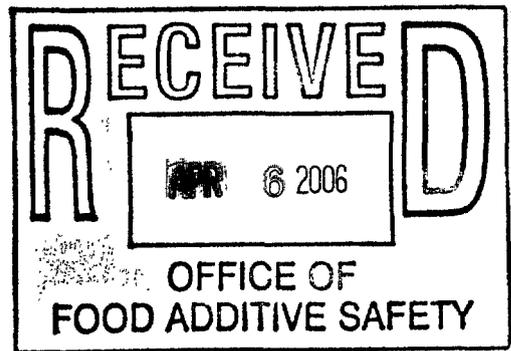


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ORIGINAL SUBMISSION

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VIA HAND DELIVERY

April 5, 2006

Office of Food Additive Safety (HFS-255)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835



RE: Submission of GRAS Notification of Bacteriophage P100

7361 Calhoun Place,
Suite 500
Rockville, Maryland 20855-2765
301.838.3120
fax: 301.838.3182

Dear Sir/Madame:

In accordance with proposed 21 CFR § 170.36 (Notice of a claim for exemption based on a GRAS determination) published in the Federal Register (62 FR 18939-18964), I am submitting in triplicate, as the agent to the notifier, EBI Food Safety, a GRAS Notification of Bacteriophage P100, formulated under the product name Listex™, for a new use in cheese to control *Listeria monocytogenes*. Also enclosed is a GRAS panel report setting forth the basis for the GRAS determination.

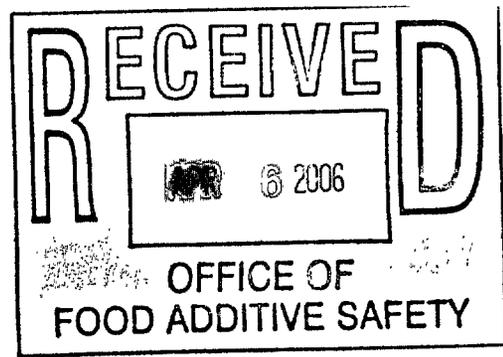
Please let me know if you have any questions.

Sincerely,

Edward A. Steele
President

Enclosures

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Listex™ P100 Bacteriophage

I. GRAS Exemption Claim

A. Claim of Exemption From The Requirement for Premarket Approval Requirements Pursuant to Proposed CFR § 170.36(c)(1)

Bacteriophage P100, formulated under the product name Listex™, has been determined to be generally recognized as safe, and therefore, exempt from the requirement of premarket approval, under the conditions of its intended use as described below. The basis for this finding is described in the following sections.

Signed,

Edward A. Steele/

Date

4/1/06

Agent for:

EBI Food Safety B.V.
Johan v. Oldenbarneveltlaan 9
2582 NE Den Haag
The Netherlands

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B. Name and Address of Notifier

Dirk DeMeester
EBI Food Safety B. V.
Johan v. Oldenbarneveltlaan 9
2582 NE Den Haag
The Netherlands
Tel: +31 (0)70-358 5008
Fax: +31 (0)70-358 5044

C. Common or Usual Name of the Notified Substance

P100 Bacteriophage

D. Conditions of Use

The intended use of the P100 bacteriophage will be for a new use in cheese to control *Listeria monocytogenes* when added in the range from 1×10^7 to 1×10^9 pfu per gram of cheese via the technical broth. P100 is added to this floral broth that is subsequently washed or smeared onto the surface of the formed cheese for maturation and characteristic flavor of a cheese.

E. Basis for the GRAS Determination

Pursuant to 21 CFR § 170.30, P100 bacteriophage has been determined to be GRAS by scientific procedures. A comprehensive search of the scientific literature was also utilized for this review.

F. Availability of Information

The data and information that serve as a basis for this GRAS are available for the Food and Drug Administration's review and copying during reasonable business hours at the offices of:

Edward A. Steele
AAC Consulting Group
7361 Calhoun Place, Suite 500
Rockville, MD 20855-2765
Telephone: 301-838-3120
Facsimile: 301-838-3182

II. Detailed Information About the Identity of the Substance

A. Identity

The P100 bacteriophage that is the subject of this GRAS notice was isolated from wastewater sources, not genetically engineered. The host and the phage identity are presented below.

Listex™ P100 Bacteriophage

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

Phage classification

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

B. Method of Manufacture

Listeria innocua is used as a host strain for the production of P100 phages. *Listeria innocua* is a non-pathogenic bacterial strain that lacks the production of endotoxins. *Listeria* cells are cultured to a certain density followed by an infection with the lytic P100 phages. Further incubation allows for the amplification of phages within the plastic bags. This is followed by a purification process that removes host cells and cell debris. The production process is a common fermentation batch process which employs normal culture media for bacterial culture and/or process additives that are GRAS.

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Specifications for P100 Bacteriophage

The specifications for the final product are given in Table 1 below.

Table 1. Product Specifications of Listex™ P100	
Physical Properties	
Description	Suspension of broad-spectrum phage preparation, formulated in propylene glycol.
Source	Fermentation derived
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 molds	Less than 10/ml
Enterobacteriaceae	Negative in 1 ml
<i>Salmonella</i>	Negative in 25 ml
<i>Listeria sp.</i>	Negative in 1 ml
<i>Staph. aureus</i>	Negative in 1 ml
<i>E. coli</i>	Negative in 1 ml

III. Self-Limiting Levels of Use

The proposed use of P100 that is the subject of this GRAS determination is as an antimicrobial ingredient for addition to cheeses such as brie, cheddar, Swiss and other cheeses that are normally aged and ripened. After a cheese is formed, the producer commonly applies a technical flora or broth of intended microorganisms to further ripen the cheese and give the cheese its characteristic taste. The purpose of P100 addition to the broth is to reduce or eliminate *Listeria monocytogenes* in the finished cheese product.

The total number of phage on 100 g of cheese is estimated at 6×10^{10} pfu or 6×10^8 pfu/g cheese. Actual use may vary in the range from 1×10^7 to 1×10^9 pfu per gram of cheese. Therefore, assuming the highest dose of 1×10^9 pfu/gram is applied, then it is estimated that a stable phage concentration of 1×10^9 pfu P100/gram of cheese would be ingested by the consumer.

According to USDA (1991), the 90th percentile consumption of cheese, excluding cottage and cream cheese, for the total U.S. population is 57 g/day (range 50-64 g/day for various age groups), with a 50th percentile intake of 28 g (range 25-32 g/day for various age groups). Total intake of P100 bacteriophage is estimated to be approximately 11.35 ug/person/day or 0.16 ug/kg/day for a 70 kg person.

The use of the product and potential intake would be self limiting by three factors. First, the cheese manufacturer would use the minimum required to achieve the technical effect of lysing *Listeria monocytogenes* contaminant bacteria due to the cost of the phage product. Secondly, intake by the general population would be limited by the amount of cheese normally consumed, an excess leading to constipation. Lastly, after the host bacteria *Listeria monocytogenes* is

depleted on the cheese, the P100 phage would no longer replicate and would gradually die back in viable numbers.

4. Summary of the Basis for the Notifier's Determination that P100 Bacteriophage is GRAS

An independent panel of recognized experts, qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was requested by EBI Food Safety to determine the Generally Recognized As Safe (GRAS) status of bacteriophage P100 (product name Listex™ P100) intended for use in cheese to control *Listeria monocytogenes*. A comprehensive search of the scientific literature was also utilized for this review.

Based on a critical evaluation of the publicly available data and information summarized in the Expert Panel GRAS S Determination document attached, the Expert Panel members have individually and collectively concluded that bacteriophage P100, meeting the specifications cited above, is generally recognized as safe (GRAS) by scientific procedures when used as an antimicrobial ingredient in cheese at levels up to 1×10^9 pfu/gram of cheese. In coming to its decision that bacteriophage P100 is GRAS, the Expert Panel relied upon the *in silico* assessment of the complete genome and gene products of P100 for allergenicity, pathogenicity or virulence, published toxicology studies and other articles relating to the safety of lytic bacteriophage which were considered to collectively demonstrate the safety of the product. It is also their opinion that other qualified and competent scientists, reviewing the same publicly available toxicological and safety information, would reach the same conclusion.

EXPERT PANEL STATEMENT

DETERMINATION OF THE GRAS STATUS OF BACTERIOPHAGE P100 AS AN ANTIMICROBIAL FOOD INGREDIENT

The undersigned, an independent panel of recognized experts (hereinafter referred to as Expert Panel), qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was requested by EBI Food Safety (EBI) to determine the Generally Recognized As Safe (GRAS) status of bacteriophage P100 (product name Listex™ P100) intended for use in cheese to control *Listeria monocytogenes*. The scientific literature for safety and toxicity information on P100 and related bacteriophages were made available to the Expert Panel. The Expert Panel independently evaluated materials submitted by EBI and other materials deemed appropriate or necessary. Following independent, critical evaluation, the Expert Panel conferred and unanimously agreed to the decision described herein.

INTRODUCTION

Listeria monocytogenes is a facultative anaerobic bacterium that is capable of growing at refrigeration temperatures. *L. monocytogenes* has been associated with a number of food-poisoning outbreaks related to foods such as soft cheeses, processed meat, poultry, and vegetables. The symptoms can range from severe diarrhea to death. 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).

Listeria does not belong to the normal flora of healthy animals or man, but is an environmental bacterium and usually contaminates foods during fermentation, processing, storage, or even packaging of foods. This includes most ready-to-eat products such as milk and cheeses (mostly soft cheese), cold-cuts (different types of meats), hot-dogs, smoked fish and seafoods, and various delicatessen items.

Most countries have adopted a zero tolerance policy for the organism in food, which has led to the recall of many products from supermarket shelves with concomitant economic losses. Food giants in the U.S. have had processing plants shut down because of deaths, which has subjected those companies to large fines. The persistence of *L. monocytogenes* in food products proves that it is difficult to eradicate this pathogen using currently available methods.

Bacteriophages can be regarded as natural enemies of bacteria, and therefore are logical candidates to evaluate as agents for the control of food borne bacterial pathogens, such as *Listeria*. The attributes of phages include the following: (i) they are designed to kill live bacterial target cells, (ii) they generally do not cross species or genus boundaries, and will therefore not affect (a) desired bacteria in foods (e.g., starter cultures), and (b) commensals in the gastrointestinal tract, or (c) accompanying bacterial flora in the environment. Moreover, (iii) since phages are generally composed entirely of proteins and nucleic acids, their eventual breakdown products consist exclusively of amino acids

and nucleic acids. Thus, they are not xenobiotics, and, unlike antibiotics and antiseptic agents, their introduction into, and distribution within a given environment may 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 self-replicating units 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 meat and meat products, more than 10^8 viable phage per gram are often present (Kennedy and Bitton, 1987). It is a fact that phages are routinely consumed with our food in quite significant numbers. Moreover, phages are also normal commensals of humans and animals, and are especially abundant in the gastrointestinal tract (Furuse, 1987; Breitbart, 2003). Thus, phages are common in sewage effluent, from which the lytic P100 phage was isolated.

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 lytic 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).

It is also 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 *Listeria* phages were experimentally shown to be able to transduce genetic markers (Hodgson, 2000), this has not been reported for the strictly virulent or lytic phages such as P100.

HOST AND PHAGE IDENTITY

Phage P100 is a bacteriophage that targets *L. monocytogenes* as well as several other species of *Listeria*. It is cultivated for commercial production in *Listeria innocua*. The phage's genome does not contain sequences that would enable its injected DNA to take up residence on the host bacterium's DNA. 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*, which 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). Similar to *Listeria* phage A511 (Loessner and Busse, 1990; Loessner, 1991, van der Mee-Marquet *et al.*, 1997), P100 features an unusually broad host range within the genus *Listeria*; more than 95% of the different strains belonging to serovar groups 1/2, 4 (*L. monocytogenes*), and 5 (*L. ivanovii*) are infected and killed (Loessner, M.J., unpublished observations).

The identity and classification of the *Listeria innocua* bacterial host is given below. This strain is considered to be non-pathogenic.

Bacterial host classification and identity

Name of host bacteria:	<i>Listeria innocua</i>
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

Phage classification

Order	Caudovirales
Family	Myoviridae
Species	P100
Host specificity	<i>Listeria monocytogenes</i> , <i>L. innocua</i> , other <i>Listeria</i> spp.

A detailed characterization of the information encoded in the phage P100 genome was conducted as described in the article by Carlton *et al.* (2005). The host for the P100 prep used for DNA extraction, sequencing, and subsequent bioinformatic 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.

P100 appears to be closely related to *Listeria* phage A511. They both feature a broad (but nevertheless different) host range within the genus *Listeria*, and belong to the same morphotype family (*Myoviridae*; Zink and Loessner, 1992). The phenotypical observations correlate well with the now available genetic data, which revealed significant nucleotide sequence homologies of P100 to the A511 genome (Loessner and Scherer, 1995; Dorscht *et al.*, submitted for publication). On an overall scale, P100 also shared 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).

SPECIFICATIONS

The food-grade formulation of P100 bacteriophage will be marketed under the trade name Listex™ P100. The specifications for the final product are given in Table 1 below. The analysis of the P100 product against specifications for three batches, as well as a table detailing the methods used, are presented in Appendix C. A tabular report of the stability of P100 product in long-term storage is also included in this Appendix. The recommended storage conditions in the production facility and for the end user are refrigerated temperatures of between 2-8°C. The P100 phage product is stable for long periods (2 years or more) at these temperatures.

Physical Properties	
Description	Suspension of broad-spectrum phage preparation, formulated in propylene glycol.
Source	Fermentation derived
Phage concentration	1x 10 ¹³ 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 molds	Less than 10/ml
Enterobacteriaceae	Negative in 1 ml
<i>Salmonella</i>	Negative in 25 ml
<i>Listeria sp.</i>	Negative in 25 ml
<i>Staph. aureus</i>	Negative in 1 ml
<i>E. coli</i>	Negative in 1 ml

PROCESS MATERIALS AND METHODS

EBI uses a certified manufacturer (CatchMabs BV; The Netherlands) who operates the laboratory under a governmental ML-1 permit with biosafety level 2-3 specifications according to the WHO Laboratory Safety Manual. The laboratory is currently implementing ISO 9001, for certification in 2006. No animal products of any kind are used in the propagation, purification or stabilization of the phage preparation. The media contains proteins that are exclusively of plant origin.

Listeria innocua is used as a host strain for the production of P100 phages. *Listeria innocua* is a non-pathogenic bacterial strain that lacks the production of endotoxins. *Listeria* cells are cultured to a certain density followed by an infection with the lytic P100 phages. Further incubation allows for the amplification of phages within the plastic bags. This is followed by a purification process that removes host cells and cell debris. The production process is a common fermentation batch process which employs normal culture media for bacterial culture and/or process additives that are GRAS. Details of the process and quality assurance measures to assure product identity and quality are given in Appendix A.

The particular culture media and manufacturing equipment used for production and purification of the P100 product are **Company Confidential** and are presented for purposes of FDA evaluation only in Appendix B.

PROPOSED USE AND ESTIMATED DIETARY INTAKE OF BACTERIOPHAGE P100

The proposed use of P100 that is the subject of this GRAS determination is as an antimicrobial ingredient for addition to cheeses such as American, Brick, Brie, Cheddar, Mozzarella, Swiss and other cheeses that are normally matured before further processing. In the cheese making process, the producer commonly applies a technical flora or broth of intended microorganisms to allow the maturation process to take place in order to give the cheese its characteristic taste. With antibiotics and other antimicrobials, it is difficult to remove pathogenic organisms from the bacterial floral broth without killing the beneficial organisms. P100 does not affect these beneficial organisms. P100 is added to the floral broth that is subsequently washed or smeared onto the surface of the formed cheese for maturation. There is usually only one application necessary to achieve the desired antimicrobial effect before the cheese is stored and aged. The purpose of P100 addition to the surface is to reduce or eliminate *Listeria monocytogenes* in the finished cheese product. As the report by Carlton *et al.* (2005) demonstrates, P100 derived from *L. innocua* host bacteria is an effective antimicrobial against *Listeria monocytogenes* in cheese when applied to the surface of the cheese.

On the types of cheeses tested to date (Carlton *et al.*, 2005), the most suitable dosage appears to be approximately 3×10^8 plaque forming units (pfu) P100 per cm^2 of surface of cheese. There are approximately 200 cm^2 of surface on 100 g of cheese. Thus the total number of phage on 100 g of cheese is estimated at 6×10^{10} pfu or 6×10^8 pfu/g cheese. Actual use may vary in the range from 1×10^7 to 1×10^9 pfu per gram 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.

Although P100 may multiply after infecting the bacterial host and release progeny phages, multiple experiments with phage application to foods have shown the phage titer to be stable over 6-10 days. For practically all cheeses, there is only a single application of phages to the cheese surface for the desired antimicrobial effect. Therefore, assuming the highest dose of 1×10^9 pfu/gram is applied, then it is estimated that a stable phage concentration of 1×10^9 pfu P100/gram of cheese would be ingested by the consumer.

According to USDA (1991), the 90th percentile consumption of cheese, excluding cottage and cream cheese, for the total U.S. population is 57 g/day (range 50-64 g/day for various age groups), with a 50th percentile intake of 28 g (range 25-32 g/day for various age

groups). Assuming a 90th percentile US consumer ingests 57 g cheese/day, then the maximum estimation of P100 ingested if all cheeses were treated would be 5.7×10^{10} (1×10^9 pfu/g cheese x 57). If each phage weighed 120 million daltons (1.2×10^8), the total weight of phage would be 6.8×10^{18} daltons. Converting this to kilograms, multiply 6.8×10^{18} daltons x 1.66×10^{-27} daltons/kg yielding 1.1×10^{-8} kg or approximately 11.35 ug/person/day. Therefore, the 90th percentile intake of cheese consumption, assuming all cheese was treated with P100, would result in an intake of P100 bacteriophage of approximately 11.35 ug/person/day or 0.16 ug/kg/day for a 70 kg person. As noted before, this number and consumption of phages is similarly comparable to the amount of mixed phage population commonly found on meat products and fresh fish (10^8 viable phages per gram).

SAFETY STUDIES

Subacute Toxicity Study

The subacute 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 in Carlton *et al.* (2005). This study was conducted according to the current OECD principles of good laboratory practice. The P100 preparation used for the feeding studies in rats came from Catchmabs B.V., the commercial supplier production facility, and was grown on *L. innocua*. Therefore, the test material was identical to the commercial P-100 product. Young Wistar albino rats were given 1.0 ml of PBS vehicle or 5×10^{11} pfu/ml phage P100 particles suspended in phosphate-buffered saline pH 7.3 (PBS) orally by gavage for a total dose of approximately 2×10^{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 a two day recovery period, all animals were anesthetized with ether, sacrificed, and exsanguinated.

All animals were examined for gross pathology. The esophagus, 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 2×10^{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. There were no abnormal physical signs or behavioral changes 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 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.

***In Silico* Assessment of Potential Pathogenicity, Virulence and Allergenicity**

After the complete sequence was assembled, genome coordinates were defined: 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 analyzed 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 analyzed *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 microorganism. Genomic data clearly indicated that P100 is related to A511, a *Listeria* specific Myovirus whose genome has recently been sequenced (Dorscht *et al.*, manuscript in preparation).

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 known 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 8×10^{-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, we conclude that gp71 has a negligible probability to act as potential immunoreactive allergen.

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 milliliter. In some fresh water sources, there are up to 10^9 phages per milliliter.

Numerous papers attest to the fact that humans are exposed to huge numbers of phages daily, through food and water, without notable evidence of any harm. Intestinal bacteriophage readily penetrate the gastrointestinal barrier, with some phages eliciting antibody production (Dabrowska *et al.*, 2005). 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

injected into patients with antibiotic-resistant bacterial disease with 80% success rate; in these patients, the phages posed no risk of toxicity or significant side effects. Although such use of lytic phage is controversial, it comprises a large body of evidence that phages can be injected into humans with no ill effects. Lytic 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 multiple 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 diarrhea 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 are 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; Chibany-Chenouffi, 2004b; Merril *et al.*, 1996). In our study, subacute dosing of rats up to 2×10^{12} pfu P100/kg did not result in any adverse effects on the gastrointestinal tract or any clinical signs of toxicity.

Further evidence that treating cheeses with phage P100 is not likely to cause harm to humans who consume such cheeses 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 likely that humans have billions of phages in their intestines at any one time. Thus, if a relatively low number of phage P100 continue to be dormant and viable on the surface of a cheese several months after being applied at the time of cheese making and are ingested by the consumer, it is unlikely to pose notable hazard because:

- Ingestion 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 and does not transduce because it lacks the necessary insertion sequences;
- *Listeria* phages such as P100 are not able to infect and kill bacteria from other genera of bacteria, and therefore are not going to upset the intestinal flora.

In conclusion, there is no reason to believe that the intake of phage with food may have any adverse effects on humans. Further, because lytic phage particles constitute non-toxic, naturally present components in our foods, they may be considered safe for intentional application in foods.

EXPERT PANEL STATEMENT OF GRAS APPROVAL

Based on a critical evaluation of the publicly available data and information summarized above, the Expert Panel members whose signatures appear below, have individually and collectively concluded that, bacteriophage P100, meeting the specifications cited above, is generally recognized as safe (GRAS) by scientific procedures when used as an antimicrobial ingredient in cheese at levels up to 1×10^9 pfu/gram of cheese. In coming to its decision that bacteriophage P100 is GRAS, the Expert Panel relied upon the *in silico* assessment of the complete genome and gene products of P100 for allergenicity, pathogenicity or virulence, published toxicology studies and other articles relating to the safety of lytic bacteriophage which were considered to collectively demonstrate the safety of the product. It is also our opinion that other qualified and competent scientists, reviewing the same publicly available toxicological and safety information, would reach the same conclusion.

Douglas L. Archer, Ph.D

3/20/06

Date

W. Gary Flamm, Ph.D., F.A.C.T., F.A.T.S.

3/9/06

Date

James W. Barnett, Jr., Ph.D., DABT

3/8/06

Date

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APPENDIX A.

Production and Quality Assurance Procedures

PRODUCTION PROCEDURE

Listeria innocua is used as a host strain for the production of P100 phages. *Listeria innocua* is a non-pathogenic bacterial strain that lacks the production of endotoxins. *Listeria* cells are cultured to a certain density followed by an infection with the lytic P100 phages. Further incubation allows for the amplification of phages within the plastic bags.

DOWN STREAM PROCESSING PROCEDURE

Cell debris is removed by a dead-end filtration step using pharma-grade filters and stainless steel and/or food grade disposable tubing. In a further step, the filtrate is concentrated using ceramic cross-flow filters and food-grade disposable tubing. In the final step, the filtrate is polished and sterilized by a serial treatment of the phage-containing fluid using Mustang filters (Pall) and a 0.2µm sterilization filter. This assures that there are no living host organisms in the final product.

FINAL PRODUCT LIQUID MEDIUM

Content **10¹³ pfu per ml of P100**
Peptone
Yeast extract
Sodium chloride
HEPES
Hydrochloric acid
Buffer: 30% glycerol, 150mM NaCl, 0.05M
phosphate buffer pH

Recommended Product Storage

Refrigerated at 2-8°C

Product Stability

Labelled for 6 months at recommended
storage temperature

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II Preparation of glycerol stock

Work must be performed in a LAF-cabinet. Keep glycerol on ice-water.

1.	Make a single colony streak out from the master glycerol stock on a LB agar plate.	done
2.	Incubate plate overnight at 30°C.	done
3.	After o/n incubation pick one individual colony and make a second single colony streak out	done
4.	Incubate plate overnight at 30°C.	done
5.	After o/n incubation pick one individual colony and inoculate into 800 ml 4xSY+50mM HEPES, pH6,8	done
6.	Grow o/n at 30°C, 200 rpm in a shaker incubator.	done
7.	Next day inoculate 70 l 4xSY+HEPES, pH6,8 with the o/n culture, start O.D. ₆₀₀ = 0.1 in 2 BPC's	done
8.	Incubate at 30°C, 200 rocks per minute in Tsunami until O.D. ₆₀₀ =0.4 is reached	done
9.	Concentrate cells using hollow fiber(400kD) until at least 1.6 liters.	done
10.	Spin down cells in 4°C pre-cooled centrifuge at 2500 rcf for 15 minutes.°CMinutesrcf
11.	Decant supernatant and resuspend cells in 1/40 volume, on ice-water chilled, 4xSY+50mM HEPES, pH 6,8 ± 10% glycerol	done
12.	Keep resuspended cells on ice-water all the time.	
13.	Aliquot 10ml cells in cryo-bpc's and immediately freeze cells with dry-ice EtOH mixture	done
14.	Label each cryo-bpc's with product, product number and batch number and place one label here.	Place here
15.	Store frozen cryo-bpc's in -80°C freezer	done
16.	Take a sample for QC1-sterility.	done

Culture Storage:

The bacterial stocks are stored at -80°C in a Nuair NU6518E -80° Freezer. The phage stocks are stored at 4°C in a Listeria dedicated Miele K2318S Fridge.

QC1: Sterility test

Dilute glycerol stock to ±1000 CFU/50_l with 4SY culture media.
Plate 50 ul in duplicate on:Colombia agar 50% sheep blood

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II Preparation of Listeria phages

All procedures must be done in a LAF-cabinet. Keep culture sterile.

1.	Listeria phages are cultured in a 2 liter shaker flask with 0,4 liter 4*SY	
2.	Pre-warm 0,4 liter 4xSY+50mM HEPES, pH6,8 to 30°C in Tsunami reactor	done
3.	When warm(30°C), inoculate bag with Listeria -80°C glycerol stock (cryo-vial) at O.D. ₆₀₀ =0.05	done O.D. ₆₀₀ =
4.	Incubate at 30°C, 30 rocks/min, 15° angle in Tsunami reactor until O.D. ₆₀₀ =0.4 is reached	done
5.	When warm(30°C) inoculate each tsunami bag with pre-culture (MP12.002) at start O.D. ₆₀₀ =0.02	done
6.	Inoculate the Tsunami bag by adding the cell suspension to the bag.	done
7.	Incubate in Tsunami reactor at 30°C, 30 rocks/min, 15° tilt O.D. ₆₀₀ = 0,1 is reached.	O.D. ₆₀₀
8.	Infect the Listeria culture with a 1:5000 phage: cell ratio. Check pH is 6,8	done µl phage stock.... pH
9.	Incubate overnight at 30°C, 30 rocks/min, 15° tilt	
10.	After culturing connect the Tsunami to the manifold. (See OP10.002 for schematics)	done
11.	Remove cell debris by filtering solution through compressed clarification filter in a filtering unit.	done
12.	Concentrate Phage solution with a 10kD concentrate until wanted concentration has been reached. Write down volume	done ml.....
13.	Filter sterilize through a 0,45µm filter by dead end filtration.	done
14.	Filter sterilize through a 0,22µm filter by dead end filtration	done
15.	Take samples for QC2 sterility and QC3 phage titer.	done

Chromogen Listeria plates

Incubate overnight at 30° C

Next day count CFU; the number of colonies on both plate types must be similar. Colonies on chromogen plates should have a green colour.

On both plates, a monoculture should be visible.

QC2: Sterility test

Plate 50 ul in duplicate on:

Colombia agar 50% sheep blood

Incubate overnight at 30°C

No cfu's should be visible.

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QC3: Phage titration

Materials

4x SY +50mM HEPES pH 6,8

4x SY +50mM HEPES Top-Agar

- Pre heat 20 ml 4xSY + 50mM hepes ph 6,8 at 30°C
- Inoculate culture media with appropriate host strain at O.D.600 = 0,1
- Incubate at 30⁰ C until O.D.600 = 0,4 is reached
- While incubating make appropriate dilutions of the phage suspension to be tested
- Melt 4x SY top-agar in the microwave, dispense 5ml in 10ml tubes, use as many tubes as needed
- Keep tubes at 48⁰ C in water bath till used.
- Infect 300 ul cells with 10 ul phage dilution
- Incubate cells + phages 30 minutes at 30°C in incubator
- Mix the cells + phages with 5ml top-agar, mix well on test tube shaker
- Pour mixture quickly into culture plate (9 cm) and let the agar solidify
- Incubate in incubator at 30⁰ C overnight
- Next day, count colonies and calculate phage titer

Genetic stability:

At present, all batches are being produced from original stocks. A P100 specific PCR is being set-up by to monitor genetic stability for commercial production.

10 distinctive Listeria strains will be used during periodic host range tests to monitor possible changes of sensitivity to P100.

Genetic stability:

Chromogen and blood agar plates will distinguish *L. innocua* from *L. monocytogenes*.

The ML-I laboratory at CatchMabs levels between Biosafety level 2 and 3 according to the third edition of the WHO Laboratory Biosafety Manual (see Table below).

BIOSAFETY LEVEL	1	2	3	4	ML-I at C M
Isolation of laboratory ^a	No	No	Yes	Yes	No
Room sealable for decontamination	No	No	Yes	Yes	No
Ventilation:					
— inward airflow	No	Desirable	Yes	Yes	Yes
— controlled ventilating system	No	Desirable	Yes	Yes	No
— HEPA-filtered air exhaust	No	No	No/Yes ^c	Yes	No
Double-door entry	No	No	Yes	Yes	Yes
Airlock	No	No	No	Yes	No
Airlock with shower	No	No	No	Yes	No
Anteroom	No	No	Yes		Yes
Anteroom with shower	No	No	Yes/No ^c	No	Yes
Effluent treatment	No	No	Yes/No	Yes	No
Autoclave:					
— on site	No	Desirable	Yes	Yes	Yes
— in laboratory room	No	No	Desirable	Yes	Yes
— double-ended	No	No	Desirable	Yes	No
Biological safety cabinets	No	Desirable	Yes	Yes	Yes
Personnel safety monitoring capability ^d	No	No	Desirable	Yes	No

a Environmental and functional isolation from general traffic.

b Dependent on location of exhaust

c Dependent on agent(s) used in the laboratory.

d For example, window, closed-circuit television, two-way communication.

Security:

The Company is located in a company-incubator building. This building has a key-card front door.

In the building, CatchMabs has its own confined office and laboratory space, which is key-card secured.

Within the facility, the fridges are inside a laboratory space which is keylocked during the night.

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Appendix B

Company Confidential Production Information

***Listeria* Phage P100 Production**

PRODUCTION MEDIUM

PRODUCTION MEDIUM

AERATION

Figure 1

End of Company Confidential Production Information

Appendix C.

Methods of Analysis Table

Report on Stability of P100 Phage after Long-Term Storage

Specification Analysis Reports of 3 Product Batches

Methods For Specification Analyses for P100 Phage

Parameter	Method number / Reference	Short Description
Total count aerobes	ANAL 10196 ISO 4833	
Yeast and mold count	ANAL 10165 ISO 7954	
<i>E. coli</i> in 1 g	CM-0746 ANAL 10247 ISO 21528-1	Enrichment in BPW, selection in E.E. broth, detection on EMB
Enterobacteria mpn <i>Salmonella</i>	ANAL 10171 Vidas	Enrichment in BPW, selection in RVS broth, enrichment in M broth, detection with VIDAS
<i>Staph. aureus</i>	CM072900 ISO 6888	
Listeria spp	ANAL10217 ISO 11290; by Rapid L mono agar (Bio Rad)	
Lead	ANAL 10014	Microwave destruction, quantification on ICP-AES
Arsenic	ANAL 10098	Microwave destruction, hydride generation with NaBH ₄ ; quantification on ICP-AES (with internal references)
Mercury	ANAL 10175	Microwave destruction, hydride generation with SnCl ₂ ; quantification with AAS at 253.7 nm

Method	Title	Application	Definition	Principle	Reporting limits (Conform NEN-7777)
ANAL-10014	Determination of cadmium, chrome, nickel and lead with ICP-AES.	This protocol describes a method for the determination of cadmium, chrome, lead, nickel and cobalt in animal feeds and feeding stuffs. The method is applicable but not accredited for other matrices.	Cadmium, chrome, lead, nickel and cobalt are elements which are present, in the matrices mentioned, by nature or as a result of pollution. These elements may have a toxic effect at higher concentrations. The amount determined in the described method will be expressed as mg/kg.	After either incineration and solution in acid or microwave (wet) destruction, samples are nebulized. The aerosol is transported to a plasma torch for excitation. Characteristic atom-line or ion-line emission spectra are produced with an inducted coupled plasma (ICP-AES).	cadmium < 0,02 mg/kg chrome < 0,5 mg/kg nickel < 0,5 mg/kg lead < 0,2 mg/kg The reporting limits are dependent on sample pretreatment and amount introduced in the test.

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ANAL-10098	Determination of arsenic with hydridegeneration and ICP-AES	This protocol describes a method for the determination of arsenic in water, animal feeds, feedingstuffs, dairy products and sludge.	Arsenic and Arsenic compound are toxic. Arsenic is found in several minerals, e.g. realgar (As ₄ S ₄), orpiment (As ₂ S ₃), arsenolite (As ₂ O ₃) arsenopyrite (FeAsS) and loallingite (FeAs ₂).	Arsenic in the sample is liberated by microwave destruction, arsenic hydride is generated with tinchloride and transported to the plasma torch. Characteristic atom-line emission spectra are produced with an inducted coupled plasma (ICP-AES).	water < 0,005 mg/L orther samples < 0,05 mg/kg
ANAL-10175	Determination of mercury (cold vapor spectrometry).	This protocol describes a method for the determination of mercury in water, animal feeds, feedingstuffs, dairy products, meat, water, additives, manures and sludge.	Mercury is a heavy metal that binds to animal tissues, in the kidneys the metal accumulates. The metal also binds to inorganic particles like sludge. The amount determined in the described method is expressed in mg/kg or mg/l.	Mercury in the sample is liberated by microwave destruction, mercury hydride is generated, lead through a cuvette and the absorbtion is measured at 253.7 nm.	< 0,01 mg/kg matrix dependent
ANAL-10196	Quantification of aerobic bacteria with plate count technique at 20, 22, 30 and 37 degrees Celcius	Horizontal method for the quantification of aerobic micro-organisms (aerobic total viable count) conforming to ISO 4833. The method is not suitable for samples known to contain antibacterial substances unless the inactivation procedure is known as well.	The aerobic total viable count (TVC) is the number of colony forming units per unit of sample developing in or on Plate Count Agar (PCA) in/at specified time and temperature. For the analysis of salted bacons and brines, PCA is supplemented with 3.5% NaCl.	When necessary suitable dilutions of the sample are made in peptone physiological salt solution. Sample aliquots are placed on or in plate count agar (PCA) with the pour-plate or spiral plate technique. After incubation, colonies are counted and the result calculated.	
ANAL-10217 000049	Determination of the presence of Listeria with UVM-broth	Presence absence test for Listeria monocytogenes in meat products, environmental samples, vegetables and fruits. Tested in 25 ml of product	See principle of the method	Listeria monocytogenes is considered present when suspect colonies, found on Rapid L mono agar after enrichment in UVM-I and UVM-II, show typical biochemical and serological reactions.	

ANAL-10165	Quantification of yeasts and molds	Horizontal method for the quantification of yeasts and molds. Discrimination between yeast and molds is possible based on differences in colony morphology. The protocol is conforming to ISO 7954.	The number of yeasts and molds is the number of colony forming units (CFU) per unit of sample developing in or on Yeast extract Glucose Chloramphenicol agar (YGC) after 4 days incubation at 25°C.	When necessary suitable dilutions of the sample are made in peptone physiological salt solution. Sample aliquods are placed on or in yeast extract chloramphenicol agar (YGC) with the pour-plate or spiral plate technique. After incubation (4 days, 25°C) colonies are counted and the result calculated.	
ANAL-10247	Determination of the presence of Enterobacteriaceae	Presence absence tests for all products and raw materials of DMV International. This protocol describes the procedure for the following analysis-codes : 0733 : 10 gr - 0734 : 750 gr - 0735 : 5 x 1 gr - 0739 : 2 x 1 gr - 0770 : 100 gr - 0771 : 4 x 1 gr - 0772 : 5 x 10 gr - 0773 : MPN.	See principle of the method	Enterobacteriaceae are considered present when after resuscitating cq enrichment in Buffered Peptone Water (BPW), overnight at 37°C, followed by selective enrichment in EE-Broth, characteristic colonies are formed on Violet Red Bile Dextrose Agar (VRBD).	
ANAL-10171	Determination of the presence of <i>Salmonella</i> spp.	Presence or absence test for all products. This protocol describes the procedure for the following analysis-codes : CM073000 Salm. in 750 gr. - CM073200 : Salm. in 50 gr. - CM078900 : Salm. in environment samples (swabs) - CM079400 Salm in 25 gr	See principle of the method	<i>Salmonella</i> is considered present when the organism is found after resuscitating cq enrichment in Buffered Peptone Water (BPW), overnight at 37°C, followed by a 24 hour selective enrichment in modified Rappaport Vassiliadis (RVS), second enrichment in M-broth, detection in the VIDAS system and confirmation according to the ISO 6579 procedures.	

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CM-0746	Determination of the presence of <i>Escherichia coli</i> .	Presence absence tests for all products and raw materials of DMV International. This protocol describes the procedure for the following analysis-codes : 0746 : 10 gr - 0747 : MPN - 0750 : 5x1 gr - 0754 : 50 gr - 0759 : 4 x 1 gr - 0763 : 750 gr - 0787 : 2 x 1 gr . - 0807 : 1 gr.	See principle of the method	<i>E. coli</i> is considered present when after resuscitating cq enrichment in Buffered Peptone Water (BPW), overnight at 37°C, followed by selective enrichment in EE-Broth, characteristic colonies are formed on Eosine Methylene Blue Agar.	
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Stability of Listeria bacteriophage P100

In this report data is presented on the stability of Listeria bacteriophage P100. A batch of phages is produced in *L.innocua* 2627, purification is done by PEG6000 precipitation and the phages are stored at 4°C in 1xPBS pH 7.4 and the batch contains 6×10^{10} pfu/ml. During storage time the batch is used as a positive control for bacteria condition and plaque formation in phage titration.

Data retrieved from titration experiments are listed in table 1

Table 1: phage titers (PFU/ml)

14/jul/2004	6.0E+10
05/oct/2004	5.4E+10
15/nov/2004	7.4E+10
12/jan/2005	5.8E+10
16/sept/2005	6.9E+10
11/oct/2005	6.4E+10
13/oct/2005	6.6E+10
20/oct/2005	6.6E+10
24/oct/2005	6.5E+10
27/oct/2005	8.0E+10
1/nov/2005	7.0E+10
7/nov/2005	6.7E+10
15/nov/2005	6.9E+10
29/nov/2005	6.8E+10
12/dec/2005	5.6E+10
15/dec/2005	7.3E+10
27/jan/2006	7.0E+10

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N.C.B.-laan 52 • 5462 GE Veghel
Postbus 107 • 5460 AC Veghel
(0413) 382633 • (0413) 392141
ccl-research@ccl.nl

Report 1000907



Filling of packages Listextm P100 for use in the food industry, batch 6001.

Veghel, 28-02-2006

Nutricontrol en CCL Research zijn onderdeel van CCL BV. Op alle diensten en leveringen zijn algemene voorwaarden van toepassing. Deze zijn gedeponeerd bij de KvK te Eindhoven. Het onderzoek is uitgevoerd in de periode tussen de datum van ontvangst monster op CCL BV en datum van rapportage. De met (D) gemerkte analyses zijn erkend door STERLAB. De geschatte onzekerheid van de vermelde resultaten is opvraagbaar. Dit rapport mag niet anders dan in zijn geheel worden gepubliceerd zonder toestemming van CCL BV. Niet elke analyse is geaccrediteerd. In het accreditatiecertificaat L 053 is beschreven welke analyses geaccrediteerd zijn. Dit certificaat is opvraagbaar.

BTW nr. NL80335054B01. Handelsregister Eindhoven 17120532, NL Rabobank Utrecht 19 23.12 895, D: ABN-AMRO Frankfurt BIZ 50230400 KoNr 1433505/001

000053

Project name : Filling of packages Listex™ P100 for use in the food
Industry, bath 6001.
Project number : CCL-311-03-V-00
Principal : EBI Food Safety
Account manager : Gerard Scheiberlich
Group CCL Research: Food Safety en Quality Systems
Project manager : Peter Enthoven
Project team : Peter Enthoven, Gerard Scheiberlich, Robert van Kaathoven
Version and date : 23-02-2006
Status : final

Agreed by project manager CCL Research

Name: P. Enthoven

Date:

Signature:

Agreed by reviewer CCL Research

Name: R. Margry

Date:

Signature:

Agreed by account manager

Name: G. Scheiberlich

Date:

Signature:

Veghel, 28 February 2006

Mailing list:

EBI Food Safety

Author: R. van Kaathoven

1. Introduction

EBI Food Safety produces, in Wageningen, bacteriophages for use in the food industry. Furthermore, EBI Food Safety conducts research in several countries for customers with the goal to establish proof of concept and proof of application of their products. EBI Food Safety is looking for a partner to fill user packages under validated conditions and to determine the chemical and microbiological characteristics of their product (Listextm P100) for use on the product specification sheet.

1.1 Goal

Filling of Listextm P100 Bacteriophage from production volumes into user packages for the food industry under validated conditions.

2. Material and methods

Filling of Listextm P100 Bacteriophage from production volumes (3 litre, batch 100-041, delivered 10-01-2006) into Nalgene® bottles, 125 ml, gamma sterile (lot 536126) supplied by EBI Food Safety was performed in a sealed safety cabinet (labconco®, chemical en carcinogen Gloveboxtm).

Using a sterile dosing pump, 27 bottles were filled with 100 ml Listextm. The product was labelled "Batch nr: 6001, Exp. date: 12-07-2006". packaged in material supplied by EBI Food Safety, and shipped on 16-01-2006 using the services of Fiege BV (SGS reference number 628,746)

To validate the filling process, contact prints were made of the disinfected (ethanol 70%) working area in the safety cabinet. (1 x sluice, 3 x worktable, 2 x rear wall, 2 x side wall, 2 x gloves) the contact prints were incubated for 44 hours at 30 °C ± 1 °C. The exterior of the materials that were introduced in the safety cabinet were disinfected using ethanol 70 %.

During the filling process, the ambient microbiological load was determined using collection plates according to protocol 10109.

Entry checks: batch 100-041 was analysed for aerobic counts (Anal-10196 Q), yeasts and moulds (Anal-10165 Q) and *Listeria monocytogenes* (Anal-10217).

Entry monitoring:

Batch: 6001 was analysed in duplicate for aerobic counts (Anal-10196 Q), yeasts and moulds (Anal-10165 Q), *Listeria* spp (Anal-10217) and single for Enterobacteriaceae (Anal-10247), *Salmonella* spp (Anal-10171), *Escherichia coli* (method-080700), *Staphylococcus aureus* (method-072900), Lead (Anal-10014), Arsenic (Anal-10098), Mercury (Anal-10175) and Sample Digestion.

Exit monitoring: batch 100-041 was analysed in duplicate for aerobic counts at 30° (Anal-10196 Q)

3. Results

Quality Insurance Hygiene

	Result	Criteria	Conclusion
Rodac contact prints, 9 samples	< 1	< 10	Within specifications
Ambient microbial load, protocol-10109	< 1	< 10	Within specifications

Entry check batch: 100-041

CL20060110.454
See: report ccl-2006003228-V01

	Result	Unit	Criteria	Conclusion
Aerobic 30°C Anal-10196 Q	< 1	cfu/ml	< 1 cfu/ml	Within specifications
Yeasts and moulds Anal-10165 Q	< 1	cfu/ml	< 1 cfu/ml	Within specifications
Listeria spp Anal-10217	n.p.	25 g	Not present	Within specifications

See report CCI2006008159-V01

Analysis	Result	Unit	Criteria	Conclusion
Aerobic 30°C Anal-10196 Q	< 1	cfu/ml	< 1 cfu/ml	Within specifications
Yeast and Moulds	< 1	cfu/ml	< 1 cfu/ml	Within specifications
E. coli method 080700	n.p.	1 g	n.p.	Within specifications
Enterococcus mpn Anal-10247	< 1	cfu/ml	< 1 cfu/ml	Within specifications
Salmonella Anal-10171	n.p.	25 ml	n.p.	Within specifications
S. aureus method 072900	n.p.	1 g	n.p.	Within specifications
Listeria spp. Anal-10217	n.p.	25 ml	n.p.	Within specifications
Lead Anal-10014	< 0,2	mg/kg	< 1 mg/kg	Within specifications
Arsenic Anal-10098	< 0,05	mg/kg	< 1 mg/kg	Within specifications
Mercury Anal-10175	< 0,01	mg/kg	< 0,5 mg/kg	Within specifications

n.p. = not present

Exit check packaged product batch: 6001

See: report ccl-2006003229-V02 and 2006003230-V02

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Sample nr	Analysis	Result	Unit	Criteria	Conclusion
CL200601112.425	Aerobic 30°C Anal-10196 Q	< 1	cfu/ml	< 1 cfu/ml	Within specifications
CL200601112.426	Aerobic 30°C Anal-10196 Q	< 1	cfu/ml	< 1 cfu/ml	Within specifications

4. Remarks

The criteria used are provisional and can be adjusted based on further experience. Based on the present results it can be concluded that no contamination has occurred during filling.

5. References

Report: CCL2006003228-V01
Report: CCL2006003230-V02

Report: CCL2006003229-V02
Report CCL2006008159-V01

N.C.B.-laan 52 • 5462 GE Veghel
Postbus 107 • 5460 AC Veghel
(0413) 382633 • (0413) 382141
ccl-research@ccl.nl



Report 1000943

Project code: CCL- 331-03-V-00

Filling of packages Listextm P100 for use in the food industry, batch 6002/6003.

Veghel, 01 March 2006

000057

Project name : Filling of packages Listex™ P100 for use in the food industry, batch 6002/6003.
Project number : CCL-311-03-V-00
Principal : EBI Food Safety
Account manager : Gerard Scheiberlich
Group CCL Research: Food Safety en Quality Systems
Project manager : Peter Enthoven
Project team : Peter Enthoven, Gerard Scheiberlich, Robert van Kaathoven
Version and date : 23-2-2006
Status : final

Agreed by project manager CCL Research

Name: P. Enthoven

Date:

Signature:

Agreed by reviewer CCL Research

Name: R. Margry

Date:

Signature:

Agreed by account manager

Name: G. Scheiberlich

Date:

Signature:

Veghel, 01 March 2006

Mailing list:

EBI Food Safety

Author: R. van Kaathoven

000058

1. Introduction

EBI Food Safety produces, in Wageningen, bacteriophages for use in the food industry. Furthermore, EBI Food Safety conducts research in several countries for customers with the goal to establish proof of concept and proof of application of their products. EBI Food Safety is looking for a partner to fill user packages under validated conditions and to determine the chemical and microbiological characteristics of their product (Listextm P100) for use on the product specification sheet.

1.1 Goal

Filling of Listextm P100 Bacteriophage from production volumes into user packages for the food industry under validated conditions.

2. Material and methods

Filling of Listextm P100 Bacteriophage from production packages (1.6 litre, batch 1045, delivered 20-01-2006) into Nalgene® bottles, 120 ml, gamma sterile (lot 536126) supplied by EBI Food Safety was performed in a sealed safety cabinet (labconco®, chemical en carcinogen Gloveboxtm).

Using a sterile dosing pump, bottles were filled with 120 ml Listextm. The product was labelled "Batch nr: 6002, Exp. date: 19-07-2006". packaged in material supplied by EBI Food Safety.

Batch 6002 is changed in batch 6003 at 02-02-2006 (see mail send by Marc Schellekens, 01-02-2006 11:13 AM)

To validate the filling process, contact prints were made of the disinfected (ethanol 70%) working area in the safety cabinet. (1 x sluice, 3 x worktable, 2 x rear wall, 2 x side wall, 2 x gloves) the contact prints were incubated for 44 hours at 30 °C ± 1 °C. The exterior of the materials that were introduced in the safety cabinet were disinfected using ethanol 70 %.

During the filling process, the ambient microbiological load was determined using collection plates according to protocol 10109.

Exit monitoring: batch:6002/6003 was analysed for aerobic counts (Anal-10196 Q), yeasts and moulds (Anal-10165 Q), Listeria spp. (Anal-10217), Enterobacteriaceae (Anal-10247), Salmonella spp (Anal-10171), Escherichia coli (method-080700), Staphylococcus aureus (method-072900), Lead (Anal-10014), Arsenic (Anal-10098), Mercury (Anal-10175) and Sample Digestion.

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3. Results

Quality Insurance Hygiene

	Result	Criteria	Conclusion
Rodac contact prints, 9 samples	< 1	< 10	<i>Within specifications</i>
Ambient microbial load, protocol-10109	3	< 10	<i>Within specifications</i>

Exit check packaged product batch: 6002/6003

Sample nr cl20060125.132

Analysis	Result	Unit	Criteria	Conclusion
Aerobic 30°C Anal-10196 Q	< 1	cfu/ml	< 1 cfu/ml	<i>Within specifications</i>
Yeast and Moulds	< 1	cfu/ml	< 1 cfu/ml	<i>Within specifications</i>
E. coli method 080700	n.p.	1 g	n.p.	<i>Within specifications</i>
Entero mpn Anal-10247	< 1	cfu/ml	<1 cfu/ml	<i>Within specifications</i>
Salmonella Anal-10171	n.p.	25 ml	n.p.	<i>Within specifications</i>
S. aureus method 072900	n.p.	1 g	n.p.	<i>Within specifications</i>
Listeria spp. Anal-10217	n.p.	25 ml	n.p.	<i>Within specifications</i>
Lead Anal-10014	< 0,2	mg/kg	< 1 mg/kg	<i>Within specifications</i>
Arsenic Anal-10098	< 0,05	mg/kg	< 1 mg/kg	<i>Within specifications</i>
Mercury Anal-10175	< 0,01	mg/kg	< 0,5 mg/kg	<i>Within specifications</i>

n.p. means not present

4. Remarks

The criteria used are provisional and can be adjusted based on further experience. Based on the present results it can be concluded that no contamination has occurred during filling.

5. References

Report: CCL-2006012471-V01

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N.C.B.-laan 52 • 6462 GE Veghel
Postbus 107 • 5460 AC Veghel
(0413) 382633 • (0413) 392141
ccl-research@ccl.nl



Report 1000944

**Filling of packages Listex™ P100 for use in the food
industry, batch 6004.**

Veghel, 28-02-2006

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Nutricontrol en CCL Research zijn onderdeel van CCL BV. Op alle diensten en leveringen zijn algemene voorwaarden van toepassing. Deze zijn gedeponneerd bij de KvK te Eindhoven. Het onderzoek is uitgevoerd in de periode tussen de datum van ontvangst monster op CCL BV en datum van rapportage. De met (Q) gemerkte analyses zijn erkend door STERLAB. De geschatte onzekerheid van de vermeldde resultaten is opvraagbaar. Dit rapport mag niet anders dan in zijn geheel worden gecopieerd zonder toestemming van CCL BV. Niet elke analyse is geaccrediteerd. In het accreditatiecertificaat L 053 is beschreven welke analyses geaccrediteerd zijn. Dit certificaat is opvraagbaar.

BTW nr. NL806335094B01, Handelsregister Eindhoven 17120632, NL: Rabobank Utrecht 19 23 12 085, D, ABN-AMRO Frankfurt Blz 50230400 KoNr 1433595/001

Project name : Filling of packages Listextm P100 for use in the food
Industry, bath 6004.
Project number : CCL-311-03-V-00
Principal : EBI Food Safety
Account manager : Gerard Scheiberlich
Group CCL Research: Food Safety en Quality Systems
Project manager : Peter Enthoven
Project team : Peter Enthoven, Gerard Scheiberlich, Robert van Kaathoven
Version and date : 24-02-2006
Status : final

Agreed by project manager CCL Research	Agreed by reviewer CCL Research
Name: P. Enthoven	Name: R. Margry
Date:	Date:
Signature:	Signature:
Agreed by account manager	
Name: G. Scheiberlich	
Date:	
Signature:	

Veghel, 28 February 2006

Mailing list:
EBI Food Safety

Author: R. van Kaathoven

000062

1. Introduction

EBI Food Safety produces, in Wageningen, bacteriophages for use in the food industry. Furthermore, EBI Food Safety conducts research in several countries for customers with the goal to establish proof of concept and proof of application of their products. EBI Food Safety is looking for a partner to fill user packages under validated conditions and to determine the chemical and microbiological characteristics of their product (Listex™ P100) for use on the product specification sheet.

1.1 Goal

Filling of Listex™ P100 Bacteriophage from production volumes into user packages for the food industry under validated conditions.

2. Material and methods

Filling of Listex™ P100 Bacteriophage from production packages (0.85 litre, batch 1047, delivered 01-02-2006) into Nalgene® bottles, gamma sterile (lot 536126) supplied by EBI Food Safety was performed in a sealed safety cabinet (labconco®, chemical en carcinogen Glovebox™).

Using a sterile dosing pump, 20 bottles were filled with 42,5 ml Listex™ and is diluted with saline solution to 100ml. The product was labelled "Batch nr: 6004, Exp. date: 27-07-2006". packaged in material supplied by EBI Food Safety.

To validate the filling process, contact prints were made of the disinfected (ethanol 70%) working area in the safety cabinet. (1 x sluice, 3 x worktable, 2 x rear wall, 2 x side wall, 2 x gloves) the contact prints were incubated for 44 hours at 30 °C ± 1 °C. The exterior of the materials that were introduced in the safety cabinet were disinfected using ethanol 70 %.

During the filling process, the ambient microbiological load was determined using collection plates according to protocol 10109.

Exit checks:

Batch: 6004 was analysed in duplicate for aerobic counts at 30° (Anal-10196 Q) and was analysed for aerobic counts (Anal-10196 Q), yeasts and moulds (Anal-10165 Q), Listeria spp (Anal-10217), Enterobacteriaceae (Anal-10247), Salmonella spp (Anal-10171), Escherichia coli (method-080700), Staphylococcus aureus (method-072900), Lead (Anal-10014), Arsenic (Anal-10098), Mercury (Anal-10175) and Sample Digestion.

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3. Results

Quality Insurance Hygiene

	Result	Criteria	Conclusion
Rodac contact prints, 9 samples	< 1	< 10	<i>Within specifications</i>
Ambient microbial load, protocol-10109	< 1	< 10	<i>Within specifications</i>

Exit check packaged product batch: 6004

Sample:20060203.401

Analysis	Result	Unit	Criteria	Conclusion
Aerobic 30°C Anal-10196 Q	< 1	cfu/ml	< 1 cfu/ml	<i>Within specifications</i>
Yeast and Moulds	< 1	cfu/ml	< 1 cfu/ml	<i>Within specifications</i>
E. coli method 080700	n.p.	1 g	n.p.	<i>Within specifications</i>
Entero mpn Anal-10247	< 1	cfu/ml	< 1 cfu/ml	<i>Within specifications</i>
Salmonella Anal-10171	n.p.	25 ml	n.p.	<i>Within specifications</i>
S. aureus method 072900	n.p.	1 g	n.p.	<i>Within specifications</i>
Listeria spp. Anal-10217	n.p.	25 ml	n.p.	<i>Within specifications</i>
Lead Anal-10014	< 0,2	mg/kg	< 1 mg/kg	<i>Within specifications</i>
Arsenic Anal-10098	< 0,05	mg/kg	< 1 mg/kg	<i>Within specifications</i>
Mercury Anal-10175	< 0,01	mg/kg	< 0,5 mg/kg	<i>Within specifications</i>

n.p. means not present

Sample nr	Analysis	Result	Unit	Criteria	Conclusion
CL20060203.256	Aerobic 30°C Anal-10196 Q	< 1	cfu/ml	< 1 cfu/ml	<i>Within specifications</i>
CL20060203.257	Aerobic 30°C Anal-10196 Q	< 1	cfu/ml	< 1 cfu/ml	<i>Within specifications</i>

4. Remarks

The criteria used are provisional and can be adjusted based on further experience. Based on the present results it can be concluded that no contamination has occurred during filling.

5. References

Report CCL-2006012472-V01
 Report CCL-2006010708-V01
 Report CCL-2006010707-V01

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