Application for Listex™ P100 as a food processing aid in Australia and New Zealand

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Executive Summary

Listeria monocytogenes has been associated with a number of food-poisoning outbreaks related to foods such as soft cheeses, processed meat, poultry, and vegetables. The disease has two manifestations, one usually associated with severe diarrhoea, the other form being an invasive disease with a high mortality rate, especially among the very young, the >60 year olds, the pregnant, and the immunocompromised.
It was estimated that approximately 2,000 hospitalizations and 500 deaths occur annually in the United States alone, as a result of the consumption of foods contaminated with *Listeria monocytogenes* (Mead, 1999). In Australia there are approximately 65 cases of listeriosis per annum or ~0.3 cases/100,000 with an average case fatality rate of 30% (CDN, 2009; Ross *et al.,* 2009). For matero-foetal infections the incidence is even higher: 4.6 infections per 100,000 births each year (Kirk *et al.*, 2003). A recent outbreak in Australia in the summer of 2009 on Virgin Blue airlines contracted 7 people with listeriosis. A recently published report estimates the total annual costs of *Listeria monocytogenes* to US residents at more than 8 billion dollars (Scharff RL, 2010).

Infection occurs almost exclusively via ingestion of contaminated dairy products, raw vegetables, or meats, and is aggravated by the ability of *Listeria monocytogenes*, a facultative anaerobic bacterium, to survive and grow at refrigerator temperatures.

Usually, foods will be contaminated during fermentation, processing, storage, or even packaging of foods. *Listeria* has been isolated from a broad variety of foods that include milk, cheese and other dairy products, meat and meat products, poultry, fish and seafood, vegetables and fruits (Farber, 1991; Ryser 1991). In rare cases infection can also occur by direct contact and during slaughter of infected animals.

Many countries have adopted a zero tolerance policy in food for the organism (*Listeria monocytogenes*), which has led to the recall of many products from supermarket shelves with concomitant economic losses. The persistence of *Listeria monocytogenes* in food products proves that it is difficult to eradicate this pathogen using currently available methods. In addition to all presently available precautionary measures, the application of bacteriophages is an attractive approach.

Bacteriophages can be regarded as natural enemies of bacteria, and therefore are logical candidates for targeted control of food borne bacterial pathogens like *Listeria*.

Important attributes of bacteriophages include:

- they kill only *bacterial* target cells (no impact on plant or animal cells);
- they do not cross species or genus boundaries; therefore they will not affect desired bacteria in foods (e.g., starter cultures for cheese and sausages), and commensals in the gastrointestinal tract, or accompanying bacterial flora in the environment;
- they are composed entirely of proteins and DNA, so their breakdown products consist exclusively of amino acids and nucleotides, both of which are present in abundance in food products.

Bacteriophages thus are not xenobiotics, and, unlike antibiotics and antiseptic agents, their introduction into, and distribution within a given environment can be seen as a natural process.
With respect to their potential application for the biocontrol of undesired pathogens in foods, feeds, and related environments, it should be considered that phages are the most abundant micro-organisms in our environment, and are present in significant numbers in water and foods of various origins, in particular fermented foods (reviewed by Sulakvelidze and Barrow, 2005). On fresh and processed dairy and meat products, more than 10^8 viable phages per gram are often present (Kennedy and Bitton, 1987). It is a fact that phages are routinely consumed with our food in high numbers. Moreover, phages are also normal commensals of humans and animals, and are especially abundant in the gastrointestinal tract (Furuse, 1987; Breitbart, 2003).
In conclusion, bacteriophages are known to be harmless for all other organisms and are species-specific" (often even specific for only a limited number of strains within this species).

In order to counteract *Listeria* contamination problems, EBI Food Safety has developed a phage product which is highly specific for *Listeria monocytogenes*: bacteriophages preparation P100; trade name Listex™ P100).
This dossier contains all available data with regard to this product.

The bacteriophage preparation P100, which has been approved by the Dutch Ministry of Health as a processing aid in June 2009 and as GRAS (Generally Recognised as Safe) for use on all foods susceptible to Listeria by the FDA in 2006 (cheese) and 2007 (all other food products):

- is a liquid culture of a specific bacteriophage (P100),
- is effective against *Listeria*, specifically *Listeria monocytogenes*,
- is intended to be used during the manufacturing process of a variety of foods, to eliminate possible contamination of *Listeria monocytogenes*, and thus prevent outgrowth at a later stage,
- (in treated foods) rapidly disintegrates into amino acids and nucleotides which are naturally present in abundance in food products,
- results in negligible amounts of residuals (amino acids and nucleotides) due to the fact that 1 gram of P100 would be sufficient to treat i.e. 2-200 million hot dogs
- has no technical effect in the finished food

Based on these features we consider P100 to be a processing aid.

Regulation concerning processing aids is laid down in Standard 1.3.3 "Processing Aids" of the Food Standards Act. Listex™ P100 falls within the scope of a processing aid, as defined by the definitions in Standard 1.3.3 but is not listed in the clauses. To get an approval for a new processing and a change in Standard 1.3.3, an application has to be made to the FSANZ.

For that reason this dossier has been prepared along the lines as laid down in the Food Standards Australia New Zealand document of December 2008: "Food Standards Australia New Zealand Application Handbook".

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1. Applicant Details

1.1 Name and address of the applicant and responsible person

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2. Procedural

2.1 Why Listex™ P100 is considered a processing aid

To establish whether Listex™ P100 is a processing aid or an additive the definitions of both substances according to Australian Standards (1.3.1 and 1.3.3) is given below.

Food Additive:
'A food additive is any substance not normally consumed as a food in itself and not normally used as an ingredient of food, but which is intentionally added to a food to achieve one or more of the technological functions specified in schedule 5. It or its by-products may remain in the food. Food additives are distinguishable from processing aids (see standard 1.3.3) and vitamins and minerals for nutritional purposes (see standard 1.3.2).'

Processing aid:
'Processing aid means a substance listed in clauses 3 to 18, where-

a) the substance is used in the processing of raw materials, foods or ingredients, to fulfil a technological purpose relating to treatment or processing, but does not perform a technological function in the final food; and

b) the substance is used in the course of manufacture of a food at the lowest level necessary to achieve a function in the processing of that food, irrespective of any maximum permitted level specified.'

Assessing Listex™ P100 based on these qualifications we believe it should be categorized as a processing aid (rather than a food additive) because:

- Listex™ P100 does not perform a technological function in the end product. As can be seen in chapter 5.3, phage P100 loses its ability to eradicate *Listeria monocytogenes* within 6-24 hours after application

- The levels of Listex™ P100 that are used in the manufacturing of a food product are the lowest levels necessary to achieve the reduction of *Listeria monocytogenes* that is wanted (1log, 2log or more). (as an example, the GMP level of Listex™ P100 is already 3,000 times lower than the GMP level of octanoic acid, qualified by FSANZ as an antimicrobial processing aid (see chapter 5.3.1).

In June 2009 the Dutch Ministry of Health confirmed in writing its viewpoint that Listex™ P100 is a processing aid (Annex 1). In 2009 the European Food Safety Authority (EFSA) released two Scientific Opinions on phages, first in April 2009, then in December 2009 (QPS update EFSA/Biohazards Panel). Though these assessments address the use of phages on food 'in general', reference is made to publications about Listex™ P100. Based on the qualifications mentioned by EFSA (Biohazards Panel) Listex qualifies as a processing aid, as confirmed by Prof Loessner (ETH, Zurich), an authority in this field (annex 5)

2.2 Assessment Procedure

in assessing the application of Listex™ P100, we believe the appropriate procedure to be adopted is the General Procedure, level 1, because the application handbook listed under General Procedure, level 1 includes 'Allowing a processing aid that is currently not permitted'.

2.3 Exclusive Capturable Commercial Benefit

The application of Listex™ P100 is expected to confer an ECCB to EBI Food Safety because:

- EBI Food Safety has a financial gain from the coming into effect of the draft standard or draft variation: When Listex™ P100 is approved as a processing aid; sales in Australia and New Zealand can take off meaning a financial gain for EBI Food Safety.
- Any other unrelated person or body would require the agreement of EBI Food Safety in order to benefit financially from the approval of Listex™ P100: The P100 bacteriophage is patented.

3. General Information on the Application

3.1 Phages

Bacteriophages are bacterial viruses that infect only bacteria, and have no effect on humans, animals or plants. Vaneechoutte *et al*. (2009) made a review for the Dutch ministry of Agriculture concluding that phages can be generally regarded as safe for the use on humans and foods for human consumption, and are efficient antibacterial agents. (Annex 2).

At any time the number of phages on the planet exceeds the number of bacteria. When a virulent phage infects a bacterium the lytic cycle is started as a method for phage replication. Many progeny phages (50-200) are liberated after lysis of the host because in order for phages not to become extinct at least one of these progeny phages needs to find a new bacterial host, often in a three-dimensional matrix, before becoming inactivated by factors such as UV-light, denaturing and proteolytic compounds or simple adsorption to particles rendering them inactive.

Bacteriophages are especially abundant in environments with a large number of bacteria. Marine and freshwater ecosystems are teeming with bacteria and as a consequence phage numbers typically reach 10^7 per ml and sometimes exceed this number 300-fold (Fuhrman 1999; Otawa *et al*., 2007; Filipini *et al*., 2006).

Furthermore many bacteria have temperate phages incorporated in their genome and a small proportion of such populations is lysed and thus sets free bacteriophages. As a matter of fact multiple lysogens in *Listeria* (host bacteria harbouring several integrated prophages) are quite common (Hagens and Loessner 2007).

3.1.1 Natural presence of phages in food

Very few foodstuffs are completely sterile. This means that most food consumed will contain bacteria and therefore phages are likely to be present.

This holds true especially for fermented products as well as unprocessed vegetables. As an example, phages can readily be isolated from Sauerkraut (Yoon *et al*., 2002; Barrangou *et al*., 2002). In one study (Lu *et al*., 2003) 26 different phages were isolated from the product of 4 commercial Sauerkraut fermentation plants.

While in most commercial cheese production settings a lot of effort has been put into ensuring that starter cultures are free of phages and to some extent resistant to phage infection, this is certainly not the case for artisanal cheeses and one might even argue that as long as timing is correct, host lysis by phages and thus liberation of the proteolytic enzymes may even be desirable. Phages infecting *Propionibacterium freudenreichii* have been isolated from Swiss cheese at levels of up to 7 $x10^5$ pfu/g (Gautier *et al*., 1995). Phages infecting thermophilic lactic acid bacteria have been isolated from Argentinean dairy plant samples at numbers of up to 10^9 pfu/ml.
More importantly, non-fermentation culture bacteriophages have also been isolated from various food sources. *E. coli* phages have been isolated from a large number of products including: fresh chicken, pork, ground beef, mushrooms, lettuce, other raw vegetables, chicken pie and delicatessen food with phage numbers as high as 10^4 per gram (Allwood *et al.,* 2004; Kennedy *et al.*, 1984, 1986).
Also *Campylobacter* phages have been isolated at levels of 4 $x10^6$ pfu from chicken (Atterbury *et al.*, 2003) and *Brocothrix thermosphacta* phages from beef (Greer 1983).
In all these cases the researchers were looking for phages infecting one particular organism, but when one considers the myriad of bacteria associated with soil and vegetables it becomes clear that in addition one would be likely to find phages associated with this multitude of other species if one were to look.
A recent article on the presence of *E. coli* and *Campylobacter* phages in New Zealand vegetables and chicken found coliphages in more than 90% of the samples at numbers of 250 per gram (Tsuei *et al.*, 2007). The investigators point out that the indicator organisms employed ensured that both male specific and other phages would be identified. Still, the incidence and numbers are likely to be an underestimate of the total coliphage population. This is because many phages are serovar-specific, recognizing features associated only with specific strains or they recognize surface proteins such as ompC or the maltose receptor, whose presence or shape may vary between strains and growth conditions.
Many listeriophages are serovar-specific, recognizing sugar substituents on the polyribitol phosphate teichoic acids. P100 recognizes the *Listeria* peptidoglycan backbone as the primary receptor (Wendlinger *et al.*, 1996), which explains its broad host range, recognizing all serovars within the genus *Listeria*, making it useful for applications in food.

3.2 Purpose

The purpose of Listex™ P100 is to eradicate or decrease *Listeria monocytogenes* on various ready to eat food products for human consumption.

3.3 Technological Need

Numerous cases of food infection resulting from the presence of *Listeria monocytogenes* have been reported, with an outbreak of listeriosis in Australia in the winter of 2006. This outbreak was caused by contaminated products from a small goods company in Adelaide. Products from this factory were linked to 3 deaths, part of the high risk population (mostly elderly). Another outbreak of listeriosis was caused by contaminated products from a factory in Ontario (Canada) in the fall of 2008. More than 20 people died, most of them part of the higher risk population.
An outbreak in Australia in the summer of 2009 on Virgin Blue airlines contracted 7 people with listeriosis. In the beginning of 2010 an outbreak was reported in Austria and Germany which by now has claimed at least 8 lives, all victims had contracted listeriosis after eating cheese contaminated with *Listeria monocytogenes*.

Despite increased insights into microbial hygiene during manufacturing, incidence of listeriosis does not seem to decrease, rather a rise in incidence can be observed in many countries around the world. Although the reason for this is not known exactly, it may well be changing eating habits: i.e. a shift towards convenience foods. In addition of course we are seeing an aging population in many developed countries.
Listeria monocytogenes is very persistent (salt, temperature) and widespread in the environment; it is present in the soil and humans can carry the bacterium without knowing it. The economic impact of production, detection and destruction of *Listeria*-contaminated food products costs the industry billions per year. In addition, the health care expenses (counting only hospitalizations) are very high (estimated at billions per year in both the US and EU). This excludes the expenses of brand damage when contaminated product reaches the market, often resulting in bankruptcy of the company involved.
A recently published report estimates the total costs of Listeria monocytogenes to US residents at more than 8 billion dollars (Scharff RL, 2010). For these reasons an effective control of *Listeria* at all stages of the food production chain is necessary.
The application of Listex™ P100 can be seen as an additional tool for control of *Listeria* in food. It can supplement GMP, HACCP and other measures aimed at the prevention of *Listeria,* though it should NOT be seen as a replacement of hygiene, but as an integral part of it. On the basis of results obtained in experimental work it can be concluded that Listex™ P100 is effective in controlling or eradicating *Listeria monocytogenes* on various food products in a dose-dependent manner.

3.4 Safety

Most phages are very specific for only one bacterial species, and therefore cannot affect or influence the natural bacterial flora of a food or raw material used to produce food or feed.
Strictly lytic (i.e., virulent) phages lack the genetic factors required for integration, will always enter the lytic cycle, and eventually kill and lyse the infected cells. In contrast to virulent phages like P100, many of the tailed phages may not be suitable for use as natural antimicrobials, since they are temperate and can integrate their genome into the host bacterial genomes, to form a lysogenic cell. This state is sometimes accompanied by undesired phenotypical changes, i.e., the integrated phage (prophage) can potentially carry and express genes encoding properties which increase pathogenicity and/or virulence of the host bacteria. In several cases, temperate phages have been identified as the carriers of toxins or regulators needed for development of full virulence of the host (reviewed by Boyd, 2005).

P100 is virulent (non-temperate) and the genetic structure of the P100 genome does not suggest any possible presence of a lysogeny module. It is preferable to select phages which are not capable of transduction, i.e., packing of host genetic material instead of phage-encoded DNA. While many temperate and virulent *Listeria* phages were experimentally shown to be able to transduce genetic markers (Hodgson, 2000), this is not the case for the family of P100 like phages. A recent paper by Klumpp *et al.*, (2008) reveals the genetic basis for the lack of transduction in these phages.

3.4.1 Sub acute toxicity study

The sub-acute toxicity study was conducted to assess gastrointestinal effects of ingestion and any clinical signs of toxicity (MB Research Laboratories, Report Number MB 05-13221.01, 2005). The study methods and results have been published by Carlton *et al.* (2005) and can be found in Annex 3. This study was conducted according to the current OECD principles of good laboratory practice.

Young Wistar albino rats were given 1.0 ml of PBS vehicle or 5 $x10^{11}$ pfu/ml phage P100 particles suspended in phosphate-buffered saline pH 7.3 (PBS) orally by gavage for a total dose of approximately 2 $x10^{12}$ pfu/kg daily for 5 days. Body weights were recorded pre-test and prior to termination. The animals were observed once daily for toxicity and pharmacological effects, and twice daily for morbidity and mortality. Food consumption was calculated at the end of the study. After euthanizing all animals were examined for gross pathology. The oesophagus, stomach, duodenum, jejunum, ileum, cecum, and colon were preserved in 10% neutral buffered formalin. Histopathologic preparation (cross sections and longitudinal sections) and examinations were performed according to standardized procedures.

Oral administration of a 2 $x10^{12}$ pfu/kg phage P100 for five consecutive days, followed by a two day recovery period in male and female Wistar albino rats, revealed no adverse effects attributable to the test material. There were no significant ($p \leq 0.05$) differences in mean body weight or food consumption between the treated and control groups.

No abnormal physical signs or behavioural changes have been noted in any animal at any observation time point. Necropsy results were normal in all animals except one of the animals of the P100 test group which showed a small red area in the mucosa at the junction of jejunum and ileum. Multiple thin sections from this area of the gastrointestinal tract were then examined, and all were within normal histological limits with no microscopic change to correlate with the gross observation. There were no treatment-related morphological changes noted in the microscopic evaluation of the gastrointestinal tract.

It was concluded by MB Research Laboratories Study Director that the histomorphologic observations in the male and female rats of both groups of this study are typical of those which occur spontaneously in laboratory rats of this strain and age, and administration of P100 phage had no effect on the type or incidence of these findings.

3.4.2 In silico assessment for pathogenicity, virulence and allergenicity

After the complete sequence was assembled, genome coordinates were defined *in silico*: nucleotide position 1 (left end of the genome) was set directly upstream of the putative terminase subunit genes. The information encoded by the P100 genome was then analysed by using the VectorNTI software (version 8; InforMax), and the annotated genome and all predicted open reading frames (ORF), gene products (gp) and secondary structures were confirmed by visual inspection. The basic prerequisites for an ORF were the presence of one of the three potential start codons ATG, TTG or GTG, a suitable ribosomal binding site (Loessner and Scherer, 1995, Loessner *et al.*, 2000), and a length of at least 40 encoded amino acids. Nucleotide and amino acid sequence alignment searches (BlastN, BlastX, and BlastP) using the ORFs and deduced gene products, respectively, were performed with Vector NTIs integrated BLAST engine which used the non-redundant database available through the NCBI web sites (http://www.ncbi.nlm.nih.gov/). Searches for specific protein domains and conserved motifs with known function were performed using the PFAM tools available online at http://pfam.wustl.edu/hmmsearch.shtml. Transmembrane domains were predicted by using the hidden Markov model (TMHMM); available at http://www.cbs.dtu.dk/services/TMHMM/. Helix-Turn-Helix-Scans (HTH) were performed using SeqWeb Version 2.1.0 (GCG package), accessed via the biocomputing services of the University of Zurich (http://www.bio.unizh.ch/bioc/). Potential tRNA genes were identified using the bioinformatics tool provided by http://www.genetics.wustl.edu/eddy/tRNAscan-SE (Lowe and Eddy, 1997). Loops and hairpins were identified using HIBIO software (Hitachi) and VectorNTI, and a preliminary graphical genetic map of P100 was constructed using VectorNTI.

In order to screen all 174 gene products predicted to be encoded by the P100 genome for possible similarities to currently known protein food allergens, another *in silico* analysis was performed based on local alignments to the amino acid sequences of the proteins contained in the FARRP (Food Allergy Research and Resource Program) allergen database at http://www.allergenonline.com.

The complete genome sequence of P100 was determined and analysed *in silico*. The bioinformatic analyses and annotations (in particular sequence alignments and motif searches) did not reveal any similarities of P100 genes or any of the 174 predicted P100 gene products to any genes or proteins or other factors known or supposed to play a direct or indirect role in pathogenicity or virulence of *Listeria monocytogenes* (Vasquez-Boland *et al.*, 2001), or any other infectious, toxin-producing or otherwise harmful micro organism. Genomic data clearly indicated that P100 is related to A511, a *Listeria* specific Myovirus whose genome has recently been sequenced (Klumpp *et al.*, 2008).

No evidence of lysogenic characteristics or integrase function was found in the bioinformatic analyses. Integration and maintenance of the lysogenic state (when a temperate phage is integrated in a bacterial chromosome) requires much more than just an integrase gene. Lysogenic activity depends on a whole set of genes and the corresponding genetic control elements including promoters, operators, terminators, attachment and integration site. These are always organized together in a so-called lysogeny control region, or lysogeny module. The genes and encoded proteins and control elements must all be present and functioning, otherwise the lysogenic state can neither be entered nor be maintained. None of these lysogeny factors are

present in the P100 genome nor do any of the sequence alignments and homology searches indicate any related gene or product. Thus, the genetic structure of the P100 genome did not suggest any possible presence of a lysogeny module.

When the predicted gene products of P100 were aligned with proteins know or suspected to be potential food allergens, one protein (gp71) showed a local similarity in its C-terminal domain to a gamma-gliadin protein of wheat. The e-value (probability index) calculated for each amino acid sequence alignment is supposed to indicate a possible immunological cross-reactivity. However, bioinformatic analyses also suggested that the e-value of 8 $x10^{-10}$ was due to a spatial accumulation of glutamine (Q) and proline (P) in specific domains of these proteins. Most importantly, sequence comparisons also showed that the Q and P-rich sequences in gp71 did not match the immunoreactive epitopes of wheat gliadin (Battais *et al.*, 2005), and there is no identical stretch of residues spanning more than 4 or 5 identical amino acids. It should also be noted that orf71 is clustered in the P100 genome with putative DNA recombination/replication elements. Therefore, gp71 is probably synthesized during the initial phase of phage infection and involved in the process of genome replication. Such proteins are not known to be components of the matured phage particle. Therefore, because of the bias in sequence alignment and based upon the predicted function of this putative protein, it is concluded that gp71 has a negligible probability to act as potential immunoreactive allergen.

3.4.3 Bacterial resistance

One might wonder whether employing P100 phages will lead to the development of resistant bacterial strains. The answer to this is simply: “No this will not happen”. In 1969 Salvador E. Luria and Max Delbrück received the Nobel Prize for medicine for their work on phage replication and interaction between phages and bacteria. In part this prize was based on the famous Luria-Delbrück experiment which unequivocally showed that resistance to bacteriophages develops independently of the presence of phages, underlining an evolutionary principle: mutations in nature occur randomly at a certain frequency (Luria and Delbrück, 1943). Therefore a mutation, for example in a molecule which a particular phage recognizes will occur once every so often. In those cases where this mutation is not fatal to the bacterium the phage may no longer be able to infect or it might infect at a lower efficiency. A realistic frequency of mutation of such a receptor molecule would be in the range of 10^{-7} (Carlton, 1999). Taking this number, in an eradication scenario one would need 10 million bacteria in order for one non-sensitive to escape treatment. For a product which aims at a 2-4 $\log_{10}$ reduction of the target bacterial numbers, this occurrence is irrelevant.

Inducing resistance and selecting for it are two very different things. On a food item with an accidental and low-number contamination, the occurrence of a non-sensitive mutant would be extremely unlikely and in any case an isolated event.

Bacteriophage preparation Listex™ P100 is meant solely for the use on foods, not as a surface decontaminant in production environments. This method of using phages lowers the risk for the come into existence of resistant *Listeria monocytogenes* strains. The intended use of Listex™ P100 is prior to packaging: if a

resistant strain originates, it will leave the production facility in the packaged product, not being able to spread.

Bacteria have developed strategies to counter phage infection, including restriction modification (R/M) systems, abortive infection mechanisms and super-infection exclusion mechanisms. In a treatment situation or pertaining to P100 these mechanisms are not relevant. Abortive infection mechanisms are not relevant in a treatment situation because they depend on host-cell suicide after infection. Ecologically this is a disaster for bacteriophages but in a treatment situation it is inconsequential because the target cell dies. Super-infection exclusion works *only for temperate phages* (see chapter 2.4, Safety-Introduction) in keeping related phages outside the host. As a strictly virulent (non-temperate) phage, P100 is not affected. R/M systems are never 100% effective, since phage DNA may be modified by host enzymes before other host enzymes degrade the DNA rendering the phage and progeny completely immune to this system and additionally the P100 genome has extremely few palindromic sites where such enzymes might act**.**
With hundreds of food-isolates tested, no insensitive strains have been found to date. While strains with a reduced sensitivity are likely to exist their occurrence on a particular food item again will be an extremely rare and isolated event.

3.4.4 Safety assessment

There are more individual bacteriophages in the biosphere than there are of any other group of organisms, including all the prokaryotes. The shape of the best studied group of phages, the tailed phages, is so distinctive that their numbers in aquatic environments were estimated simply by centrifuging them onto an electron microscope sample grid and counting them. In coastal seawater, there are typically as many as 10^7 tailed phages per millilitre. In some fresh water sources, there are up to 10^9 phages per millilitre.

Numerous papers attest to the fact that humans are exposed to huge numbers of phages daily, through food and water, without evidence of any harm. Gorski and Weber-Dabrowska (2005) have also presented evidence that phages are helpful to humans by exerting immunosuppressive activity in the gut to control local inflammatory and autoimmune reactions and act in concert with the immune system in immunosurveillance against bacteria and viruses. These reviewers cited thousands of cases where phages have been used in treatment of patients suffering from diseases caused by antibiotic resistant bacteria, either by injection or oral administration, resulting in an 80% success rate. No negative side effects of phage administration were observed. While these studies and therefore conclusions about the beneficial treatment effects did not follow western standards (there were no double blind trials) this massive body of evidence shows conclusively that phage administration, either orally or by injection, has no ill effects. Virulent bacteriophages have been used as prevention or treatment for many bacterial diseases including sepsis for years. Although much of the literature comes from studies in Eastern Europe and the Soviet Union, Western nations are becoming more aware of the possibilities of phage treatment of bacteria that have become resistant to antibiotics (Sulakvelidze, 2005). No allergic reactions in humans have been reported despite evidence that phage enter circulation (Matsuzaki *et al.*, 2005).

Human volunteers have been fed *E. coli* phage T4 phage with no harmful effects noted in a controlled study; and no phage or phage-specific antibodies could be detected in the serum of the human subjects (Bruttin and Brussow, 2005). The authors propose that use of such phages may be a useful therapy for acute diarrhoea caused by *E. coli* worldwide (Brussow, 2005).

Bacteriophages have been purposefully placed in the food chain, particularly used as treatment or prevention of gastrointestinal diseases of poultry (Carillo *et al.,* 2005; Berchieri *et al.*, 1991). These phages obviously will be present on the food following slaughter.

Other studies on the application of phages to animals also reported no adverse or unexpected effects of bacterial phages in animals (Biswas *et al.*, 2002; Cerveny *et al.*, 2002; Chibani-Chenouffi, 2004b; Merril *et al.*, 1996).

All this is in line with the results of our sub acute study with rats with a cumulative dosage of phage P100 up to 2 $x10^{12}$ pfu P100/kg (see Part 2.4.1) which did not reveal any adverse effects on the gastrointestinal tract or any clinical signs of toxicity.

Further evidence that treating food products with phage P100 is not likely to cause harm to humans who consume such foods is the abundance of bacteriophages of many genera and species in the human intestine. Given that the intestines are colonized by vast numbers of bacteria and that bacteria are often infected with phages; it is therefore estimated that humans have billions of phages in their intestines at any one time.

Thus, if a small number of phage P100 continues to be present on the surface of a food product after application at the time of product making, and is ingested by a consumer, it is impossible that these phages pose any hazard because:

- Large numbers would not be hazardous
- *Listeria* phages both like and unlike to P100 are ingested regularly
- A large proportion of any phage will be destroyed in the stomach
- Ingestion of the number of P100 phages is relatively small compared to the billions of phage particles of other species already present
- Phage P100 does not contain genetic elements harmful to humans nor does it have any other undesirable properties such as the ability to lysogenize and or transduce host DNA
- *Listeria* phages such as P100 are not able to infect and kill bacteria from other genera of bacteria, and therefore will not disturb the intestinal flora
- Listex™ has been used by our customers for years to their satisfaction

3.5 Cost/Benefits

As mentioned above, apart from health damage (estimated at 2.5 billion per year for the US, for only hospitalizations caused by *Listeria monocytogenes)*, and the suffering for those involved, high economic damages are likely to originate from a

listeriosis outbreak. Economic damages such as production, detection and destruction of contaminated products, as well as product recalls, reputational damage and anxiety for those concerned, including those responsible for quality control, all add to the costs for society.
A cost-benefit analysis by Ivanek *et al*. (2004) compared the costs of *Listeria* control (costs of recalls etc.) to the benefits of *Listeria control* (prevented medical costs, willingness to pay more for a safer product). Results from this study show that the estimated benefits per prevented case far exceed the estimated costs per prevented case. This implies that, to reach an optimal level of food safety, more food safety measures are warranted.
The actual benefits might be even higher because a listeriosis outbreak causes reputational damage not only for the company involved, but often for an entire country or industry concerned. As such, measures against *Listeria* should be considered 'pre-competitive', and a comprehensive part of good corporate citizenship.

3.6 Efficacy

Listex™ P100 is very effective against *Listeria monocytogenes* in various food products. Several highly credible institutes around the world have conducted scientific trials with Listex™. In a study by Guenther *et al.* (2009) it is shown that bacteriophages P100 causes a reduction up to 3 logs in several foods like hot dogs, cabage, mixed seafood and salmon. Another study performed by Carlton *et al.* (2005) shows that P100 can totally eradicate Listeria monocytogenes in smeared cheeses. Holck *et al.* (2009) showed that P100 is successful in reducing Listeria monocytogenes on cooked ham: alone or as an hurdle technology.

3.7 Authorized applications in other countries

On the 14th of July 2009, the Dutch ministry of Public Health stated that Listex P100 is considered a processing aid for the use on all foods in the Netherlands. The official document can be found in Annex 1. This is in line with an extensive review, published by Von Jagow and Teufer in Zeitschrift für das Gesamte Lebensmittelrecht (ZLR / European Food Law, 2007/01) which establishes a legal classification for P100 in the EU as a processing aid (Annex 4a).
According to another publication, in European Food and Feed Law Review (EFFL) 2007/03, the use of bacteriophages as a processing aid is exempted from EU regulation and free from labelling requirements, provided the manufacturer observes the mandatory safety requirements pursuant to Art 14 Regulation EC Nr 178/2002 (Annex 4b).

Per June 2007 SKAL, the designated Public Inspection Authority of The Netherlands, has issued a formal confirmation that in conformity with EU Regulation (EEC) nr. 2092/91 Annex VI Section B, Listex™ P100 is a processing aid which can be used also in all *organic* food products. SKAL surveys the organic production in accordance with the public law, based on EU-Regulation (EEC) nr. 2092/91. This occurs by order of the Dutch Ministry of Agriculture, Nature and Food Quality.

Products certified as organic by an EU recognized inspection body can be marketed as such within the entire EU (Annex 4c).

In the USA the bacteriophage preparation P100 has been approved as GRAS (Generally Recognized As Safe, FDA approved per October 17th 2006 for its intended use in cheese referenced as GRN 000198). Per June 22, 2007 the FDA and the USDA extended the approval for use of Listex™ P100 to include *all* food products susceptible to *Listeria monocytogenes* (GRN 000 218) (Annex 4d).

In June 2008 the Swiss Bundesamt für Gesundheit (BAG) approved the use of Listex™ against *Listeria monocytogenes* on cheese (Annex 4e).

3.8 Opinion European Food Safety Authority (EFSA)

The European Food Safety Authority (EFSA) panel on Biological Hazards (BIOHAZ) recently published two documents in which bacteriophages are discussed.

The first document, released on the 22nd of April 2009 (EFSA 2009), focuses on the use and the mode of action of bacteriophages in food production. The conclusion of this report is that virulent (lytic) bacteriophages have demonstrated to be very effective in the targeted elimination of specific pathogens like *Listeria monocytogenes*. In this specific report it is stated that bacteriophage P100 can completely eradicate Listeria monocytogenes in various cheeses.

The second document in which phages are discussed is the QPS (qualified presumption on safety) update of (EFSA, 2009).In this document it is concluded that notification from QPS for bacteriophages are not expected based on different genera and species. Instead, phages have to be reviewed in a case by case situation taking into account the safety and the mode of action. Regarding the safety: the phage has to be strictly lytic and unable to transduce bacterial DNA. In several studies (Loessner and Busse, 1990 and Carlton *et al.,* 2005) it is proven that bacteriophage P100 is strictly lytic and does not contain genes involved with virulence, toxins and integrase activity nor is it able to transduce bacterial DNA(Hodgson, 2000).
Regarding the mode of action, many biocontrol experiments show that phage (including P100) activity stops after 6 to 24 hours in a variety of foodstuffs such as cheese (Abuladze *et al*., 2008; Atterbury *et al*., 2003; Leverentz *et al*., 2003; Guenther *et al*., 2009; Holck and Berg, 2009).

The fulfilment of the above criteria to bacteriophage P100 are also reviewed by a scientific expert in the field of bacteriophages, Professor Loessner from the ETH in Zürich. He comes to the conclusion that, in line with EFSA's requirements (safety, working mechanism (EFSA 2009, QPS), bacteriophage P100 is safe for use in foods and qualifies as a processing aid in solid foodstuffs. The full review can be found in Annex 5.

4. Technical Information on the Processing Aid

4.1 Type of processing aid

Bacteriophage preparation Listex™ P100 does not fall within the categories mentioned in Standard 1.3.3 – Processing Aids, but in our opinion can be considered an antimicrobial against *Listeria monocytogenes* which fits in the category L, *miscellaneous functions*.

4.2 Identity

- The final product is an opaque liquid containing: 2 x10^{11} plaque forming units per ml product in buffered saline.
- The product consists of a purified strictly virulent (lacking lysogenic activity) bacteriophage (phage) specific against *Listeria monocytogenes*.
- The phage is deposited at, and assigned an identifying code by, a scientifically recognized culture collection centre.
- The agent is produced from cell cultures of *Listeria innocua* in a safe and suitable nutrient medium.

4.3 Physical and chemical properties

Phages comprise a number of proteins enveloping nucleic acids. pI's (isoelectric point) of phage structural proteins are generally low, and all P100 structural proteins exhibit pI's of around 4. Addition of phages during treatment occurs at such low levels that food properties are not changed at all. Listex™ P100 has an effect only when *Listeria monocytogenes* is actually present: it has no effect on the foodstuff itself. The amount of proteins and nucleic acids present in the added dose of bacteriophages is negligible compared to the level of proteins and nucleic acids already existing in the foodstuff. In addition, bacteriophage residues are is essence identical to food compounds.

4.4 Manufacturing process

The production process to obtain the phage P100 consists of the following steps:

- Fermentation batch process culturing *Listeria innocua*, a safe and non-pathogenic Class I bacterial species, employing normal culture media for bacterial culture and/or raw materials that are food grade quality, free from animal, GMO and allergenic components.
- Phage amplification: infection of the bacterial culture with lytic P100 phages.
- Downstream processing steps:
    - Clarification; in this step host cells and cell debris is removed by filtration.
    - Concentration
    - Sterile filtration

- Standardization; the product is standardized to a specific phage content with sterile water.

After this, the product is bottled in HDPE bottles.

More details on the manufacturing process and quality assurance measures to assure product identity and quality are given below.

Fermentation and Phage amplification process
Bacteriophage P100 is produced on a growing host culture of *Listeria innocua,* a safe, non-pathogenic class I bacterial species that lacks all genes for production of toxins. The culture is fed with plant peptone and yeast extract. When the culture reaches the targeted optical density it is first infected with lytic phage P100 and then harvested.

Down-stream processing
After completion of the phage propagation the following down stream processing steps take place:

- Clarification
  In this step the phages are separated from host cells and cell debris by using filtration steps.
- Concentration
  The clarified phages are concentrated.
- Filter Sterilization
  The concentrated phages are filter sterilized by filtration with a 0.2µm pore size absolute filter.
- Standardization
  Phage concentration in the product is standardized with sterile water.
- Packing
  The product is bottled in 100 ml bottles. Specific packaging volumes and phage concentrations can be chosen for specific customers.

Storage
The product is stored at 4°C in a refrigerator, which is located in a phage-dedicated lab.

More details on the manufacturing process are given in the following subchapters:

4.4.1 Overview of the Manufacturing Process

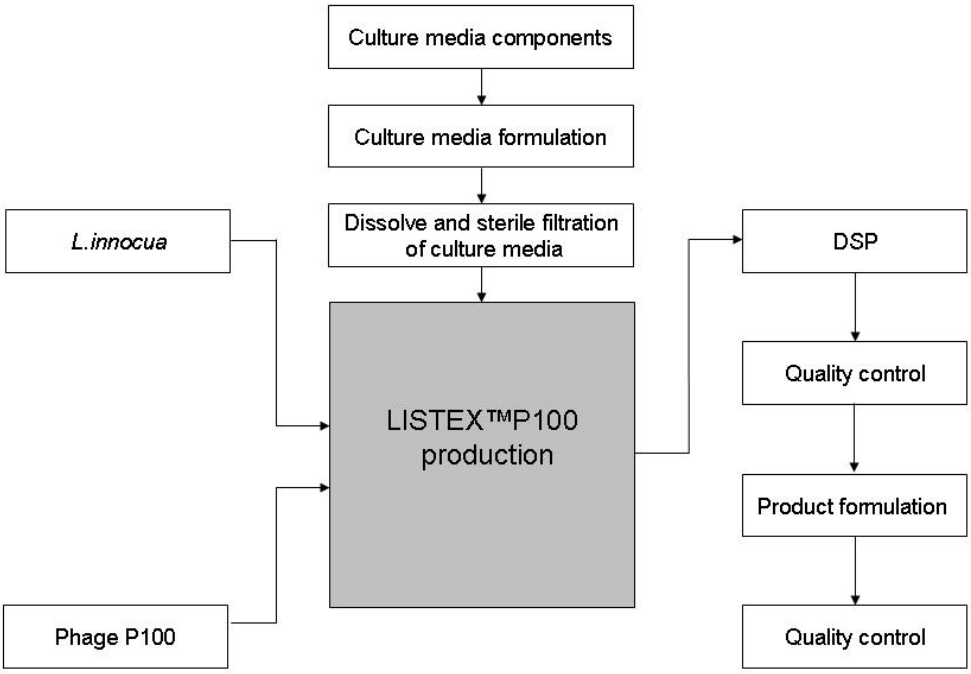

Fig. 1: Production flow sheet

The production of Listex™ P100 consists of several steps:

Culture media components

The culture media components are tested for functionality in the production of phages. With each delivery of culture media components a product sheet containing product specifications, such as batch and lot number, composition, production date, etc is received. The product sheets are stored in a central storage space.

Culture media formulation

The culture media used for the propagation of phages consist of several components. The culture media is composed according to standard operation procedures (SOP) and the component volume is recorded in a SOP datasheet, together with the components article, batch and/or lot number, expiry date, operator and composition date.

Preparation of culture media

The formulated culture media is dissolved in the appropriate amount of water according to a SOP, the amount of water added is recorded on a SOP datasheet.
After the media has been dissolved, the culture media is filter-sterilized via 0.2µm absolute membrane filter into the bioreactor. The filter supplier, type, article number, batch and/or lot number and surface area are recorded in a SOP datasheet.

Phage P100 bacteriophage production

The sterile filtrated culture media is heated in the bioreactor to a set temperature. Organic and sterile sunflower oil is added to serve as antifoam. When the required temperature is reached *Listeria innocua* is added. The cells are incubated in the bioreactor. During the fermentation step, culture samples are taken to monitor the optical density. When culture reaches the required density, phage P100 is added to the culture, the incubation of the culture proceeds under the same conditions as during cell growth. Data recorded on the SOP datasheet: Sunflower oil: supplier, article number, expiry date, added amount. Culture conditions: air flow, temperature, agitation motion, start density. Bacterial information: bacterial strain, expiry date, density, calculation of amount of cells needed for start. Phage information: phage strain, expiry date, phage particles per ml, infection ratio in the culture, calculation for the amount of phages, added amount of phages.

Downstream processing (DSP)

After the incubation period, the culture is harvested in a tank. The filtration module is connected to the tank. The micro filtration (MF) process separates the phages from larger particles such as host bacteria and cell debris. The DSP of phages is a continuous process. While the MF process is running, the phages are concentrated using cross flow ultra filtration (UF). During the MF, water is used for diafiltration. Finally the concentrated phages are sterile-filtered with a 0.2 µm sterilization filter; the filter-sterilized phages are stored in a storage bag at $+4^0$C at a phage-dedicated lab. Pump speed, pressure settings, diafiltration volume and phage titer are recorded in a SOP datasheet.

Quality control

A test report of each manufactured batch of phages is stored at a central storage. The number of plaque forming units of the concentrate is determined in house and recorded on the DSP SOP datasheet.

Formulation of Listex™P100

After product release based on the analytical results, the phages are packaged in standard 100ml HDPE bottles containing $2x10^{11}$ pfu per ml (other packaging volumes are produced on request). To this end the phages are diluted using filter sterilized water to the appropriate concentration. Customer specific packing volumes, concentration or buffers can also be formulated. Dilution calculations and packing volume are recorded on a formulation datasheet. All packages are labelled with expiry date, batch number and item number.

Quality control

Per 10 packages a 10 ml sample is taken and sent to an external analytical lab and tested for yeasts, moulds and total aerobic count. Test reports are stored in a central storage. All apparatus used in the production of Listex™P100 are marked with an apparatus number which is recorded on the SOP datasheets used in that specific step together with an expiry date, if applicable. All SOP datasheets are stored in a central location and copies of SOP datasheets which belong to a specific production batch are bundled and stored per batch. All SOP datasheet are checked and approved by a second person who is not involved in the production process.

4.4.2 Manufacturing Equipment

Fermentation and phage propagation step

For the fermentation and propagation step a specific Bio Process Reactor (BPC) is used. The culture media formulation is prepared in a polyethylene bioreactor and phage P100 is produced in the closed, food grade plastic bioreactor. The tubing used for air flows, feed lines and sampling tubes are made from food grade platinum cured silicone. All air filters and connector appendages connected to the BPC are food- or pharma-grade. All equipment used for DSP for food processing applications are pharma-grade. A peristaltic pump equipped with single use platinum cured silicone tubing and sterilization filter is used for the sterile filtration of the phages.

Filling & Finishing
Standardization

All fill and finish procedures are performed in a laminar air flow hood. The sterile filtered P100 is standardized by adding sterile water. To get a final phage concentration of 2 x 10^{11}pfu/ml, the water buffered phage mixture is mixed thoroughly. The standardized solution is divided in 100 ml aliquots using a sterile dose pump. After every 10^{th} bottle a sample of approximately 10 ml is taken and sent in for quality control by an external laboratory.

4.4.3 Information on the Bacterial Strain used

The identity and classification of the *Listeria innocua* bacterial host is given below. The strain is a safe, non-pathogenic, class 1 organism.

Bacterial host classification and identity

Name of host bacteria:	*Listeria innocua*
Authors:	Seeliger 1983
Status:	New Species
Literature:	Int. J. Syst. Bacteriol. 33:439
Risk group:	1 (German classification)
Type strain and Registry numbers:	ATCC 33090, DSM 20649, NCTC 11288, SLCC 3379

4.4.4 Information on the Bacterial Phage P100

Phage P100 is a bacteriophage which targets *L. monocytogenes* as well as several other species of *Listeria.* It is cultivated for commercial production in *Listeria innocua.*

Phage classification

Order	Caudovirales
Family	Myoviridae
Species	P100[1]
Host specificity	*Listeria monocytogenes, L. innocua*, other *Listeria* spp

Phage identity

The phage's genome does not contain sequences which would enable its injected DNA to be incorporated in the host genome. Therefore, it is a purely lytic phage, as opposed to being a temperate phage. Phage P100 is one of the few known virulent phages for the genus *Listeria*. Such phages are strictly lytic and therefore are invariably lethal to a bacterial cell once an infection has been established.

P100 has been discovered in a culture while screening *Listeria* isolates from food processing plants (Loessner, M.J., unpublished data). P100 features an unusually broad host range within the genus *Listeria*; more than 95% of strains belonging to serovar groups 1/2, 4 (*L. monocytogenes* and others), 5 (*L. ivanovii*) and 6 *(L. innocua*)are infected and killed (Loessner, M.J., unpublished observations).

A detailed characterization of the information encoded in the phage P100 genome was conducted as described in the article by Carlton *et al.*. The host for the P100 prep used for DNA extraction, sequencing, and subsequent bioinformatics analyses, was *L. monocytogenes* strain WSLC 1001 (serovar 1/2a). The complete DNA genome sequence of P100 of 131,384 base pairs was assembled from a highly redundant set of 1,756 single sequence reads with an average length of 800 bp, yielding a total of 1,405,715 base pairs (corresponding to $>$ 10-fold average coverage). The fully annotated sequence has been deposited in GenBank, under accession number DQ004855. A total of 174 open reading frames were identified, predicted to encode gene products (proteins) ranging from 5 kDa (gp61) to 146 kDa (gp35). In addition, P100 encodes a total of 18 tRNAs, located at the right end of the genome (nucleotide position 123,714 – 129,372). Solely on the basis of sequence similarities, putative functional assignments could be made to 25 of the predicted products, whereas the other proteins represent new entries in the database.

[1] P100 featurse a broad host range within the genus *Listeria*, and belongs to the morphotype family *Myoviridae* (Zink and Loessner, 1992). On an overall scale, P100 shares some sequence similarities with other known *Myoviridae* phages infecting Gram-positive bacteria of the low G+C cluster, such as *Staphylococcus aureus* phage K (O'Flaherty *et al.*, 2004) and *Lactobacillus plantarum* phage LP65 (Chibani-Chennoufi *et al.*, 2004a)

4.4.5 Raw Materials used in the Manufacturing Process

Raw materials are considered confidential. A separate document covering this subchapter is supplied.

4.4.6 Hygiene and Quality Control

Hygiene

For optimal phage production, it is very important that hygiene conditions during the fermentation process and down stream processing are strictly controlled. Microbial contamination would immediately result in reduced growth of the production organism and consequently in a too low yield of product.

In addition to the microbial hygiene, it is also important that the raw materials and processing aids used during manufacturing are of sound quality and do not contain contaminants such as pesticides or heavy metals, which might affect the optimal growth of the production organism and the specifications of the final phage product.

In that respect the company has its own HACCP plan. All procedures are performed according to Good Laboratory Practices (GLP).

The quality of the stock culture and the strict control of parameters as temperature and aeration during fermentation are also of the utmost importance for optimal phage production and yield.

Quality control and control measures:

Host bacteria

The identity of the used host bacteria is controlled and guaranteed by using the same route of stock production at all times. The master stock, which is stored at -80^{0}C, is used to produce a seed stock. The seed stock is tested on being a monoculture and all test data are recorded in a SOP datasheet. The same rules apply for the production of the working stock. All bacteria stocks are stored at -80^{0}C, to ensure the highest stability possible.. In figure 3 a schematic overview of the route from master stock to production is given.

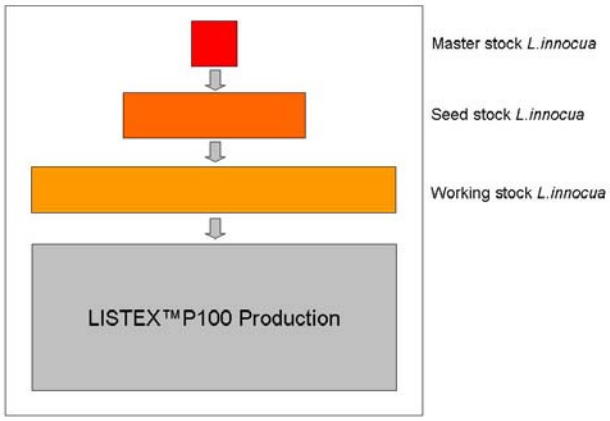

Fig. 3: schematic overview of host bacteria working stock production

Only certified seed stocks are used for working stock production. Only certified working stock batches are used for production purposes.

Phages

The identity of the phages used is controlled and guaranteed by using the same route of stock production at all times. The master stock is used to produce a working stock. The working stock is tested on *Listeria* serovars 1 / 2, 3, 4, 5 and 6 to ensure its broad spectrum, and a DNA digestion profile is made to compare the master and working stock. Working stock production, as well as all tests, is performed according to a SOP and data is recorded in a SOP datasheet. Figure 4 shows a schematic overview of phage master stock production.

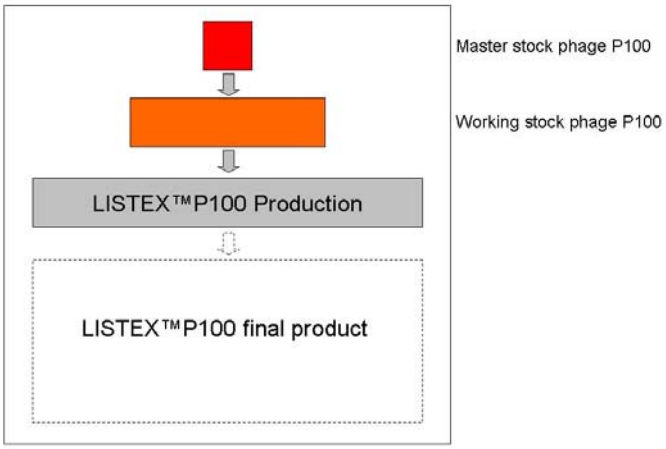

Fig. 4: schematic overview of phage working stock production

Raw materials

The raw materials used in the fermentation process are checked to be of suitable purity and free of harmful substances. The ingredients used are tightly controlled to avoid contaminants that would inhibit growth of the production organism.

Each new batch of raw materials is tested on its performance, and in case of a deviation, the materials supplier is contacted and consulted.

4.5 Specification for Identity and Purity

Listex™ P100 is a bacteriophage that belongs to the order of Caudovirales, family of Myoviridae, genus SPO1-like viruses. The size of one particle is approximately 300 nm and it consists of linear double stranded DNA surrounded by a protein shell. The genome of P100 consists of 131,384 basepairs predicted to encode for 174 gene products and 18 tRNAs. The fully annotated sequence of P100 is deposited in GenBank under accession number DQ004855. All possible gene products from P100 were analysed *in silico* for possible similarities with known (food) allergens. One protein showed a local similarity to wheat gliadin but further analysis showed that it has no anticipated probability to act as a potential immunoreactive allergen, as can be read in chapter 2.3. The in silico assessment of P100 did not reveal any similarities to genes or proteins believed to play a role in pathogenicity (toxins) and virulence of *Listeria monocytogenes* or any other harmful micro organism. Bacteriophage P100 is a *Listeria* specific lytic particle, and therefore invariably lethal to a bacterial Listerial cell once an infection has been established.

Details on the purity of Listex™ P100 are provided in the table 1.

Table 1. Product Specifications of Listex™ P100

Physical Properties	
Description	Suspension of broad-spectrum phage preparation, formulated in phosphate buffered saline.
Source	Fermentation derived
Phage concentration	2 x10^{11}phage/ml
Chemical Properties	
Heavy metals (as lead)	<10 ppm
Lead	<1 ppm
Arsenic	<1 ppm
Mercury	<0.5 ppm

Microbiological Properties	
Standard plate count	Sterile
Yeasts and moulds	Less than 10/ml
Enterobacteriaceae	Negative in 1 ml
Salmonella	Negative in 25 ml
Listeria sp.	Negative in 25 ml
Staph. aureus	Negative in 1 ml
E. coli	Negative in 1 ml

The analysis of the P100 product against external specifications for three batches, as well as a table detailing the methods used, is presented respectively in Annex 6 and 7.

A tabular report on the stability of P100 in long term storage is included in Annex 8. The recommended storage conditions are temperatures between 2-8°C. At these temperatures the phage product P100 is stable for long periods of time (designated shelf life: 6 months).

5. Dietary Exposure to the Processing Aid

5.1 Food or food groups to be treated

Listex™ P100 can be used on several ready to eat food products on which *Listeria monocytogenes* might be a present such as meat, fish and cheese. A detailed description for the application of Listex™ P100 on meat, fish and cheese is described below but Listex™ P100 can also be used on other RTE foods like cut vegetables for example.

In the meat industry, the highest risk products are meats contaminated with *Listeria* after a heating step intended to sterilize the meat product. For these products Listex™ P100 can be used as a post-lethality treatment. Since *Listeria* is able to grow under conditions of high salt and low temperature, a sporadic *Listeria* infection of a product after the heating step can lead to high numbers in the cooling chain. The best way of applying Listex™ P100 on the surfaces of meats, like ham and hotdogs for example, is as close as possible to the moment of possible contamination of the product. A method to incorporate Listex™ P100 into meat is to spray it on hot dogs just before the hot dogs are inserted in their packaging. In Annex 9 the incorporation of Listex™ P100 in hot dog manufacturing is visualized. A control module, a supply module and a delivery module have to be inserted in the production line. Listex™ P100 is sprayed on the hot dogs, they are packaged and the packaging is vacuumed. The vacuum distributes the Listex™ P100 evenly over the hotdogs.

Contamination with *Listeria monocytogenes* is one of the most prominent microbial problems in the fish industry. This because *Listeria* occurs naturally in coastal and river waters and is naturally present on living fish. The *Listeria* is mainly concentrated in the gills and can be transferred when the fish is slaughtered and filleted. Since Listex™ P100 needs to be applied as close to the expected moment of contamination as possible (as the phages rapidly lose their function due to structural degredation and adsorption), the best moment for the incorporation of Listex™ P100 into the processing of fish is after the most intensive handling like the filleting, skinning and trimming but before smoking, marinating and salting. The wash or spray containing Listex™ P100 should be applied in such a way that it is distributed evenly over the surface and on and in the crevices of the fish fillets.

In cheese, Listex™ P100 may be part of the surface cultures to eliminate *Listeria* contaminations on the cheese surface or in the smear cultures itself. Also Listex™ P100 may be added to the brine in order to protect the brine from becoming a source of contamination.
Alternatively, Listex™ P100 can be used in raw milk or in the filtrate of raw milk in order to eliminate possible contaminations which make the milk unfit for the safe production of raw milk cheeses. (While raw milk is tested by analysis of samples, *Listeria* contaminations below levels of detection may still make finished cheeses unfit for consumption, necessitating their destruction). Depending on the type of cheese, Listex™ P100 is added to the raw milk or, at a later stage, in combination with the used bacterial cultures or rind washes which are essential in the production

process of specific types of cheeses. As such it can prevent the rind washes or brine bath from becoming a source of contamination of entire batches.

5.2 Application dosages

The optimal concentration of Listex™ P100 depends on the food product and on the targeted reduction. To achieve a reduction of 2 logs a higher dose is needed than for a 1 log reduction. As can be seen in Annex 10, the use of 3 $x10^8$ pfu/g causes a total eradication in some cases and reduction of ~2 logs in most of the food products.

5.3 Levels of residue and technological function

A processing aid needs to conform to a number of definitions. According to the Standard 1.3.3 a food processing aid is a substance (listed in the clauses):

- used in the processing of raw materials, foods or ingredients, to fulfil a technological purpose relating to treatment or processing, but does not perform a technological function in the final food
- used in the course of manufacture of a food at the lowest level necessary to achieve a function in the processing of that food, irrespective of any maximum permitted level specified

To get Listex™ P100 approved as a processing aid, Listex™ has to comply with the two criteria described above. In the next subchapters these requirements are addressed.

5.3.1 Phage residues

Processing aids, the use of which inadvertently leads to the substance remaining in a foodstuff, should no longer have a technical function in the final food product and have to be present at the lowest level necessary to achieve a function in the processing of that food.

Phages can be compared to antimicrobials that have been approved as processing aids in Australia and New Zealand in Standard 1.3.3, for example octanoic acid. Octanoic acid is approved with a maximum permitted level that has to be in line with GMP. In the initial assessment report on octanoic acid as a processing aid, it can be found that GMP levels correspond with **220 ppm** (FSANZ, 2004).
Phages, because of their one-on-one activity against their bacterial host, are described by the number of particles used, instead of by weight. GMP levels of phages reach up to 1 $x10^9$ pfu/g. This number may seem large but converted to weight it is very definitely insignificant: the total weight of the phages would be **0.0001993 ppm**[1]. Compared to octanoic acid, phages would be present at a level of more than **1,1 million times lower**. In actual use, the amount of phages used is even lower because this particular concentration does not always correspond with the doses needed to achieve the desired reduction in *Listeria monocytogenes* levels.

Phages are of course larger than the chemical molecules but in terms of molecules applied to a food item the discrepancy becomes even clearer. 220 ppm octanoic acid corresponds to 0.22 grams/kg of product. 0.22 grams of octanoic acid corresponds with ~9.2 $x10^{20}$ molecules per kilogram of final food product. In comparison, phages, at the highest dosage, would be present at 1.0 $x10^{12}$ particles per kilogram, or put

differently in terms of active particles at a concentration **920 million times lower than octanoic acid**.
Both in terms of weight per final product and in molecule numbers, the remaining phage concentration is very low. Therefore we believe the use of phages during the processing of food products can be considered a processing aid.

Table 2: Comparison of residual levels of P100 phage to an approved processing aid

	Octanoic acid	**P100 Phage**
w/w basis	220 ppm	0.0001993 ppm
Molecule or pfu/Kg basis	~9.2 $x10^{20}$ molecules/Kg	~1.0 $x10^{12}$ pfu /Kg

[1]weights and measures: Phage P100 = 1.2 $x10^{8}$ D (Dalton) per phage particle, 1D= 1.66 $x10^{-27}$ Kg
octanoic acid $_{Mw}$= 144.21: 1M (6.022 $x10^{23}$ molecules)= 144.21g

5.3.2 Loss of function

Processing aids are not allowed to have a technical function in the final product. Listex™ P100 is inactivated within 24 hours after addition to the food (Guenther et al., 2009). This inactivation is caused by various factors such as adsorption of phages to particles, proteolytic degradation of the phage particle by chemicals and enzymes, temperature, salts and light (Suttle and Chen 1992; Garza and Suttle 1998; Hurst *et al*., 1980) (Annex 11). Eventually, phages will fall apart into amino acids and nucleotides. The experimental data shown in Annex 12 clearly show that after a significant initial reduction of *Listeria*, just after application of the phages, regular growth of the remaining *Listeria* resumes within 6-24 hours after application in all foodstuffs. In other words, the phages lose their function within hours after application.

5.3.3 Phage adsorption

Rapid phage inactivation is caused largely by adsorption of the pages to the food matrix. It is commonly known that proteins adsorb to surfaces (Ruggiero *et al.*, 2005) and since phages consist of a protein hull containing DNA also phages are likely to adsorb. There are several interactions between the phage and the food surface that contribute to the strong binding:

- Hydrophobic interactions: the side chain of several amino acids is non-polar and hence interacts poorly with polar molecules like water. When non-polar residues are exposed at the surface of two different molecules, it is energetically more favourable for their non-polar surfaces to approach each other closely, displacing the water from between them.
- Ionic interaction: proteins contain both positively and negatively charged amino acids. These interact with and bind to other, oppositely charged groups.
- Hydrogen bonds: a strongly electronegative atom (e.g., oxygen, nitrogen) approaches a hydrogen atom which is covalently attached to a second strongly electronegative atom. These can be formed in the case of phages and foodstuffs between the –C=O group and the H-N- groups, and between –C=O groups and H-O- groups proteins and sugars.

Individually these bonds are much weaker than covalent bonds (typically about 20 times), but many of them together can have formidable strength. The first bond to occur brings the phage closer and holds it to the food surface, increasing the likelihood of additional bonds to form. This is the reason why adsorption only becomes stronger over time. Any one bond can be broken with relative ease, but for phages to desorb, all bonds must be broken simultaneously which is impossible in the commercial setting. Detailed information on phage adsorption can be found in annex 12.

5.4 Average Daily Intake

The proposed use of Listex™ P100 that is the subject of this dossier is for it to be used during the manufacturing process of a variety of foods, to eliminate possible contamination of *Listeria monocytogenes,* and thus prevent outgrowth at a later stage.

The total number of phage P100 on a food product depends on the surface/weight ratio. For cheeses this has been tested by Carlton *et al.* (2005). On the types of cheeses tested to date, the most suitable dosage appears to be approximately 5×10^7 plaque forming units (pfu) P100 per cm^2 of surface of cheese. There are approximately 200 cm^2 of surface on a small 100 g cheese. Thus the total number of phage on 100 g of cheese is estimated at 1×10^{10} pfu or 1×10^8 pfu/g of cheese.
The weight of small biological particles such as phages is usually given in Daltons, which are equivalent to one atomic unit. A Dalton weighs 1.66×10^{-27} kg. The P100 phage has about 133 K base pairs. The mass of the DNA is approx. 5×10^7 Daltons. One then adds the phage particle itself, the protein packaging, weighing approximately 7×10^7 Daltons. Therefore, the total mass of a particle is approximately 1.2×10^8 Daltons. An estimate of 120 million Daltons is therefore used for the weight of a single P100 phage.

For arguments sake and assuming the high dose of 1×10^8 pfu/gram is applied and without the subsequent degradation of phages, a phage concentration of 1×10^8 pfu/gram of food would be ingested by the consumer.

To calculate the average daily intake for Listex™ P100, the values for the annual intake for different food products from “Apparent Consumption of Foodstuffs Australia 1997-98 and 1998-99” from the Australian Bureau of Statistics was used. Since meat and poultry, seafood and cheese are food groups likely to be treated with Listex™, the average daily intake was calculated in case these food groups are treated.

The average annual meat and poultry consumption in the years 1998-99 was 102.4 Kg which is the same as 280.55 g/day. Assuming that the average Australian consumer with a mean bodyweight of 60 Kg ingests 280.55 grams of meat and poultry per day and all the meat and poultry is treated with Listex™ P100 and moreover all the phages are still present on the product, the amount of phage ingested will be 280.55×10^8 pfu. If each phage weighs 120 million Daltons (1.2×10^8) the total weight of the phages would be 3.37×10^{18}. Converting this to kilograms, multiply 3.37×10^{18} Daltons by 1.66×10^{-27} Daltons/Kg yielding 5.5942×10^{-9} Kg. This value is equal to 0.093 µg/Kg bodyweight/day.

Applying the above calculations to the other food groups on which Listex™ P100 is likely to be used (seafood and cheese) gives the following equations and values.
The estimated daily intake of seafood according to the Australian Bureau of Statistics is 29.86 g/person/day. If all of the consumed seafood is treated with Listex™ and all of the phages are still present on the seafood when consumed the amount of phage ingested will be 29.86 $x10^8$ pfu which corresponds with 3.58 $x10^{17}$ Daltons or 0.0098 µg/Kg bodyweight/day.

For cheese the estimated daily intake is 29.32 g/person/day. If all consumed cheese is treated with Listex™ and all phages are still present, the amount of phages ingested will be 29.32 $x10^8$ pfu which corresponds with 3.52 $x10^{17}$ Daltons of 0.0097 µg/Kg bodyweight/day.

Taken all of the food products that can be treated with Listex™ P100 together, the maximum daily intake of phage P100 is 0.1125 µg/Kg bodyweight/day. However, this value is a huge overestimate because of two reasons. First, the amount of phages added to a product is not the same as the amount ingested: as described in chapter 4.4 phages adsorb to the food product and fall apart into amino acids and nucleotides quickly after application. Secondly, the applied dose used in the calculations above is 1 $x10^8$ pfu/gram. The actual dose a food producer is going to use depends on the log reduction the manufacturer wants to achieve and on the economical aspect. It is likely to be lower than 1 $x10^8$ and therefore the actual consumption is also lower.

5.5 Percentage of market likely to use the processing aid

In Australia and New Zealand there is a zero tolerance policy towards the presence of *Listeria monocytogenes* in foods. Processors of foods which have a heightened risk of *Listeria* presence or foods with a heightened public health risk are the designated part of the market to use Listex™ P100. It is predicted that innovative companies, which are looking for a more natural solution to control *Listeria monocytogenes*, will be the first to use Listex™ P100. Another part of the industry, expected to be among the first, are food producers for which other methods of fighting *Listeria monocytogenes* do not work or are not applicable because they cause changes in taste, colour or odour of the food.
In the future it is expected that bacteriophage applications like Listex™ P100 will become a standard in food production.

6. Statutory Declaration

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