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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

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Abstract

PL73 (LM) 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 lysine. The recipient organism *E. coli* K-12S B-7 is considered to be safe. The traits introduced in the final modified strain *E. coli* FERM BP-10941 are mainly limited to the overproduction of lysine. 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 the biomass regarding the genetic modification of the strain. Although considering the zootechnical end-points only, the maximum safe level for dairy cows would be 6% PL73 (LM) of feed dry matter (~ 5% in complete feed) and for pigs for fattening up to 6% PL73 (LM), the unexplained effects on blood coagulation, on plasma lipoproteins in dairy cows and on total plasma bilirubin and liver weight in pigs prevent a clear conclusion of safe dietary levels for ruminants and pigs for fattening. PL73 (LM) is safe for salmonids up to a dietary concentration of 13%. The toxicological data indicate adverse effects of PL73 (LM) on blood coagulation and liver, which also occur in target species. As a consequence, the FEEDAP Panel is unable to conclude on the safety for the consumer of products derived from animals receiving feed containing PL73 (LM). PL73 (LM) 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 PL73 (LM) for other protein-rich feed materials will not adversely affect the environment.

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

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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.

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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 the safety and the nutritional value of a biomass produced by fermentation with *Escherichia coli*, a by-product of the production of lysine, as a feed material for pigs, ruminants and salmonids.

PL73 (LM) is a dried, killed bacterial biomass produced by fermentation of the genetically modified *Escherichia coli* FERM BP-10941. In this strain, genes for the overexpression of enzymes for L-lysine 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 strain.

Considering the zootechnical endpoints only, the maximum safe level for dairy cows would be 6% PL73 (LM) of feed dry matter (~ 5% 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 6% PL73 (LM). These levels would not influence the sensory quality of tissues and products of animals. However, the unexplained effects on blood coagulation, on plasma lipoproteins in dairy cows and on total plasma bilirubin and liver weight in pigs prevent a clear conclusion of safe dietary levels for ruminants and pigs for fattening. Consequently, no safe feed concentration of PL73 (LM) could be established for complete feed for ruminants and pigs for fattening. PL73 (LM) is safe for salmonids up to a dietary concentration of 13%.

The toxicological data indicate effects of PL73 (LM) on blood coagulation and liver, which are considered to be adverse and which also occur in target species. 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 PL73 (LM).

PL73 (LM) 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.

PL73 (LM) 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 PL73 (LM) 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 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-10941) (PL73 (LM)) 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 (PL73 (LM)) produced by the genetically modified *E. coli* FERM BP-10941 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 PL73 (LM) 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 PL73 (LM) 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 (PL73 (LM)), which is a by-product of L-lysine production by fermentation using a genetically modified strain of *E. coli* K-12 (FERM BP-10941). 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 PL73 (LM). It also contains some data relative to another biomass produced by a previous strain of *E. coli* (PL73 (LYS)). When data from this biomass has been used in the assessment, this will be indicated in the opinion.

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.

[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-2008-61. FEED dossier reference: FAD-2008-046.
[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.

After removal of the L-lysine, 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 the 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-10941[5]

The microorganism of which PL73 (LM) is made is a genetically modified derivative of *E. coli* K-12, deposited in the Japanese International Patent Organism Depositary and National Institute of Advanced Industrial Science and Technology with accession number FERM BP-10941. 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 29 antibiotics including 7 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-10941. The strain was found not to contain plasmids, transposons, sex factors or prophages.

The recipient strain, *E. coli* K-12S B-7, is a derivative of *E. coli* K-12 obtained by mutagenesis. *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 seven batches of the product. Two of the batches derived from small-scale pilot production (produced in 2006 and 2008), four from an industrial scale produced in 2008–2009 and one industrial scale produced in 2015. Four additional batches produced in 2015 were used for the analysis of dry matter (DM), crude protein (CP), antifoaming agent residues, purine and pyrimidine bases and endotoxin content. The results for the four industrial scale batches produced in 2008–2009 and those produced in 2015 are considered below in detail.

The product is characterised by its high content of CP (approx. 85%). The chemical composition is shown in Table 1.

Table 1: Chemical composition of the industrial batches of PL73 (LM) produced in 2008–2009 (4 batches) and 2015 (1 batch). Values are given in % of dry matter (DM) (except DM: % of the product)

	2008–2009[(a)]		2015[(b)]	
	Average	**Range**	**Average**	**Range**
Dry matter	93.8	91.7–95.6	95.9	94.4–97.1
Ash	2.1	1.6–2.5	2.2	–
Crude protein	84.9	83.2–87.1	85.9	84.5–87.4
Crude fat	11.0	9.5–13.6	7.8	–
Starch	0.1	–	–	–
Sugars	0.5	–	–	–

(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.

[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.

The total amino acid content of the four industrial batches (produced in 2008–2009) was between 69.4% and 71.0% (DM basis) (Table 2), coming mainly from bacterial protein, with free amino acids (other than lysine) representing only a minor fraction (0.123–1.15%). Free lysine amounted to 9.8% (DM basis) which is approximately 70% of total lysine (Table 2). The concentrations of the main amino acids (including asparagine and glutamine) showed little variation between the batches, except for a lower amount of L-lysine in one batch. Amino acid analyses performed with one batch produced in 2015 gave essentially the same results.

Table 2: Amino acids in the industrial scale batches of PL73 (LM) produced in 2008–2009. Values are given as % of dry matter

Amino acid	% (Dry matter basis)	Range
Lysine	13.73	11.59–15.13
Glutamic acid/glutamine	7.81	7.46–8.51
Aspartic acid/asparagine	6.79	6.50–7.20
Leucine	5.72	5.47–5.87
Alanine	4.71	4.48–5.05
Arginine	4.11	3.90–4.35
Valine	3.90	3.56–4.49
Threonine	3.33	3.07–3.72
Isoleucine	3.18	3.06–3.30
Glycine	3.18	3.01–3.41
Phenylalanine	2.86	2.80–2.96
Tyrosine	2.57	2.45–2.76
Serine	2.38	2.26–2.49
Proline	2.19	2.08–2.44
Methionine	1.68	1.60–1.73
Histidine	1.38	1.33–1.44
Tryptophan	1.06	1.01–1.10
Cystine	0.42	0.40–0.44
Total amino acids	**70.96**	**69.44–71.04**

Ammonium N (as NH_4) was 1.70% and 'true protein' (calculated by subtracting ammonia N from total N) was 76.6 for the four batches produced between 2008 and 2009. Low levels of nitrates ($\leq$ 0.1%) and nitrites ($\leq$ 0.003%) were detected, while betaine levels were 0–0.55%. Slightly lower values for ammonium N (1.12%) and higher values for true protein (85.8%) were obtained for the batch produced in 2015.

Three of the industrial batches (2008–2009) were analysed for levels of DNA + RNA which were 0.004–0.008%. The levels of nucleotides (purine and pyrimidine monophosphates) showed high variation between the production batches varying from below the level of detection[6] in one batch to 2.02% in another. Purine and pyrimidine bases were not measured in the industrial batches produced in 2008–2009. Values for the five batches produced in 2015 showed that purine bases were on average 0.6% DM while pyrimidine bases were on average 0.4%.

Three of the industrial batches produced in 2008–2009 were analysed for biogenic amines (putrescine, cadaverine, histamine, spermidine, agmatine, spermine, tyramine and phenylethylamine) with the following results (expressed on DM basis): 0.007–0.012% putrescine and 0.025–0.163% cadaverine; values for the other amines were below 0.005% with the exception of phenylethylamine and tyramine for which values were below the limit of detection of 0.01%. Results in one of the batches produced in 2015 were lower for all amines.

Analysis of organic acids (lactic acid, acetic acid, propionic acid, formic acid, pyruvic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid) showed very low levels in three of the batches produced in 2008–2009 (< 0.0001% DM basis). In the remaining batch, total organic acids represented < 1% DM, with acetic acid predominating. One of the batches produced in 2015 was also analysed and the total amount of organic acids was 0.03%.

[6] Limit of detection: 0.5 g/kg.

The most significant inorganic substances detected were sulfates (6–9% DM, equivalent to about 2–3% S). Other minerals are shown in Table 3.

Table 3: Content of minerals and trace elements of four industrial scale batches of PL73 (LM) produced in 2008–2009. Unless otherwise stated values are given as % dry matter

	Average	Range
Sodium	0.12	0.08–0.21
Potassium	0.22	0.16–0.26
Calcium	0.01	0–0.04
Magnesium	0.01	0.01–0.02
Chlorides	0.15	0.02–0.82
Sulfates	7.34	5.95–9.00
Phosphates	0.11	0.06–0.18
Iron (mg/kg product)	147	140–160
Copper (mg/kg product)	2.8	2.5–3.2

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 1.4×10^6 to 2.9×10^7 EU/g.[7]

3.3.2. Impurities

Heavy metals (Pb, Hg and Cd) and arsenic were measured in three industrial batches (2008–2009) and found to be well below the maximum authorised limits for feed materials. Residues of pesticides, dioxins and dioxins plus dioxin-like polychlorinated biphenyls (PCBs) were analysed in one batch and found to be below the limits of quantification (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 PL73 (LM) (produced in 2015) showed that the levels of antifoaming agent were approximately 20 g/kg.

The microbiological analysis of four industrial batches (2008–2009) showed that *Salmonella*, staphylococci, *Clostridium perfringens*, sulfite-reducers, coliforms and faecal streptococci were below the limit of detection. Yeasts were detected in one batch (500 CFU/g) and filamentous fungi were detected in three batches (60–5,000 CFU/g). All fermentation substrates are obtained with a specification that meets 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-10941 from the product PL73 (LM) 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). For both methods, 1 g of the inactivated fermentation product was tested.

The presence of recombinant DNA from *E. coli* FERM BP-10941 was investigated in two batches of PL73 (LM) by PCR, capable of amplifying a 454 bp fragment of recombinant DNA. Results indicated the presence of trace amounts of DNA from the production strain in the final product. Another PCR assay, targeting a 930 bp fragment, did not show any amplification.

3.3.3. Physical properties

PL73 (LM) is a slightly brown pelleted product (pellet size approx. 6–8 mm diameter) with an apparent bulk density of 664 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 3.6% w/w of the total product.

[7] 1 EU corresponds to 0.1 ng of the international reference endotoxin standard coming from *Escherichia coli* (amount present in 10^5 bacteria).

It was shown that 41% 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 689, 275 and 27 mg/kg product which would result in a classification of PL73 (LM) as of low-moderate dusting potential.

3.3.4. Storage life

In a stability trial, PL73 (LM) (one batch) was stored for 12 months under three different conditions (25°C/60% relative humidity (RH), 40°C/30% RH and 40°C/60% RH). Moisture content, water activity, crude nutrients, ammonium N/ammonia and biogenic amines were monitored. Additionally, microbiological quality was assessed by aerobic count, counts of *Staphylococcus aureus*, *Salmonella*, *Bacillus cereus,* yeasts, filamentous fungi and Enterobacteriaceae. Pellet quality was also measured. The parameters measured showed no deterioration of PL73 (LM) in any of the conditions studied.

PL73 (LM) was included in a complementary feed for dairy cows at 13.3%. Samples were stored at 5, 25 or 40°C with a RH of 60% for 3 months in punctured nylon polyethylene (PE)-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 PL73 (LYS) 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-10941

The strain *E. coli* FERM BP-10941, from which the product is made, was confirmed to have been derived from the recipient strain *E. coli* K-12S B-7. 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. The production strain was shown to be sensitive to all antibiotics tested. 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 (LM), obtained from *E. coli* BP-10941, 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 PL73 (LM)

3.5.1. Conditions of use

PL73 (LM) 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 7% 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 DM.

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 PL73 (LM). Some studies were made with the biomass PL73 (LYS) also derived from cells of *E. coli* K-12. As nutrient composition shows the PL73 (LYS) biomass is sufficiently similar to PL73 (LM) that results obtained with PL73 (LYS) can be considered in the assessment of PL73 (LM) (Table 4). Since some essential nutrient characteristics of PL73 (LYS) are inferior to those of PL73 (LM) (i.e. sum of amino acids, energy, ammonium N, sulfate), tolerable dietary levels derived from studies with PL73 (LYS) can be taken as a conservative estimate for PL73 (LM).

Table 4: Comparison of the composition of the biomasses PL73 (LM) and PL73 (LYS) (on % dry matter basis)

Parameter	PL73 (LM)[(a)]	PL73 (LYS)[(b)]
Dry matter	93.8	90.6
Crude protein	84.9	84.8
Ammonium N	1.4	3.1
Sum of total amino acid	71.0	61.8
Crude fat	11.0	6.3
Ash	2.1	2.6
Potassium	0.22	0.22
Sodium	0.12	0.29
Chlorides	0.15	0.84
Sulfates[(c)]	7.34	8.9
Phosphates (PO_4)	0.11	0.65

(a): Average of four batches (Section 3.3.1).
(b): One batch.
(c): Equivalent to 2.44–2.96% S.

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 (LM). The results showed an organic matter digestibility of 82%, protein solubility of 30% (of total CP), rumen escape protein (42–48% of CP).

The same exercise applied to the former biomass (PL73 (LYS)) showed the similarity of the two products (organic matter digestibility (76%), protein solubility (28% of CP) and rumen escape protein (35% of CP).

These data confirm the conclusion already made from the crude nutrient composition of both products that *in vivo* data from the former product can be used in the assessment of the product under evaluation.

These data support the assumption that the nutritive value of the PL73 (LYS), in terms of sum of amino acids and crude fat, is somewhat lower than that of the product under assessment. Therefore, the *in vivo* studies with that product would provide a conservative estimate of the nutritive value/tolerance of the target species to the product under assessment (PL73 (LM)).

An *in situ* study with three rumen fistulated dairy cows showed an effective rumen degradability of the protein fraction of PL73 (LM) of 85% based on passage rate of 6%/h.

3.5.2.2. *In vivo* digestibility with PL73 (LYS)

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% PL73 (LYS))/day), respectively. The apparent digestibility was 90% for CP and 66% for crude fat.

Pigs

The study was conducted on nine intact (for faecal digestibility) and nine fistulated (for ileal digestibility) pigs (barrows, body weight at start 28–30 kg) for 9 weeks to determine the apparent ileal and faecal digestibility of PL73 (LYS). 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/kg feed. The test diet contained 15% PL73 (LYS).

The apparent ileal (faecal) digestibility coefficients were 78 (86)% for DM, 83 (88)% for CP, and 87 with a range from 79 to 92% for the sum of amino acids. Digestible energy was 18 MJ/kg DM and net energy 8.5 MJ/kg DM.

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 (fishmeal (60%), gelatinised wheat starch (24%), fish oil (12%), supplemented with chromic oxide (1%) as an inert marker) or the test diet consisting of 70% basal diet and 30% PL73 (LYS), respectively. Faeces were collected for the second week of a 2-week feeding period. The apparent digestibility of CP from PL73 (LYS) was 84%. Digestible energy was 17 MJ PL73 (LYS)/kg DM.

3.5.2.3. Feeding studies in target animals – Dairy cows

Studies with PL73 (LM)

Study 1

To investigate the maximum incorporation rate of PL73 (LM) in diets for ruminants, a study was conducted on a total of 64 dairy cows (Dutch Holstein-Friesian type). After a 4-week adaptation period to 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 or 150 g of PL73 (LM) DM per kg TMR DM, referred to as RPL00, RPL05, RPL10 and RPL15, 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 PL73 (LM) (0%, 10.7%, 21.3% and 32.0%) at the expense of soybean meal, palm oil and urea. It contained 6.9 MJ net energy and about 30% CP. The content of intestinal digestible protein was calculated with 120, 141, 162 and 182 g for the concentrates with increasing amounts of PL73 (LM). 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 PL73 (LM) 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 7, individual blood samples were taken to evaluate the effect of PL73 (LM) on prothrombin time and concentrations of alkaline phosphatase, total protein, albumin, cholesterol, phospholipids, inorganic phosphate, packed cell volume and haemoglobin. Counts were made on total leukocytes, neutrophilic, basophilic and eosinophilic granulocytes, lymphocytes, monocytes, erythrocytes and thrombocytes.

Feed intake, milk production and milk composition data were analysed with the restricted maximum likelihood method (REML). Blood values from week 7 were analysed by analysis of variance (ANOVA).

In the first week of the experimental period, one cow each of group RPL05 and of group RPL15 were taken out of the trial, both because of severe lameness. Another cow of group RPL05 was taken out of the trial in week 3 of the experimental period after she stopped eating and was diagnosed with peritonitis. A fourth cow (group RPL10) was euthanised because of paratuberculosis in week 5.

Feed intake was significantly lower for treatment RPL15 than for the other treatments (Table 5), probably due to the high S content of the diet. Addition of 5%, 10% and 15% PL 73 added 1.2; 2.4 and 3.6 g S/kg DM and exceeded the maximum of S-intake by NRC (2005). Overall milk production declined during the experiment, but the decrease was most notable for treatment RPL15. Milk production and persistency was significantly lower for treatment RPL15 when compared to the other groups.

Milk fat concentration was significantly higher in group RPL10 from the start of the experiment, whereas other treatments were not significantly different from RPL00. Protein concentration was significantly higher in group RPL10 when compared to RPL00 or other treatments. Lactose concentration was significantly lower for treatment RPL15 when compared to other treatments. Table 5 informs on milk yield and composition in week 8.

Table 5: Feed intake, milk production (only data of week 8 of the experimental period are given) and composition and some blood parameters of dairy cows fed different levels of PL73 (LM)[1]

Group	RPL00	RPL05	RPL10	RPL15
PL73 (LM) Calculated (g/kg DM[2])	0	50	100	150
PL73 (LM) Actual (g/kg DM)	0	50	98	143
DM-intake (kg/day)	22.8^{a}	22.5^{a}	22.5^{a}	18.9^{b}
PL73 (LM)-intake (kg DM/day)	0	1.16	2.23	2.74
Milk yield (kg/day)	34.2^{b}	37.3^{a}	30.7^{b}	27.3^{b}
FPCM[3] (kg/day)	33.8	36.5	31.2	24.4
Protein (g/kg milk)	34.2^{b}	34.1^{b}	35.5^{a}	35.0^{a,b}
Protein (kg/day)	1.16	1.27	1.09	0.95
Fat (g/kg milk)	39.4^{a,b}	37.5^{b}	40.8^{a,b}	42.6^{a}
Fat (kg/day)	1.33	1.40	1.29	1.16
Lactose (g/kg milk)	44.3^{a,b}	45.1^{a}	44.1^{b}	43.3^{c}
Lactose (kg/day)	1.51	1.69	1.35	1.19
Urea (mg/100 mL milk)	42^{a}	39^{b}	37^{c}	32^{d}
Cholesterol (mmoL/L blood)	7.1^{a}	6.3^{a}	4.5^{b}	3.3^{c}
Phospholipids (mmoL/L blood)	3.9^{a}	3.5^{b}	2.6^{c}	2.0^{d}

(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,c,d: Means within a row with different superscript letters are significantly different ($p \leq 0.05$).

Concentrations of cholesterol and phospholipids decreased significantly with increasing inclusion level of PL73 (LM). Phosphate, prothrombin time, thrombocyte count and differential leukocyte counts showed significant differences between treatments but differences were not dose related; however, prothrombin time was significantly shorter in all PL73 (LM) groups compared to the control group. All other blood parameters (e.g. total protein, albumin, alkaline phosphatase, erythrocyte count, haemoglobin) were not significantly different between treatments.

In summary, the inclusion of 143 g PL73 (LM) DM/kg TMR DM (group RPL15) reduced feed intake, milk, fat, protein and lactose yield. Also 98 g PL73 (LM) DM/kg TMR DM (group RPL10) negatively affected fat, protein and lactose yield. It reduced dose related the plasma concentrations of cholesterol and phospholipids, prothrombin time was shortened for all treatment groups. Considering only the zootechnical endpoints, 50 g PL73 (LM) DM/kg TMR DM (group RPL05) can be considered as the maximum tolerated level for dairy cows.

Study 2

In this study, palm oil and soybean meal, were exchanged by different levels of PL73 (LM), beet pulp and urea in a TMR for dairy cows, containing grass silage, maize silage, grass seed hay and concentrates. The maximum level used in this experiment was 60 g of PL73 (LM) DM per kg ration DM to prevent an excessive consumption of intestinal digestible protein and rumen-degradable protein balance.

PL73 (LM) was tested at three dosage levels (20, 40 and 60 g DM per kg ration DM) in comparison to a negative control treatment. Diets were fed to 24 multiparous dairy cows, 40–120 days in lactation at the start of the trial. Effects of dietary treatments on feed intake and milk performance were tested in a complete Latin square design with four treatments and four periods of 4 weeks each. In the last 2 weeks of each period, individual feed intake and milk yield were recorded daily. Feed and milk samples were taken and analysed. Feed was analysed to estimate the intake of DM, organic matter, CP, net energy for lactation and digestible intestinal protein. Milk samples were analysed for fat, protein, lactose and urea concentration. The effect of treatment was analysed statistically by ANOVA; the quantitative effect of dosage level of PL73 (LM) by polynomial regression analyses. Although the individual feeding periods were shorter than required by the guidance (EFSA FEEDAP Panel, 2011b), the FEEDAP Panel considers that this study provides evidence to support the practical application of PL73 (LM).

The average daily DM intake on each treatment ranged between 25.1 and 25.6 kg/day. Crude protein intake ranged between 3.4 and 3.6 kg/day. Exchanging soybean meal and palm oil by PL73

(LM), beet pulp and urea had no effect on DM and net energy intake. CP intake and digestible intestinal protein intake decreased linearly with increasing inclusion level of PL73 (LM) due to the decreasing concentration of CP and digestible intestinal protein in the experimental concentrates.

Exchanging soybean meal and palm oil by PL73 (LM), beet pulp and urea had no effect on milk yield (average 36.9 kg/day), concentrations of milk protein (average 35.7 g/kg), lactose (average 46.4 g/kg) and urea (average 17.6 mg/100 mL), nor on yields of milk fat (average 1.64 kg/day), protein (average 1.34 kg/day) and lactose (average 1.75 kg/day). Milk fat concentration increased linearly with increasing inclusion level of PL73 (LM).

Studies with PL73 (LYS)

Study 1

Ten groups of two dairy cows (black and white Holstein Friesian type, 2–6 years old, from 93 to 241 days in lactation at start, parity: from first to fourth calf) were used in an incomplete Latin square design with three experimental periods. Each experimental period lasted 4 weeks. Each pair of cows received three different experimental diets. 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 PL73 (LYS), the intake of crude protein increased (12.5%, 15.6%, 18.9%, 21.9% and 24.9% CP in DM of the diets (TMR) with 0%, 5%, 10%, 15% and 20% PL73 (LYS), respectively. High protein feed materials should be compared on an isonitrogenous basis. Therefore, this study was not further considered.

Study 2

In a second study with 24 dairy cows (2–10 years old, parity: from first to eighth calf, 65–152 days in lactation at start), four levels of PL73 (LYS) (0%, 3.3%, 6.7% and 10.0% DM) were tested in an incomplete Latin square design with three 5-week periods. The cows received the ration as TMR of which the DM was based on 20% grass silage, 30% maize silage and 50% concentrate, the concentrate containing the varying levels of PL73 (LYS). The diets were isonitrogenous (18.8% CP) and isocaloric (7.0 MJ Net energy for lactation/kg DM). Although the design of the study shares some of the weaknesses described above, the use of isonitrogenous and isocaloric diets allows some comparison between treatments. Individual feed intake and milk yield were recorded daily, during the last 2 weeks of each experimental period, milk samples (four consecutive milkings each week) were collected and analysed for protein, fat, lactose and urea. In the last two experimental periods, blood samples were taken and analysed for prothrombin time, alkaline phosphatase, total protein, albumin, cholesterol, phospholipids, inorganic P, packed cell volume, haemoglobin and blood cell composition.

Actual inclusion levels were close to the intended values. The main results are given in Table 6. DM intake decreased with increasing PL73 (LYS) in the diet. Concomitant with the linear decrease in DM intake, the intake of CP and net energy decreased linearly. However, when the treatment with the highest inclusion level of PL73 (LYS) was excluded from the statistical analyses, no significant effect could be observed. The authors discuss the possibility that the ammonia smell of PL73 (LYS) may have reduced palatability of the PL73 (LYS) containing diets.

Table 6: Milk production and composition of cows feed graded levels of PL73 (LYS)

PL73 (LYS) (% dry matter)	0	3.3	6.7	10.0	LSD
Dry matter intake (kg/day)	20.6^{b}	$20.0^{a,b}$	$20.1^{a,b}$	19.5^{a}	0.64
Milk (kg/day)	31.1	31.1	30.9	30.2	1.2
Fat and protein corrected milk yield (kg/day)	31.6	31.1	31.6	30.4	1.6
Fat (g/kg milk)	41.3	40.8	40.6	41.1	1.3
Fat yield (g/day)	1.26	1.23	1.25	1.21	0.07
Protein (kg/kg milk)	35.9	35.9	36.2	36.1	0.5
Protein yield (kg/day)	1.09	1.07	1.10	1.07	0.08
Lactose (g/kg milk)	45.0^{b}	45.0^{b}	$44.7^{a,b}$	44.3^{a}	0.3
Lactose yield (kg/day)	1.39	1.37	1.38	1.32	0.07
Calcium (g/kg milk)	1.17^{c}	$1.15^{b,c}$	$1.13^{a,b}$	1.12^{a}	0.02

a,b,c: Means within a row with different superscript letters are significantly different ($p \leq 0.05$).

PL73 (LYS) did not significantly affect milk yield and yield of milk fat, milk protein and lactose, however, the concentrations of lactose and Ca decreased with increasing PL73 (LYS). The blood parameters determined gave no indications for any PL73 (LYS) effect.

Conclusions for dairy cows and other ruminants

From the three studies assessed and taking into consideration the weaknesses in study design, it can be concluded that commonly used protein-rich ingredients in rations for high-yielding dairy cattle can be replaced by biomass PL73 (LM) when using the current guidelines for dairy cow nutrition in practice, the chemical composition and the nutritive value of PL73 (LM) and other ration ingredients.

The FEEDAP Panel concludes that the biomass PL73 (LM) can be used in formulating diets for ruminants as any other protein-rich feed material up to level of 6% in DM. The levels of S resulting from the incorporation of this biomass at 6% in the ration DM would be well within the maximum concentration of sulfur in the diets for ruminants recommended by NRC (2005).[8]

3.5.2.4. Feeding studies in target animals – Pigs

Studies with PL73 (LYS)

Study 1

A total of 40 pigs (20 gilts, 20 boars, 32 kg body weight (bw) at start) were fed diets containing 0%, 5%, 10%, 15% and 20% PL73 (LYS), respectively. Four pigs per sex were allocated to each group and were individually housed and fed a grower diet for 6 weeks and a finisher diet for at least 4 weeks. The diets consisted mainly of maize, tapioca, soybean meal and wheat middlings. The PL73 (LYS) was included mainly at the expense of soybean meal, the diets were calculated to be isocaloric. K_2CO_3 and $NaHCO_3$ (at the expense of NaCl) were supplemented to prevent dietary electrolyte imbalances in the diets containing the PL73 (LYS).

Dietary CP content in the diets increased with increasing amounts of the PL73 (LYS) (for the diets with 0%, 5%, 10%, 15% and 20% PL73 (LYS), CP levels of 17.2%, 18.5%, 19.5%, 20.7% and 21.7% in the grower and 14.9%, 16.3%, 17.4%, 18.4% and 19.3% 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, faeces consistency were recorded in regular intervals. At day 54, blood samples were taken for haematology and routine blood chemistry.[9] 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.

One animal (5% PL73 (LYS)) was excluded due to pneumonia and subsequent poor performance, another of the same group due to slow growth rate. Overall performance during the trial was good (control group: body weight gain 985 g/day; feed intake 2.34 kg/day, feed to gain 2.38).

Feed intake, and as a result, body weight gain were found to be reduced over the whole experimental period at inclusion levels of 10–20% PL73 (LYS). Feed to gain ratio was not significantly affected by the experimental treatments ($p > 0.05$).

Faeces consistency was reduced with increasing levels of PL73 (LYS) in the diet. Water intake increased by up to 17% in treatments with 15 and 20% PL73 (LYS) compared to treatments with 0, 5 and 10% PL73 (LYS) in the grower phase and up to 32% in treatments with 15 and 20% PL73 (LYS) compared to the control treatment in the finisher period. These changes may be the result of an unbalanced anion/cation ratio, particularly of an excess of sulfur.

Haematological parameters were not affected by treatment. Clinical blood chemistry parameters were not affected by treatment except for bilirubin levels which were significantly higher in the 10% and 15% PL73 (LYS) groups (2.3 and 2.4 μmol/L for 10% and 15% PL73 (LYS), respectively),

[8] NRC values are 3 g S/kg DM concentrate and 5 g S/kg DM roughage. Assuming that the diet consists of equal quantities of concentrate and roughage, the corresponding value for complete feed for ruminants would correspond to 4 g S/kg DM complete feed. PL73 (LM) contains about 26 g S/kg DM. Incorporation of the biomass at 6% of the ration would result in an inclusion of 1.6 g S/kg DM complete feed.

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

compared to the control group (1.5 μmol/L). Organ weights were normal except for absolute and relative liver weights, which were increased at the 15 and 20% dose levels.

Study 2

Four groups of 64 pigs (four replicates with eight boars and four replicates with eight gilts, about 30 kg body weight at start) were fed for 10 weeks (6 weeks grower diets and 4 weeks finisher diets) diets containing 0%, 6%, 9% and 12% PL73 (LYS), respectively. The control diet was based on wheat, barley, maize, tapioca, soybean meal, peas and wheat middlings were incorporated in the diets at the expense of soybean meal and peas. The diets were formulated to be isoenergetic in terms of net energy and to meet the requirements for the levels of apparent ileal digestible lysine, methionine and cysteine, threonine and tryptophan. The diets containing PL73 (LYS) were supplemented with $NaHCO_3$ and K_2CO_3 to prevent dietary electrolyte imbalances. 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.

Dietary CP was analytically shown to be approximately equal in grower (diets with 0%, 6%, 9% and 12% PL73 (LYS), CP levels of 18.6%, 18.7%, 18.7% and 18.6%, respectively) and finisher diets (diets with 0%, 6%, 9% and 12% PL73 (LYS), CP levels of 17.0%, 17.0%, 17.1% and 17.0%, respectively).

Data of the control group (feed intake 2,272 g/day, weight gain 932 g/day and feed to gain ratio 2.44) are indicative of a well-designed and conducted study. Feed intake was not significantly affected by increasing amounts of PL73 (LYS) up to 12% for the total period, but decreased for the 12% PL73 (LYS) group in the grower period. Average daily gain was significantly reduced and feed to gain ratio increased in the groups receiving diets containing 9% and 12% PL73 (LYS). These effects were most pronounced in the grower period.

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.

Conclusions for pigs

The FEEDAP Panel concludes that PL73 (LYS) can be used in formulating diets for pigs for fattening up to a level of 6%. The Panel considers that this conclusion applies to PL73 (LM). Particular consideration should be given to the electrolyte balance including S of diets containing PL73 (LM).

3.5.2.5. Feeding studies in target animals – rainbow trout

Study with PL73 (LYS)

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 fishmeal (65.2%) was replaced by 10%, 20%, 40% and 60% PL73 (LYS), corresponding to actual levels of PL73 (LYS) in feed of 5.8%, 11.7%, 23.3% and 35.0%. Fish were fed twice a day by hand near to visual satiety. Water was supplied from natural springs at a constant temperature of 17 ± 1°C. The content of CP of the control diet was 50.5% in DM, the diets with 5.8%, 11.7%, 23.3% and 35.0% PL73 (LYS) contained 50.0%, 50.0% 49.9% and 49.8% CP in DM. Gross energy (17.6 MJ/kg DM in the control diet) rose with increasing amounts of PL73 (LYS) up to 20.2 MJ/kg DM in the 35.0% PL73 (LYS) 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, 3 and 24 h after slaughter.

Survival rate was high (> 90%) and not affected by diet. Feed intake (values given in graphical form only), expressed as g/fish per day, declined significantly ($p < 0.05$) in the group with 35% dietary PL73 (LYS) after the first 2 weeks. Final body weight of trout fed diets with 5.8%, 11.7% and 23.3% PL73 (LYS) (403, 412 and 396 g, respectively) did not differ from the control group (409 g), however 35% PL73 (LYS) led to a significant growth depression (final weight 338 g). Gain to feed DM ratio was also significantly reduced at 35% PL73 (LYS) (1.09, 1.06, 0.97, 0.99 and 0.94 gain/feed DM for the diets with 0%, 5.8%, 11.7%, 23.3% and 35% PL73 (LYS), respectively). There were no significant differences between groups for the viscero-somatic 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, 5.8%, 11.7% and 23.3% PL73 (LYS)) 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 PL73 (LYS), especially at 23.3% PL73 (LYS).

As regards whole body composition, trout fed the 35% PL73 (LYS) diet had a significantly lower protein content (16.2%) as compared to the other groups (control group: 17.0% CP). Protein and energy retention were significantly higher in trout fed the control diet and the diet with 5.8% PL73 (LYS) than in trout fed the other experimental diets (protein retention for the groups with 0%, 5.8%, 11.7%, 23.3% and 35% PL73 (LYS) 36%, 34%, 31%, 32% and 29%, and energy retention 58%, 60%, 49%, 51% and 42%, respectively).

Conclusions for salmonids

The results of the feeding study with PL73 (LYS) in rainbow trout support the recommendation of the applicant for the use of PL73 (LM) up to 13% in the diet for salmonids.

3.5.3. Product quality

Morning milk samples were obtained in the first study with dairy cows reported (see Study 1 with PL73 (LM)). Milk was homogenised and pasteurised before a taste panel evaluation. 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 6.1 and 6.3, with no significant differences between treatment groups. No off-flavours were observed.

Sensory properties of meat samples of seven pigs taken randomly from the control group and the group with 12% dietary PL73 (LYS) from the experiment described above (see Study 2 in pigs) were evaluated. A taste panel considered 23 sensory attributes divided into the categories appearance, odour, texture, taste and aftertaste according to ISO standard 6564 (1985). For most attributes (except for 'tough' in the texture category), no significant differences between the meat from control pigs and the 12% PL73 (LYS) group were found. The 'tough' parameter was marginally improved in the meat from animals given PL73 (LYS) diet.

Although sensory attributes can be quite subtle, it is probable that the results seen with PL73 (LYS) would also apply to comparable diets with PL73 (LM).

3.6. Toxicological characterisation

The toxicological studies were made with various batches (from pilot scale to industrial scale) which were all considered representative of the product. Duplicate studies were also made with the former biomass PL73 (LYS).

3.6.1. Genotoxicity and mutagenicity studies

As the bacterial biomass PL73 (LM) is an insoluble material, aqueous extracts were used for *in vitro* genotoxicity tests. Suspensions of PL73 (LM) 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 PL73 (LM) 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* WP2*uvr*A, 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 PL73 (LM) 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). Four concentrations (625, 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 two highest dose levels 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 (1,000, 1,500 and 3,000 μg/mL) were analysed. The pulse treatment with S9-mix was slightly cytotoxic to the cells at all dose levels, while the continuous treatment without S9-mix induced dose related cytotoxicity and the two higher dose levels (4,000 and 5,000 μg/mL) could not be analysed, due to lack of metaphases. In the first test, the highest concentration of the test substance without metabolic activation induced a slight but statistically significant increase in the number of the aberrant cells, when compared to the control cultures. However, the observed increase was within the historical data of the testing facility and was not confirmed in the second test;, therefore, it was not judged to be biologically relevant. 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 PL73 (LM) 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 PL73 (LM) evaluated for mutagenicity in the presence of S9-mix was equivalent to 5,000 μg/mL. In the absence of S9-mix, the highest concentration evaluated was equivalent to 1,200 and 5,000 μg/mL after 24 and 4 h treatment, respectively. The extract of PL73 (LM) was toxic to the cells, especially in the absence of S9-mix after extended (24 h) treatment. In the absence of S9-mix the relative total growth (RTG) was decreased at and above 398 and 1,200 μg/mL equivalent, after 24 and 4 h treatment, respectively. The RTG values at the highest concentrations evaluated for mutagenicity were 6% and 19%, respectively. In the presence of S9-mix, the RTG was decreased at and above a concentration of 1,200 μg/mL equivalent. The RTG value at the highest concentration evaluated for mutagenicity was 21%. 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 former biomass PL73 (LYS) also gave negative results.

3.6.2. Toxicity studies with laboratory animals

3.6.2.1. Repeated dose subchronic oral toxicity study

Study 1

A study was conducted with a sample of a pilot batch of PL73 (LM) which was administered in the diet (at the expense of casein) to groups of ten rats of each sex for 90 days at concentrations of 0%, 3% (low), 8% (mid) or 15% (high) in compliance with OECD guideline 408. Animals were monitored at least once daily and examined in detail once weekly. Body weight and food intake were monitored weekly throughout the study. Water intake was measured during 5-day periods in weeks 1, 6 and 11 only. Ophthalmic examinations were made at the start and end of the study. Functional observations were made in the 12th week of treatment. A renal concentration test and urinalysis were carried out shortly before the end of treatment. Haematology and blood chemistry were carried out on blood collected from the aorta at necropsy. All animals were subject to full necropsy with organ weights with tissues preserved. Tissues were processed and examined histologically only for control and high-dose groups apart from liver and spleen which were examined from all groups. Sperm analysis was carried out on samples collected at necropsy and for females the oestrous cycle was examined over a three-week period at the end of the study.

There was no effect of treatment on clinical condition, ophthalmoscopy, functional observations, and body weight or food intake. Overall intake of PL73 (LM) was calculated for low-, mid- and high-dose groups, respectively to be 1.4, 4.0 and 7.8 g/kg bw per day for males and 1.5, 4.2 and 8.1 g/kg bw per day for females. Water intake was generally increased in the two highest dose groups of both sexes throughout the study.

Thrombocyte counts were reduced in a dose-related manner in females. Prothrombin time was significantly increased in all male treated groups. A higher reticulocyte count was seen in high dose males.

Cholesterol and phospholipids were reduced in all male groups while creatine and aminoaspartate transferase (ASAT) were higher in high-dose males than controls. There were trends towards higher triglyceride levels and lower urea and glucose levels in females and higher sodium and inorganic phosphorus in both sexes.

Urinary pH was increased in the male high-dose groups otherwise there were no treatment–related differences in the results of urine examinations.

At necropsy, relative liver weights were increased in mid and high-dose groups of both sexes.

Brown pigmentation was seen in the spleen of females but this was investigated and determined to be an artefact, unrelated to treatment. In the liver, the degree of mononuclear cell aggregates/necrotic hepatocytes was slightly increased in all groups of males but was not considered by the contract laboratory to be treatment-related. In the kidneys, the incidence of unilateral pelvic dilatation was significantly higher in the high-dose females (5 out of 10). Because the dilatation was unilateral and not accompanied by any evidence of pathological changes, it was not considered to be related to treatment.

Study 2

A second repeated dose oral toxicity study was conducted to provide evidence on the possible mechanism of the observed generic effects (an increase in water intake and liver weight, reduction of cholesterol, phospholipids and prolonged blood coagulation time). Three groups of 15 male Wistar WU (Crl:WI(WU), outbred) rats received control feed, PL73 (LM) at 5% or 15%. Observations were conducted similarly to those described for the previous study and the new study confirmed those effects observed previously (prolonged prothrombin time, lower levels of plasma cholesterol and phospholipids, increased 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, 102,000–107,000 for the 5% group and 220,000–235,000 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% PL73 (LM) 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 PL73 (LM) cannot be ascribed to circulating endotoxins or their derivatives.

Study with PL73 (LYS)

An earlier study made with the old biomass (PL73 (LYS)) at levels of 3%, 8% and 15% also showed many of the effects observed in the studies done with the current product (PL73 (LM)). 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 subchronic oral toxicity studies

Many of the effects seen in the subchronic studies with PL73 (LM) were also seen with other bacterial biomass products, both the earlier form of this product (PL73 (LYS)) and related products, also produced by fermentation with *E. coli,* PT73 (TM) and PT73 (THR) (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 PL73 (LM) and for all other rat studies with biomasses; a similar effect was also seen in pigs. This effect at high doses is most likely explained by the higher intake of sulfate and electrolytes, with sulfate playing the major part. 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 all male rats fed PL73 (LM). A similar finding is seen with related biomasses (PL73 (LYS), PT73 (TM) and PT73 (THR)) in rats and with PL73 (LM) in dairy cows. 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 explain the decreased blood cholesterol and phospholipids levels observed in laboratory animals fed diets containing PL73 (LM).

Prothrombin time was prolonged and thrombocyte counts reduced by treatment with PL73 (LM) and by all the other related biomass products. Although the 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 PL73 (LM) and similar findings are reported for PT73 (TM), PL73 (LYS) and PT73 (THR). Histologically, the enlargement was associated with increased incidence of necrotic hepatocytes and 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 (alanine transaminase (ALAT) increased in high-dose males) were observed. Liver weight was also increased in pigs fed PL73 (LYS). 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 PL73 (LM) or any *E. coli*–derived biomass in feed cannot be sufficiently established from the available data.

3.6.2.2. Prenatal developmental toxicity

PL73 (LM) was included in the feed of groups of 24 mated female rats from day 0 to day 21 at concentrations of 0, 3.2, 8.4 and 15.8% in accordance to OECD guideline 414. A reference control group was also included receiving basal diet plus 1.2% $(NH_4)_2SO_4$. 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 foetuses 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, 23, 19, 24 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 foetal 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 PL73 (LYS).

3.6.3. Conclusions on toxicological studies with PL73 (LM)

The genotoxicity studies conducted provide sufficient reassurance of the lack of genotoxicity of PL73 (LM) and leave no concerns in this respect for target species, consumer or user safety.

Additionally, the studies conducted on foetal development demonstrate no evidence of adverse effects on the offspring; thus, there are no specific concerns regarding effects on foetal development.

The two 90-day studies conducted with PL73 (LM) showed several reproducible effects, which were reduced cholesterol and phospholipid levels, increased water intake and liver weight 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 the 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 2,000 mg/kg feed. This would correspond to an exposure of animals of about 30, 60 and 90 mg/kg body weight 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 target species

The two products PL73 (LM) and PL73 (LYS) are considered sufficiently similar to allow the use of the studies performed with either product for the assessment of PL73 (LM).

From the studies with dairy cows, pigs for fattening and rainbow trout and considering production endpoints only, it would appear that PL73 (LM) 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 PL73 (LM) are properly taken into account. The maximum safe level for dairy cows would be 6% PL73 (LM) of feed dry matter (~ 5% 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 6% PL73 (LM).

Based on the studies on the influence of PL73 (LM) and PL73 (LYS) on the sensory characteristics of milk and pork, the FEEDAP Panel concludes that an influence of PL73 (LM) up to the levels considered safe in the diet would not influence the sensory quality of tissues and products of animals.

However, unexplained effects on blood coagulation (reduced prothrombin time) and less relevant on plasma lipoproteins (reduced cholesterol and phospholipids) in the 56-day cow study, and on total plasma bilirubin and absolute and relative liver weight (both showed a dose related increase) in the pig study (≥ 70 days of experiment) prevent a clear conclusion of safe dietary levels for ruminants and pigs for fattening. These concerns are supported by toxicological data in laboratory animals indicating effects of PL73 (LM) on blood coagulation and liver, which are considered to be adverse.

Consequently, no safe feed concentration of PL73 (LM) can be established for complete feed for ruminants and pigs for fattening.

From all data obtained (including plasma metabolites and morphometry) in an 84-day study with PL73 (LYS) in rainbow trout. it is concluded that PL73 (LM) is safe for salmonids up to a dietary concentration of 13%.

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 in rats indicate effects of PL73 (LM) on blood coagulation and liver which are considered to be adverse and which may occur in the target species following use of PL73 (LM) 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 PL73 (LM).

3.9. Safety for the user

All the studies performed to assess the safety for the user were done with PL73 (LYS). However, considering the similar production and composition, the FEEDAP Panel considers that the hazards for the user would not be significantly different for PL73 (LM).

3.9.1. Effects on eyes and skin

PL73 (LYS) 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.

PL73 (LYS) was tested in three rabbits for acute eye irritation potential following OECD guideline 405. Effects on the conjunctiva were seen initially but these had cleared by 72 h after exposure. Based on the results of this study the product is classified as not irritating to the human eyes.

No data were provided on the skin sensitisation potential. However, the material safety data sheet for the product states the product to be 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.

A group of five rats of each sex was exposed to PL73 (LYS) nose only for 4 h at a concentration of 5.26 g/m^3 following OECD guideline 403. Some slight breathing difficulties were seen during exposure and the general condition of animals was below normal for 3 days 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 2.9×10^7 EU/g), the Panel considers that any exposure to dust represents a serious risk to users.

3.9.3. Conclusions regarding user safety

PL73 (LM) 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 PL73 (LM) product does not contain viable cells of the production strain *E. coli* FERM BP-10941. 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 PL73 (LM) 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[10] and Good Manufacturing Practice.

5. Conclusions[11]

The recipient organism *E. coli* K-12S B-7 is considered to be safe. The traits introduced in the production strain *E. coli* FERM BP-10941 are mainly limited to the overproduction of lysine. 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, as a result of the genetically modified strain from which it is made.

Considering the zootechnical end-points only, the maximum safe level for dairy cows would appear to be 6% PL73 (LM) of feed dry matter (~ 5% in complete feed). This value could be extended to other ruminants (from the beginning of rumination). Complete feed for pigs for fattening may contain

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

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

up to 6% PL73 (LM). These levels would not influence the sensory quality of tissues and products of animals. However, the unexplained effects on blood coagulation, on plasma lipoproteins in dairy cows and on total plasma bilirubin and liver weight in pigs prevent a clear conclusion of safe dietary levels for ruminants and pigs for fattening. Consequently, no safe feed concentration of PL73 (LM) could be established for complete feed for ruminants and pigs for fattening. PL73 (LM) is safe for salmonids up to a dietary concentration of 13%.

The toxicological data indicate effects of PL73 (LM) on blood coagulation and liver, which are considered to be adverse and which also occur in target species. 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 PL73 (LM).

PL73 (LM) 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 PL73 (LM) 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 PL73 (LM) intended to be placed on the market as feed material, by-product of the production of L-lysine by fermentation using the strain *E. coli* K12 No19E (FERM BP-10941). July 2008. Submitted by Ajinomoto Eurolysine S.A.S.
2) Application for authorisation of the dried killed bacterial biomass PL73 (LM) intended to be placed on the market as feed material, by-product of the production of L-lysine by fermentation using the strain *E. coli* K12 No19E (FERM BP-10941). Supplementary information. March 2012. Submitted by Ajinomoto Eurolysine S.A.S.
3) Application for authorisation of the dried killed bacterial biomass PL73 (LM) intended to be placed on the market as feed material, by-product of the production of L-lysine by fermentation using the strain *E. coli* K12 No19E (FERM BP-10941). Supplementary information. April 2013. Submitted by Ajinomoto Eurolysine S.A.S.
4) Application for authorisation of the dried killed bacterial biomass PL73 (LM) intended to be placed on the market as feed material, by-product of the production of L-lysine by fermentation using the strain *E. coli* K12 No19E (FERM BP-10941). Supplementary information. October 2016. Submitted by Ajinomoto Eurolysine S.A.S.

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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
N	nitrogen

OECD	Organisation for Economic Co-operation and Development
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
PE	polyethylene
QPS	Qualified Presumption of Safety
REML	restricted maximum likelihood method
RH	relative humidity
RNA	ribonucleic acid
RTG	relative total growth
TMR	total mixed ration
UV	ultraviolet