviation for intermediate precision
RSDr	relative standard deviation for repeatability
SCAN	Scientific Committee on Animal Nutrition
WGS	whole genome sequence
WHO	World Health Organization

Appendix A – Safety for the user

The effects of the endotoxin inhalation and the exposure limits have been described in a previous opinion (EFSA FEEDAP Panel, 2015).

Calculation of maximum acceptable levels of exposure from feed additives

The likely exposure time according to EFSA guidance (EFSA FEEDAP Panel, 2012) for additives added in premixtures assumes a maximum of 40 periods of exposure per day, each comprising 20 s, equal to = 800 s per day. With an uncertainty factor of 2, maximum inhalation exposure would occur for 2 × 800 = 1,600 s (0.444 h per day). Again, assuming a respiration volume of 1.25 m^3/h, the inhalation volume providing exposure to potentially endotoxin-containing dust would be 0.444 × 1.25 = 0.556 m^3 per day. This volume should contain no more than 900 IU endotoxin, so the dust formed from the product should contain no more than 900/0.556 = 1,619 IU/m^3.

Calculation of endotoxin content of dust

Two key measurements are required to evaluate the potential respiratory hazard associated with endotoxin content of the product (the dusting potential of the product, expressed in g/m^3; the endotoxin activity of the dust, determined by the *Limulus* amoebocyte lysate assay (expressed in IU/g)). If data for the dust are not available, the content of endotoxins of the product can be used instead. If the content of endotoxins of the relevant additive is IU/g and the dusting potential is b g/m^3, then the content of endotoxins of the dust, c IU/m^3, is obtained by the simple multiplication a × b. This resulting value is further used for calculation of potential inhalatory exposure by users to endotoxin from the additive under assessment (Table A.1) (EFSA FEEDAP Panel, 2012).

Table A.1: Estimation of user exposure to endotoxins from the additive L-isoleucine produced by *Escherichia coli* FERM ABP-10641 including consideration of using filter half mask (FF P2 or FF P3)[42] as a preventative measure

Calculation	Identifier	Description	Amount	Source
	a	Endotoxin content IU/g product	911	Technical dossier
	b	Dusting potential (g/m^3)	2.01	Technical dossier
a × *b*	*c*	Endotoxin content in the air (IU/m^3)	1831.11	
	d	No of premixture batches made/working day	40	EFSA FEEDAP Panel (2012)
	e	Time of exposure (s)/production of one batch	20	EFSA FEEDAP Panel (2012)
d × *e*	*f*	Total duration of daily exposure/worker (s)	800	
	g	Uncertainty factor	2	EFSA FEEDAP Panel (2012)
f × *g*	*h*	Refined total duration of daily exposure (s)	1,600	
h/3 600	*i*	Refined total duration of daily exposure (h)	0.44	
	j	Inhaled air (m^3)/eight-hour working day	10	EFSA FEEDAP Panel (2012)
j/8 × *i*	*k*	Inhaled air during exposure (m^3)	0.56	
c × *k*	*l*	**Endotoxin inhaled (IU) during exposure/eight-hour working day**	**1017.28**	
	m	Health-based recommended exposure limit of endotoxin (IU/m^3)/eight-hour working day	90	Health Council of the Netherlands (2010)
m × *j*	*n*	**Health-based recommended exposure limit of total endotoxin exposure (IU)/eight-hour working day**	**900**	
l /10		Endotoxins inhaled (IU)/eight-hour working day reduced by filter half mask FF P2 (reduction factor 10)	102	
l /20		Endotoxins inhaled (IU)/eight-hour working day reduced by filter half mask FF P3 (reduction factor 20)	51	

[42] Filtering face piece or filtering half mask according to European standard EN 149. They are graded from 1 to 3 depending on their filtering capacity.

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for L-isoleucine produced by *Escherichia coli* K-12 - FERM ABP-10641 for all animal species

In the current application authorisation is sought under Article 4(1) for L-isoleucine produced by fermentation with a strain derived from *Escherichia Coli* K-12 FERM ABP-10641 under the category/ functional groups 2(b) 'sensory additives/flavouring compounds' and under Article 14 as 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 93.4% (expressed on the dry matter basis). As nutritional feed additive, L-isoleucine is intended to be added directly into feedingstuffs or through premixtures and water for drinking. As sensory feed additive, L-isoleucine is intended to be added into feedingstuffs through flavouring premixtures. The Applicant did not propose any minimum or maximum content of L-isoleucine in feedingstuffs. However, when authorised as sensory additive, it is recommended to use the amino acid at a level of 25 mg/kg complete feed.

For the quantification of L-isoleucine in the feed additive and premixtures the Applicant submitted the ring-trial validated method EN ISO 17180:2013 specifically designed for the determination of lysine, methionine and threonine in products containing more than 10% of amino acid. This standard method is based on ion-exchange chromatography coupled to visible or fluorescence detection (IEC-VIS/FLD). It does not distinguish between the salts of amino acids and cannot differentiate between enantiomers. The Applicant presented results from validation and verification studies demonstrating the extension of the scope of the above mentioned ISO method for the determination of L-isoleucine in the feed additive and premixtures. The following performance characteristics are reported: a relative standard deviation for repeatability (RSDr) ranging from 0.3 to 2.9%, a relative standard deviation for intermediate precision (RSDip) ranging from 0.6 to 3.2% and a recovery rate from 93 to 108%.

For the quantification of L-isoleucine in feedingstuffs the Applicant submitted the ring-trial validated European Union method (Commission Regulation (EC) No 152/2009) based on IEC coupled to photometric detection (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: a RSDr ranging from 2.0 to 5.4% and a relative standard deviation for reproducibility (RSDR) ranging from 6.8 to 14.3%. Furthermore, limits of quantification between 30 and 350 mg/kg feedingstuffs are specified for various amino acids. Nevertheless, when L-isoleucine is used as sensory feed additive, the Applicant recommended its maximum content in feedingstuffs of 25 mg/kg. Therefore, the EURL is unable to recommend the European Union method for the official control of L-isoleucine in feedingstuffs when it is used as sensory additive.

For the quantification of isoleucine in water the Applicant submitted the ring-trial validated EN ISO 13903:2005 method (equivalent to the European Union one). In the frame of the stability studies the Applicant presented experimental data obtained when analysing the feed additive in water according to this method, thus demonstrating its applicability for the determination of isoleucine in 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; (ii) the ring-trial validated method EN ISO 17180:2013 based on ion-exchange chromatography coupled to visible or fluorescence detection (IEC-VIS/FLD) to quantify free isoleucine in the feed additive and premixtures; and (iii) the ring-trial validated European Union method based on IEC-VIS for the quantification of isoleucine in premixtures, feedingstuffs and water (only when L-isoleucine is used as nutritional feed additive).

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.