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## Safety and efficacy of L-arginine produced by fermentation with *Escherichia coli* NITE BP-02186 for all animal species

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### Abstract

L-Arginine is considered as a non-essential amino acid for most adult mammalian species, but it is classified as essential for birds, fish, possibly reptiles and also for strict carnivores. L-Arginine produced by fermentation with *Escherichia coli* NITE BP-02186, genetically modified to enhance the production of L-arginine, is intended to be used in feed and water for drinking for all animal species and categories. The product under assessment does not give rise to any safety concern with regard to the genetic modification of the production strain. Its use as a nutritional additive is safe for target species when supplemented to diets in appropriate amounts. The use of L-arginine as a feed flavouring agent is unlikely to pose any concern. No risks are expected for the consumer from the use of the product under assessment as a feed additive. It is not irritant to skin or eyes, nor a skin sensitiser. Although the presence of endotoxin activity is of no concern, the available exposure and toxicological data indicate that the additive may pose a risk to users by inhalation. The use of this additive in animal nutrition does not pose a risk to the environment. The additive is an effective source of arginine for all species. L-Arginine is considered efficacious when used as a flavouring compound in animal nutrition.

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## Summary

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on L-arginine produced by fermentation with *Escherichia coli* NITE BP-02186 when used as nutritional additive and sensory additive for all animal species.

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of L-arginine was in line with the principles laid down in Regulation (EC) No 429/2008 and the relevant EFSA guidance documents. The FEEDAP Panel used the data provided by the applicant together with data from other sources, such as previous risk assessments by the European Food Safety Authority (EFSA) or other expert bodies, peer-reviewed scientific papers, other scientific reports and experts' knowledge, to deliver the present output.

L-Arginine is considered as a non-essential amino acid for most adult mammalian species, but it is classified as essential for birds, fish, possibly reptiles and also for strict carnivores.

Neither the production strain *E. coli* NITE BP-02186 nor its recombinant DNA was detected in the final product. The product L-arginine, manufactured by fermentation with *E. coli* NITE BP-02186, does not give rise to any safety concern with regard to the genetic modification of the production strain.

The use of L-arginine produced by *E. coli* NITE BP-02186 as a nutritional additive is safe for target species when supplemented to diets in appropriate amounts. There are no safety concerns arising from ruminal L-arginine metabolism. The use of L-arginine as a feed flavouring agent is unlikely to pose any concern.

No risks are expected for the consumer from the use of L-arginine produced by *E. coli* NITE BP-02186 as a feed additive.

The product under assessment is not irritant to skin or eyes, nor a skin sensitiser. Although the presence of endotoxin activity is of no concern, the available exposure and toxicological data indicate that the additive may pose a risk to users by inhalation.

The use of the product under assessment in animal nutrition does not pose a risk to the environment.

The additive is an effective source of the amino acid arginine for all species. For the supplemental L-arginine to be as efficacious in ruminants as in non-ruminant species, it requires protection against microbial degradation in the rumen. L-Arginine is considered efficacious when used as a flavouring compound in animal nutrition.

The FEEDAP Panel made a recommendation concerning the description of the additive.

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## 1. Introduction

### 1.1. Background and Terms of Reference

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

The European Commission received a request from Ajinomoto Eurolysine S.A.S.<sup>2</sup> for authorisation of the product L-arginine, feed grade when used as a feed additive for all animal species. The application is for use in feed and in water for drinking. Two different uses of the additive are proposed: (i) under category: nutritional additives; functional group: amino acids, their salts and analogues and (ii) under category: sensory additives; 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). The particulars and documents in support of the application were considered valid by EFSA as of 18 July 2017.

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-arginine, when used under the proposed conditions of use (see Section 3.1.7).

### 1.2. Additional information

The product L-arginine, feed grade, produced by the genetically modified strain of *Escherichia coli* NITE BP-02186 has not been previously authorised as a feed additive in the European Union (EU).

L-Arginine (98%) produced by *Corynebacterium glutamicum* strains ATCC 13870, KCTC 10423BP or KCCM 80099 is currently authorised as a nutritional feed additive for all animals without any restrictions by Commission Regulation (EC) No 1139/2007<sup>3</sup> and Commission Implementing Regulation (EU) 2016/972<sup>4</sup>, respectively.

The EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) issued three opinions on the safety and efficacy of the product containing L-arginine produced by fermentation using *C. glutamicum* (strains ATCC 13870, KCTC 10423BP or KCCM 80099, respectively) for all animal species (EFSA, 2007; EFSA FEEDAP Panel, 2016, 2017). The FEEDAP Panel issued one opinion on the safety and efficacy of the use of amino acids (chemical group 34) when used as flavourings for all animal species (EFSA FEEDAP Panel, 2014).

The EU Scientific Committee for Food (SCF) found acceptable the use of L-arginine as a food for particular nutritional purposes (European Commission, 1999). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) issued an opinion on the safety evaluation of certain food additives prepared by the sixty-third meeting of this committee (WHO, 2006) that included L-arginine.

The EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA Panel) delivered two opinions related to the substantiation of health claims related to L-arginine (EFSA NDA Panel, 2011a,b).

L-Arginine, like other amino acids and other nitrogen compounds, is authorised for food use according to Commission Regulation (EC) No 1243/2008 for infant formulae and follow-on formulae.<sup>5</sup> According to Commission Regulation (EC) No 953/2009 and Commission Directive 2001/15/EC, amino acids such as L-arginine may be added in all dietary foods for particular nutritional uses including foods

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

<sup>2</sup> Ajinomoto Eurolysine S.A.S., 153 rue de Courcelles, 75817 Paris Cedex 17, France.

<sup>3</sup> Commission Regulation (EC) No 1139/2007 of 1 October 2007 concerning the authorisation of L-arginine as a feed additive. OJ L 256, 11, 2.10.2007, p. 3.

<sup>4</sup> Commission Implementing regulation (EU) 2016/972 of 17 June 2016 concerning the authorisation of L-arginine produced by *Corynebacterium glutamicum* KCTC 10423BP as feed additive for all animal species. OJ L 161/18, 18.6.2016, p. 3.

<sup>5</sup> Commission Regulation (EC) No 1243/2008 of 12 December 2008 amending Annexes III and VI to Directive 2006/141/EC as regards compositional requirements for certain infant formulae. OJ L 335 25, 13.12.2008, p. 18.

for particular nutritional uses intended for special medical purposes.<sup>6</sup> L-Arginine and related compounds are also registered as an ingredient in cosmetic products (Commission Decision 2006/257/EEC).<sup>7</sup> L-Arginine is registered as pharmaceutical grade (for total parenteral nutrition) in many European countries and is described in a monograph of the European Pharmacopoeia (European Pharmacopoeia, 2014). According to Commission Regulation (EU) No 37/2010, L-arginine is also listed as pharmacologically active substance in veterinary medicinal products and is not subjected to maximum residue levels when used in food producing animals.<sup>8</sup>

A risk assessment has been made for L-arginine in food (Shao and Hathcock, 2008); the authors concluded that arginine at intakes up to 20 g/day was safe for normal healthy adults.

## 2. Data and methodologies

### 2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier<sup>9</sup> in support of the authorisation request for the use of L-arginine, feed grade, produced by *E. coli* NITE BP-02186 as a feed additive (nutritional and sensory) for feed and water for drinking. The technical dossier was prepared following the provisions of Article 7 of Regulation (EC) No 1831/2003, Regulation (EC) No 429/2008<sup>10</sup> and the applicable EFSA guidance documents.

The FEEDAP Panel used the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA or other expert bodies, 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-arginine in animal feed. The Executive Summary of the EURL report can be found in Annex A.<sup>11</sup>

### 2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of L-arginine, feed grade, is in line with the principles laid down in Regulation (EC) No 429/2008 and the relevant guidance documents: Guidance on nutritional additives (EFSA FEEDAP Panel, 2012a), Guidance for the preparation of dossiers for sensory additives (EFSA FEEDAP Panel, 2012b), Technical guidance: Tolerance and efficacy studies in target animals (EFSA FEEDAP Panel, 2011), Technical Guidance for assessing the safety of feed additives for the environment (EFSA, 2008a), Guidance for establishing the safety of additives for the consumer (EFSA FEEDAP Panel, 2012c), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012d), Technical Guidance: Microbial Studies (EFSA, 2008b), Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance (EFSA FEEDAP Panel, 2012e), Guidance for the preparation of dossiers for additives already authorised for use in food (EFSA FEEDAP Panel, 2012f) and Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use (EFSA GMO Panel, 2011).

## 3. Assessment

The product subject of this application is L-arginine ( $\geq 98\%$  on dry matter basis) produced by fermentation with a genetically modified strain of *E. coli* K-12. It is intended for use in feed and water for drinking for all animal species and categories, under the category 3c of Regulation EC 1831/2003

<sup>6</sup> Commission Directive 2001/15/EC of 15 February 2001 on substances that may be added for specific nutritional purposes in foods for particular nutritional uses. OJ L 52, 22.2.2001, pp. 19–25. Commission Regulation (EC) No 953/2009 of 13 October 2009 on substances that may be added for specific nutritional purposes in foods for particular nutritional uses. OJ L 269 9, 14.10.2009, 11 pp.

<sup>7</sup> Commission Decision of 9 February 2006 amending Decision 96/335/EC establishing an inventory and a common nomenclature of ingredients employed in cosmetic products. OJ L 97, 5.4.2006, pp. 1–528.

<sup>8</sup> Commission Regulation (EU) No 37/2010, of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. OJ L 15, 20.1.2010, pp. 72.

<sup>9</sup> FEED dossier reference: FAD-2017-0028.

<sup>10</sup> Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.

<sup>11</sup> The full report is available on the EURL website: [https://ec.europa.eu/jrc/sites/jrcsh/files/finrep\\_fad-2017-0028\\_arginine.pdf](https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2017-0028_arginine.pdf)

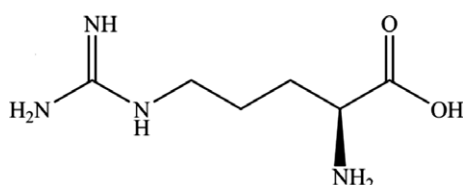
'amino acids, their salts and analogues'; and the category 2.b 'flavouring compounds'. Although authorisation is sought for these two classifications, the additive is strictly identical, with the same characterisation and manufacturing process. The present assessment will follow the structure of the nutritional additives' scientific opinions and will incorporate, when needed, the corresponding sections for sensory additives (flavouring compounds).

L-Arginine is considered as a non-essential amino acid for most adult mammalian species including humans, but it is classified as essential for birds, fish, possibly reptiles and also for strict carnivores. For mammalian neonates, it is also considered to be essential.

### 3.1. Characterisation

#### 3.1.1. Characterisation of the active substance

L-Arginine (International Union of Pure and Applied Chemistry (IUPAC) name: (S)-2-amino-5-guanidinopentanoic acid; synonym 2-amino-5-guanidinovaleric acid), is a compound identified with the Chemical Abstracts Service (CAS) No 74-79-3, and the European Inventory of Existing Commercial chemical Substances (EINECS) No 200-811-1). It has a molecular mass of 174.2 Da. The molecular formula of L-arginine is  $C_6H_{14}N_4O_2$ . The structural formula is given in Figure 1.



**Figure 1:** Molecular structure of L-arginine

#### 3.1.2. Characterisation of the production organism

The additive is produced by a genetically modified strain of *E. coli* K-12 (AJ111230), which is deposited in the National Institute of Technology and Evaluation (NITE) of Japan with accession number NITE BP-02186.<sup>12</sup> The strain was identified as *E. coli* K-12 by biochemical tests, molecular serotyping and multilocus sequence typing (MLST) using data obtained by whole genome sequence (WGS).<sup>13</sup>

*E. coli* NITE BP-02186 was tested for antibiotic susceptibility using culture broth suspension microdilution. The battery of antibiotics tested was that recommended by EFSA (EFSA FEEDAP Panel, 2012a–f) for *E. coli*. All minimum inhibitory concentration (MIC) values were below the corresponding cut-off values defined by the FEEDAP Panel. In addition, no acquired antimicrobial resistance genes were found by searching the WGS against relevant databases.<sup>13</sup> WGS analysis also indicated the absence of known *E. coli* virulence factors, including genes encoding enterotoxins, shiga toxins, adhesion and invasion factors.<sup>13</sup>

##### 3.1.2.1. Information relating to the genetically modified microorganism

###### *Characteristics of the recipient or parental microorganism*

The recipient strain is *E. coli* K-12S B-7, derivative of *E. coli* K-12 obtained by conventional mutagenesis (UV irradiation). *E. coli* K-12 is well-characterised and its safety (non-pathogenicity) has been documented extensively (Gorbach, 1978). Its genome is fully sequenced (Hayashi et al., 2006).

###### *Characterisation of the donor organism*

[REDACTED]

<sup>12</sup> Technical dossier/Section II/Annex Supp II.1.1 Deposition Certificate NITE BP-02186.

<sup>13</sup> Technical dossier/Section II/Annex Supp II.6.

### 3.1.2.2. Description of the genetic modification

The objective of the genetic modification was to increase the production of L-arginine in *E. coli* strain

[REDACTED]. The genes of interest were [REDACTED]

The strain K-12S B-7 was modified [REDACTED] to obtain the production strain *E. coli* NITE BP-02186:

As a result of the genetic modifications, [REDACTED]

During the genetic modification processes to construct the production strain NITE BP-02186, [REDACTED]

[REDACTED] The absence of all full-length antibiotic resistance genes used during the genetic modification from the final production strain was demonstrated by Southern analysis<sup>14</sup> and comparison of the WGS with relevant databases.<sup>15</sup> [REDACTED]

[REDACTED]. The production strain was susceptible to all of the antibiotics involved.

### 3.1.3. Manufacturing process

L-Arginine is produced in a fed-batch fermentation process with *E. coli* NITE BP-02186. After fermentation, the fermentation broth is inactivated [REDACTED]

The fermentation medium contains [REDACTED]

[REDACTED]<sup>16</sup>

No antibiotic is used during fermentation process.<sup>17</sup>

<sup>14</sup> Technical dossier//Section II/Annex\_Supp\_II\_38.

<sup>15</sup> Technical dossier/Section II/Annex\_Supp\_II\_6.

<sup>16</sup> [REDACTED]

<sup>17</sup> Technical dossier/Section II.2.3.1.2.1.



### 3.1.4. Characterisation of the additive

According to the specification, the additive contains  $\geq 98\%$  L-arginine on dry matter basis. Other components not quantified are moisture and other amino acids. The analysis of five industrial pilot batches (from the ultimate phase of the manufacturing process development before routine industrial implementation) showed an average value of L-arginine of 98.4% on 'as is' basis (range 98.1–98.8%).<sup>18</sup> Moisture average was 0.9% (range 0.4–1.3%) and no quantifiable free amino acids other than arginine could be detected. The content of arginine in the additive was 99.3% on dry matter basis (range 99.1–99.4). Sum of quantifiable ammonium, nitrates, nitrites and betaine was on average 0.33% (0.21–0.44%). Sum of quantifiable organic acids was on average 0.2% (0.04–0.41%).<sup>19</sup> Sum of quantifiable inorganic cations and anions was on average 0.03% (0.02–0.03%).<sup>20</sup> The amount of identified material was 99.85% on dry matter basis.

Analytical data on specific optical rotation of three batches showed a range + 26.9° to + 27.0°, which is within the range described in the European Pharmacopoeia for this amino acid (+ 25.5° to + 28.5°).<sup>21</sup>

#### 3.1.4.1. Impurities

Three batches were analysed for impurities. Cadmium, lead, mercury and arsenic were below the limit of detection (LOD).<sup>22</sup> Other elements or compounds analysed were chromium, copper, nickel, phosphorus and fluorine (in five batches), and melamine and cyanic acid (in three batches), all them with values below the respective LODs. Iron (analysed in five batches) ranged from below LOD to 0.2 mg/kg. The sum of dioxins (polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans (PCDD/F)) and dioxin-like polychlorinated biphenyls (DL-PCBs) ranged from 0.0486 to 0.0542 ng WHO-PCDD/F-PCB TEQ per kg (Van den Berg et al., 2006, upper limit). Non dioxin-like PCBs (IECS-6) ranged from 0.051 to 0.067 ng/kg TEQ (Van den Berg et al., 2006, upper limit).<sup>23</sup> Residues of organochlorine (including pyrethroids) and organophosphorus pesticides (analysed in three batches) were all below the limit of quantification.<sup>24</sup>

The microbial analyses of six batches of the product showed the absence of *Salmonella* spp. (25 g samples); total germ count (at 30°C) < 100 CFU/g; coliforms, *Staphylococcus* coagulase positive, yeast and moulds < 10 CFU/g; and *Bacillus cereus* (at 30°C) < 100 CFU/g.<sup>25</sup> As regards the mycotoxins, analysis of three 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 respective limits of quantification (LOQ).<sup>26</sup>

Endotoxin activity was measured in three batches of the additive (*Limulus* amoebocyte lysate test) and the values ranged from 0.4 to 2.9 IU/g.<sup>27</sup>

No viable cells of the production strain were found in three batches of the product (each tested in triplicate). Tests were done by (i) filtering 100 mL of a 10 g/L solution of the product and incubating the filter on non-restrictive solid medium at 37°C for 2 days, and (ii) incubation of 1 g of product in non-restrictive, liquid medium at 37°C for 3 days and subsequent plating in the same conditions as before.<sup>28</sup>

No recombinant DNA was detected in three batches of L-arginine by polymerase chain reaction (PCR),

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<sup>18</sup> Technical dossier/Section II/Annex II.2.1 to II.2.5. Method derived from NF ISO 17180 expanded to arginine, see Annex II.4.

<sup>19</sup> Technical dossier/Section 2.1.3.2.5. Organic acids analysed were lactate, formate, pyruvate, acetate, propionate, butyrate and isovalerate.

<sup>20</sup> Technical dossier/Section 2.1.3.2.6. Anions analysed were chlorides, sulphates and phosphates; cations analysed were potassium, calcium, magnesium and sodium.

<sup>21</sup> Technical dossier/Section II/Annex II.1.2.

<sup>22</sup> Technical dossier/Section II/Annex II.9.1 to II.9.5. LOD in mg/kg was 0.005 for mercury; 0.05 for lead; 0.01 for cadmium; 0.1 for arsenic, copper, iron and nickel; 0.5 for melamine; 1.5 for hydrocyanic acid; 3 for phosphorus and 20 for fluorine. Chromium had three batches analysed with a LOD of 0.2 and other two batches with a LOD of 0.05.

<sup>23</sup> Technical dossier/Section II/Annex II.9.1 to II.9.3.

<sup>24</sup> Technical dossier/Section II/Annexes II.9.1 to II.9.3 and Annex II.10. LOQ in mg/kg for the 107 organochlorinated pesticides (including pyrethroids) ranged from 0.002 to 0.01; and for the 116 organophosphorus pesticides ranged from 0.01 to 0.03.

<sup>25</sup> Technical dossier/Section II/Annexes II.9.1 to II.9.3 and II.11.10 to II.11.12.

<sup>26</sup> Technical dossier/Section II/Annex II.9.1 to II.9.3. LOQ in µg/kg was 0.1 for aflatoxins B1, B2 and G1; 0.2 for aflatoxin G2 and ochratoxin A; 20 for zearalenone, T2 and HT-2 toxins and the fumonisins; and 50 for deoxynivalenol.

<sup>27</sup> Technical dossier/Section II/Annex II.16.

<sup>28</sup> [Redacted]

The absence of antimicrobial activity of the production strain was investigated (three batches of the additive) using the method described in EFSA 'Technical Guidance on Microbial studies'.<sup>29</sup> Antimicrobial effect against one of the five strains (*Pseudomonas aeruginosa* ATCC 27853) was observed at a MIC of 9.1% after 24–48 h incubation at 37°C. The FEEDAP Panel considers that if a MIC is equal to or below four times the maximum additive concentration in feed/water, there are no safety concerns. The normal use level ranges from 0.01% to 0.2% arginine in feed. Four times the highest value of this range is 0.8%, one order of magnitude below 9.1% and consequently the antimicrobial activity observed does not raise safety concerns at normal use levels.

### 3.1.4.2. Physical characteristics

The additive is a white or pale yellow crystalline powder. Its solubility in water at 20°C is 148 g/L, it has a pH ranging from 10.5 to 12.0 (5% solution in water),<sup>30</sup> and its aerated bulk density is 590–662 kg/m<sup>3</sup>.<sup>31</sup>

The particle size distribution was analysed in five batches by laser diffraction (v/v) and the fractions having diameters < 10, < 50 and < 100 µm were 0–0.4%, 0.2–2.5% and 1.2–5.6%, respectively.<sup>32</sup>

The applicant provided analytical data on dusting potential (Stauber–Heubach method) of four batches of the additive and the results ranged from 0.41 to 1.71 g/m<sup>3</sup>.<sup>33</sup> The applicant calculated the dust fractions (inhalable, thoracic and respirable) of each batch. Inhalable fraction ranged from 0.30 to 1.27 g/m<sup>3</sup>; thoracic fraction from 0.13 to 0.64 g/m<sup>3</sup> and the respirable fraction from 0.03 to 0.15 g/m<sup>3</sup>.

### 3.1.5. Stability and homogeneity

#### 3.1.5.1. Shelf life

The shelf life of the additive (three batches) was studied when stored in closed plastic bottles at 25 and at 40°C during 12 months.<sup>34</sup> No losses were observed.

#### 3.1.5.2. Stability in premixtures

The stability of the additive (three batches) in three types of poultry premixtures (for layers and starter and grower for chickens for fattening) was studied at 25°C and 30% relative humidity (RH) or at 40°C and 60% RH, when stored packed in sturdy nylon polyethylene (PE) bags punctured at the top (to allow exchange of oxygen and humidity of the atmosphere) for 6 months.<sup>35</sup> The supplemented levels in the premixtures of layers, starter and grower for chickens for fattening were 2.2%, 2.1% and 2.7% L-arginine, respectively. The premixtures contained vitamins and minerals, including choline chloride (1.6–2% depending on the premixture). In premixtures stored at 25°C and 30% RH losses ranged from 0% to 1.5%. Losses in the premixtures stored at 40°C and 60% RH ranged from 15% to 23%.

#### 3.1.5.3. Stability in feedingstuffs

The stability of the additive (three batches) in three types of poultry feeds (for layers (crumb), and starter (mash) and grower (pelleted) feed for chickens for fattening) was studied at 25°C and 30% relative humidity (RH) or at 40°C and 60% RH, when stored packed in sturdy nylon PE-bags punctured at the top for 3 months.<sup>36</sup> The basal diet was made of maize, wheat and soybean meal and the supplemented levels in the compound feed of layers, starter and grower for chickens for fattening were 0.09%, 0.11% and 0.14% L-arginine, respectively. In feeds stored at 25°C and 30% RH losses ranged from 0% to 0.5%. In the feeds stored at 40°C and 60% RH, losses ranged from 6% to 15%.

The stability of the additive was studied during feed processing (pelleting consisting of pre-heating mash at 60°C for 5 s, pelleting at 80°C for 1 s and cooling to ambient temperature in few minutes) and no losses were observed.<sup>37</sup>

<sup>29</sup> Technical dossier/Section II/Annex II.18.

<sup>30</sup> Technical dossier/Section II/Annex II.13.

<sup>31</sup> Technical dossier/Section II/Annex II.16 and supplementary information December 2017/Annex 2.

<sup>32</sup> Technical dossier/Section II/Annex II.15.

<sup>33</sup> Technical dossier/Supplementary information December 2017/Annex 2.

<sup>34</sup> Technical dossier/Section II/Annex II.29.

<sup>35</sup> Technical dossier/Section II/Annex II.31.

<sup>36</sup> Technical dossier/Section II/Annex II.32.

<sup>37</sup> Technical dossier/Supplementary information December 2017/Annex 3, table 4.3.

#### 3.1.5.4. Stability in water for drinking

The stability of the additive (three batches) in water for drinking was measured at 25°C when diluted at 0.5, 2.5 and 5 g/L of local tap water.<sup>38</sup> The solutions were stored in bottles up to 50 h. No losses were observed.

#### 3.1.5.5. Homogeneity

The capacity of the additive to distribute homogeneously in premixtures was studied in the poultry premixtures described above.<sup>35</sup> Ten subsamples of each premixture were analysed and the coefficients of variation (CV) ranged from 2.4% to 2.6%.

The capacity of the additive to distribute in feedingstuffs was studied in the poultry feeds described above.<sup>36</sup> Ten subsamples of each feed were analysed and the CVs ranged from 0.7% to 2.1%.

#### 3.1.6. Physicochemical incompatibilities

No physicochemical incompatibilities are expected with other additives, medicinal products or the components of feeds.

#### 3.1.7. Conditions of use

As a nutritional additive, it is proposed that L-arginine will be used in feeds to achieve an adequate amino acid profile and to meet the L-arginine requirements for all animal species. It can be added directly to feedingstuffs or complementary feedingstuffs, or via premixture. It is also proposed to use the additive in water for drinking, either in addition to quantities provided by the feedingstuffs or in place of these ones.<sup>39</sup> No inclusion levels have been proposed, as the requirements, in quantitative terms, depend 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 proposes a supplemental use in complete feedingstuffs ranging from 0.5 to 2 g arginine/kg feed.<sup>40</sup>

As flavouring compound, flavouring premixtures can be added to mash feed or water for drinking, or sprayed on pelleted or mash feed. Liquid flavouring premixtures can be applied directly or after dilution using an appropriate solvent. The applicant proposed a use up to the maximum concentration of 25 mg/kg complete feed although in practice it is included at levels ranging from 0.1 to 2 mg/kg.<sup>39</sup> No indications of the concentration used in water for drinking were given by the applicant.

### 3.2. Safety

#### 3.2.1. Safety aspects of the genetic modification<sup>41</sup>

The recipient organism *E. coli* K-12S B-7 is considered to be safe. The production strain NITE BP-02186 contains

. None of these introduced modifications raise a safety concern. There are no genes in the final production strain remaining from the genetic modification process.

The applicant provided sufficient information that neither the production strain nor its recombinant DNA is present in the final product. The final product, manufactured by fermentation with *E. coli* NITE BP-02186, does not give rise to any safety concern with regard to the genetic modification of the production strain.

#### 3.2.2. Safety for the target species

Tolerance studies with essential and conditionally essential amino acids 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. This statement is, in principle, also applicable to non-essential amino acids since a well-balanced dietary protein should have a certain ratio between essential and non-essential

<sup>38</sup> Technical dossier/Section II/Annex II.33.

<sup>39</sup> Technical dossier/Section II.2.5.1.

<sup>40</sup> Technical dossier/Section IV/Section 4.2.

<sup>41</sup> This section has been amended following the confidentiality claims made by the applicant.

amino acids for optimal performance and low nitrogen emissions per product (Baker, 2004). Nevertheless, for 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 purity is > 99% and the amount of identified material represents > 99% on a dry matter basis. Therefore, the FEEDAP Panel considers that safety concerns for target species are highly unlikely to arise from L-arginine produced by *E. coli* NITE BP-02186.

The classification of L-arginine as a dispensable or an indispensable amino acid, its dietary requirements, the adverse effects of excess of arginine in the diets and the lysine-arginine antagonism have been discussed in a previous opinion (EFSA FEEDAP Panel, 2016). In that opinion, it was reported that feeding weaned piglets (age: 3–4 weeks; live weight 7 kg) in short-term experiments (3–4 weeks) with 0.67%, 1.6% and 2.0% supplemental L-arginine decreased weight gain and feed intake, but had variable effects on feed/gain and no effect on the nitrogen balance (Southern and Baker, 1982; Anderson et al., 1984), whereas a moderate L-arginine supplementation (0.22%) did not affect performance of growing piglets (Rosell and Zimmerman, 1983). More recent research (Hu et al., 2015; Wu et al., 2016), however, revealed a higher tolerance to L-arginine in pig diets. The authors assessed the safety of long-term L-arginine supplementation (0%, 1%, 1.5% and 2%) to a typical maize–soybean meal diet (1.35% arginine as background) in pigs between 30 and 121 days of age, based on general observations (e.g., behaviour, skin health, and hair appearance), feed intake, growth, body composition, as well as haematological and blood chemistry measurements. Results of all of the measured variables in the pigs were within physiological ranges and were not adversely affected by the L-arginine supplementation.

In ruminants, arginine is extensively catabolised. The initial products of L-arginine degradation by ruminal microorganisms are ornithine,  $\delta$ -aminovaleric acid, and putrescine (Lewis and Emery, 1962), which are then either converted to volatile fatty acids or incorporated into microbial cell biomass. As these products have no recorded deleterious effects to the host animal, there are no safety concerns arising from ruminal L-arginine metabolism when supplemented to diets in appropriate amounts.

The use of L-arginine produced by *E. coli* NITE BP-02186 is safe for target species when supplemented to diets in appropriate amounts. There are no safety concerns arising from ruminal L-arginine metabolism.

The applicant carried out a literature review and reports data on safety of arginine supplementation in pigs for fattening, piglets, gestating sows, sheep and cattle, poultry, cats, dogs, rats and fish.<sup>42</sup> The data showed that (i) potential adverse effects may occur at high supplementation levels (usually beyond a level of 2% arginine supplementation in the diet) and these may vary between and within species; (ii) the observed adverse effects usually consist of reduced feed intake and weight gain.<sup>43</sup> Although a study by Li et al. (2010) detected reduced embryonic survival and plasma progesterone in sows during gestation at diets containing a total of 1.5% arginine in the diet (0.8% of which was supplemented), other authors reported beneficial effects on embryonic/foetal survival in sows and survival of offspring with diets containing up to a total of 1.7% arginine in the diet (up to 1% of which was supplemented) (Mateo et al., 2007, 2008; Li et al., 2015; Palencia et al., 2018). The FEEDAP Panel notes that this literature review, while providing some additional information, does not modify the previous assessments concerning arginine supplementation in target species (EFSA, 2007; EFSA FEEDAP Panel, 2016, 2017).

The use of L-arginine as a feed flavouring agent is proposed at the maximum level of 25 mg/kg complete feed. The FEEDAP Panel considers that such inclusion level is unlikely to have any impact on the arginine-lysine ratio when the amino acid composition of the diet is sufficiently balanced.

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

### 3.2.2.1. Conclusions on safety for the target species

The use of L-arginine produced by *E. coli* NITE BP-02186 at the doses proposed (0.5–2 g arginine/kg feed) is safe for target species when supplemented to diets in appropriate amounts. There are no safety concerns arising from ruminal L-arginine metabolism. The use of L-arginine as a feed flavouring agent is unlikely to pose any concern at the proposed maximum level of 25 mg/kg complete feed.

<sup>42</sup> Technical dossier/Section III.3.1.1.

<sup>43</sup> Technical dossier/Section III.3.1.1.2.

### 3.2.3. Safety for the consumer

The absorption and metabolic fate of arginine were described in a previous opinion (EFSA, 2007).

As a general principle, conventional toxicology studies are considered to be inappropriate for amino acids.

The product under assessment is produced by fermentation. Concerns for the consumer would derive not from the amino acid itself, which will be incorporated into animal protein, but from possible residues from the fermentation. Considering that the additive is highly purified ( $\geq 99\%$  L-arginine on dry matter basis), no additional toxicological data are required (EFSA FEEDAP Panel, 2012a–f).

Amino acids supplemented to feed will be incorporated into proteins of tissues and/or products of target animal species and any of their potential excess will be metabolised and excreted. Therefore, the composition of tissues and products of animal origin will not be changed by the use of L-arginine in animal nutrition.

#### 3.2.3.1. Conclusions on safety for the consumer

The composition of edible tissues and products of animal origin will not be changed by the use of L-arginine in animal nutrition. Considering the high purity of the product under assessment, no risks are expected for the consumer from the use of L-arginine produced by *E. coli* NITE BP-02186 as a feed additive.

### 3.2.4. Safety for the user

The test item used in the following studies to support the safety for the user was the product under assessment.

#### 3.2.4.1. Effects on the respiratory system

The particle size and the dusting potential of the additive indicates that exposure by inhalation of the user is likely (see Section 3.1.4.2).

An acute inhalation test was carried out with the additive under assessment on CrI:WI(Han) rats (five males and five females) in accordance with OECD guideline 403.<sup>44</sup> The FEEDAP Panel notes that the batch used in this study had a lower potential for exposure of the respiratory system compared to other batches of the additive (dusting potential 0.11 g/m<sup>3</sup>; no particles < 10  $\mu\text{m}$ , 2.5% < 50  $\mu\text{m}$  and 5.6% of particles < 100  $\mu\text{m}$ ). After 4 h exposure at the dose of 5.1 g L-arginine/m<sup>3</sup>, some adverse effects were observed: increased breathing rate during exposure in all animals, which resolved shortly after exposure and sparse haired areas in two female rats which persisted up to the end of the post-treatment observation period (14 days). At necropsy (performed 2 weeks after exposure), two out of five females and four out of five males in the group exposed to L-arginine showed localised hyaline spots on the lungs with partial collapse of the tissue in the affected region. In addition, one male had red spots in the thymus. No such changes were present in the control group. Histopathology was not performed; based on the data available, the observed changes should be regarded as treatment-related adverse effects that can persist after a single exposure. The study design did not allow to set a no observed adverse effects concentration (NOAEC). Considering the potential inhalation exposure and the observation of persistent adverse effects, the FEEDAP Panel considers that the additive may pose a risk by inhalation.

The production species, *E. coli*, is a Gram-negative bacterium. Although the K-12 strain and its derivatives are safe from the point of view of enterotoxins and other virulence factors (Gorbach, 1978; EPA 1997, Bauer et al., 2007), *E. coli* K-12 retains lipopolysaccharide in its cell envelope (Luchi and Morrison, 2000; Svensson et al., 2005; Gao et al., 2006) which potentially may result in endotoxin activity in the final product. Inhalation exposure to endotoxins is a concern for user safety (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 the Appendix A. The health based recommended threshold for the quantity of inhaled endotoxins per working day is 900 IU, derived from provisional occupational exposure limits given by the Dutch Expert Committee on Occupational Safety (DECOS) (Health Council of the Netherlands, 2010) and the UK Health and Safety Executive (HSE, 2013). Based upon the calculation of the potential endotoxin content in dust, the inhalation exposure could be up to 2.75 endotoxin IU per working day, indicating no risk of exposure to endotoxins for persons handling the additive.

<sup>44</sup> Technical dossier/Section III/Annex III.3.159.

### 3.2.4.2. Effects on skin and eyes

The additive under assessment has a high pH (10.5–12) in aqueous solution.

In an *in vitro* dermal irritation test based on reconstructed skin membranes in accordance with OECD Guideline 439, the skin membranes were topically exposed to the additive under assessment for 60 min. Viability of the epidermal cells was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test 43 h after exposure. The positive and negative controls performed as expected. The mean viability of the skin membranes was 78% compared to the negative control group.<sup>45</sup> The additive was considered not irritant for the skin.

In an acute eye irritation study using the isolated chicken eye test in accordance with OECD 438, 30 mg of the product under assessment were applied for 10 s to chicken eyes, followed by a 20 mL saline rinse. The eyes were examined periodically up to 240 min after exposure. Corneal swelling and corneal opacity were very slight and fluorescein retention slight to moderate. At microscopic evaluation, only very slight vacuolation was observed in the top layer of the corneal epithelium. The product was considered not irritant for the eye.

In a skin sensitisation study using local lymph node assay in the mouse, in accordance with OECD Guideline 439, the product under assessment was tested topically at 10, 25 or 50% w/w (25 µL/ear). All auricular lymph nodes of the animals of the experimental and control groups were considered normal size. Since the test item elicited a stimulation index < 3 when tested up to 50%, the additive was not considered to be a skin sensitiser.<sup>46</sup>

### 3.2.4.3. Conclusions on safety for the user

L-Arginine produced by *E. coli* NITE BP-02186 is not irritant to skin or eyes, nor a skin sensitiser. Although the presence of endotoxin activity is of no concern, the available exposure and toxicological data indicate that the additive may pose a risk to users by inhalation.

### 3.2.5. Safety for the environment

Neither the production strain *E. coli* NITE BP-02186 nor its recombinant DNA was detected in the final product. The final product does not pose any environmental safety concern associated with the genetic modification of the production strain.

L-Arginine is a natural component of animals and plants whose use in animal nutrition would not lead to any localised increase of its concentration in the environment. It is mainly not excreted as such, but as urea or uric acid and carbon dioxide. The FEEDAP Panel concludes that the use of the product L-arginine produced by *E. coli* NITE BP-02186 in animal nutrition would not pose a risk to the environment.

## 3.3. Efficacy

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

Since L-arginine is used in food as flavouring,<sup>47</sup> it is to be expected that it can provide a similar function in feed. Therefore, 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<sup>48</sup> and Good Manufacturing Practice.

<sup>45</sup> Technical dossier/Section III/Annex III.168.

<sup>46</sup> Technical dossier/Section III/Annex III.169.

<sup>47</sup> L-Arginine (FL No 17.003) is included in the 'Union list of flavourings and source materials,' Annex I of Regulation (EC) No 1334/2008 on flavourings for use in and on foods.

<sup>48</sup> Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

## 4. Conclusions

Neither the production strain *E. coli* NITE BP-02186 nor its recombinant DNA was detected in the final product. The product L-arginine, manufactured by fermentation with *E. coli* NITE BP-02186, does not give rise to any safety concern with regard to the genetic modification of the production strain.

The use of L-arginine produced by *E. coli* NITE BP-02186 as a nutritional additive is safe for target species when supplemented to diets in appropriate amounts. There are no safety concerns arising from ruminal L-arginine metabolism. The use of L-arginine as a feed flavouring agent is unlikely to pose any concern.

No risks are expected for the consumer from the use of L-arginine produced by *E. coli* NITE BP-02186 as a feed additive.

The product under assessment is not irritant to skin or eyes, nor a skin sensitiser. Although the presence of endotoxins is of no concern, the available exposure and toxicological data indicate that the additive may pose a risk to users by inhalation.

The use of the product under assessment in animal nutrition does not pose a risk to the environment.

The additive is an effective source of arginine for all species. L-Arginine is considered efficacious when used as a flavouring compound in animal nutrition.

## 5. Recommendations

The description of the additive should contain the statement 'L-arginine produced by *Escherichia coli* NITE BP-02186'.

### Documentation provided to EFSA

- 1) L-Arginine produced using strain NITE BP-02186. June 2017. Submitted by Ajinomoto Eurolysine SAS.
- 2) L-Arginine produced using strain NITE BP-02186. Supplementary information. December 2017. Submitted by Ajinomoto Eurolysine SAS.
- 3) Evaluation report of the European Union Reference Laboratory for Feed Additives on the Methods(s) of Analysis for L-arginine produced by fermentation with *Escherichia coli* NITE BP-02186.
- 4) Comments from Member States.

## References<sup>49</sup>

Anderson LC, Lewis AJ, Peo Jr ER and Crenshaw JD, 1984. Effects of excess arginine with and without supplemental lysine on performance, plasma amino acid concentrations and nitrogen balance of young swine. *Journal of Animal Science*, 58, 369–377.

Baker DH, 2004. Animal models for human amino acid responses. *Journal of Nutrition*, 134, 1646–1650.

Bauer AP, Dieckmann SM, Ludwig W and Schleifer KH, 2007. Rapid identification of *Escherichia coli* safety and laboratory strain lineages based on multiplex-PCR. *Federation of European Microbiological Societies Microbiology Letters*, 269, 36–40.

EFSA (European Food Safety Authority), 2007. Opinion of the scientific Panel on Additives and Products or Substances used in Animal Feed on the safety and efficacy of the product containing L-arginine produced by fermentation from *Corynebacterium glutamicum* (ATCC-13870) for all animal species. *EFSA Journal* 2007; 5(5):473, 19 pp. <https://doi.org/10.2903/j.efsa.2007.473>

<sup>49</sup> This section has been amended following the confidentiality claims made by the applicant.

- EFSA (European Food Safety Authority), 2008a. 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 (European Food Safety Authority), 2008b. Technical Guidance: microbial Studies. *EFSA Journal* 2008;6(10):836, 3 pp. <https://doi.org/10.2903/j.efsa.2008.836>
- 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: [www.efsa.europa.eu/efsa-journal](http://www.efsa.europa.eu/efsa-journal)
- EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2011. Technical guidance: tolerance and efficacy studies in target animals. *EFSA Journal* 2011;9(5):2175, 15 pp. <https://doi.org/10.2903/j.efsa.2011.2175>
- EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012a. Guidance for the preparation of dossiers for nutritional additives. *EFSA Journal* 2012;10(1):2535, 14 pp. <https://doi.org/10.2903/j.efsa.2012.2535>
- EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012b. Guidance for the preparation of dossiers for sensory additives. *EFSA Journal* 2012;10(1):2534, 26 pp. <https://doi.org/10.2903/j.efsa.2012.2534>
- EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012c. Guidance for establishing the safety of additives for the consumer. *EFSA Journal* 2012;10(1):2537, 12 pp. <https://doi.org/10.2903/j.efsa.2012.2537>
- EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012d. 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), 2012e. 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), 2012f. Guidance for the preparation of dossiers for additives already authorised for use in food. *EFSA Journal* 2012;10(1):2538, 4 pp. <https://doi.org/10.2903/j.efsa.2012.2538>
- 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), 2016. Scientific Opinion on the safety and efficacy of L-arginine produced by *Corynebacterium glutamicum* KCTC 10423BP for all animal species. *EFSA Journal* 2016;14(1):4345, 17 pp. <https://doi.org/10.2903/j.efsa.2016.4345>
- 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, Wester P, Costa L, Dierick N, Leng L, Glandorf B, Herman L, Karenlampi S and Wallace RJ, 2017. Scientific Opinion on the safety and efficacy of L-arginine produced by *Corynebacterium glutamicum* KCCM 80099 for all animal species. *EFSA Journal* 2017;15(6):4858, 15 pp. <https://doi.org/10.2903/j.efsa.2017.4858>
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2011. Scientific Opinion on Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use. *EFSA Journal* 2011;9(6):2193, 54 pp. <https://doi.org/10.2903/j.efsa.2011.2193>
- EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2011a. Scientific Opinion on the substantiation of health claims related to: anthocyanidins and proanthocyanidins (ID 1787, 1788, 1789, 1790,1791); sodium alginate and ulva (ID 1873); vitamins, minerals, trace elements and standardised ginseng G11 extract (ID 8, 1673, 1674); vitamins, minerals, lysine and/or arginine and/or taurine (ID 6, 1676, 1677); plant-based preparation for use in beverages (ID 4210, 4211); *Carica papaya* L. (ID 2007); "fish protein" (ID 651); acidic water-based, non-alcoholic flavoured beverages containing calcium in the range of 0.3 to 0.8 mol per mol of acid with a pH not lower than 3.7 (ID 1170); royal jelly (ID 1225, 1226, 1227, 1228, 1230, 1231, 1326,1328, 1329, 1982, 4696, 4697); foods low in cholesterol (ID 624); and foods low in trans-fatty acids (ID 672,4333) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA Journal* 2011;9(4):2083, 34 pp. <https://doi.org/10.2903/j.efsa.2011.2083>



- EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2011b. Scientific Opinion on the substantiation of health claims related to L-arginine and 'immune system functions' (ID 455, 1713), growth or maintenance of muscle mass (ID 456, 1712, 4681), normal red blood cell formation (ID 456, 664, 1443, 1712), maintenance of normal blood pressure (ID 664, 1443), improvement of endothelium-dependent vasodilation (ID 664, 1443, 4680), 'physical performance and condition' (ID 1820), 'système nerveux' (ID 608), maintenance of normal erectile function (ID 649, 4682), contribution to normal spermatogenesis (ID 650, 4682), 'function of the intestinal tract' (ID 740), and maintenance of normal ammonia clearance (ID 4683) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. EFSA Journal 2011;9(4):2051, 30 pp. <https://doi.org/10.2903/j.efsa.2011.2051>
- EPA (US Environmental Protection Agency), 1997. *Escherichia coli* K-12 derivatives final risk assessment. Available online: [http://epa.gov/biotech\\_rule/pubs/fra/fra004.htm](http://epa.gov/biotech_rule/pubs/fra/fra004.htm)
- European Commission, 1999. Opinion on substances for nutritional purposes which have been proposed for use in the manufacture of foods for particular nutritional purposes. Available online: [https://ec.europa.eu/food/sites/food/files/safety/docs/sci-com\\_scf\\_out31\\_en.pdf](https://ec.europa.eu/food/sites/food/files/safety/docs/sci-com_scf_out31_en.pdf)
- European Pharmacopoeia (PhEur), 2014. Arginine, Monograph 07/2014:0806, 8th Edition Council of Europe. (COE)—European Directorate for the Quality of Medicines, Strasbourg, France.
- Gao B, Wang Y and Tsan MF, 2006. The heat sensitivity of cytokine-inducing effect of lipopolysaccharide. Journal of Leukocyte Biology, 80, 359–366.
- 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, 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.
- 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.
- Hu S, Li X, Rezaei R, Meininger CJ, McNeal CJ and Wu G, 2015. Safety of long-term dietary supplementation with L-arginine in pigs. Amino Acids, 47, 925–936.
- Lewis TR and Emery RS, 1962. Intermediate products in the catabolism of amino acids by rumen microorganisms. Journal of Dairy Science, 45, 1363–1368.
- Li X, Bazer FW, Johnson GA, Burghardt RC, Erikson DW, Frank JW, Spencer TE, Shinzato I and Wu G, 2010. Dietary supplementation with 0.8% L-arginine between days 0 and 25 of gestation reduces litter size in gilts. The Journal of Nutrition, 140, 1111–1116.
- Li J, Xia H, Yao W, Wang T, Piao X, Thacker P, Wu G and Wang F, 2015. Effects of arginine supplementation during early gestation (day 1 to 30) on litter size and plasma metabolites in gilts and sows. Journal of Animal Science, 93, 5291–5303.
- Luchi M and Morrison DC, 2000. Comparable endotoxic properties of lipopolysaccharides are manifest in diverse clinical isolates of Gram-negative bacteria. Infection and Immunity, 68, 1899–1904.
- Mateo R, Wu G, Bazer F, Park J and Shinzato I, 2007. Dietary L-arginine supplementation enhances the reproductive performance of gilts. Journal of Nutrition, 137, 652–656.
- Mateo RD, Wu G, Moon HK, Carroll JA and Kim SW, 2008. Effects of dietary arginine supplementation during gestation and lactation on the performance of lactating primiparous sows and nursing piglets. Journal of Animal Science, 86, 827–835.
- Palencia JYP, Lemes MAG, Garbossa CAP, Abreu MLT, Pereira LJ and Zangeronimo MG, 2018. Arginine for gestating sows and foetal development: a systematic review. Journal of Animal Physiology and Animal Nutrition, 102, 204–213.
- Rosell VL and Zimmerman DR, 1983. Effects of ingestion of high levels of threonine and arginine on weanling pigs. Journal of Animal Science, 57, 266.
- Rylander R, 1999. Health effects among workers in sewage treatment plants. Occupational and Environmental Medicine, 56, 354–3571.
- Shao A and Hathcock JN, 2008. Risk assessment for the amino acids taurine, L-glutamine and L-arginine. Regulatory Toxicology and Pharmacology, 50, 376–399.
- Southern LL and Baker DH, 1982. Performance and concentration of amino acids in plasma and urine of young pigs fed diets with excesses of either arginine or lysine. Journal of Animal Science, 55, 857–866.
- Svensson M, Han L, Silfersparre G, Haggstrom L and Enfors SO, 2005. Control of endotoxin release in *Escherichia coli* feed-batch cultures. Bioprocess and Biosystems Engineering, 27, 91–97.
- Thorn J, 2001. The inflammatory response in humans after inhalation of bacterial endotoxin: a review. Inflammatory Response, 50, 254–261.

- Van den Berg M, Birnbaum LS, Denison M, De Vito M, Farland W, Feeley M, Fiedler H, Hakansson H, Hanberg A, Haws L, Rose M, Safe S, Schrenk D, Tohyama C, Tritscher A, Tuomisto J, Tysklind M, Walker N and Peterson RE, 2006. The 2005 World Health Organization Reevaluation of Human and Mammalian Toxic Equivalency Factors for Dioxins and Dioxin-Like Compounds. *Toxicological Sciences*, 93, 223–241. <https://doi.org/10.1093/toxsci/kfl055>
- WHO (World Health Organization), 2006. Safety evaluation of certain food additives. Joint FAO/WHO Expert Committee on Food Additives (JECFA), WHO Food Additives Series: 54. International Programme on Chemical Safety, WHO, Geneva, Switzerland.
- Wu Z, Hou Y, Hu S, Bazer F, Meininger C, McNeal C and Wu G, 2016. Catabolism and safety of supplemental L-arginine in animals. *Amino Acids*, 48, 1541–1552.

## Abbreviations

ATCC	American type culture collection
CAS	Chemical Abstracts Service
CFU	colony forming unit
CV	coefficient of variation
DECOS	Dutch Expert Committee on Occupational Safety
DL-PCB	dioxin-like polychlorinated biphenyl
EINECS	European Inventory of Existing Commercial chemical Substances
EURL	European Union Reference Laboratory
FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
GMO	Genetically modified microorganism
IUPAC	International Union of Pure and Applied Chemistry
LLNA	local lymph node assay
LOD	limit of detection
LOQ	limit of quantification
MIC	minimum inhibitory concentration
MLST	multilocus sequence typing
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NDA	EFSA Panel on Dietetic products, Nutrition and Allergies
NITE	National Institute of Technology and Evaluation
NOAEC	no observed adverse effects concentration
OECD	Organisation for Economic Co-operation and Development
PCDD/F	polychlorinated dibenzo- <i>p</i> -dioxins and polychlorinated dibenzofurans
PCR	polymerase chain reaction
PE	polyethylene
pH	hydrogen potential
RH	relative humidity
SCF	Scientific Committee of Food
WGS	whole genome sequence
WHO	World Health Organization

## Appendix A – Safety for the user

The effects of 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 probable exposure time according to EFSA guidance (EFSA FEEDAP Panel, 2012d) for additives added in premixtures assumes a maximum of 40 periods of exposure per day, each comprising 20 s =  $40 \times 20 = 800$  s/day. With an uncertainty factor of 2, maximum inhalation exposure would occur for  $2 \times 800 = 1,600$  s = 0.444 h/day. Again, assuming a respiration volume of 1.25 m<sup>3</sup>/h, the inhalation volume providing exposure to potentially endotoxin-containing dust would be  $0.444 \times 1.25 = 0.556$  m<sup>3</sup>/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<sup>3</sup>.

### Calculation of endotoxin content of dust

Two key measurements are required to evaluate the potential respiratory hazard associated with the endotoxin content of the product (the dusting potential of the product, expressed in g/m<sup>3</sup>, and 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 taken instead. If the content of endotoxins of the relevant additive is  $a$  IU/g and the dusting potential is  $b$  g/m<sup>3</sup>, then the content of endotoxins of the dust,  $c$  IU/m<sup>3</sup>, is obtained by simple multiplication,  $a \times b$ . This resulting value is further used for calculation of the potential inhalatory exposure of users to endotoxins from the additive under assessment (Table A.1) (EFSA FEEDAP Panel, 2012d).

**Table A.1:** Estimation of user exposure to endotoxins from the additive L-arginine produced by *Escherichia coli* NITE BP-02186, including consideration of using a filter mask FF P2 or FF P3 as a preventative measure

Calculation	Identifier	Description	Amount	Source
	$a$	Endotoxin content IU/g product	2.9	Technical dossier
	$b$	Dusting potential (g/m <sup>3</sup> )	1.706	Technical dossier
$a \times b$	$c$	Endotoxin content in the air (IU/m <sup>3</sup> )	4.95	
	$d$	No of premixture batches made/ working day	40	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012d)
	$e$	Time of exposure (s) per production of one batch	20	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012d)
$d \times e$	$f$	Total duration of daily exposure/ worker (s)	800	
	$g$	Uncertainty factor	2	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012d)
$f \times g$	$h$	Refined total duration of daily exposure/worker (s)	1,600	
$h/3,600$	$i$	Refined total duration of daily exposure (h)	0.44	
	$j$	Inhaled air (m <sup>3</sup> ) per 8-h working day	10	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012d)
$j/8 \times i$	$k$	Inhaled air during exposure (m <sup>3</sup> )	0.56	
$c \times k$	$l$	<b>Endotoxin inhaled (IU) during exposure per 8-h working day</b>	<b>2.75</b>	
	$m$	Health-based recommended exposure limit of endotoxin (IU/m <sup>3</sup> ) per 8-h working day	90	Health Council of the Netherlands (2010)
$m \times j$	$n$	<b>Health-based recommended exposure limit of total endotoxin exposure (IU) per 8-h working day</b>	<b>900</b>	

Calculation	Identifier	Description	Amount	Source
I/10		Endotoxins inhaled (IU) per 8-h working day reduced by filter mask FF P2 (reduction factor 10)	0	
I/20		Endotoxins inhaled (IU) per 8-h working day reduced by filter mask FF P3 (reduction factor 20)	0	

## **Annex A – European Reference Laboratory evaluation report on the analytical methods submitted with the application for authorisation of L-arginine produced by fermentation with *Escherichia coli* NITE BP-02186**

In the current application authorisation is sought under Article 4(1) for L-arginine produced by fermentation with *Escherichia coli* NITE BP-02186, under the category/functional group 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. L-arginine is already authorised as nutritional feed additive under Commission Regulation (EC) No 1139/2007 and Commission Implementing Regulation (EU) 2016/972.

For the characterisation of the feed additive, the EURL identified the "L-arginine monograph" of the Food Chemical Codex (FCC).

For the quantification of arginine in the feed additive and premixtures, the Applicant submitted a validated and further verified method derived from the ring trial validated method described in EN ISO 17180:2013. The method is based on ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-VIS). It is specifically intended for lysine, methionine and threonine in commercial feed additives and premixtures. The method does not distinguish between the salts of amino acids and it cannot differentiate between enantiomers. The corresponding method performance characteristics, recalculated by the EURL from the experimental data provided, are in agreement with those reported in the standard thus demonstrating the applicability of the slightly modified protocol to the quantification of arginine in the feed additive and premixtures.

For the quantification of L-arginine in premixtures and feedingstuffs the Applicant submitted the ring-trial validated Community method (Commission Regulation (EC) No 152/2009). The method was further ring-trial validated by CEN resulting in EN ISO 13903:2005. The method is based on ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-VIS). This method does not distinguish between the salts and the amino acid enantiomers. The following performance characteristics were reported for the quantification of total arginine: a relative standard deviation for repeatability (RSDr) ranging from 2.3% to 3.3% and a relative standard deviation for reproducibility (RSDR) ranging from 7.2% to 9.7%.

For the quantification of L-arginine in water the Applicant suggested a procedure based on the standard EN ISO 13903:2005. The Applicant did not perform a validation and verification study, but provided, in the frame of stability studies, the detailed experimental protocol used supported by experimental data. From the data reported the EURL calculated the following performance characteristics: RSDr ranging from 0.6% to 0.8% and relative standard deviation for intermediate precision (RSDip) ranging from 0.6% to 1.0%. The performance characteristics obtained are in agreement with those stated in the EN ISO 13903:2005 standard thus demonstrating the applicability (extension of the scope) of the slightly modified protocol to the quantification of arginine in water.

Based on the performance characteristics available, the EURL recommends for official control: (i) the "L-arginine monograph" of the Food Chemical Codex (FCC) for the identification of L-arginine in the feed additive; (ii) the validated and further verified IEC-VIS analytical method for the quantification of arginine in the feed additive; (iii) the Community method based on IEC-VIS for the quantification of arginine in premixtures and feedingstuffs; and (iv) the IEC-VIS analytical method to quantify arginine 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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.