Supporting document 1

Risk assessment report

Application A1045

Executive summary

Introduction

Listex P100 bacteriophage preparation (hereafter referred to as the P100 preparation) is proposed for use on solid ready-to-eat (RTE) food products[1] for the purpose of reducing numbers of *Listeria monocytogenes*. The types of food where the P100 preparation may be used include all solid RTE food products.

The Applicant proposes the P100 preparation would be used in combination with good hygienic practices (GHP) currently applied in food processing to control contamination of food with *L. monocytogenes*. It is intended to complement existing GHPs, not as a replacement for GHP. It is designed for use as a spray or dip for targeted application to surface treat food products and not as a surface disinfectant or general bactericide within the processing facility. The stated purpose and technological function of the P100 preparation may be consistently achieved when process validation has been undertaken for each food product, under commercial conditions, and when the defined protocols are followed.

FSANZ has assessed the safety and the proposed technological function of the P100 preparation. The efficacy (ability to reduce *L. monocytogenes* on contaminated food) and the ongoing technological function (ability to continuously limit growth of *L. monocytogenes*) under proposed conditions of use have been assessed.

Characterization

Question: Is the P100 bacteriophage preparation well characterised?

The identity of the P100 bacteriophage has been determined as belonging to the Order *Caudovirales,* Family *Myoviridae,* subfamily *spounaviridae*, genus twort-like and species *Listeria* phage P100. The host (production) organism is a non-pathogenic type strain of *Listeria innocua* (ATCC 33090, DSM 20649, NCTC 11288, SLCC 3379). The bacteriophage P100 and production organism are completely characterised. P100 has been recently reclassified by the International Commission for Taxonomy of Viruses (ICTV).

[1] A ready-to-eat food is defined as any food which is normally eaten in its raw state or any food handled, processed, mixed, cooked, or otherwise prepared into a form which is normally eaten without further preparation.

Technological function

Question: Does the P100 preparation achieve its stated technological purpose?

FSANZ has made an assessment of the efficacy and the possibility of an ongoing technological function when the P100 preparation is used for the stated purpose. The P100 is effective in reducing numbers of *L. monocytogenes* in treated foods. P100 has been assessed to perform the technological function to reduce and control the levels of *L. monocytogenes* contamination on solid RTE foods by dipping or spraying the surfaces of the food with a large excess of phage particles compared to the *L. monocytogenes* contamination concentration. For effective treatment the P100 dosing concentration may need to be greater than 10^3 – 10^4 times greater than *L. monocytogenes* concentration. It is also important to treat the food before the levels of *L. monocytogenes* have grown too large.

The overall weight of evidence, noting the restricted functionality of the bacteriophage in commercial conditions and in solid food matrices, supports the conclusion that P100 has no ongoing technological function in solid RTE food (with the exclusion of those solid foods visibly covered or immersed in a liquid phase) according to the use and levels proposed by the Applicant. This is explained by P100 particles being bound to the surfaces of solid food and so not being mobile to find and eliminate *L. monocytogenes* after the initial treatment. The bound P100 particles may not all be completed destroyed or inactivated but being bound they no longer have any function to reduce *L. monocytogenes* levels.

It is important to note that P100 cannot be assumed to be a complete single treatment that will destroy and eliminate all *L. monocytogenes* from treated food. It should be considered only as additional technology food manufacturers can use along with their current processes to control *L. monocytogenes.* Food manufacturers will need to determine appropriate process optimisation and SOP's (Standard Operating Practices) to establish efficacy on a case-by-case basis for different foods and different production plants and to monitor efficacy consistently.

The risk assessment reviewed the information on the possibility of emergence of bacteriophage-resistant strains of *L. monocytogenes.* The conclusion from the scientific evidence, supported by experts in the field and international regulators, is that when using bacteriophages to treat food, the development of resistance in food processing environments is minimal, provided adequate information on the use, application and disposal of unsold product is provided to food manufacturers, and that manufacturers have regard to that information. This is no different to resistance developed by bacteria as a stress response to other bactericidal treatments applied during food processing. Treated products are not expected to re-enter the processing facility. Adherence to GHP ensures phage treated product that is not appropriate to be processed for commercial sale needs to be removed from the production facility on a regular basis, along with appropriate cleaning regimes to ensure there is no build-up of bacteriophage reservoirs in the facility. Continuous screening and monitoring of host susceptibility and phage resistance development in food premises using the P100 preparation, is being maintained by the Applicant.

Safety Assessment

No food safety issues were identified from the available toxicity data. This conclusion is supported by the absence of biologically significant homology between the P100 proteins and any known allergens or toxins.

P100 bacteriophage is only effective against bacteria of the genus *Listeria.* It cannot infect plant, animal or human cells. Ingestion or contact with P100 does not present a public health risk.

P100 meets two critical safety criteria in that it is both lytic and non-transducing (these terms are explained in the Section 1 - Introduction) which ensures it cannot be a vector for the transfer of virulent genes between host bacteria.

Dietary exposure assessment

Since there are no identifiable hazards, an exposure assessment was considered unnecessary.

Conclusions of the risk assessment

The use of the P100 bacteriophage preparation in solid RTE foods as proposed by the Applicant is safe and technologically justified. There is no ongoing technological function performed by the P100 preparation in solid RTE foods (with the exclusion of those solid foods visibly covered or immersed in a liquid phase).

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1 Introduction

An application submitted by EBI Food Safety Ltd seeks approval to permit the use of a bacteriophage (often abbreviated as phage) preparation, designated Listex P100, as a processing aid to reduce *Listeria monocytogenes* numbers in ready-to-eat foods. The bacteriophage is prepared as a liquid culture of P100. There are no current permissions for the use of bacteriophage as a processing aid in the *Australia New Zealand Food Standards Code* (the Code).

The Applicant claims the P100 preparation could be used as a technology in combination with other good hygienic practices (GHP) to be applied during the processing of food to reduce numbers of *L. monocytogenes*. It is intended for use as a spray or dip for surface treatment of food products prior to packaging.

The P100 preparation is proposed for use within a food processing facility in a controlled environment such that the application is typically targeted on the surface of the product. It is not designed for use as a surface disinfectant or general bactericide within the processing facility. The treated product is not expected to re-enter the food processing premises once it leaves the factory for subsequent distribution and sale. Similar to other food processing technologies, the stated purpose and technological function of the P100 preparation may be consistently achieved when process optimisation has been undertaken, followed and continuously monitored for each product under commercial conditions.

1.1 Bacteriophages and their mode of action

Bacteriophages are viruses that attach to and replicate only in bacteria. They are ubiquitous, occupying every environmental niche and are present in large numbers in the environment, including food. Bacteriophages are highly specific to the bacterial species they infect and cannot infect plant, animal or human cells. Ingested bacteriophages pass through the gut without causing any hazard to humans.

Bacteriophages are non-motile, lacking the ability to actively locate bacterial cells. They rely on diffusion to randomly encounter and attach to host bacterial cells. Once attached to the host cell, bacteriophage can follow two pathways; the lytic[2] cycle and the lysogenic[3] cycle. Those that can only follow the lytic cycle are known as virulent bacteriophage, while those that can follow the lysogenic cycle are known as temperate bacteriophage.

1.1.1 Virulent (lytic) bacteriophages

Inactivation of host bacterial cells can occur by the following mechanisms: 'lysis from within', 'lysis from without' and abortive infection mechanisms, which are explained below. Lysis from within is not relevant to the P100 preparation and is included here for explanation only.

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[2] The lytic cycle is where bacteriophage undergoes replication within the bacterial host cell, with release of phage particles upon rupturing of the host cell. This cycle does not integrate phage genetic material into the bacterial chromosome.

[3] The lysogenic cycle is where the genetic material of the bacteriophage integrates with the chromosome of the bacterial host, enabling it to lie dormant and to release phage particles when conditions are suitable. The lysogenic cycle provides a mechanism whereby toxin genes may be spread/exchanged between bacteria, altering their virulence properties.

a) Lysis from within

- The bacteriophage adsorbs (binds irreversibly) onto peptidoglycan receptors on the surface of the phage-susceptible bacterial cell using its tail fibres and other components.
- Replication and maturation – Bacteriophage enzymes are used for the insertion of phage genetic material into the host cell. Once this occurs, a series of molecular events are initiated and host synthesis machinery is hijacked and intracellular resources diverted to produce materials for phage replication.
- Lysis – Bacteriophage particles proliferate within the host cell, leading to breakdown of the cell wall and release of the new phage particles.

b) Lysis from without

Alternatively, several phage particles may attach to the cell wall receptors of the host, altering the host cell permeability to cause cell death. This phenomenon is termed "lysis from without" and causes bacterial cell lysis, without the need for phage replication. However, Molineux (2006) states that "most bacteriophages do not cause lysis-from-without; more often, high multiplicities of infection simply overwhelm all capacity for efficient macromolecular synthesis and the cell simply dies". In food related pathogen control, this mechanism may be exploited by applying large numbers of bacteriophage relative to target bacteria.

c) Abortive infection mechanisms

Host cell inactivation can also be caused by abortive infection mechanisms, where the bacterial defence mechanisms cause cell suicide upon attachment of a phage particle. The latter mode of action is perhaps prevalent in applications where relatively high phage doses are applied to achieve host destruction at first infection.

1.1.2 Temperate (lysogenic) bacteriophages

In contrast, temperate bacteriophages can integrate their genetic material into the host bacterium to form a lysogenic cell. The integrated phage (prophage) can potentially carry and express genes encoding toxicogenic proteins which increase the pathogenicity (ability to cause infection and disease) of the host pathogen. In several instances, temperate bacteriophages have been identified as the carriers of toxins or toxin genes (Boyd et al. 2001; Holck and Berg 2009).

1.2 Bactericidal properties of bacteriophage in food-related applications

The bactericidal properties of bacteriophage are related to many factors including the ratio of bacteriophage to host cell numbers, adsorption characteristics (the mechanism and properties by which bacteriophage adsorbs to the host cell surface) and the physical and chemical characteristics of the food (e.g. diffusion of bacteriophage in the water phase and ability to passively locate host cells).

At low cell concentrations, Kasman et al. (2002) demonstrated that every phage particle which is irreversibly bound to a host cell (due to adsorption) is capable of successful transfer of phage DNA into the host cell, which results in cell death. In food, bacterial dispersion is non-uniform and the lack of active host seeking mechanisms by bacteriophages complicates the estimation of host/phage ratios. It is recommended that, under such conditions, it is best to use concentrations of bacteriophage sufficient to kill host cells at the first dose.

The use of an appropriate concentration avoids the need to rely on active bacteriophage replication to achieve host cell death (Abedon 2009). Non-replicating bacteriophages inject phage genomic material which expresses bacterial suicide genes leading to host cell death.

In food-related applications, the adsorption properties, rather than the replication properties of the bacteriophages, are utilised. The adsorption constant (a property characteristic of each bacteriophage/host combination which determines the efficiency of adsorption of the phage cells onto the host cell wall) is therefore the most important criterion for such applications. Adsorption characteristics vary between phage types and are influenced by other factors such as growth phase of the host bacterial cells, presence of divalent ions, organic compounds in the food matrix, agitation, temperature, cell size, and density of available surface receptors on the host. These parameters therefore influence the effectiveness of bacteriophages in food products, resulting in a degree of variability depending on the food matrix and processing conditions. Such variability is expected of biological food ingredients.

In foods with low bacterial numbers ($<10^4$ cfu/mL), as in the case of food borne pathogens, initial bacterial cell numbers are not critical. Bigwood et al. (2009) demonstrated that it is not necessary to know the concentration of pathogens present beforehand to achieve bacterial reduction as long as there are sufficient phage particles available. It is therefore necessary to supply an excess of phage to achieve a substantial bacterial kill (Abedon, 2009). Using approximations of bacterial survival and modelling approaches, it has been estimated that 10^8 or more phage per mL and an exposure time (contact time) longer than 2 minutes is required to achieve this outcome (Abedon 2009).

Bacteriophage lack the ability to actively locate host bacterial cells. They do so by mass kinetics and diffusion. Therefore there are major differences in their activity in liquid and non-liquid foods. In liquid foods bacteriophages are able to diffuse and therefore gain access to the bacterial cells whereas in non-liquid foods their mobility is limited. In addition to the adsorption properties specific to bacteriophages, other physicochemical conditions such as pH and salinity of food could influence the infectivity and therefore the technological function of bacteriophages.

1.2.1 Safety considerations

Several biological characteristics need to be considered in the use and application of bacteriophages to food.

1.2.1.1 Lysogenic activity

It is recommended that bacteriophages used in food processing are purely virulent, or lytic, bacteriophages rather than those with lysogenic properties. The absence of the ability to cause lysogeny ensures that there is no potential for the bacteriophage to transfer genes to the host bacterium that may increase pathogenicity or virulence in humans (Hagens and Loessner 2010). Some lytic bacteriophages are capable of switching to the lysogenic mode, unlike the purely lytic bacteriophages such as P100. Lytic bacteriophages are invariably lethal to the bacterial cell once infection has been established.

1.2.1.2 Transduction

Transduction is the mechanism whereby bacterial genetic material is transferred between bacteria through a bacteriophage vector. Transduction is facilitated through either the lytic cycle or the lysogenic cycle of bacteriophages. Transduction occurs in the natural environment, where phages are numerous, and is known to be a key driver of bacterial evolution. However, in applications relating to the control of bacterial pathogens by phages, it is important that *non-transducing* phages are selected.
For a more detailed description of generalised and specialised transduction, and methods to determine transduction potential, readers are referred to Fineran et al. (2009) and Waddell et al. (2009).

2 Objectives of the assessment

In proposing to amend the Code to include the P100 preparation as a processing aid, a pre-market assessment is required.

The objectives of this risk assessment are to determine whether:

- the P100 preparation achieves its stated technological function
- any potential health and safety concerns may arise from the use of P100 as a processing aid

3 Risk assessment questions

The following risk assessment questions have been developed to address the objectives of the assessment:

- Is the P100 bacteriophage preparation well characterised?
- Does the P100 preparation achieve its stated technological purpose?
  - Has the technological need been articulated clearly?
  - Is the preparation added in a quantity and form which is consistent with delivering the stated purpose?
  - Can development of resistance render the P100 preparation ineffectual?
- Does the P100 preparation present any food safety issues?
  - Are there potential allergens present in the P100 preparation?
  - Are there toxicological safety issues?

4 Characterisation of P100

4.1 Identity of the bacteriophage

Order:	*Caudovirales*
Family:	*Myoviridae*
Subfamily	*Spounaviridae*
Genus	Twort-like
Species	*Listeria* phage P100
Host specificity:	Specific to a large number of strains of *L. monocytogenes*, and *L. innocua*
Marketing name:	Listex P100

P100 is a purified, non-transducing, strictly virulent (lacking in lysogenic activity) bacteriophage. It has an unusually broad host range and is able to infect species within the genus *Listeria*. It has been found to successfully lyse 95% of *Listeria* spp. tested including *L. monocytogenes,* serovars 1/2 and 4; serovar 5 of *L. ivanovii* and serovar 6 of *L. innocua* (Loessener, unpublished in Carlton et al. 2005).

4.2

4.2 Chemical and physical properties

A detailed characterisation of the P100 genome has been carried out by Carlton et al. (2005), with a fully annotated sequence deposited in GenBank under accession number DQ004855.

4.3 Production of P100

FSANZ has used the confidential information provided by the Applicant to assist in its safety assessment.

Standard fermentation procedures are employed for the production of P100 which take place within bioreactors and consist of the following steps:

L. innocua Seeliger is grown in ATCC medium 44: brain heart infusion agar or broth at 37°C. It is classified as Biosafety Level 1 by ATCC as it is not known to cause disease in healthy adult humans (lacks virulence determinants). The P100 phage preparation does not contain any allergens that are required to be labelled under the requirements of clause 4 of Standard 1.2.3 – Mandatory Warning and Advisory Statements and Declarations.

a) Fermentation

Bacteriophage P100 is produced in a growing host culture of *L. innocua* (further information on the host organism is provided in Section 4.4). The culture is maintained using standard batch culture media prepared according to standard operating procedures (SOPs), incubated within a bioreactor and infected with P100 once the host culture reaches a target concentration.

b) Downstream processing

Following incubation, the culture is harvested and subjected to a microfiltration process where the bacteriophages are separated from host cells and cell debris. The phages are then concentrated using cross flow ultra-filtration. The concentrated bacteriophages are filter sterilised through a 0.2 µm pore sterilisation filter, and standardised to obtain a final phage concentration of $2x10^{11}$ pfu (plaque forming units)/mL using sterile water. All equipment used for downstream processing applications are made of food or pharmaceutical grade material. The standardised solution is divided into 100 mL aliquots and every 25th bottle is sent to an external laboratory for quality control testing.

c) Quality Assurance

The Applicant has described quality assurance methods, SOPs and sampling protocol adhered to during procurement of raw materials and production. Tests for the stability of the microorganism are carried out using standardised methods (Annex 8 of Application) and results of stability tests have been provided by the Applicant. Up to date record keeping is maintained under the SOPs. A 10 mL sample is taken per 25 packages and externally analysed for *Listeria* species, yeasts, moulds and total aerobic plate count.

4.4 Identification of the host (production) organism

Name of host organism:	*Listeria innocua*
Literature:	Seeliger HP (1983) International Journal of Systematic Bacteriology 33:439
Risk group:	ATCC Biosafety Level 1
Type strain and registry numbers:	ATCC 33090, DSM 20649, NCTC 11288, SLCC 3379

4.5 Analysis and specifications

As discussed in Section 1.1, the activity of P100 is defined by the ability to destroy host bacterial cells and therefore expressed as the reduction of bacterial numbers. To analyse for the presence of P100 in bacteriophage treated food products, a standard agar overlay method can be employed. A dilution or suspension of the food sample (containing the bacteriophage) is mixed in a small volume of molten agar containing host bacteria (e.g. *L. innocua*) and poured onto the surface of a nutrient agar plate. Following overnight incubation, the host bacterial cells have grown uniformly throughout the top agar layer (forming a bacterial 'lawn') and bacteriophage are enumerated by assaying plaques caused by cell lysis, being expressed as plaque forming units (pfu) per g of the initial solid food.

The Application contains information relating to a polymerase chain reaction (PCR) analytical method applicable for determining the presence of P100 bacteriophage on treated food. To confirm the presence of P100, a PCR method is applied using the following primers: Forward: 5'-ccttcacgcatctttgttacag (binds P100 genome bp: 108867-108888); reverse: 5'-cagggttgtatttaggtactc (binds P100 genome bp: 109957-109937). This analytical method is available and could be used by analytical laboratories for enforcement purposes if required.

It is stated in the Application that the bacteriophage preparation is produced using appropriate Good Manufacturing Practices (GMP) controls and processes to ensure the finished product does not contain any impurities of a hazardous or toxic nature.

The Applicant has provided specifications for the P100 preparation (see Table 1) and three certificates of analysis which demonstrate conformance to the stated specifications (Appendix 6 in the Application).

Table 1 *Specifications for P100 bacteriophage preparation (as provided by the Applicant)*

Physical Properties	**Specification**
Description	Suspension of broad spectrum[4] phage preparation formulated in phosphate buffered saline
Source	Fermentation derived
Phage concentration	$2x10^{11}$ phage/mL
Chemical Properties	
Heavy metals (as Lead)	<10 mg/kg
Lead	<1 mg/kg
Arsenic	<1 mg/kg
Mercury	<0.5 mg/kg
Microbiological Properties	
Standard plate count	sterile
Yeasts and moulds	<10/mL
Enterobacteriaceae	Negative in 1 mL
Salmonella	Negative in 25 mL
Listeria spp.	Negative in 25 mL
Staphylococcus aureus	Negative in 25 mL
Escherichia coli	Negative in 25 mL

[4] Broad host range bacteriophages are capable of infecting a wide range of host strains within the bacterial species they infect.

A report on the stability of the P100 preparation under long term storage is included in the Application. The recommended storage temperature is 2 to 8°C. At these storage temperatures, the designated shelf life of the P100 preparation is six months.

5 Technological function

5.1 Technological function of P100

L. monocytogenes occurs ubiquitously in processing environments and can contaminate and multiply in certain food products, even at refrigeration temperatures (Zago et al. 2007; Yates 2011). Analysis of food recall data by FSANZ (2011) reports that the detection of *L. monocytogenes* was the leading cause of recalls (125 of 260 recalls) due to microbial contamination in Australia between 1 January 2000 and 31 December 2010. Many of the recalls during this period were RTE foods and include soft cheeses, smoked fish, and meat products. The concentration of *L. monocytogenes* found in recalled foods was generally not reported.

Ross et al. (2011) reported *L. monocytogenes* concentration data (1997-2003) for processed meats in Australia that had tested positive after enrichment culture. The majority of samples (71.8%) had <3 MPN (Maximum Possible Number)/g, while 4% of samples had concentrations greater than 100 MPN/g and 2.3% greater than or equal to 1100 MPN/g. Although the data quoted by Ross et al. (2011) is now historical, and *Listeria* management by the food industry is expected to have improved since 2003, the food recall data suggests that *L. monocytogenes* is still an ongoing problem for the food industry.

The Applicant claims the stated purpose (technological function) of their phage preparation is to reduce levels of *L. monocytogenes* in contaminated ready-to-eat food treated during the manufacture and processing of the food. It is intended for use as a spray or dip for application on food products prior to packaging as a complement to existing Good Hygienic Practices (GHP).

5.2 Evaluation of efficacy of phage preparation and its stated purpose

FSANZ has investigated how the P100 preparation performs its technological function when used as proposed by the Applicant. In assessing the technological function, both efficacy (ability to reduce numbers of *L. monocytogenes* on application) and ongoing technological function (ability to *continuously* reduce bacterial numbers) were considered.

A number of recent scientific papers received from the Applicant as well as studies identified by relevant literature searches were included in the assessment. It is noted that the bacteriophage A511, known to be similar to P100 in biology and function, has been used in some experiments and results compared to P100 (Carlton et al. 2005; Guenther et al. 2009).

The types of food treated with P100 (and comparable phage preparations) that have been studied are provided in Table 2 and represent a good cross section of solid RTE food.

All studies include monitoring of *L. monocytogenes* numbers in food samples treated with either the P100 or the A511 bacteriophage preparations[5]. These studies are further described in the following sections.

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[5] The activity of P100 is measured by monitoring the change of the host cell (in this case *L. monocytogenes*) numbers. Viable bacterial counts (expressed as cfu/g) can be measured over the period of application subsequent to being treated with the bacteriophage preparation.

Table 2 *Food categories from phage treatment studies*

Food category	Reference
Ham, chocolate milk, mozzarella cheese brine hot dogs, sliced turkey meat, smoked salmon, mixed seafood, sliced cabbage and lettuce leaves (P100 and A511)	Günther (2007) Guenther et al. (2009)
Soft ripened white mould and red-smear cheese (P100)[6]	Guenther and Loessner (2011)
Red-smear cheese (P100)	Carlton et al. (2005)
Brazilian fresh sausage (P100)	Rossi et al. (2011)
Surface ripened (Munster) cheese (P100)	Schellekens et al. (2007)
Salmon fillet (P100)	Soni and Nannapaneni (2010)
Catfish fillets (P100)	Soni et al. (2010)
Cooked sliced ham (P100)	Holck and Berg (2009) EBI Annex 12 of the Application

5.2.1 Analysis of studies by Günther (2007) and Gunther et al (2009)

FSANZ analysed data from Günther (2007) (included in Annex 12 of the Application) and Guenther et al. (2009) in greater detail since they covered a large variety foods. In these studies, samples were inoculated with *L. monocytogenes* at levels of 10^3 cfu/g or 10^3 cfu/mL for solid and liquid foods respectively. Bacteriophage was added to the foods at concentrations between $3x10^6$ to $3x10^8$ pfu/g (or mL for liquid foods). The following discussion is limited to trials using the initial concentration of $3x10^8$ pfu/g (or mL) as lower initial phage densities did not result in significant reductions of *L. monocytogenes*. The samples were stored for 6 days at 6°C.

FSANZ used a regression model which is explained in Annex 1 for this purpose. Some background to the analysis and discussion of results is given in 5.2.2.

5.2.2 Statistical approach to the assessment of efficacy and ongoing technological function

FSANZ's statistical analysis assessed claims made by the Applicant on the technological function of the P100 preparation using the following approach:

- The degree of efficacy was assessed by the initial reduction obtained due to bacteriophage treatment, represented by the difference of the y-axis intercept of the graphs.
- The claim that there is no ongoing technological function in foods was assessed by observing the ongoing growth rate, following treatment, represented by the slope of the graphs during the course of the study[7].

The above parameters were determined from the bacterial concentrations observed in the bacteriophage treatment studies.

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[6] During the ripening period, cheese is repeatedly smeared with salty water to enable specific microflora to develop on the surface.

[7] The slope of the treated samples would be equal to (no ongoing technological function) or less than (ongoing technological function) the slope of the untreated foods. Therefore, the difference between the slopes of the untreated vs treated samples is expected to be zero for non-ongoing technological function. Where there is an ongoing technological function, the difference in slope will be negative.

Bacteria respond in different ways to bacteriophages depending on the properties of food matrices and the bacteriophage concentrations. Four possible responses are presented in Figure 1:

(A) No efficacy or ongoing technological function – both treatment and untreated lines are identical
(B) No efficacy but an ongoing technological function – same intercept, but a decreased slope of treatment line
(C) Effective but no ongoing technological function – difference in intercepts but lines are parallel
(D) Effective and an ongoing technological function – difference in slope and continuously declining treatment line.

In order to be considered a processing aid, the bacteria should respond as Case (C).

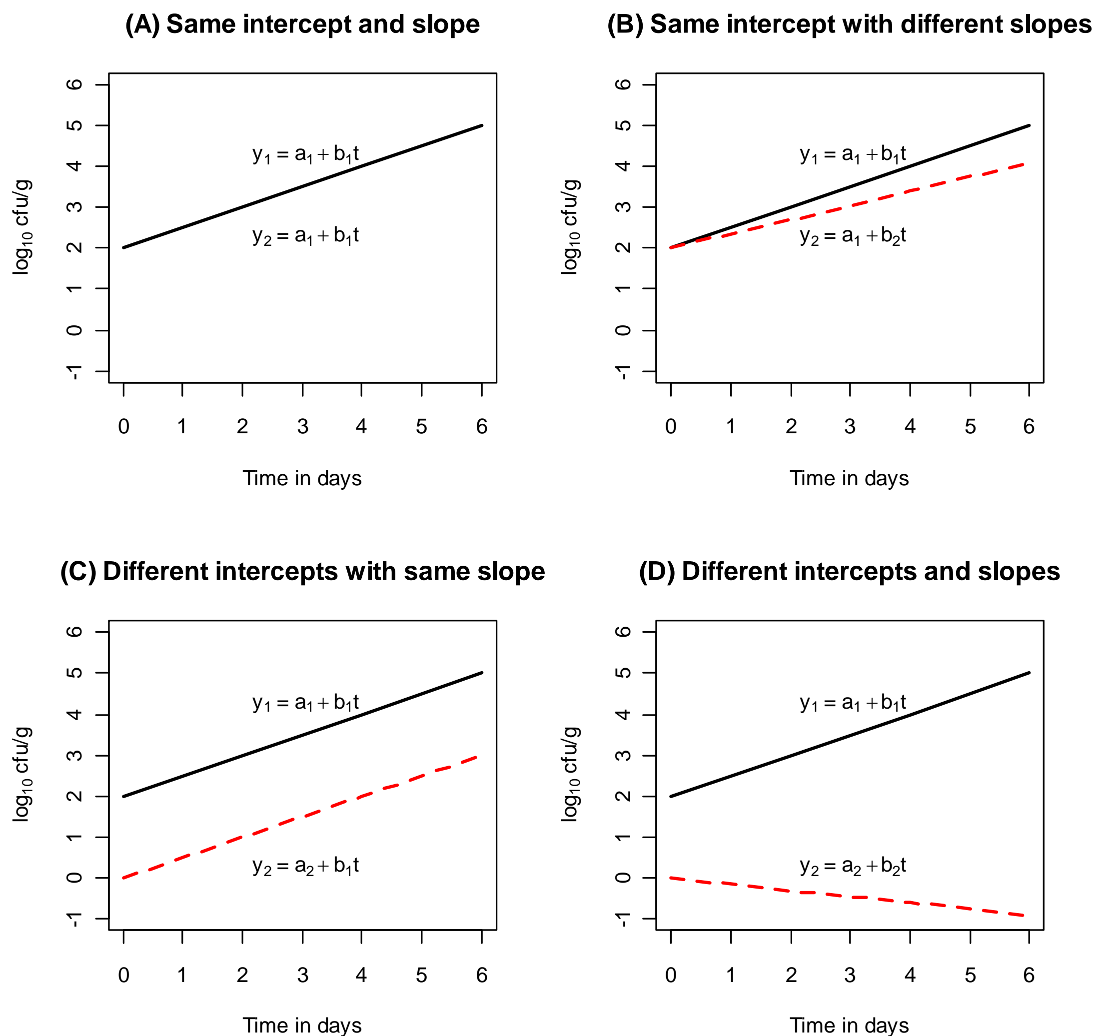


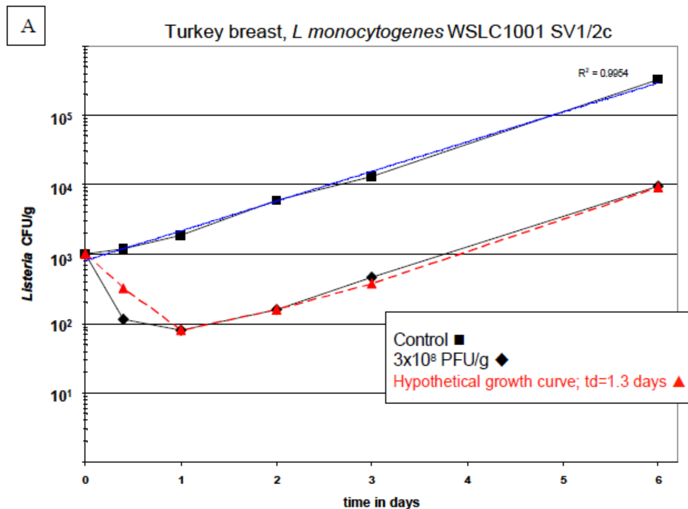
Figure 1 *Possible responses of bacteria following treatment with bacteriophage. The continuous and dashed lines represent the growth of the bacteria in the untreated and phage treated experiments respectively*

Example illustrating Case C

The changes in concentration for *L. monocytogenes* (strain WSLC1001 of serovar 1/2c) on turkey breast, treated with $3x10^8$ pfu/g of bacteriophage A511 is used as an example to illustrate the typical response of *L. monocytogenes* to treatment in non-liquid foods. Experimental data (as provided by the Applicant) is given in Figure 2A. The untreated experimental data is indicated by the solid square symbols. The solid straight line fitted to the logarithm of the *L. monocytogenes* concentration indicates that the cells are growing exponentially at a rate dependant on the food properties and temperature.

Following treatment with bacteriophage preparation the *L. monocytogenes* concentration (solid diamond symbols) is initially reduced by around 1 log unit after six hours with only a small additional reduction up to 1 day. No further reduction is observed after 1 day and the surviving *L. monocytogenes* cells grow exponentially at a rate similar to the untreated experiment (indicated by the dashed lines from 1 to 6 days).

Results of FSANZ's statistical analysis of these same data (using the linear regression model) is given in Figure 2B. Here, the continuous line is the fitted line for the untreated experiment and the dashed line is the fitted line for the phage-treated experiment, as generated by the linear regression model. The growth rates for both experiments are identical and are represented by parallel lines. The analysis of efficacy and ongoing technological function of this example is given in Annex 1.



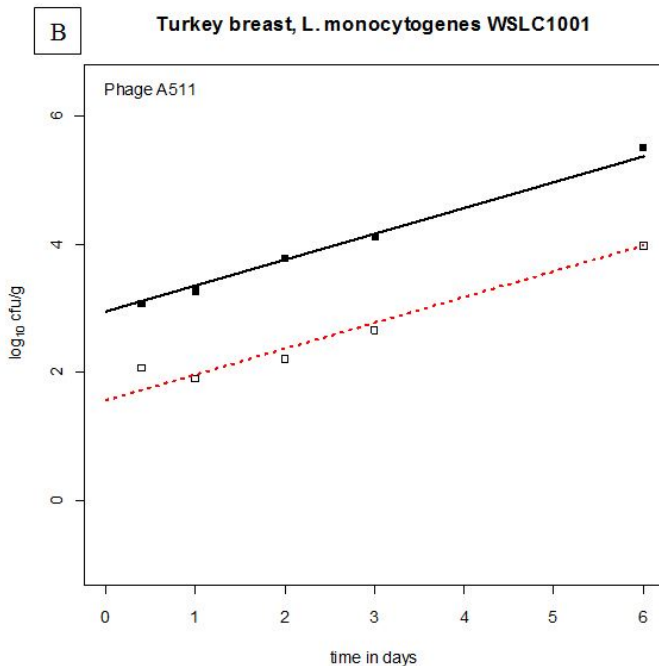


Figure 2 *Comparison of the Applicant's (A) and FSANZ (B) analysis of experimental data for turkey breast treated with an initial concentration of 3x10^{8} pfu of bacteriophage A511 per gram of turkey breast stored at 6°C. The continuous and dashed lines represent the growth of* L. monocytogenes *in the untreated and phage treated turkey breast experiments respectively. The doubling time, td, from the FSANZ analysis is 1.1 days.*

5.2.2.1 Solid foods

Results of the statistical analysis are given in Table 3. A total of eight solid foods: ham, turkey breast, sliced smoked turkey breast, hot dogs, mixed seafood, smoked salmon, Iceberg lettuce and cabbage representing 17 experiments were extracted for evaluation. All untreated foods supported the growth of *L. monocytogenes* strains Scott A and WSLC1001 at 6°C.

Graphs illustrating the statistical representation of non-liquid foods covered in the experiments are given in Figure 3. Statistical interpretations on efficacy and ongoing technological function is summarised below:

a) Efficacy

Most of the solid foods fell into the typical pattern illustrated by case C of Figure 1 and Figure 2A. Statistically significant efficacies (reductions in bacterial concentrations on initial treatment) were achieved for all non-liquid foods analysed (see Column 4 of Table 3). Further illustrations and descriptions relating to specific non-liquid foods are given in Annex 1.

b) Ongoing technological function

Ongoing technological function was assessed by comparing the growth rates of the untreated and bacteriophage treated experiments. In 12 of 15 experiments the growth rates (slopes) were found to be the same in both the untreated and treated foods, following the pattern of Figure 1C (indicated by "Yes" in column 5 in Table 3).

The results with both *L. monocytogenes* strains were consistent and support the claim of no ongoing technological function for four foods tested (ham, turkey breast, sliced smoked turkey breast and Iceberg lettuce). Further explanation of the statistical evaluation is provided in Annex 1.

Table 3 *Summary table of the statistical analysis of experimental data for phage A511 treatment of different foods.*

Food Matrix	***L. monocytogenes*** **strain**	**slope (Untreated) (log_{10}/day)**	**Efficacy (Δ log reduction extrapolation to t = 0 days)**	**Same slope**	**Difference in slope (Treated) (log_{10}/day)**	**Comment**
Solid foods						
Ham	Scott A	0.37	-1.26	Yes	0	
Ham	WSLC1001	0.29	-1.43	Yes	0	
Turkey breast	Scott A	0.4	-1.4	Yes	0	
Turkey breast	WSLC1001	0.4	-1.39	Yes	0	
Sliced smoked turkey breast	Scott A	0.38	-1.27	Yes	0	
Sliced smoked turkey breast	WSLC1001	0.22	-1.2	Yes	0	
Hot dogs	Scott A	0.17	-2.62	Yes	0	
Hot dogs	WSLC1001	0.2				Insufficient data
Hot dogs	WSLC1001	0.15				P100 Insufficient data
Mixed seafood	Scott A	0.56	-1.64	No	-0.18	Growth rate of phage treated sample was positive but not parallel
Mixed seafood	WSLC1001	0.22	-2.16	Yes	0	
Smoked salmon	Scott A	0.27	-0.51	No	-0.27	Growth rate of phage treated sample was zero
Smoked salmon	WSLC1001	0.11	-0.31	Yes	0	
Smoked salmon	WSLC1001	0.081	-0.55	Yes	0	P100
Iceberg lettuce	Scott A	0.14	-2.45	Yes	0	
Iceberg lettuce	WSLC1001	0.12	-2.17	Yes	0	
Cabbage	Scott A	0.33	-2.45	No	-0.07	Growth rate of phage treated sample was positive but not parallel
Cabbage	WSLC1001	0.08				Insufficient data
Liquid Foods						
Chocolate milk	Scott A	0.33				Insufficient data
Chocolate milk	WSLC1001	0.33	-1.65	No	-2.24	
Mozzarella cheese	Scott A	0.24	-2.85	No	-0.36	

Food Matrix	*L. monocytogenes* strain	slope (Untreated) (log_{10}/day)	Efficacy (Δ log reduction extrapolation to t = 0 days)	Same slope	Difference in slope (Treated) (log_{10}/day)	Comment
brine						
Mozzarella cheese brine	WSLC1001	0.25	-1.48	No	-1.89	

c) Differences in ongoing technological function

Three experimental results of the 18 experiments conducted on solid foods showed a difference in the slopes between the treated samples and untreated controls and are indicated by "No" in column 5 of Table 3. These results were observed only with the Scott A strain and seen in:

1. Mixed seafood
2. Cabbage; and
3. Smoked salmon

The graphs relating to these foods are given in Figure 3 of Annex 1.

For mixed seafood, the *L. monocytogenes* Scott A strain grew strongly (0.38 log/d), but with a growth rate slower than in the untreated food (0.56 log/d). The slopes are not parallel. However, this difference in growth rates, although statistically significant, is not considered to be of practical significance as it was only a slight difference and the results for WSLC1001 showed parallel lines

The growth rate of *L. monocytogenes* Scott A on treated cabbage was weak at 0.07 log/d. The concentrations observed during the six days of the study were all close to the limit of detection. Similar observations were noted for strain WSLC1001. The high efficacy of the phage treatment on cabbage is demonstrated in this case, with very low numbers of bacterial colonies surviving below detection limits. Such data are not appropriate to be statistically analysed according to the model.

This is an example of a study where additional information on the number of "independent experiments" (Guenther et al. 2009) and the number of detections at each sampling time would be needed to fully evaluate the data.

L. monocytogenes Scott A did not grow in the bacteriophage A511 treated smoked salmon during the six days of the study. The remaining colonies following bacteriophage treatment continue to grow but at a slower rate than non-treated controls. This is similar to the pattern of Figure 1D, but with a very small but statistically significant difference in slopes. The concentration of the Scott A strain after bacteriophage treatment (6 hours to 6 days) was 2.17 log (range 1.79 – 2.44 log cfu/g). These concentrations are not close to the detection limit and are unlikely to be an artefact of the experimental method.

For smoked salmon, the Scott A strain result is different to both WSLC1001 strain experiments when both bacteriophages A511 and P100 were used. In these two studies there was also limited efficacy (<1 log) but the slopes of the experiments were found to be parallel, indicating no ongoing technological function. In smoked salmon, the reason for the difference in response between the Scott A and WSLC1001 strains is not immediately clear, but may be attributed to biological strain differences often encountered in bacteria.

It should be noted that the experimental data analysed by FSANZ from Guenther et al. (2009) covers only the bacteriophage concentration of $3x10^8$ pfu/g.

The use of higher bacteriophage concentrations may change the findings for *L. monocytogenes* Scott A in smoked salmon with respect to the potential for assessing ongoing technological function.

5.2.2.2 Liquids

The typical response of bacteria to bacteriophages in liquid foods is illustrated in Figure 1D. Experiments in mozzarella cheese brine demonstrated reductions in *L. monocytogenes* concentration throughout the six days of the studies, with up to a 2.85 log reduction overall (Scott A strain). In the case of chocolate milk the bacteriophage reduced the *L. monocytogenes* concentrations to below the detection limit after six hours for strain Scott A and after 24 hours for the WSLC1001 strain (see Figure 4) suggests an ongoing technological function of phage in liquid foods due to mobility and diffusion as discussed above.

For chocolate milk, reductions in cell concentrations were only observed when the bacteriophage concentration was increased to $3x10^7$ pfu/mL, with an immediate effect plus an ongoing decline in concentrations being observed when $3x10^8$ pfu/mL was used.

The continuous decline in the slope of Figure 4 illustrates an ongoing technological function in liquid foods.

5.2.3 Summary of statistical analysis

A reduction in *L. monocytogenes* numbers within the range of 0.31 log to >3 log is achieved in solid RTE food samples treated with $3x10^8$ pfu/g (or mL) of either the P100 or the A511 bacteriophage preparations. These reductions are depicted by the reductions in intercept that are statistically significant. None of the samples produced responses depicted in Figures 1A and 1B, as statistically significant efficacies were observed in all treated samples.

It is unlikely that high initial pathogen concentrations used in the experimental situations are replicated under commercial situations. This result is not considered to be of practical significance. However, some differences in response to treatment may occur due to biological strain differences.

Based on the results of the statistical analysis, it is demonstrated that there is no ongoing technological function of the A511 bacteriophage, known to be similar to P100 in biology and function, in solid foods. Where possible comparisons between A511 and P100 were made (hot dogs and smoked salmon) and the results were found to be comparable (see Table 3 and Annex 1).

Comments on experimental design and data

The Günther (2007) and Guenther et al. (2009) experimental data have some elements that need to be considered in the interpretation of the statistical analysis. The first point is that the *L. monocytogenes* concentration data in the figures of Guenther et al. (2009) appear to be the logarithm of the arithmetic mean. This finding is apparent due to the asymmetry of the error bars. An alternative approach would be to present the mean of the logarithm of the concentrations. The second point is the presentation of error bars around concentrations where all experimental results at a sampling time are presented as “not detected”. It is not apparent how error bars could be calculated in this situation. The third point is the lack of information on the number of “independent experiments” for each food and *L. monocytogenes* strains combination. Guenther et al. (2009) states that between 2 and 5 ‘independent experiments’ were performed.

The fourth point is to recognise the inherent difficulties of performing experiments on solid surfaces. In these experiments both the *L. monocytogenes* and bacteriophage cultures must be applied homogeneously to the surface of the solid foods in order to achieve repeatable results. For liquid foods this can easily be achieved by shaking or mixing.

5.2.4 Additional challenge studies using the P100 preparation

While the experiments described in 5.2.3 covered data obtained using mainly A511, the following summarises experimental results obtained using the P100 preparation.

Sausages

The P100 preparation has been used in studies investigating the reduction of *L. monocytogenes* on Brazilian fresh sausage (Rossi et al. 2011). For these studies, the initial inoculum of *L. monocytogenes* was $2.1x10^4$ cfu/g, while the phage treatment was $3.0x10^7$ pfu/g (greater by a factor of 10^3). Both the inoculum and phage treatment were applied to the prepared raw meat containing pork meat, lard, nitrate, flavouring and spices before the sausage casing was applied.

The treated sausages had counts reduced by approximately 2.5 log compared to controls at both 0 and 10 days (shelf life limit) stored at 4°C. However, after 10 days storage the treated sausages still contained *L. monocytogenes* at an increased level to that determined after the initial phage treatment, indicating the treatment did not completely eliminate the bacteria upon treatment, and it was subsequently able to grow.

An outcome of the study was that higher phage concentrations (minimum 10^8 pfu/g of food product) may be needed to reduce *L.monocytogenes* numbers below detectable concentrations. In the case of sausages eaten uncooked (as with Brazilian sausages), the authors suggest the addition of P100 just before casing.

Fish fillets

Studies have been undertaken on two different types of fish fillets – raw salmon fillet (Soni and Nannapaneni 2010) and catfish (Soni et al. 2010) – using the P100 preparation for surface treatment. These studies were conducted using a 2-strain mix of equal volumes of *L. monocytogenes* (Scott A and EGD).

For salmon fillet, high phage concentration treatment of 10^8 pfu/g produced reductions of 1.8, 2.5 and 3.5 log cfu/g of *L. monocytogenes* from initial loads of 2.0, 3.0, and 4.5 log cfu/g respectively, when stored at 4° or 22°C. The authors noted that the phage preparation remained quite stable on the salmon fillet surface over a 10 day storage period, where there was only a small loss of 0.6 log pfu/g of phage numbers from the initial treatment of 10^8 pfu/g.

A second fish fillet study by Soni et al. (2010), using catfish fillets, assessed the influence of four parameters on the effectiveness of the P100 preparation: i) contact time, ii) concentration, iii) storage temperature and iv) storage duration. The study concluded that the success of treating artificially contaminated fillets with phage preparation was influenced by both phage contact time and initial phage dose, regardless of subsequent storage temperature. A phage contact time of 30 minutes was adequate to yield greater than 1 log cfu/g reduction of *L. monocytogenes* (initially inoculated at 4.3 log cfu/g) when treated with P100 at $2.7x10^7$ pfu/g.

Cheese

A number of studies have been conducted on cheese using both the P100 preparation and a A511 preparation. For example Carlton et al. (2005) undertook a 'proof of concept' study on the Applicant's P100 preparation to treat *L. monocytogenes* intentionally added to cheese. Trials were conducted studying the effects of phage dosage concentrations and frequency of phage dosing on the *L. monocytogenes* concentrations with storage times. The studies indicated high concentrations of the Applicant's phage preparation could decrease the levels of *L. monocytogenes* contamination on cheese surfaces compared to untreated control samples by at least 3.5 log, or even produce complete elimination. The authors reported recovery of phage preparations from the cheese surfaces over a period of 6 days with no significant decrease or increase in phage concentrations.

The Applicant's phage preparation has been found to produce a significant reduction of *L. monocytogenes* on Munster (surface ripened) cheese surfaces of approximately 3.5 log compared to untreated controls (Schellekens et al. 2007). The study investigated the effects of timing, frequency and dosage of the phage preparation. *L. monocytogenes* levels on the cheese surfaces could be reduced to below detection limits even after 21 days storage of the final cheese by treating frequently with high phage doses during the cheese production process. The researchers determined that the phages can remain active on the cheese surface for several days. The most important conclusion from the study was that an appropriate high concentration (to be determined by plant trials) of phage is required for initial treatment to successfully eliminate all bacteria.

While investigating the efficacy of an A511 bacteriophage preparation on reducing *L. monocytogenes* on soft ripened white mould and red-smeared cheeses, Guenther and Loessner (2011) also concluded that:

(i) The initial phage concentration needs to be sufficiently large (in the order of 10^8 pfu/cm^2 for surface treatment). The appropriate concentration needs to be optimised for each product and production process.

(ii) The phage preparation needs to be applied early in the production process before the *L. monocytogenes* population has grown too large.

Cooked ham

Two bacteriophage treatment studies were undertaken on cooked ham: Holck and Berg (2009) and EBI (2011).

The Holck and Berg (2009) study investigated the combined effect of the application of bacteriophage P100 together with a protective culture of *Lactobacillus sakei* TH1. A single experiment where P100 was applied at 5×10^7 pfu/cm^2 without the *Lactobacillus* culture achieved a 1 log reduction in *L. monocytogenes* numbers. No ongoing effect on the *L. monocytogenes* numbers was observed during the 28 days of the study as bacterial growth was resumed.

The EBI study, provided in Annex 12 of the Application, applied the P100 bacteriophage at two levels: 5×10^6 and 1×10^7 pfu/cm^2, together with three combinations of organic acid salts (lactate and diacetate) on cooked ham. Survival studies showed the additional of the organic acids had no effect on the number of viable bacteriophage during the three days of the trial.

The experimental results from this study were analysed using the same statistical methods used on the Günther (2007) and Guenther et al. (2009) studies (see Section 5.2.3 and Annex 1).

The mean efficacy achieved by the P100 was -0.76 and -2.17 log at the lowest and highest concentrations, respectively. These results highlight that small changes in bacteriophage concentration, in this case a doubling, can have large effects on *L. monocytogenes* numbers resulting in more than ten times greater reduction.

The slopes were parallel, indicating no ongoing technological function in two of the three experiments at the lowest bacteriophage dose of $5x10^6$ pfu/cm^2. When the bacteriophage dose was increased to $1x10^7$ pfu/cm^2, all three experiments showed no ongoing technological function.

5.3 Phage-resistant bacterial strains

The efficacy of any bacteriophage-based preparation would be reduced in the presence of phage-resistant bacterial strains. Bacterial resistance development can occur as a stress response to any bactericidal treatment applied in food processing, such as the use of sanitisers, high pressure treatment and cleaning agents.

The Applicant maintains efficacy of the formulation by monitoring phage susceptibility in the food processing facilities where the P100 preparation is used. To date, no P100 bacteriophage-resistant *L. monocytogenes* strains have been identified in any of the food-related strains they have isolated.

A single mutation has been observed involving a cell wall component of serovar 1/2 and 3 strains. However, P100 has been shown to adapt epigenetically in response to this change in the host to continue to be effective in lysing host cells.

The only restriction/methylation (RM) system reported for *Listeria* is an isoshizomer of SauA1[8] which recognizes the genetic sequence GATC which is completely absent in the P100 genome. The P100 genome contains very few sites recognised by restriction enzymes, possibly as an adaptive response to the above mentioned RM resistance mechanisms present in the host bacteria. The likelihood of new resistance mechanisms arising due to recognition by bacterial restriction enzymes is therefore minimal for the P100 bacteriophage. Additionally the vigilant screening of factory derived hosts and continuous stability and efficacy testing carried out provides adequate assurance that phage resistance phenomena is understood and addressed by the Applicant in an ongoing manner.

The European Food Safety Authority (EFSA) also acknowledges that while bacteria have developed specialised bacteriophage-defence mechanisms, bacteriophages also continue to adapt to these altered host systems (EFSA 2009). Bacterial restriction enzyme systems (endonucleases) recognize and cut foreign DNA. Phages can protect themselves against these enzymes by modifying their own DNA or by modifying the genetic sequences recognised by these enzymes.

It has been demonstrated that spontaneous mutations conferring bacteriophage resistance may actually have deleterious effects on these bacteria, and do not necessarily confer an evolutionary advantage in the absence of phages. Studies have demonstrated that such mutations occur at the same rate in bacterial populations whether phages are present or not (Carlton 1999). However, mutations are detrimental in general and any effects related to phage resistance may disappear when the phage presence is negated either by reversion to the non-mutant form, or by higher survival due to fitness of the non-mutant bacteria. In one study bacteriophage-insensitive mutants reverted to phage sensitivity in the absence of

[8] Restriction endonucleases that recognize the same sequence

selective pressure (O'Flynn et al. 2004).

Further, acquiring resistance to phage infection by mutation generally leads to a loss in fitness of the bacterium in its natural environment (Molineux 2006).

Carlton et al. (1999) state that phage resistant mutations will lead to the expression of low efficacy of the bacteriophage, but these events are of a low frequency, in the order of 10^{-7} (i.e. one non-sensitive bacterium could occur in 10 million bacteria). The Applicant contends that at the frequency of pathogens typically present in food, where the aim is to generally achieve 2-4 log reductions of target bacterial numbers, these occurrences are infrequent enough to be of no relevance.

EFSA highlights a number of strategies that have been indicated in the literature that may be used to overcome, or limit, development of resistance, These include the prevention of the recycling of the bacteriophages in the reservoir of the pathogen by alternating use of different bacteriophages (either in a cocktail of several bacteriophages or in consecutive treatments). In a factory setting it is therefore important to prevent the re-entry of phage-treated product/packaging into the processing factory. The presence of *L. monocytogenes* in factory environments is minimised by effective cleaning and sanitising programs.

The P100 formulation is designed solely for use on foods and not as a surface decontaminant in production environments. Therefore the risk of encounter of P100 with environmental *L. monocytogenes* strains is reduced. Additionally, the intended use of P100, which is prior to packaging, means that any resistant organism which could emerge on a rare occasion would leave the processing premises enclosed within the package, thereby preventing re-entry and spread of this organism within the production facility.

In summary, bacterial resistance can occur naturally or be acquired via normal stress-response mechanisms following exposure to any bactericidal treatment (biological, chemical or physical). Given the nature of application (high dosage of bacteriophage to low numbers of target bacteria), and use of GHPs in the production facility, the potential for reduced efficacy of the P100 preparation due to the presence of phage-resistant *L. monocytogenes* is minimal. This view is consistent with that of other international regulators regarding the application of bacteriophages in food manufacture.

5.4 Conclusion

The stated purpose for this bacteriophage preparation, namely for use as a processing aid to reduce or eliminate *L. monocytogenes* in a range of foods, is clearly articulated in the Application. The evidence presented to support the proposed uses, provides adequate assurance that the P100 bacteriophage preparation, in the form and prescribed amounts, is technologically justified and has been demonstrated to be effective in achieving its stated purpose.

Data presented by the Applicant and analysed by FSANZ demonstrate the efficacy and non-ongoing technological function of the P100 (or equivalent) preparation across a range of different solid RTE foods. These foods include processed meat products (hot dogs, turkey breast and ham), processed dairy products (mould ripened cheese), fish products (smoked salmon, mixed seafood) and horticultural products (salads). FSANZ considers this range sufficient to demonstrate the technological function as described, and confirmed by the additional challenge studies based on the P100 preparation alone.

Guenther et al (2009) noted that active bacteriophage could be isolated up to 6 days after application when stored at 6°C but while the bacteriophage may be intact, they are immobilised soon after addition to solid food. Therefore they are incapable of performing an ongoing technological functional due to limited diffusion.

The authors note that phage treatment is less successful for solid food with uneven surface where bacteria may be able to find refuge and escape contact with phage preparations. Bacteriophages are also sensitive to light and may be inactivated under commercial conditions. The reduction in moisture content of the samples appears to significantly reduce the continuous activity of the bacteriophage according to this analysis.

Considering the results of the statistical analysis performed by FSANZ, the functionality of bacteriophages in commercial conditions and the weight of evidence, it is concluded that the P100 does not perform an ongoing technological function in treated foods.

The phage preparation cannot be assumed to be a complete single treatment that will destroy and eliminate all *L. monocytogenes* from treated food; it should be considered an extra technology that can assist in reducing *L. monocytogenes* levels in combination with GHP. If the *L. monocytogenes* population has not been completely eliminated by this treatment, the population can regrow at the same rate as the untreated samples, but from lower initial levels. Like other treatment technologies, food manufacturers will need to conduct their own trials to determine the efficacy of phage treatments to account for strain differences in their products and make a determination if it would be a beneficial hurdle technology taking into account a range of other factors (see section 1.2), and cost of the treatment.

The risk of the potential presence of phage-resistant *L. monocytogenes* strains in the processing environment (and subsequent reduced efficacy of the P100 preparation) would be minimised through application of GHP. The use of the P100 preparation is not a replacement, rather an additional hurdle used in the production of ready-to-eat foods to reduce levels of *L. monocytogenes*.

6 Hazard Assessment

EFSA (2009) evaluated the use and mode of action of bacteriophage in food production and concluded that each bacteriophage application should be considered on a case-by-case basis, to enable the assessment of a) the absence of a lysogenic cycle (temperate phages), b) the absence of any potential virulence factors and c) the inability to facilitate general transduction (exchange of genetic material) between bacteria.

The hazards of the P100 bacteriophage preparation were assessed by considering the following:

(1) the biological and chemical characteristics of the production and host organisms, including the use of P100 in food production processes

(2) potential allergenicity of the encoded proteins

(3) genomic and bioinformatic studies on potential toxicity of the encoded proteins

(4) potential for lysogeny

(5) potential for transduction

(6) potential to increase phage resistance in *L. monocytogenes*

6.1 Hazard of the production and host organisms

6.1.1 P100

6.1.1.1 Lysogeny

No evidence of lysogenic characteristics or integrase[9] functions was found in the bioinformatic analysis (Carlton et al., 2005). The information coded within the P100 genome was analysed using Vector BTI software (version 8; InforMax) and the annotated genome and all predicted open reading frames (ORF), gene products and secondary structures confirmed by visual inspection. Nucleotide and amino acid sequence alignment searches (BlastN, BlastX and BlastP) were performed with Vector NTIs integrated BLAST engine which used the non-redundant databases available through NCBI websites.

No lysogenic 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 Applicant has demonstrated that potential lysogenic modules are absent in the P100 genome.

6.1.1.2 Transduction

The inability of the family of P100 bacteriophages to undergo transduction has been demonstrated by Klumpp et al. (2008). Therefore the information submitted by the Applicant has demonstrated the inability of P100 to mediate generalised or specialised transduction.

6.1.2* Safety of the production organism *(L. innocua)

The safety of the production organism is an important consideration in the safety assessment of bacteriophage production. The primary issue is the toxicogenic potential of the production organism, that is, possible synthesis of toxins by the production strain, and the potential for the carryover of these into the bacteriophage preparation (Pariza and Johnson 2001).

The Applicant describes the procedures adhered to in procurement and handling of the host organism. Certified stocks are obtained and all stock and working solutions are stored at -80°C to ensure the stability of the host organism. The seed stock is tested to ensure that it is a monoculture and all records are kept, with adherence to GMPs and SOPs in handling, use and storage of the host organism.

There are no safety concerns in the unlikely event that residual *L. innocua* remains present in the P100 preparation as *L. innocua* is not a pathogen. Additionally, no safety concerns are raised with any other residual materials that may remain in the P100 preparation as manufactured by the process supplied by the Applicant.

6.2 Assessment of toxicity and allergenicity

Information submitted:

Carlton R, Noordman WH, Biswas B, de Meester ED, Loessner MJ (2005) Bacteriophage P100 for control of *Listeria monocytogenes* in foods: Genome sequence, bioinformatic analyses, oral toxicity study, and application. Regulatory Toxicology and Pharmacology 43: 301 – 312.

[9] An enzyme that facilitates integration or excision of prophage into the bacterial chromosome.

6.2.1 Potential toxicity

The assessment focuses on whether:

- there is any evidence, from bioinformatic analysis, of the proteins expressed in P100 having amino acid sequences that have similarity with sequences in known protein toxins;
- an oral toxicity study raises any safety concerns

6.2.1.1 Bioinformatic analyses

Bioinformatic analyses are useful for assessing whether proteins share any amino acid sequence similarity with known protein toxins. The sequence of the P100 double-stranded genome was obtained using a basic 'shotgun' cloning strategy supplemented with primer walking. The fully annotated sequence is available from GenBank (http://www.ncbi.nlm.nih.gov/nuccore/DQ004855) Accession No. DQ004855.

Commercial software (Vector NTI, version 8) was used to analyse the entire genome for potential open reading frames (ORFs). This approach is valid since there are no introns present in the P100 genome. An ORF was defined as a region following a start codon (ATG, TTG or GTG) with a suitable ribosomal binding site and with a minimum size coding for 40 amino acids. A total of 174 putative protein coding regions were identified along with the protein products that ranged from 5 kDa to 146 kDa.

Alignment searches were then carried out for each of the 174 putative ORFs and protein products using BLAST (Basic Local Alignment Search Tool) searches of the non-redundant database available through the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). BLAST finds regions of local similarity between sequences by comparing nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. Three BLAST algorithms were utilised, BLASTN (which searches a nucleotide database using a nucleotide query), BLASTP (which searches a protein database using an amino acid sequence query) and BLASTX (which searches a protein database using a translated nucleotide query).

For 72 of the ORFs, similarities were found to genes or gene products of other phages, particularly *Listeria* phage A511 (Loessner and Scherer 1995; Klumpp et al. 2008) and *Staphylococcus aureus* phage K (O'Flaherty et al. 2004). There are no similarities between P100 genes or gene products and any genes or proteins known to play a direct or indirect role in the pathogenicity of *L. monocytogenes* or any other infectious, toxin-producing or otherwise harmful organisms.

6.2.1.2 Oral toxicity study

A repeat dose oral toxicity study was conducted over a total period of eight days in order to examine the potential toxicity of P100. The test species was Wistar rats that were approximately eight weeks old on the day of treatment. One mL of $5x10^{11}$ pfu/mL P100, suspended in phosphate buffered saline (PBS), was administered daily by gavage to five female and five male rats for five days followed by a 3-day recovery phase.

A control group of five female and five male rats were treated with 1 mL PBS under the same conditions. The rats were observed daily for clinical signs. On the eighth day of the study all animals were sacrificed and examined for macroscopic changes in organs.

There were no deaths, clinical signs, macroscopic changes or a reduction in body weight gain.

6.2.1.3 *Endotoxins*

Endotoxins are lipopolysaccharide complexes found in the outer membrane of the cell wall of Gram-negative bacteria. They remain associated with the cell wall until disintegration of the bacterium (e.g. following bacteriophage-induced lysis), and their release can elicit a variety of adverse effects in animals (http://www.textbookofbacteriology.net/endotoxin.html). *L. monocytogenes* is a Gram-positive bacterium. A report that this microorganism may contain an endotoxin-like compound (Wexler and Oppenheim 1979) was subsequently questioned (Maitra et al. 1986). No safety concern is raised regarding effects on humans as a result of the lysis of target *L. monocytogenes* cells by P100 phage.

6.2.2 Potential allergenicity

The amino acid sequences of the 174 potential gene products predicted to be encoded by the P100 genome (refer to section 6.2.1.1) were each compared with all known allergen sequences contained in a reference allergen database (Food Allergy Research and Resource Program – FARRP) using AllergenOnline (http://www.allergenonline.org). The criterion used to indicate potential allergenicity was a full length identity (measured by E-value[10]) with an allergenic protein.

One match was found – a 419 amino acid polypeptide (gp71) which showed a local similarity (E-value = 8×10^{-10})[11] in its C-terminal domain to an amino acid sequence in wheat ɣ-gliadin. Gliadins are one type of protein found in wheat flour that are involved in food allergy to wheat (Battais et al. 2005). A sequence comparison of the relevant region of the gp71 with the known immunoreactive epitopes of wheat gliadin indicated that there was no match and that there was no identical stretch of residues spanning more than five identical amino acids. The gene sequence coding for gp71 is also similar to gene sequences in other phages and is therefore not unique to P100.

Additional to this, the location of orf71 in the P100 genome with putative DNA recombination/replication elements suggested that the protein is involved in DNA replication and, as such, is unlikely to be present in the mature phage particle.

6.2.3 Conclusion

Based on the weight of evidence, no toxicity or allergenicity concerns are raised with the use in food of P100, as prepared by the manufacturing process specified by the Applicant.

7 Dietary Exposure

[10] The Expectation value (E) is a calculated value that reflects the degree of similarity of the query protein to its corresponding matches.

[11] Comparisons between highly homologous proteins yield E-values approaching zero, indicating the very low probability that such matches would occur by chance. A larger E-value indicates a lower degree of similarity. Commonly, for protein-based searches, hits with E-values of 10^{-3} or less and sequence identity of 25% or more are considered significant although any conclusions reached need to be tempered by an investigation of the biology behind the putative homology (Baxevanis 2005).

Processing aids perform their technological function during the manufacture of food. Information contained in the Application on the use of P100 as a processing aid supports the conclusion that negligible levels would be present in the final food. A dietary exposure is considered unnecessary for this assessment. Any ingested bacteriophages pass through the human gut without causing harm.

8 Response to Risk Assessment Questions

Is the P100 bacteriophage preparation suitably well characterised?

The P100 bacteriophage has been identified as belonging to the Order *Caudovirales,* Family *Myoviridae*, subfamily *spounaviridae*, genus *twortlike* and species *listeria* phage P100. The host (production) organism is a non-pathogenic type strain of *Listeria innocua* (ATCC 33090, DSM 20649, NCTC 11288, SLCC 3379). The bacteriophage P100 and production organism are completely characterised.

Does the P100 preparation achieve its stated technological purpose?

FSANZ has made an assessment of the efficacy and the possibility of an ongoing technological function when the P100 preparation is used for the stated purpose. The P100 is effective in reducing numbers of *L. monocytogenes* in treated foods. The overall weight of evidence, noting the restricted functionality of the bacteriophage in commercial conditions and in non-liquid food matrices, supports the conclusion that P100 has no ongoing technological function in solid RTE food according to the use and levels proposed by the Applicant.

It is important to note that P100 cannot be assumed to be a complete single treatment that will destroy and eliminate all *L. monocytogenes* from treated food. It should be considered only as additional technology food manufacturers can use along with their current processes to control *L. monocytogenes.* Food manufacturers will need to determine appropriate process optimisation and SOP's (Standard Operating Practices) to establish efficacy on a case-by-case basis for different foods and different production plants and to monitor efficacy consistently.

The risk assessment reviewed the information on the possibility of emergence of bacteriophage-resistant strains of *L. monocytogenes.* The conclusion from the scientific evidence, supported by experts in the field and international regulators, is that when using bacteriophages to treat food, the development of resistance in food processing environments is minimal, provided adequate information on the use, application and disposal of unsold product is provided to food manufacturers, and that manufacturers have regard to that information. This is no different to resistance developed by bacteria as a stress response to other bactericidal treatments applied during food processing. Treated products are not expected to re-enter the processing facility. Adherence to GHP ensures phage treated product that is not appropriate to be processed for commercial sale needs to be removed from the production facility on a regular basis, along with appropriate cleaning regimes to ensure there is no build-up of bacteriophage reservoirs in the facility. Continuous screening and monitoring of host susceptibility and phage resistance development in food premises using the P100 preparation, being maintained by the Applicant.

Does the P100 preparation present any food safety issues?

No food safety issues were identified from the available toxicity data. This conclusion is supported by the absence of biologically significant homology between the P100 proteins and any known allergens or toxins.

P100 bacteriophage is only effective against bacteria of the genus *Listeria.* It cannot infect plant, animal or human cells. Ingestion or contact with P100 does not present a public health risk.

9 Conclusion

The risk assessment has considered the technological suitability, the potential hazards and any public health and safety issues of using the Applicant's phage preparation, P100 to treat food.

P100 bacteriophage preparation is unlikely to pose any health risk when used to treat solid RTE food. It was further concluded that the proposed use of P100, to reduce the levels of *L. monocytogenes* in a range of RTE foods, was technologically justified in the form and prescribed amounts, and demonstrated to be effective. There is no ongoing technological function performed by the P100 preparation in solid RTE foods.

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Annex 1

Statistical analysis of Günther (2007), Guenther et al. (2009) and Annex 12 of the Application

In order to investigate the behaviour of *L. monocytogenes* after treatment with bacteriophage a statistical analysis of experimental data was undertaken. The Applicant argues that the growth rate (and doubling time) of the bacteriophage treated and untreated experiments is the same and that the concentration of the *L. monocytogenes* is reduced within the first 24 h after treatment.

In this analysis it is assumed that there is no lag time and that growth is exponential. A number of responses of bacteria to the treatment with bacteriophage are possible and are presented in Figure 3. The "worst-case" is that the bacteriophage has no effect. In this case the plot of the logarithm of the bacterial concentration versus time would be the same for the untreated and treated experiments (case (A) in Figure 3) and the intercepts (a_i) and slopes (b_i) would be identical.

A second case (B) could be that the phage does not reduce the initial concentration, but has an ongoing effect on the bacterial population as indicated by a reduced growth rate.

A third case (C) is where the phage initially reduces the bacterial concentration and then the growth rates are identical.

A fourth case (D) is where there is an initial reduction in the concentration of the bacteria followed by an ongoing effect. In this case the effect is strong enough to cause an ongoing reduction in concentration.

In order to statistically assess each of these cases a linear regression followed by an analysis of variance (ANOVA) was performed on the experimental data. For case (C) where the test is to evaluate if the slopes are the same, but with different intercepts the hypothesis may be written as the following:

Null hypothesis: H_o
(Equal slopes of the growth curves)

$$y_1 = a_1 + b_1 t$$
$$y_2 = a_2 + b_1 t$$

Alternative hypothesis: H_1
(Unequal slopes of the growth curves)

$$y_1 = a_1 + b_1 t$$
$$y_2 = a_2 + b_2 t$$

The y_i's are the logarithm of the bacterial concentrations while the a_i and b_i's are the estimates of the intercepts and slopes of the growth curve.

The linear regression and ANOVA was performed using the statistical software R (R Development Core Team 2011).

Example of strain WSLC 1001 on Turkey breast

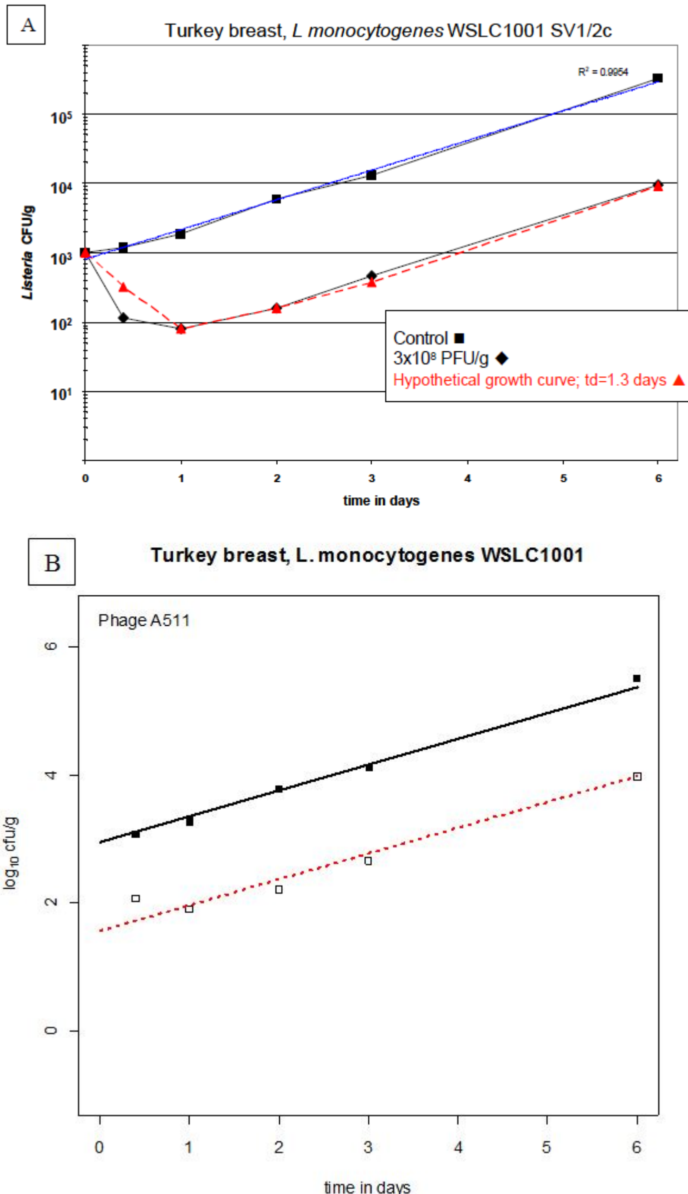

Figure 2 *Comparison of the Applicant's (A) and FSANZ (B) analysis of experimental data for turkey breast treated with an initial concentration of $3x10^8$ pfu of bacteriophage A511 per gram of turkey breast stored at 6°C. The continuous and dashed lines represent the growth of* L. monocytogenes *in the untreated and phage treated turkey breast experiments respectively. The doubling time, td, from the FSANZ analysis is 1.1 days.*

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Analysis of efficacy

In this example, the efficacy of the A511 bacteriophage can be determined by comparing the difference in the y-axis intercepts between the bacteriophage treated and untreated samples. In this experiment the intercept for the untreated group was approximately 3 log, while the intercept for the bacteriophage treated group was extrapolated back to 1.56 log, thereby indicating a reduction of 1.39 log in the treated samples. The analysis of variance found that the difference in intercepts between the bacteriophage treated and untreated experiments

was highly significant ($p<0.05$).

The analysis therefore concludes that the phage treated turkey breast produced an approximate reduction of 1.39 log of *L. monocytogenes* after the initial treatment compared to the untreated control.

Analysis of ongoing technological function

Ongoing technological function was assessed by an analysis of variance testing the null hypothesis that the slopes in the bacteriophage treated and control untreated groups were identical (parallel lines). In this case (Figure 1B) the null hypothesis that the slopes are identical is not rejected ($p=0.23$), supporting the argument that there is no ongoing technological function of the bacteriophage. The exponential growth rate was found to be 0.4 log/day, with a doubling time, td, of 1.1 days.

The resulting equations for the logarithm of the *L. monocytogenes* concentration in the untreated and treated experiments for turkey breast are therefore:

Untreated: $y_1 = 3 + 0.4t$

Treated: $y_2 = 1.56 + 0.4t$

These findings support the alternative statistical approach to evaluate both the efficacy and ongoing technological function of the bacteriophage using linear regression in preference to the comparison of concentrations of bacteria in the treated and untreated samples at the end of the study period as described by Guenther et al. (2009).

Table 3 *Summary table of the statistical analysis of experimental data for phage A511 treatment of different foods.*

Food Matrix	***L. monocytogenes*** **strain**	**slope (Untreated) (log_{10}/day)**	**Efficacy (Δ log reduction extrapolation to t = 0 days)**	**Same slope**	**Difference in slope (Treated) (log_{10}/day)**	**Comment**
Non-liquid foods						
Ham	Scott A	0.37	-1.26	Yes	0	
Ham	WSLC1001	0.29	-1.43	Yes	0	
Turkey breast	Scott A	0.4	-1.4	Yes	0	
Turkey breast	WSLC1001	0.4	-1.39	Yes	0	
Sliced smoked turkey breast	Scott A	0.38	-1.27	Yes	0	
Sliced smoked turkey breast	WSLC1001	0.22	-1.2	Yes	0	
Hot dogs	Scott A	0.17	-2.62	Yes	0	
Hot dogs	WSLC1001	0.2				Insufficient data
Hot dogs	WSLC1001	0.15				P100 Insufficient data
Mixed seafood	Scott A	0.56	-1.64	No	-0.18	Growth rate of phage treated sample was positive but not parallel
Mixed seafood	WSLC1001	0.22	-2.16	Yes	0	

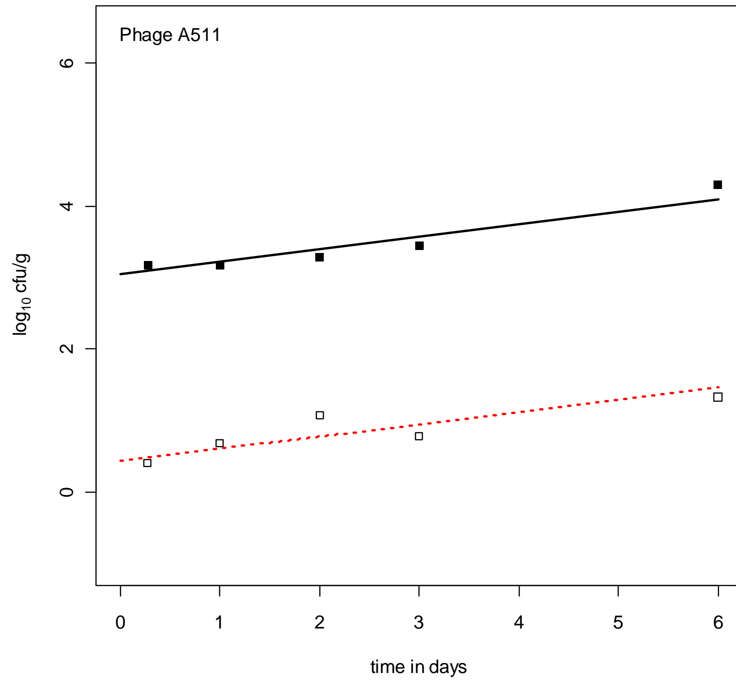
Food Matrix	*L. monocytogenes* strain	slope (Untreated) (log_{10}/day)	Efficacy (Δ log reduction extrapolation to t = 0 days)	Same slope	Difference in slope (Treated) (log_{10}/day)	Comment
Smoked salmon	Scott A	0.27	-0.51	No	-0.27	Growth rate of phage treated sample was zero
Smoked salmon	WSLC1001	0.11	-0.31	Yes	0	
Smoked salmon	WSLC1001	0.081	-0.55	Yes	0	P100 bacteriophage
Iceberg lettuce	Scott A	0.14	-2.45	Yes	0	
Iceberg lettuce	WSLC1001	0.12	-2.17	Yes	0	
Cabbage	Scott A	0.33	-2.45	No	-0.07	Growth rate of phage treated sample was positive but not parallel
Cabbage	WSLC1001	0.08				Insufficient data
Liquid Foods						
Chocolate milk	Scott A	0.33				Insufficient data
Chocolate milk	WSLC1001	0.33	-1.65	No	-2.24	
Mozzarella cheese brine	Scott A	0.24	-2.85	No	-0.36	
Mozzarella cheese brine	WSLC1001	0.25	-1.48	No	-1.89	

Figures 3 and 4 present a graphical summary of the findings of the statistical analysis for solids and liquid foods, and illustrate some of the differences in responses to bacteriophage shown by *L. monocytogenes* strains on different food matrices.

In experiments involving *L. monocytogenes* WSLC1001 strain on hot dogs (P100 and A511) and cabbage (A511) the bacteriophage was so effective at reducing the numbers that there was insufficient data to perform the statistical analysis. Low level detections of *L. monocytogenes* as seen in the hot dog and cabbage experiments were found to be inconsistent e.g. *L. monocytogenes* was detected after one day, but was not detected at six hours, two, three or six days of storage on hot dogs. These low level detections are considered to be sporadic and represent artefacts of the experimental method. The efficacy for both of these experiments was greater than -3 log.

The corresponding efficacy for the *L. monocytogenes* Scott A strain on hotdogs and cabbage was -2.62 and -2.45 log, respectively (see Table 3 in Annex 1). The efficacy results for both strains are consistent and demonstrate substantial reductions in numbers after treatment.

Greater than 2 log reductions (-2.45 log for Scott A and -2.17 log for WSLC1001) were also observed in Iceberg lettuce. The efficacy of bacteriophage A511 on *L. monocytogenes* Scott A was the same for both cabbage and Iceberg lettuce. For the mixed seafood experiments efficacies of -1.64 and -2.16 log were achieved. The results for ham, turkey breast and sliced smoked turkey breast were consistent with efficacies ranging between -1.20 and -1.43 log. The lowest efficacy was observed with the smoked salmon experiments with reductions of -0.31 and -0.5 log for bacteriophage A511 and -0.55 log for bacteriophage P100.



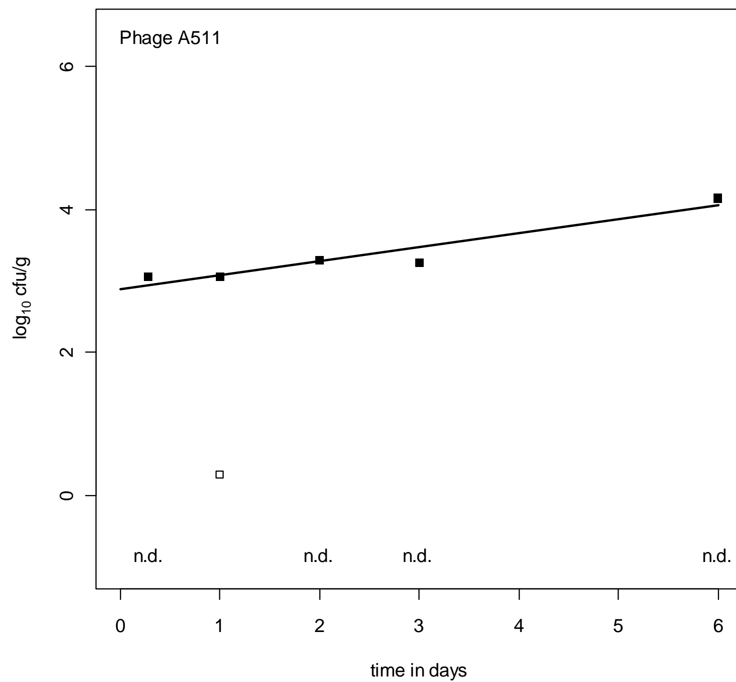
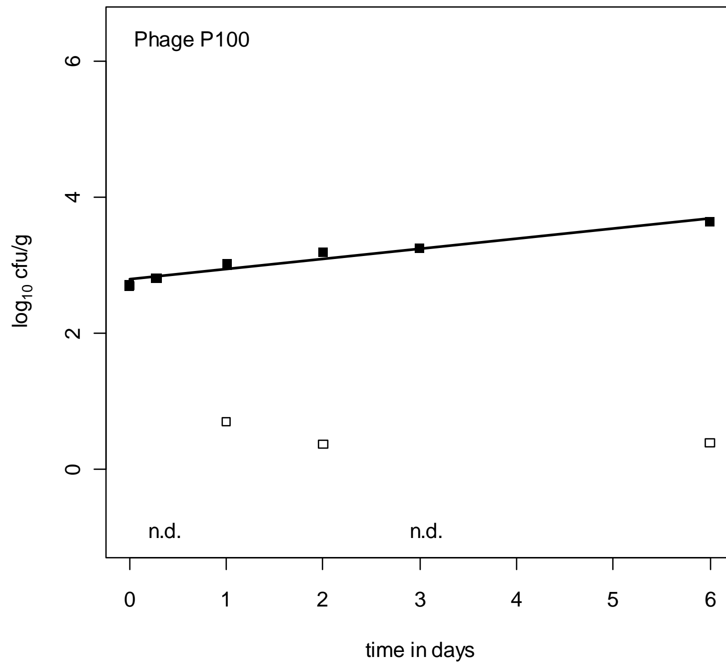
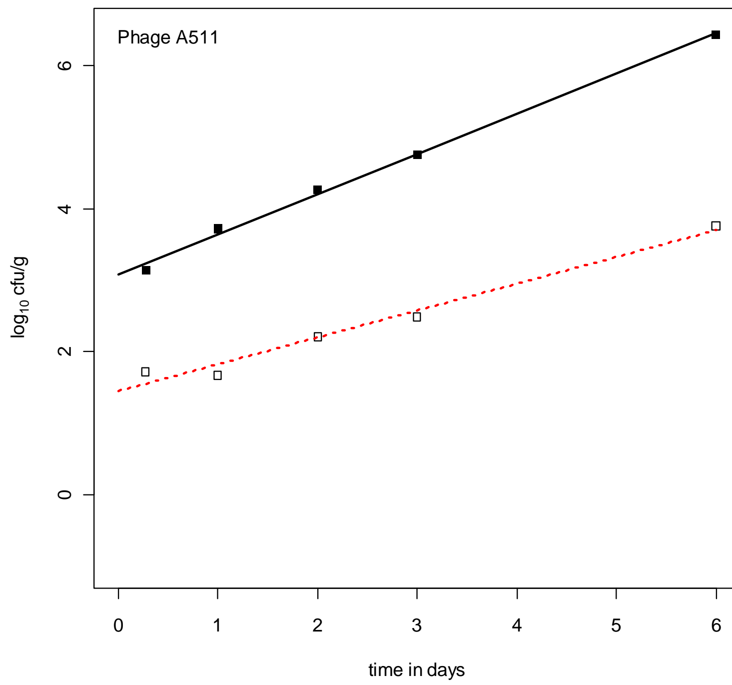
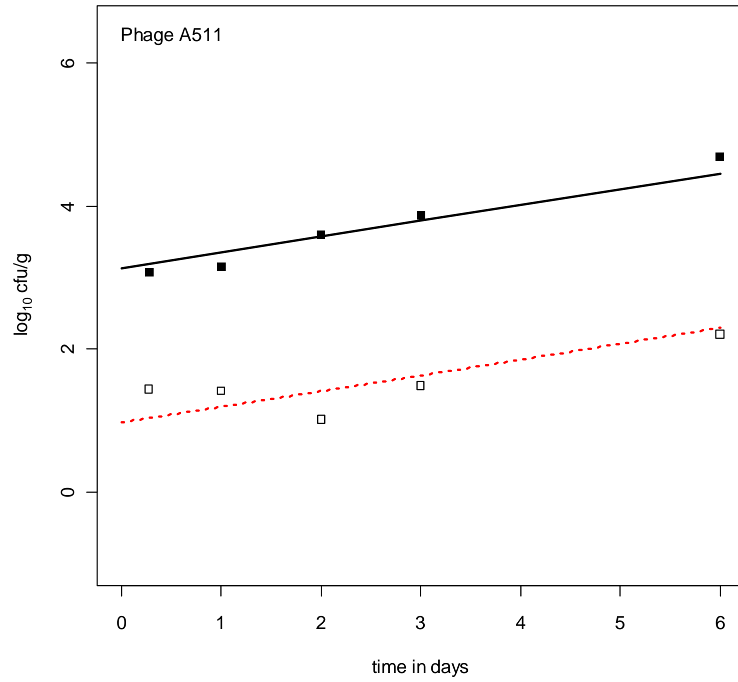
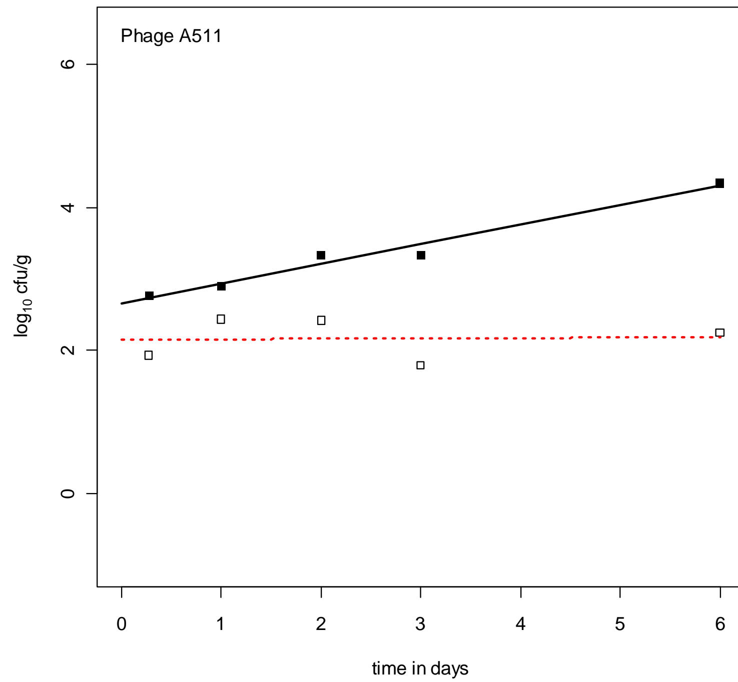


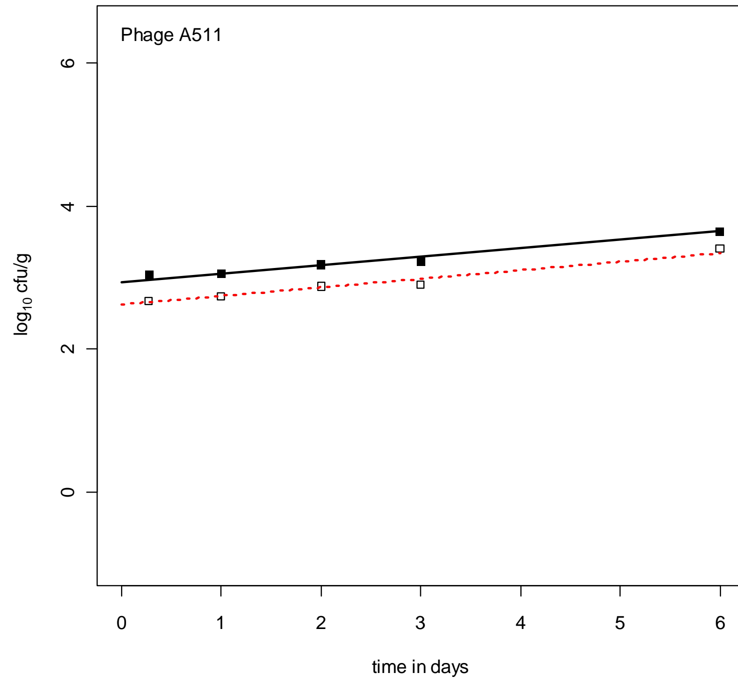
Figure 3 *Behaviour of* L. monocytogenes *in solid foods after treatment with bacteriophage A511 and P100. The continuous and dashed lines represent the growth of L. monocytogenes in the untreated and treated foods from the statistical analysis. n.d. – not detected.*


Hot dogs, Listeria monocytogenes WSLC1001
Phage P100
log10 cfu/g
0
2
4
6
n.d.
n.d.
0
1
2
3
4
5
6
time in days


Mixed seafood, L. monocytogenes Scott A
Phage A511
log10 cfu/g
0
2
4
6
0
1
2
3
4
5
6
time in days


Mixed seafood, L. monocytogenes WSLC1001
Phage A511
log10 cfu/g
0
2
4
6
0
1
2
3
4
5
6
time in days


Smoked salmon, L. monocytogenes Scott A
Phage A511
log10 cfu/g
0
2
4
6
0
1
2
3
4
5
6
time in days


Smoked salmon, L. monocytogenes WSLC1001
Phage A511
log10 cfu/g
time in days
0
2
4
6
0
1
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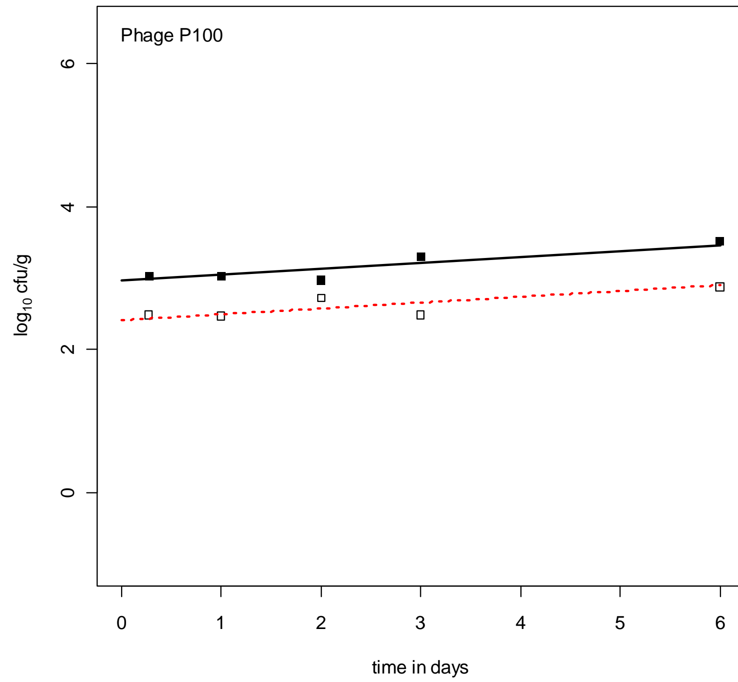
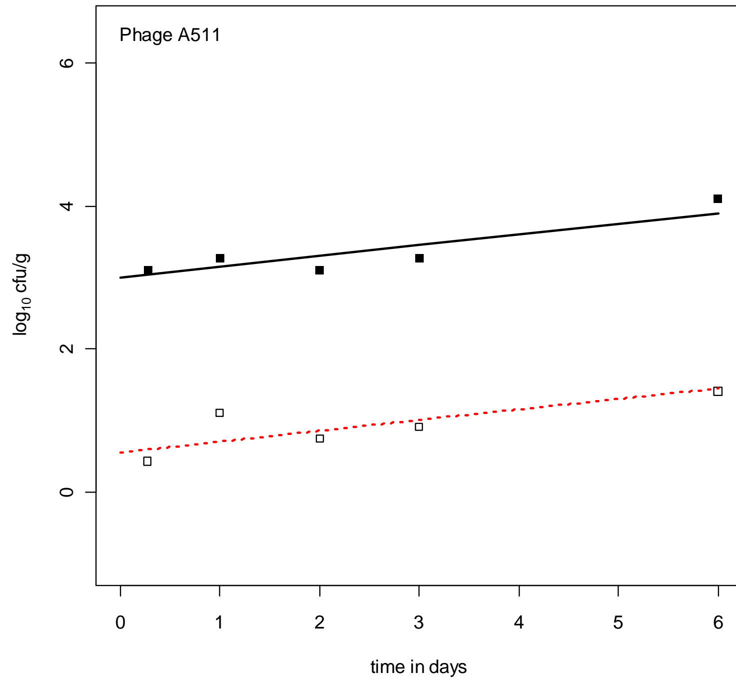
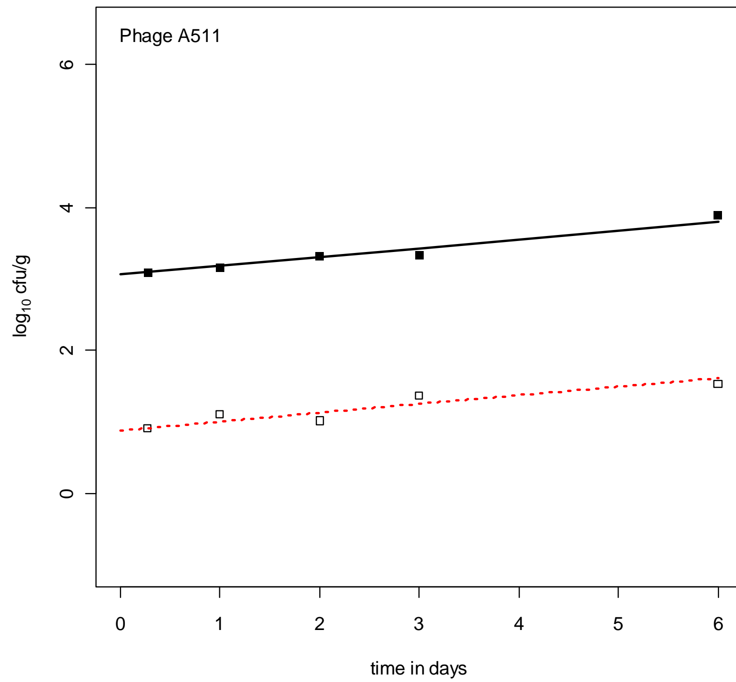
4
5
6


Smoked salmon, L. monocytogenes WSLC1001
Phage P100
log10 cfu/g
time in days
0
2
4
6
0
1
2
3
4
5
6


Iceberg lettuce, L. monocytogenes Scott A
Phage A511
log10 cfu/g
time in days
0
2
4
6
0
1
2
3
4
5
6


Iceberg lettuce, L. monocytogenes WSLC1001
Phage A511
log10 cfu/g
time in days
0
2
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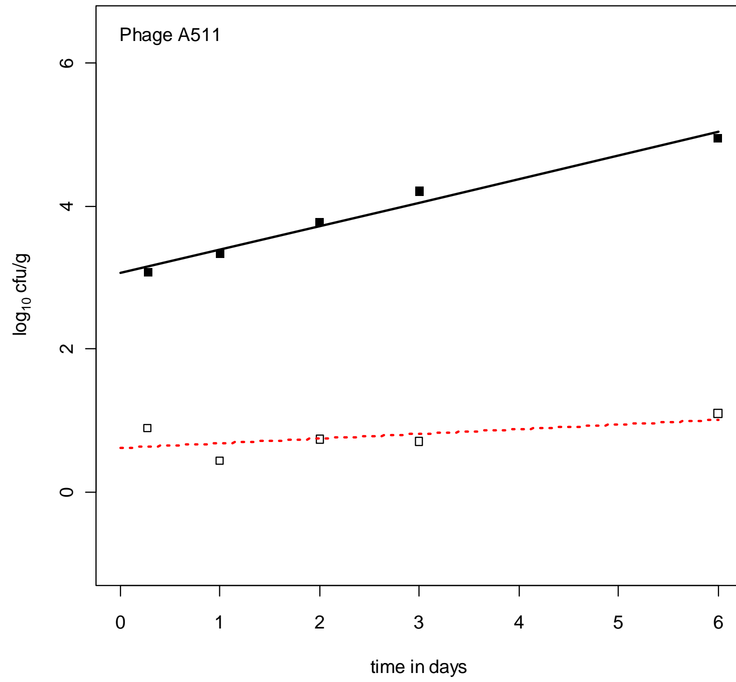
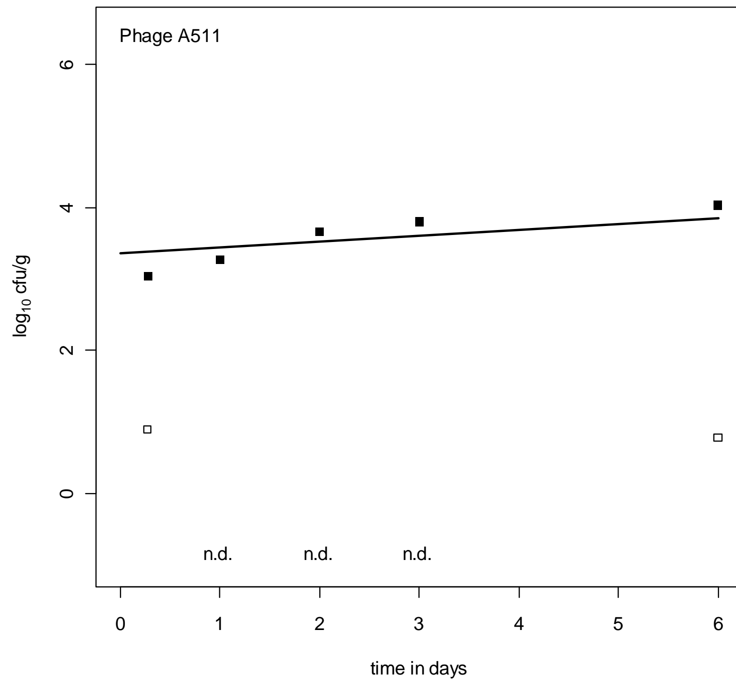
5
6


Cabbage, L. monocytogenes Scott A
Phage A511
log10 cfu/g
0
2
4
6
0
1
2
3
4
5
6
time in days


Cabbage, L. monocytogenes WSLC1001
Phage A511
log10 cfu/g
0
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n.d.
n.d.
n.d.
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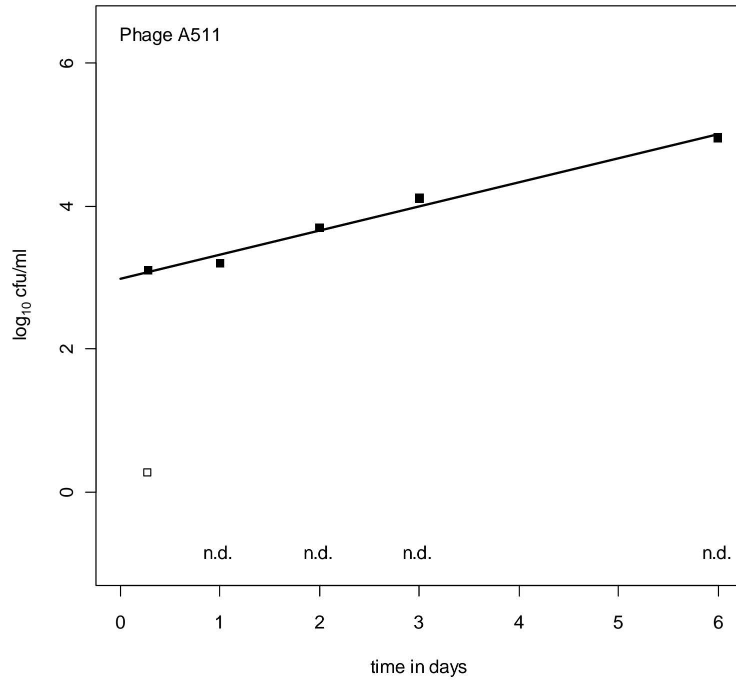
6
time in days



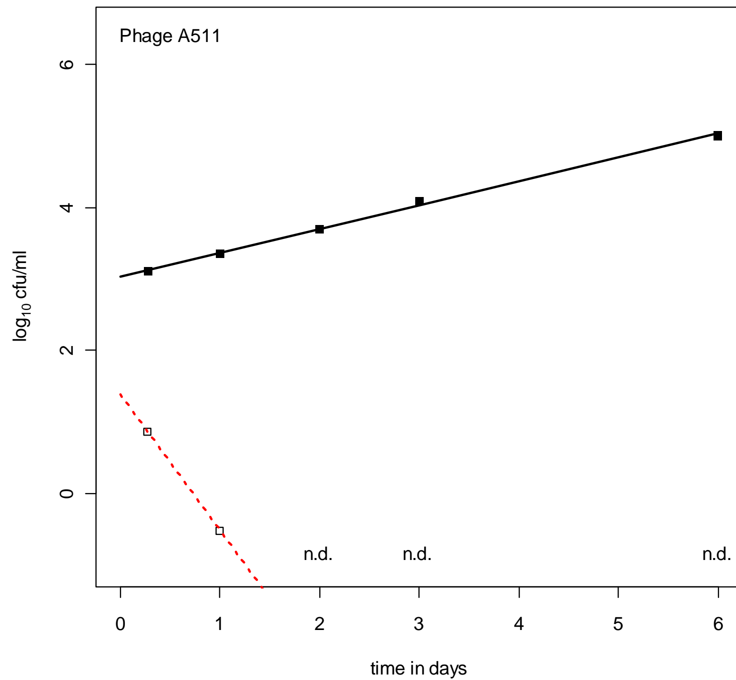
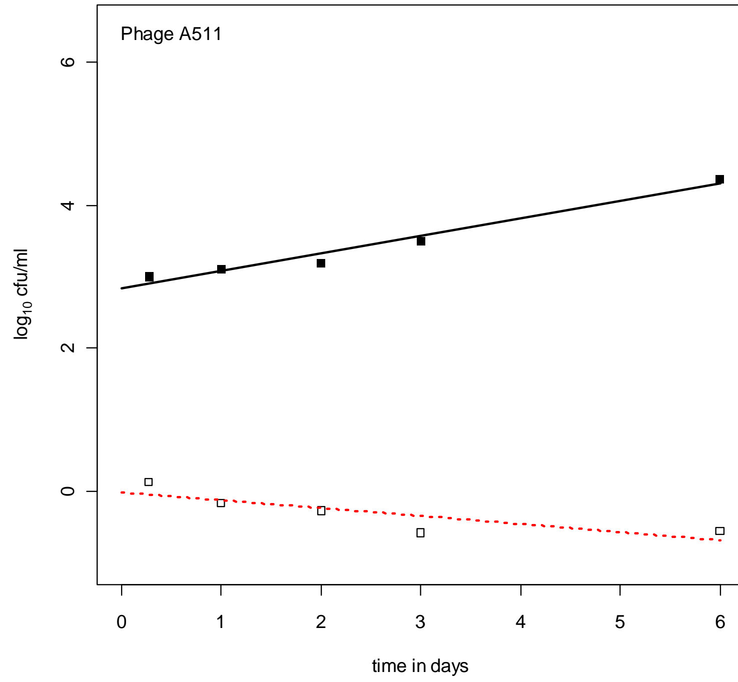
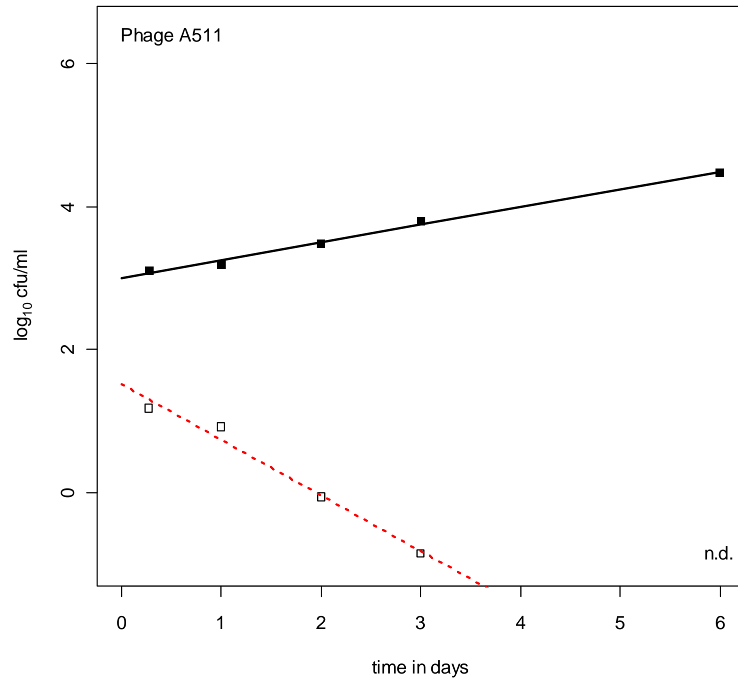


Figure 4 Behaviour *of* L. monocytogenes *in liquid foods after treatment with bacteriophage A511. The continuous and dashed lines represent the growth of L. monocytogenes in the untreated and treated foods from the statistical analysis. n.d. – not detected.*


Mozzarella cheese brine, L. monocytogenes Scott A
Phage A511
log10 cfu/ml
0
2
4
6
0
1
2
3
4
5
6
time in days


Mozzarella cheese brine, L. monocytogenes WSLC1001
Phage A511
log10 cfu/ml
n.d.
0
2
4
6
0
1
2
3
4
5
6
time in days