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Safety and efficacy of L-leucine produced by fermentation with *Escherichia coli* NITE BP-02351 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-leucine produced by fermentation with *Escherichia coli* NITE BP-02351 when used as nutritional additive or as feed flavouring compound in feed and water for drinking for all animal species. The product under assessment is L-leucine produced by fermentation with a genetically modified strain of *E. coli* (NITE BP-02351). The production strain and its recombinant DNA were not detected in the final products. L-Leucine, manufactured by fermentation with *E. coli* NITE BP-02351, does not give rise to any safety concern to the production strain. The use of L-leucine produced with *E. coli* NITE BP-02351 is safe for the target species when used to supplement the diet in appropriate amounts. It is safe at the proposed use level of 25 mg/kg when used as flavouring compound for all animal species. The use of L-leucine produced by fermentation with *E. coli* NITE BP-02351 in animal nutrition raises no safety concerns for consumers of animal products. The additive is not irritating to the skin or eyes and is not a skin sensitiser. There is a risk for persons handling the additive from the exposure to endotoxins by inhalation. The use of L-leucine produced by *E. coli* NITE BP-02351 as feed additive does not represent a risk to the environment. The additive L-leucine produced by *E. coli* NITE BP-02351 is regarded as an effective source of the amino acid L-leucine when used as nutritional additive. For the supplemental L-leucine to be as efficacious in ruminants as in non-ruminant species, it requires protection against degradation in the rumen. It is also considered efficacious as feed flavouring compound under the proposed conditions of use.

Keywords: leucine, *Escherichia coli* NITE BP 02351, amino acid, nutritional additive, flavouring, sensory additive

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1. Introduction

1.1. Background and Terms of Reference

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

The European Commission received a request from Ajinomoto[2] for authorisation of the product L-leucine, when used as a feed additive for all animal species (category: nutritional additives; functional group: amino acids, their salts and analogues; and category: sensory additives; functional group: flavourings).

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 20 August 2018.

According to Article 8 of Regulation (EC) No 1831/2003, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment and on the efficacy of the product L-leucine produced by fermentation with *Escherichia coli* NITE BP-02351, when used under the proposed conditions of use (see Section 3.1.4).

1.2. Additional information

L-Leucine has not been assessed as feed nutritional additive. The EFSA Panel on Additives and Products or Substances used in Animal Feed published an opinion 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-leucine (EFSA FEEDAP Panel, 2014). The Panel on Dietetic Products, Nutrition and Allergies (NDA) of EFSA issued a scientific opinion on the substantiation of health claims related to several amino acids including L-leucine (EFSA NDA Panel, 2014).

L-Leucine is currently not authorised as nutritional additive in feed. L-Leucine produced by chemical synthesis or protein hydrolysis is currently authorised as a sensory additive (functional group flavouring compounds) for use in all animal species with a maximum content of 25 mg/kg complete feedingstuff.[3] This authorisation does not include L-leucine produced by fermentation.

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

L-Leucine is described in a monograph of the European Pharmacopoeia (2017), monograph 01/2017:0771.

[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 Eurolysine S.A.S., Rue de Courcelles, 153, 75817, Paris Cedex 17, France.

[3] 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, -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, 23.0.2008, p. 134.

[4] Regulation (EU) No 609/2013 of the European Parliament and of the Council of 12 June 2013 on food intended for infants and young children, food for special medical purposes and total diet replacement for weight control and repealing Council Directive 92/52/EEC, Commission Directives 96/8/EC, 1999/21/EC, 2006/125/EC and 2006/141/EC, Directive 2009/39/EC of the European Parliament and of the Council and Commission Regulations (EC) No 41/2009 and (EC) No 953/2009, OJ L 181, 29.6.2013, p.35.

[5] Commission Decision of 9 February 2006 amending Decision 96/335/EC establishing an inventory and a common nomenclature of ingredients employed in cosmetic products. OJ L 97, 5.4.2006, pp. 1–528.

[6] Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. OJ L 15, 20.1.2010, p. 1.

[7] Regulation (EC) No 470/2009 of the European Parliament and of the Council of 6 May 2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin, repealing Council Regulation (EEC) No 2377/90 and amending Directive 2001/82/EC of the European Parliament and of the Council and Regulation (EC) No 726/2004 of the European Parliament and of the Council. OL L 152, 16.6.2009, p. 11.

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier[8] in support of the authorisation request for the use of L-leucine produced by fermentation with *E. coli* NITE BP-02351 as a feed additive.

The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) Panel used the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA, peer-reviewed scientific papers, other scientific reports and experts' knowledge, to deliver the present output.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of L-leucine produced by fermentation with *E. coli* NITE BP-02351 in animal feed. The Executive Summary of the EURL report can be found in Annex A.[9]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of L-leucine produced by fermentation with *E. coli* NITE BP-02351 is in line with the principles laid down in Regulation (EC) No 429/2008[10] and the relevant guidance documents: Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2018), Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2017a), 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 on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012a), Guidance for assessing the safety of feed additives for the environment (EFSA, 2008b), Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018).

3. Assessment

The product subject of this application is L-leucine (≥ 98% on a dry matter (DM) basis) produced by fermentation with a genetically modified strain of *E. coli*. It is intended to be used as nutritional additive and as sensory additive to feed and water for drinking in all animal species and categories. L-Leucine produced by fermentation with *E. coli* NITE BP-02351 is not currently authorised for use as a nutritional additive or as sensory additive in the European Union.

3.1. Characterisation

3.1.1. Characterisation of the production organism

The additive is produced by a genetically modified strain of *Escherichia coli* K-12 [redacted], which is deposited in the National Institute of Technology and Evaluation (NITE) of Japan with accession number NITE BP-02351.[11] The strain was identified as *E. coli* K-12 by molecular serotyping, and multi-locus sequence typing (MLST) using data obtained by whole genome sequencing (WGS).[12]

The production strain *E. coli* NITE BP-02351 was tested for antibiotic susceptibility using culture broth suspension microdilution. The battery of antibiotics tested was that recommended by EFSA for *E. coli* (EFSA FEEDAP Panel, 2012b) and *Enterobacteriaceae* (EFSA FEEDAP Panel, 2018).[12] All minimum inhibitory concentration values were below the corresponding cut-off values defined by the FEEDAP Panel. In addition, no complete antibiotic resistance genes were found by searching the WGS against relevant databases. WGS analysis also indicated the absence of known *E. coli* virulence factors, including genes encoding enterotoxins, shiga toxins, adhesion and invasion factors.[12]

[8] FEED dossier reference: FAD-2018-0041.
[9] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2018-0041_l-leucine.pdf
[10] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.
[11] Technical dossier/Section II/Annex Supp II.1.
[12] Technical dossier/Section II/Annex Supp II.6.

3.1.1.1. Information relating to the genetically modified microorganism

Characteristics of the recipient or parental microorganism

The recipient strain is [redacted] *E. coli* K-12 is well characterised and its safety (non-pathogenicity) has been documented extensively (Gorbach, 1978) and its genome is fully sequenced (Hayashi et al., 2006).

Characteristics of the donor organism

[redacted]

Description of the genetic modification process

[redacted]

[redacted]

3.1.2. Manufacturing process

L-Leucine is obtained by fed-batch fermentation with *E. coli* NITE BP-02351. After fermentation, the fermentation broth is inactivated [redacted][15]

The applicant declared that no antimicrobials are used in the manufacturing of the additive under assessment.[16]

3.1.3. Characterisation of the active substance/additive

L-Leucine (International Union of Pure and Applied Chemistry (IUPAC) name: 2-amino-4-methylpentanoic acid; synonyms: 2-amino-4-methylvaleric acid, alpha-aminoisocaproic acid; (S)-2-amino-4-methylpentanoic acid) has the Chemical Abstracts Service (CAS) No 61-90-5, European Inventory of Existing Commercial Chemical Substances (EINECS) No 200-522-0 and the EU Flavour Information System (FLAVIS) number [17.012]. It has a molecular weight is 131.18 g/mol and a nitrogen content of 10.7%. The chemical formula is $C_6H_{13}NO_2$, and the structural formula is given in Figure 1.

According to the specification, the product contains ≥ 98% L-leucine on a DM basis. The analysis of five batches from industrial pilot production of L-leucine[17] showed an average content of leucine of

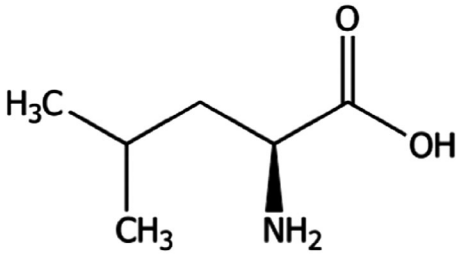

Figure 1: Structural formula of L-leucine

98.2% on 'as is' basis (range 97.3–98.7%), moisture ranged 0.8–1.8%, substances other than leucine ranged 1.3–2.7%. On a DM basis, the content of leucine was on average 99.2% (range 99.0–99.5%).[18]

In three out of the five batches, the free amino acids [redacted] were quantified. The sum of quantifiable free amino acids amounted to 0.4–0.7% (excluding leucine) and to 97.9–99.1% (including leucine) on 'as is' basis. The sum of quantifiable nitrogen compounds (ammonium, nitrites, nitrates and betaine) in three batches was ≤ 0.05% and oxalic acid (the only organic acid found) was < 0.02%.

[13] Technical dossier//Section II/Annex_Supp_II_38.
[14] Technical dossier/Section II/Annex_Supp_II_6.
[15] Technical dossier/Section 2.3.
[16] Technical dossier/Section II/2.3.1.2. Manufacturing process, p.79.
[17] Technical dossier/Section II/Annex II_10. ISO17180:2013 method for lysine, methionine and threonine expanded to leucine for in-house and for official controls.
[18] Technical dossier/Section II/Annex_II_4.

Sugars were not detected. The sum of inorganic anions and cations (chloride, sulfate, phosphates, calcium, magnesium and sodium) in three samples ranged 0.5–0.9%, and crude ash 0.2–0.3%.[19] The amount of identified material is, on average, 100.7% (range 100.5–100.8%) on DM basis.

The specific optical rotation of five batches of the final product was on average 15.2° (range 15.1–15.3°),[18] which is within the range described in the European Pharmacopoeia (+14.5 to +16.5°) for this amino acid and confirms the identity of the L-enantiomer.[20]

3.1.3.1. Impurities

Three batches of the final product were analysed for heavy metals (lead, cadmium and mercury) and arsenic. All analytical values were below the respective limit of detection (LOD).[21] Dioxins (polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs)) and dioxin-like polychlorinated biphenyls (PCBs) were analysed in three batches of the final product and were found below the LODs.[22] Mycotoxins were analysed in three batches of the final product. Aflatoxins (B1, B2, G1 and G2), ochratoxin A, zearalenone, deoxinivalenol, T2-toxin and HT2-toxin and fumonisins (B1, B2 and B3) had concentrations below the corresponding LODs.[23]

A multiresidue analysis of organochlorine and organophosphorus pesticides showed all pesticides were below the corresponding LODs.[24]

Among biogenic amines, cadaverine [redacted], spermine [redacted], putrescine [redacted] and spermidine [redacted] were detectable in three out of five batches. Other analysed impurities were melamine [redacted], hydrocyanic acid [redacted], nitrates [redacted] and nitrites [redacted].

The concentrations of the undesirable substances described above do not raise safety concerns.

Analysis of microbial contamination of the final product (five batches) indicated that *Salmonella* spp. was absent in 25 g samples, *Staphylococcus* was $< 10^2$ colony-forming unit (CFU)/g, coliforms and *Enterobacteriaceae* < 10 CFU/g, total bacterial count, $< 10^2$ CFU/g, yeasts and moulds $< 10^1$ CFU/g.[25]

The final product (two batches) was tested for the presence of antimicrobial substances using the methods described in the EFSA Guidance on Microbial studies (EFSA, 2008a,b) on *Escherichia coli, Staphylococcus aureus, Enterococcus faecalis, Bacillus subtilis and Pseudomonas aeruginosa* at concentrations below 9.1% (range 0.3–9.1%) after 24 and 48 h incubation.[26] No inhibition was detected at 24 or at 48 h of incubation.

The endotoxin activity was measured in five batches (*Limulus* amoebocyte lysate assay).[27] Four batches showed comparable values (range 18–78 EU/g), whereas a fifth batch contained a higher amount of endotoxins (4,362 EU/g).

No viable cells of the production strain were found in three batches (each tested in triplicate).[28] Test were done by (i) filtering 100 mL of a 10 g/L solution of the product and incubating the filter on non-restrictive medium at 37°C for 2 days, and (ii) incubation of 10 g of product in 1 L non-restrictive, liquid medium at 37°C for 3 days followed by plating on restrictive medium and incubation at 37°C for 2 days.

No recombinant DNA was detected in three batches of L-leucine (1 mg of product analysed in triplicate) [redacted]

3.1.3.2. Physical characteristics

The product under assessment is a white crystalline powder. The pH in 1% aqueous solution at 20°C ranges from 5.5 to 6.5.[29] The bulk density ranges from 644 to 652 kg/m^3. Its water solubility is

[19] Technical dossier/Section II/Annex II.4.
[20] European Pharmacopoeia monograph 1/2017:0771.
[21] Technical dossier/Section II/Annex II_5. LOD in μg/kg were 5 for mercury, 10 for cadmium, 50 for lead and 100 for arsenic.
[22] Technical dossier/Section II/Annex II.5. LOD (in ng/kg) was 0.0223–0.0445 for PCDD/F and 0.278–5.56 for PCBs
[23] Technical dossier/Section II/Annex II.5. LOD (in μg/kg) was 0.1 for aflatoxins B1, B2 and G1;0.2 for aflatoxin G2 and ochratoxin A; 20 for zearalenone, fumonisins (B1, B2 and B3), T2-toxin and HT2-toxin; 50 for deoxynivalenol.
[24] Technical dossier/Section II/Annex II.5. LOD (in μg/kg) was 2-30 depending on the pesticide considered.
[25] Technical dossier/Section II/Annex II.8.
[26] Technical dossier/Supplementary information December 2018/Supp Info Annex 4.
[27] Technical dossier/Section II/Annex II.33 and supplementary information December 2018/Supp Info Annnex 3.
[28] Technical dossier/Section II/Annex Supp II.41.
[29] Technical dossier/Section II/Annex_II_30.

24.3 g/L at 25°C.[30] The pKa values is 2.32 (carboxyl function) and 9.58 (amino group)[31] and the octanol/water partition coefficient (Log Kow) is −1.8.[32]

The dusting potential of the additive (three batches analysed by Stauber–Heubach method) showed values ranging from 2.90 to 8.33 g/m^3.[33] The particle size of the collected dust (when dusting potential was measured) showed an average particle size (v/v) of 21 μm, with 10% of particles < 9.1 μm, 50% < 19.2 μm, 90% < 35.5 μm and 100% < 55.6 μm.[34]

Particle size distribution of the additive was analysed by laser diffraction in five batches and 10% (v/v) of the particles showed a particle size (v/v) < 33 μm diameter; 50% of the particles a particle size < 74 μm diameter; and 90% of the particles a particle size < 259 μm diameter.[35]

3.1.3.3. Stability and homogeneity

The shelf-life of three batches of the additive was tested at 25°C and at 40°C when packed in sealed nylon-polyethylene (PE) bags for 9 months.[36] No losses were observed.

The stability of three batches of the additive was tested in three different vitamin/mineral premixtures for piglets, sows and chickens for fattening (containing 0.8%, 1.6% and 2% choline chloride, respectively) at 25°C and at 40°C when packed in sealed nylon-PE bags for 6 months.[37] The supplementation rate was 3% in piglet and sow premixtures and 5% in chicken for fattening premixture. No losses were seen in sow premixtures. In piglet premix, no losses were seen at 25°C and losses up to 2% were seen at 40°C. In chicken for fattening premix, losses were of 4% and 5% at 25 and 40°C, respectively.

The stability of three batches of the additive was tested in pelleted complete feed for piglets, sows and chickens for fattening when stored at 25°C and 40°C in sealed nylon-PE bags for 3 months. The basal diet of the piglet feed consisted on wheat, soybean meal, barley and maize; that of the sow on barley, wheat and maize; and the chicken feed on wheat, maize and sunflower meal. The supplementation rate was 0.15% in feed for piglets and 0.25% in feed for sows and chickens for fattening. After mixing, the complete feed for piglets, sows and chickens for fattening was preconditioned at 62, 59 and 65°C and pelleted at 89, 76 and 73°C, respectively. Pelleting caused a loss of 1.4, 3.8 and 0.8%, respectively. After the 3-month period, losses observed after storage at 25°C were 0.9, 4.7 and 10.8%, respectively, whereas losses after storage at 40°C were 2.2, 11.2 and 7.8%, respectively.[38]

The capacity of the additive to distribute homogeneously in the premixtures described above was studied by analysing 10 subsamples. The coefficient of variation (CV) was 1.2% for the chicken premixture, 3% for the sow premixture and 3.3% for the piglet premixture.[37]

The capacity of the additive to distribute homogeneously in a pelleted complete feed for piglet, sows and chicken for fattening was studied analysing 10 subsamples (nine in the chicken for fattening feed). The CV was 4.4, 4.1 and 3.7%, respectively.[39]

The stability of the additive in water for drinking (three batches) was measured at three concentration levels (0.5, 2.5 and 5 g/L, one batch was used for each concentration) when stored at 25°C for 50 h. Losses ranged from 0 to 1.1%, depending on the concentration tested.[40]

3.1.3.4. Physico-chemical incompatibilities

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

3.1.4. Conditions of use

As nutritional additive, L-leucine is proposed to be used in feeds or in water for drinking in order to achieve the adequate amino acid profile and meet the L-leucine requirements for all animal species. It can be added directly to the feedingstuffs/complementary feedingstuffs or via premixture and via water for drinking. No inclusion levels for use in feed are proposed, as the requirements in quantitative terms depend

[30] Technical dossier/Section II/Annex II_12.
[31] Technical dossier/Section II/Annex II_31.
[32] Technical dossier/Section II/Annex_II_32.
[33] Technical dossier/Section II/Annex II_.3.
[34] Technical dossier/Section II/Annex II.6.
[35] Technical dossier/Section II/Annex II_6.
[36] Technical dossier/Section II/Annex II_52.
[37] Technical dossier/Section II/Annex II_54.
[38] Technical dossier/Section II/Annex II_56.
[39] Technical dossier/Section II/Annex II_56, Appendix C.
[40] Technical dossier/Section II/Annex II_59.

on the species, the physiological state of the animal, the performance level, the environmental conditions and the amino acid composition of the unsupplemented diet.[41] The applicant states that supplementation of L-leucine via water for drinking is at levels ranging from 0.5 to 5 g/L of water for drinking.[42]

When used as feed flavouring, L-leucine is proposed to be used at a maximum recommended level of inclusion of 25 mg/kg feed.

3.2. Safety

3.2.1. Safety of the genetic modification

The recipient strain [redacted] is considered to be safe. [redacted] None of the introduced modifications raise a safety concern. [redacted] The production strain and its DNA were not detected in the final additive.

3.2.2. Safety for the target species

Tolerance studies with essential amino acids, such as L-leucine, cannot be designed in accordance with the protocols of conventional toxicity experiments because high dietary concentrations of a certain amino acid will result in amino acid imbalances and depression of feed intake and, hence, impaired performance. Nevertheless, in the case of nutritional additives produced by fermentation, the risks associated with the residues of the fermentation process in the final product need to be assessed. In this specific product, the active substance represents > 99% of the additive on a DM basis. The level of endotoxin activity in the product (up to 4,362 IU/g) is similar to that observed in other feedingstuffs (Cort et al., 1990) and is therefore of no concern for the target species. No safety concerns were identified as regards the genetic modification of the production strain. The data submitted allow to exclude the presence of recombinant DNA in the final product.

Depending on the animal species and the genetics, sex and physiological state of the animal, the requirements for L-leucine in feed range in pigs from 0.50 to 1.50% (NRC, 1998); in poultry (NRC, 1994), 0.60–1.10% (chickens for fattening) and 0.5–1.9% (turkeys for fattening); and in fish (NRC, 2011), 1.50–1.90%. Common cereal feed materials contain 0.7–1.0% L-leucine, cereal based by-products 3.0 (distilled dried grains with solubles (DDGS))–12.0% (maize gluten meal), oilseed by-products 2.0–4.0% and animal-derived feed materials 5.0–12.0% (feather meal, fish meal, blood meal, spray-dried blood cells), while compound feeds for pigs and poultry normally contain between 1.5 and 2.5% background L-leucine (NRC, 1998; CVB, 2011). Therefore, typical compound feeds provide already the requirements of L-leucine. Under EU conditions, with a more diverse spectrum of available feed materials than in the USA, L-leucine seems to be the seventh limiting amino acid after lysine, threonine, sulfur (S)-amino acids, tryptophan, valine and isoleucine. According to the applicant, typical supplementation levels of L-leucine to compound feeds, particularly those with lower crude protein, are in the range 0.01–0.2%.[43]

The strong antagonism between the branched chain amino acids (BCAAs as L-valine, L-isoleucine and L-leucine) 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 in animals has already been discussed in EFSA FEEDAP Panel (2013). In general, the BCAAs are well tolerated when provided in great excess (Baker, 2005). Dietary overdoses of L-valine and L-isoleucine are better tolerated by almost all animal species than an excess of L-leucine; this could be ascribed to their metabolic fate (L-valine glucogenic, L-isoleucine both glucogenic and ketogenic, L-leucine only ketogenic).

Edmonds and Baker (1987a,b) reported no negative effects in growth performance for weanling pigs and chickens for fattening when adding 4% of L-leucine to a basal diet that contained already 1.74% L-leucine as background, but a detrimental growth effect was reported when L-leucine was added at 6% to the same basal diet. Waldroup et al. (2002) found no negative effects in chickens for fattening feeding up to 3.3% total L-leucine, but not 3.7% in diets, if requirements for L-isoleucine and L-valine are met. In a trial where piglets were fed a low-protein diets (16.9% crude protein) supplemented with 0, 0.27 or 0.55% L-leucine (total leucine contents in the diets being 1.34, 1.61 or 1.88%, respectively), Yin et al. (2010) reported no adverse effects with the highest L-leucine levels.

[41] Technical dossier/Section II.2.5.1.
[42] Technical dossier/Section IV.4.5.
[43] Technical dossier/Section IV/Subsection 3.5.

Growth depressing effects of L-leucine by using pig diets with high background levels of L-leucine at or higher than 4–5% L-leucine (spray dried blood cells; maize gluten meal) have indeed been described; however to counteract the BCAAs antagonism, sufficient levels of L-valine and L-isoleucine should be available in such diets (Kerr et al., 2004; Fu et al., 2006; Hinson et al., 2007; Fruge et al., 2009). Baker (2005) in his review on tolerance for L-leucine in animals and humans, also concluded that a rather large dietary excess of L-leucine, above requirement, is well tolerated when present in diets providing adequate levels of protein and the other two BCAAs (L-isoleucine, L-valine).

In a first experiment, Choo et al. (1991) fed diets containing 1.1, 1.5, 2.2, 2.7, 3.5, 4.5, 6.0 and 6.5% L-leucine in wheat germ meal-crystalline amino acid diets to rainbow trout. Diets containing up to 6.5% L-leucine did not inhibit weight gain or feed intake. In a second experiment, fish were fed similar diets containing 3.3, 6.2, 9.2 and 13.4% leucine. After 10–11 week of feeding, gross lesions including scoliosis, deformed opercula, scale deformities, scale loss, spongiosis of epidermal cells and scale regeneration were observed in 20% of the fish fed diets containing 13.4% L-leucine. No such effects were observed at diets containing 9.2% leucine.

Based on the above, it can be concluded that L-leucine content up to 3% in the diet will not have negative effects on performance of pigs, poultry and fish, provided that the requirements for L-isoleucine and L-valine are met or the concept of the 'ideal protein' (Emmert and Baker, 1997; Van Milgen and Dourmad, 2015; Miles and Chapman, 2007) was applied in the feed formulation. Furthermore, the usual supplementation levels are very small compared to the background L-leucine content already present in feed materials and compound feeds.

The initial product of L-leucine degradation by ruminal microorganisms is isovaleric acid (Blackburn et al., 1965). Isovaleric acid may be beneficial to ruminal fibre breakdown, because of the growth requirement that cellulolytic ruminal bacteria have for straight- and branched-chain C-4 and C-5 fatty acids (Dehority et al., 1967). As isovaleric acid has no recorded deleterious effects to the host animal, there are no safety concerns arising from ruminal L-leucine metabolism.

Therefore, the FEEDAP Panel concludes that L-leucine produced by *E. coli* NITE BP-02351 is safe for the target species when used as nutritional additive to supplement the diet in appropriate amounts.

Since the levels proposed for the use of L-leucine as flavouring compound (25 mg/kg complete feed) are substantially lower than the animal requirements, the FEEDAP Panel considers L-leucine produced with *E. coli* NITE BP-02351 safe for the target species when used as a flavouring compound.

The FEEDAP Panel, in its previous statement (EFSA FEEDAP Panel, 2010), identified risks of nutritional imbalances and hygienic concerns for amino acids when administered in water for drinking.

3.2.2.1. Conclusions on safety for the target species

The use of L-leucine produced with *E. coli* NITE BP-02351 is safe for the target species when used as nutritional additive to supplement the diet in appropriate amounts. The proposed use level as sensory additive (25 mg/kg feed) is safe for all animal species.

3.2.3. Safety for the consumer

The applicant reviewed the absorption and metabolic fate of L-leucine and other branched chain amino acids (isoleucine and valine).[44] Overall, with the only possible exception of extreme, unphysiological overdosing, ingested leucine enters the physiological metabolic pools and it does not give rise to any deposition in animal tissues or products. Like other amino acids supplemented to feed, leucine will be incorporated into proteins of tissues and/or products of animal origin and any of their potential excess will be metabolised and excreted. Therefore, the composition of tissues and products of animal origin will not be affected by the use of L-leucine in animal nutrition.

The product under assessment is produced by fermentation. The concerns for the consumer would not derive from the amino acid itself, which will be incorporated into the proteins of the animal tissues/products, but from the possible residues from the fermentation process. Considering that the product originating from *E. coli* NITE BP-02351 is highly purified (> 99% L-leucine and < 1% unidentified material on a DM basis), no concerns for the consumers would arise from the use of this additive in animal nutrition.

3.2.3.1. Conclusions on safety for the consumer

The use of L-leucine produced by fermentation with *E. coli* NITE BP-02351 in animal nutrition presents no concern to consumers of animal products.

[44] Technical dossier/Section III/Annex III.1.

3.2.4. Safety for the user

The applicant provided an acute inhalation toxicity test, an eye irritation test, a skin irritation test and a dermal sensitisation test all performed with L-leucine produced by the strain under assessment (*E. coli* NITE BP-02351).

3.2.4.1. Effects on the respiratory system

Dusting potential (measured in three batches) was up to 8.33 g/m^3 and the dust contains about 10% of particles having a diameter < 10 μm diameter. Therefore, workers may be exposed by inhalation.

In an acute inhalation toxicity study in accordance with the Organisation for Economic Co-operation and Development (OECD) Guideline 403,[45] a group of 10 Crl:WI(Han) strain rats (5 males and 5 females) were exposed to a concentration of 5.2 mg L-leucine/l air for 4 h (nose only exposure system). The signs observed (decreased respiratory rate, shallow breathing and restlessness) disappeared within an hour after exposure. No mortality occurred and no macroscopic lesions were observed at the necropsy. The lethal concentration that would kill 50% of the rat population (LC_{50}) for acute inhalation toxicity after 4 h exposure is considered to be > 5.2 mg/L (g/m^3).

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, 2012b), 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 upon calculations of the content of endotoxins in dust, exposure by inhalation would be 20,182 IU per 8-h working day, indicating a risk of exposure to endotoxins for people handling the additive (see Appendix A).

3.2.4.2. Effects on skin and eyes

In an *in vitro* isolated chicken eye (ICE) assay in accordance with OECD Guideline 438, 30 mg of L-leucine (98.7% purity) was applied to adult chicken corneas for 10 sec.[46] Negative (physiological saline, 30 μL) and positive control (sodium hydroxide, 30 mg) items were tested concurrently. The three endpoints measured, corneal swelling, opacity and fluorescein retention were combined in an empirically derived formula to obtain an *in vitro* irritation index. As the index for the test item was 0, no classification is required. The controls performed as expected.

In an *in vitro* skin irritation study using reconstructed human epidermis model (EpiDerm™) in accordance with OECD Guideline 439, 25 mg of L-leucine were applied (triplicate tissues) topically on the epidermal surface for 35 min, rinsed and followed by a post-exposure incubation period of 42 h.[47] Potential cytotoxicity of the test item was measured by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay and compared with negative (30 μL of phosphate-buffered saline solution) or positive (30 μL of sodium dodecyl sulfate 5% w/v) controls. The relative mean viability of the test item treated tissues was 108% after 42 h post-exposure incubation period. The controls performed as expected. The test item was considered not irritant for the skin.

In a *in vivo* skin sensitisation study (local lymph node assay in mouse) in accordance with OECD Guideline 429, L-leucine (98.35% purity) caused no signs of toxicity, visual local skin irritation or irritation indicated by an ≥ 25% increase in mean ear thickness.[48] Consequently, the additive was classified as non skin sensitiser.

3.2.4.3. Conclusions on safety for the user

The product is not irritating to the skin or eyes and is not a skin sensitiser. There is a risk for persons handling the additive from the exposure to endotoxins by inhalation.

3.2.5. Safety for the environment

Regarding the production strain, none of the introduced modifications raise a safety concern. There are no full-length antimicrobial resistance genes in the final production strain remaining from the

[45] Technical dossier/Section III/Annex_III_42.
[46] Technical dossier/Section III/Annex III.3.03.
[47] Technical dossier/Section III/Annex III_52.
[48] Technical dossier/Section III/Annex III.53.

genetic modification process. The production strain and its DNA were not detected in the final product. Consequently, no safety concerns for the environment arise regarding the production strain.

The amino acid L-leucine is a physiological and natural component of animals and plants. When consumed, it will be absorbed, and the non-absorbed fraction will be incorporated into the intestinal microbial mass and excreted as such. The absorbed L-leucine will be incorporated into body protein or excreted as urea/uric acid and as carbon dioxide. The use of amino acids in water for drinking, when given in addition to complete diets with a well-balanced amino acid profile, would disturb the nitrogen balance and increase nitrogen excretion via urine. The use of the product L-leucine in animal nutrition would not lead to any localised increase in the concentration in the environment. It is concluded that the use of the additive under assessment, produced by *E. coli* NITE BP-02351 as a feed additive does not represent a risk to the environment.

3.3. Efficacy

Efficacy studies are not required for amino acids naturally occurring in the proteins of plants and animals. The nutritional role of the amino acid L-leucine is well established in the scientific literature.

Free leucine is degraded by ruminal microorganisms (Chalupa, 1976; Broderick and Balthrop, 1979; O'Connor et al., 1993; Velle et al., 1997; Schwab et al., 2005). Accordingly, a great part of free L-leucine provided to ruminants would be expected not to reach the abomasum intact and be absorbed. Measures such as encapsulation are recommended by animal nutritionists to protect the amino acid from microbial degradation and thereby to ensure efficient uptake by the animal.

In monogastric, leucine supplementation is envisaged mainly when feeding regimens provide a relatively low content of crude proteins, e.g. when main feed materials are represented by cereals rather than by soy.

The additive L-leucine produced by *E. coli* NITE BP-02351 when used as nutritional additive is regarded as an effective source of the amino acid L-leucine.

As L-leucine is used in food as flavouring compound, it is expected that it can provide a similar function in feed and no further demonstration of efficacy is necessary when used at concentrations up to 25 mg/kg complete feed.

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[49] and Good Manufacturing Practice.

4. Conclusions

The production strain and its recombinant DNA were not detected in the final products. The product L-leucine, manufactured by fermentation with *Escherichia coli* NITE BP-02351, does not give rise to any safety concern to the production strain.

L-Leucine produced with *E. coli* NITE BP-02351 is safe for the target species when used to supplement the diet in appropriate amounts. L-Leucine produced with *E. coli* NITE BP-02351 is safe at the proposed use level of 25 mg/kg when used as flavouring compound for all animal species.

The use of L-leucine produced by fermentation with *E. coli* NITE BP-02351 in animal nutrition raises no safety concerns for consumers of animal products.

The product is not irritating to the skin or eyes and is not a skin sensitiser. There is a risk for persons handling the additive from the exposure to endotoxins by inhalation.

The use of L-leucine produced by *E. coli* NITE BP-02351 as feed additive does not represent a risk to the environment.

The additive L-leucine produced by *E. coli* NITE BP-02351 is regarded as an effective source of the amino acid L-leucine when used as nutritional additive. For the supplemental L-leucine to be as efficacious in ruminants as in non-ruminant species, it requires protection against degradation in the rumen. It is also considered efficacious as feed flavouring compound.

[49] 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.

Chronology

Date	Event
25/06/2018	Dossier received by EFSA. Feed grade L-leucine produced with *Escherichia coli* NITE BP-02351. Submitted by Ajinomoto Eurolysine S.A.S.
06/07/2018	Reception mandate from the European Commission
20/08/2018	Application validated by EFSA – Start of the scientific assessment
09/10/2018	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterisation of the additive, production process and characterisation of the genetic modification.*
20/11/2018	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
21/11/2018	Comments received from Member States
18/12/2018	Reception of supplementary information from the applicant - Scientific assessment re-started
02/04/2019	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

References

Baker DH, 2005. Tolerance for branched-chain amino acids in experimental animals and humans. Journal of Nutrition, 135, 1585–1590.

Blackburn TH, 1965. Nitrogen metabolism in the rumen. In: Physiology of Digestion in the Ruminant. Ed. By Dougherty et al., Butterworths, Washington, 1965. 322–334.

Broderick GA and Balthrop JE, 1979. Chemical inhibition of amino acid deamination by ruminal microbes in vitro. Journal of Animal Science, 49, 1101–1111.

Chalupa W, 1976. Degradation of amino acids by the mixed rumen microbial population. Journal of Animal Science, 43, 828–834.

Choo P-S, Smith TK, Cho CY and Ferguson HW, 1991. Dietary excesses of leucine influence growth and body composition of rainbow trout. The Journal of Nutrition, 121, 1932–1939. https://doi.org/10.1093/jn/121.12.1932

Cort N, Fredriksson G, Kindahl H, Edqvist LE and Rylander R, 1990. A clinical and endocrine study on the effect of orally administered bacterial endotoxin in adult pigs and goats. Journal of Veterinary Medicine, A-Physiology Pathology, Clinical Medicine., 37, 130–137.

CVB (Centraal Veevoederbureau), 2011. Livestock feed tables (Veevoedertabel); Productschap Diervoeding, Lelystad, The Netherlands.

Dehority BA, Scott HW and Kavaluk P, 1967. Volatile fatty acid requirements of cellulolytic rumen bacteria. Journal of Bacteriology, 94, 537–543.

Edmonds MS and Baker DH, 1987a. Amino acid excesses for young pigs: effects of excess methionine, tryptophan, threonine or leucine. Journal of Animal Science, 64, 1664–1671.

Edmonds MS and Baker DH, 1987b. Comparative effects of individual amino acid excesses when added to a corn-soybean meal diet: effects on growth and dietary choice in the chick. Journal of Animal Science, 65, 699–705.

EFSA (European Food Safety Authority), 2008a. Technical Guidance: Microbial Studies. EFSA Journal 2008;6(10):836, 3 pp. https://doi.org/10.2903/j.efsa.2008.836

EFSA (European Food Safety Authority), 2008b. Technical Guidance of the Scientific Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) for assessing the safety of feed additives for the environment. EFSA Journal 2008;6(10):842, 28 pp. https://doi.org/10.2903/j.efsa.2008.842

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2010. Scientific opinion on the use of feed additives authorised/applied for use in feed when supplied via water. EFSA Journal 2010;8(12):1956, 9 pp. https://doi.org/10.2903/j.efsa.2010.1956. Available online: http://www.efsa.europa.eu/efsajournal

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012a. Guidance on studies concerning the safety of use of the additive for users/workers. EFSA Journal 2012;10(1):2539, 5 pp. https://doi.org/10.2903/j.efsa.2012.2539

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012b. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA Journal 2012;10(6):2740, 10 pp. https://doi.org/10.2903/j.efsa.2012.2740

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2013. Scientific Opinion on the safety and efficacy of L-valine produced by Corynebacterium glutamicum (KCCM 80058) for all animal species, based on a dossier submitted by CJ Europe GmbH. EFSA Journal 2013;11(10):3429, 20 pp. https://doi.org/10.2903/j.efsa.2013.3429

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2014. Scientific Opinion on the safety and efficacy of the use of amino acids (chemical group 34) when used as flavourings for all animal species. EFSA Journal 2014;12(5):3670, 19 pp. https://doi.org/10.2903/j.efsa.2014.3670

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2015. Scientific Opinion on the safety and efficacy of L-lysine monohydrochloride produced by fermentation with *Escherichia coli* for all animal species based on a dossier submitted by HELM AG on behalf of Meihua Holdings Group Co. Ltd. EFSA Journal 2015;13(3):4052, 16 pp. https://doi.org/10.2903/j.efsa.2015.4052

EFSA FEEDAP Panel (EFSA Panel on additives and products or substances used in animal feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, Lopez-Alonso M, Lopez Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Anguita M, Galobart J and Innocenti ML, 2017a. Guidance on the identity, characterisation and conditions of use of feed additives. EFSA Journal 2017;15(10):5023, 12 pp. https://doi.org/10.2903/j.efsa.2017.5023

EFSA FEEDAP Panel (EFSA Panel on additives and products or substances used in animal feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, Lopez-Alonso M, Lopez Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Anguita M, Galobart J, Innocenti ML and Martino L, 2017b. Guidance on the assessment of the safety of feed additives for the target species. EFSA Journal 2017;15(10):5021, 19 pp. https://doi.org/10.2903/j.efsa.2017.5021

EFSA FEEDAP Panel (EFSA Panel on Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, Lopez-Alonso M, Lopez Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Anguita M, Dujardin B, Galobart J and Innocenti ML, 2017c. Guidance on the assessment of the safety of feed additives for the consumer. EFSA Journal 2017;15(10):5022, 17 pp. https://doi.org/10.2903/j.efsa.2017.5022

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, Lopez-Alonso M, Lopez Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Glandorf B, Herman L, Karenlampi S, Aguilera J, Anguita M, Brozzi R and Galobart J, 2018. Guidance on the characterisation of microorganisms used as feed additives or as production organisms. EFSA Journal 2018;16(3):5206, 24 pp. https://doi.org/10.2903/j.efsa.2018.5206

EFSA NDA Panel (Panel on Dietetic Products, Nutrition and Allergies), 2014. Scientific Opinion on the substantiation of a health claim related to a combination of L-threonine, L-valine, L-leucine, L-isoleucine, L-lysine plus chromium picolinate and reduction of post-prandial glycaemic responses pursuant to Article 13(5) of Regulation (EC) No 1924/2006. EFSA Journal 2014;12(7):3752, 8 pp. https://doi.org/10.2903/j.efsa.2014.3752

Emmert JL and Baker DH, 1997. Use of the ideal protein concept for precision formulation of amino acid levels in broiler Diets. Journal of Applied Poultry Science, 6, 462–470.

European Pharmacopoeia (PhEur), 2017. Leucine, monograph 01/2017:0771, 9th Edition. Strasbourg, France. Council of Europe (COE) – European Directorate for the Quality of Medicines.

Fruge ED, Bidner TD and Southern LL, 2009. Effect of incremental levels of red blood cells on growth performance and carcass traits of finishing pigs. Journal Animal Science, 87, 2853–2859.

Fu SX, Fent RW, Allee GL and Usry JL, 2006. Branched chain amino acid interactions increase isoleucine requirement in late-finishing pigs. Journal of Animal Science, 84, 283–284.

Gorbach SL, 1978. Risk assessment of recombinant DNA experimentation with *Escherichia coli* K12. Proceedings from a workshop at Falomuth. Massachusetts. Journal of Infectious Diseases, 137, 613–714.

[redacted]

Hayashi K, Morooka N, Yamamoto Y, Fujita K, Isono K, Choi S, Ohtsubo E, Baba T, Wanner BL, Mori H and Horiuchi T, 2006. Highly accurate genome sequences of *Escherichia coli* K-12 strains MG1655 and W3110. Molecular Systems Biology, 2, 2006.0007. https://doi.org/10.1038/msb4100049

Health Council of the Netherlands, 2010. Endotoxins. Health-based recommended occupational exposure limit. Publication no 2010/04OSH, 100 pp.

Hinson RB, Allee GL and Crenshaw JD, 2007. Use of spray-dried blood cells and isoleucine supplementation in pig starter diets. Journal Animal Science, 85, 93.

HSE (Health and Safety Executive), 2013. Occupational hygiene implications of processing waste at materials recycling facilities (MRFs). RR977 Research Report, HSE, London, UK. 41 pp.
Kerr BJ, Kidd MT, Cuaron JA, Bryant KL, Parr TM, Maxwell CV and Weaver E, 2004. Utilization of spray-dried blood cells and crystalline isoleucine in nursery pig diets. Journal of Animal Science, 82, 2397–2404.
Miles RD and Chapman FA, 2007. The concept of ideal protein in formulation of aquaculture feeds. The institute of food and agricultural sciences (IFAS) of the University of Florida. Document FA144.
[redacted]
NRC (National Research Council), 1994. Nutrient requirements of poultry. 9th, revised edition. The National Academies Press, Washington, DC. p. 176.
NRC (National Research Council), 1998. Nutrient requirements of swine. 10th, revised edition. National Academy Press, Washington, DC. p. 178.
NRC (National Research Council), 2011. Nutrient requirements of fish and shrimp. The National Academies Press, Washington, DC, USA. p. 376.
O'Connor JD, Sniffen CJ, Fox DG and Chalupa W, 1993. A net carbohydrate and protein system for evaluating cattle diets: IV. Predicting amino acid adequacy. Journal of Animal Science, 71, 1298–1311.
Rylander R, 1999. Health effects among workers in sewage treatment plants. Occupational and Environmental Medicine, 56, 354–357.
Schwab CG, Huhtanen P, Hunt CW and Hvelplund T, 2005.Nitrogen requirements of cattle. In: Pfeffer E and Hristov AN (eds.). Nitrogen and phosphorus nutrition of cattle. CABI International: Wallingford, Oxfordshire, UK. pp. 13–70.
Thorn J, 2001. The inflammatory response in humans after inhalation of bacterial endotoxin: a review. Inflammatory Response, 50, 254–261.
Van Milgen J and Dourmad JY, 2015. Concept and application of ideal protein for pigs. Journal of Animal science and Biotechnology., 6, 15.
Velle W, Sjaastad OV, Aulie A, Gronset D, Feigenwinter K and Framstad T, 1997. Rumen escape and apparent degradation of amino acids after individual intraruminal administration to cows. Journal of Dairy Science, 80, 3325–3332.
Waldroup PW, Kersey JH and Fritts CA, 2002. Influence of Branched-Chain Amino Acid Balance in Broiler Diets. International Journal of Poultry Science, 1, 136–144.
[redacted]
Yin Y, Yao K, Liu Z, Gong M, Ruan Z, Deng D, Tan B, Liu z and Wu G, 2010. Supplementing L-leucine to a low-protein diet increases tissue protein synthesis in weanling pigs. Amino Acids, 39, 1477–1486.

Abbreviations

CAS	Chemical Abstracts Service
CFU	colony-forming unit
CV	coefficient of variation
DM	dry matter
EINECS	European Inventory of Existing Commercial Chemical Substances (EINECS)
EURL	European Union Reference Laboratory
FCC	Food Chemical Codex
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
FLAVIS	Flavour Information System
ICE	isolated chicken eye
IEC-VIS/FLD	ion exchange chromatography coupled to visible or fluorescence detection
IUPAC	International Union of Pure and Applied Chemistry
LOD	limit of detection
MLST	multi-locus sequence typing
NDA	Dietetic Products, Nutrition and Allergies
NITE	National Institute of Technology and Evaluation
OECD	Organisation for Economic Co-operation and Development
PCDDs	polychlorinated dibenzodioxins
PCDFs	polychlorinated dibenzofurans
RSDr	relative standard deviation for repeatability
RSDip	relative standard deviation for intermediate precision
WGS	whole genome sequencing

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, 2012b) 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 a 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, 2012b).

Table A.1: Estimation of user exposure to endotoxins from the additive L-leucine (4,362 IU endotoxin activity/g) produced by *Escherichia coli* NITE BP-02351 including consideration of using filter half mask (FF P2 or FF P3)[50] as a preventive measure.

Calculation	Identifier	Description	Amount	Source
	a	Endotoxin content IU/g product	4,362	Technical dossier
	b	Dusting potential (g/m^3)	8.33	Technical dossier
a × *b*	*c*	Endotoxin content in the air (IU/m^3)	36,327	
	d	Number of premixture batches made/working day	40	EFSA FEEDAP Panel (2012b)
	e	Time of exposure (s)/production of one batch	20	EFSA FEEDAP Panel (2012b)
d × *e*	*f*	Total duration of daily exposure/ worker (s)	800	
	g	Uncertainty factor	2	EFSA FEEDAP Panel (2012b)
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)/8-h working day	10	EFSA FEEDAP Panel (2012b)
j/8 × *i*	*k*	Inhaled air during exposure (m^3)	0.56	
c × *k*	*l*	**Endotoxin inhaled (IU) during exposure/8-h working day**	**20,182**	
	m	Health-based recommended exposure limit of endotoxin (IU/m^3)/ 8-h working day	90	Health Council of the Netherlands (2010)
m × *j*	*n*	**Health-based recommended exposure limit of total endotoxin exposure (IU)/8-h working day**	**900**	

[50] 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.

Calculation	Identifier	Description	Amount	Source
//10		Endotoxins inhaled (IU)/8-h working day reduced by filter half mask FF P2 (reduction factor 10)	2,018	
//20		Endotoxins inhaled (IU)/8-h working day reduced by filter half mask FF P3 (reduction factor 20)	1,009	

Annex A – Executive summary of the evaluation report of the European Union Reference Laboratory on the analytical methods submitted for the L-leucine produced using strain NITE BP-02351

In the current application, authorisation is sought under Article 4(1) for L-leucine produced using the bacteria strain NITE BP-02351, under the category/functional groups 3(c) 'nutritional additives'/ 'amino acids, their salts and analogues' and 2(b) 'sensory additives/flavouring compounds' according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for all animal species.

According to the Applicant, L-leucine has a minimum purity (mass fraction) of 98%. As nutritional feed additive, L-leucine is intended to be added directly into feedingstuffs or through premixtures and water for drinking. As sensory feed additive, L-leucine is intended to be added into feedingstuffs and water for drinking through flavouring premixtures. However, the Applicant did not propose any minimum or maximum content of L-leucine in feedingstuffs.

For the quantification of leucine in the feed additive and premixtures, the Applicant submitted the ring-trial validated method EN ISO 17180:2013 specifically designed for 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 leucine in the feed additive and premixtures. The following performance characteristics were reported: a relative standard deviation for repeatability (RSDr) ranging from 0.7 to 2.7%, a relative standard deviation for intermediate precision (RSDip) ranging from 0.6 to 3.2% and a recovery rate from 98 to 105%. In addition, the EURL identified the 'L-leucine monograph' of the Food Chemical Codex (FCC) for the identification of L-leucine in the feed additive.

For the quantification of L-leucine in premixtures and feedingstuffs, the Applicant submitted the ring-trial validated Community method (Commission Regulation (EC) No 152/2009) based on IEC coupled with photometric detection (VIS). The method, designed only 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 by 23 laboratories, resulting in the EN ISO 13903:2005 method. The following performance characteristics were reported for the quantification of total leucine: RSDr ranging from 1.7 to 2.7% and RSDr ranging from 6.3 to 7.6%. In the frame of the stability studies, the Applicant presented experimental data obtained analysing the feed additive in water according to ISO 13903:2005 thus demonstrating its applicability for the determination of leucine in water.

In the frame of this authorisation, the EURL recommends for official control (i) the 'L-leucine monograph' of the FCC based on infrared absorption for the identification of L-leucine in the feed additive; (ii) the ring-trial validated method EN ISO 17180:2013 based on IEC-VIS/FLD to quantify free leucine in the feed additive and premixtures (containing more than 10% leucine); (iii) the ring-trial validated Community method based on IEC-VIS for the quantification of leucine in premixtures and feedingstuffs; and (iv) the ring-trial validated EN ISO 13903:2005 method based on IEC-VIS for the quantification of leucine in water.

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