

SCIENTIFIC OPINION

Scientific Opinion on the safety and efficacy of L-isoleucine for all animal species¹

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) and EFSA Panel on Genetically Modified Organisms (GMO)^{2,3}

European Food Safety Authority (EFSA), Parma, Italy

SUMMARY

Following a request from the European Commission, the European Food Safety Authority (EFSA) was asked to deliver a scientific opinion on the efficacy and safety of the product L-isoleucine for all animal species.

The product L-isoleucine contains on a dry matter basis not less than 93.4 % L-isoleucine, about 5 % other amino acids and less than 1 % unidentified impurities. It is produced by a genetically modified *Escherichia coli* K12 strain. The introduced genes do not trigger any safety concerns. The final product does not contain any cultivable production organism and the level of the recombinant DNA is below the limit of detection of the method used.

Based on the available knowledge and studies in piglets, it is concluded that L-isoleucine is a source of available isoleucine and is safe for all animal species, when added to diets to cover the animal requirement.

L-isoleucine contains less than 1 % unidentified impurities. Considering also data from additional toxicity studies supporting its safety and the fact that the consumer will not be exposed to additional isoleucine, it is concluded that no safety concerns for the consumer would result from the use of L-isoleucine as feed additive.

The product L-isoleucine is considered to be non irritant to skin and eyes and not to be a dermal sensitiser. The only detectable risk for the user could be derived from the dustiness of the product, but on the basis of an acute inhalation toxicity study, this is expected to be minor.

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² FEEDAP Panel members: Gabriele Aquilina, Georges Bories, Paul Brantom, Francesca Caloni, Andrew Chesson, Pier Sandro Cocconcelli, Noël Dierick, Mikolaj Gralak, Jürgen Gropp, Ingrid Halle, Joop de Knecht, Reinhard Kroker, Nils-Gunnar Ilbäck, Lubomir Leng, Sven Lindgren, Anne-Katrine Lundebye Haldorsen, Alberto Mantovani, Miklós Mézes, Derek Renshaw and Maria Saarela. Correspondence: FEEDAP@efsa.europa.eu

GMO Panel members: Hans Christer Andersson, Salvatore Arpaia, Detlef Bartsch, Josep Casacuberta, Howard Davies, Patrick du Jardin, Gerhard Flachowsky, Lieve Herman, Huw Jones, Sirpa Kärenlampi, Jozsef Kiss, Gijs Kleter, Harry Kuiper, Antoine Messéan, Kaare Magne Nielsen, Joe Perry, Annette Poeting, Jeremy Sweet, Christoph Tebbe, Atte Johannes von Wright and Jean-Michel Wal. Correspondence: GMO@efsa.europa.eu

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L-isoleucine is a physiological amino acid and a natural component of animals and plants whose use in animal nutrition would not lead to any localised increase in concentration in the environment. It is concluded that the use of the product as a feed additive does not present a foreseeable risk to the environment.

KEY WORDS

Nutritional additives, amino acids and their salts and analogues, L-isoleucine, bioavailability, safety, genetically modified microorganisms.

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BACKGROUND

Regulation (EC) No 1831/2003⁴ 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 the company Ajinomoto Eurolysine⁵ for authorisation of the product “L-isoleucine-Feed Grade”, L-isoleucine, to be used as a feed additive for all animal species (category: Nutritional; functional group: amino acids their salts and analogues) under the conditions mentioned in Table 1. 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). EFSA received directly from the applicant the technical dossier in support of this application.⁶ According to Article 8 of that Regulation, 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. The particulars and documents in support of the application were considered valid by EFSA as of 28 April 2009.

The additive L-isoleucine is a preparation of L-isoleucine produced by the genetically modified micro-organism *Escherichia coli* (FERM ABP-10641). This product has not been previously authorised in the Community.

TERMS OF REFERENCE

According to Article 8 of Regulation (EC) No 1831/2003, EFSA shall 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 animal(s), consumer, user and the environment and the efficacy of the product L-isoleucine, when used under the conditions described in Table 1.

⁴ OJ L 268, 18.10.2003, p.29

⁵ Ajinomoto Eurolysine, 153, rue de Courcelles, 75817 Paris Cedex, 17, France

⁶ EFSA Dossier reference: FAD-2009-001

Table 1. Description and conditions of use of the additive as proposed by the applicant

Additive		L-isoleucine		
Registration number/EC No/No (if appropriate)				
Category of additive		Nutritional additive		
Functional group of additive		Amino acids, their salts and analogues		
Description				
Composition, description	Chemical formula	Purity criteria (if appropriate)		Method of analysis (if appropriate)
		<p>L-isoleucine content: not less than 92 %, when expressed on product 'as is' (equivalent to: not less than 93.4 %, when expressed as Dry Matter basis)</p> <p>Substances other than L-isoleucine and residual water/moisture: not more than 6.5 % expressed on product 'as is' (equivalent to substances other than L-isoleucine: not more than 6.6 % when expressed as Dry Matter basis)</p> <p>Moisture: not more than 1.5 %</p> <p>Strain <i>Escherichia coli</i> K-12 3149 – FERM ABP-10641</p>		
Trade name (if appropriate)		L-isoleucine (Feed Grade)		
Name of the holder of authorisation (if appropriate)				
Conditions of use				
Species or category of animal	Maximum Age	Minimum content	Maximum content	Withdrawal period (if appropriate)
		mg/kg of complete feedingstuffs		
All species	Not Applicable	Not Applicable	Not Applicable	None
Other provisions and additional requirements for the labelling				
Specific conditions or restrictions for use (if appropriate)		None		
Specific conditions or restrictions for handling (if appropriate)		Like for any products which may generate dust, wear a mask and safety goggles		
Post market monitoring (if appropriate)		Not considered necessary in view of adequate monitoring and operating conditions during the manufacturing process to ensure quality and safety of the additive (Ajinomoto Eurolysine has been certified ISO and FAMI-QS). A questionnaire for reporting problems faced by clients may be distributed together with product's MSDS.		
Specific conditions for use in complementary feedingstuffs (if appropriate)		None		
Maximum Residue Limit (MRL) (if appropriate)				
Marker residue	Species or category of animal	Target tissue(s) or food products	Maximum content in tissues	
-	-	-	-	

ASSESSMENT

1. Introduction

The applicant requested authorisation for the product L-isoleucine under the category nutritional additives, functional group amino acids and their salts and analogues. L-isoleucine is a purified fermentation product produced by a genetically modified (GM) strain of *Escherichia coli* (FERM ABP-10641). The additive under assessment is intended to be used as a source of the essential amino acid L-isoleucine to optimise the amino acid pattern by supplementing animal diets.

The objective of feed supplementation with essential amino acids is to complete the amino acid supply of the diet in order to closely meet the requirement of individual amino acids of the animals or to compensate for potential imbalances. The supplementation of feedingstuffs with amino acids is a conventional tool in improving the protein quality and utilisation. This supplementation became even more important since protein reduced diets were introduced in animal husbandry for economic and environmental reasons. The branched chain amino acid (BCAA) L-isoleucine is well recognised as an essential amino acid which may become limiting under specific feeding conditions.

Currently, the amino acid L-isoleucine is used in human nutrition in a 'pharmaceutical' grade form (with purity not less than 98.5 % on a dry matter basis) for parenteral nutrition and as a food ingredient (nutritional supplement) for infant formulae and follow-on formulae.⁷

2. Characterisation

2.1. Identity of the additive

The product L-isoleucine is a crystalline powder of white creamish colour with appearance of waxy, shiny, rhombic leaflets. Its melting point is 168 to 170°C and its density 1.23 kg/L. The product contains not less than 92 % L-isoleucine and a maximum moisture content of 1.5 % (equivalent to not less than 93.4 % on DM basis). Data on product composition are available for three batches from a bench pilot scale production made in 2004 and for an additional fourth batch made in 2006.⁸

The first three batches showed a low variability. The average moisture content was less than 0.1 %. The following mean values were calculated: isoleucine 93.8 % (93.2 to 94.4), valine 2.8 %, α -amino-butyric acid 2.1 % and other amino acids 0.6 %, amounting together to 99.3 %. Crude fat was <0.1 % and crude ash <0.1 %. Less than 1 % of the product remains unidentified. DNA and RNA contents in product were <80 $\mu\text{g}/\text{kg}$. The fourth batch from the later pilot production contained 97.4 % isoleucine.⁹

Control measures are in place to ensure that mycotoxins are not present in the fermentation substrate. Arsenic, mercury, cadmium and nickel were analysed in the three first batches (2004 pilot fermentation) (<10, 1, 2 and 50 $\mu\text{g}/\text{kg}$, respectively). The concentrations of Pb varied between 69 and 81 $\mu\text{g}/\text{kg}$, Cr between 50 and 64 $\mu\text{g}/\text{kg}$, Cu between 100 and 560 $\mu\text{g}/\text{kg}$ and Zn between 590 and 650 $\mu\text{g}/\text{kg}$.¹⁰

The total maximum content of dioxins analysed in two batches was found to be 0.079 ng WHO-TEQ/kg. The PCB congeners (PCB-28, -52, -101, 118, 138, 153 and 180) analysed in one batch were <1 $\mu\text{g}/\text{kg}$ each. The total concentration of polyaromatic hydrocarbons in one batch was found to be

⁷ Annex III to Commission Directive 91/321/EEC of 14 May 1991 on infant formulae and follow-on formulae

⁸ Technical Dossier/ Section II, Page 56.

⁹ Technical Dossier/ Section II, Annexes.

¹⁰ Technical Dossier/ Section II., Page 65, Annex.

equivalent to 1.22 µg Benzo(a)Pyrene/kg. Organochlorine and organophosphorus pesticides were below detection levels.¹¹

Batch four was tested for microbial contamination.¹² *Salmonella* was absent while *Staphylococcus*, *Clostridium perfringens*, sulfite reducing bacteria, coliforms and Enterobacteriaceae were <10 CFU/g product. Enterococci (“faecal streptococci”) were found at 80 CFU/g product.

A particle size analysis (laser diffraction analysis) showed that 21 % of the particles had a size <50 µm and 5.6 % <10 µm.¹³ Dustiness was determined by rotating drum (CEN, 2006). The critical fractions determined in dust were 841 (<50 µm), and 52 (<10 µm) mg/kg, respectively. The measured values allow the dustiness of the product to be classified as moderate. However, the relative standard deviation was high.¹⁴

2.2. Characterisation of the active substance

L-isoleucine (synonyms: 2-amino-3-methylvaleric acid; α-amino-β-methylvaleric acid; (2S,3S)-2-amino-3-methylpentanoic acid) CAS RN number: 73-32-5, EINECS number: 200-798-2 is an essential BCAA. The molecular formula is C₆H₁₃NO₂; the molecular weight is 131.17. The structural formula is given in Figure 1.

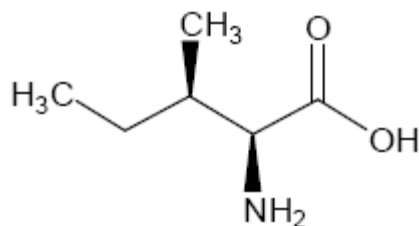


Figure 1. Structural formula of L-isoleucine

2.3. Characterisation of the production organism

2.3.1. Information relating to the genetically modified microorganism

2.3.1.1. Characteristics of the recipient or parental microorganism

The recipient strain is *Escherichia coli* K12S B-7 (also named VKPM B-7). This strain is derived from *E. coli* K12 by mutagenesis (UV irradiation).

E. coli K12 is non-pathogenic and non-toxic, as verified by numerous studies. Furthermore, *E. coli* K12 is listed as an example of a non-pathogenic and biologically-contained host microorganism (Good Industrial Large-Scale Practice microorganism) by the OECD (OECD, 1986).

In addition, the applicant confirmed previous observations of the non-pathogenic nature of *E. coli* K12 by providing PCR data on the absence from the recipient strain K12S B-7 of genes encoding ten

¹¹ Technical Dossier/ Section II. Page 66, Annex.

¹² Technical Dossier/ Section II, Page 73, Annex.

¹³ Technical Dossier/ Section II. Paragraph 2.1.5.1, Annex.

¹⁴ Technical Dossier/ Section II. Paragraph 2.1.5.2, Annexes.

different virulence factors (heat-labile toxin, heat-stable toxin, verotoxin, enterohaemolysin A, intimin, adherence factor, invasivity factor, adhesins, enterotoxin 1, aerobactin).¹⁵

2.3.1.2. Characteristics of the donor organisms

The donor organisms are *E. coli* K12 strains and *E. coli* H155. The applicant provided tests indicating the absence of factors of adhesion, invasion, survival in tissues, cytotoxicity or cytotoxicity in *E. coli* H155. Furthermore, the absence of the production of diarrhoeagenic enterotoxins (*i.e.* heat-labile enterotoxins, heat-stable enterotoxins) and verotoxins was confirmed. Thus, *E. coli* H155 can be considered a non-pathogenic strain.¹⁶

2.3.1.3. Description of the genetic modification process

Several cassettes were integrated into the chromosome of the final production strain *E. coli* K12 AG3149 (FERM ABP-10641) by the use of the Mu system or transduction with phage P1, in order to deregulate the expression of the enzymes of precursors of the isoleucine synthesis pathway, increase the metabolic flow towards isoleucine synthesis and increase the production of L-isoleucine. Southern analysis of the production strain confirmed the presence of five to seven copies of each cassette.¹⁷

In addition, several genes have been deleted to increase the metabolic flow towards the synthesis of L-isoleucine and other genes of interest for the production of this amino acid have been introduced in a single copy.

During the construction of the production strain, two antibiotic resistance marker genes were introduced in intermediate strains. Both marker genes were, subsequently, excised (one gene after having been, previously, inactivated). The excision of the two antibiotic resistance marker genes was confirmed by PCR analysis.^{18, 19, 20}

2.4. Manufacturing process

The product L-isoleucine is obtained by fermentation using the genetically modified strain of *E. coli* (K-12 3149 FERM ABP-10641) in aerobic and discontinuous procedure in large aerated aseptic containers. After fermentation, the cells in the broth are inactivated. Bacteria are separated from the broth by continuous ultrafiltration technique. The effluent is treated with decolourisation resins and concentrated up to the crystallisation point of L-isoleucine and cooled. After filtration, re-suspension in water and centrifugation, the crystals are dried.²¹

2.5. Information relating to the production process

The production of L-isoleucine of feed grade has not yet started on an industrial scale. The products used for carrying out the different analyses and studies for the preparation of the dossier were produced at a “bench-pilot” scale.²²

¹⁵ Confidential information / Confidential Annexes.

¹⁶ Technical Dossier/ Section II

¹⁷ Supplementary information October/ 2009

¹⁸ Supplementary information October/ 2009

¹⁹ Confidential information/ Section II

²⁰ This section has been edited following the provisions of Article 8.6 and 18 of Regulation (EC) No 1831/2003

²¹ Technical Dossier/ Section II/ Paragraph 2.1.3.1.

²² Technical Dossier/ Section II

2.6. Information relating to the product purification process

The L-isoleucine of feed grade was extracted and purified on a “bench-pilot” scale.

Three batches (extracted and purified from two fermentations) did not contain any colonies corresponding to *E. coli* K12 AG3149 (FERM ABP-10641) in 1 g samples of the product. This was verified by an independent laboratory for one batch.

Possible presence of recombinant DNA from *E. coli* K12 AG3149 was investigated by PCR. A primer set targeting a cassette present in six copies and amplifying a fragment of 506 bp was used. No recombinant DNA was detected in three batches of the product with the detection limit provided by the applicant.^{23, 24}

2.7. Physical-chemical and technological properties of the additive

2.7.1. Stability

A blend of the first three batches of the product L-isoleucine was kept for 12 months at 5°C, 25°C and 40°C and at 25°C and 40°C at 60 % and 30 % relative humidity (RH). No change in the content of L-isoleucine was observed; only a small reduction in levels of other minor amino acids was detected after 12 months. Since the batches tested were produced one year before the start of stability measurements, the applicant claims that the shelf-life of the product L-isoleucine could be two years.²⁵

L-isoleucine is known to be not particularly sensitive to light (Santos Ramos *et al.*, 1990), or to oxidation in air (Mudd *et al.*, 1969) and to be stable under a wide range of pH.²⁶

Experimental evidence on stability of the product L-isoleucine in premixtures was not provided.

Stability of L-isoleucine (the same blend of three batches) was tested in pelleted complete piglet diets prepared for a tolerance study. Five months storage with different levels of L-isoleucine at 5°C and 60 % RH, 25°C and 60 % RH, and 40°C and 60 % RH resulted in free L-isoleucine recoveries of 102, 100 and 96 %, respectively.

2.7.2. Homogeneity

The applicant intended to demonstrate the homogeneity of the product L-isoleucine in mash feed based on consecutive sampling of mixed feed during the mixing process in 50-seconds intervals.²⁷ This method is not considered suitable to conclude on homogeneity.

2.7.3. Physico-chemical incompatibilities or interactions

No physico-chemical incompatibilities are expected with other additives, medicinal products or the components of feedingstuffs. Furthermore, the applicant refers to the fact that no incompatibilities or interactions have been reported to date, with respect to the use of the pharmaceutical grade of L-isoleucine for food applications.²⁸

²³ Confidential information/ Confidential Annexes.

²⁴ This section has been edited following the provisions of Article 8.6 and 18 of Regulation (EC) No 1831/2003

²⁵ Technical Dossier/ Section II/ Paragraph 2.4.1.1/ Annex.

²⁶ Technical Dossier/ Section II/ Section II/ Paragraph 2.4.1.1/ Annex.

²⁷ Technical Dossier/ Section II/ Paragraph 2.4.2/ Annex

²⁸ Technical Dossier/ Section II/ Paragraph 2.4.4

2.8. Conditions of use

The product L-isoleucine is proposed to be supplemented to feedingstuffs which are deficient in the essential amino acid L-isoleucine.

2.9. Evaluation of the analytical methods by the Community Reference Laboratory (CRL)

EFSA has verified the CRL report as it relates to the methods used for the control of the product L-isoleucine in animal feed. The Executive Summary of the CRL report can be found in the Appendix.

3. Safety

3.1. Safety aspects of the genetic modification

3.1.1. Information relating to the GMM and comparison of the GMM with its conventional counterpart

a) Description of the genetic trait(s) or phenotypic characteristics and any new trait which can be expressed or no longer expressed

The identity of the production strain *E. coli* K12 AG3149 (FERM ABP-10641) was studied by ribotyping and serotyping, confirming its derivation from *E. coli* K12.²⁹ The production strain shows a significant increase of metabolic flux towards L-isoleucine synthesis, compared with its conventional counterpart *E. coli* K12S B-7. This results from modifications allowing: 1) the increase of the metabolic flow towards the synthesis of precursors of the isoleucine synthesis pathway; 2) the increase of the metabolic flow towards the synthesis of L-isoleucine; 3) deregulation of the expression of enzymes of precursors of the isoleucine synthesis pathway; 4) deletion or inactivation of enzymes of the degradation pathway of isoleucine precursors and 5) integration of other genes of interest for the synthesis of isoleucine.³⁰

b) Structure and amount of any vector and/or donor nucleic acid remaining in the final construction of the modified microorganism

E. coli K12 AG3149 contains a mutated operon, six copies of the cassette constructed to increase the metabolic flow towards isoleucine synthesis via some of its precursors, seven and five copies of two cassettes constructed to increase the production of L-isoleucine and one copy of other genes of interest for the production of this amino acid. This has been confirmed by Southern analysis.³¹

No antibiotic resistance marker genes are present in the final production strain *E. coli* K12 AG3149 as confirmed by PCR and phenotypic analysis.^{32, 33}

3.1.2. Conclusions regarding the genetic modification

It was considered that the genetic modifications present in the production strain do not trigger safety concerns regarding the final product. All inserted DNA was derived from non-pathogenic *E. coli* strains and no sequences which cause concern were added to the production strain *E. coli* K12 AG3149. Any toxic consequences of unanticipated alterations in metabolic flow would have been revealed by the toxicological studies that were undertaken on the product.

²⁹ Technical Dossier/ Section II

³⁰ Confidential information/ Confidential Annexes.

³¹ Supplementary information October/ 2009

³² Supplementary information October/ 2009

³³ This section has been edited following the provisions of Article 8.6 and 18 of Regulation (EC) No 1831/2003

3.2. Safety for the target species

Tolerance studies with nutrients like the essential amino acid L-isoleucine cannot be designed along the lines of conventional toxicity experiments since the appearance of amino acid imbalances at higher dosages will restrict the desired margin of safety.

The tolerance of animals to overdoses of amino acids varies with the amino acid (substance and isomeric form), the dietary protein content and the animal species.

Moreover, it is known (Harper *et al.*, 1984) that the BCAA isoleucine, valine and leucine exert a strong antagonism on each other, 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. Relative excesses of isoleucine or valine are better tolerated by almost all animal species than dietary overdoses of leucine. The interaction of BCAAs fed at excessive levels has already been described in the former FEEDAP opinion on L-valine (EFSA, 2008).

Tolerance studies are normally not required for highly purified amino acids even if produced by fermentation. Essential amino acids are generally considered safe. Species differences in the sensitivity to amino acids excess are known.

The applicant provided one experiment with piglets fed diets with two supplementation levels of the product L-isoleucine.

3.2.1. Experiment with piglets

The experiment was conducted with four groups of 16 piglets each, to study the effect of excessive additions (10-fold) of L-isoleucine and L-valine alone or in combination to a grain/soybean meal starter diet. The source of the product L-isoleucine in the study was a mixture of the first three batches.³⁴ Feed intake and body weight were recorded weekly. Blood samples were taken at the end of the experimental period (21 days) after an overnight fast, from a total of 20 piglets (5 per group). The plasma samples were analyzed for amino acids concentrations. The same piglets were slaughtered and necropsy was performed. The weight of the carcass, liver and kidney was recorded. Statistical analyses are based on 4 groups of 16 animals each (5 piglets per group for the plasma amino acids).

For the purpose of assessing L-isoleucine tolerance, only two groups will be considered further (Table 2). The feed intake, weight gain and feed/gain ratio and the plasma concentration of isoleucine and valine are summarised in Table 2.

³⁴ Technical Dossier/ Section II/ Paragraph 2.1.3.2/Annexes.

Table 2. Effects of L-isoleucine in a tolerance test in piglets (12 to 21.4 kg bw; 21 days)

	Control	Ile 10 fold
L-Ile addition (%)	0.0760	0.7605
L-Val addition (%)	0.1019	0.1019
Free L-Ile (%) analysed	0.074	0.643
Free L-Val (%) analysed	0.111	0.126
Total L-Ile (%) analysed ³⁵	0.69	1.26
Total L-Val (%) analysed	0.81	0.83
Feed intake (g/day)	767	786
Weight gain (g/day)	459	475
Feed/gain (kg/kg)	1.73	1.67
Plasma Val (mg/L)	23.7	23.7
Plasma Ile (mg/L)	15.9	20.6
Plasma Leu (mg/L)	23.0	20.3

An increase of L-isoleucine supplementation from the product by the factor ten compared to control diet, resulting in L-isoleucine concentrations of about twice the requirement of piglets (0.63 % Ile for piglets of 10 – 20 kg bw (NRC, 1998)), slightly improved feed intake, weight gain and feed efficiency in piglets, however the differences were not significant. The plasma levels of leucine and valine were not significantly influenced by high L-isoleucine.

3.2.2. Conclusions on the safety for target species

The product L-isoleucine contains less than 1 % of unidentified compounds and is therefore considered safe for the target species. The results of the short-term study support the above general statement.

3.3. Safety for the consumer

As a general principle, conventional toxicology studies are considered to be inappropriate for testing pure substances which are (essential) dietary nutrients. Such substances have a physiological concentration which is optimum for health and performance.

Dietary intakes of such substances, that lead to amounts significantly below or above that which is optimum for health and performance, will inevitably cause a physiological imbalance and consequent adverse effects. This principle applies to substances where the purity is well established, with the source and method of production sufficiently well characterised for reassurance that no toxic contaminants will be present in the product. The testing appropriate to such substances will need to be judged on a case-by-case basis, but in circumstances where the use of the substance leads to no increase in human intake there is no requirement for further toxicity data.

For a product which is a physiological nutritional substance and shows less than 1 % of unknown substances it is assumed that it will behave as a normal nutrient. Thus there is no requirement for studies to provide evidence of consumer safety. The applicant has submitted a range of studies which confirm this assumption by testing in laboratory animals. Since these studies are not required for the assessment, these are reported in Appendix B.

3.3.1. Conclusion on consumer safety.

L-isoleucine will be incorporated in the body's protein of the animal. The body protein composition will not be changed. Free isoleucine will not be stored in the tissues. Based on the purity of the

³⁵ Technical Dossier/ Section III/ Paragraph 3.1.1.1

product, it is concluded that no safety concerns for the consumer would result from the use of L-isoleucine as feed additive. This conclusion is further supported by the additional toxicity studies provided.

3.4. Safety for the user

The studies submitted concerning user safety have been conducted with one of the batches of the bench pilot scale production of L-isoleucine made in 2004 (Batch 1).

3.4.1. Effects on the respiratory system

A group of five rats of each sex was exposed by nose-only inhalation to a limit concentration of $5.41 \pm 0.20 \text{ g/m}^3$ for 4 hours.³⁶ The study was conducted in compliance with OECD guideline 403 and animals were observed for 14 days after exposure. There were some slight adverse effects seen in posture and appearance during and immediately after exposure but no other clinical abnormalities were recorded during the observation period. Body weight gain was reduced during the first week after treatment. At necropsy, grey discolouration of the lungs was noted.

3.4.2. Effects on the eyes and skin

A skin irritation study was conducted in three rabbits according to OECD guideline 404 and Method B.4 of the Annex part B of Directive 92/69/EEC.³⁷ There was no evidence of irritant effects at any point thus the product L-isoleucine is classified as not irritating to human skin.

An eye irritation study was conducted in three rabbits according to OECD guideline 405 and Method B.5 of the Annex part B of Directive 92/69/EEC.³⁸ There were slight effects immediately after treatment which had resolved fully by 48 h, thus the product L-isoleucine is classified as not irritating to human eyes.

Potential for dermal sensitisation was assessed in local lymph node assay conducted in mice according to OECD guideline 429.³⁹ Although there was a significant difference between L-isoleucine and the vehicle group in ³H-thymidine incorporation, the difference fell well below the threshold for a positive result, thus the product L-isoleucine is not considered able to act as a skin sensitiser under the conditions of the test.

3.4.3. Conclusions on user safety

The product L-isoleucine is considered to be non irritant to skin and eyes and not to be a dermal sensitiser. The product is moderately dusty. The minor changes that were seen in the lungs of rats after acute inhalation exposure to dust are not considered of toxicological concern.

3.5. Safety for the environment

No environmental impact from the use of this product is expected on the basis of the sequences incorporated. The absence of production microorganism was demonstrated and the recombinant DNA in the product is below the limit of detection of the method used. Therefore, no further environmental risk assessment is required.

³⁶ Technical Dossier/ Section III/ Paragraph 3.3.1.1 and Annexes.

³⁷ Technical Dossier/ Section III/ Paragraph 3.3.1.2.1 and Annexes.

³⁸ Technical Dossier/ Section III/ Paragraph 3.3.1.2.2 and Annexes.

³⁹ Technical Dossier/ Section III/ Paragraph 3.3.1.2.3 and Annexes..

L-isoleucine is a physiological amino acid and a natural component of animals and plants whose use in animal nutrition would substitute/complement for isoleucine occurring in dietary protein and would not increase excretion into the environment. Consequently the use of the product as a feed additive does not represent a risk to the environment.

4. Efficacy

Published studies were submitted with the dossier showing the benefits of L-isoleucine supplementation to blood product containing diets and to protein-reduced diets for pigs. Under field conditions, L-isoleucine deficiency or amino acids imbalances which can be corrected by L-isoleucine addition may be expected in diets considerably reduced in protein and already supplemented with first (more) limiting amino acids. Low protein diets reduce the nitrogen excretion and provide therefore benefit to the environment. The availability of limiting amino acids in synthetic form opens the possibility to compose diets with equivalent nutritional value and provides more flexibility to the feed compounder.

A short-term study is required to support efficacy for amino acids salts and analogues not already authorised as feed additives. Bioavailability studies may be used as short-term studies to demonstrate the utilisation of a novel additive (e.g., amino acids). Only one trial is required in a single animal species/category including laboratory animals. An assessment of the efficacy of L-isoleucine feed grade can therefore be based on a study of L-isoleucine bio-equivalence.

4.1. Bioequivalence study

Altogether, 48 weaned piglets (gilts and barrows; Piétrain x German Landrace; initial weight 8 kg; ten animals per treatment, except highest inclusion levels: nine animals) were assigned according to weight and sex to five treatments for 42 days, after an adaptation period of three days.⁴⁰ Each animal was housed individually. The piglets were fed a control diet (wheat, corn, barley, blood cells, corn gluten feed; first-limiting in isoleucine) or diets supplemented with L-isoleucine either from feed grade (0.102 %; 0.206 %; batch B4-2006, containing 97.1 % L-Ile) or pharmaceutical grade (0.100 %; 0.200 %; batch 050402 containing 99.9 % L-Ile). Isoleucine supplementation was confirmed by analysis. Feed and water were provided *ad libitum*. Health status was checked daily. Weight gain and feed intake was recorded weekly. The overall results are described in Table 3.

Table 3. Effects of L-isoleucine in a bioequivalence test in piglets (0 - 42 days)

L-Ile (%) supplementation	Feed-grade			Pharma-grade	
	0	0.102	0.206	0.100	0.200
Free L-Ile (%) analysed	<0.02	0.105	0.192	0.105	0.198
Total L-Ile (%) analysed	0.41	0.49	0.59	0.50	0.59
Feed intake (g/day)	289 ^c	478 ^b	748 ^a	509 ^b	693 ^a
Weight gain (g/day)	161 ^c	306 ^b	499 ^a	330 ^b	465 ^a
Feed/gain (kg/kg)	1.87 ^a	1.57 ^b	1.50 ^b	1.55 ^b	1.50 ^b
Feed/gain (kg/kg)	1.87 ^a	1.57 ^b	1.50 ^b	1.55 ^b	1.50 ^b

^{a, b, c}: Means in a row not sharing a common superscript are significantly different (P < 0.05)

The piglets appeared to be in good health during the experiment. Significant increases in feed intake and weight gain of the piglets were observed with increasing L-isoleucine supply. The differences in performance parameters between the two L-isoleucine sources were not statistically different. Therefore bio-equivalence of feed grade L-isoleucine to pharmaceutical grade L-isoleucine is established.

⁴⁰ Technical Dossier/ Section IV and Annexes.

4.2. Conclusions on efficacy for all species

The product L-isoleucine is considered as a source of available isoleucine for all animal species.

5. Post-market monitoring

No risks associated with the use of the product are foreseen. It is considered that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation⁴¹ and Good Manufacturing Practice.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

The introduced genes do not trigger safety concerns in terms of toxins or transferable antibiotic resistance. The final preparation contains no cultivable production organisms and the level of the newly introduced DNA is below the limit of detection.

It is concluded that the product L-isoleucine is safe for all animal species when added to diets to cover the isoleucine requirement.

The product L-isoleucine contains less than 1 % unidentified impurities and therefore it has no safety concerns for the consumer.

The product L-isoleucine is considered to be non irritant to skin and eyes and not to be a dermal sensitiser. The only detectable risk for the user could be derived from the dustiness of the product, but on the basis of an acute inhalation toxicity study, this is expected to be minor.

The product L-isoleucine is a physiological amino acid and a natural component of animals and plants whose use in animal nutrition would substitute/complement for isoleucine occurring in dietary protein and would not increase excretion into the environment. Consequently the use of the product as a feed additive does not present a foreseeable risk to the environment.

Based on the available knowledge and a bioequivalence study in piglets, it is concluded that the product L-isoleucine is a source of available isoleucine for all animal species.

RECOMMENDATIONS

The FEEDAP Panel recommends that:

The purity of the product should reflect the minimum L-isoleucine content (93.4 %) and specify a maximum of 1 % unidentified material, both on a dry matter basis.

The deposition number of the production micro-organism only should be included in the specifications of the additive (e.g., only FERM ABP-10641).

When considering protective measures in the use of the product L-isoleucine, its dustiness should be taken into account particularly for persons handling the product, as proposed by the applicant.

DOCUMENTATION PROVIDED TO EFSA

1. L-isoleucine. January 2009. Submitted by Ajinomoto Eurolysine.

⁴¹ OJ L 35, 8.2.2005, p.1

2. Supplementary Information received on October 2009. Submitted by Ajinomoto Eurolysine.
3. Evaluation report of the Community Reference Laboratory for Feed Additives on the methods of analysis for L-isoleucine.
4. Comments from Member States received through the ScienceNet.

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http://www.efsa.europa.eu/EFSA/Scientific_Opinion/feedap_op_ej695_valine_en.pdf?sbinary=true
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APPENDICES

APPENDIX A

Executive Summary of the Evaluation Report of the Community Reference Laboratory for Feed Additives on the Method(s) of Analysis for L-isoleucine for all animal species.

In the current application authorisation is sought for L-isoleucine under the category 'nutritional additives', functional group 'amino acids, their salts and analogues', according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use L-isoleucine for supplementing feed for all animal species. The product is a crystalline powder with a minimum content of 92 % L-isoleucine. The feed additive is intended to be included into premixtures and feedingstuffs at a final concentration depending on the concentration of L-isoleucine already present in the feed components and on the nutritional requirements of the different animal species.

For the determination of the active substance (L-isoleucine) in the *feed additive*, *premixtures*, and *feedingstuffs* the applicant proposes the official Community and fully ring-trial validated method for determination of amino acids [Commission Regulation (EC) No 152/2009]. The method is applicable for both the determination of free (synthetic and natural) and the determination of total (peptide-bound and free) amino acids including L-isoleucine, using an amino acid analyser or High Pressure Liquid Chromatography (HPLC) combined with post-column derivatisation using ninhydrin as derivatisation agent and photometric detection at 570 nm. The same method is adopted by ISO and described in the ISO standard 13903:2005 [Animal feedingstuffs – determination of amino acids content], which additionally reports the results from a second intercomparison study performed on different premixtures and feeds [Llames & Fontaine, J. of AOAC Int., Vol. 77, No. 6, 1994]. Performance characteristics for the target analyte (L-isoleucine) include the relative standard deviation for repeatability (RSD_r) ranging from 2.00 to 5.38 % and relative standard deviation for reproducibility (RSD_R) ranging from 6.84 to 14.62 %, depending on the matrix. The method is suitable for official controls for the determination of free and total L-isoleucine in *feedingstuffs*. Although performance characteristics for the *feed additive* itself and, for *premixtures* are not available, the method can be considered suitable also for official control of active substance in these matrices. It is not suitable to differentiate between the salts or D- and L-forms of amino acids, or between naturally occurring and added L-isoleucine. Alternatively, validated methods based on the same techniques, such as the method 4.11.6 of the Association of German Agricultural Analytical and Research Institutes (VDLUFA) [Methodenbuch III, 5. Erg. 2004, VDLUFA – Verlag, Darmstadt] and the similar AOAC Method 999.13 [Fontaine and Eudaimon, J. of AOAC Int., Vol. 83, No. 4, 2000] can complement the official Community method for the determination of L-isoleucine in the *feed additive* and in *premixtures* and therefore are considered suitable for official control purposes in the frame of the authorisation. Further testing or validation by the CRL is not considered necessary.

Appendix B

Consumer safety studies

Studies submitted concerning mutagenicity and toxicity in laboratory animals have been conducted with the product L-isoleucine Batch 1, unless otherwise specified.

i. Acute toxicity studies in rats

In an acute limit test (OECD 423), two groups of three rats were given 2000 mg L-isoleucine/kg by gavage and monitored for 14 days before being examined at necropsy.⁴² No adverse effects were observed in any of the animals at any time. From these results the product L-isoleucine is considered not harmful if swallowed.

ii. Genotoxicity studies including mutagenicity

The product L-isoleucine was tested for mutagenic activity (OECD guideline 471) in four strains of *Salmonella* Typhimurium (TA100, TA98, TA1535 and TA1537) and *Escherichia coli* WP2_{uvrA} both with and without metabolic activation, at concentrations ranging from 62 to 5000 µg/plate.⁴³ The test showed no evidence of mutagenicity.

The product L-isoleucine was examined for potential to induce gene mutations using mouse lymphoma L5178Y cells (OECD 476) both with and without metabolic activation. The dose tested under both conditions ranged from 0.076 to 1.25 mg/mL.⁴⁴ No increases in mutant frequency were seen at any dose thus it is concluded that L-isoleucine is not mutagenic in this assay under the conditions used.

The product L-isoleucine was tested in a chromosome aberration assay using Chinese Hamster Ovary cells (OECD 473) both with and without metabolic activation at doses up to the maximum of 1.31 mg/mL.⁴⁵ There was no significant increase in chromosome aberrations under either condition thus it is concluded that L-isoleucine is not clastogenic under the conditions of the test.

iii. Sub-chronic repeated dose oral toxicity studies

Groups of ten rats of each sex were given the product L-isoleucine at constant concentrations of the active ingredient of 0, 2000, 10000 or 50000 mg/kg feed for 90 days, equivalent to approximately 0, 100, 600 and 3000 mg/kg bw of active ingredient, respectively.⁴⁶ The study was conducted in accordance with OECD guideline 408 and B.26 of Annex 5D of Directive 2001/59/EC and included the full range of required observations plus evaluation of oestrous cycle and sperm characteristics.

The only differences between treated and control groups were a slight increase in red blood cells and haemoglobin concentration in high-dose males, a reduction in thrombocyte count of high-dose females and a significant increase in the mean length of the longest oestrous cycle in the same group. Otherwise there were no differences which could be attributed to treatment with L-isoleucine.

Although the differences seen are of questionable toxicological significance the NOAEL is set at the intermediate dose of 10000 mg/kg feed or 600 mg/kg bw of active ingredient.

⁴² Technical Dossier/ Section III/ Paragraph 3.2.2.1.1 and Annexes.

⁴³ Technical Dossier/ Section III/ Paragraph 3.2.2.2.1 and Annexes.

⁴⁴ Technical Dossier/ Section III/ Paragraph 3.2.2.2.2 and Annexes.

⁴⁵ Technical Dossier/ Section III/ Paragraph 3.2.2.2.3 and Annexes.

⁴⁶ Technical Dossier/ Section III/ Paragraph 3.2.2.3.1 and Annexes.

iv. Chronic oral toxicity studies

Groups of 50 rats of each sex received diets containing L-isoleucine (purity 100 %) at 0, 25000 or 50000 mg/kg feed for 104 weeks.⁴⁷ Animals were examined once daily; body weight and feed intake were measured regularly throughout the study. Urinalysis was conducted on ten rats per group during weeks 26, 52, 78 and 104. Blood samples collected at necropsy were used for haematological measurements and to prepare serum for clinical chemistry analyses. All animals were subject to a post-mortem examination and tissues preserved for histological examination.

Survival rate at 104 weeks for the males was 86 %, 82 % and 74 % for the groups receiving 0, 25000 or 50000 mg/kg feed, respectively. For females the figures were 84 %, 86 % and 76 %. The body weight of treated rats showed an increase compared with controls particularly towards the end of the study, although feed intake was similar in all groups. Urinalysis and haematology results did not differ between the groups at any time during the study. Some differences were seen in the results of clinical chemistry measurements but these were mostly confined to one sex and to the highest dose. Relative kidney weight was increased in high-dose males, while testes weight was decreased in the same group. There was no difference histologically between kidneys of treated and control groups, however the reduced testes weights were associated with evidence of atrophy. Tumour incidences were not significantly different between the groups, apart from a lower incidence of C-cell adenoma of thyroid in high-dose males. The NOAEL is concluded to be 924.7 mg kg bw/d.

v. Reproduction toxicity studies

A one-generation reproduction study (OECD guideline 415) was conducted in groups of 28 rats of each sex receiving diets containing L-isoleucine at 0, 10000, 20000, 30000, 40000 or 50000 mg/kg feed.⁴⁸ Rats received the diets for 14 days prior to mating. The dietary levels were calculated from body weight and feed intake data to be equivalent to 0, 550, 1080, 1580, 2110 or 2720 mg/kg bw for males and 880, 1750, 2610, 3430, 4280 mg/kg bw for females. There were no treatment-related effects on fertility or reproductive performance or on the size, sex ratio or weight of litters. At the highest dose there were significantly more pups classified as runts at day 21, compared with controls and other treatment groups. Although the authors conclude that the effect seen was not to be regarded as adverse since mean weights were not affected, the highest dose in this study could not be considered to be fully tolerated during the reproductive cycle.

A prenatal developmental toxicity study in rats compared groups receiving diets containing L-isoleucine at 0, 2000, 10000 or 50000 mg/kg feed from fertilisation to day 21.⁴⁹ The study was conducted according to OECD guideline 414 and B.31 of Annex 2F of Directive 2004/73/EC.

Body weight was unaffected by treatment but feed intake was decreased in animals at the highest dose over days 7-10 and 14-17. Test substance intake was calculated from feed intake and body weight data to be 0, 90-150, 480-750 and 2320-3640 mg/kg bw for the groups from control to high dose respectively. No differences were seen in any of the observations made during the study.

⁴⁷ Technical Dossier/ Section III/ Annexes.

⁴⁸ Technical Dossier/ Section III/ Paragraph 3.2.2.5.1 and Annexes.

⁴⁹ Technical Dossier/ Section III/ Paragraph 3.2.2.5.2 and Annexes.