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Specializing in FDA Regulatory Matters

December 21, 2006

REC'D DEC 21 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 for Use in Foods, Generally.

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 use in foods, generally, to control *Listeria monocytogenes*.

Please let me know if you have any questions.

Sincerely,

Edward A. Steele
President

Enclosures



REC'D DEC 21 2006

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 12/21/06

Agent for:

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

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LISTEX™ P100 Bacteriophage

B. Name and Address of Notifier

Edward A. Steele
President

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1940 Duke Street, Suite 200
Alexandria, VA 22314

(877) 327 - 9808 Toll Free

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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 foods, generally, to control *Listeria monocytogenes* when added in the range from 1×10^7 to 1×10^9 pfu per gram of food.

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 at EBI Food Safety B.V. (address below) and will be sent to the Food and Drug Administration upon request.

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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.

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.

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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LISTEX™ P100 Bacteriophage

Specifications for P100 Bacteriophage

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

Physical Properties	
Description	Suspension of broad-spectrum phage preparation, formulated in propylene glycol.
Source	Fermentation derived
pH	6.0-7.0
Specific gravity	1.05 g/ml (+/- 0.5%)
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 foods that are susceptible to *Listeria monocytogenes*. The purpose of P100 addition is to reduce or eliminate *Listeria monocytogenes* in the finished product.

The use of the product and potential intake would be self limiting by two factors. First, the 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. P100 phage has no effect on spoilage bacteria or any ongoing effect on the food after packaging. Secondly, after the host bacteria *Listeria monocytogenes* is depleted on the food, the P100 phage would no longer replicate and would gradually die back in viable numbers and degrade.

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

EBI Food Safety has determined that the use of P100 on food is generally recognized as safe (GRAS) based on scientific procedures. Previously, 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 GRAS status of bacteriophage P100 (product name LISTEX™ P100) intended for use in cheese to control *Listeria monocytogenes*. (See GRN 000198) A comprehensive search of the scientific literature was also utilized for this review.

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LISTEX™ P100 Bacteriophage

Based on a critical evaluation of the publicly available data and information summarized in the document attached, we conclude that bacteriophage P100, meeting the specifications cited above, is generally recognized as safe (GRAS) by scientific procedures when used as an antimicrobial ingredient at levels consistent with current good manufacturing practices. In coming to this conclusion, EBI Food Safety relied on the information considered by the earlier Expert Panel, including an *in silico* assessment of the complete genome and gene products of P100 for allergenicity, pathogenicity or virulence, and published toxicology studies and other articles relating to the safety of lytic bacteriophage.. It is our position that other qualified and competent scientists, reviewing the same publicly available toxicological and safety information, would reach the same conclusion.

Attachments:

Basis for GRAS determination including reference citations

Appendix A. Production and Quality Assurance Procedures

Appendix B. Company Confidential Production Information

Appendix C: Methods of Analysis Table and Report on Stability of P100 Phage after Long-Term Storage

Appendix D. Prepublication Article by Prof. Martin J. Loessner (Confidential)

Appendix E. PowerPoint Slides Showing Efficacy (Confidential)

Appendix F. Persistence and inactivation of bacteriophages in the environment.

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

The discussion below is based on a previous evaluation by 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. It 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* and submitted with GRN 000198. As part of this determination, the original panel's conclusion document is incorporated by reference. This document provides the rationale for concluding that bacteriophage P100 is GRAS for use in foods generally, as a processing aid for those foods that are susceptible to *L. monocytogenes*.

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 all related to susceptible foods such as soft cheeses, processed meat, poultry, and vegetables. While *Listeria* is very sensitive to heating, it is capable of growth over a wide pH and salt range and is able to grow at refrigeration temperatures. Thus circumstances usually able to prevent outgrowth of microorganisms during storage have little effect on *L. monocytogenes*. 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 susceptible products such as milk and cheeses (mostly soft cheese), cold-cuts (different types of meats and poultry, or meat and poultry products), sausages such as hot-dogs, smoked fish and other 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, and have been subjected 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 are 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

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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 occurs naturally. With respect to their potential application for the biocontrol of undesired pathogens in foods, feeds, and related environments, 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). This is not true of strictly lytic phages, however.

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 effluence. (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 known to be non-pathogenic.

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.

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.*, in preparation 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).

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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, is 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.

Table 1. Product Specifications of Listex™ P100	
Physical Properties	
Description	Suspension of broad-spectrum phage preparation, formulated in propylene glycol.
Source	Fermentation derived
Phage concentration	2x 10 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 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 Food Safety propagates bacteriophages under GLP guidelines. HACCP and Halal certification is expected in July 2007.

Listeria innocua is used as a host strain for the production of P100 phages. *Listeria innocua* is a non-pathogenic bacterial strain. *Listeria* cells are cultured to a certain density in single use, closed, food grade plastic bioprocess containers followed by an infection with the lytic P100 phages. Further incubation allows for the amplification of phages. 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.

USE OF BACTERIOPHAGE P100

The use of P100 that is the subject of this GRAS determination is as an antimicrobial ingredient for addition to susceptible products, generally. While phage P100 effectively eliminates *L. monocytogenes*, it does not affect any other bacteria. Thus, flora that serve as an indicator for shelf life, such as lactic acid bacteria, are not affected and there is no shelf-life prolonging effect as would be the case with antibiotics and other antimicrobials. There is usually only one application necessary to achieve the desired antimicrobial effect before the products are packaged and stored. The purpose of P100 addition to the surface when risk of contamination is highest is to reduce or eliminate *Listeria monocytogenes* in the finished 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 food products.

Appendices D-F demonstrate efficacy on a variety of products as well as duration of activity for P-100. These documents are confidential at this time and are not pivotal to the determination of safety. Appendix D is a prepublication document provided by Professor Martin Loessner, from ETH Zurich, demonstrating the efficacy of phage, and P100 in particular, on a wide selection of food categories. Because publication depends on it not being made public earlier, it should be considered confidential until published.

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 doses in other foods would depend on surface area but would be of the same order of magnitude.

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 susceptible products, including ready-to-eat (RTE) products, a single application of phages to the surface will suffice for the desired antimicrobial effect. Excessive use would provide no advantage and would be precluded for economic reasons. 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 product would be ingested by the consumer.

For the reasons discussed below, under Safety Studies and Safety Assessment, the precise exposure to P100 is not important to the safety assessment. P100 shows no potential for acute or chronic toxicity and can be treated like any other common protein and nucleic acid. There is no basis for setting an upper level for safety as is done with an acceptable daily intake. Thus, as with other safe sources of protein and nucleic acids, such as meat, the common EDI/ADI safety assessment model is not applicable for this product.

SAFETY STUDIES

Subacute Toxicity Study

The subacute toxicity study was conducted to assess potential for 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 body weight 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. MB Research Laboratories' study director 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,

attachement 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

In the environment, phages are widely distributed; several estimates suggest that their total number on this planet (all environmental niches together) exceed 10^{31} virus particles (Chibani-Chennoufi *et al.*, 2004c). 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 some 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

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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; Cervený, *et al.*, 2002; Chibany-Chenouffi, 2004b; Merrill *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 susceptible and RTE products with phage P100 is not likely to cause harm to humans who consume such food 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 product several days after being applied at the time of production 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.

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Appendix A - Production and Quality Assurance Procedures

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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. Listeria cells are cultured to a certain density in single use, closed, food grade plastic bioprocess containers followed by an infection with the lytic P100 phages. Further incubation allows for the amplification of phages

DOWN STREAM PROCESSING PROCEDURE

Cell debris is removed by a cross flow filtration device containing food-grade filters and stainless steel and/or food grade disposable tubing. In a further step, the filtrate is concentrated by a cross flow filtration device containing food-grade filters and stainless steel and/or food grade disposable tubing. In the final step, the concentrate is filter sterilized by treatment of the phage-containing fluid by a 0.2 µm sterilization filter. This assures that there are no living host organisms in the final product.

LIQUID CULTURE MEDIA

Ingredients:

Peptone
Yeast extract
Hepes acid free (buffer)
Hydrochloric acid or sodium hydroxide

FINAL PRODUCT

Ingredients:

Content: 2×10^{11} plaque forming units per ml
product in phosphate buffered saline

RECOMMENDED PRODUCT STORAGE

Refrigerated at 2-8°C

PRODUCT STABILITY

Labelled for 6 months at recommended
storage temperature



STORAGE OF BACTERIA AND PHAGES

The bacterial stocks are stored at -80°C in a Sanyo MDF-U72V Freezer.

The phage stocks are stored at 4°C in a Liebherr KB4250, which is located in a phage dedicated lab.

The final Listex P100 product is stored at 4°C in a Liebherr KB4250, which is located in a phage dedicated lab.

QUALITY CONTROL

The phage containing solution that is filter sterilized after concentration is stored in sterile containers. 30 ml from the concentrated phage solution is sent to CCL Nutricontrol (The Netherlands) and checked on *Listeria* spp., aerobe and anaerobe bacteria, yeasts and molds. The amount of plaque forming units is determined in house by using an in house developed standard operation procedure. After release (no vegetative organisms found) the phage containing solution is diluted to product specifications using sterile PBS (pH 7) and dispensed in 100 ml units. From the dispensed units 10 ml samples are collected during the dispensing and sent in to CCL Nutricontrol for analyses on aerobe and anaerobe bacteria, yeasts and molds.

Released (no vegetative organisms found) can be shipped to customers.

From all produced batches detailed production batch records are stored together with the quality controls performed by CCL Nutricontrol.

SECURITY

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

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

Within the facility, the refrigerators are inside a laboratory space which is key locked during the night.

Appendix B - Company Confidential Production Information

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Appendix C - Methods of Analysis Table and Report on Stability
of P100 Phage after Long-Term Storage

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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	Enrichment in BPW, selection in E.E. broth, detection on EMB
Enterobacteria mpn	ANAL 10247 ISO 21528-1	
<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	
<i>Listeria</i> 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 NaBH4 ; quantification on ICP-AES (with internal references)
Mercury	ANAL 10175	Microwave destruction, hydride generation with SnCl2 ; 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 Celsius	Horizontal method for the quantification of aerobic microorganisms (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	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.	

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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 chlooramphenicol 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 4⁰C in 1xPBS pH 7.4 and the batch contains 6x10¹⁰ 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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Appendix D - Prepublication Article by Prof. Martin J.
Loessner (Confidential)

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Appendix E - PowerPoint Slides Showing Efficacy
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Appendix F - Persistence and Inactivation of Bacteriophages
in the Environment.

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Persistence and inactivation of bacteriophages in the environment and possible consequences for application in foods

The purpose of Listex-P100 application is the eradication of contamination with *Listeria monocytogenes* during food processing. When an infective phage particle encounters a susceptible host bacterium, the encounter will eventually result in the death of the bacterium. In order to achieve this purpose on a food surface, a critical number of phages which ensures the likelihood of the phage-host encounter has to be applied at a specifically selected point in time. It is clear that the highest level of efficacy is obtained before phages become gradually inactivated, and this obviously is shortly after application/addition of phages.

Once the number of infective phages drops below a critical value due to inactivation the efficacy of this type of processing aid is no longer maintained. A summary of the various inactivation factors and their relevance in a food environment is given further below.

Reaching and maintaining the critical number of phages is essential because of the spatial distributions on the sub-microscopic scale as illustrated in Figure 1.

As an example, it is assumed that a 100 cm² area is treated with 10⁷ PFU/cm² P100. Approximately 100 *Listeria* cells present (a likely contamination scenario) are now infected by phages and each produces 50 progeny phages. This would result in a net increase of 49 phages per infection and a total increase of 4900. This would constitute a total increase of approximately 0.0005% of phages present, and can be considered negligible.

Numerous environmental factors can contribute to inactivation of functional phages. Among these are: adsorption of phages to particles, proteolytic degradation of the phage virion by chemicals and enzymes, temperature, salts and also light which damages the DNA (Suttle and Chen 1992; Garza and Suttle 1998; Hurst *et al.*, 1980).

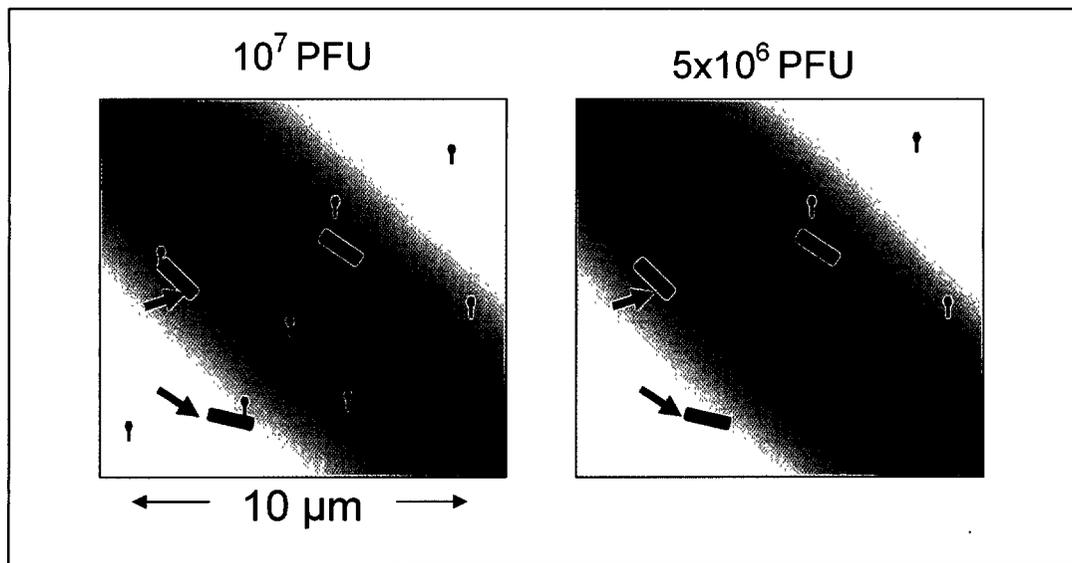


Figure 1. The figure shows possible distributions of *Listeria* cells and phages on 1 millionth of a square centimeter, with two phage concentrations differing only by a factor 2 (drawn to scale indicated). The red phage represents a phage already inactivated by adsorption to a particle. The two bacterial cells indicated by arrows face very different situations in the two panels. Those in the right panel will likely be able to replicate, leading to outgrowth while those in the left panel are likely to encounter an active phage and thus be eradicated. Since bacterial growth is not linear but exponential the drop in phage numbers below the critical value results in exponentially diminished efficacy over time. The progeny phages released from cells infected in or on the food will not significantly contribute to the number already present.

The decay in infectivity rates of phages infecting cyanobacteria in seawater are typically measured at around $1\% \text{ h}^{-1}$ (Noble and Fuhrman 1997), which indicates a rather rapid loss of infectivity. In marine and soil environments, adsorption to particles constitutes a major factor inactivating phages (Suttle and Chen 1992; Garza and Suttle 1998; Hurst *et al.*, 1980). While the phages remain structurally intact they are no longer physically able to interact with their host bacteria. This phenomenon will also occur in foods, regardless of its nature.

Enzymes from the microbial flora in soil also contribute to more rapid inactivation of phages (Nasser *et al.*, 2002). In fermented foods, proteolytic enzyme levels may be especially high and even non-fermented foods of animal or plant origin may contain enzymes from the organism as well as organic acids and other inhibitory agents.

Other acids and chemicals may be present due to production techniques inherent to certain foods. Whatever the individual contributions of the various mechanisms in any particular food, the number of active phages will constantly decline from the moment of application. While absorption to particles will constitute a relatively similar drain in two closely related products, variations in the microbial flora found in two different cheese factories will result in different

enzymes and different enzyme levels. This makes any prediction on the various speeds of inactivation almost impossible and will differ from case to case.

In summary it can be concluded that active phage numbers will decline from the moment of application. This necessitates achieving the critical dose before packaging. Increasing this dosage would not make sense because:

a) It would increase cost of application.

b) *Listeria* is an opportunistic pathogen and not a spoilage-associated bacterium, and Listex P100 is not designed to help in extending shelf-life in any way.

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Specializing in FDA Regulatory Matters

December 22, 2006

DEC 26 2006

Robert L. Martin, PhD.
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

Dear Dr. Martin:

I am providing you confirmation that the GRAS Notification of Bacteriophage P100 submitted on December 21, 2006 on behalf of EBI Food Safety is for use in foods, generally, including meat and poultry products. Therefore, at your request an additional copy of the notice is enclosed for review by the US Department of Agriculture.

Please let me know if you need any additional information.

Sincerely,

Edward A. Steele
President

Enclosure

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SUBMISSION END

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Specializing in FDA Regulatory Matters

Via email: Robert.merker@fda.hhs.gov and Mail

May 26, 2007

Robert Merker, Ph.D.
Division of Biotechnology and GRAS Notification HFS-255
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: GRN 000218

Dear Dr. Merker:

You will find enclosed a report and data sheets from organoleptic testing of bacteriophage P 100. This information was requested by Bill Jones and Jeff Canavan of USDA/FSIS to document the suitability of this agent for use on meat and poultry. As shown in the report, no difference could be determined between meat or poultry treated with a suspension of this bacteriophage and that treated with plain water. We are also sending a copy directly to USDA/FSIS to expedite their review.

Please let me know if you need any additional information.

Very Truly, /

(b)(6)

Edward A. Steele
President

Enclosures

Agent for:

EBI Food Safety B.V.
Johan v. Oldenbarneveltlaan 9
2582 NE Den Haag
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EBI FOOD SAFETY

Sensory perception of LISTEX™ treated RTE meat products

Introduction

This experiment was conducted in order to determine the influence of LISTEX™ on the organoleptic properties of ready to eat (RTE) meat products. Influence on taste, appearance and smell were determined in a blind test with ten subjects on three different RTE meat products: roasted chicken breast, cooked ham and salami.

Material and Methods

Pre-cut Roasted chicken breast, cooked ham and salami slices with a thickness of 2-3 mm were purchased at a local retailer (for product details please see Fig. 1).

For the experiments slices were treated with $1\mu\text{l}/\text{cm}^2$ of undiluted LISTEX™ and $1\mu\text{l}/\text{cm}^2$ of tap water respectively (Dutch tap water is not chlorinated).

This dosage represents double the amount of LISTEX™ that would be deposited on slices at the highest envisaged treatment dose. Slices were placed on plastic plates marked A and B and test subjects were asked to indicate which product they preferred and why, starting with appearance followed by tasting and olfactory impression (please see Fig.2). Use of the letters A and B on plates for the samples was randomized per foodstuff and sensory category i.e. all treated grilled chicken-samples for taste perception were on plates designated A, while treated salami samples were on plates designated B, and treated chicken samples for smelling were on plates designated B, etc (see Appendix I).

A total of ten people sampled the foodstuffs. 5 test persons were EBI Food Safety employees, the other five volunteers were employees of different companies working in the same building.

For the taste experiment each subject was asked to eat a small piece of white bread, followed by a sip of water prior to tasting each of the samples. The order of sampling was: chicken breast, ham and salami.



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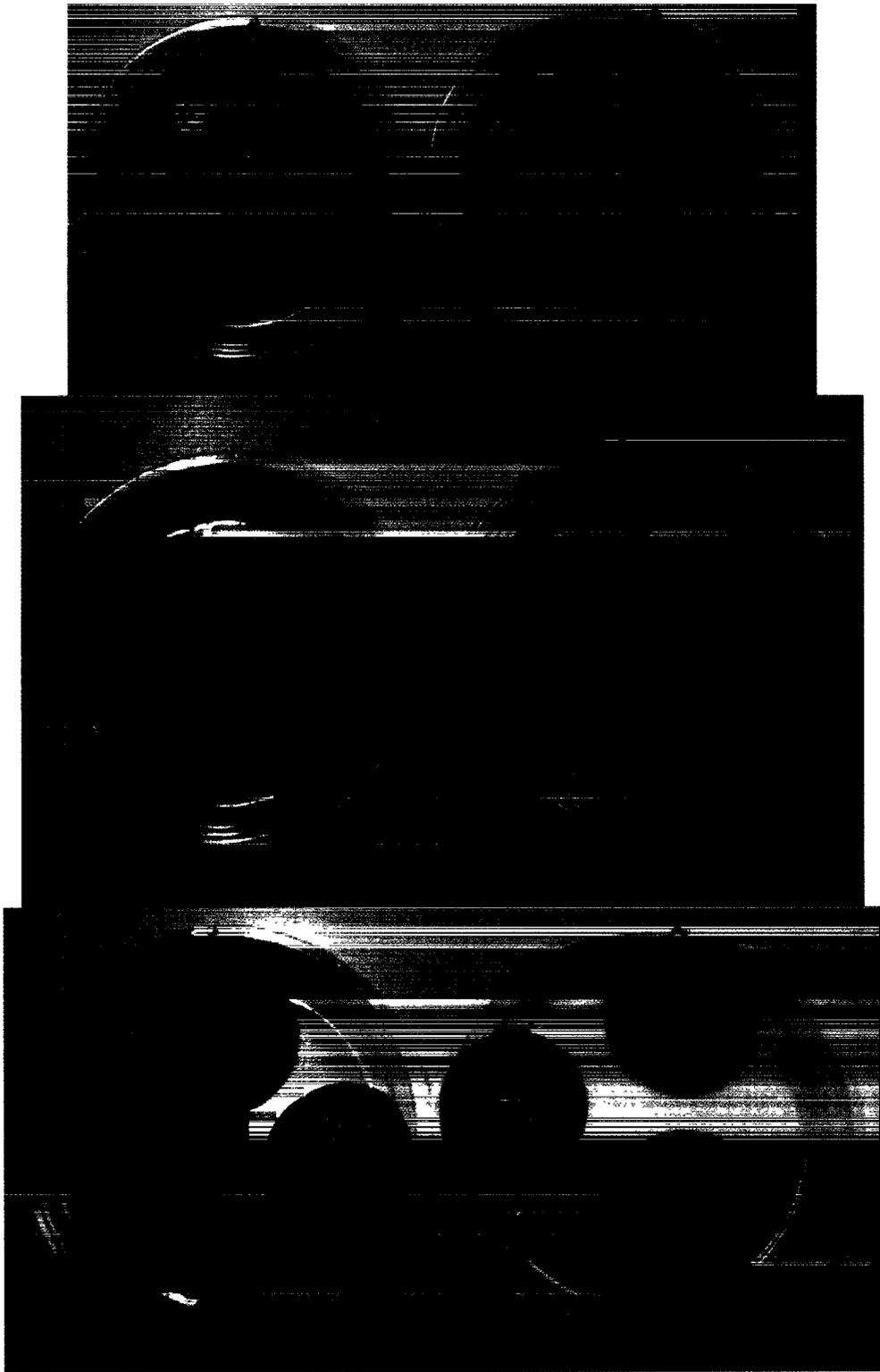


Fig 2. Samples as used in sensory perception study.



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Results and discussion

The test subjects' responses for all categories are depicted in Table 1. The data obtained from ten people does not lend itself for exhaustive statistical analysis, nonetheless it is deemed sufficient to prove that treatment of RTE meats with LISTEX™ has no effect on the organoleptic properties of the foods.

Table 1: Preferences of the test subjects per food category and sensory category

	Preference for Treated	Preference for Untreated	Preference for Neither
Ham			
Appearance	3	2	5
Taste	2	2	6
Smell	1	3	6
Chicken			
Appearance	3	0	7
Taste	2	3	5
Smell	0	1	9
Salami			
Appearance	1	2	7
Taste	1	3	6
Smell	1	2	7
Total	14 (16%)	18 (20%)	58 (64%)

In an overview of all sensory categories and all foodstuffs the most common answer (64%) was that the test subjects had no preference for either treated or untreated samples. By far the most common reason given for this was that the test subject did not perceive any difference (see APPENDIX I – Questionnaires)

Overall preference for treated and untreated samples was 16% and 20% respectively.

In the category appearance results were 23% for treated samples, 17% for untreated samples and 60% no preference.



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Most reasons given for preferences are impossible to relate to the presence or absence of LISTEX™. Examples include: “*more red*” (the cooked ham has both darker and lighter areas and differences in the fat layer), “piece is broken” and “looks fatty”.

Again the most common answer in this category was “no difference” or “same”.

In the category taste 16% preferred treated, 27% untreated and 57% had no preference. Again the most common reason given was an inability to perceive a difference. Two subjects who preferred untreated chicken to the treated sample state that to them the treated product tasted drier. The same amount of liquid was applied to both samples and it appears difficult to correlate this impression to the use of LISTEX™. Two test subjects consistently preferred the taste of treated and untreated samples respectively but another preferred the treated sample in one case and the untreated sample in another. Another test subject stated the presence of more fat in the treated ham sample as the reason for preferring that sample.

In the category smell test 6% of test subjects preferred treated samples, 20 % preferred untreated samples and 74% had no preference. Again, the most common reason given is an inability to perceive any difference.

No test subject consistently preferred either treated or untreated samples. One subject motivated preference for one ham sample with “creamier smell”, an attribute not likely to be conveyed either by water or LISTEX™.

All questionnaires are attached and a key for telling which samples were treated is given further bellow.

The results of this test can be summarized as follows:

- “No preference” is the most common answer
- “No difference” in perception is the most common motivation
- No consistency can be found in cases were preference is stated
- Motivations for preferences seem to correlate more easily to inherent qualities of a particular sample than properties conveyed by a phage preparation or a minute amount of water

Therefore it can be concluded that neither presence nor absence of LISTEX™ can readily be detected. Setting up professional sensory perception tests is a science in itself, with efforts made to distinguish between those people who actually perceive a difference and those who cannot, before assessing preferences. Within the limited scope of this trial the results are exactly as one would expect if no effect on sensory perception is expected from an application: The most common result



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being no perception of difference with the remainder divided roughly equally between preferring either treated or untreated sample.

This conclusion is corroborated by a scientific line of argumentation.

Applying LISTEX™ at the highest envisaged dose on a thinly sliced product such as cooked ham, with a diameter of 10 cm, a thickness of 2-3 mm, a weight of ~20g and a surface area of approx. 160 cm² results in application of 7.8×10^{10} bacteriophages per slice. With a weight of approximately 10^{-15} g for a single phage the total added weight is roughly 4 ppm with numbers being lower in most applications. Such a concentration is below the human perception threshold even for most compounds having extremely distinct taste in a taste-neutral background such as water.

Phages consist of proteins and nucleic acids which are not noted for having any particular effect on sensory perception. While the phages on the product become gradually inactivated by adsorption to particles and small structural changes in the host recognizing tail fibers, the phages remain structurally intact for long periods of time. Nonetheless, even complete degradation into peptides and oligo-nucleotides would not likely have an effect on taste.



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Appendix I Questionnaires and key

LISTEX™/Water control sample key.

		LISTEX™	Water
Appearance			
	Chicken	A	B
	Ham	A	B
	Salami	B	A
Smell			
	Chicken	B	A
	Ham	A	B
	Salami	B	A
Taste			
	Chicken	A	B
	Ham	A	B
	Salami	B	B



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Which product do you prefer?

HAM A B Neither Why ?

Appearance

Taste

Smell

A is a bit darker than B, but this does not influence my choice
The same product.
Both smell like deer meat

CHICKEN A B Neither Why ?

Appearance

Taste

Smell

No visual difference
A tastes drier and less texture
Small difference, but I don't prefer one.

SALAMI A B Neither Why ?

Appearance

Taste

Smell

No vis. diff.
No diff.
Smells a bit faster



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Which product do you prefer?

HAM A B Neither Why ?

Appearance

No difference

Taste

No difference

Smell

Smells the same

CHICKEN A B Neither Why ?

Appearance

Slice is broken

Taste

No perceivable difference

Smell

Similar

SALAMI A B Neither Why ?

Appearance

No difference

Taste

Same

Smell

The same



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Which product do you prefer?

HAM A B Neither Why ?

Appearance

Taste

Smell

PIECE A HAD MORE FAT

CHICKEN A B Neither Why ?

Appearance

Taste

Smell

SALAMI A B Neither Why ?

Appearance

Taste

Smell



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Which product do you prefer?

HAM

A

B

Neither

Why ?

Appearance

Taste

Smell

looks a little bit darker
stronger taste
better smell

CHICKEN

A

B

Neither

Why ?

Appearance

Taste

Smell

looks the same
more tasteful
more chicken smell

SALAMI

A

B

Neither

Why ?

Appearance

Taste

Smell

looks darker
spicier taste
more smell



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Which product do you prefer?

HAM A B Neither Why ?

Appearance

Taste

Smell

<i>Chemicals both.</i>

CHICKEN A B Neither Why ?

Appearance

Taste

Smell

SALAMI A B Neither Why ?

Appearance

Taste

Smell



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Which product do you prefer?

HAM A B Neither Why ?

Appearance

Taste

Smell

<i>Butter more real no black spots</i>
<i>More creamy saize</i>

CHICKEN A B Neither Why ?

Appearance

Taste

Smell

<i>Was more Frischer Flesh color more fresh</i>
<i>more schärf less schärf</i>
<i>Sitz</i>

SALAMI A B Neither Why ?

Appearance

Taste

Smell

<i>Lyfetter Look's more fatty</i>
<i>less schärf</i>



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Which product do you prefer?

HAM A B Neither Why ?

Appearance

Taste

Smell

B looks Fatty
B has a taste
A smells very strong

CHICKEN A B Neither Why ?

Appearance

Taste

Smell

A is dryer

SALAMI A B Neither Why ?

Appearance

Taste

Smell

A is more tasteful



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Which product do you prefer?

HAM

A

B

Neither

Why ?

Appearance

Taste

Smell

<i>less smell</i>

CHICKEN

A

B

Neither

Why ?

Appearance

Taste

Smell

SALAMI

A

B

Neither

Why ?

Appearance

Taste

Smell

<i>less fat visible</i>
<i>less a half.</i>



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Which product do you prefer?

HAM A B Neither Why ?

Appearance

Taste

Smell

CHICKEN A B Neither Why ?

Appearance

Taste

Smell

A was more tasty

SALAMI A B Neither Why ?

Appearance

Taste

Smell

A had a stronger taste.

The Use of LISTEX™ Bacteriophage as a Processing Aid

Introduction

Several studies have shown that the use of EBI Food Safety's LISTEX™ can result in a 2-3 log₁₀ decrease in Listeria contamination in a variety of foods. (Study performed at the Swiss Federal Institute of Technology [ETH], Zürich) The anti-Listeria effect of LISTEX™ is due to the encounter of bacteria with the bacteriophages. The time when this encounter is likely to happen is shortly after bacteriophage application. The phages are applied to food in a liquid solution; at this stage the phages have some limited mobility by passive diffusion. After a short time the phages quickly become immobilized. This is eventually followed by inactivation. Immobilization reduces the likelihood of phage-bacteria encounter; inactivation completely abolishes residual effects of bacteriophages.

Life Cycle of LISTEX™

Close analysis of the efficacy on various foodstuffs demonstrates that there is virtually no residual activity of the bacteriophage after the initial Listeria reduction. This loss of activity is due, initially, to the rapid phage immobilization followed by a decline in the number of viable phages.

Any initial reduction in the bacterial population will result in a delayed resumption of the growth of surviving cells. Of course, if the initial reduction is complete there will not be a resumption of growth at all. The period after reduction until resumption of growth is defined by the time necessary for the bacterial population to return to the original numbers.

It is possible to calculate hypothetical growth curves that plot Listeria growth after such the initial reduction. This is possible because the growth rate of bacteria under the given conditions can be calculated by analyzing the growth curve of the untreated control.

Exponential growth of bacterial cultures follows the following equation:

$$N = (N_0) e^{kt}$$

In a semi-logarithmic graph k is represented by the slope of growth

$$k = \frac{\text{actual rise}}{\text{actual run}} = \frac{\ln Y_2 - \ln Y_1}{t_2 - t_1}$$

Values Y_2 and Y_1 are established by taking any two y-values of the slope at two time-points t_2 and t_1

The doubling time of the bacterial population is therefore:

$$t_d = \frac{\ln 2}{k}$$

While it is theoretically possible to add phages in sufficient quantity to effect *Listeria* growth throughout a product's shelf-life, the inherent characteristics of meat and poultry products and the rapid decrease in functionality of phages due to immobilization and inactivation make it more likely that there will be no lasting effect. As noted above, the phages must be mobile to interact with the bacteria. As the surface of the meat dries, the phage becomes immobile losing its ability to interact.

A number of factors contribute to phage permanent inactivation. In essence phage inactivation can be divided into two distinct parts, degradation and adsorption. Several compounds and circumstances can lead to inactivation by structural degradation of the phage particles, among which are proteolytic enzymes, UV-light, acids, etc. Adsorption to particles on the food surface renders the phages unable to interact with their target bacteria.

Of the causes leading to structural degradation of phages in foodstuffs the presence of proteolytic enzymes is likely to be the most relevant. In cooked, ready to eat meat products the concomitant flora will contribute to the presence of such enzymes and together with adsorption of phages to particles cause inactivation.

Detailed Analysis Of The Anti-Listerial Effects Of LISTEX™ P100 In Meat Products

Fig. 1 shows the effect of phage treatment on minced meat. In addition, a hypothetical growth curve showing the development of a bacterial population with identical growth characteristics as the untreated control but which starts with cell numbers identical to those of the treated samples following the initial reduction. In other words this curve represents the growth of the population if it had been reduced to that level by a treatment known to have no residual effect such as heat treatment or irradiation. It is obvious from this graph that growth of bacteria that have survived initial treatment is not impeded by the presence of the phages.

Fig. 2 and 3 shows the similar effect of phage treatment on ham and cooked turkey breast. All data (including that previously submitted to FDA and USDA in GRASN's 198 and 218) were obtained from Swiss Federal institute of Technology Zürich, where phage treatment of a large variety of foods was investigated in the framework of a PhD thesis. A large part of the data was recently submitted for publication (Günther et al. submitted to Applied and Environment Microbiology).

Hypothetical growth curves following an initial reduction have also been included in Figures 2 and 3 showing the effect of phage treatment on ham and turkey breast artificially contaminated with high numbers of two different *L. monocytogenes* strains. Doubling times of the bacterial population were calculated based on the growth of the untreated controls. In all cases, resumption and rate of growth after treatment follow the hypothetical growth

curves. The *Listeria* cells remaining after initial reduction grow almost exactly as those in the untreated control. The highest reduction is obtained after approximately 24 hours; after that no remaining residual activity can be observed.

The surface texture of foods may effect the length of time that the initial phage treatment remains effective. Figure 4 shows the effect of P100 treatment on the growth of *Listeria* on hot dogs, a model for foods with smooth surfaces.

The treated hotdogs shows no *Listeria* growth within 6 days. It is difficult to establish whether this is caused solely by longer phage activity. A high initial reduction could also leave bacterial numbers at a level below the detection threshold, or a combination between the two factors, low residual *Listeria* and residual phage activity, may explain the observation.

Treatment with a tenfold lower dose achieves a slightly lower initial reduction of $\sim 2.5 \log_{10}$ and resumption of growth can be observed throughout 6 days (Fig. 4). In reality this lower reduction is more than effective in combating *Listeria* contamination because this product will be essentially free from *Listeria* after cooking and the contamination level used for this evaluation, 10^3 CFU/g, is much higher than the contamination levels that are anticipated in plants operating within functional GMP and HACCP programs.

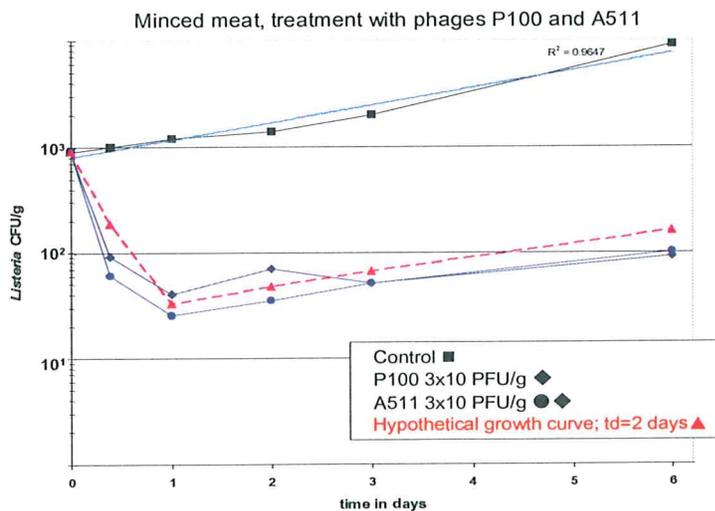


Figure 1

Fig. 1: Effect of phage treatment on *Listeria* growth with phages P100 and A511 in minced meat. The red line shows hypothetical growth after a $1.5 \log_{10}$ reduction and a doubling time as observed in the untreated control.

Figure 2A

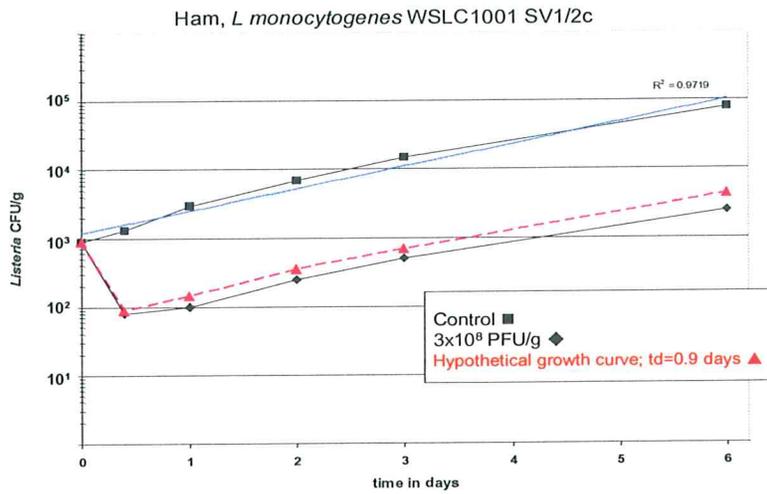
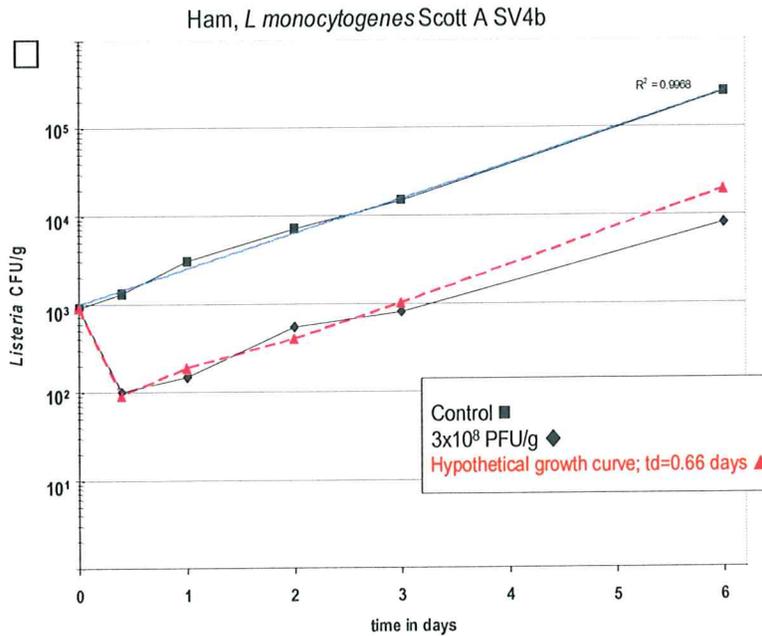


Fig. 2: Effect of phage treatment on growth of *Listeria* strain WSLC1001 (A) and strain Scott A (B) on ham.



The red lines show hypothetical growth after a 1log₁₀ reduction and a doubling time as observed in the untreated controls.

Figure 3

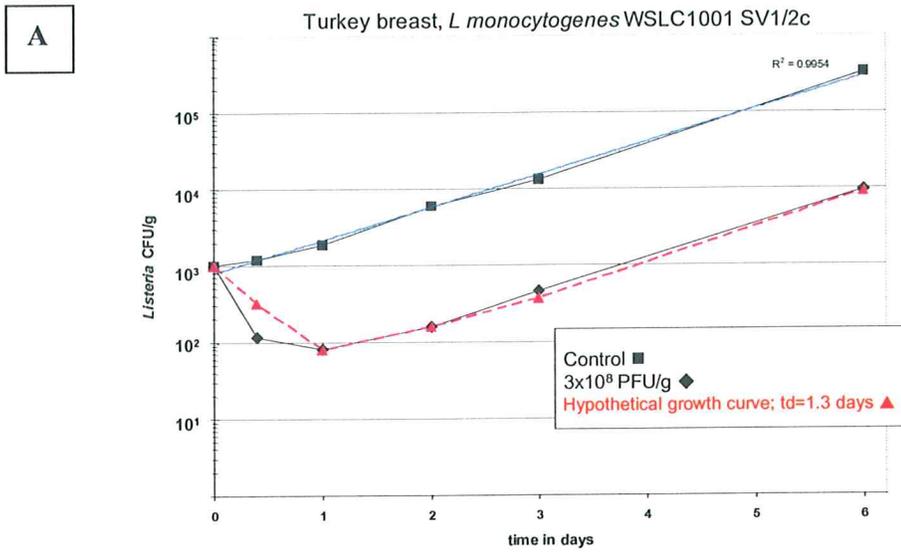


Fig. 3: Effect of phage treatment on *Listeria* growth of strain WSLC1001 (A) and strain Scott A (B) on turkey breast. The reds line shows a hypothetical growth after a $1\log_{10}$ reduction and a doubling time as observed in the untreated controls.

Figure 4

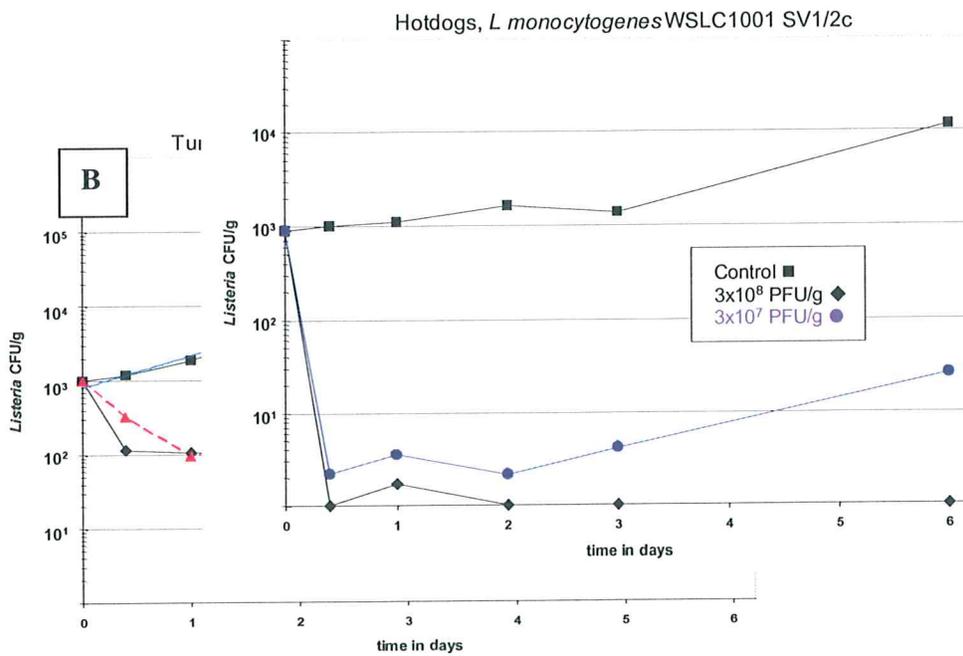


Fig. 4: Effect of two different phage doses on an artificial *Listeria* contamination of hotdogs. While a similar level of reduction can be observed the higher dose of phages either leads to immediate eradication of the entire bacterial population or remains active for a slightly longer period of time keeping bacterial numbers below the detection threshold.

Phage inactivation as cause for loss of function in the final product

Depending on dosage a certain initial reduction of bacterial numbers is achieved. Surviving cells will eventually resume growth. In the case of phage treatment there may be a residual activity which is quickly lost over a short period of time.

The residual activity is explained by growing colonies encountering bacteriophages very early in colony development. At this point in time all the cells in the colony are metabolizing, allowing infection and subsequent release of progeny phage and eradication of all cells in the colony.

If the growing edge of a colony encounters a bacteriophage at a later stage, only the metabolizing cells at the edge of a colony will be infected and release progeny phage, whereas those cells in the center of the colony are not metabolizing, and will not release progeny phages. These cells will form a barrier against the bacteriophages thus “protecting” cells at the other growing edge from being infected. This effect is visualized in figure 6 which shows normal colonies and colonies growing on plates which had been inoculated with phages (1.5×10^4 PFU/cm²).

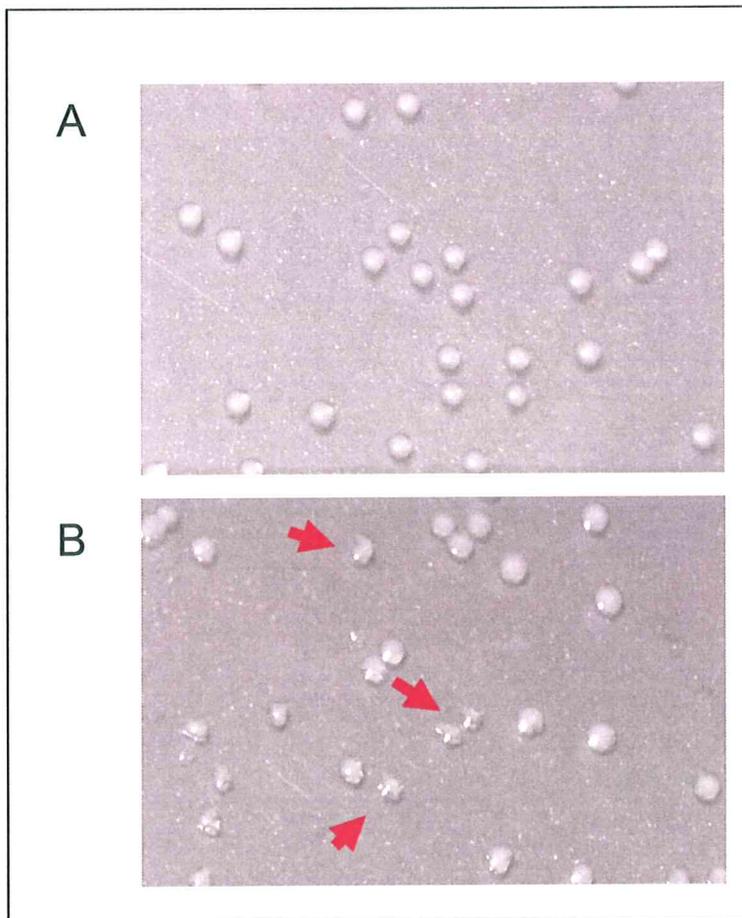


Fig. 6 A: Typical morphology of *Listeria* cells on a BHI agar plate. B: Altered colony morphology of some colonies on BHI agar plate after plating in combination with 1.5×10^4 PFU/cm² of bacteriophage P100. Phages can be recovered from material of these irregular colonies.

Depending on the initial phage dosage, some cells surviving initial treatment colonies might not encounter active phages before nutrients in the vicinity become exhausted. Consequently, to achieve a significant residual effect throughout the shelf-life of the product a sufficiently high number of phages would have to be administered and remain active on a treated surface to ensure infection in the early developmental stages of bacterial survivors. A higher dose would at the same time increase the initial level of reduction making residual activity superfluous.

A good phage treatment should therefore target initial eradication of possible contamination rather than establish residual activity. While increasing phage dose is one option for increased residual activity, increasing phage dose will not be as effective unless the phages can remain mobile. In meat and poultry products, however, excess fluid is not tolerated and therefore this route is not feasible. Moreover, if the target of eradicating a possible contamination is achieved there is also no real need to ensure continued activity. Use of phages to ensure activity beyond opening of packaging is impossible. LISTEX™ is used to treat surfaces. If a treated product is sliced new surfaces are created which would not be protected regardless of the initial phage dose used.

Additionally, on any given food matrix the number of active phages will decline over time. The speed at which phages are degraded in different foods varies. It will also vary between identical products from different factories or from identical products in the same factory at different time-points. While the irreversible adsorption of phages to particles will be similar in the same food product, structural degradation by enzymes will differ, depending on the concomitant flora which always varies.

In one experiment on red-smear cheese, a one \log_{10} (90%) reduction in recoverable phages was observed in a 14 day period. In an experiment on white mold cheese, a two \log_{10} (99%) was observed after 14 days (data not shown).

An experiment on sterile ham showed a one \log_{10} or 90% reduction of viable bacteriophage P100 over a 90-day period (data not shown). Since an absence of other bacteria ensures that little or no degrading enzymes are present, this decrease probably represents the percentage of phages that permanently adsorbed to particles.

It is impossible to distinguish between structural degradation and irreversible adsorption to particles. Phages may also be inactivated by reversible interaction with particles, their orientation in the matrix no longer allowing them to infect target bacteria. This effect is likely to increase over time but is impossible to quantify. These circumstances would influence the phage dose necessary to ensure continued activity of phages beyond the initial reduction.

Conclusions

Phage doses which are effective in reducing *Listeria* contaminations (2-3 \log_{10}) in meat and poultry products and thus ensure food safety in a highly effective manner do not have a residual effect in the final product. Bacteriophages are quickly immobilized and subsequently inactivated by structural degradation or irreversible and reversible adsorption to particles in such foodstuffs.

Higher doses of phages would need to be added to extend the period of residual activity. However, this would not be feasible or practical for meat or poultry products.

Processing aids include substances that are present in finished food at insignificant levels and do not have any technical or function effect. Phage doses which are effective in reducing *Listeria* contamination (2-3 log) meet this definition. Processing aids also include substances added during processing that are converted to constituents normally present in food at insignificant levels.

Bacteriophages are comprised solely of proteins and DNA and their degradation products are therefore amino acids and nucleic acids, both of which are abundantly present in the products to be treated. Therefore phages, used to achieve a reduction of unwanted pathogens within a post lethality treatment, should be considered a processing aid.