EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Scientific Opinions
C2 - Management of scientific committees II; scientific co-operation and networks

Opinion of the Scientific Committee on Animal Nutrition on the use of Tryptosine™ 15/70

(Adopted on 16 October 2002)

1. BACKGROUND

A request for inclusion in point 3 of the Annex of Directive 82/471/EEC concerning certain products used in animal nutrition of a blend of L-lysine-HCl (70%°) with L-tryptophan (15-20%) and its residues of fermentation with *Escherichia coli K-12* has been submitted by ADM Ölmühlen Beteiligungsgesellschaft mbH. The name of the product is Tryptosine™ 15/70 (identified hereafter as Tryptosine™) and is intended for use in piglets, pigs for fattening and chickens for fattening.

2. TERMS OF REFERENCE (NOVEMBER 2000)

The Scientific Committee for Animal Nutrition is therefore requested to give an opinion on the following questions:

2.1. Does the blend of L-lysine-HCl (70%°) with L-tryptophan (15-20%) and its residues of fermentation with *Escherichia coli K-12,* under the conditions proposed in feedingstuffs for piglets, pigs for fattening and chickens for fattening, have nutritional value?

2.2. Does the use of a blend of L-lysine-HCl (70%°) with L-tryptophan (15-20%) and its residues of fermentation impair the organoleptic characteristics of animal products?

2.3. Is it safe

- for the target animal categories (piglets, pigs for fattening and chickens for fattening)
- for the consumer?
- for the workers and the user?
- for the environment?

2.4. Does the manufacturing process of tryptophan executed by fermentation of a genetically modified bacteria (*Escherichia coli* K-12) and its products of

fermentation have detrimental effect on human or animal health or on the environment?

3. OPINION OF THE COMMITTEE

The dossier consists of 196 pages and contains large sections in German and English.

3.1. Characteristics of the product

Tryptosine™ is a blend of commercially available L-lysine HCl (according to Directive 82/471/EEC) with an L-tryptophan containing ultrafiltrate from a batch fermentation process. It consists of 70 % L-lysine HCl (min. 78 % lysine), 15-20 % L-tryptophan and 7.5-15.2 % soluble fermentation residues (fermentation nutrients) on a 98 % dry matter basis. The feed grade L-lysine HCL and the ultrafiltrated fermentation broth are dried together.

An approximate nutrient analysis gives averages of 92 % crude protein (14.4-15.2 % N), 0.1-0.15 % ether extract, 0.10-0.25 % crude fibre, 1.0-1.5 % ash and 1.0-3.0 % glucose. The product also contains traces of other amino acids (between 0.06 and 0.28 % for the individual amino acids threonine, serine, glycine, alanine, cysteine, valine, isoleucine, histidine, ornithine and glutamic acid).

The ash fraction contributes to 0.23 % Ca, 0.10 % Na, 1.40 % K and 0.05 % P as macro elements in the product, to 0.01 % Fe, 0.01 % Zn, 0.13 mg Pb, 0.01 mg Hg, 0.02 mg Cd, 0.025 mg As and <0.025 mg Se/kg product as trace elements.

Tryptosine™ is free from Salmonella and *E.coli*, it contains less than 3 colony forming units per plate counts yeast and mould as well as coliforms per g, less than 5 Enterobacteriaceae/g and less than 10 Staphylococci/g. The production strain could not be found.

3.1.1. Fermentation product

L-tryptophan is produced by the batch fermentation of a genetically engineered strain of *E. coli* K-12, grown on a medium containing glucose, soya flour, cornsteep liquor, vitamins and minerals (including inorganic nitrogen). After fermentation, the broth is heat-treated, and then passed through a series of ultrafiltration, separation and concentration processes to yield a product which, at this stage, typically contains 55% (range 50-80%) of the total dry matter as L-tryptophan. The remainder of the product consists of low molecular weight products derived from the spent medium. The heat treatment applied is said to be sufficient to kill the production strain and all cell debris is removed by the ultrafiltration. The product is then mixed with L-lysine HCl and dried to give the final formulation. No DNA is detectable in the final formulation (detection limit 1 ng/g additive).

The containment conditions used are said to meet the requirements of the United States National Institute of Health safety standards and to be

consistent with those of Annex II, IV and V of 90/219/EEC, the European Directive regulating the contained production of genetically modified micro-organisms. However, the site of manufacture is not provided and it is unclear what regulatory scrutiny has been applied to the production process or to the engineered strain.

3.1.2. The parent strain and its modification

E. coli K-12 is one of the most thoroughly studied of all strains of bacteria and was one of the first to have its genome fully sequenced and proteome described (www.genome.wisc.edu/). Because of the lack of any virulence factors and because it has never been associated with any clinical symptoms, K-12 is often the strain of choice both for laboratory investigations and for industrial purposes. A modified strain of K-12 is one of the sources of recombinant human insulin.

The parent strain was originally isolated from the human gut and it survives poorly in other environments. Although isolated from the gut, K-12 is unable to compete with other strains of *E. coli* when reintroduced into the human or animal digestive tract. The underlying mechanisms for this inability to colonise has been extensively studied and has been ascribed to defects in the lipopolysaccharide structure of the cell wall (defective *rfb* gene cluster), the absence of functional fimbriae and an inability to express K antigens *in vivo*. This general lack of pathogenicity led the United States Environmental Protection Agency (EPA) to exempt K-12 strains and derivatives from parts of the EPA safety review.

The strain of K-12 used was transformed with plasmid p5LTRPS-2 made commercially available by Genencor International. The plasmid of approximately 14 kb has been fully sequenced and shown to contain the tryptophan operon, a set of five structural genes (*trpA* to *trpE*) expressed as a polycistronic mRNA, encoding the five enzymes required for the synthesis of L-tryptophan from chorisamate. These genes were derived from another strain of K-12. The plasmid also carries a *tetR* gene as selection marker, which confers resistance to tetracycline by an efflux mechanism and *aroG* encoding DAHP synthase involved in the regulation of aromatic amino acid production. It is unclear whether this form of DAHP synthase is resistant to feedback inhibition.

3.1.3 Stability

Storability of the product Tryptosine™ is two years from date of manufacturing considering the level of lysine and tryptophan. If incorporated in a mixed feed, stability could be proven for two months considering the dietary tryptophan concentration. Longer intervals were not tested.

3.2. Efficacy

Tryptosine™ is a source of the essential amino acids lysine and tryptophan which are used to improve the quality of dietary protein for pigs and poultry.

Lysine is essentially significant in supplementing pig diets and particularly of protein reduced diets (for environmental reasons). All pig diets will not contain enough lysine from the natural feed ingredients to allow maximum pig growth and protein deposition. Lysine is generally considered as the first limiting amino acid in pig diets.

For corn based diets with a low amount of soybean meal also tryptophan will become limiting.

All experiments to demonstrate the efficacy of lysine and tryptophan in Tryptosine™ are performed under the assumption that Tryptosine™ contains 15 % L-tryptophan and 70 % L-lysine HCl.

The company recommends adding a minimum of 0.1 % Tryptosine™ to diets for piglets, growing pigs and for broiler from 0 to 42 days corresponding to 0.055 % lysine and 0.015 % tryptophan.

3.2.1. Chicken

In a first experiment Tryptosine™ was fed to 3 x 3 male chicks per group from day 10 to day 22 post hatching. Tryptosine™ was compared on a lysine and tryptophan deficient diet after supplementation of lysine and tryptophan with L-tryptophan and L-lysine HCL, respectively. Two fortification levels for each amino acid were used (0.1 and 0.2 % L-lysine HCl, 0.015 and 0.030 % L-tryptophan, either from Tryptosine™ or as feed grade amino acids) both below the requirement.

The responses (weight gain, feed intake and feed conversion) were similar for the amino acids, independent from the source.

In a second experiment tryptophan from Tryptosine™ was compared with the same feed grade amino acid for 4 weeks starting on day 9 post hatching with 4 x 12 male chickens. The tryptophan deficient diet contained 0.16 % tryptophan (calculated from the analysed ingredients), the fortification levels were 0.03 and 0.06 % tryptophan, respectively. The total number of animals on which the final results are based is not known (appendix mentioned but not presented).

Both tryptophan sources improved equally weight gain and feed conversion and protein deposition (calculated from body composition).

Health status measured by macroscopic examination of liver, kidneys and heart complex revealed no differences between the treatments.

3.2.2. Piglet/pig

On 4 x 10 piglets (about 10 kg bw after a 15 days pre-test period) two tryptophan sources (feed grade amino acid and Tryptosine™) were compared at two supplementation levels (0.03 and 0.06 %, respectively) for 4 weeks. The basal diet was tryptophan deficient (0.16 %) but satisfactory in lysine (1.19 % at 18 % crude protein).

No mortality occurred during the trial, but 12 pigs were removed or excluded from the statistical analysis due to serious health (leg) problems.

Both tryptophan levels improved weight gain and feed/gain ratio. The differences in weight gain and feed conversion between the two tryptophan sources were insignificant.

In a second experiment 14 x 2 pigs (36 kg bw at start) were fed for 4 weeks a diet (wheat, barley soybean meal and gluten) fortified with L-lysine HCL, DL-methionine, L-tryptophan and L-threonin. 0.66 % L-lysine HCL and 0.06 % L-tryptophan were added to the control diet, 0.42 % Tryptosine™ (corresponding to 0.29 % L-lysine HCl and 0.06 % L-tryptophan) was added to the treatment diet (in which the L-lysine HCl supplementation was reduced to 0.37 %).

Growth rate of the control and the Tryptosine™ group was 1,090 g/d and 1,060 g/d, feed conversion 2.37 and 2.42 g feed/g gain, respectively. The differences were not statistically significant at the 0.01 % probability level.

3.2.3. Conclusions

Tryptosine™ (blend of crystalline L-lysine-HCl (70%°) with L-tryptophan (15-20%) and its residues of fermentation with *Escherichia coli K-12*) is a source of available amino acids under the conditions proposed in feedingstuffs for piglets, pigs for fattening and chickens for fattening.

The amino acids lysine and tryptophan present in Tryptosine™ show obviously the same feeding value (and availability) as the feed grade amino acids approved under Directive 82/471/EEC. However, the efficacy data submitted are rather poor concerning the number of trials (piglet: 1, pig: 1, chicken: 2), the number of animals (replicates) per treatment in the experiments with poultry, the duration of the experiments and the status of the reports and the fact, that lysine from Tryptosine™ was not tested in poultry.

But an exception from the number of trials (and number of animals) set in comparable guidelines seems permissible because the principal efficacy of adding amino acids to amino acid deficient diets had not to be shown by the data submitted. The aim of the studies was to test if an equivalent effect could be expected from the amino acids contained in Tryptosine™ as it was shown for amino acids already approved.

3.3. Safety aspects

3.3.1. Safety to the target animal

A tolerance study was performed on 3 x 4 male chicks per group during the period 10 to 22 d post hatching. Graded levels of L-lysine HCl and L-tryptophan (1.0 + 0.29, 2.0 + 0.58 and 4.0 + 1.16 %) were supplemented to a standard corn-soybean meal diet either from feed grade amino acids or from Tryptosine™.

Excess lysine and tryptophan caused a gradual amino acid imbalance resulting in depressed feed intake and reduced growth. No differences between the feed grade amino acids and Tryptosine™ could be observed. Already 1.4 % Tryptosine™ showed a slight body weight gain depression.

In a second experiment (only progress report presented) Tryptosine™ levels of 0, 0.167, 0.835, 1.67 and 3.34 % were added to a tryptophan deficient corn, feather meal, soy-bean meal type diet. The diets were fed to 5 replicates of an unknown number of 8 day old female chicks for 14 days. 0.0167 % Tryptosine™ improved weight gain from 227 g to 286 g, feed intake from 390 g to 436 g and feed efficiency from 0.582 g gain/g feed to 0.655 g/g. No further increase was obtained with 0.835 % Tryptosine™, higher levels of Tryptosine™ depressed the gain/feed ratio. The conclusion to the authors that Tryptosine™ is well tolerated by chicks up to 1.67 % or 10 times the normal supplemented level can not be accepted.

One trial was performed on 4 x 3 piglets per group for 2 weeks (no final report presented, only a summary, a research protocol and a preliminary report in tables). Tryptosine™ was added to a tryptophan deficient diet with 0, 1.67, 3.33 and 6.67 %, respectively. The addition of 1.67 % and 3.33 % Tryptosine™ increased ($P < 0.01$) weight gain, feed intake and feed efficiency in comparison to the non supplemented group, but 3.33 % Tryptosine™ at a lesser extent than 1.67 %. The experiment is not well designed, so that the conclusion of the authors (the threshold level appears to reside between 1.67 and 3.33 %) can not be followed. The tolerance level may at the 1.67 % inclusion level or higher but less than 3.33 %, it could also be lower because 1.67 % was the lowest level tested.

3.3.2. Safety of the modified strain

Although the wild-type K-12 strains are considered non-toxigenic, the engineered production strain (GICC0498) and the permeate from its culture were examined for heat labile enterotoxin, heat-stable enterotoxin, verotoxin and endotoxin production. As would be expected of a Gram negative organism, the production strain proved positive for endotoxin (lipopolysaccharide) production and the permeate and ten-fold concentrated permeate weakly positive. All other tests for toxin production proved negative. The appropriate positive and negative controls were included and in each case behaved as expected.

The *tetR* gene conferring antibiotic resistance is expressed in the production strain. However as no viable organism or fragments from the production organism are retained in the final product, and as the product has been demonstrated free of detectable DNA, this does not pose a hazard.

3.3.3. Metabolites of tryptophan

In 1998, an outbreak of eosinophilia-myalgia syndrome (EMS) in humans, an autoimmune disease, was traced back to a single commercial source of L-tryptophan produced by fermentation (*Bacillus* spp.). Six metabolites of tryptophan were found related to the incidence of EMS, of which five have been identified (1,1'-ethylidenebis L-tryptophan, 3-phenylaminoalanine, 2-

(3-indoylmethyl)-L-tryptophan, 3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrro-loindole-2-carboxylic acid, and 2-(2-hydroxindoline)-tryptophan).

Several batches of tryptophan produced from the modified *E.coli* K-12 were included in a survey of tryptophan metabolites in commercial sources of tryptophan produced by fermentation (including the original problem source). None of the metabolites associated with EMS were detected (Simat *et al*., 1999).

Although the production of tryptophan is routinely monitored by HPLC, this seems only to be used for measuring concentration. No routine measures appear in place for the monitoring for any metabolites of tryptophan produced during the fermentation or by oxidation during the heat treatment and subsequent purification stages of manufacture. This could be introduced in conjunction with the routine assay of tryptophan in the fermentation medium and in the permeate after ultrafiltration.

3.3.4 Conclusions

The studies on target animals do not show results which could not be attributed to unphysiological overdoses of amino acids to feed, resulting in amino acid imbalances. But only one experiment (of 3) is designed that such a comparison could really be made. As expected, a margin of safety for Tryptosine™ cannot be established because it depends from the (i) respective amino acid content of the feed and (ii) the amount the total level of the respective amino acid exceeds the requirement in relation to the other essential amino acids. From this point of view, Tryptosine™ can be handled like another amino acid approved.

The engineered production strain was positive for endotoxin production. The permeate which is used for Tryptosine™ was only weakly positive, which is not considered as a risk. Because the permeate does not contain viable organism nor fragments nor detectable DNA, no hazard is expected from the use of the product.

Considering the potential occurrence of EMS in pigs, pure tryptophan is obviously safe at excesses estimated at 30 times the maximal dose that would be used in practice (Chung *et al.*, 1991). Tryptophan produced from the modified *E.coli* K-12 apparently does not contain the relevant tryptophan metabolites implicated in EMS. However a routine assay for tryptophan metabolites in the fermentation medium and in the permeate after ultrafiltration is recommended.

The tryptophan (with its residues of fermentation) should be tested for genotoxicity in a *Salmonella*/microsome reverse mutation assay and in an *in vitro* assay for chromosome aberrations.

3.4. Safety for the worker

No aerosol formation is concluded from the absence of particles < 10 μm. Skin irritation and sensitisation tests were not performed. Safety for the worker can therefore not be sufficiently assessed.

4. CONCLUSIONS

4.1. Nutritional value

The blend of L-lysine-HCl (70%°) with L-tryptophan (15-20%) and its residues of fermentation with *Escherichia coli K-12* has nutritional value under the conditions proposed in feedingstuffs for piglets, pigs for fattening and chickens for fattening.

The amino acids lysine and tryptophan present in Tryptosine™ show the same value as the feed grade amino acids approved under Directive 82/471/EEC. There is no doubt on that, although the efficacy data per se are rather poor.

The advantage of a combination of L-lysine and L-tryptophan in one product seems rather doubtful. The requirement for lysine and tryptophan for piglets and growing pigs shows a differing ratio during growth. In contrast to lysine which is primarily needed for body protein synthesis (protein deposition), several intermediary functions (*e.g.* nicotinamid and serotonin formation) can be attributed to tryptophan. This is the reason for a higher maintenance requirement of tryptophan. The brain concentration of tryptophan (for serotonin formation) depends on the plasma tryptophan concentration, but the influx into the brain is based on one carrier (LAT) which also shows a high affinity to the large neutral amino acids (LNNA = valin, isoleucine, leucine, phenylalanine, tyrosine). The tryptophan supply of the brain will therefore depend on the dietary supply with tryptophan but also considering the concentration of LNNA which may impair the tryptophan transport to the brain by competitive mechanism.

No reason is given in the document why the condensed tryptophan containing permeate is dried together with L-lysine HCl. From a scientific aspect SCAN would prefer individual amino acids being available for supplementing feedingstuffs and adjusting each amino acid independently from each other to the dietary level wanted.

4.2. Influence on organoleptic characteristics

The use of Tryptosine™ with L-lysine-HCl (70%°) and L-tryptophan (15-20%) is not expected to impair the organoleptic characteristics of animal products, although data are not presented.

It is also considered as plausible that the residues of fermentation detected in the product will not impair the organoleptic characteristics of animal products.

4.3. Safety

The product is considered safe for the target animal categories (piglets, pigs for fattening and chickens for fattening) as far as amino acids are concerned. Concerning its fermentation residues, animal safety cannot be assessed.

The fermentation permeate incorporated in the product does not contain viable organisms, recognisable fragments of the production strain or detectable DNA. It may contain fragments of LipoPolySaccharide in low concentration. These findings do not implicate any hazard.

The absence of tryptophan metabolites related to Eosinophilia-Myalgia Syndrome is claimed for tryptophan produced from modified *E. coli* K-12. However, some doubts remain in assessing safety for human and animal health, because SCAN is only able to draw conclusions from literature data. Therefore SCAN would prefer viewing at least one expert analysis confirming the absence of the problematic tryptophan metabolites in the permeate used for Tryptosine™ production. SCAN also recommends the establishment of a routine assay for tryptophan metabolites in the fermentation medium and in the permeate after ultrafiltration. The tryptophan source (with its residues of fermentation) in Tryptosine™ is also not tested for genotoxicity.

The tryptophan (with its residues of fermentation) should be tested for toxicity in a rat feeding study (*e.g.* 90-days). The test material should be given at a range of doses up to the maximum practicable dose. Particular attention should be given to the design of the study and the composition of the test and control diets. The use of synthetic diets is recommended.

Safety for the consumer needs not to be studied additionally if general safety is doubtlessly given.

Safety for the worker and the user has not been studied, and therefore cannot be assessed. Studies are required.

Although safety for the environment has not been studied, SCAN would not expect detrimental effects on the environment from the use of Tryptosine™. In addition, following the guidelines, for this kind of product, such an assessment is not needed.

5. LITTERATURE

Simat, T.J., Kleeberg, K.K., Müller, B. and Sierts, A. 1999. Synthesis, formation and occurrence of contaminants in biotechnologically manufactured L-tryptophan. In: T*ryptophan, Serotonin and Melatonin: Basic Aspects and Applications* (Heuther et al. eds) pp 469-480, Kluwer Academic/Plenum Publishers, New York.

Chung, T.K., Gelberg, H.B., Domer, J.L. and Baker, D.H. 1991. Safety of L-tryptophan for pigs. J. Anim. Sci, 69:2955-2960