SCIENTIFIC OPINION

EFSA Journal

ADOPTED: 5 July 2017

AMENDED: 10 November 2017

doi: 10.2903/j.efsa.2017.4936

Safety and nutritional value of a dried killed bacterial biomass from *Escherichia coli* (FERM BP-10942) (PT73 (TM)) as a feed material for pigs, ruminants and salmonids

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), Guido Rychen, Gabriele Aquilina, Giovanna Azimonti, Vasileios Bampidis, Maria de Lourdes Bastos, Georges Bories, Pier Sandro Cocconcelli, Gerhard Flachowsky, Jürgen Gropp, Boris Kolar, Maryline Kouba, Marta López-Alonso, Secundino López Puente, Alberto Mantovani, Baltasar Mayo, Fernando Ramos, Maria Saarela, Roberto Edoardo Villa, Robert John Wallace, Pieter Wester, Paul Brantom, Mikolaj Antoni Gralak, Lieve Herman, Sirpa Kärenlampi, Jaime Aguilera, Jaume Galobart, Orsolya Holczknecht and Andrew Chesson

Abstract

PT73 (TM) is a dried, heat-inactivated bacterial biomass used as a feed material produced from an *Escherichia coli* K-12 strain, which was genetically modified to overproduce threonine. The recipient organism *E. coli* MG 1655 is considered to be safe. The traits introduced in the final modified strain *E. coli* FERM BP-10942 are mainly limited to the overproduction of threonine. No full-length antibiotic resistance genes or other sequences of concern remain in the modified strain. In conclusion, the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP): does not identify risks for human and animal health or the environment from this biomass regarding the genetic modification of the strain. The proposed recommended use level for dairy cows (8% PT73 (TM) of feed dry matter (~ 7% in complete feed)) and salmonids (13%) is considered safe for these target animals. The conclusion form dairy cows could be extended to other ruminants (from the beginning of rumination). Complete feed for pigs for fattening may contain up to 10% PT73 (TM). The toxicological data indicate effects of PT73 (TM) on blood coagulation and liver, which are considered to be adverse. As a consequence, the FEEDAP Panel is unable to conclude on the safety for the consumer of products derived from animals receiving feed containing PT73 (TM). PT73 (TM) is not considered a skin/eye irritant but should be considered as a potential skin and respiratory sensitiser. Moreover, any exposure of users to dust from the product via the inhalation route should be considered a serious risk. The FEEDAP Panel considers that substitution of PT73 (TM) for other protein-rich feed materials will not adversely affect the environment.

Keywords: heat-inactivated biomass, *Escherichia coli*, safety, nutritional value, genetically modified microorganism

Requestor: Competent Authority of France

Question number: EFSA-Q-2008-412b

Correspondence: feedap@efsa.europa.eu

Panel members: Gabriele Aquilina, Giovanna Azimonti, Vasileios Bampidis, Maria de Lourdes Bastos, Georges Bories, Andrew Chesson, Pier Sandro Cocconcelli, Gerhard Flachowsky, Jürgen Gropp, Boris Kolar, Maryline Kouba, Marta López-Alonso, Secundino López Puente, Alberto Mantovani, Baltasar Mayo, Fernando Ramos, Guido Rychen, Maria Saarela, Roberto Edoardo Villa, Robert John Wallace and Pieter Wester.

Note: The content of this opinion may be edited once a decision on confidentiality is received from the European Commission, in accordance with Article 30(2) of Regulation (EC) No 1829/2003.

Acknowledgements: The Panel wishes to thank the members of the former GMO Panel Working Group on Genetically Modified Microorganisms: Niels Bohse Hendriksen, Michael Gasson, Nickolas Panopoulos and Christoph Tebbe for the preparatory work on this scientific output.

Amendment: This scientific opinion has been amended in the authors list to include Mikolaj Antoni Gralak, a member of the Working Group on Feed Materials of the FEEDAP Panel 2015–2018. This scientific output, published on 10 November 2017, replaces the earlier version.

Suggested citation: 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, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Brantom P, Gralak MA, Herman L, Kärenlampi S, Aguilera J, Galobart J, Holczknecht O and Chesson A, 2017. Scientific Opinion on the safety and nutritional value of a dried killed bacterial biomass from *Escherichia coli* (FERM BP-10942) (PT73 (TM)) as a feed material for pigs, ruminants and salmonids. EFSA Journal 2017;15(8):4936, 22 pp. https://doi.org/10.2903/j.efsa.2017.4936

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

Summary

Following a request from the Competent Authority of France, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on subject.

PT73 (TM) is a dried, killed bacterial biomass produced by fermentation of the genetically modified *Escherichia coli* strain FERM BP-10942. In this strain, genes for the overexpression of enzymes for L-threonine production are introduced. The molecular characterisation of the strain does not indicate a safety concern. No DNA sequences of concern including those conferring antibiotic resistances are present in the production strain.

The maximum safe level for dairy cows would be 7% PT73 (TM) of feed dry matter (≈ 6% in complete feed). This value could be extended to other ruminants (from the beginning of rumination). Complete feed for pigs for fattening may contain up to 10% PT73 (TM). PT73 (TM) is safe for salmonids up to a dietary concentration of 13%.

The toxicological data indicate effects of PT73 (TM) on blood coagulation and liver, which are considered to be adverse. Since the causative agent and mechanism of these effects is unknown, there is no way of determining whether residues exist in animal products sufficient to cause similar effects in consumers. As a consequence, the FEEDAP Panel is unable to conclude on the safety for the consumer of products derived from animals receiving feed containing PT73 (TM).

PT73 (TM) is not considered a skin/eye irritant but should be considered as a potential skin and respiratory sensitiser. The FEEDAP Panel considers that due to the endotoxin content any exposure of users to dust from the product via the inhalation route should be considered a risk.

PL73 (TM) does not contain viable recombinant cells but contains trace amounts of recombinant DNA. No risks associated with a theoretically possible horizontal gene transfer of this recombinant DNA to environmental bacteria have been identified. The FEEDAP Panel considers that substitution of PT73 (TM) for other protein-rich feed materials will not adversely affect the environment.

Since the risks identified do not relate to the genetic modification but to the product itself (biomasses from fermentation with *E. coli* and potentially other Gram-negative bacteria), the Panel recommends that similar non-genetically modified products used as a feed material should also be assessed for safety.

Table of contents

1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1829/2003[1] establishes the rules governing the Community authorisation of genetically modified food and feed. In particular, Article 17(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 that Article.

On 7 August 2008, the European Food Safety Authority (EFSA) received from the French Competent Authority an application for authorisation of a dried killed genetically modified (GM) bacterial biomass from *Escherichia coli* (FERM BP-10942) (PT73 (TM)) submitted by Ajinomoto Eurolysine SAS[2] within the framework of Regulation (EC) No 1829/2003 on GM food and feed.

According to Regulation (EC) No 1829/2003, this scientific opinion is to be seen as the report requested under Article 18(6) of that Regulation and thus will be part of the EFSA overall opinion in accordance with Article 18(5).

According to Article 17 of Regulation (EC) No 1829/2003, EFSA shall determine whether the feed complies with the conditions laid down in Article 16. EFSA shall deliver an opinion on the safety for humans, animals and the environment and on the nutritional quality of the dried killed bacterial biomass (PT73 (TM)) produced by the genetically modified *E. coli* (FERM BP-10942) when used as a feed material under the conditions described in Section 3.5.1.

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[3] in support of the authorisation request for the use of PT73 (TM) as a feed material.

The FEEDAP Panel used the data provided by the applicant together with data from other sources, such as peer-reviewed scientific papers and other scientific reports to deliver the present output.

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and nutritional value of PT73 (TM) is in line with the principles laid down in the relevant guidance documents: Guidance on the assessment of microbial biomasses for use in animal nutrition (EFSA FEEDAP Panel, 2011a), Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use (EFSA GMO Panel, 2011), Technical guidance: Tolerance and efficacy studies in target animals (EFSA FEEDAP Panel, 2011b), Guidance for establishing the safety of additives for the consumer (EFSA FEEDAP Panel, 2012a) and Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012b).

3. Assessment

The current application concerns the use of an inactivated *E. coli* biomass (PT73 (TM)), which is a by-product of L-threonine production by fermentation using a genetically modified strain of *E. coli* K-12 (FERM BP-10942). It is intended to be used as a feed material for pigs, ruminants and salmonids.

The data contained in the dossier relates to different batches from pilot scale production to full scale industrial production of the biomass PT73 (TM). It also contains some data relative to another biomass produced by a previous strain of *E. coli* (PT73 (THR)). When data from this biomass has been used in the assessment, this will be indicated in the opinion.

[1] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. OJ L 268, 18.10.2003, p. 1.
[2] Ajinomoto Eurolysine SAS. 153, rue de Courcelles, 75817 PARIS Cedex 17 (France).
[3] Application EFSA-GMO-FR-2008-59. FEED dossier reference: FAD-2008-035.

3.1. Manufacturing process[4]

The fermentation process has been described in detail in the dossier with full information on all of the substances used during the production process.

After removal of the L-threonine, the solids are concentrated and dried. The resulting biomass powder is granulated/pelleted, cooled and sieved to remove broken pellets and fine dust which are recycled to pellet production.

An antifoaming agent is added during the fermentation which results in residues in the final feed material (see Sections 3.3.2 and 3.6.4).

3.2. Characterisation of the genetically modified strain *E. coli* FERM BP-10942[5]

The microorganism of which PT73 (TM) is made is a genetically modified derivative of *E. coli* K-12, deposited at the Japanese International Patent Organism Depositary and National Institute of Advanced Industrial Science and Technology with accession number FERM BP-10942. The strain was identified as *E. coli* K-12 by molecular serotyping of the *rfb* cluster and the *fli*C gene and comparison with databases. Antibiotic susceptibility was tested using agar diffusion against of 31 antibiotics including 8 out of the 10 antibiotics recommended by EFSA (EFSA FEEDAP Panel, 2012a,b) for *E. coli*. Minimum inhibitory concentration (MIC) values were provided. The strain was found sensitive to all antibiotics tested. Polymerase chain reaction (PCR) analysis indicated the absence of virulence genes including genes encoding enterotoxins, Shiga toxins, and adhesion and invasion factors in the genome of *E. coli* FERM BP-10942. The strain was found not to contain plasmids, transposons, sex factors or prophages.

The recipient strain *E. coli* MG 1655 is a derivative of *E. coli* K-12 obtained after ultraviolet (UV) irradiation followed by mutagenic treatment (Bachmann, 1987). *E. coli* K-12 is a Gram-negative, non-sporulating bacterium. It is well-characterised and its safety (non-pathogenicity) has been reviewed extensively (Gorbach, 1978). The genome of *E. coli* K-12 is fully sequenced (Hayashi et al., 2006).

The dossier contains detailed and sufficient information regarding the origin and function of the different genetic elements introduced in the production strain, the genetic modification process and the genetic and phenotypic traits introduced.

3.3. Characterisation of the final feed material

3.3.1. Composition

The applicant has provided information on the composition of six batches of the product. One of the batches derived from a small scale pilot production (produced in 2006), four from industrial scale production (produced in 2009 and 2013) and one industrial scale produced in 2015. Four additional batches produced in 2015 were used for the analysis of dry matter, crude protein (CP), antifoaming agent residues, purine and pyrimidine bases and endotoxin content. The results for the industrial scale batches produced in 2009, 2013 and 2015 are considered in detail below.

The product is characterised by its high content of CP (~ 84%). The chemical composition of the four industrial and the pilot scale batches is shown in Table 1.

Table 1: Chemical composition of the industrial batches of PT73 (TM) produced in 2009 and 2013 (4 batches) and 2015 (1 batch). Values in % of dry matter (DM) (except DM: % of the product)

	2009–2013[(a)]		2015[(b)]	
	Average	**Range**	**Average**	**Range**
Dry matter	93.2	88.6–96.8	96.1	95.6–96.6
Ash	4.9	1.3–8.0	1.1	–

[4] This section has been amended following the confidentiality claims made by the applicant on the basis of Article 30(1) of Regulation (EC) No 1829/2003.

[5] This section has been amended following the confidentiality claims made by the applicant on the basis of Article 30(1) of Regulation (EC) No 1829/2003.

	2009–2013[a]		2015[b]	
	Average	**Range**	**Average**	**Range**
Crude protein	86.1	82.8–89.6	82.3	81.1–83.1
Crude fat	8.1	7.76–8.6	8.5	–
Starch	1.0	–	–	–
Sugars	0.8	–	–	–

(a): Dry matter, ash, crude protein and crude fat were determined in four batches. Starch and sugars were determined in one batch.
(b): Dry matter and crude protein were determined in five batches. Ash and crude fat were determined in one batch.

The total amino acid content of the four industrial batches (produced in 2009 and 2013) was 76.6%, (DM basis) (Table 2), coming mainly from bacterial protein, with free amino acids (other than threonine) representing only a minor fraction (0–0.4%, total approximately 4%). The amounts of the main amino acids revealed little variation between the batches. Amino acid analyses made with one batch produced in 2015 gave essentially the same results.

Table 2: Amino acids in the four industrial scale batches of PT73 (TM) produced in 2009 and 2013. Values are given as % of dry matter

Amino acid	**Average**	**Range**
Glutamic acid/glutamine	9.62	8.90–10.49
Aspartic acid/asparagine	7.88	7.38–8.36
Threonine	7.27	6.71–7.75
Leucine	6.91	6.66–7.22
Alanine	5.78	5.37–6.22
Arginine	4.89	4.61–5.20
Lysine	4.84	4.29–5.28
Valine	4.54	4.16–5.07
Glycine	3.85	3.57–4.15
Isoleucine	3.83	3.62–4.03
Phenylalanine	3.35	3.17–3.50
Tyrosine	3.01	2.80–3.13
Serine	2.96	2.82–3.24
Proline	2.60	2.39–2.88
Methionine	2.01	1.91–2.09
Histidine	1.65	1.45–2.04
Tryptophan	1.14	1.10–1.20
Cystine	0.46	0.41–0.49
Total amino acids	**76.58**	**72.35–81.83**

Ammonium N (as NH_4) ranged between approximately 0.8% and 2% and 'true protein' (calculated by subtracting ammonia N from total N) was 82.8 for the four industrial batches. Low levels of nitrates (≤ 0.03%) and nitrites (≤ 0.06%) were detected, while betaine levels were 0–0.18%. Slightly lower values for ammonium N (0.6%) were obtained for the batch produced in 2015.

Three of the industrial batches (2009) were analysed for levels of DNA and RNA which were 0.0003–0.0043% and 0.002–0.0049%, respectively. The levels of nucleotides (purine and pyrimidine monophosphates) varied between 1.29% and 3.57%. Purine and pyrimidine bases were not measured in the industrial batches produced in 2009 and 2013. Values for the five batches produced in 2015 showed that purine bases were on average 1.4% DM while pyrimidine bases were on average 1.0%.

Analysis of biogenic amines (putrescine, cadaverine, histamine, spermidine, agmatine, spermine, tyramine and phenylethylamine) in the three industrial batches produced in 2009 had the following results (expressed on DM basis): 0.003–0.007% putrescine, 0.011–0.032% cadaverine and 0.0015–0.0031% spermidine while the other amines were below the respective limits of quantification (LOQ). Similar values were obtained with the batches produced in 2013 and 2015.

Total crude fat, measured in four batches (2009 and 2013) showed a mean value of 7.76%. The most abundant fatty acids were C14:0 (0.42% in DM), C16:0 (1.99%), C16:1*cis* (0.40%) and C18:1*cis* (0.72%), typical of Gram-negative bacteria. A similar pattern was seen in the analysis of one batch produced in 2015.

Organic acids (acetic, propionic, butyric, lactic, formic, pyruvic and isovaleric acid) were analysed in the four industrial batches. The sum of organic acids (% as is) was around 0.5–0.9%. One of the batches produced in 2015 was also analysed and the total amount of organic acids was 0.03%.

The most significant inorganic substances detected were sulfates and sodium, but for some of them there was considerable variation between the batches (Table 3).

Table 3: Content of minerals and trace elements of four industrial scale batches of PT73 (TM) produced in 2009 and 2013. Unless otherwise stated values are given as % dry matter

	Average	Range
Sodium	0.86	0.16–1.78
Potassium	0.03	0.02–0.06
Calcium	0.005	0.002–0.009
Magnesium	0.005	0.003–0.01
Chlorides	0.05	0.02–0.09
Sulfates	1.36	0.87–2.71
Phosphates	1.09	0.02–3.58
Iron (mg/kg product)	123	84–180
Copper (mg/kg product)	1.2	1.0–1.3

Analysis of one industrial batch produced in 2015 showed levels of water soluble vitamins expected for a material deriving from bacterial biomass. Vitamins A, D and E, provitamin A and other carotenoids were below levels of quantification.

The contents of endotoxins (lipopolysaccharides (LPS)) were analysed in duplicate in the five batches of the product produced during 2015. Values ranged between 2.3×10^5 and 1.0×10^6 EU/g.[6]

3.3.2. Impurities

Heavy metals (Pb, Hg and Cd) and arsenic were measured in four batches and found to be well below the maximum authorised limits for feed materials.[7] Residues of pesticides, dioxins and dioxins plus dioxin-like polychlorinated biphenyls (PCBs) were analysed in two batches and found to be below the LOQ for pesticides and below the maximum levels set by Directive 2002/32/EC for dioxins and dioxins plus dioxin-like PCBs.

Residues of the antifoaming agent added during the fermentation were analysed in the final feed material. Data in three batches of PT73 (TM) (produced in 2015) showed that the levels of antifoaming agent were approximately 15 g/kg.

The microbiological analysis of the four batches showed that *Salmonella*, staphylococci, *Clostridium perfringens*, sulfite-reducers, coliforms and faecal streptococci were below the limit of detection. Yeasts were detected in one batch (< 40 CFU/g) and filamentous fungi were detected in three batches (80–260 CFU/g). All fermentation substrates are obtained with a specification that meets European Union (EU) legislation on maximum limits for aflatoxin B1, and consequently, no analyses were performed in the final product.

The absence of viable cells of *E. coli* FERM BP-10942 from the product PT73 (TM) was demonstrated by (i) liquid culturing, allowing the growth of stressed cells, followed by selective plating in three batches of the final product, each tested in triplicate; and (ii) direct selective plating of two batches of the final product (20 replicates per batch). In both liquid and solid culture methods, 1 g of the inactivated fermentation product was tested.

The presence of recombinant DNA from *E. coli* FERM BP-10942 was investigated in 13 batches of PT73 (TM) product by PCR, capable of amplifying a 454 bp fragment of recombinant DNA. Results

[6] 1 EU corresponds to 0.1 ng of the international reference endotoxin standard coming from *Escherichia coli* (amount present in 10^5 bacteria).

[7] Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. OJ L 140, 30.5.2002, p. 10.

indicated the presence of trace amounts of DNA from the production strain in about eight out of the 13 batches tested. Another PCR assay, targeting a 930 bp fragment, did not show any amplification.

3.3.3. Physical properties

PT73 (TM) is a brown pelleted product (pellet size ~ 6–8 mm diameter) with an apparent bulk density of 638 kg/m^3. The particle size determination (laser diffraction) and the dusting potential measurement (rotating drum technique) were carried out on the residual fine dust fraction obtained by sieving the pellets. This fraction (< 1,400 μm) represented 6.3% w/w of the total product. It was shown that 15% of that sample had a particle size below 45 μm. The results of dustiness obtained for the fine powder fraction were recalculated to the original biomass pellets. Values showed that the inhalable, thoracic and respirable fraction were 1,092, 471 and 48 mg/kg product which would result in a classification of PT73 (TM) as of low-moderate dusting potential.

3.3.4. Storage life

In a stability trial, PT73 (TM) (one batch) was stored for 12 months under three different conditions (25°C/60%, 40°C/30% and 40°C/60%). Moisture content, water activity, crude nutrients, ammonium N/ammonia and biogenic amines were monitored. Additionally, microbiological quality was assessed by aerobic count, and counts of counts of *Staphylococcus aureus*, *Salmonella*, *Bacillus cereus,* yeast, filamentous fungi and Enterobacteriaceae. Pellet quality was also measured. The parameters measured showed no deterioration of PT73 (TM) in any of the conditions studied.

PT73 (TM) was included in a complementary feed for dairy cows at a rate of 13.3%. Samples were stored at 5, 25 or 40°C with a relative humidity (RH) of 60% for three months in punctured nylon bags to simulate practical storage conditions. The parameters evaluated included gross composition, biogenic amines, microbiological counts and physical properties. No significant differences were observed in the parameters tested after storage.

The above findings were supported by a study in pig feed with PT73 (THR) with levels of inclusion of 5% and 20% up to 6 months.

3.4. Safety aspects of the genetically modified strain *E. coli* FERM BP-10942

The strain *E. coli* FERM BP-10942, from which the product is made, was confirmed to have been derived from the recipient strain *E. coli* MG 1655. The recipient strain *E. coli* K-12S B-7 is considered to be safe.

The traits introduced are well known and do not raise safety concern. Southern or PCR analysis confirmed the absence of all full-length antibiotic resistance genes used during the entire genetic modification. Bioinformatic analysis did not show any biologically relevant similarity to known allergens or toxins for any of the putative peptides that might be produced from the open reading frames spanning the junctions between the Mu sequences and genomic DNA. Therefore, the product PL73 (TM), obtained from *E. coli* BP-10942, does not give rise to any safety concern with regard to the genetically modified strain from which it is made.

3.5. Nutritional characterisation of PT73 (TM)

3.5.1. Conditions of use

The product PT73 (TM) is intended to be used as a feed material, a source of protein for pigs for fattening, ruminants (for meat and milk production from the beginning of rumination) and salmonids. The maximum use levels recommended in complete feed are 12% for pigs and 13% for salmonids (or replacement of 20% of fish meal in a complete diet containing 65% fish meal). For ruminants, the maximum recommended level is 7.3% biomass DM in complete feed dry matter.

3.5.2. Nutritional studies

In order to show the nutritional value of the biomass under assessment, the applicant submitted a series of *in vitro*/*in vivo* digestibility studies and long-term feeding trials with the target animals. Not all of the studies were performed with the biomass PT73 (TM). Some nutritional studies were made with the biomass PT73 (THR) also derived from cells of *E. coli* K-12 modified to increase threonine production. As nutrient composition shows the PT73 (THR) biomass is sufficiently similar to PT73 (TM)

that results obtained with PT73 (THR) can be considered in the assessment of PT73 (TM) (Table 4). Since some essential nutrient characteristics of PT73 (THR) are inferior to those of PT73 (TM) (i.e. CP, sum of amino acids, energy, sulfate), tolerable dietary levels derived from studies with PT73 (THR) can be taken as a conservative estimate for PT73 (TM).

Table 4: Main composition parameters of the biomasses PT73 (TM) and PT73 (THR) (on % dry matter basis)

Composition parameter	PT73 (TM)[(a)]	PT73 (THR)[(b)]
Dry matter	87.3	88.9
Crude protein	86.1	77.4
Ammonium N	0.98	0.95
Sum of total amino acid	76.6	62.0
Crude fat	8.1	7.5
Crude fibre	–	0.7
Crude ash	4.9	5
Potassium	0.03	0.27
Sodium	0.86	0.34
Chlorides	0.05	0.87
Sulfates	1.36	2.99
Phosphates	1.09	3.7

(a): Average of four batches (Table 1).
(b): One batch.

3.5.2.1. *In vitro* digestibility

A battery of *in vitro* tests simulating ruminal and/or gastrointestinal conditions was used to estimate the digestibility of different fractions of PL73 (TM). The results showed organic matter digestibility (81–84%), rumen protein solubility (2830% of CP), rumen escape/undegraded protein (34–67% of CP), CP digestibility in monogastrics (63%).

3.5.2.2. *In vivo* digestibility PT73 (THR)

Pigs

The study was conducted on 2 × 9 pigs (barrows, body weight at start 28–30 kg) for 9 weeks to determine the apparent ileal and faecal digestibility of PT73 (THR). The study was designed according the 'difference technique'. The standard diet was mainly based on wheat, barley, soybean meal, wheat middlings and tapioca and was supplemented with 0.25 g chromium oxide as inert marker per kg feed. The test diet contained 15% PT73 (THR), which was 'contaminated' with another *E. coli* biomass (PL73 (LYS)) most probably at a level between 5% and 10%.

The apparent ileal (faecal) digestibility coefficients were 82 (90)% for DM, 84 (91)% for CP, and 91 with a range from 76% to 95% for the sum of amino acids. Sulfate is apparently largely absorbed. Digestible energy was found to be 19, net energy 10 MJ/kg DM.

Sheep

In a digestion trial, two groups of six sheep (wethers, body weight at start 70–100 kg) were offered 900 g basal diet (chopped hay, sugar beet pulp and molasses)/day and 1,000 g test diet (75% standard diet, 25% PT73 (THR))/day), respectively. The apparent digestibility was measured for CP with 93%, for crude fat with 89% and for nitrogen free extract with 96%.

The digestibility coefficient of CP from PT73 (THR) is in the same range as that of other protein-rich feedstuffs like rape seed or soybean expeller.

Rainbow trout

The study was conducted on a total of 90 rainbow trout (*Oncorhynchus mykiss*) (body weight at start about 100 g) divided in six replicates (three per treatment of 15 fish each). Fish were fed by hand to apparent satiation a basal diet (fish meal (60%), gelatinised wheat starch (24%), fish oil (12%), supplemented with chromic oxide (1%) as an inert marker) and the test diet consisting of 70% basal diet and 30% PT73 (THR), respectively. Faeces were collected for the second week of a 2-week

feeding period. The apparent digestibility of CP from PL73 (THR) was 87%. Digestible energy was 17 MJ/kg PT73 (THR) DM.

3.5.2.3. Feeding studies in target animals – Dairy cows

Studies with PT73 (TM)

Study 1

To investigate the maximum incorporation rate of PT73 (TM) in diets for ruminants, a study was conducted on a total of 64 dairy cows (Dutch Holstein-Friesian type). After a 5-week adaptation period to the total mixed ration (TMR), four groups of 16 cows equalised for milk production, feed intake and lactation stage, were fed diets containing 0 (control), 50, 100 and 150 g of PT73 (TM) DM per kg TMR DM, referred to as RPT00, RPT05, RPT10 and RPT15, respectively. The experimental period lasted 8 weeks (56 days).

The basal TMR consisted of grass silage, forage maize silage, grass seed hay and a concentrate. The concentrate consisted of increasing amounts of PT73 (TM) (0%, 14.2%, 28.9% and 43.2%) at the expense of maize gluten meal, soybean meal, palm oil and urea. It contained 7.6 MJ net energy and about 40% CP. The content of intestinal digestible protein was calculated with 262, 260, 259 and 256 g for the concentrates with increasing amounts of PT73 (TM). All rations were offered *ad libitum*. Animals were fed at a non-limiting protein level (around 20% CP in ration DM), ensuring that any effect of the inclusion of PT73 (TM) in the ration is caused by characteristics other than its feeding value for protein.

Throughout the experiment, feed intake and milk yield were recorded daily for each animal. Each week, milk was sampled and concentrations of fat, protein, lactose and urea were determined. In week 0, 2, 4 and 8, individual blood samples were taken to evaluate the effect of PT73 (TM) prothrombin time, packed cell volume and haemoglobin, total leukocytes, neutrophilic, basophilic and eosinophilic granulocytes, lymphocytes, monocytes, erythrocytes and thrombocytes as well as alkaline phosphatase, total protein, albumin, cholesterol, phospholipids, inorganic phosphorus, non-esterified fatty acids, triglycerides, transferrin, 25-hydroxycholecalciferol and 1,25-dihydroxycholecalciferol.

Feed intake, milk production and milk composition data were analysed with the restricted maximum likelihood method. Blood values were analysed by an analysis of variance (ANOVA).

In week 6 of the experimental period, one cow of RPT05 had to be removed because of a large hematoma from the mammary vein and one cow of RPT15 died of liver abscess.

No significant effects were observed on feed intake, milk yield and milk composition between the different treatment groups (Table 5). Feed intake decreased linearly over the whole experimental period including the 5-week adaptation period, with a slight tendency for recover intake during the last 3 weeks.

Table 5: Feed intake, milk production (only data of week 8 of the experimental period are given) and composition and some blood parameters of cows fed graded levels of PT73 (TM)[(1)]

	RPT00	RPT05	RPT10	RPT15
PT73 (TM) calculated (g/kg DM[(2)])	0	50	100	150
PT73 (TM) actual (g/kg DM)	0	54	109	167
DM intake (kg/day)	22.1	22.2	22.0	22.2
PT73 (TM) intake (kg DM/day)	0.0	1.2	2.4	3.7
Milk yield (kg/day)	35.5	36.8	35.3	35.8
FPCM[(3)] (kg/day)	34.6	36.0	34.5	35.4
Protein (g/kg milk)	32.6	34.5	36.0	36.6
Protein (kg/day)	1.15	1.26	1.26	1.30
Fat (g/kg milk)	38.6	38.2	37.6	38.2
Fat (kg/day)	1.36	1.38	1.30	1.35
Lactose (g/kg milk)	47.2	46.6	45.8	46.2
Lactose (kg/day)	1.67	1.72	1.61	1.65
Urea (mg/100 mL milk)	37	37	35	39
Cholesterol (mmoL/L blood)	5.0	4.8	4.8	4.8
Phospholipids (mmoL/L blood)	3.1	2.9	3.0	2.9

	RPT00	RPT05	RPT10	RPT15
25-Hydroxycholecalciferol, (nmol/L blood)	104[a]	96[b]	98[a,b]	94[b]

(1): No statistics were provided for FPCM, protein, fat and lactose yield (kg/day).
(2): Dry matter.
(3): Fat (40 g/kg) and protein (33 g/kg) standardised milk.
a,b: Means within a row with different superscript letters are significantly different ($p \leq 0.05$).

Haematology did not show significant differences between groups except a slight but not dose-related changes on thrombocytes and segmented and basophilic granulocytes.

There was a significant reduction in plasma vitamin D metabolites. Plasma 25-hydroxycholecalciferol levels in the PT groups were significantly lower than in the control group. However, plasma cholesterol was not influenced by the treatment.

Study 2

In a study with 24 dairy cows, PT73 (TM) was tested at four levels (50, 100, 150 and 200 g of DM per kg of ration DM) in comparison to a zero control treatment. This resulted in five experimental treatments. An incomplete Latin square design was used with three 4-week periods. The cows received the ration as TMR of which the DM was based on 16.7% grass silage, 34.5% forage maize silage and 48.8% concentrate, the concentrate containing the varying levels of PT73 (TM). The FEEDAP Panel has considerable reservations about the experimental design selected, since not all cows were subject to all treatments, the experimental period was short (4 weeks/treatment) and the potential effect of the biomass was measured against reducing performance (reduced milk yield). Moreover, with increasing inclusion of PT73 (TM), the intake of CP increased (from 17.6% to 26.3% CP in DM of the diets (TMR) with 0% and 20% PT73 (TM), respectively. High-protein feed materials should be compared on an isonitrogenous basis. Therefore, this study was not further considered.

Study 3

To investigate the maximum incorporation rate of PT73 (TM) in diets for ruminants, a study was conducted on a total of 24 dairy cows (Dutch Holstein-Friesian type) Six groups of cows were formed. In each group, four cows at a comparable stage of lactation and with a comparable milk yield were selected. Cows within each group were randomly distributed over the four experimental treatments in period I. The 21-week experiment was set up as an incomplete Latin square design with four treatments and three periods. After a 5-week adaptation period (3 weeks TMR and commercial concentrate), the cows were offered four diets containing 0 (control), 26.6, 53.3 and 80.0 g of PT73 (TM) DM per kg complete feed DM, referred to as PT00, PT27, PT53 and PT80, respectively. The experimental periods lasted 5, 5 and 6 weeks.

The basal TMR consisted of grass silage, forage maize silage, grass seed hay and a concentrate. The concentrate consisted of increasing amounts of PL73 (TM) (0%, 6.7%, 13.3% and 20.0%) at the expense of soybean meal, palm oil and urea. It contained 7.53–7.60 MJ net energy for lactation and 23.3–24.4% CP. The content of intestinal digestible protein was calculated with 150, 163, 170 and 173 g for the concentrates with increasing amounts of PT73 (TM). All rations were offered *ad libitum* (24 kg DM complete feed/day).

Throughout the experiment, feed intake and milk yield were recorded daily for each animal. In the fourth and fifth week of each experimental period, milk was sampled and concentrations of fat, protein, lactose and urea were determined.

The effects of treatments on feed intake, milk production and milk composition were analysed by ANOVA. Effects of the level of incorporation were tested by polynomial regression analyses.

In the first week of the second experimental period, one cow broke its leg and was replaced by other one. During statistical analyses the data for Period I for this cow were defined as missing values.

Daily feed intake of the diets was lower (22.4–22.9 kg DM) than the projected feed intake of 24 kg DM per cow used for ration formulation. No statistical differences in DM intake and intake of Net energy for lactation (NEL) between treatments. For treatment PT27, the intake of CP was significantly lower than for treatments PT00 and PT80.

No differences between treatments were observed in production of milk (32.4–32.7 kg/day), milk fat, milk lactose and milk urea. A tendency was observed for a treatment effect on milk protein concentration ($p = 0.07$). Polynomial analyses showed a significant positive linear effect of rate of incorporation of PT73 (TM) on milk protein concentration ($p = 0.02$).

Conclusions for dairy cows and ruminants

Commonly used protein-rich ingredients in rations for high-yielding dairy cattle can be replaced by biomass PT73 (TM) when using the present guidelines for dairy cow nutrition in practice, the chemical composition and the nutritive value of PT73 (TM) and other ration ingredients.

The FEEDAP Panel concludes that the biomass PT73 (TM) can be used in formulating diets for ruminants as any other protein-rich feed material at the level recommended by the applicant (8% in complete feed DM).

3.5.2.4. Feeding studies in target animals – Pigs for fattening

Studies with PT73 (THR)

Study 1

A total of 40 pigs (20 gilts, 20 boars, 32 kg body weight (bw) at start) were fed for 9 weeks diets containing 0%, 5%, 10%, 15% and 20% PT73 (THR), respectively. Four individually housed pigs per sex were allocated to each group and fed a grower diet for 36 days and a finisher diet for 27 days. The diets consisted mainly of maize, tapioca, soybean meal and wheat middlings. The PT73 (THR) was included mainly at the expense of soybean meal, the diets were calculated to be isocaloric. K_2CO_3 was supplemented to prevent dietary electrolyte imbalances in the diets containing the PT73 (THR). Dietary CP content in the diets increased with increasing amounts of the PT73 (THR) (for the diets with 0%, 5%, 10%, 15% and 20% PT73 (THR), CP levels of 18.5%, 19.15, 20.4%, 21.6% and 22.0% in the grower and 14.9%, 16.0%, 17.0%, 18.0% and 19.2% in the finisher diet were found, respectively). The FEEDAP Panel has reservations regarding the formulation of the experimental diets; high protein feed materials should be compared on an isonitrogenous basis.

Body weight, feed and water intake, and faeces consistency were recorded in regular intervals. At day 54, blood samples were taken for haematology and routine blood chemistry.[8] At post-mortem, kidneys, liver, spleen and the pars oesophagea and fundus region of the stomach were examined, and the weight of the kidneys, liver, spleen and empty stomach was recorded. Data were analysed using an ANOVA with sex and treatment as factors. Differences between treatments were evaluated by least significant difference (LSD) test.

Overall performance during the trial was good (control group body weight gain, 1,086 g/day, feed intake 2.46 kg/day, feed to gain 2.27). These parameters did not significantly differ between groups for the entire 9-week period, but in the grower period, all PT73 (THR) groups had a significantly higher feed to gain ratio, and body weight gain of the 15% and 20% PT73 (THR) groups was significantly less than that of the control.

Water and water to feed intake ratio were not significantly affected by the PT73 (THR) diets. Faeces consistency of the 15% and 20% PT73 (THR) groups was significantly reduced in the three other groups (grower phase and complete experimental period).

Clinical blood chemistry parameters were not affected by treatment with the exception of a dose-related increase in bilirubin levels which were significantly higher in the 15% and 20% PT73 (THR) groups, compared to the control group. No significant differences were observed with regard to haematological parameters, with the exception of the content of haemoglobin and the packed cell volume that were slightly higher in the 10% and 15% groups compared to the control group. As no dose–response relationship was observed, these increases are considered to be incidental.

Body weight at slaughter, back fat thickness and meat percentage of the carcass were not affected by increasing dietary PT73 (THR). Organ weights were not affected by treatment with the exception of absolute kidney weights which were slightly but significantly increased at the 10%, 15% and 20% levels compared to the control group. These findings may be related to increasing renal urea excretion with increasing dietary protein.

Study 2

Four groups of 64 pigs (4 replicates with 8 boars and 4 replicates with 8 gilts, about 30 kg bw at start) were fed for 10 weeks (6 weeks grower diets and 4 weeks finisher diets) diets containing 0%, 6%, 9% and 12% PT73 (THR), respectively. The control diet was based on wheat, barley, maize,

[8] Including: prothrombine time, alkaline phosphatase, total protein, albumin, total bilirubin, cholesterol, inorganic phosphate and phospholipids.

tapioca, soybean meal, peas and wheat middlings. PT73 (THR) was incorporated in the diets at the expense of soybean meal and peas.

The diets were formulated to be equal in net energy content and to meet the requirements for the levels of apparent ileal digestible lysine, methionine and cysteine, threonine and tryptophan. The diets containing PT73 (THR) were supplemented with $NaHCO_3$ and K_2CO_3 to prevent dietary electrolyte imbalances. Dietary CP was analytically shown to be approximately equal in grower (18.7–18.9%) and finisher diets (17.0–17.2%). General health, individual body weight and feed intake (per pen) were monitored thorough the study. At the end of the study, the pigs were killed and carcass weight, meat percentage and backfat and muscle thickness were measured. Data were analysed by ANOVA. Differences between means were evaluated by LSD test.

Data of the control group (feed intake 2,272 g/day, weight gain 932 g/day and feed to gain 2.44) are indicative of a well-designed and conducted study. Feed intake, average daily gain and feed to gain ratio were not significantly affected by increasing amounts of PT73 (THR) up to 12% in the diet in either the grower or finisher phase.

Body weight at slaughter and carcass characteristics (carcass weight, carcass percentage, meat percentage of the carcass, back fat and muscle thickness) were not markedly affected by the experimental treatments. However, backfat thickness (control value 16.3 mm) was increased (significantly for the groups with 6% and 9% PT73 (THR)).

Conclusions for pigs for fattening

The FEEDAP Panel concludes that PT73 (THR) can be used in formulating diets for pigs for fattening up to a level of 10% on the basis of the apparent increased sensitivity of animals in the grower phase. The Panel considers that this conclusion applies to PT73 (TM). Particular consideration should be given to the electrolyte balance of diets containing PT73 (TM).

3.5.2.5. Feeding studies in target animals – rainbow trout

Study with PT73 (THR)

A total of 15 replicates (tanks) of 40 rainbow trout (*O. mykiss*) each (body weight at start 99 g) were allocated to five experimental treatments. Fish were fed for 12 weeks diets in which 10%, 20%, 40% and 60% of total fish meal (65.2% in the control group) was replaced by PT73 (THR), corresponding to actual levels of PT73 (THR) in feed of 6.6%, 13.1%, 26.3% and 39.4%. Fish were fed twice a day by hand near to visual satiety. Water was supplied from natural springs at a constant temperature of 17 $\pm$ 1°C. CP of the control diet was 50.5% in DM, the diets with 6.6%, 13.1%, 26.3% and 39.4% PT73 (THR) contained 50.3%, 50.0%, 50.2% and 48.9% CP in DM. Gross energy (17.6 MJ/kg DM in the control diet) rose with increasing amounts of PT73 (THR) up to 22.1 MJ/kg DM in the 39.4% PT73 (THR) diet. Every 3 weeks fish were kept unfed for 24 h, counted and weighed in groups. At termination of the trial, blood was sampled from 15 fish per replicate 18 h after the last meal for analysis of ammonia, urea and uric acid in plasma. Fish were subsequently dissected for morphometric measurements. Another five fish per replicate were taken for whole body composition. Rigor index and muscle pH were measured in six fish per treatment at 0, 2, 2 and 24 h after slaughter.

Survival rate was high (> 90%) and not affected by diet. Feed intake (values given in figures only), expressed as g fish/day, declined significantly ($p < 0.05$) in the group with 39% dietary PT73 (THR) after the first 2 weeks. Final body weight of trout fed diets with 6.6% and 13.1% PT73 (THR) (434 and 398 g, respectively) did not differ from the control group (409 g); however, 26.3% and 39.4% PT73 (THR) led to a significant growth depression (350 and 228 g, respectively). Feed to gain ratio was significantly reduced in groups fed diets with 13.1–39.4% PT73 (THR) (1.09, 1.09, 0.98, 0.93 and 0.62 for the diets with 0%, 6.6%, 13.1%, 26.3% and 39.4% PT73 (THR), respectively). Protein efficiency ratios followed similar trends.

There were no significant differences between groups for the viscerosomatic index, gutting index and liver to body weight ratio or in plasma metabolites.

For the post-mortem muscle characteristics, only those groups having similar final mean body weights (control, 6.6% and 13.1% PT73 (THR)) were retained. Muscle pH changes followed similar patterns in all groups. Rigor index was initiated earlier in trout fed the fishmeal-based diet and was slower in trout fed the diets containing PT73 (THR), especially at 13.1% PT73 (THR).

As regards whole body composition, trout fed the 39.4% PT73 (THR) diet had a significantly decreased protein (15.9%), fat (5.9%) and energy content (6.4 KJ/g) as compared to the other groups (control group: 17.0% CP, 10.4% fat and 8.3 KJ/g). Protein and energy retention were

significantly higher in trout fed the control diet and the diet with 6.6% PT73 (THR) than in trout fed the other experimental diets (protein retention for the groups with 0%, 6.6%, 13.1%, 26.3% and 39.4% PT73 (THR) 36%, 34%, 31%, 30% and 18%, and energy retention 58%, 55%, 46%, 39% and 20%, respectively).

Conclusions on salmonids

The results of the feeding study with PT73 (THR) in rainbow trout indicate that a concentration of 13% in the diet does not adversely affect performance of salmonids.

3.5.3. Product quality

Morning milk samples were obtained in the study with dairy cows fed with PT73 (TM). Samples of milk (2.5 L) were pooled from four cows, homogenised and pasteurised before a taste panel evaluation (N = 12). The overall score of sensory characteristics derived from 18 different descriptors (e.g. sweet, sour, bitter). In the 8-point scale used, an overall score of 6 describes milk of a sufficient quality. Scores observed in this study varied between 6.03 and 6.41, with no significant differences between treatment groups. There were neither obvious off-flavours nor defects noticed by any of the panel members.

Sensory properties of meat from samples (*m. longissimus lumborum*) of seven random pigs of the control group and of the group with 12% dietary PT73 (THR) from the experiment described above (Section 3.5.2.4, study 2) were evaluated (23 sensory attributes, divided into the categories appearance, odour, texture, taste and aftertaste) by a Taste Panel according to ISO standard 6564 (1985). For all attributes, no significant differences between the meat from control pigs and the 12% PT73 (THR) group were found.

Although sensory attributes can be quite subtle, it is probable that the results seen with PT73 (THR) would also apply to comparable diets with PT73 (TM).

3.6. Toxicological characterisation

The genotoxicity/mutagenicity studies and the subchronic oral toxicity studies were performed with one of the pilot scale batches while the oral prenatal developmental study was made with one of the industrial scale batches of PT73 (TM). Duplicate studies were also made with the former biomass PT73 (THR).

3.6.1. Genotoxicity and mutagenicity studies

As the bacterial biomass PT73 (TM) is an insoluble material, aqueous extracts were used for *in vitro* genotoxicity tests. Suspensions of PT73 (TM) in saline solution (bacterial test) or in culture medium without serum (tests in mammalian cells) were sonicated for 15 min and incubated for 24 h at 37°C under agitation. These extracts were centrifuged for 20 min at 1,200*g*. The supernatants were sterilised by passage through a micropore filter (0.45 μm). The initial suspensions used to prepare the extracts tested in the three genotoxicity studies had different concentrations: 10 mg/mL, 50 mg/mL and 12.5 mg/mL for the bacterial test, the chromosome aberration test and the gene mutation test, respectively. The nominal concentration of each final extract was considered equivalent to the concentration of the initial suspension and the tested concentration levels reported in the studies reflect this nominal concentration.

3.6.1.1. Bacterial reverse mutation assay

An aqueous extract of PT73 (TM) was tested at five concentrations ranging from 62 to 5,000 μg/plate in *Salmonella* Typhimurium strains TA1535, TA1537, TA100, TA98 and in *E. coli* WP2uvrA, in the absence and presence of a liver fraction of Aroclor 1254-induced rats for metabolic activation (S9-mix) using the plate incorporation method, in compliance with OECD guideline 471. No cytotoxicity was reported. The test item was not mutagenic under the conditions employed in this study while the positive controls gave the expected increase in the mean number of revertant colonies.

3.6.1.2. *In vitro mammalian* chromosome aberration test

An aqueous extract of PT73 (TM) was tested in a chromosomal aberration study in Chinese hamster ovary cells, in both the absence and presence of a metabolic activation system (S9-mix) in compliance with OECD guideline 473 (revision 1997). Two independent chromosomal aberration tests

were conducted. In the first test, a 4-h treatment time and an 18-h harvesting time were applied (pulse treatment). Three concentrations (1,250, 2,500 and 5,000 μg/mL) were tested with and without metabolic activation system (S9-mix). The test substance was slightly cytotoxic to the cells at the highest dose level in the presence of metabolic activation system. In the second test, the pulse treatment schedule in the presence of S9-mix was applied and the tested concentrations were 3,000, 4,000 and 5,000 μg/mL. In the absence of S9-mix, the treatment time was 18 h (continuous treatment) and three dose levels of the test substance (3,000, 4,000 and 5,000 μg/mL) were analysed. The pulse treatment with S9-mix was not cytotoxic to the cells at any dose levels, while the continuous treatment without S9-mix was slightly cytotoxic to the cells at the mid dose and clearly cytotoxic at the highest dose level (5,000 μg/mL). In both the first and second chromosomal aberration test, the test substance did not induce a statistically significant increase in the number of aberrant cells at any of the concentrations and time-points analysed. The positive control substances induced the expected statistically significant increases in the incidence of structural chromosomal aberrations.

3.6.1.3. *In vitro* gene mutation test

An aqueous extract of PT73 (TM) was tested for its potential to induce gene mutations at the TK-locus of cultured mouse lymphoma L5178Y cells, in both the absence and the presence of a metabolic activation system (S9-mix) in compliance with OECD guideline 476. Two assays were conducted. In the first assay, single cultures were treated for 24 and 4 h in the absence and presence of S9-mix, respectively. In the second assay, single cultures were treated for 24 and 4 h in the absence of S9-mix. The highest concentration of the extract of PT73 (TM) evaluated for mutagenicity in both the absence and presence of S9-mix was equivalent to 5,000 μg/mL. The extract of PT73 (TM) was slightly toxic to the cells, especially in the absence of S9-mix after extended (24 h) treatment. At the highest concentration tested the relative total growth (RTG) was 46% and 77% in the absence and presence of S9-mix, respectively. In both the absence and presence of S9-mix, no relevant increases in the mutant frequency were observed at any concentration, while the positive controls performed as expected.

Comparable studies with the PT73 (THR) biomass also gave negative results.

3.6.2. Toxicity studies with laboratory animals

3.6.2.1. Repeat dose subchronic oral toxicity study

Study 1

PT73 (TM) was administered in the diet to groups of 10 Wistar rats of each sex for 90 days at concentrations of 0%, 5%, 10% or 20%, the highest dose equivalent to 10.1–10.9 g/kg bw per day. The proteinaceous test article (partly) substituted casein in the diet. The study was conducted according to OECD guideline 408. Observations included daily clinical inspection, weekly neurobehavioral testing, ophtalmoscopy, body weight, food and water consumption, haematology, clinical chemistry, urinalysis including renal concentration test, oestrus cyclicity, sperm analysis, organ weights and gross and histopathology in the control and high-dose groups, and grossly observed lesions in the intermediate dose groups.

No treatment-related effects were found in clinical and neurobehavioral observations, food consumption, gross pathology, oestrus cyclicity, sperm analysis.

Effects observed included occasional increase in water intake in mid- and high-dose males, a downward trend in thrombocytes in females, prolonged prothrombin time in all dosed male (but within historical control range), increase in total white blood cells and monocytes in high-dose females, lower percentage of lymphocytes and increase in neutrophils in the high dose (both sexes). Urine production showed an upward trend in males.

In relative and absolute organ weights increase was found in kidney, liver and spleen notably in the high-dose females, and in clinical chemistry various blood parameters were increased or decreased. Notably cholesterol and phospholipids were decreased (males dose related, females high dose only) while alanine transaminase (ALAT) was increased in high-dose females. In histopathology, a slight increase in focal mononuclear aggregates was observed in the liver of all treated males and hepatocellular vacuolation was slightly increased in all treated females and high-dose males. Increased brown pigment was observed in the spleen of all treated animals, identified as a preservation (formalin) artefact, the relation with treatment being unknown.

Study 2

A second repeated dose oral toxicity study was conducted to provide evidence on the possible mechanism of the observed generic effects. Three groups of 15 male Wistar WU (Crl:WI(WU), outbred) rats received control feed, PT73(TM) at 5% or 15%. Observations were conducted similarly to those described for previous studies and the new study confirmed those effects observed previously (prolonged prothrombin time, lower levels of plasma cholesterol and phospholipids, increased aspartate transaminase (ASAT) or alkaline phosphatase (ALP) activity and increased kidney weights).

Some of the adverse effects observed in this study and the previous one might be compatible with the absorption of endotoxins (LPS) from the intestinal tract. The levels of LPS in the diets (EU/g) were 566–803 for the control, 36,100–27,000 for the 5% group and 109,000–119,000 for the 15% group. Despite the sensitive detection limit for endotoxins (< 66 ng/mL), LPS was not detected in the serum of any rat after receiving feed containing 5% or 15% PT73 (TM) for 4 or 13 weeks. Also, there were no relevant changes in first-phase reactants (C-reactive protein, haptoglobin). It was concluded that orally administered endotoxins did not become systemically available.

The FEEDAP Panel concludes that the new data provide sufficient evidence that the generic effects seen with PT73 (TM) cannot be ascribed to circulating endotoxins or their derivatives.

Study with PT73 (THR)

An earlier study made with the biomass PT73 (THR) at levels of 3%, 8% and 15% also showed many of the generic effects observed in the studies done with the current product (PT73 (TM)), such as the increase in prothrombin time, increase in water intake and urine production, decrease in cholesterol and phospholipids, increase in liver, spleen and kidney weight, and no associated histopathological findings. Thrombocyte counts were significantly lower than controls in the high-dose group of both sexes and in mid-dose males. Prothrombin time was significantly increased in high-dose males. Serum alkaline phosphatase was increased in both sexes at mid and high doses. Cholesterol was significantly decreased in male mid- and high-dose groups, and phospholipids were reduced in the high-dose males only. Urinary volume was increased in the male mid- and high-dose groups. At necropsy, relative weights of adrenals and kidneys were significantly increased in mid- and high-dose males. Relative liver weights were increased in all male groups and the female high-dose only. Absolute and relative uterus weights were increased in high-dose females.

Discussion on Repeated dose oral toxicity studies

Many of the effects seen in the subchronic studies with PT73 (TM) were also seen with other bacterial biomass products, both the earlier form of this product (PT73 (THR)) and related products, also produced by fermentation with *E. coli,* PL73 (LM) and PL73 (LYS) (EFSA FEEDAP Panel, 2017). Since they are reproducibly related to exposure to these products it may be concluded that those effects are causally related to treatment. The main effects seen were an increase in water intake and liver weight, reduction of cholesterol, phospholipids and prolonged blood coagulation time.

Water intake was noted to be increased by treatment of rats with PT73 (TM) and for all other rat studies with biomasses. This effect at high doses is most likely explained by the higher intake of electrolytes. Renal weight changes, changes in faecal consistency and an increase in urine output may also be associated with such an effect (Lina and Kuijpers, 2004).

Serum cholesterol and phospholipid levels were decreased in dose dependent way in male rats (in females at the highest dose only) fed PT73 (TM). A similar finding is seen with related biomasses (PT73 (THR), PL73 (LM) and PL73 (LYS)) in rats. This reduction in cholesterol is attributed to the dietary compositional changes caused by the use of high levels of bacterial biomass. Although diets for rat studies were adjusted to be isoproteic, animal proteins are generally considered to be more cholesterolaemic than other proteins. The hypercholesterolaemic effect of casein/casein-based diets, compared to diets containing other sources of proteins, in rats and other species such as hamsters, rabbits, mice, pig and humans has been reported in numerous studies (Potter, 1995; Balmir et al., 1996; Morita et al., 1997; Nagaoka et al., 1999; Greaves et al., 2000; Tomotake et al., 2000; Ascensio et al., 2004; Esteves et al., 2011). Thus, the substitution in the experimental rat diets of casein by a protein of non-animal origin may suffice to explain the decreased blood cholesterol and phospholipids levels observed in laboratory animals fed diets containing PT73 (TM).

Prothrombin time was prolonged and thrombocyte counts reduced by treatment with PT73 (TM) and by all the other related biomass products. Although nutritional studies have occasionally shown effects of diet on one or other of the above, there is insufficient evidence to conclude that these are

nutritionally related rather than specific effects of the biomass. Although the effects are small, they are reproducible and must be considered adverse effects of treatment. Without knowing the origin of these effects, it cannot be identified whether or not they are relevant to consumer or target species risk.

Liver weight and relative liver weight are increased by treatment with PT73 (TM) and similar findings are reported for PT73 (THR), PL73 (LM) and PL73 (LYS). Histologically, the enlargement was associated with increased incidence of mononuclear cell aggregates. Such changes are normally present in rats but were increased by treatment. The histological and weight changes were not associated with a pattern of enzyme/clinical biochemistry changes normally associated with hepatic damage but some such changes (ALAT increased in high-dose females) were observed. Although the applicant suggests that the liver weight increase was due to increasing levels of ammonium and CP, there is no convincing evidence from other studies of dietary variation to support this hypothesis. As such the safety for both the consumer and target species of incorporating PT73 (TM) or any *E. coli*-derived biomass in feed cannot be sufficiently established from the available data.

3.6.2.2. Prenatal developmental toxicity

PT73 (TM) was included in the feed of groups of 24 mated female rats from day 0 to day 21 at concentrations of 0%, 4.9%, 9.8% and 19.6%. A reference control group was also included receiving basal diet plus 1.3% $(NH_4)_2SO_4$ in order to match the increased ammonium level in the high dose. Each rat was monitored daily; body weight and food intake were monitored regularly throughout gestation. Females were killed on day 21 and subject to gross necropsy with detailed examination of the uterus and contents. Half of the fetuses were examined for soft part abnormalities and the remainder for skeletal abnormalities. These examinations were carried out blind.

There were no effects of treatment on body weight or food intake of females. The number of females apparently pregnant was 17, 21, 22, 19 and 22 of control, low, mid, high and reference group respectively, but one high dose and one reference control female had no viable fetuses. No differences were observed between groups in gestation index, fecundity index, corpora lutea, implantation sites, pre- and post-implantation loss, live and dead fetuses, resorptions or sex ratio. External fetal and placental observations and weights did not reveal any treatment-related effects. No treatment-related effects were observed on visceral malformations, anomalies and variations, and on skeletal malformations, anomalies, variations and retardations.

Similar findings were observed with the former biomass PT73 (THR).

3.6.3. Conclusions on toxicological studies with PT73 (TM)

The genotoxicity studies conducted provide sufficient reassurance of the lack of genotoxicity of PT73 (TM) and leave no concerns in this respect for target species, consumer or user safety. Additionally, the studies conducted on fetal development demonstrate no evidence of adverse effects on the offspring; thus, there are no specific concerns regarding effects on fetal development.

The 90-day studies conducted with PT73 (TM) showed several reproducible effects, which were reduced cholesterol and phospholipid levels, increased water intake and liver weight (one study) and extended coagulation time. Of these, the effects on liver and coagulation time were considered by the Panel to be both adverse and unexplained.

3.6.4. Toxicological studies with antifoaming agent

During the manufacturing process, an antifoaming agent is used. The highest dose of the antifoaming agent tested (1,000 mg/kg in feed or 94 mg/kg bw per day) in a 28-day rat study showed no adverse effects. A 10% inclusion level in complete feed would result in a concentration of 1,500 mg/kg feed. This would correspond to an exposure of animals of approximately 23, 45 and 68 mg/kg bw of salmonids, dairy cows and pigs, respectively. The quantitative contribution of the antifoaming agent to the diet is not considered to represent a risk and is not further considered in this assessment.

3.7. Safety for the target species

The two products PT73 (TM) and PT73 (THR) are considered sufficiently similar to allow the use of the studies performed with either product for the assessment of PT73 (TM).

From the studies with dairy cows, pigs for fattening and rainbow trout it is concluded that PT73 (TM) can be used as any other protein-rich feed material in diet formulation when the guidelines for nutrition and the chemical composition and nutritive value of PT73 (TM) are properly taken into account. The study in dairy cows support the proposal of the applicant that inclusion of 8% PT73 (TM) of feed dry matter (~ 7% in complete feed) is safe for dairy cows. This value could be extended to other ruminants (from the beginning of rumination). Complete feed for pigs for fattening may contain up to 10% PT73 (TM). From the data obtained in an 84-day study with PT73 (THR) in rainbow trout, it is concluded that PL73 (TM) is safe for salmonids up to a dietary concentration of 13%.

Based on the studies on the influence of PT73 (TM) and PT73 (THR) on the sensory characteristics of milk and pork, the FEEDAP Panel concludes that an influence of PT73 (TM) up to the levels considered safe in the diet would not influence the sensory quality of tissues and products of animals.

3.8. Safety for the consumer

For animal feed ingredients or additives, assessment of the safety for the consumer is based upon the toxicological data and the relevance of those data to consumer exposure. The toxicological data indicate effects of PT73 (TM) on blood coagulation and liver which are considered to be adverse and which may occur in the target species following use of the product in animal feed. Since the causative agent(s) and mechanism(s) of these effects are unknown, there is no way of determining whether residues exist in animal products sufficient to cause similar effects in consumers. As a consequence, the FEEDAP Panel is unable to conclude on the safety for the consumer of products derived from animals receiving feed containing PT73 (TM).

3.9. Safety for the user

All the studies performed to assess the safety for the user were done with PT73 (THR). However, considering the similar production and composition, the FEEDAP Panel considers that the hazard for the user would not be significantly different for PT73 (TM).

3.9.1. Effects on eyes and skin

PT73 (THR) was tested in three rabbits for acute dermal irritation potential following OECD guideline 404. Since no effects were observed, the product is classified as not irritating to the human skin.

PT73 (THR) was tested in three rabbits for acute eye irritation potential according to OECD guideline 405. Effects on the conjunctiva were seen initially but these had cleared by 7 days after exposure. Based on the results of this study the product is classified as not irritating to the human eyes.

Although no data are provided on sensitisation potential the applicant states, the product to be both a respiratory and a dermal sensitiser.

3.9.2. Effects on the respiratory system

Due to the proteinaceous nature of the product, it should be considered as a respiratory sensitiser.

An acute inhalation toxicity study according to OECD guideline 403 was performed on a group of 5 rats of each sex was exposed to PT73 (THR) nose only for 4 h at a concentration of 5.25 g/m^3. Some slight breathing difficulties were seen during exposure and the general condition of animals was below normal for 1 day afterwards but had returned to normal by this time without any other signs of adverse effect or any mortality. No effects of treatment were seen at necropsy. The study conducted would probably not detect the respiratory inflammation effects typical of LPS and thus such effects in users cannot be excluded. Considering the levels of LPS present in the product (up to 1×10^6 EU/g), the Panel considers that any exposure to dust represents a serious risk to users.

3.9.3. Conclusions regarding user safety

PT73 (TM) is not considered a skin/eye irritant but should be considered as a potential skin and respiratory sensitiser. The FEEDAP Panel considers that due to the endotoxin content any exposure of users to dust from the product via the inhalation route should be considered a serious risk.

3.10. Potential environmental impact

The applicant provided data demonstrating that the PT73 (TM) product does not contain viable cells of the production strain *E. coli* FERM BP-10942. The heating and the inactivation conditions described in the dossier are considered to be efficient so that no viable production strain cells would be present in the final product.

No full-length antibiotic resistance gene sequences or other sequences of concern remained in the production strain and no DNA fragments of sufficient size to encode a gene were detected. Therefore, no environmental impact from the use of this product is expected regarding the recombinant DNA sequences possibly remaining in the product.

The FEEDAP Panel considers that substitution of PT73 (TM) for other protein-rich feed materials will not adversely affect the environment.

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[9] and Good Manufacturing Practice.

5. Conclusions[10]

The recipient organism *E. coli* MG 1655 is considered to be safe. The traits introduced in the production strain *E. coli* FERM BP-10942 are mainly limited to the overproduction of threonine. No full-length antibiotic resistance gene sequences or other sequences of concern remained in the production strain. In conclusion, the FEEDAP Panel did not identify risks for human and animal health or the environment from the heat-inactivated biomass, regarding the genetically modified strain from which it is made.

The proposed recommended use level for dairy cows (8% PT73 (TM) of feed DM (~ 7% in complete feed)) and salmonids (13%) is considered safe for these target animals. The conclusion form dairy cows could be extended to other ruminants (from the beginning of rumination). Complete feed for pigs for fattening may contain up to 10% PT73 (TM).

The toxicological data indicate effects of PT73 (TM) on blood coagulation and liver, which are considered to be adverse. Since the causative agent(s) and mechanism(s) of these effects are unknown, there is no way of determining whether residues exist in animal products sufficient to cause similar effects in consumers. As a consequence, the FEEDAP Panel is unable to conclude on the safety for the consumer of products derived from animals receiving feed containing PT73 (TM).

PT73 (TM) is not considered a skin/eye irritant but should be considered as a potential skin and respiratory sensitiser. The FEEDAP Panel considers that due to the endotoxin content any exposure of users to dust from the product via the inhalation route should be considered a serious risk.

The FEEDAP Panel considers that substitution of PT73 (TM) for other protein-rich feed materials will not adversely affect the environment.

6. Recommendation

Since the risks identified do not relate to the genetic modification but to the product itself (biomasses derived from fermentation with *E. coli* and potentially other Gram negative bacteria), the Panel recommends that similar non-genetically modified products used as feed materials should also be assessed for safety.

Documentation provided to EFSA

1) Application for authorisation of the dried killed bacterial biomass PT73 (TM) intended to be placed on the market as feed material, by-product of the production of L-threonine by fermentation using the strain *E. coli* K12 FERM BP-10942 (FERM BP-10942). April 2008. Submitted by Ajinomoto Eurolysine S.A.S.

[9] OJ L 35, 8.2.2005, p. 1.

[10] This section has been amended following the confidentiality claims made by the applicant on the basis of Article 30(1) of Regulation (EC) No 1829/2003.

2) Application for authorisation of the dried killed bacterial biomass PT73 (TM) intended to be placed on the market as feed material, by-product of the production of L-threonine by fermentation using the strain *E. coli* K12 FERM BP-10942 (FERM BP-10942). Supplementary information. March 2012. Submitted by Ajinomoto Eurolysine S.A.S.
3) Application for authorisation of the dried killed bacterial biomass PT73 (TM) intended to be placed on the market as feed material, by-product of the production of L-threonine by fermentation using the strain *E. coli* K12 FERM BP-10942 (FERM BP-10942). Supplementary information. July 2012. Submitted by Ajinomoto Eurolysine S.A.S.
4) Application for authorisation of the dried killed bacterial biomass PT73 (TM) intended to be placed on the market as feed material, by-product of the production of L-threonine by fermentation using the strain *E. coli* K12 FERM BP-10942 (FERM BP-10942). Supplementary information. February 2013. Submitted by Ajinomoto Eurolysine S.A.S.
5) Application for authorisation of the dried killed bacterial biomass PT73 (TM) intended to be placed on the market as feed material, by-product of the production of L-threonine by fermentation using the strain *E. coli* K12 FERM BP-10942 (FERM BP-10942). Supplementary information. April 2013. Submitted by Ajinomoto Eurolysine S.A.S.
6) Application for authorisation of the dried killed bacterial biomass PT73 (TM) intended to be placed on the market as feed material, by-product of the production of L-threonine by fermentation using the strain *E. coli* K12 FERM BP-10942 (FERM BP-10942). Supplementary information June 2014. Submitted by Ajinomoto Eurolysine S.A.S.
7) Application for authorisation of the dried killed bacterial biomass PT73 (TM) intended to be placed on the market as feed material, by-product of the production of L-threonine by fermentation using the strain *E. coli* K12 FERM BP-10942 (FERM BP-10942). Supplementary information. October 2016. Submitted by Ajinomoto Eurolysine S.A.S.

References

Ascensio C, Torres N, Isoard-Acosta F, Gomez-Pérez FJ, Hernandez-Pando R and Tovar AR, 2004. Soy protein affects serum insulin and hepatic SREBP-1 mRNA and reduces fatty liver in rats. Journal of Nutrition, 134, 522–529.

Bachmann BJ, 1987. Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12. In: Neidhart FC, Ingraham JL, Low KB, Magasanik B, Schaechter M and Umbarger HE (eds.). *Escherichia coli* and Salmonella typhimurium Cellular and Molecular Biology. American Society for Microbiology, Washington, DC. pp. 1190–1219.

Balmir F, Staack R, Jeffrey E, Berber Jimenez MD, Wang L and Potter SM, 1996. An extract of soy flour influences serum cholesterol and thyroid hormones in rats and hamsters. Journal of Nutrition, 126, 3046–3053.

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2011a. Guidance on the assessment of microbial biomasses for use in animal nutrition. EFSA Journal 2011;9(3):2117, 8 pp. https://doi.org/10.2903/j.efsa.2011.2117

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2011b. 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 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), 2012b. 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) Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos M, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Brantom P, Herman L, Kärenlampi S, Aguilera J, Galobart J, Holczknecht O and Chesson A, 2017. Scientific Opinion on the safety and nutritional value of a dried killed bacterial biomass from Escherichia coli (FERM BP-10941) [PL73 (LM)] as a feed material for pigs, ruminants and salmonids]. EFSA Journal 2017;15(7):4935, 29 pp. https://doi.org/10.2903/j.efsa.2017.4935

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

Esteves EA, Bressan J, Costa NMB, Martino HSD, Donkin SS and Story JA, 2011. Modified soybean affects cholesterol metabolism in rats similarly to a commercial cultivar. Journal of Medicinal Food, 14, 1363–1369.

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

Greaves K, Wilson A, Rudel MD, Williams LL and Wagner JD, 2000. Consumption of soy protein reduces cholesterol absorption compared to casein protein alone or supplemented with an isoflavone extract of conjugated equine estrogen in ovariectomized Cynomolgus monkeys. Journal of Nutrition, 130, 820–826.

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

Lina BAR and Kuijpers MHM, 2004. Toxicity and canrcinogenicty of acidogenic or alkalogenic diets in rats; effects of feeding NH_4Cl, $KHCO_3$ or KCL. Food and Chemical Toxicology, 42, 135–153.

Morita T, Oh-hashi A, Takei K, Ikai M, Kasaoka S and Kiriyama S, 1997. Cholesterol-lowering effects of soybean, potato and rice proteins depend on their low methionine contents in rats fed a cholesterol-free purified diet. Journal of Nutrition, 127, 470–477.

Nagaoka S, Miwa K, Eto M, Kuzuya Y, Hori G and Yamamoto K, 1999. Soy protein peptic hydrolysate with bound phospholipids decreases micellar solubility and cholesterol absorption in rats and Caco-2 cells. Journal of Nutrition, 129, 1725–1730.

Potter SM, 1995. Overview of proposed mechanisms for the hypocholesterolemic effect of soy. Journal of Nutrition, 125, 606S–611S.

Tomotake H, Shimaoka I, Kayashita J, Yokoyama F, Nakajoh M and Kato N, 2000. A buckwheat protein product suppresses gallstone formation and plasma cholesterol more strongly than soy protein isolate in hamsters. Journal of Nutrition, 130, 1670–1674.

Abbreviations

ALAT	alanine transaminase
ALP	alkaline phosphatase
ANOVA	analysis of variance
ASAT	aspartate transaminase
bw	body weight
CFU	colony forming unit
CP	crude protein
DM	dry matter
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
GM	genetically modified
LOQ	limit of quantification
LPS	lipopolysaccharides
LSD	least significant difference
MIC	minimum inhibitory concentration
NEL	Net energy for lactation
OECD	organisation for economic co-operation and development
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
RH	relative humidity
RNA	ribonucleic acid
RTG	relative total growth
TMR	total mixed ration
UV	ultraviolet