Application under article 17 of Regulation 1829/2003

PL73 (LM)

**Dried killed bacterial biomass,
by-product of L-Lysine production by fermentation
using a genetically modified strain of *E. coli* K12,
intended to be used as feed material**

PART II

Summary

**Dossier prepared using, as guidance,
the 'Guidance Document for the risk assessment
of genetically modified microorganisms and
their derived products intended for food and feed use'
(adopted on 17 May 2006, The EFSA Journal 374)**

AJINOMOTO EUROLYSINE S.A.S.
June 2008

SUMMARY OF APPLICATION FOR THE GM PRODUCT PL73 *E.COLI* (LYS) FOR FEED USE

A. GENERAL INFORMATION

1. Details of application

a) Member State of application France
b) Application number Not yet allocated to the Applicant at the time of the remittance of the dossier to the French competent authorities.
c) Name of the product (commercial and other names) -For the purpose of this dossier: PL73 (LM) -Commercial name: PROT-AEL-L or 'PROT-AEL' (*not completely fixed yet and subject to the confirmation that it may be acceptable as registered trade mark*)
d) Date of acknowledgement of valid application Validity of the application to be established by EFSA.

2. Applicant

a) Name of applicant Ajinomoto Eurolysine S.A.S, contact person: Philippe GUION
b) Address of applicant 153, rue de Courcelles 75817 PARIS Cedex 17 France
c) Name and address of the person established in the Community who is responsible for the placing on the market, whether it be the manufacturer, the importer or the distributor, if different from the applicant (Commission Decision 2004/204/EC Art 3(a)(ii)) The person established in the Community responsible for the placing on the market will be the Applicant.

3. Scope of the application

☐ GM microorganisms and/or derived products for food use

■ GM microorganisms and/or derived products for feed use

☐ GM microorganisms and/or derived product(s) belonging to Group 1, as defined in Chapter II, 2. of this Guidance

■ GM microorganisms and/or derived product(s) belonging to Group 2, as defined in Chapter II, 2. of this guidance

☐ GM microorganisms and/or derived product(s) belonging to Group 3, as defined in Chapter II, 2. of this guidance

☐ Import and processing (Part C of Directive 2001/18/EC)

4. Is the product being simultaneously notified within the framework of another regulation?

Yes ☐	No ■
If *yes*, specify	

5. Has the GM microorganism been notified under Part B of Directive 2001/18/EC and/or Directive 90/220/EEC?

Yes ☐	No ■
If *no*, refer to risk analysis data on the basis of the elements of Part B of Directive 2001/18/EC	

6. Has the GM microorganism or derived products been previously notified for marketing in the Community under Part C of Directive 2001/18/EC or Regulation (EC) 258/97?

Yes ☐	No ■
If *yes*, specify	

7. Has the product been notified in a third country either previously or simultaneously?

Yes ☐	No ■
If *yes*, specify	

8. General description of the product

a) Name of the recipient or parental microorganism and the intended function of the genetic modification

The product PL73 (LM), subject of the present application, consists of the dried killed cells of a genetically modified strain of *Escherichia coli* K-12 (*E. coli* K-12), named strain N°19E. The strain N°19 E is intended to be used by the Applicant for the production of L-lysine by fermentation of substrates of agricultural origin.

Strain N°19 E has been constructed from a specific mutant strain of *E. coli* K-12 – strain *E. coli* K-12S B-7 - in several steps using conventional and modern techniques of genetic modifications. The purpose of the genetic modifications is to obtain a high production rate of L-lysine.

b) Types of products planned to be placed on the market according to the authorization applied for

The product PL73 (LM) (dried killed bacterial biomass) mentioned in a), will be a by-product of the L-lysine manufacturing process using strain N°19E.

c) Intended use of the product and types of users

Considering its high nitrogen content, this dried killed bacterial biomass may serve as a direct or indirect source of protein for animals. Therefore, a use as feed material - a concentrated source of crude protein - for compound feedingstuffs formulated for pigs, salmonids and ruminants (inter alia dairy cows) is considered.

The product will be sold in pellet form (minimization of dust production) and in 'bulk' to feed mills only.

d) Specific instructions and/or recommendations for use, storage and handling, including mandatory restrictions proposed as a condition of the authorization applied for

Handling
Instructions for handling, mentioned in the material safety data sheet, are standard precautions for powdered products or products generating fine dust.
In particular:

- Avoid dust dispersion during grinding
- Provide mechanical exhaust or ventilation
- Clean and eliminate dust from equipments regularly
- Use anti-static equipment
- Avoid heat sources and sparks

The product may cause sensitisation by inhalation and skin contact (as any protein-containing product). It may also cause feelings of discomfort.
In particular, wear:

- A face shield with skin contact, or mask (paper mask) and safety goggles with skin contact
- Gloves
- Wear protective clothes covering skin (discard or wash protective clothes after exposition to dust of the product).

Storage
The product shall be stored at dry conditions in standard silos and kept away from ignition and heat sources

Use in compound feedingstuffs:

* Pigs (for fattening, grower- finisher)
Maximum incorporation rate in the feed: 6 % on DM basis (or ~7% on 'as is' basis).

* Dairy cows (for milk production) & ruminants (in general for meat and milk production as from the beginning from rumination)
Maximum incorporation rate in the feed: 7.3% on dry matter basis (~8% on 'as is' basis).

* Salmonids
Maximum incorporation rate in the feed: 13% on 'as is' basis (or replacement of 20% of fish meal, feedingstuff containing 65% fish meal).

e) Any proposed packaging requirements

Except pelletisation the product will be sold as such in bulk to feed mills. There are no proposed packaging requirements.

f) A proposal for labeling in accordance with Articles 13 and Articles 25 of Regulation (EC) 1829/2003. In the case of GMOs, food and/or feed containing or consisting of GMOs, a proposal for labeling has to be included complying with the requirements of Article 4, B(6) of Regulation (EC) 1830/2003 and Annex IV of Directive 2001/18/EC

a) As feed material

- Feed material
- *(Name)*: Bacterial protein, by-product from the production of L-lysine, produced from genetically modified microorganism
- Nitrogen expressed as crude protein
- Moisture: (commercial specification retained: maximum 12%)
- Crude Ash
- Approval number (Regulation (EC) No 183/2005): **α FR 80 021 090**
- Batch number
- Expiry date
- Net quantity
- Name and address of the producer (which is also the person placing the product on the market)

b) Declarations to be made on the label or packaging of compound feeding stuffs

- *Name of the feed material*: 'Bacterial protein, by-product from the production of L-lysine, produced from genetically modified micro-organism'
- Amount of the product contained in the feedingstuff.
- Percentage of the total crude protein provided by non-protein nitrogen

As the product will be delivered in bulk to feed mills (delivery by means of tank trucks), the information corresponding to labelling will be provided to customers by means of the commercial documents preceding or accompanying the delivery of the product (taking into account the official language of the country of destination) and the commercial technical sheet corresponding to this product.

g) Unique identifier for the GM microorganism in accordance with Regulation (EC) 65/2004

Not applicable.
Strain N°19E is used for production in containment only.

The manufacturing process of L-lysine and of the by-product/ dried killed bacterial biomass PL73 (LM) ensures that the final product will not contain viable cells or transferable DNA of the L-lysine producer GM microorganism.

h) If applicable, geographical areas within the EU to which the product is intended to be confined under the terms of the authorization applied for. Any type of environment to which the product is unsuited

The authorization sought by the Applicant for a use as feed material of the product 'PL73 (LM)' concerns the EU market.

Environment to which the product is unsuited: not applicable.

9. Measures suggested by the applicant to take in case of unintended release or misuse as well as measures for disposal and treatment

The product PL73 (LM) does not contain viable cells and no transferable DNA of the L-lysine-producer GM microorganism. Therefore, it is not necessary to take measures, because no unintended release or misuse is expectable.

B. INFORMATION RELATING TO THE GMM

1. Characteristics of the recipient or (when appropriate) parental organism

1.1 Identity

a) Common name Strain *Escherichia coli* K-12S B-7 (also named VKPM B-7)
b) Strain designation Strain designation is that provided in the literature. The strain is only a laboratory strain used in the case of the applicant, for the construction of the final L-lysine producer microorganism (i.e. strain N°19E).
c) Source of the strain *E. coli* K-12
d) Accession number from a recognized culture collection Available in several culture collections

1.2 Taxonomy

a) Genus *Escherichia*
b) Species *Escherichia coli*
c) Subspecies Not applicable
d) Strain K-12S B-7 (or VKPM B-7, mutant of *E. coli* K-12) Confirmation of the taxonomic position of the strain *E. coli* K-12S B-7 (and of the final lysine producer: strain N°19E) was made using ribotyping and serotyping.

1.3 Other names

VKPM B-7.

1.4 Phenotypic and genetic markers

a) Phenotypic and genotypic information relevant to identification, genetic stability and safety

- Single cells, which are Gram-negative rods and not sporulating
- Colony: size around 2 to 3 mm, round, rough and whitish with a clear edge
- Glucuronidase activity
- *E. coli* K-12S B-7 strain has the following genotype: F^- , $P1^-$, λ^-, supE and relA
- No evidence of instability of the recipient strain

b) Information on pathogenicity

As a mutant of *E. coli* K-12, the body of knowledge concerning the absence of pathogenicity of *E. coli* K-12 also applies to the strain *E. coli* K-12S B-7
Confirmation of the taxonomic position of the strain *E. coli* K-12S B-7 was made using ribotyping and serotyping
The results of the molecular typing study performed on *E. coli* K-12S B-7 (and the lysine producer: strain N°19E) are described in B.1.11.d of this summary.

c) Biological properties

Following nutritional and physicochemical demands apply to the development of the *E. coli* K-12S B-7 strain:

- Facultative anaerobic micro-organism with optimum growth temperature of 37 °C and optimum growth pH between 6.8 and 7.2,
- *E. coli* K-12 requires a supply of organic carbon which can be provided by the addition of carbon/carbohydrates-containing sources to the culture medium,
- *E. coli* K-12 requires a supply of nitrogen which can be provided either a mineral form (ammonia or ammonium sulphate) or in an inorganic form (amino acids).

1.5 Degree of relatedness between recipient and donor(s), when appropriate

Except for two genes, the sequences introduced or modified in the final strain N°19E are all coming from *E. coli* K-12 genomes or vectors/ transposons developed from *E coli* K-12 strains. Additional information is considered confidential.

1.6 Description of identification and detection techniques

Ribotyping and serotyping are used as identification and detection techniques. However, it is not considered relevant to develop specific detection techniques for the parental strain *E. coli* K-12S B-7 considering that it is a laboratory strain well described in the literature.

1.7 Sensitivity, reliability and specificity of the detection techniques

This section is not relevant for the recipient strain *E. coli* K-12S B-7, as it is a laboratory strain which has been well described in the literature. The ribotyping and serotyping techniques mentioned in 1.6, with serotyping being based on PCR are sensitive, reliable and specific.

1.8 Source and natural habitat of the recipient microorganism

Not applicable for GM product falling within Group 2 according to the EFSA guidance document for this application.

1.9 Organisms with which transfer of genetic material is known to occur under natural conditions

The recipient strain *E. coli* K-12S B-7 has the following genotype: F^- , $P1^-$, λ^-. This strain does not have conjugative plasmids or self-transmissible plasmids. Therefore, the possibility of natural transfer is expected to be very low.

1.10 Information on the genetic stability of the recipient microorganism

No evidence of instability of the recipient strain.

1.11 Pathogenicity, ecological and physiological traits

a) Classification of hazard according to the current Community legislation

E. coli K-12 strains may be categorized in Group 1 according to Directive 2000/54/EC. Microorganisms in this group are biological agents, which are unlikely to cause human disease.

b) Information on the doubling time and of the mode of reproduction

E. coli K-12 has a doubling time of less than one hour. The mode of reproduction is the vegetative form.

c) Information on survival, ability to form spores or other survival structures

E. coli K-12 as well as the recipient strain do not produce spores.
The literature reports that *Salmonella*, *Campylobacter*, *Escherichia*, *Shigella*, *Vibrio* species, and species from other genera can exist in a state where they are viable but cannot be cultured by normal microbiological methods. This differentiation of vegetative cells into a dormant ‘’viable but non-culturable’’ (VNC) state was considered as a survival strategy for many non-sporulating species.
Studies performed on an *E. coli* K-12 strain in soil and water show that the *E. coli* cells were disappearing from the non sterile microcosms studied (perhaps consumed by indigenous microorganisms) and raising the question of whether the VNC state would be irrelevant in natural environments. From these elements the

conclusion may be drawn that a significant increase of *E. coli* K-12 survival ability through the VNC state is far from being clearly established.

d) Infectivity

- *E. coli* K-12 is listed as a non-pathogenic micro-organism.

- Safety has been extensively reviewed and *E. coli* K-12 is known as non-toxigenic.

- A molecular typing study performed on *E. coli* K-12S B-7 (and the lysine producer N°19E), based on the detection by PCR of the genes encoding the virulence factors of *E. coli,* showed the absence of these factors in strain *E. coli* K-12S B-7 (and strain Nº19E). The host strain *E. coli* K-12S B-7 and the lysine producer N° 19E do not contain genes of virulence factors.

- *E. coli* strains have not been reported to cause allergic reactions.

On the basis of the information available in the literature on the safety of *E. coli* K-12 in general, on the recipient strain *E. coli* K-12S B-7 and the additional studies performed on SB7 (and the final lysine producer: strain Nº19E) the absence of pathogenicity of the recipient strain is considered to be sufficiently proven.

e) Presence of genes that confer antibiotic resistance

The strain *E. coli* K-12S B-7 has not been reported to present antibiotic resistance.

f) Involvement in environmental processes

Not applicable, because this concerns a strain which is only used at laboratory level.

1.12 Information on indigenous mobile genetic elements

To the knowledge of the applicant the recipient strain *E. coli* K-12S B-7 has no plasmids, sex factors and prophages such as lambda and P1. Like strain *E. coli* K-12, several mobile insertion elements are present in the genome of the recipient strain.

1.13 Description of its history of use

The recipient strain *E. coli* K-12S B-7 has not been used *per se* and is only an intermediate strain/starting point for the construction of the L-lysine-producing strain N°19E. As regards the general use of *E. coli* K-12 strains see B.1.11.

1.14 History of previous genetic modifications

The recipient strain *E. coli* K-12S B-7, mutant of *E. coli* K-12, was obtained from *E. coli* K-12 wild type by techniques of mutagenesis (by UV radiation and mutagenic agent treatment).

2. Characteristics of the donor organism(s)

Except two genes, the sequences introduced or modified in the final strain N°19E are all coming from *E. coli* K-12 derivative strains. For these *E. coli* K-12 derivative strains the elements of this section are already described in part B.1 of this summary. Below, only the non-confidential characteristics of the other donor organisms are described. Most information of this section is considered as confidential information and is, therefore, not provided in this summary.

2.1 Identity

a) Common name (of the non *E. coli* K-12 donor microorganism)

- 1st donor organism: *E. coli* H155, used at Ajinomoto laboratory level only.
- 2nd donor organism: This information is considered as confidential information. Therefore, no information is provided in this summary.

b) Strain designation (of the non *E. coli* K-12 microorganism)

- 1st donor organism: *E. coli* H155
- 2nd donor organism: This information is considered as confidential information. Therefore, no information is provided in this summary.

c) Source of the strain

This information is considered as confidential information. Therefore, no information is provided in this summary.

d) Accession number from a recognized culture collection

- 1st donor organism: the strain *E. coli* H155, used at Ajinomoto laboratory level, has not been deposited in any culture collection.
- 2nd donor organism: deposited in a culture collection, but the accession number is considered as confidential information.

2.2 Taxonomy

1st donor organism

- Class: Scotobacteria
- Order: Eubacteriales
- Family: Enterobacteriaceae
- Genus: Escherichia
- Species: *Escherichia coli*
- Strain: H155 not clearly defined

2nd donor organism: considered as confidential information

2.3 Other names

1st donor organism: No other name for the donor microorganism (*E. coli* H155).

2.4 Phenotypic and genetic markers

Different characteristics of the two donor organisms are considered as confidential information. Therefore, no details are provided in the summary.

2.5 Description of identification and detection techniques

It is not relevant to develop specific identification and detection techniques for the two donor strains. Only the final amino acid producing strains are relevant in this respect.

2.6 Sensitivity, reliability and specificity of the detection techniques

This section is not relevant for the donor strains considering they are laboratory strains.

2.7 Source and habitat of the organism

According to Chapter III, Part E (summary of the risk assessment requirements, table 1) of the EFSA GMMs Guidance document, this section is not applicable to final products deriving from GMM falling within **Group 2**.

2.8 Pathogenicity traits

For the donor organism *E. coli* H155, the absence of any factor of adhesion, invasion, survival in tissues, cytotonicity or cytotoxicity was shown in studies. Therefore, it was concluded not to be pathogenic to humans. The second donor organism is recognized as a non-pathogenic microorganism. Detailed information is considered as confidential information and is, therefore, not included in this summary.

2.9 Description of its history of use

As regards the first donor organism (*E. coli* H155), the applicant is not aware of information about its usage beyond its own usage and the studies it has performed..

The second donor organism has a long history of use.

3. Description of the genetic modification process

For gene integration and amplification, the applicant used its own technique. It allows the stable integration and amplification of genes of interest in random position without the presence of any marker gene of antibioresistance in the final strain.

The advantages of this method are:
- The constructed producing strains are stable. The number of copies of the integrated genes is expected not to change during bacterial growth and storage.
- The integration of several copies of genes without marker genes of resistance to antibiotics is obtained after a few steps.

Further information is considered as confidential information.

3.1 Characteristics of the vector

Several vectors were prepared and used as intermediate vectors for the genomic construction. They were well characterised. Further information is considered as confidential. Therefore, no details are provided in the summary.

3.2 Information relating to the genetic modification

Conventional and modern recombinant DNA techniques/methods were used to construct the strain N°19E. This information is considered as confidential information. Therefore, no details are provided in the summary.

4. Identification of the conventional counterpart microorganism and its characteristics

Not applicable for GM product falling within Group 2 according to the EFSA guidance document for this application.

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

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

As indicated in section B.1, studies using ribotyping and serotyping were performed with the lysine producing strain N°19E in comparison with the host strain *E. coli* K-12S B-7. They confirm that strains N° 19E and *E. coli* K-12S B-7 derive from the same ancestral strain *E. coli* K-12.

A study performed to confirm the antimicrobial susceptibility of the strain N°19E showed that this one was susceptible to all antibiotics tested.

A significant increase of the metabolic flow towards L-lysine synthesis for the production of L-lysine is observed in strain N°19E, compared with the host strain *E. coli* K-12S B-7.

The other differences between strain N°19E and the host strain resulting from the construction of the former are considered as confidential information and are, therefore, not included in this summary.

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

In order to guarantee that none of the antibiotic resistance genes used for intermediate constructions could be present in the genome of strain N°19E, southern hybridization experiments were performed for these antibiotic resistance genes. For all the antibiotic resistance genes tested, no band was observed in the lane corresponding to the DNA of strain N°19E, thus showing the absence of these antibiotic resistance genes in the final construction/strain. These results are in good accordance with the data obtained concerning the research of antimicrobial susceptibility in B.5.1.

The other pieces of information relating to this section are considered as confidential and are, therefore, not included in this summary.

5.3 Stability of the microorganism in terms of genetic traits

To follow the phenotypic stability, the ability to produce lysine is verified by fermentation tests after several generations corresponding to the industrial process using different strain stocks.
A stability study was also performed, at laboratory scale, by analysis of the genomic structure by hybridization after cultivation of the strain N°19E during 15 to 20 generations, including L-lysine production. Genomic DNA was prepared before and after 15 to 20 generations and digested by restriction enzymes. The DNA fragments were then separated on agarose gel and transferred onto membrane. The same pattern of hybridization was observed before and after culture. This shows the stability of the copies integrated in the genome, even after 15 to 20 generations.

Other information relating to this section is considered as confidential and, therefore, not provided in this summary.

5.4 Rate and level of expression of the new genetic material

Not applicable for GM product falling within Group 2 according to the EFSA guidance document for this application.

5.5 Description of identification and detection techniques

For strain N°19E two techniques of detection are available:

- The first one is also used as traceability method for PL73 (LM) consisting of the dried killed cells of strain N° 19E and is described in Part V of the application dossier;
- The second one is the monitoring of the integration cassettes in the genome by southern hybridization.

5.6 Information on the ability to transfer genetic material to other organisms

To limit any potential transfer of genetic material to other organisms, the strategy of construction for the strain N°19E strain was based on:

- Chromosomal insertion for the genes introduced, rather than using extrachromosomal elements;
- Avoiding that, genes useless for lysine production, such as marker genes of antibioresistance, which could confer a selective advantage to the recipient microorganism, remain at the end of the construction.

Moreover, except for one modification, the only genes transferred in the final strain N°19E correspond to the over-expression of existing metabolic enzymes for the synthesis/production of amino acids.

Other information relating to this section is considered as confidential information. Therefore, no details are provided in the summary.

5.7 Information on the interaction of the GMM with other organisms

Not applicable for GM product falling within Group 2 according to the EFSA guidance document for this application.

5.8 History of previous releases or uses of the GMM

Not applicable to the GMM used to produce PL73 *E. coli* (LYS). This microorganism has not been subject to any release.

5.9 Safety for humans and animals

a) Information on any toxic, allergenic or other harmful effects on human or animal health

Considering that the genetic modifications made resulting in strain N°19E, with one exception, exclusively correspond to the over-expression of existing metabolic enzymes for the synthesis/production of amino acids, this section is not applicable.

A molecular typing study was performed on the initial host strain and on the derived L-lysine-producing strain Nr 19E to detect by PCR the genes encoding the virulence factors of *E. coli*. None of the genes encoding the virulence factors investigated were found to be present in the L-lysine-producing strain Nr 19E.

b) Potential for DNA transfer or any capacity for enhanced gene transfer

To limit any potential transfer of genetic material to other organisms, the strategy of construction for the strain N°19E strain was based, among others, on:

- Chromosomal insertion for the genes introduced, rather than using extrachromosomal elements;
- Avoiding that, genes useless for lysine production, such as marker genes of antibioresistance, which could confer a selective advantage to the recipient microorganism, remain at the end of the construction.

Moreover, except for one modification, the only genes transferred in the final strain Nr 19E correspond to the over-expression of existing metabolic enzymes for the synthesis/production of amino acids.

A part of this section is also answered in section C.4 (risk assessment for DNA transfer).

Other information relating to this section is considered as confidential information. Therefore, no details are provided in the summary.

c) Viability and residence time of the GMM in the alimentary tract

Not applicable to PL73 (LM) deriving from the GMM as this biomass does not contain viable cells of the GMM.

d) Information on any impact of the GMM on the microbiota of the human or animal gastrointestinal tract

Not applicable to PL73 (LM) deriving from the GMM as this biomass does not contain viable cells of the GMM.

5.10 Information on monitoring, control, waste treatment and emergency response plans

This section is not applicable to final products deriving from GMM falling within Group 2 according to the EFSA guidance document of this application.

C. INFORMATION RELATING TO THE GM PRODUCT

'PL73 (LM)', intended to be used as feed material (concentrated source of protein), is a by-product of the manufacturing process of L-lysine by fermentation. It consists of the dried killed cells of the microorganism producing L-lysine (*E. coli* K-12 strain N°19E).
Ajinomoto Eurolysine has already applied, under article 17 of Regulation 1829/2003, for the authorisation of such a *E. coli* K-12-lysine derived bacterial biomass – 'PL73 *E. coli* (LYS)', under the reference EFSA-GMO-FR-2007-40 - however deriving from another *E. coli* K-12 strain (i.e. strain No10S).

Compared with the previous *E. coli* K-12-lysine derived bacterial biomass 'PL73 *E. coli* (LYS)', besides the change of strain (described in section B), the manufacturing processes of L-lysine and of its derived bacterial biomass are basically kept unchanged.
Only minor changes, rather fine-tunings, were made concerning operating conditions for the fermentation phase: adjustments to the needs of strain N°19E.
A certain number of adjustments of operating conditions (considered as confidential information) have also concerned the steps of the manufacturing process downstream of the inactivation treatment of the fermentation broth for the production of L-lysine and the corresponding derived bacterial biomass PL73 (LM)

Considering that:

- the safety of strain N°19E being established, no additional risks / hazards are introduced in the manufacturing processes of lysine and of the bacterial biomass because the raw materials and equipments used are not modified, and
- the intrinsic characteristics of the bacterial biomass (accessibility to the digestive enzymes, digestibility, etc.) are not changed even if the content in certain components of the bacterial biomass may vary between PL73 (LM) and PL73 *E. coli* (LYS),

data collected for PL73 *E. coli* (LYS) regarding nutritional value and safety are regarded as applicable for the assessment of PL73 (LM).

Nevertheless, additional genotoxicity tests and a 13-week sub-chronic toxicity study in rat were specifically performed on the dried killed bacterial biomass PL73 (LM) to confirm its safety for animals and consumers of animal derived products. Their results are in accordance with those obtained with PL73 *E. coli* (LYS).

For these reasons, different studies performed on PL73 *E. coli* (LYS), derived from the corresponding sections of the PL73 *E. coli* (LYS) application dossier under reference EFSA-GMO-FR-2007-40 and considered as applicable to PL73 (LM) as well, are included in the different sections of this dossier instead of performing new studies on PL73 (LM) .

1. Information relating to the production process

PL73 (LM) is a by-product of the manufacturing process of L-lysine by fermentation. L-lysine is produced by fermentation process ('fed-batch fermentation') of a selected strain of *E. coli* K-12, which has been modified to produce L-lysine. The fermentation culture medium consists of carbon sources, nitrogen sources, salts, amino acids and vitamins. The production of lysine and, then, PL73 (LM) consists of the following steps (chronological): strain preservation, culturing of seeds, fermentation using the sterilised raw materials of the fermentation culture medium, ammonia and filtrated air. Afterwards the broth is inactivated and subjected to further processing containing the following steps (chronological): recovery and washing of the inactivated cells of the L-lysine producer microorganism, decantation and concentration of the bacterial cells, cell drying, pelletisation, cooling, sieving and storage.

2. Information relating to the product purification process

2.1 Technique used to remove microbial cells from the product

Not applicable since the microbial cells are not removed from the product. PL73 (LM), in essence, contains inactivated and denaturated microbial cells.

2.2 Information on the technique used to kill the microbial cells

The micro-organism inactivation procedure has been defined on the basis of bibliographic data on the sensitivity of *E. coli* to heat. As all vegetative cells, *E. coli* shows a high sensitivity to heat treatment, contrary to spore-forming organisms.

The possible presence of viable cells of strain N°19E in PL73 (LM) after the inactivation procedure is investigated by detection of bacterial growth of viable cells after plating on *E. coli* specific media adapted to this strain. No viable N°19E cell was detected in different production samples/batches tested

The efficacy of the inactivation treatment developed by AEL and applied to the 'lysine fermentation broth' to effectively kill the cells of the *E. coli* K-12 strain producing L-lysine had already been confirmed, as described in the dossier for the application for authorisation of PL73 *E. coli* (LYS) and confirmed by the French competent authority 'Commission de Génie Biomoléculaire'.

As described for 'PL73 *E. coli* (LYS)', besides cultures on specific medium additional studies were performed on the inactivated broth, which were based on the FISH and DVC FISH methods. FISH (fluorescence in situ hybridization) detects viable bacteria and bacteria which died in the last 48h. DVC FISH (direct viable count FISH) allows to identify and detect metabolically active bacteria. All bacteria (viable, viable but non culturable, and dead bacteria) can be detected by DAPI (DiAmidinoPhenylIndole) staining.

The results of these methods were compared and confirmed the efficacy of the inactivation treatment. These results are also considered to be representative for PL73 (LM).

2.3 Information on the process used to purify the product from the microbial growth medium

This section, mainly introduced for 'purified' products, such as amino acids, enzymes, etc., is not really applicable to products such as PL73 (LM).
It may be indicated that the inactivated bacterial cells making up PL73 (LM) are partly washed during their recovery.

3. Description of the product

3.1 Designation of the product

PL73 (LM) is a dried killed bacterial biomass, a by-product of L-lysine production by fermentation using a genetically modified strain of *E. coli* K-12. Like the previous *E. coli* K-12-lysine derived bacterial biomass PL73 *E. coli* (LYS), it is intended to be used as feed material (concentrated source of protein). PL73 (LM) has a complex nature and does not contain viable cells or transferable DNA of the GMM. The proposed commercial name will be 'PROT-AEL-L' or 'PROT-AEL' (standing for 'Protein- Ajinomoto Eurolysine ').

The product will be delivered in bulk to feed mills. The information corresponding to labelling will be provided to customers by means of the commercial documents preceding or accompanying the delivery of PL73 (LM) and the commercial technical sheet corresponding to this product.

3.2 Intended use and mode of action

The product is intended to be used as a feed material supplying protein (concentrated source of protein) in compound feeding stuffs for pigs (grower-finisher), fish (salmonids), ruminants (in particular dairy cow).

3.3 Composition

-A compositional analysis was performed for several parameters to determine its main and minor components (also in view of assessing its nutritive value) and the occurrence of potential contaminants. Some analyses of micro-components and potential contaminants (dioxins, dioxin-like PCBs, PAHs, residues of pesticides) previously performed on PL73 *E. coli* (LYS) in the framework of the application under the reference EFSA-GMO-FR-2007-40 were not carried out again on PL73 (LM) as they were considered applicable to the latter as well. In this case, the values found for PL73 *E. coli* (LYS) were used for PL73 (LM). Compositional analysis was performed on:

* Nitrogen components (total and free amino acids, ammonium N, amide N, urea N, biogenic amines, nitrates and nitrites, nucleic acids)
* Crude fat, fatty acids
* Carbohydrate fraction
* Organic acids
* Inorganic components
* Vitamins
* Potential contaminants: heavy metals, organochlorine and organophosphorus pesticides, dioxins, PCBs, polyaromatic hydrocarbons

Compared with the lysine-derived bacterial biomass PL73 *E. coli* (LYS) obtained using the strain No10S, it may be observed that, on a dry matter basis, the lysine biomass PL73 (LM) shows a lower ammonium N content (by 2.3 to 2.6%), a lower total N content, but a higher 'true protein' content. Compared with PL73 *E. coli* (LYS), PL73 (LM) also shows a lower content in sulphates and a higher content in crude fat.
The crude ash content remains comparable between these two *E. coli* K-12-lysine derived bacterial

biomasses.

The differences of composition between PL73 (LM), produced using strain N°19E, and PL73 *E. coli* (LYS), produced using strain N°10S also reflect the variations in the operating conditions (fermentation step, downstream steps) which took place across the different production trials at industrial scale aiming at defining their optimal level during the scaling up of the manufacturing of lysine using a strain of *E. coli* K-12 (first with strain No10S, then with strain N°19E) instead of a strain of *Brevibacterium lactofermentum/Corynebacterium glutamicum*..

The differences of composition between the two *E. coli* K-12-lysine derived bacterial biomasses PL73 (LM) and PL73 *E. coli* (LYS) are considered to affect the composition of the biomass only on non-essential characteristics (levels of minerals or ammonium N) without modifying its intrinsic nature (concentrated source of protein, accessibility to digestive enzymes, digestibility of proteins).

The reduction of the levels of ammonium N and sulphates and increase of the levels of 'true protein' and energy value (higher fat content) in the bacterial biomass PL73 (LM) [strain N°19E], compared with the bacterial biomass PL73 *E. coli* (LYS) [strain N°10S], is considered to favorably influence the composition of the *E. coli* K-12-lysine derived bacterial biomass and its nutritive value.

3.4 Physical properties

The results obtained with PL73 *E. coli* (LYS) are applicable to PL73 (LM) as these characteristics mainly result from the very nature of the product (*E. coli* K-12 derived bacterial biomass) and of its manufacturing process (kept basically unchanged), such as drying conditions and pelletisation conditions.

PL73 (LM) is a brown solid product with a bulk density of 0.66 kg/L and a pH of 3.5 to 4..5 (in 10 % w/v suspension).

- Electrostatic properties (mJ): $810 < MIE^{1} < 1200$
- Auto-ignition: 540 ^{o}C
- Thermoanalysis: 247 ^{o}C (Classified as 'among most reactive dusts')
- Explosivity:
  Pmax: 7.3 bar
  MRPmax: 280 bar/s
  Kst: 76 bar
  Explosion class: St1

3.5 Technological properties

The results obtained with the *E. coli* K-12-lysine derived bacterial biomass 'PL73 *E.coli* (LYS)' are applicable to PL73 (LM) as these characteristics mainly result from the very nature of the product (*E. coli* K-12 derived bacterial biomass) and of its manufacturing process (kept basically unchanged), such as drying conditions and pelletisation conditions.

It is reminded that a large quantity of PL73 *E. coli* (LYS) was produced for carrying all the studies necessary for the corresponding application dossier (under reference EFSA-GMO-FR-2007-40). To minimize any risk of degradation, especially microbial degradation, of this quantity of PL73 *E. coli* (LYS) until parts were taken to prepare the various experimental diets for the studies to evaluate its safety and nutritive value or for carrying

out stability studies, it was stored in refrigerated conditions at 2-3°C with 70% humidity in big bags of about 1 ton each. This quantity was subject to regular monitoring and microbiological analyses over a total period of 23 months. PL73 *E. coli* (LYS) was stable from a microbiological perspective during the storage period of 23 months at 2-3 °C. Therefore, the chemical composition and the nutritive value of the product were not altered due to the activity of microorganisms during this storage.

The effects of different climatic conditions - combinations of different temperatures and relative humidity (RH) - on the behaviour and stability of the *E. coli* K-12-lysine derived bacterial biomass PL73 *E. coli* (LYS), as such, or of compound feedingstuffs containing it (compound feedingstuffs prepared to evaluate its nutritive value) were investigated:

- PL73 *E. coli* (LYS) was chemically and physically stable during 12 months storage at 5 different climatic conditions, covering a wide range of moderate and subtropical conditions. PL73 *E. coli* (LYS) demonstrated a good microbiological quality during the storage period and can, therefore, be considered as microbiologically safe feed material.
- Pig feeds and dairy concentrates containing max. 20% PL73 *E. coli* (LYS) were chemically stable during 6 months storage at 3 different climatic conditions, covering a realistic range of moderate and subtropical conditions. Furthermore, they did not contain pathogenic microorganisms at hazardous levels.

Using a quantified and controlled sample preparation procedure of the pellets of PL73 *E. coli* (LYS) a fraction of < 1400 µm was obtained, suitable for the measurements of the particle size distribution and classification according to dustiness. Particle size analyses of samples of the fraction < 1400 µm show that the fraction < 25 µm represents about 1% of the original samples used. Applying dustiness measurements according to CEN standard EN 15051 the sample of the fraction < 1400 µm was classified as moderate for dustiness (based on all fractions (inhalable, thoracic and respirable dust).

4. Assessment of the presence of recombinant DNA and of the potential risk of gene transfer

In this section it is shown that the dried killed bacterial biomass – PL73 (LM) – obtained using the *E. coli* K-12 strain N°19E and the manufacturing process described in the dossier neither consists of, nor contains GMOs, within the meaning of the definitions of 'GMO' and 'organism' provided by Directive 2001/18/EC (on the deliberate release of genetically modified organisms into the environment).

No viable cells of strain N°19E was detected in the samples of PL73 (LM) tested.

Measures by conventional and Real Time PCR were performed, with sets of primers/primers-probe targeting specific DNA sequences of the N°19E genome and used to amplify DNA fragments of 454bp (or more) and 930bp (or more) in the case of conventional PCR and 83 bp (or more) in the case of RT-PCR.. With conventional PCR, amplification of the DNA fragment of 0.454kb was detected in PL73 (LM), however only in trace form. Larger fragments of DNA were not detected. By Real Time PCR, the DNA fragment of 83pb was detected, with a quantity of DNA fragments (83bp) significantly higher (by 1 000 times) than the one detected by the conventional PCR method (454bp).
These results confirm that the genomic DNA of strain N°19E was degraded extensively during the inactivation procedure.

<u>*General conclusion*</u>
In summary, PL73 (LM) contains recombinant DNA fragments. However, these are small size. Therefore, gene transfer from PL73 (LM) is considered very unlikely.

5. Comparison of the GM product with its conventional counterpart

This section is not applicable to PL73 (LM), intended to be placed on the market as feed material, because no biomass resulting from the production of lysine using a conventional strain of *E. coli* K-12 has been previously manufactured and placed on the market. A comparative risk assessment with a conventional counterpart is thus not possible. A specific risk assessment was therefore carried out. The data previously collected for the *E. coli* K-12-lysine derived bacterial biomass PL73 *E. coli* (LYS) were largely used for the risk assessment of PL73 (LM).

6. Considerations for human health and animal health of the GM product

6.1 Toxicology

As already mentioned, between the *E. coli* K-12-lysine derived bacterial biomasses PL73 (LM) and PL73 *E. coli* (LYS), subject of a previous application for authorisation, only the following changes were made:

- Use of the lysine producer strain *E. coli* K-12 N°19E (for the former) instead of *E. coli* K-12 No 10S (for the latter),
- Fine-tunings of operating conditions for the fermentation step and for steps of the manufacturing process downstream the inactivation of the fermentation broth for lysine extraction and separation of the cells of the microorganism from the fermentation broth.

The information supplied on the genetic modifications applied to the recipient strain to result in the lysine producing strain N°19E on the one hand, and the studies performed on the recipient, donor and final strains on the other hand allow establishing the safety of strain N°19E. PL73 (LM), like PL73 *E. coli* (LYS), does not contain any viable organisms, or transferable DNA from the producer strain.
No additional risks / hazards are introduced in the manufacturing processes of lysine and of the bacterial biomass because the raw materials and equipments used are not modified.
Therefore, it is considered that the data collected for PL73 *E. coli* (LYS) can largely be used for the risk assessment of PL73 (LM). In this summary section, the data for PL73 *E. coli* (LYS) and PL73 (LM) are considered interchangeable. Nevertheless, to sustain this approach mutagenicity tests and a 13-week sub-chronic toxicity study in rats were performed on PL73 (LM) in addition to those carried out on PL73 *E. coli* (LYS).

6.1.5.1 Acute toxicity studies

- Acute oral toxicity of the *E. coli* K-12-lysine derived bacterial biomass PL73 *E. coli* (LYS) in rats: LD_{50} > 2000 mg/kg body weight. PL73 *E. coli* (LYS) is considered not harmful when ingested. This value is considered applicable to PL73 (LM) as well.

- Acute inhalation toxicity of PL73 *E. coli* (LYS) in rats: LD_{50} > 5.26 g/m^3. PL73 *E. coli* (LYS) is not harmful when inhaled. This value is considered applicable to PL73 (LM) as well.

- PL73 *E. coli* (LYS) is not irritating to the skin and to the eyes. These results for PL73 *E. coli* (LYS) are considered applicable to PL73 (LM) as well.

- As any protein containing material, the *E. coli* K-12-lysine derived bacterial biomass, PL73 *E. coli* (LYS) or PL73 (LM) is a potential sensitizer to the skin and by inhalation. As it will be delivered in bulk, information

corresponding to the risk phrases R42/43 ('May cause sensitization by inhalation and skin contact') according to Directive 2001/59/EC[1] will be provided through the product's MSDS and in the document accompanying the delivery.

Overall, it is concluded that the *E. coli* K-12-lysine derived bacterial biomass (PL73 *E. coli* (LYS) or PL73 (LM)) has a low acute toxicity. The product may be a sensitizer (risk phrase R42/43).

6.1.5.2 Subchronic and genetic toxicology studies

- Bacterial Reverse Mutation (Ames) test, chromosome aberrations testing in mammalian cells and gene mutation testing in mammalian cells were carried out on the *E. coli* K-12-lysine derived bacterial biomass PL73 (LM). PL73 (LM) is not mutagenic. These results with PL73 (LM) are in line with those obtained with PL73 *E. coli* (LYS) for the same tests.

- In a 13-week oral toxicity study in rats, the l*E. coli* K-12-lysine bacterial biomass PL73 (LM) was tolerated without obvious signs of toxicity at dietary levels up to 15% (equivalent to ca 7.8 (males) – 8.1 (females) g/kg bw/d). These results with PL73 (LM) are in line with those obtained with PL73 *E. coli* (LYS).

- No effects on reproduction are expected on the basis of the reproduction parameters, which were found to be normal in the subchronic feeding studies in rats.

- The results of a developmental toxicity study in the rat with the *E. coli* K-12-lysine bacterial biomass PL73 *E. coli* (LYS) indicated that no effects on development are to be expected from feeding the lysine bacterial biomass to pregnant animals up to dietary levels of 15%. These results are considered to be applicable to PL73 (LM) as well.

6.1.5.3 Target animals

Studies carried out with PL73 *E. coli* (LYS) showed that:

- Cows can tolerate feed supplemented with up to 10% PL73 *E. coli* (LYS) without negative effects on feed intake and milk production and concentrations of protein, fat and lactose in milk.

- In pigs no specific toxic or detrimental effect of the use of PL73 *E. coli* (LYS) as dietary protein source is seen except for a lower faecal consistency. On the basis of the tolerance study it is concluded that PL73 *E. coli* (LYS) can be included in the diet of pigs (grower – finisher) as a protein source up to a level of at least 6% (dry matter basis) without effects on the zootechnical performances or health of pigs.

- No effects on fertility or reproduction in the target animals are to be expected from the intended use of PL73 *E. coli* (LYS) in animal feed on the basis of the fertility and fecundity parameters in experimental animal studies.

- No effects on microflora in the gastrointestinal tract, colonisation of pathogens in the GI tract, or increased antibiotic resistance are to be expected from the intended use of PL73 *E. coli* (LYS) in animal feed.

[1] Commission Directive 2001/59/EC of 6 August 2001 adapting to technical progress for the 28th time Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances (Official Journal of the E.U., L225, p.1, 21-8-2001)

- No residues from heavy metals, pesticides, PAHs, PCBs, dioxins, and mycotoxins, originating from the raw materials used in the manufacturing process of PL73 *E. coli* (LYS) or that may be formed during this one, are expected in edible commodities or excreta of animals fed this by-product.

These results are considered applicable to PL73 (LM) produced using strain N°19E as well.

It is noted that the *E. coli* K-12-lysine derived bacterial biomass is a complex mixture of components for which the following considerations apply:

- the components, other than some of the proteins of this *E. coli* K-12 bacterial biomass, are for most usual components/ nutrients present in feed materials (of which metabolism is known),
- the microbial strain (N°19E) producing L-lysine, the dried killed cells of which make up the lysine bacterial biomass are well identified and characterised (do not produce toxins),
- the product does not contain contaminants of toxicological concern at the levels present.

Considering these aspects it was concluded that studies on the metabolism of the *E. coli* K-12-lysine derived bacterial biomass, here PL73 (LM), were not considered of additional value as not providing additional information.

Conclusions

Overall, it is concluded that, based on studies performed on target and experimental animals as well as on supplemental information and toxicological considerations, the intended use of the *E. coli* K-12-lysine derived bacterial biomass, PL73 (LM) in animal feed is not expected to result in undesirable biological consequences for target animals or the environment. On the basis of the studies performed with PL73 E. coli (LYS), target animals can tolerate a maximum incorporation rate of *E. coli* K-12-lysine derived bacterial biomass, in the daily ration of 6 and 10% (DM) for pigs and cows, respectively. Workers handling the product are advised to take protective measures, which are described in the MSDS.

6.2 Risk assessment of newly expressed proteins

The proteins expressed, as a result of the construction of the strain *E. coli* K-12 N°19E producing L-lysine, are proteins/enzymes of the general metabolism of *E. coli*/*E. coli* K-12 or of the metabolic pathways leading to lysine production and promoting it. It may also be noted that the strain *E. coli* K-12 N°19E is devoid of any marker gene of antibiotic resistance, as confirmed by different studies, and, thus, of the corresponding expression protein.
On this basis, proteins, which are part of the lysine bacterial biomass, are not considered to be of health relevance.

6.3 Testing of new constituents other than proteins

PL73 (LM) is not known to contain new constituents.

6.4 Information on natural food and feed constituents

PL73 (LM) is a complex product and its constituents (other than some proteins) are found in a number of other feed materials. As a new feed material, without any conventional counterpart to which it could be compared to, even at least partially, PL73 (LM) was assessed as such regarding its safety (section C.6.1. of the dossier) taking into account results from studies performed on PL73 (LM) itself or on PL73 *E. coli* (LYS).

6.5 Testing of the whole GM product

The following studies were performed on the whole *E. coli* K-12–lysine derived bacterial biomass:
- acute toxicity studies [on PL73 *E. coli* (LYS)]
- (sub)chronic [on both PL73 (LM) and PL73 *E. coli* (LYS)]
- developmental toxicity studies [on PL73 *E. coli* (LYS)]
- genetic toxicology testing [on both PL73 (LM) and PL73 *E. coli* (LYS)]
- studies on target species: cows, pigs and fish [with PL73 *E. coli* (LYS)]
  * tolerance studies
  * performance studies
  * digestibility studies

The results are presented in C.6.1 and C.6.9 of this summary.

6.6 Allergenicity

As PL73 (LM) is intended for use in feed, it is noted that regarding animal health, allergenicity is not an issue that needs to be addressed specifically.

6.7 Assessment of allergenicity of newly expressed protein

According to the EFSA Guidance document, Section III. C.6.8 ‘Regarding animal health, allergenicity is not a significant issue that needs to be addressed specifically’.
Therefore, although PL73 (LM) may contain newly expressed proteins as a result of the construction of L-lysine producer strain Nr 19E, this section was not specifically addressed.

6.8 Assessment of allergenicity of the whole GM product

As PL73 (LM) is intended for use in feed, it is noted that regarding animal health, allergenicity is not an issue that needs to be addressed specifically.

6.9 Nutritional assessment of GM feed

E. coli K-12-lysine derived bacterial biomasses can be described as biomasses with a high crude protein content: (780 to 797 g/kg DM for PL73 (LM) and 837 g/kg DM for PL73 *E. coli* (LYS)) and, thus, as concentrated sources of protein. Approximately 5.5 to 7.6 % of the nitrogen is present in the form of ammonium-nitrogen in PL73 (LM) (10 % in the case of PL73 *E. coli* (LYS)). The remaining part of the nitrogen-containing fraction consists mainly of protein and amino acids.

The *in vitro* digestibility study (performed on PL73 *E. coli* (LYS)), in combination with the digestibility in pigs and sheep and performance studies in pigs, carried out with PL73 *E. coli* (LYS), indicate that *E. coli* K-12-(lysine)derived bacterial biomasses, of which PL73 (LM), are suitable protein sources in the diet for pigs (monogastrics), ruminants and fish.

The maximum incorporation level in feedingstuffs for pigs of PL73 (LM), based on the results obtained with PL73 *E. coli* (LYS), is up to a level of 60 g/kg DM (~ 7 % on 'as is' basis). The overall eating quality of meat from pigs fed with a diet containing the lysine bacterial biomass at this level at this level is not found to be different from the control group fed a standard diet without the test product. The meat was only found to be slightly less tough in comparison with the control group.

The digestibility study in sheep and the performance study in cows indicate that the *E. coli* K-12-lysine derived bacterial biomass, based on the results obtained with PL73 *E. coli* (LYS), is a suitable protein source in the diet for ruminants. The maximum incorporation level of the lysine derived bacterial biomass in feedingstuffs for ruminants is up to a level of 73 g/kg DM (or ~800 g/kg on as is basis).

Feeding cows a diet supplemented with PL73 *E. coli* (LYS) up to a level of 20% does not result in significant alterations in the composition of the milk with regard to fat, protein, and lactose content. All the milk samples have good smell and taste characteristics. There are no obvious off-flavours, nor defects.

The digestibility and growth studies in fish (rainbow trout) indicate that the *E. coli* K-12-lysine derived bacterial biomass, based on the results obtained with PL73 *E. coli* (LYS), is a suitable source of protein that can be substituted to fishmeal in trout diet. Substitution of fishmeal by up to 20% PL73 *E. coli* (LYS) (in a fish feed containing 65% fishmeal) did not alter growth performances of the trout nor the animal characteristics.

6.10 Post-market monitoring of GM products

The applicant has the opinion that no post marketing monitoring of PL73 (LM) is necessary for the following reasons;

- As shown in section C.4, this product does not contain viable cells or transferable DNA of the lysine-producing GMM (strain N°19E).
- The product is intended to be placed on the market as feed material only.

- A somewhat comparable dried killed bacterial biomass resulting from the production of lysine using *Corynebacterium glutamicum/Brevibacterium lactofermentum*[2] has been placed on the market as feed material since 1976 as a product obtained using conventional strains of *B. lactofermentum*, and since January 1998 as a product obtained using GM strains of *B. lactofermentum* (*) without any report of whatsoever adverse effects.

(*): notified as 'existing product' according to article 20 (1) of Regulation (EC) 1829/2003 and for which an application for renewal of authorization - dossier under the reference EFSA-GMO-RX-PL73 – has been remitted to Authorities).

[2]These are two names for the same species: *Brevibacterium lactofermentum* has been re-classified as *Corynbacterium glutamicum*