A study of natural lytic *Listeria* phages with decontaminating properties for use in seafood processing plants

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Dedication

The thesis is dedicated to my loving mother and late father,
my four siblings and aunties,

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Abstract

Listeria monocytogenes is a major cause of illness, associated with seafood, therefore it is important to control this pathogen in seafood processing environments. Sporadic listeriosis outbreaks and seafood recalls indicate that current treatments to control this pathogen may be inadequate. The ability to adapt to harsh environmental conditions, develop resistance and form biofilms makes this environmental pathogen difficult to control using regular disinfectants. Bacteriophages (phages) could serve as effective alternative biocontrol agents. The main objective of this study was to isolate and characterize natural lytic *Listeria* specific phages and examine their effectiveness against *L. monocytogenes* under conditions mimicking those found in seafood processing environments.

Among a group of phages isolated from a seafood waste treatment unit, three phages (LiMN4L, LiMN4p and LiMN17) were selected based on plaque morphology and their source. The three phages were distinguished by morphology, efficiency of plating (EOP) in citrate agar and differences in EOP using different *L. monocytogenes* host strains. Three phages which were found as strictly virulent by whole genome sequence analysis, had broad host ranges at 15 °C and each phage also infected either *L. ivanovii* or *L. innocua*. These phages were unstable at 60 °C for 10 min suggesting psychrotrophic properties. The three phages showed low burst sizes indicating their potential suitability as passive biocontrol agents.

Low counts of *L. monocytogenes* strains (19O9, 19DO3 and 19EO3) in late exponential phase, metabolically injured/stressed by heat and salt, lysed by the three phages at 15 ^{o}C in 30 min. The results suggested that the three virulent phages may be good candidates as biocontrol agents against *L. monocytogenes* under conditions commonly found in seafood processing plants.

The phages LiMN4L, LiMN4p and LiMN17, used as single phage or a cocktail of three phages, lysed cells adhered to stainless steel conditioned with soluble fish protein and on clean stainless steel coupons (SSC). The phage cocktail also eradicated low cell counts of about 2 log CFU/cm^2 adhered to SSC surfaces in the presence of fish proteins at 15 °C in 15 min and no re-growth of cells was observed from phage infected surfaces. This study suggested that a biofilm matrix shielded the bacterial cells from phage infection as three consecutive repeat applications of phages did not efficiently lyse undisturbed biofilm cells. Biofilm cells, once removed from the surface, showed

similar to sensitivity to that of exponential phase planktonic cells. Therefore, disruption of the biofilm structure may be required for effective phage treatments.

Phages suspended in phosphate buffered saline survived refrigeration for at least twelve months and were stable for at least 6 h under likely application conditions such as ambient temperatures and under fluorescence lighting. The three phages, either individually or as a cocktail, showed a high lytic efficacy indicating their potential to serve as bio-decontaminating agents in seafood processing environments.

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List of Manuscripts

The chapters 3, 4, 5, 6 and 7 were presented as manuscripts. Each manuscript has been formatted according to the specifications of each journal.

Table1. List of manuscripts

Chapter number	Title of manuscript (Journal)
3	Host range and *in vitro* lysis of *Listeria monocytogenes* seafood isolates by bacteriophages (Submitted to Food Science and Technology International)
4	Characteristics of three listeriaphages isolated from New Zealand seafood environment (Submitted to Journal of Applied Microbiology)
5	Preliminary investigation of bacteriophages mediated lysis of physiologically stressed *L. monocytogenes* (To be submitted to Food Science and Technology International)
6	Effectiveness of phages in the decontamination of *Listeria monocytogenes* adhered to clean stainless steel, stainless steel coated with fish protein, and as a biofilm (Submitted to Journal of Industrial Microbiology & Biotechnology)
7	Bacteriophages for the control of low levels of *Listeria monocytogenes* in a simulated fish processing environment (To be submitted to International Journal of Food Microbiology)

The following three manuscripts published with some amendments are included in the Appendix 1.

1. Host range and *in vitro* lysis of *Listeria monocytogenes* seafood isolates by bacteriophages (Submitted to Food Science and Technology International)
2. Characteristics of three listeriaphages isolated from New Zealand seafood environment (Submitted to Journal of Applied Microbiology)
3. Effectiveness of phages in the decontamination of *Listeria monocytogenes* adhered to clean stainless steel, stainless steel coated with fish protein, and as a biofilm (Submitted to Journal of Industrial Microbiology & Biotechnology)

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Chapter 1: General Introduction

Listeria monocytogenes is an important food-borne pathogen which can be transmitted to humans through the consumption of contaminated food (Farber & Peterkin, 1991; Gandhi & Chikindas, 2007). The majority of listeriosis outbreaks have been linked to the consumption of ready-to-eat (RTE) foods including milk products (Lunden, Tolvanen, & Korkeala, 2004; Makino et al., 2005), meat, poultry, fish (Gottlieb et al., 2006; Gudbjornsdottir et al., 2004; Rocourt, Jacquet, & Reilly, 2000), cooked cereal, and fresh vegetable products (Allerberger & Guggenbichler, 1989; Aureli et al., 2000; Beuchat, 1996). An outbreak of listeriosis linked with the consumption of smoked mussels contaminated with *L. monocytogenes* was reported in New Zealand in 1992 (Brett, Short, & McLauchlin, 1998). In this incident, *L. monocytogenes* were recovered from the implicated mussel products and three affected patients (Brett et al., 1998). Smoked finfish and mussels have been identified under a risk category of foods associated with listeriosis outbreaks (WHO/FAO, 2004).

Healthy individuals normaly only develop a mild gastroenteritidis, while immunocompromised individuals who have been affected by cancer, diabetes and HIV-infection; have undergone organ transplants; eldery persons and pregnant women together with their infants are more susceptible to listeriosis with a mortality rate of 20-30% (Swaminathan & Gerner-Smidt, 2007). Pregnant women are about 20 times more susceptible to be infected with *L. monocytogenes* than normal healthy individuals and listeriosis may result in abortion, premature delivery, still birth or neonatal infection (Abram et al., 2006; Donnelly, 2001; Schuchat, Swaminathan, & Broome, 1991). Other symptoms of the disease in adults include sepsis and meningitis (Mook et al., 2011). The management of listeriosis outbreaks and clinical cases is a costly exercise in addition to the loss of lives (Lake, Cressey, Campbell, & Oakley, 2009; Scharff, 2012). The infectious dose of *L. monocytogenes* remains unknown, but illness has been reported with about 10^3 CFU/g (Tompkin, Scott, Bernard, Sveum, & Gombas, 1999). Stringent food standards are implemented to control this fatal food-borne pathogen. European Union implements a tolerance level of *L. monocytogenes* as $<10^2$ CFU/g in RTE foods which do not support the growth of this pathogen during storage, while the United States implements a zero tolerance limit for all RTE foods (Klontz et al., 2008; Tompkin et al., 1999). In New Zealand, zero tolerance standards of *L. monocytogenes*

are applied for processed RTE Greenshell™ mussels including other RTE foods (Food Standards Australia New Zealand, 2013a).

Seafoods are a valuable healthy food that contains proteins of high biological value; unsaturated fatty acids, particularly omega-3, minerals and vitamins (Wen et al., 2011). The world fish industry is rapidly expanding due to an increase in production and the development of distribution channels (Fisheries and Aquaculture Department, 2012). The New Zealand economy benefits from fish exports with fish products ranked seventh among the export commodities (Ministry of Primary Industries, 2013). The seafood exports of New Zealand mainly comprise farmed Greenshell™ mussel, rock lobster, hoki, squid and salmon. About 3 million tons of seafood products worth NZ$ 1.56 billion were exported in 2010-2011 (Ministry of Fisheries, 2012).

L. monocytogenes is microaerophilic (Jemmi & Stephan, 2006) with ability to survive and grow under refrigeration temperaures (Chan & Wiedmann, 2008; Huss et al., 2000; Rorvik, Yndestad, & Skjerve, 1991), a wide pH range (4.5-9.0) (Barker & Park, 2001; Cheroutre-Vialette, Lebert, Hebraud, Labadie, & Lebert, 1998; Guenther, Huwyler, Richard, & Loessner, 2009; Takhistov and George, 2005), and to tolerate up to 10% w/v sodium chloride (Farber, Coates, & Daley, 2008). This bacterium can, therefore, adapt and survive under harsh conditions in many different types of food matrices and food processing environments.

Seafoods such as lightly preserved fish such as hot and cold-smoked fish (<6% w/v salt and pH >5) and lightly salted fish products (brined cooked shrimp, herring, and marinated products) stored under refrigeration are most susceptible to *Listeria* contamination (Huss et al., 2000). Raw fish can be a vehicle for *L. monocytogenes* entering into fish processing plants (Eklund et al., 1995; Huss, Jorgensen, & Vogel, 2000). Fish processing plants often report both sporadic and persistent *Listeria* strains (Borucki, Peppin, White, Loge, & Call, 2003; Lappi et al., 2004; Moretro & Langsrud, 2004). *L. monocytogenes* becomes difficult to eradicate from food processing environments due to its ability to form biofilms (Moretro & Langsrud, 2004; Pan, Breidt, & Kathariou, 2006; Purkrtova, Turonova, Pilchova, Demnerova, & Pazlarova, 2010). This pathogen can also develop resistance to chemical disinfectants (Lou & Yousef, 1997), contributing to its establishment in food processing environments, which may lead to the cross-contamination of food during processing (Chmielewski & Frank, 2006, Tompkin, 2002).

Contamination of foods with *L. monocytogenes* has made costly food recalls

globally (Mead et al., 2006; Wen, Anantheswaran, & Knabel, 2009; Wong, Street, & Delgado, 2000). About 23 food recalls connected to *L. monocytogenes* contaminated RTE food were reported between July 2006 and May 2009 in New Zealand, which included mussel and smoked fish products (Crerar, Castle, Hassel, & Schumacher, 2011). Moretro & Langsrud (2004) suggested the need of developing alternative biocides for improved sanitaion approaches in processing plants together with a better understanding of the ecology of specific *L. monocytogenes* strains in seafood processing plants.

Consumer demands for the use of natural antimicrobial agents in food processing environments is driven by concerns of the potential health hazards of synthetic chemicals (Goodridge & Bisha, 2011; Leonard, Virijevic, Regnier, & Combrinck, 2010). This concern is supported by Wirtanen and Salo (2003) who reported the presence of chemical residues on food contact surfaces after sanitation procedures. Biocontrol agents are generally recognized as safe (GRAS) and eco-friendly (Leonard et al., 2010) alternatives and may be even more economic than synthetic chemicals. Some essential oils of plant extracts, nisin, and lactic acid bacteria (LAB) cultures significantly inactivated *L. monocytogenes* biofilm cells (Bower, McGuire, & Daeschel, 1995; Leonard et al., 2010; Leriche, Chassaing, & Carpentier, 1999).

Lytic bacteriophages (phages) are also a type of effective bactericidal agent that can be used in a variety of food systems (Hagens & Loessner, 2010; Hudson, Billington, Carey-Smith, & Greening, 2005). The lytic phages are safe to use in foods as they are lethal only to their targeted bacteria and therefore not harmful to humans (Sulakvelidze & Pasternack, 2010), do not alter the inherent quality of food and are biodegradable (Hagens & Loessner, 2010). Two food grade commercial phage products are currently available for use to control *L. monocytogenes* contamination. Micreos Food Safety (2013) has introduced Listex™ containing *Listeria* phage P100 which was isolated from effluents of dairy plant in southern Germany (Carlton, Noordman, Biswas, De Meester, & Loessner, 2005). The United States Food and Drug Administration (FDA) have approved Listex™ for the GRAS status (FDA, 2007) and also as a food additive to be used in poultry and cheese products (Codex-Standard, 1987; Garcia, Rodriguez, Rodriguez, & Martinez, 2010; Micreos Food Safety, 2013). ListShield™ is a cocktail of six *Listeria* phages produced by Intralytix (2013) and these six phages have been isolated from inner harbour sea water in Baltimore in USA (Pasternack & Sulakvelidze, 2009). The cocktail has gained approval by the FDA to be used as a food additive in

RTE poultry products (Bren, 2007). The characteristics of these commercial phages comply with food safety regulations of the FDA (FDA, 2006). Some of the biosafety features of phages include the absence of genes that encode pathogenic exotoxins, the inability to participate in transduction, are non-toxic and do not produce human allergenic reactions (Carlton et al., 2005; Pasternack & Sulakvelidze, 2009). The high efficacy of bacteriophages to control the *L. monocytogenes* and other pathogens has been demonstrated in a variety of foods (Garcia et al., 2010; Hudson et al., 2005; Mahony, McAuliffe, Ross, & Van Sinderen, 2011). Bacteriophages are also effective in decontaminating food contact surfaces. ListShield^{TM} has been approved by the Environmental Protection Agency (EPA) to control *L. monocytogenes* contamination on food processing surfaces (Sulakvelidze & Pasternack, 2010).

Roy, Ackermann, Pandian, Picard and Goulet (1993) reported that initial counts of *L. monocytogenes* (4-5 logs) adhered on polypropylene and stainless steel could be reduced by 3.5-3.7 log CFU/cm^2 after exposure to a three listeriaphage cocktail with a titre of 3.5 x 10^8 PFU/ml at room temperature (26 °C) for 1 h. Hibma, Jassim and Griffiths (1997) reported that attachment of cells (biofilm formation) did not occur on the stainless steel at 30 °C for 6 h when coupons were immersed in an L-form cell suspension (≈10^5 CFU/ml) and 10^9 PFU/ml of a phage modified by bacteriophage recombineering with electroporated DNA (BRED). In another experiment, a six hour intervention of BRED phage (10^{10} PFU/ml) on an 18 h old L-form *Listeria* biofilm reduced the biofilm cells of by ≈3 log CFU/cm^2 (Hibma et al., 1997). The phage P100 at 10^9 PFU/ml, reduced initial counts of a two-day and a seven-day biofilm (≈7 and 6.6 log CFU/cm^2, respectively) of *L. monocytogenes* on stainless steel by ≈5.4 and 3.5 log CFU/cm^2, respectively at 22-24 ^{o}C after 24 h (Soni & Nannapaneni, 2010b). Two dose levels (10^7 and 10^8 PFU/ml) of phage P100 decreased initial cell numbers (≈4.3 log CFU/cm^2) of biofilm by ≈3.4 log CFU/cm^2 after 8 h. The cell numbers decreased further a non-detectable level (detection limit <0.25 log CFU/cm^2) at 22 °C after 48 h (Montanez-Izquierdo, Salas-Vazquez, & Rodriguez-Jerez, 2012).

There is little information on the lysis efficacy of phages against many of *L. monocytogenes* strains isolated from seafood processing plant environments and information on the efficacy of phages to control *L. monocytogenes* on processing surfaces at low temperature (≈15 °C) is not reported. The lytic effectiveness of phages has been studied on exponential phase *L. monocytogenes* strains, however, it is known that organisms are often damaged and undergo lag or stationary phase in the processing

environment (Busch & Donnelly, 1992). They may be present in the final product and processing surfaces in low numbers as injured cells (Dykes & Moorhead, 2002; FDA, 2001; Gill, 2010). Therefore, information on the lytic efficacy of phages against injured cells is important to ensure the efficacy of bacteriophage treatments in food processing environments.

Listex^{TM} has recently been approved to be used as a food additive in the New Zealand food industry (Food Standards Australia New Zealand, 2013b). The application of phage based products to control *L. monocytogenes* in the New Zealand seafood industry has not been reported. However, the sector has expressed interest in evaluation of the efficacy of phages in fish processing environments. The formulation of lytic phage solutions using indigenous phages is seen as an exciting new area for the development of uniquely New Zealand phage-based products. Therefore, the aim of the present study was to determine the lytic efficacy of phages against *L. monocytogenes* strains on food contact surfaces in fish processing plants, with the following objectives: i) Isolation of natural lytic *Listeria* specific phage, that are effective at low temperatures, from fish processing environments in New Zealand, ii) Characterization of the best performing phage isolates by physical, biological and molecular methods, iii) Evaluation of the lytic activity of phage against seafood-borne, physiologically damaged *L. monocytogenes* strains and iv) Evaluation of the efficacy of phages against *L. monocytogenes* adhered to surfaces and in biofilms and stability of phages under conditions representing fish processing plants.

References

Given in Chapter 2.

Chapter 2: Literature Review

2.1 Introduction

L. monocytogenes causes listeriosis which is a major food-borne illness supported by date from regular surveillances. Strict *Listeria* control strategies are developed and implemented to prevent contamination of foods (Crerar, Castle, Hassel, & Schumacher, 2011; Klontz et al., 2008). Current decontamination treatments can only reduce pathogenic bacteria to acceptable levels but cannot completely eliminate them from food contact surfaces and processing environments (Goodridge & Bisha, 2011; Hagens & Loessner, 2010; Tompkin, 2002). The chemical disinfectants used in food processing environments may be toxic and corrosive at their most effective levels (Sulakvelidze & Pasternack, 2010). The residues that potentially remain after an application, can pose health risks and environmental pollution. Therefore, there is consumer demand for alternate more natural anti-bacterial agents that are non-toxic (Goodridge & Bisha, 2011; Leonard, Virijevic, Regnier, & Combrinck, 2010). Another confounding issue is the increasing resistance of bacteria to antibiotics used to treat human illness. This makes human illness more difficult to treat (Ahmed, Kaderbhai, & Kaderbhai, 2012) and supporting the development of alternative methods to prevent human illnesses.

Biocontrol (biological control) is the use of a natural living organism that is antagonistic to another organism and this antagonistic effect can be used to suppress the target population (Duche, Tremoulet, Glaser, & Labadie, 2002; Stephens, Roberts, Jones, & Andrew, 2008). Therefore, the use of bacteriophages to control pathogenic or contaminant bacteria in a given system can be viewed as a biocontrol approach. More specifically, *L. monocytogenes* is a microorganism that is targeted for biocontrol. Biocontrol of pathogenic bacteria has advantages over other control measures. Natural lytic bacteriophage preparations has been approved in the category of generally recognized as safe (GRAS) by United States Food and Drug Administration (FDA) (Hagens & Loessner, 2010). Lytic bacteriophages have been accepted as one of the alternate natural biocontrol agents (Goodridge & Bisha, 2011; Hudson, Billington, Carey-Smith, & Greening, 2005). This review aims to discuss mainly the significance of *L. monocytogenes* as a food-borne pathogen in the seafood industry and the isolation,

characterization and applications of bacteriophages, referring mainly to phages that infect *Listeria.*

2.2 Bacteriophage ecology

2.2.1 Historical background of bacteriophages

Bacteriophages of *Mircococcus* and *Shigella* were discovered independently by Frederick Twort, a British microbiologist, in 1915 and Felix d'Herelle, a French-Canadian microbiologist in 1917 respectively (Duckworth, 1976). Nevertheless, there were two previous incidences that reported bacterial lysis by unidentified agents in cultures (Sulakvelidze, Alavidze, & Morris, 2001). In 1896, the lysis of *Vibrio cholera in vitro* was observed by Ernest Hankin, a British bacteriologist and the other report two years later, a similar lethal effect against *Bacillus subtilis* was noticed by Gamaleya, a Russian bacteriologist. However unlike previous microbiologists, d'Herelle had continued the investigations further in *Shigella* strains and proposed that the bacteria killing agent could be a virus. Then he named the "virus" as a bacteriophage by the combination of two words, "bacteria" and "phagein" which means "to eat" in Greek (Sulakvelidze et al., 2001). After this early discovery, d'Herelle attempted to use phage as a therapeutic agent and treated successfully a 12 year old boy affected with severe bacterial dysentery (Sulakvelidze et al., 2001). Phages were later investigated for use in other applications such as phage typing of bacteria strains (Loessner & Busse, 1990) and bicontrol of bacterial pathogens in agriculture and farm animals (Ahmed et al., 2012; Hoopes et al., 2009; Loessner, Rees, Stewart, & Scherer, 1996). Phages have been trialed in a variety of biotechnological approaches such as delivery vehicles for protein and DNA vaccines; as gene therapy delivery vehicles; as alternatives to antibiotics; for the detection of pathogenic bacteria; and as tools for screening libraries of proteins, peptides or antibodies (Clark & March, 2006; Hoopes et al., 2009; Loessner et al., 1996).

2.2.2 Existence in nature

Phages are the tiniest of parasites in the ecosystem. The phages are the most abundant biological particles on earth (Abedon, 2009; Breitbart, Wegley, Leeds, Schoenfeld, & Rohwer, 2012) with 10 times more phage than the number of bacteria in the

environment (Chibani-Chennoufi, Bruttin, Dillmann, & Brussow, 2004). Being the largest population on earth, the total number of phages are estimated to be about 10^{31} (Hatfull, 2008; Hendrix, 2003; Rohwer & Edwards, 2002). Phages are omnipresent in the ecosystem (Bergh, Borsheim, Bratbak, & Heldal, 1989; Mahony, McAuliffe, Ross, & Van Sinderen, 2011) since they are found in every niche, including extreme environmental conditions such as extreme cold, hot springs and animal guts (Ackermann, 2003; Borriss, Helmke, Hanschke, & Schweder, 2003; Breitbart et al., 2004; Sulakvelidze et al., 2001). Phages consist of core nucleic acid (DNA or RNA) covered with protein coat (capsid) or lipid containing envelope and this structure is referred to as a head. Additionally, some phages consist of a tail or other appendages (Ackermann, 2009; Ahmed et al., 2012). Phages differ in their morphology and dimensions as well (Ackermann, 2009). The diameter of the head is about 43-100 nm and the length of a tail measures about 3-825 nm (Ceyssens & Lavigne, 2010; Petrovski, Dyson, Seviour, & Tillett, 2012). Bacteriophages are obligate parasites of bacteria that lack an independent metabolism (Ceyssens & Lavigne, 2010). They are co-evolving with bacteria in nature and this has been demonstrated by comparative genomic studies (Brussow, Canchaya, & Hardt, 2004; Hagens & Lossner, 2007).

2.2.3 Behaviour of phages and interactions with bacteria

In natural environments, phages infect and interact with bacteria (host) in different ways (Ackermann, 2009; Weinbauer, 2006) for their survival. After a phage reaches a suitable host surface it is adsorbed onto the cell surface receptors of the host. The adsorbed phage starts its infection process by injecting the phage DNA into the cell and then, phages follow either a lytic or lysogenic life cycle inside the host (Raya & Herbert, 2009; Weinbauer, 2006).

Lytic life style: This process results in the generation of new phage progeny through a phage replication cycle and is explained as a single step replication (growth). Several sequential distinct stages can be identified in the complete lytic cycle: i) Adsorption and attachment of a phage to the specific receptors of bacterial cell wall, ii) Injection of phage genetic material into bacterial cell cytoplasm through a perforation made on the bacterial cell wall and membranes, iii) Generation of phage DNA and capsid/envelop materials of the progeny by altering bacterial biosynthetic machinery (eclipse period), iv) Assembly of phages (post eclipse), and v) Release of progeny by lysis of the cell. The amount phage released per one cycle (burst size) may vary from a few to 500 in

numbers depending on the phage strain, the bacterial strain and its metabolic state (Hyman & Abedon, 2009; Weinbauer, 2006). The release of progeny is accomplished by the action of phage encoded holin (a hydrophobic polypeptide) and lysin. Holin makes holes in cell membrane and lysin degrades peptidoglycan structures resuting in cell lysis (Ceyssens & Lavigne, 2010). Single step growth of *E. coil* phages were studied in the decade of 1940 (Delbruck, 1940). Considering the basic principles of these early studies, systematic approaches and protocols of single step growth of phages are described (Carlson, 2005; Hyman & Abedon, 2009).

Lysogenic life style: In the lysogenic life cycle, the genome is ejected by the phage and then it integrates with the bacterial genome at a specific site in the genome or remains as plasmid DNA (Ceyssens & Lavigne, 2010; Weinbauer, 2006). The phage genome, which is fused into the genome of the host, is called a prophage or the temperate phage while the bacterium bearing prophage DNA is called the lysogen. The expression of repressor genes of the prophage prevents the progression of a subsequent lytic life cycle. The integrated prophage is replicated along with division of the lysogen bacterium. However, it has been found that under some harsh environmental conditions, such as exposure to UV/ionizing radiation, mutagenic chemicals and heat etc., the dormant state of temperate phage is terminated, the phage genome is activated inside the lysogen and this subsequently causes the host bacterial cell to undergo lysis with release of a new phages similar to the lytic phages. Many of the *Listeria* phages, currently, reported are temperate phages (Loessner, Krause, Henle, & Scherer, 1994; Zink & Loessner, 1992).

Pseudolysogenic style: In this phenomenon, the phage DNA injected into cytoplasm undergoes a dormant state in the host bacterium without following either the lytic or lysogenic path (Gill, 2010). Ripp and Miller, (1998) demonstrated that *Pseudomonas aeruginosa* specific phages enter into a pseudolysogenic state when the starved cells of *Pseudomonas* were infected with phages. When the starved pseudolysogenic bacteria are resuscitated with nutrient rich medium, the dormant phage is activated to follow either a temperate or lyic pathway. The researchers suggest that this dormant state is a natural mechanism of survival of phages in harsh environments where bacteria does not have sufficient energy sources to spend on the phage's lytic cycle or temperate status (Ripp & Miller, 1998). Thus, some dormant phage-DNA in bacteria could be passed on to new bacteria generations during bacterial division (Gill, 2010).

Lysogenic conversion: The transfer of some genes from a phage (prophage) genome to bacterium (lysogen) genome is called lysogenic conversion during the temperate life style (Weinbauer, 2006). Expression of some prophage genes and transfer of prophage genes (morons) to the genome of the lysogen are believed to help increase the survival fitness of the lysogen in the natural environment (Weinbauer, 2006). For example, some prophages transfer genes encoding for the production of toxins in the lysogen, thereby, pathogenicity (virulence) is established in the lysogen. Some of toxins which are reported to be pathogenic for humans in *Corynebacterium diphtheriae*, *Clostridium botulinum*, *Streptococcus pyogenes*, *Staphylococcus aureus* and *E. coli* (Shiga toxin) are prophage genes (Brussow et al., 2004). In addition, the expression of repressor genes during the temperate state prevents the lysogen from subsequent super-infection by other phages. Lysogeny is a commonly prevailing phenomenon in bacterial ecology (Raya & Herbert, 2009) and also common among *Listeria* strains (Loessner, Inman, Lauer, & Calendar, 2002).

Transduction: Transduction refers the transfer of DNA of bacteria to other new bacteria through the mediation of a phage and two types of transductions are explained (Weinbauer, 2006). Generalized transduction occurs by packing a DNA fragment of the bacterial genome into a newly generated empty phage capsid during the phage replication inside the host bacterium. The bacterial DNA containing phage is called a transducing phage particle which behaves similarly to the new phage particle and transfers the bacterial DNA to another new host (recipient bacterium) upon infection. In specialized transduction, both a fragment of host DNA near the integration site of the host genome and regenerated phage DNA are packed into one capsid and then transferred to a new bacterium. (Thierauf, Perez, & Maloy, 2009) The recombinant novel bacterial DNA of the recipient genome may encode different characteristics expressible in the recipient bacterium.

2.3 Different applications of phage and phage products

2.3.1 Typing phage

Listeria phages are used as a typing tool to identify *L. monocytogenes* strains since the recognition of the first *Listeria* phage in 1945 (Loessner & Busse, 1990). Different groups of phages selectively attack particular *Listeria* strains (Loessner, 1991). Eugster,

Haug, Huwiler and Loessner (2011) described the distinguishable differences among the serotypes based on the carbohydrate subunits of the cell wall teichoic acid (TA). Different groups of phages bind to specific cell wall receptors (different carbohydrate moieties) of *Listeria* strains that belong to a particular serotype (Eugster et al., 2011). Therefore, the binding affinity of a phage group on *Listeria* strains specifically within a serotype provides the basis for an additional sub-typing system (Loessner, 1991). Phage based typing is economical and can be performed in laboratories with only basic facilities. This method is very useful in discriminating the strains linked to food poisoning outbreaks and epidemiological studies (Capita, Alonso-Calleja, Mereghetti, Moreno, & Garcia-Fernandez, 2002; Loessner & Busse, 1990; Pasternack & Sulakvelidze, 2009; Van der Mee-Marquet, Loessner, & Audurier, 1997). Loessner and Busse (1990) pointed out that early typing work was based on solely the lysogenic phage and typing sets were expanded by isolating new phages, including A511 from environmental samples.

2.3.2 Use of phages in rapid detection of *L. monocytogenes* in food

Bacteriophage A511 was modified to contain the gene *luxAB* which is naturally present in *Vibrio harveyi* (Loessner et al., 1996). This Luciferase-reporter-bacteriophage A511::*luxAB* (LRB A511::*luxAB*) was evaluated using the samples spiked with *L. monocytogenes.* The low count cells ($5x10^2$ CFU/ml) of *L. monocytogenes* were able to be detected in the samples by LRB A511::*luxAB* assay in 2 h compared to conventional detection methods requiring several days (Loessner et al., 1996). The results from the LRB A511::*luxAB* assay performed on *Listeria* contaminated field samples which included a 24 h pre-enrichment were similar to the results obtained on sample by performing the conventional 4 day plating method used detect to *L. monocytogenes* (Loessner, Maier, Daubek-Puza, Wendlinger, & Scherer, 1997). The LRB A511::*luxAB* assay was more effective in detecting *L. monocytogenes* in milk and environmental samples than in meat and poultry samples (Loessner et al., 1997).

2.3.3 Lysins

Bacteriophage lysins are also called lysosymes, endolysins, muralytic or mureolytic enzymes (Loessner, 2005) and are a highly effective group of bio-molecules that degrade the ploypeptidoglycan structure (Weidenmaier & Peschel, 2008) leading to cell lysis. Lysin has been suggested as a useful antimicrobial agent in the food industry,

biotechnology and medicine due to the high specificity of lysin for the target bacterium and high efficacy (Loessner, 2005). Lysins work best on Gram positive bacteria since they have no external cell envelope. Lysin consists of two major subunits: the N-terminal domain, which has catalytic function and C-terminal domain that binds specifically to the cell wall. A purified recombinant lysin of ply511 (100 µl/ml) showed complete lysis of *L. monocytogenes* in 150 s (Loessner, 2005). *Lactococcus lactis* cloned with lysin genes (*ply*118 gene of phage A118 and *ply*115 gene of phage A115) was found to lyse *L. monocytogenes* cells in broth (Gaeng, Scherer, Neve, & Loessner, 2000). Another recombinant lysin expressed by gene *LYS*25 of *Listeria* phage FWLLm3 showed a broad lytic spectrum. The purified recombinant enzyme (50 mg at 60 U/ml) caused lysis of lawns of 18 *L. monocytogenes* strains (serotypes 1/2a, 1/2b, and 4a), one *L. welshimeri* (serotype 6a) and one *L. innocua* (serotype 6a). This purified protein (40 U/ml) lysed cell counts to an undetectable level by reducing the initial counts by >4 log CFU/ml in soya milk at 4 °C in 3 h (Zhang, Bao, Billington, Hudson, & Wang 2012). Lysins can be combined with other antibacterial agents in order to get synergistic effect on less sensitive bacterial cells (Djurkovic, Loeffler, & Fischetti, 2005). Purified lysin has been suggested for use as a therapeutic agent either alone or in combination with known antibiotics (Loessner, 2005).

2.3.4 Monocins

Monocins have been described as bacteriocin like substances and some have been shown to lyse *Listeria* strains (Bradley & Dewar, 1966; Zink, Loessner, Glas, & Scherer, 1994). Monocins are described as defective prophages that can be induced by mitomycin C or Ultra Violet (UV) light (Zink, Loessner, & Scherer, 1995). The Transmission electron microscopy (TEM) images of monocins (obtained by inducing under UV light) appear as phage tails (Kalmokoff, Daley, Austin, & Farber, 1999; Zink et al., 1995). Zink et al. (1995) determined that monocins contain lytic enzymes in the tail end from which it bind to the cell wall of *Listeria* strains. Monocins that had a broad lytic spectrum were used as a supplementary tool in phage based typing of *Listeria* strains including *L. innocua and L. ivanovii* but *L. grayi* was not sensitive to monocins (Zink et al., 1994). Stability of different types of monocins also has been studied. A monocin type (M1040) is stable at 30-40 °C, at 2 °C for several weeks, storage at -20 °C, at pH 3-11 but is not stable at 50 °C (Zink et al., 1995). Curtis and Mitchell (1992) showed some monocins are trypsin-resistant and also heat labile at 50 °C. Monocins

with a broad lysis spectrum, strain specificity and stability under a wide range of conditions may have potential as control agents for *L. monocytogenes*.

2.4 *L. monocytogenes* as a food-borne pathogen

2.4.1 Prevalence of *L. monocytogenes*

This organism is a major food-borne pathogen causing listeriosis in humans. Listeriosis outbreaks have occurred through nearly every type of RTE foods contaminated with *L. monocytogenes* such as dairy, meat, fish poultry, fruits and vegetables (Gudbjornsdottir et al., 2004; Schlech III & Acheson, 2000; WHO, 1988). A significant rise in the incidences of listeriosis occurred in the European Union from 2003 to 2006 (Allerberger & Wagner, 2009). The mortality rate of ≈20-30% is reported among listeriosis cases (Mook, O'Brien, & Gillespie, 2011; Vazquez-Boland et al., 2001). High costs are involved in managing this disease (Coffey, Mills, Coffey, McAuliffe, & Ross, 2010). The RTE foods contaminated with *Listeria* has made costly food recalls globally (Mead et al., 2006; Wong, Street, & Delgado, 2000). In 2007, 280 tones of green lipped mussel meat that was found contaminated with *L. monocytogenes* were recalled globally by a New Zealand company ("*Listeria* sparks", 2007).

This bacterium was originally isolated from laboratory rabbits and guinea pigs in 1926 and named as listerella (Fish & Schroder, 1949; Schultz, 1945). Later, listerella was named *L. monocytogenes* (Gibbons, 1972). *L. monocytogenes* is prevalent in nearly every niche in nature, being found in soil, water and animals (Tompkin, 2002). It is also carried in the human gut of up to 10% of individuals (Hodgson, 2000). This bacterium is a Gram-positive, non-spore forming bacillus with flagella and has dimensions (length and width) measuring ≈0.5-2.0 and 0.4-0.5 μm, respectively. *L. monocytogenes* is micro-aerophilic (Jemmi & Stephan, 2006), psychrotrophic and survives at 1-45 ^{o}C, halo-tolerant surviving in up to 10% sodium chloride (FDA, 2001; Takhistov & George, 2005), capable of growing over a pH range from 4.5 to 9.0 (Takhistov & George, 2005) and therefore capable to survive in wide range of environments (Chmielewski & Frank, 2006; WHO, 1988). *L. monocytogenes* is one species among eight *Listeria* species. The other species of *Listeria* are *innocua, seeligeri, welshimeri, ivanovii*, *grayi* (Hitchins & Jinneman, 1998), *marthii* (Graves et al., 2010) and *rocourtiae* (Leclercq et al., 2010). *L. monocytogenes* has been reported as the main species causing food-borne listeriosis despite the pathogenic potential of *L. ivanovii* and *L. seeligeri* (Hitchins & Jinneman, 1998).

2.4.2 Fish industry and food safety

Fish and fishery products are gaining popularity as a source of healthy food containing proteins with high biological value, fatty acids and essential micronutrients such as minerals and vitamins (Wen, et al., 2011). Fish is referred to as a collective term which includes fin fish, molluscs, crustaceans and any aquatic animal harvested (Fisheries and Aquaculture Department, 2013). The world fish food supply is increasing dramatically due to an increase in production and the development of the distribution channels (Fisheries and Aquaculture Department, 2012). The fish food supplies were about 148 million tons in 2010 and 154 million tons in 2011. In 2010, more than 86% of the total fish supply was used for direct human consumption and the remaining amount (20.2 million tons) was utilized in non-food uses (Fisheries and Aquaculture Department, 2012). Food fish was destined as live, fresh and chilled form (46.9%), frozen fish (29.3%) and cured and other form of processed food (23.8%) in 2010. The non-food uses of fish includes fish meal and fish oil (75% of total of non food fish), ornamental, fingerling and fry in culture fishery, bait, feed in aquaculture and farm animals, and pharmaceuticals (Fisheries and Aquaculture Department, 2012).

The fish production in New Zealand (NZ) is expanding under strategic plan of Fisheries 2030 that aims to maximize the benefits from the use of fisheries for New Zealanders within environmental limits (Ministry of Primary Industries, 2013). NZ is ranked at 25th among the world fish product exporting nations and contributed approximately 1.2% of world fish products exports (Ministry of Primary Industries, 2013). Fish products are becoming the seventh export commodity in NZ with 310,000 tones of total seafood exports worth about NZ$ 1.56 billion in 2010-2011 (Ministry of Fisheries, 2012). The farmed Green shell mussels, salmon, rock lobster, hoki and squid contribute to seafood products exported by NZ. Fish is harvested from different types of natural and constructed or managed aquatic environments. In some instances, human pathogenic bacteria are reported in the aquatic resources where fish are harvested. Being high moisture and proteinaceous materials rich in soluble nutrients, the harvested raw fish products can serve easily as sources for the pathogenic flora, such as *L. monocytogenes,* and to enter fish processing plants. Food safety, therefore, is a high priority for the seafood industry.

2.4.3 Types of *Listeria monocytogenes* isolates linked to seafood contaminations

L. monocytogenes strains have been divided into 13 different serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7) (FDA 2001; Neves, Lourenco, Silva, Coutinho, & Brito, 2008; Norton et al., 2001; Rebuffo-Scheer, Schmitt, & Scherer, 2007). Many studies have demonstrated that the variety of *L. monocytogenes* strains is more often unique to the individual processing environment under consideration (FDA, 2001). Studies on *L. monocytogenes* isolated from food-borne outbreaks and food processing environments have shown that >90% of human listeriosis are caused by serotypes 1/2a, 1/2b, 1/2c and 4b (FDA, 2001; Jemmi & Stephan, 2006; McLauchlin, 1990; Neves et al., 2008; Rebuffo-Scheer et al., 2007). *L. monocytogenes* (serotypes 1/2a, 1/2b and 4b) are linked with >90% of human listeriosis cases (McLauchlin, 1990). *L. monocytogenes* serotypes 1/2a and 1/2b are more predominant than serotype 4b in turkey processing plants (Kim, Siletzky, & Kathariou, 2008).

Most of the seafood *L. monocytogenes* strains belong to the serotype 1/2a (Chou & Wang, 2006; Cruz & Fletcher, 2011). About 80% of the *L. monocytogenes* isolated from seafood retail products were serotype 1/2a strains and included roe of cod and salmon (Handa et al., 2005). Miya et al. (2010) reported 54% of 39 isolates of *L. monocytogenes* recovered from different seafoods (14 minced tuna, 7 salmon roe and 15 cod roe samples) were the serotype 1/2a, while others belonged to serotypes 3a, 1/2b, 3b and 4b. One *L. monocytogenes* isolate was found in serotype 3b which is rarely reported in seafoods. In another study, 15 strains of *L. monocytogenes* isolated from semi-preserved RTE fish semi-preserves belonged to four different serotypes (1/2a, 4b, 1/2b and 4b) constituting ≈73, 33, 67 and 6%, respectively (Gambarin et al., 2012). Cruz and Fletcher (2011) found 96% of *L. monocytogenes* strains isolated from mainly mussel processing premises in NZ belonged to serotype 1/2a or 3a and the remaining 4% included serotype 1/2b, 3b or 7, similar to the previous findings of seafood premises. Interestingly, ≈74% of the serotype 1/2a strains belonging to three PFGE based sub typing groups were found recurrent in more than one processing plant. However, the authors suggest that the recurrent nature of these strains may be linked to the colonization ability of the strains of *L. monocytogenes* or continuous entering of *L. monocytogenes* into the processing plant environment (Cruz & Fletcher, 2011). Cruz and Fletcher (2011) reported the same *L. monocytogenes* strain which was linked to a smoked mussel outbreak in 1992 (Brett, Short, & McLauchlin, 1998), among the strains

isolated from environmental samples from mussel processing plants during sampling of 2007-2009. Rorvik, Aase, Alvestad and Caugant (2000) demonstrated six sub types (Multilocus enyme electrophoresis) belonging to serotype 1 and 4, which were isolated from patients and were also isolated from fish products and RTE fish products (i.e. cold smoked salmon, marinated salmon, cold smoked mackerel and raw fish fillet).

Incidences of listeriosis reported with contaminated seafoods such as smoked mussels, smoked salmon and undercooked fish have been reported (Brett et al., 1998; Ericsson et al., 1997; Gudbjornsdottir et al., 2004; Tham, Ericsson, Loncarevic, Unnerstad, & Danielsson-Tham, 2000). The seafoods that are lightly preserved such as fish such as hot and cold smoked fish (<6% salt, pH >5), and lightly salted fish (brined cooked shrimp, herring and marinated products) are more likely to be contaminated with *L. monocytogenes* (Huss, Jorgensen, & Vogel, 2000). These investigations indicate potential risks of listeriosis outbreaks due to seafood contaminated with *L. monocytogenes*. Smoked fin fish and mussels have been identified under a risk category of food associated with listeriosis outbreaks (FAO/WHO, 2004). Due to the severity of the disease, many food safety regulatory authorities have set stringent tolerance limits for *L. monocytogenes* in foods. Limited evidence is available to indicate that food containing <100 CFU/g causes health risks to normal individuals (Jorgensen & Huss, 1998). A zero tolerance for *L. monocytogenes* in all RTE food is specified in the United States by FDA (Klontz et al., 2008). Food Standards Australia New Zealand (2013a) also implements zero tolerance limits for *L. monocytogenes* for RTE mussel products. In the European Union, the tolerance level of *L. monocytogenes* between zero and <100 CFU/g is allowed for RTE which do not support the growth during storage (Koutsoumanis & Angelidis, 2007; Tompkin, Scott, Bernard, Sveum, & Gombas, 1999).

The ability of *L. monocytogenes* strains to grow under refrigeration conditions, the development of resistance to chemical disinfectants and the ability to form biofilms in food processing plants may lead to the persistence in processing environments (Folsom & Frank, 2006; Kim et al., 2008; Lunden, Autio, Markkula, Hellstrom, & Korkeala, 2003; Moretro & Langsrud, 2004; Purkrtova, Turonova, Pilchova, Demnerova, & Pazlarova, 2010). The most common niches in processing plants are the narrow or covered surfaces that are difficult to clean and sanitize properly, such as hollow rollers on conveyors, cracked tubular support rods on equipment, the space between close-fitting metal-to-metal or metal-to-plastic parts, worn or cracked rubber

seals around doors, on-off valves and many other points (Tompkin et al., 1999). Such common surfaces and locations of food processing plants have been highlighted in detailed (Tompkin et al., 1999).

The widely used disinfectants in food processing plants are quaternary ammonium compounds, chlorine based compounds, ethanol, and peracetic acid (Aarnisalo, Lunden, Karkalla, & Wirtanen, 2007; Gram, Bagge-Ravn, Ng, Gymoese, & Vogel, 2007; Holah, Taylor, Dawson, & Hall, 2002). Experiments have revealed the high efficacy of natural biochemical and organisms in controlling *L. monocytogenes* on processing surfaces. These components include essential oils of plant extracts, nisin (a commercially available bacteriocin) that has been approved by FDA, essential oils and lactic acid producing bacteria (LAB) cultures (Bower, McGuire, & Daeschel, 1995; Leonard et al., 2010; Leriche, Chassaing, & Carpentier, 1999). However, limitations of the efficacy of each class of biocides in different applications, warrant alternate approaches and the efficacy of bacteriohages in food systems are discussed in this review.

2.5 Isolation of phages from environments

2.5.1 Isolation of natural lytic phages from the environments

Phages are most likely to be isolated from the habitats of the host bacterium and both phage strains and host strains have been isolated from the same location (Hodgson, 2000; Kim et al., 2008; Walakira et al., 2008). The new phages were recovered using different protocols depending on the type of sample, temperature and the potential abundance of particular phages. The phage isolation protocols related to the variety of sample types including water, soils, sludge and environmental swabs are described (Carlson, 2005; Kim et al., 2008; Loessner & Busse, 1990; Walakira et al., 2008). *Listeria* specific phages have been isolated from different environments including sea water (Pasternack & Sulakvelidze, 2009), municipal sewage (Loessner & Busse, 1990), dairy plant sewage (Carlton, Noordman, Biswas, De Meester, & Loessner, 2005), different locations (floor drains, conveyer belts, boots, aprons) of a turkey processing plant (Kim et al., 2008), human sewage effluent (Dykes & Moorhead, 2002), sheep feces (Lee, 2008), and silage (Hodgson, 2000; Schmuki, Erne, Loessner, & Klumpp, 2012).

2.5.2 Impact of the protocol used for the phage isolation

Kim et al. (2008) isolated phage from environmental swab samples following the protocol given below. The swab samples were eluted in Brain Heart Infusion by vortexing, the eluant was filtered (0.2 µm filters), the filtrate was enriched with an indicator host strain at 37 °C for overnight, and then filter sterilized enrichment was assayed for plaque formation (Kropinski, Mazzocco, Waddell, Lingohr, & Johnson, 2009) at 37 °C. Phages were isolated from sewage by analyzing the sewage effluents at 30 °C (Loessner & Busse, 1990). The sewage effluents were centrifuged at 2,500 x g for 10 min, the supernatant was then filtered (0.22 µm), the 10 ml sample filter was inoculated with 0.5 ml of exponential phase *L. monocytogenes* (indicator strain) and incubated at 30 °C for 5-6 h in shaker incubators, again centrifuged and filtered and then assayed for the presence of phages following the drop testing method (Mazzocco, Waddell, Lingohr, & Johnson, 2009). Phages remain firmly adhered in some environments and therefore, they cannot be eluted easily (Mark R Liles, personal communication, December 20, 2010; Parsley et al., 2010).

Different reagents have been investigated in order to elute phages from abiotic substrates and thereby increase the potential of phage isolation. In an experiment to evaluate the viral metagenome in activated sludge, bacteriophages were eluted from sludge samples using 10% meat extract (Mark R Liles, personal communication, Parsley et al., 2010). The researchers reported that the elution contained 95% of tailed viruses (Order *Caudovirales*). The results indicate that the elution process was effective over the total viral community since it has been estimated that approximately the same percentage (96%) of total phages are represented by tailed phages in the natural environment (Hagens & Loessner, 2007). Bitton, Chou, and Farrah (1982) achieved about 40% elution efficacies for poliovirus virus from marine sediments using urea-lysine and trichloroacetate-glycine (pH 9.0). Monpoeho et al. (2001) reported a high yield of virus from sludge by using two eluant systems: a) Beef extract (10% w/v) at pH 9 followed by sonication, and b) Sodium chloride (0.3 M) and beef extract (7% w/v) at pH 7.5 followed by Freon (fluorinated hydrocarbon) treatment. Most of the *Listeria* phage isolations follow an enrichment step in the protocol (Kim et al., 2008; Lee, 2008; Loessner & Busse, 1990). In some trials, the enrichment step was repeated twice (Eisenstark, 1967; Jochen Klummp, personal communication, January 3, 2013). The initial sample may have very few numbers of phages and/or insufficient elution in the steps prior to the enrichment. Nevertheless, Hodgson (2000) reported that some *Listeria*

phages were picked up directly by drop testing of filtrates of TM buffer which were obtained by washing silage. The drops of silage-washed TM Buffer were placed on *L. monocytogenes* lawns, incubated at room temperature and the resultant plaques were picked up (Hodgson, 2000). However, the silage samples used had already been identified to be infected with *L. monocytogenes.*

Due to high binding affinity, phages are lost on filters, nevertheless low protein binding filters made with polytetrafluoroethylene, polypropyelene, polycarbonate, polysulfone and cellulose acetate are recommended to use in phage filtration work (Twest & Kropinski, 2009). Eisenstark (1967) indicated the high impact of phage loss on filters which have the high phage binding affinity, in situations where very low numbers of phages are present in the field samples. The author suggests passing a protein solution through the fresh filter before filtering the sample enrichment in order to reduce the phage loss. The author also suggests increasing the plating volume of the field sample enrichments. Chloroform ($CHCl_3$) is used to lyse the bacteria in sample enrichments for phage detection (Walakira et al., 2008). Some *Myoviruses,* however, have been shown to be sensitive to $CHCl_3$ (Ackermann, 2009; Hodgson, 2000;). Some isolates of a *Listeria* phage collection isolated from filter-sterilized samples were found sensitive to $CHCl_3$ (Hodgson, 2000). Therefore, the use of $CHCl_3$ instead of filtering the samples will narrow the diversity of phage isolation.

The use of divalent ions such as Mg^{+2} and Ca^{+2} (1-10 mM) in enrichments of soil and water samples is recommended because sample phages effectively adsorb and replicate in the presence of divalent cations (Twest & Kropinski, 2009). The *Listeria* phages have been isolated using Ca^{2+} in sample enrichments at 1.25 mM (Lee, 2008) and 10 mM (Hodgson, 2000; Kim et al., 2008). In addition, 10 mM $MgSO_4$ has also been used (Hodgson, 2000).

2.5.3 Effect of temperature of phage isolation

The phages are isolated at temperatures that relate to the growth temperature of the host bacterium and/or the temperature tolerance of the phage being detected. Hodgson (2000) isolated *Listeria* phages at room temperature (24 °C). Olsen, Metcalf, and Todd (1968) isolated psychrotrophic and mesophilic phages specific for *Pseudomonas* by screening the field samples with psychrophilic and mesophilic *Pseudomonas* strains at 20-25 °C and 37 °C, respectively. The psychrophilic *Pseudomonas* phages isolates replicated between 3.5 and 32 °C while mesophilic phages replicated between 25 and 37

°C (Olsen et al., 1968) and they suggested that replication of phages may be influenced by temperature sensitive enzymes.

Nearly all *Listeria* phages have been isolated from environments at 30 °C or above except for a collection of phages, including phage P35 (Hodgson, 2000) which were isolated at room temperature (Table 2.1). Phages replicate optimally *in vitro* at temperatures from which they are isolated. The temperature tolerance and the replication capabilities of phages are attributed to their ecology. There may be more chances of discovering high productive *Listeria* phages at low temperatures using *L. monocytogenes* from a low temperature environment. Phages with high replication potential will be good candidates for bicontrol in foods stored under refrigeration.

Table 2.1 Characteristics of lytic *Listeria* phages belonging to the order *Caudovirales* and families *Myoviridae* and *Siphoviridae*

Phage	Source of isolation/ country	Isolation (°C)	Taxonomic order or family	Size of genome (kb)	Diameter x length of head (nm)	Tail length (nm)	Infecting serotypes of *Listeria*	Reference
P100	Diary processing effluent/ Germany	30	*Myoviridae*	133	89§[u]	198	1/2, 4 and 5 of *L. ivanovii* *	Carlton et al., 2005; Klumpp et al., 2008
A511	Sewage effluent/ Germany	30	*Myoviridae*	137.6	87[§u]	199	1/2, 4 and *L. ivanovii* *	Zink and Loessner, 1992; Klumpp et al., 2008,
List-1	Baltimore inner harbour water/USA	30	*Caudovirales*	42.8	60 x 50[§n]	245	1/2a, 1/2b, 1/2c, 3a, 4a, 3b, 4b, 4d ¶	Sulakvelidze, Sozhamannan, & Voelker, 2004; Pasternack & Sulakvelidze, 2009
List-2		30	*Caudovirales*	43	56 x 44[§n]	272		
List-3		30	*Caudovirales*	40	53 x 48[§n]	292		
List-4		30	*Caudovirales*	40.4	50 x 46[§n]	205		
List-36		30	*Caudovirales*	131.9	72 x 68[§n]	167		
List-38		30	*Caudovirales*	39.3	60 x 65[§n]	258		
P35 or ΦLMUP35	Silage/USA	25	*Siphoviridae*	35.8	58 [§p]	110	1/2a	Hodgson, 2000; Dorscht et al., 2009; Klumpp et al.,
P40	Sewage effluent/ Germany	n	*Siphoviridae*	35.6	56 [§u]	108	1/2a, 4, 5, 6 *	Dorscht et al., 2009

Table 2.1 (Continued)

Characteristics of lytic *Listeria* phages belonging to the order *Caudovirales* and families *Myoviridae* and *Siphoviridae*

Phage	Source of isolation/ country	Isolation (°C)	Taxonomic order or family	Size of genome (kb)	Diameter x length of head (nm)	Tail length (nm)	Infecting serotypes of *Listeria*	Reference
P70	Grass silage/ Switzerland	n	*Siphoviridae*	67.1	57 x 218[§u]	141	1/2a, 1/2b, 1/2c, 4a 4c, 4d, 4e, 5, 6a, 6b*	Schmuki, Erne, Loessner & Klumpp, 2012
FWLLm1	Sheep feces/ New Zealand	30	*Myoviridae*	n	98[§p]	225	1/2a, 2a, 3a, 4b*	Lee, 2008; Bigot et al., 2011
FWLLm3	Sheep feces/ New Zealand	30	*Myoviridae*	n	108[§p]	226	1/2a or 3a , 1/2b, 4b, 4d, 4e *	Lee, 2008
FWLLm5	Sheep feces/ New Zealand	30	*Myoviridae*	n	100[§p]	213	1/2a, 2a, 3a , 4b*	Lee, 2008
20422-1	Turkey processing plant/ USA	37	n	n	n	n	1/2a, 3a, 1/2b , 3b, 4b, 4d, 4e*	Kim et al., 2008
805405-1	Turkey processing plant/ USA	37	n	n	n	n	1/2a, 3a, 1/2b, 3b, 4b, 4d, 4e*	Kim et al., 2008

[¶]: Host range is based lysis zone formed by 10 µl of 10^9 PFU/ml of cocktail of six phages on bacterial streak 37 °C for 16 h.

[*]: Lyse other *Listeria* species in addition to *L. monocytogenes* strains

[§]: Negatively stained with: u: uranyle acetate (2% w/v), p: phosphotungstic acid (2% w/v)

n: Not given

2.6 Characterisation of phage isolates

2.6.1 Different parameters to distinguish new phage isolates

Newly discovered phage isolates are differentiated by characterizing with different parameters. The appearance of plaques is helpful in preliminary differentiation of lytic phages from temperate phages and generally lytic phages form clear plaques while temperate phages makes turbid plaques (Adams, 1959). However, culture based characteristics are subjective and influenced by many experimental parameters and therefore, phages need to be characterized with additional features which involve in more precise determination methods such as the analysis of complete genome sequences (Sulakvelidze & Pasternack, 2010). There have been exceptions to the discrimination of lytic and temperate nature of phages based on plaque morphology. *Listeria* phage P35 forms a turbid plaque (Hodgson, 2000) but this phage has been characterized as strictly lytic phage by genome sequencing (Dorscht et al., 2009).

Some biological, physical and genomic methods that are easy to perform with few laboratory facilities have been reported to distinguish a variety of phages. These methods include the presence of Ca^{+2} in broth cultures (Walakira et al., 2008); stability at 60 °C; efficiency of plating (EOP) in citrate agar (Oslen et al., 1968); size of plaques over different gel concentrations (Serwer et al., 2004); and host ranges (Hodgson, 2000; Kim et al., 2008; Lee, 2008; Loessner & Busse, 1990; Pasternack & Sulakvelidze, 2009; Walakira et al., 2008). The phage isolates were discriminated using the images obtained by Transmission Electronic Microscope (TEM) and restriction digestion profiles of the phage DNA. The distinct restriction digestion profiles of phage DNA were obtained using ClaI, SacI, and SpeI for some *Listeria* phages (Kim et al., 2008; Loessner et al., 1994; Pasternack & Sulakvelidze, 2009).

Ackerman et al. (1978) described guidelines for the characterization of phages using a set of criteria under three categories (low, medium and high). The low level criteria include plaque size, adsorption velocity, efficiency of plating, latent time, burst size and pH sensitivity. The host range, stability of phages to physical and chemical agents such as chloroform, ether, heat and ultraviolet light are included in the medium level criteria. Under high level criteria, the morphology and dimensions of phages using TEM images, determining types and percent of phage proteins, type and number of strands of nucleic acids of phage are recommended.

Bacteriophages discovered from different places can be found deposited in reference laboratories including the Felix d'Herelle Reference Center, University of Laval, Canada (http://www.phage.ulaval.ca/en/phage_deposit); American Type Culture Collection (ATCC), 10801 University Boulevard, USA (http://www.atcc.org/) and JSC Biochimpharm, Georgia (http://www.biochimpharm.ge/).

2.6.1.1 Host range of phages

The host range is one of the main criteria for the selection of a lytic phage for its suitability for biocontrol. The lytic *Listeria* phages have broad host ranges and they were reported to infect *Listeria* species in addition to *L. monocytogenes* (Table 2.1). Phage P100 has a broad host range (Carlton et al., 2005) and it infects >95% of *Listeria* strains (approximately 250 food-borne *Listeria* isolates) that include *L. monocytogenes* (serotypes 1/2 and 4) and *L. ivanovii* (serotype 5). Phage A511 (Klumpp et al., 2008) also has broad host range and infects ≈95% of *L. monocytogenes* strains (serotypes 1/2 and 4) and 100% of *L. ivanovii* strains. LMP-102 (a six phage cocktail) is sensitive to over 89% of the 160 *L. monocytogenes* strains belonging to serotypes 1/2a, 1/2b, 1/2c, 3a, 4a, 3b, 4b and 4d (Pasternack & Sulakvelidze, 2009). Some strains of *L. monocytogenes* are not sensitive to the phages.

The lack of phage receptors on the host cell wall is one of the reasons for resistance to phages (Hyman & Abedon, 2010). *L. monocytogenes* strain WSLC 1442, (serotype 1/2a) is not infected by many of the phage strains including phage A511 and phage P35 (Eugster et al., 2011; Wendlinger, Loessner & Scherer, 1996). Investgations on cell wall receptors found that this strain lacks N-acetylglucosamine on the cell wall TA (Eugster et al., 2011). The other phage resistant mechanisms of bacteria are the uptake blocks (prevention of the phage genome reaching the bacterial cytoplasm after phage adsorption on the cell wall), restriction modification, and clustered regularly interspaced short palindromic repeats (CRISPR) (Labrie, Samson & Moineau, 2010; Hyman & Abedon, 2010).

2.6.1.2 Taxonomy of Listeria phages

The present phage classification system of the International Commission of Taxonomy of Virus (ICTV) is based on the scheme given by Bradley in 1967 (Ackermann, 2009). The majority of phages have been classified under one Order (*Caudovirales*). *Caudovirales* is the largest phage group (>90% of total known viruses) and consists of tailed phages found to infect both Eubacteria and Archaea. Ackermann (2009) suggests

the use of term “Prokaryote viruses” which include both Eubacteria and Archaea, in place of the term “phage” which was originally given as a term for bacterial viruses (Sulakvelidze et al., 2001). The current diversity of phage is expanding due to the discovery of new phages with enormous structures that include the tailed phages that infect halophilic and methanogenic archaea. Varied phages of hyperthermophiles are quite dissimilar to the conventional phages (Ackermann, 2009). Phages belonging to the order *Caudovirales* consist of double stranded DNA, icosahedral or elongated head, a tail with appendages like base plates, tail fibers and spikes. The phage DNA is packed into preformed capsid during replication, and there is no outer envelope (Ackermann, 2009). The order *Caudovirales* includes three phage families (*Myoviridae, Siphoviridae* and *Podoviridae*) which are described based on the nature of the tail. *Myoviridae* has long rigid contractile tail (e.g. phage A511), *Siphoviridae* has a long non contractile tail (e.g. phage A118) and *Podoviridae* has a short non contractile tail (e.g. *E. coli* phage T7) (Ackermann, 2009).

A phage that could infect *Listeria* was first reported in 1947 (Schultz, 1945). About 400 *Listeria* phages, both temperate and lytic strains, were reported in 2005 (Hagens & Loessner, 2007). The lytic *Listeria* phages reported to date are given in Table 2.1. All known *Listeria* phages belong to the taxonomic family *Myoviridae* or *Siphoviridae.* The species of phage are classified based on a group of characteristics and all of these characteristics may or may not find in a member of the species. This approach is known as the polythetic-species concept (Ackermann et al., 1992). According to the ICTV classification, phage A511 and P100 belong to the subfamily, *Spounavirinae*; and Genus, *Twortlikevirus*. The species names of the two phages are *Listeria phage A511* and *Listeria phage P100*, respectively (ICTV, 2011).

2.6.1.3 Bio-safety characterization of lytic phages

The main bio-safety characteristics of a phage which is intended to be used in food systems include the inability of transduction and lack of genes encoding for exotoxins or allergins (proteins) and these features are assessed by performing bioinformatics analysis on whole phage genome sequences (Carlton et al., 2005; Hagens & Loessner, 2010; Klumpp et al., 2008). The phages that have large linear permuted terminal repeats of various sizes in the genome and lack of bacterial 16S-rRNA genes are described as non-transducing phages (Carlton et al., 2005; Klumpp et al., 2008; Sulakvelidze & Pasternack, 2010). Six *Listeria* phages of ListShield^{TM} lack undesirable genes that

encode exotoxins including genes of *Staphylococcus aureus*, *Corynebacterium diphtheriae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Clostridium botulinum* and *Streptococcus pyogenes* (Pasternack & Sulakvelidze, 2009). Also, P100 was assured to be non toxic for consumption by performing oral toxicity bioassays in mice (Carlton et al., 2005).

The FDA has approved ListShield™ to be used in food to control *L. monocytogenes* based on several criteria including the biosafety aspects, in brief: a) The safety of use of the phage preparation (lethal only to *L. monocytogenes* and not for other living organisms, b) Level of potential residues of *L. monocytogenes* toxins (haemolysin) are well below the acceptable limits, c) Phage does not contain undesirable genes such as those conferring production of toxigenic compounds and is unable to transduce, d) High phage titre, e) Absence of *L. monocytogenes* and other pathogens and f) Content of the total organic carbon in the phage formulation (FDA, 2006).

2.6.1.4 Phage binding receptors on the L. monocytogenes cell wall

The binding of bacteriophages occurs on different receptors on the bacteria cell wall. Teichoic acids (TA) of cell wall peptidoglycan were described as the components of phage receptors of the Gram positive bacteria such as *Bacillus* spp. *Lactobacillus* spp and *Listeria* strains (Givan et al., 1982; Wendlinger et al., 1996; Yokoura, 1977). In addition to the cell wall peptidoglycan, some phage strains bind to different appendages or other structural components associated with cell wall. For example, *P. aeruginosa* bacteriophage D3112 binds onto pili (Roncero, Darzins, & Casadaban, 1990), *B. subtilis* bacteriophage SP3 binds onto flagella (Shea & Seaman, 1984), *E. coli* bacteriophage K29 attach to the capsule (Bayer, Thurow, & Bayer, 1979), T7 phage binds onto lipopolysaccharide (LPS) and T5 phage onto surface proteins (Furukawa & Mizushima, 1982).

Wendlinger et al. (1996) identified cell wall components of *L. monocytogenes* that serve as receptors for three phage strains. They demonstrated that cell wall TA together with rhamnose and glucosamine serve as receptors for phage A118 (a temperate phage). The cell wall TA was the receptor for phage A500. The both phage A511 and P35 which are broad host range lytic phages, bind to the *N*-acetylglucosamine of petidoglycan of the *L. monocytogenes* cell wall (Eugster et al., 2011; Wendlinger et al., 1996). The presence or absence of *N*-acetylglucosamine in the cell wall can be visualized by

staining *Listeria* strains with fluorescently labeled wheat germ agglutinin (WGA) (Nir-Paz, Eugster, Zeiman, Loessner, & Calendar, 2012).

2.6.1.5 Adsorption rate constant (k value)

The rate of attachment of phages on bacteria is depend on the number of phages and bacteria present, effective radius of the phage particle, rate of diffusion of the phage, temperature applied, ionic strength of reaction medium and number of receptors on the host cell wall (Hyman & Abedon, 2009). Kasman et al. (2002) indicated the magnitude of phage k values is influenced by cell wall receptor density by comparing the k values of two *E. coli* phages (T4 and M13) against the same bacteria strain. Phage T4 has a high k value (2.4×10^{-9} ml/min) since there are ≈300 number of phage T4-specific receptors on the bacterium. The k value of M13 is $\approx 3 \times 10^{-11}$ ml/min due to the presence of only ≈2-3 M13-specific receptors on the bacterium. Adsorption capabilities of a given *E. coli* phage population are heterogeneous and therefore, a non-adsorbing fraction of phages (≈5%) is found at the end of about a 15 min adsorption assay period (Schlesinger, 1932). Gallet, Lenormand and Wang (2012) also demonstrated that a residual phage fraction (un-adsorbed *E. coli* phage) does exist in a given phage population. They suggest that an un-adsorbed phage fraction may help to prevent the lytic phages from extinction in the natural environment in harsh conditions where productive infections cannot be progressed after the phage adsorption. Moldovan, Chapman-McQuiston, and Wu (2007) found that the adsorption rate of phage Lambda onto *E. coli* Ymel strain dropped at around 4 °C compared with temperatures between 15 and 40 °C. They demonstrated that the phage binding receptors of bacterial cell wall were coagulated at low temperatures. However, the effect of low temperatures on the k value of psychotropic *Listeria* phages has not been reported.

The k value is a useful parameter to determine mutants of both phage and bacterial strains and the influence of different cofactors on phage binding (Hyman & Abedon, 2009). The k value is also an important measurement in phage based lysis kinetics (Abedon, 2009; Bigwood, Hudson, & Billington, 2009; Hagens & Loessner, 2010; Kasman et al., 2002).

2.7 Phage application strategies

2.7.1 Different approaches of phage application

Optimum lytic effectiveness of phages against given cell population can be achieved by either active or passive biocontrol approaches (Abedon, 2009; Gill, 2010). In the active biocontrol strategy, a low phage dose that is not sufficient to infect all cells, is introduced initially in a given system and sufficient number of phages to infect total cells are expected to produce by self-replication over time and therefore this approach involves a comparatively long treatment time. The rise in the numbers of phage during replication is associated with a decrease in bacterial counts. Theoretically if phages are not lost by other factors in the system and a high phage yield results from replication, all remaining cells will be subject to lysis. Some phage challenge experiments, which had used initially low phage titres, achieved a significant reduction of target cells to non-detectable levels over time (Leverentz, Conway, Janisiewicz, & Camp, 2004; Montanez-Izquierdo, Salas-Vazquez, & Rodriguez-Jerez, 2012).

In the passive bicontrol strategy, the initial dose itself contains a sufficient number of phages which can infect all target bacteria by adsorbing a one phage, at least on each bacteria within a comparatively short time (Gill, 2010). The time required for passive biocontrol path may be shorter than a lysis time (latent time) of a phage, because the minimum time required to kill the cells from this approach will be up to the successfully initiation of phage infection that occurs following the adsorption of a phage and then the ejection of phage DNA. After the initiation of a phage infection in a bacterium, the infected bacterium loses its own synthetic pathways (Abuladze et al., 2008). The intervention time is relatively shorter than for the active control approach which relies on phage replication over time (Abedon, 2009). The initial phage dose to lyse the total cells can be predicted by using an equation given by Kasman et al., (2002) which makes asumptions which will be discussed in another section below. A study which had used sufficiently high phages reported complete elimination of a *Yersinia* strain cell contamination on hard surfaces in ≈5 min (Rashid et al., 2012). However, the optimum time required for infection (lysis) of bacteria may depend on properties of a given treatment environment.

Another passive mode of cell lysis described as *lysis from without* (LWO), also causes cells to lyse without forming new phages (Abedon, 2011). In this phenomenon,

simultaneous adsorption of multiple phages causes the damage of the cell wall and then lysis of the cell (Abedon, 2011). The adsorbed phages are also destroyed along with lysis of the cells. Delbruck (1940) demonstrated that cell lysis occurs in a relatively short time (≈10 min) when ≈10^8 CFU/ml cells were mixed with 200 times more *E. coil* phages. Nevertheless, some bacterial strains are not sensitive towards LWO (Abedon, 2011). The possible reasons for resistance have been explained as the expression of certain genes (gene *sp*) of the prophage (Abedon, 2011). An experiment showed that *Staphylococcus aureus* was not sensitive to LWO with phages alone but the supply of phages and lysin together showed lysis of cells (Ralston, Baer, Lieberman, & Krueger, 1957; Ralston & McIvor, 1964). In some instances, cell lysis induced by lysin alone is also called LWO (Abedon, 2011).

Phages could be used as anti-bacterial agents targeting one or both of the above lysis strategies depending on the scope of the application. The active strategy will be more suitable if the action of the phage is expected to remain for over the course of time such as for the degradation of complex biofilms and therapy in human infection (Lu & Collins, 2007; Sutherland, Hughes, Skillman, & Tait, 2006). For the application of phages as surface decontamination agents in processing plants etc, the LWO method would be more appropriate since it could achieve fast killing of cells without running the risk of a productive infection that could lead to the emergence of phage resistant bacteria. One disadvantage of this approach is the need for high phage titres.

2.7.2 Kinetic modelling of phage application

Kinetic models are useful to estimate phage doses or the efficacy of a phage treatment under different dosing times and target levels of cell populations in different environments. The important parameters in assessing the efficacy of phage based lysis kinetics are host population, phage adsorption rate constant (k), phage dose and phage application time (Hagens and Loessner, 2010). An MOI of 10 given in mathematical model of Schelsinger (equation 1 of Kasman et al., 2002) has been used as the main model for many early phage challenge experiments (Kasman et al., 2002).

The MOI of 10 was found to fit for experiments that used high count cell cultures. The high level of cell lysis was achieved by using 10 times more phages. However, low count cell cultures (≈10^3 CFU/ml) cannot be effectively lysed using ten times more phages as explained by Hagens and Loessner (2010). When one reactant occurs at a

very low level, the other reactant must be at sufficiently high enough for a collision to occur to allow the reaction to proceed (Hagens & Loessner 2010).

The reasons for the failure of phage-based lysis of low count cells by following Schlesinger's model, was also elaborated using a new model (equation 2 of this reference) which was a derivative of Schlesinger's model (Kasman et al., 2002). The new model defined the multiplicity (MOI) of 10 as MOI_{actual} and shows a relationship between MOI (MOI_{input}) that is a ratio of the phages to host at the start of the treatment. An MOI_{actual} of 10 brings ≈99.999% cell infection, assuming a Poisson distribution (Abedon, 2009; Kasman et al., 2002). In order to maintain a MOI_{actual} of 10, the value of MOI_{input} or size the input phage dose should be manipulated in terms of the target host cell counts, k value and length of phage dosing time. According to the new model, a phage dose containing a considerably high titre is required to completely lyse the low count culture in a short time. This was also demonstrated experimentally by Kasman et al. (2002). This model makes the assumptions that all cells are sensitive to phage and the cell concentration remains constant during the phage dosing. Therefore, these assumptions are fairly valid in phage applications with a short intervention time. Based on this new model, another equation (equation 4 given by the authors) was elucidated that could be used to calculate an effective input phage dose against a given cell population (Kasman et al., 2002). The efficacy of phages should be assessed against low counts of bacteria since food processing plants are operated under hygienic regimes (Hagens & Loessner, 2010).

2.7.3 Commercial phage products for the food industry

Phage P100 has received GRAS status and approval to be used as a food additive in ready-to-eat (RTE) meats by FDA (Micreos Food Safety, 2013). Intralytix (2013) has manufactured three food grade bacteriophage products: ListShield™, EcoShield™ and SalmoFresh™ which can be used to control contaminations of *L. monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella* strains highly pathogenic for humans. ListShield™ is a phage cocktail of six phages received approval by the FDA as a food additive in RTE meat and poultry products (Intralytix, 2013; Sulakvelidze & Pasternack 2010).

2.7.4 *Listeria* phage-based biocontrol in food systems

Gunether et al. (2009) investigated phage A511 in different food types contaminated with two *L. monocytogenes* strains (WSLC1001 and Scott A) in separate trials. Different phage titres (3 x 10^6 - 3 x 10^8 PFU/g) were assessed. The phage A511 at 3 x 10^8 PFU/g was effective in reducing ≈10^3 CFU /g of initial *L. monocytogenes* counts in different food types at 6 °C for 6 days. The viable cell counts of both *Listeria* strains in cabbage and iceberg lettuce were dropped by >2 log units after one day followed by slow growth (<1 log unit) during six days compared with untreated control which showed final counts of 3-4 log CFU/g. The initial contamination level (≈3 log units) of both strains in chocolate milk and mozzarella cheese brine, was reduced to a non-detectable level after 1 and 3 days, respectively and re-growth did not occur while *L. monocytogenes* counts of control chocolate milk and mozzarella brine reached to ≈6 and 4.5 log units, respectively. In hot dogs, cells of both strains were decreased by <1 log unit in day 1. Strain WSLC 1001 infected with phage remained non-detectable for 2-6 days in hotdogs. However, ScottA grew slowly over 6 days up to ≈1.5 log CFU/g compared with the control which reached over 4 log units CFU/g in 6 days. In mixed seafood (cooked shrimp, mussel and calamari) and sliced cooked turkey meat, initial contamination reduced by ≈1 log unit in day 1 and then cell counts increased but remained low compared with counts of the controls which reached to >6 and >5.5 log CFU/g, respectively after 6 days. The least effective product to be treated was smoked fish with a ≈1 log unit reduction of strain WSLC 1001 cells in day 1 after which the levels remained static for 6 days. With Scott A, the initial reduction was <0.5 log units with no further reduction over 6 days (Guenther et al., 2009). The low efficacy of phages in treating smoked fish and meat may be due to the dryness of the surfaces and limited diffusion of phage and shielding of the host in the matrix of uneven surfaces (Guenther et al., 2009). The efficacy of phage can be increased using high phage doses and larger liquid volumes on such surfaces. These investigations suggest the need to find suitable protocols to optimize the phage efficacy for particular foods (Guenther et al., 2009).

A six phage cocktail (LMP102) against *L. monocytogenes* LCDC81-861 (a strain implicated in a processed cabbage outbreak) was investigated in cut pieces of honey dew melon (Leverentz et al., 2004). In one assay, melons were contaminated with ≈1.5 log CFU/cm^3) of *L. monocytogenes* and then sprayed with a phage cocktail (10^8

PFU/ml) immediately. The viable counts reduced to a non-detectable level in 2 days and then a re-growth of cells occurred to ≈0.4 and 1.8 log CFU/cm^3 at 10 °C after 5 and 7 days, respectively. When phages were applied to the melon 30 min after contamination with *L. monocytogenes* (≈1.4 log CFU/cm^3), the cell counts reduced and remained non-detectable for 2 days. Later, the contamination level increased slowly to ≈3.2 and 4.6 log CFU/cm^3 at 10 °C after 5 and 7 days, respectively. However, the rise of cells was significantly lower than in growth on the control melon (7 log CFU/cm^3) after 7 days. In cut surfaces of apples, phages failed to reduce the *Listeria* contamination and it was believed to be due to the rapid loss of phages to a non-detectable level in 30 min due to the high acidity (pH ≈4.4) of apple pieces (Leverentz et al., 2003; Leverentz et al., 2004). About 10^8 PFU/ml was necessary to reduce the pathogen populations to a non detectable level and phages needed to be sprayed onto cut pieces of honey dew melon the immediately after cutting in order to control the *L. monocytogenes* growth on this product (Leverentz et al., 2004).

The lysis effect of P100 (6 x 10^7 PFU/cm^2) was evaluated against a low count contamination of *L. monocytogenes* LmC (serotype 1/2c), isolated from a dairy plant, on red smear cheese at 14 °C for 10 days followed by packed storage at 6 °C for 5 days (Carlton et al., 2005). Initial contamination of ≈2 x 10^1 CFU/cm^2 was decreased to a non-detectable level (<5 CFU/cm^2) by phage P100 over 6 days and re-growth did not occur for another 15 days. In the uninfected control cheese, the level of contamination increased from ≈10^1 to 10^7 CFU/cm^2 during 21 days. The elimination of surface contamination of *L. monocytogenes* was confirmed by a protocol that included a pre-enrichment step followed by subsequent enumeration of cells using a plating method (Carlton et al., 2005).

The phage-based biocontrol of *L. monocytogenes* during cheese ripening occurred at 12-13 °C in 11 days followed by 6 °C for 11 days (Guenther & Loessner, 2011). *L. monocytogenes* strains (Scott A and CNL 103/2005) that are linked to a cheese listeriosis outbreak were challenged with phage A511 (3 x 10^8 PFU/cm^2). Scott A was inoculated on white mold soft cheese (≈10^3 CFU/cm^2) and phages were applied after 1 h. The cell counts were decreased by 2.5 log units after the cheese ripening period (21 days) compared with counts in the untreated control cheese. More or less similar efficacy was also shown when phages were applied on cheese after 1 and 20 h of the *Listeria* contamination (repeat dosing). The application of a single treatment containing

a high phage dose (≈1 x 10^9 PFU/cm^2) after 1 h of *Listeria* contamination was able to reduce growth of *Listeria* contamination at a significant level than above phage treatments during ripening period. The phage A511 at 3 x 10^8 PFU/cm^2 was able to reduce the initial Scott A contaminations (10^1 and 10^2 CFU/cm^2) in unripened cheese to a not detectable level (<1 CFU/g) after 1 and 6 days, respectively and remained so for 21 days compared with high counts (7 log units) in untreated control after 21 days. Sufficient phages are needed in the initial single dose treatment as the phages are not believed to be able to effectively penetrate the microbial consortium on ripening rind as a complex biofilm develops (Guenther & Loessner 2011). The *L. monocytogenes* CNL 103/2005 had a comparatively fast growth rate in the cheese model showing the adaptability of this strain to cheese processing environments.

Holck and Berg (2009) studied the effectiveness of P100 (5 x 10^7 PFU/cm^2) combined with *Lactobacillus sakei* THI (10^3 CFU/cm^2) in controlling contamination with two strains of *L. monocytogenes* [strain 2230/92 (serotype 1) and strain 167 (serotype 4b)] on cooked ham under vacuum, stored at 10 °C for 28 days. The ham slices were contaminated with mixed *Listeria* cultures containing ≈10^3 CFU/cm^2, and phage were applied after 1 h at 20 °C. *L. sakei* THI were then inoculated onto the ham after 1 h of the phage treatment. The ham samples were stored at 10 °C for 28 days. A rapid reduction of contamination by 1 log CFU/cm^2 occurred in samples treated with phage alone or both phage and *L. sakei* within the first day. But in phage treated ham, the *Listeria* grew gradually up to ≈8 logs over 28 days, similar to the final counts of control ham. There was a 2 log units reduction of cells in ham which received both treatments compared with phage alone application during the 28 day storage. The initial counts of 10^4 CFU increased to 7 and 8 log CFU/cm^2 in 7 and 14 days, respectively then counts remained static in the control untreated samples up to 28 days. The results demonstrated a synergistic effect of the combined treatments in mitigating the growth of *L. monocytogenes* during the storage of hams compared with the application of phage alone.

Bigot et al. (2011) investigated the efficacy of phage FWLLm1 to control *L. monocytogenes* contamination in vacuum packed chicken meat during storage at 5 °C. They used *L. monocytogenes* 2004/47 strain isolated from meat and linked to an outbreak (Sim et al., 2002). The phages at 2.5 x 10^7 PFU/cm^2 controlled the initial contamination (10^3 CFU/ cm^2) of *L. monocytogenes* close to a non-detectable level at 5

°C for 21 days. The phage P100 was assessed for the control of a two strain mix of *L. monocytogenes* [EGD (1/2a) and Scott A (4b)] in catfish tissues (Soni et al., 2010). The initial contamination of cells (≈4.3 log CFU/g) was reduced by ≈0.4 and 1.6 log CFU/g by the application of phage 2 x 10^5 and 2 x 10^7 PFU/g, respectively at 22 °C for 2 h. When the phages were used at a higher level (2 x 10^7 PFU/g) this resulted in a decrease of ≈0.8 and 1.3 log CFU/g after 15 and 30 min, respectively at 22 °C, with no further significant reduction for ≈1.5 h ($p > 0.05$). This experiment showed the cat fish tissues should be treated with phages for at least 30 min for the best control of *L. monocytogenes* (Soni et al., 2010). The effectiveness of phage P100 (2 x 10^7 PFU/g) against *L. monocytogenes* contamination (4.3 log CFU/g) in cat fish fillets was assessed during storage at 4 and 10 °C. The phages reduced the initial contamination to ≈2.7 log in 30 min followed by slow growth of the remaining cells to reach 3.8 log CFU/g at 4 °C for 10 days compared with growth in controls to 5.2 log CFU/g. At 10 °C, the initial counts decreased to 2.5 log CFU/g in 30 min following phage treatment followed by growth to a lower level (4.3 log CFU/g) in 10 days compared with counts (6.3 log CFU/g) in the untreated control (Soni et al., 2010).

A series of phage P100 doses were evaluated against a two strain mix of *L. monocytogenes* (EGD and Scott A strain) contamination in salmon fish fillets at 4 and 22 °C for 2 h (Soni & Nannapaneni, 2010a). A phage dose of 10^8 PFU/g resulted in a decrease in the initial cell contaminations of ≈2, 3 and 4.5 log CFU/g on salmon fillets by 1.8, 2.5 and 3.5 log CFU/g, respectively in 30 min either at 4 or 22 °C. The contamination on salmon fillets (≈1.6 log CFU/g) was reduced to 0.3 log CFU/g by phage treatment (10^8 PFU/g) after day 1 and then remained static at 4°C over 10 days, while contamination of untreated control fillets increased to 2.6 log CFU/g in 10 days (Soni & Nannapaneni, 2010a).

2.7.5 Phage treatment of abiotic surfaces

Listeria phages significantly reduce bacterial contamination on abiotic surfaces (Roy, Ackermann, Pandian, Picard, & Goulet, 1993; Soni & Nannapaneni, 2010b). The time involved in the phage-based cell lysis is longer than exposure time of the antimicrobial (chemicals) in categories defined by the EPA (Sulakvelidze & Pasternack, 2010). A chemical sanitizer should be able to reduce 5 log units of cells (99.999%) in 30 s (Sulakvelidze & Pasternack, 2010). Therefore, EPA has classified the bacteriophages intended to be used in environmental applications under the category of microbial

pesticides (EPA, 2013) and Listhield^{TM} has been approved by the EPA as a microbial pesticide to be used on food contact or other surfaces.

The lysis of *L. monocytogenes* adhered onto stainless steel and polypropylene was investigated by using three *Listeria* phage strains (2671, H38 and H387-A) (Roy, Ackermann, Pandian, Picard, & Goulet, 1993) Two *L. monocytogenes* strains [10401 (serotype 4), and 8427 (serotype unknown)] were investigated separately. The lysis efficacy of individual phage and a cocktail of three phages were evaluated at 3.5 x 10^8 PFU/ml. The cells adhered onto stainless steel and polypropylene were in the range of ≈4-5 log CFU/ml. Single phage or cocktail phages reduced the adhered cell counts by 99-99.9% at 26 °C in 1 h with the highest efficacy was shown by the phage cocktail. The investigators also reported that the combined application of a phage cocktail (10^8 PFU/ml) and 40 ppm quatal [10.5% N-alkyldimethyl-benzylammonium, HCl (40% C-12, 50% C-14, 10% C-16) and 5.5% glutaraldehyde] completely lysed the cells on both surfaces indicating a strong synergistic effect. The combined use of disinfectants will help to reduce the use of synthetic chemicals in food processing environments (Roy et al., 1993). Hibma, Jassim and Griffiths (1997) investigated the control of the cell-wall deficient *L. monocytogenes* (L-form) that are reported to cause meningitis was investigated using a phage modified by a process called bacteriophage recombineering with electroporated DNA (BRED) phage. The L-form *L. monocytogenes* were formed using *L. monocytogenes* ATCC 23074 (serotype 4b), containing a *luxAB* gene expression plasmid, [pSB331). A BRED phage was obtained by a chemical treatment of a *L. monocytogenes* phage ATCC 23074-B1. The attachment of L-form cells on stainless steel coupons (SSC) was prevented by the BRED phage when SSC were immersed in an L-form cell suspension (≈10^5 CFU/ml) containing ≈10^9 PFU/ml of BRED phage at 30 °C for 6 h. In the control experiment, the cells were attached onto SSC that were incubated in a cell suspension without phage (Hibma et al., 1997).

2.7.6 Effectiveness of phage in biofilms

Soni and Nannapaneni (2010b) examined the efficiency of P100 in the degradation of two and seven day biofilms formed under static conditions on stainless steel at 22 °C using five *L. monocytogenes* strains (two 1/2a strains and three 4b strains) on SSC (1 cm^2). The *Listeria* culture used to inoculate the stainless coupons was replaced with fresh TSB at 2 day intervals during the formation of the biofilms. Both 2 and 7 day old biofilms were treated with 1 ml of phage (10^9 PFU/ml) for 24 h followed by a count of

surviving *Listeria*. The cells of the 2 day biofilm (≈7 log CFU/cm^2) were reduced by ≈5.4 units and from the 7 day bioflm (6.6 log CFU/cm^2) were reduced by ≈3.5 log units (Soni & Nannapaneni, 2010b). This investigation suggests that cell lysis due to phage treatment is affected by the age of the biofilm.

Montanez-Izquierdo et al. (2012) evaluated the effect of P100 on a 3 day biofilm formed by *L. monocytogenes* CCUG 15526 on stainless steel at 22 °C. Phage suspensions of 100 µl containing 10^5, 10^6, 10^7 and 10^8 PFU/ml were used to treat the biofilm and surviving cells were enumerated after 2, 8, 24 and 48 h. The two phage treatments (10^7 and 10^8 PFU/ml) for 8 h, resulted in a reduction of biofilm cells (≈4.3 log CFU/cm^2) by ≈3.4 log units. Biofilm cell counts reached non-detectable levels after 48 h of treatment (Montanez-Izquierdo et al., 2012). A phage (10^{10} PFU/ml) significantly reduced the cells of an 18 h old L-form biofilm by ≈3 log CFU/cm^2 at 30 °C over 6 h (Hibma et al., 1997). They reported a similar level of cell reduction using lactic acid (pH 3.2) at 130 ppm over 6 h.

2.7.7 Stability of phages

The phages should be stable under conditions used to store phage stocks and conditions under which the phage is used as a biocidal agent (Guenther, Huwyler, Richard, & Loessner, 2009; Hudson et al., 2005). Listex^{TM} (P100 suspended in saline) is stable and active at 4 °C, pH ranges of 5.5-9.5, in saturated NaCl and water activity range of 0.92-0.99, while it is inactivated at >50 °C, <pH 3 and low levels of HOCl solutions (Micros Food Safety, 2013). ListShield^{TM} phage cocktail is contained in phosphate buffered saline at pH 7.0-7.5, UV sensitive and stable at 2-6 °C in the dark during storage (Intralytix, 2013). The number of phage A511 on fresh sliced cabbage and lettuce reduced by ≈0.6-1.2 and ≈2 log PFU/g at 6 and 20 °C, respectively during a 6 day trial (Guenther et al., 2009). Researchers suggested natural compounds present in vegetable leaves such as organic acids and tannins may have damaged the phages during storage. The phage numbers reduced only by <0.6 log PFU/g in sausages, cooked turkey breast meat, smoked salmon, mixed seafood (cooked and chilled cocktail of shrimp, mussels and calamari), chocolate milk (pasteurized, 3.5% fat) and mozzarella cheese brine at 6 °C over 6 days. The results imply that phage P100 is stable in coagulated proteinacious materials and salt solutions. The viability of phage A511 in red-smear ripened cheese and white mold ripened cheese decreased by <1 and ≈1.5 log CFU/cm^2, respectively over 14-6 °C with variation of pH 5-8 over for 21 days and the phage loss on cheese

might be due to proteiolytic enzymes of starter cultures and the ripening flora on cheese (Guenther et al., 2009). The infectivity of phage P100 reduced by 5.5 and 5.2 log PFU/g on catfish fish fillet tissues at 4 and 10 °C, respectively over 10 days (Soni, Nannapaneni, & Hagens, 2010). The decrease of phage P100 in salmon fillets was only 0.6 log PFU/g at 4 °C for 10 days (Soni & Nannapaneni, 2010a). A six phage cocktail (LMP102) survived well at pH 5.7 in honey dew melon at 10 °C for 10 days (Leverentz et al., 2003). In apple tissues, a *Listeria* phage cocktail was reduced to a non-detectable level in 30 min due to the acidity (pH 4.37) of apples tissues (Leverentz et al., 2003). The varying survival profiles of phage on different conditions in above studies suggest that phage stability is attributed to the properties of the substrate.

2.7.8 Development of phage resistant mutants

Phage resistant bacterial mutants have been isolated in some phage challenge trials. O'Flynn, Ross, Fitzgerald and Coffey (2004) reported the emergence of bacteriophage insensitive mutants (BIMs) of *E. coli* O157:H7 at rate of about 10^{-6} in phage challenge experiments. However, the mutant phenotype reverted to the wild-phenotype when mutants were removed from phage exposure for 50 generations (O'Flynn et al., 2004). Phage insensitive colonies of *L. monocytogenes* 103/2005 strain were recovered in cheese which was treated with phage A511 (≈3 x 10^8 PFU/cm^2) after ripening of cheese for 22 days (Guenther & Loessner, 2011). However, bacteriophage insensitive mutants (BIM) were not found with *L. monocytogenes* ScottA contaminations in the same environment. Similarly, in other studies BIMs were not found when the same phage was applied against other *L. monocytogenes* strains in different foods (Guenther et al., 2009). Therefore, the investigators interpreted that the development of BIMs is a property of the target bacterial strain (Guenther & Loessner, 2011).

The BIMs may be due to various adaptations including a mutation of the binding cell wall receptors of bacteria (Guenther & Loessner, 2011) and restriction modification (Enikeeva, Severinov, & Gelfand, 2010). When phage are used as environmental cleaning agents, there may be better chances to evolve spontaneous bacterial mutants and adaptation of resistance against the phages in environmental niches with repeated exposure to phages (Hagens & Loessner, 2010). These phage resistant strains should be targeted with novel phages. The use of cocktails containing the two or more phage

strains, the rotation of the phages or antimicrobial agents and the use of phages combined with other types of antimicrobial compounds are suggested to prevent BIMs (Abuladze et al., 2008; Carlton et al., 2005; Hagens & Loessner, 2010; Roy et al., 1993; Tanji et al., 2004). Each phage strain of a cocktail should have different binding ligands that specifically attach onto the different cell wall receptors of a target bacteria cell (Tanji et al., 2004). Roy et al. (1993) demonstrated a high synergistic effect of *Listeria* phages in combination with a chemical disinfectant (Quatal at 40 ppm). The chance for the emergence of resistant mutants against many antimicrobials simultaneously is very unlikely (Roy et al., 1993). An alternate remedy would be the isolation of new phages from the environment as replacements for less effective phages owing to BIMs (Sulakvelidze et al., 2001).

2.8 Conclusion

L. monocytogenes is recognized as a significant food-borne pathogen that can persist in seafood processing environments. Food recalls and *L. monocytogenes* positive samples indicate that current lethal treatments are inadequate to control contamination in seafood processing plants. Bacteriophages have been reported as potential alternative surface decontaminating agents for the control of *Listeria*. Most of the virulent listeriaphages which have been isolated from different locations including New Zealand were not fully characterized.

A little is known about the efficacy of phages including current two commercial listeriaphage products against the isolates of *L. monocytogenes* from the seafood industry or under conditions likely to be found in seafood processing plants. *L. monocytogenes* cells are most likely to be physiologically stressed or damaged in a seafood processing environment and it is unknown how this will affect their sensitivity to bacteriophage treatment. Phage-insensitive *L. monocytogenes* strains that may emerge during bacteriophage treatment are a potential concern; therefore, further phages need to be isolated and characterized. Considering the intellectual property rights and potential limitations on planning some experiments with listeriaphages belonging to other laboratories, including the commercialized phages, this project aimed to isolate

and characterize novel phages active against *L. monocytogenes* under simulated seafood environment conditions along the objectives mentioned in the introduction.

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Chapter 3: Host range and in vitro lysis of *Listeria monocytogenes* seafood isolates by bacteriophages

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Abstract

Bacteriophages that infect *Listeria* (listeriaphages) can be used to control *Listeria monocytogenes* in the food industry. However, the sensitivity of many of seafood-borne *Listeria* strains to phages has not been reported. This study investigated the host ranges of three listeriaphages (FWLLm1, FWLLm3 and FWLLm5), the *in vitro* lysis kinetics of listeriaphage FWLLm3 and predicted the phage titres required to lyse host cells. The host ranges of the phages were determined by using 50 *Listeria monocytogenes* strains, of which 48 were isolated from the seafood industry and two from clinical cases. Based on the formation of discrete plaques or lytic zones (host kill zones), the host ranges phages FWLLm1, FWLLm3 and FWLLm5 were ≈87%, 81% and 87%, respectively, at 25 °C. Six *L. monocytogenes* strains previously isolated from the seafood processing environment were observed to be insensitive to all three phages tested. The adsorption rate constant (k value) of listeriaphage FWLLm3 was between $1.2x10^{-9}$ and $1.6x10^{-9}$ ml/min across four host strains in tryptic soy broth at 25 °C. The cultures (3-4 $\log_{10}$ CFU/ml) were lysed to a non-detectable level (< 1 $\log_{10}$ CFU/ml) when cultures were infected with FWLLm3 at > 8.7 $\log_{10}$ PFU/ml for 30 min. Re-growth of phage-infected cultures was not detected after 24 h. The effective empirical phage titre was similar to the calculated titre using a kinetic model. Results indicate the potential use of the three phages for controlling *L. monocytogenes* strains in seafood processing environments.

Keywords Host range, listeriaphages, seafood, *Listeria monocytogenes,* adsorption rate constant

3.1 Introduction

New Zealand earns considerable revenue from seafood exports that mainly comprise farmed Greenshell™ mussel, rock lobster, hoki, squid and salmon. About 300,000 tons of seafoods worth NZ$ 1.56 billion were exported in 2010-2011 (Ministry of Fisheries, 2012). In the seafood industry, like many other food sectors around the world, *Listeria monocytogenes* is a fastidious environmental pathogen which causes food-borne listeriosis through contaminated foods (Farber and Peterkin, 1991). Immunocompromised individuals (with cancer, diabetes, HIV-infected and organ transplants), eldery persons and pregnant women together with their infants are more susceptible to listeriosis with a mortality rate of 20-30% while healthy individuals normally only develop a mild gastroenteritidis (Swaminathan & Gerner-Smidt, 2007). High costs are incurred in management of listeriosis outbreaks and clinical cases while the loss of lives of the affected is incomparable (Lake, et al., 2009; Scharff, 2012).

Listeriosis outbreaks associated with contaminated smoked mussels, smoked salmon and undercooked fish are reported (Brett et al., 1998; Ericsson et al., 1997; Gudbjornsdottir et al., 2004; Tham et al., 2000). A small listeriosis outbreak occurred in New Zealand 1992 due to consumption of contaminated smoked mussel and three affected patients were reported (Brett et al., 1998). *L. monocytogenes* serotype 1/2a strain (KM92 strain) linked to this outbreak (Brett et al.,1998) was also included in this study. *L. monocytogenes* contaminated RTE food caused about 23 food recalls, which also included mussel and smoked fish products between July 2006 and May 2009 in New Zealand (Crerar et al., 2011).

Currently, a variety of listericidal products are being used or investigated including physical methods such as steam treatment, and high pressure processing (Bremer et al., 2002; Fletcher et al., 2008; Keklik et al., 2009; Ozer and Demirci, 2006) and chemical methods (Crapo et al., 2004; Kastbjerg et al., 2009; Kim and Young, 2009; Kim et al., 1999). The recurrent and persistent nature of *L. monocytogenes* (Cruz and Fletcher, 2011) resulting from the development of resistance to disinfectants, its ability to form biofilms and quorum sensing (Gandhi and Chikindas, 2007; Moltz and Martin, 2005; Norwood and Gilmour, 2000), and growth at refrigeration temperatures challenges the existing listericidal strategies used in food processing environments.

As an alternative strategy, the use of listeriaphages to control *L. monocytogenes* has been investigated in a variety of settings including broth cultures (Dykes and Moorhead, 2002; Soni and Nannapaneni, 2010a), simulated biofilms (Soni and Nannapaneni, 2010b), fish products (Guenther et al., 2009; Soni and Nannapaneni, 2010a; Soni et al., 2010), meat (Bigot et al., 2011; Bigwood et al., 2008; Dykes and Moorhead, 2002;), cheese (Carlton et al., 2005; Guenther and Loessner, 2011) and fruits (Leverentz et al., 2004). The use of phages as a food safety strategy is desirable as they are often very specific towards a pathogen of concern, yet they do not affect the existing commensal microflora or alter desirable food properties and are biodegradable (Hagens and Offerhaus, 2008). The use of listeriaphages as bactericidal agents could also reduce the use of chemicals in the food industry. Currently, two commercial listeriaphage products: a) Listex™P100 containing listeriaphage P100 from Micreos Food Safety, formerly EBI, (Micreos Food Safety, 2013) and b) ListShield™ from Intralytix, Inc., a cocktail of six listeriaphages (Intralytix, 2013) are available for use in the food industry (Monk et al., 2010).

There are several studies on effective phage doses for maximum bactericidal effect in food systems (Bigwood et al., 2009; Kasman et al., 2002) as well as reports on theoretical modelling work (Abedon, 2009; Cairns et al., 2009; Gill, 2010; Hagens and Loessner, 2010; Kasman et al., 2002). The relevant theoretical values of input multiplicity of infection (MOI) pertaining to the infection of total cells of culture have been demonstrated by mathematical modelling (Kasman et al., 2002). Several authors have reported the efficacy of listeriaphage *in vitro* lysis of cells based on the ratios of phage titres and host counts (Bigot et al., 2011; Dykes and Moorhead, 2002; Soni and Nannapaneni, 2010a), but no studies have been reported on the use of mathematical models to estimate the effective input listeriaphage titres *in vitro*. At present, there is no published information on the sensitivity of many seafood-borne *L. monocytogenes* strains to phages.

The listeriaphages used in this study (FWLLm1, FWLLm3 and FWLLm5) were isolated from sheep feces and have also been reported to lyse species of *Listeria* other than *L. monocytogenes* (Lee, 2008). The objectives of the present study were to: a) assess the host range of three listeriaphages against *L. monocytogenes* strains previously isolated from the seafood processing environment, b) determine experimental ratios of host and phage involved in total cell lysis *in vitro*, c) determine the adsorption rate constant (k value) and d) estimate the minimum listeriaphage titres for total host cell

lysis using a mathematical model (Kasman, et al., 2002). We also compared the conformity of the empirical and estimated phage titres relevant to total host cell lysis *in vitro*.

3.2 Materials and methods

3.2.1 *L. monocytogenes* strains and listeriaphages

Fifty *L. monocytogenes* strains (Tables 3.1 and 3.2) mainly isolated from seafood environments were obtained from different laboratories in New Zealand comprising four strains from the University of Otago, Dunedin; one strain from Massey University, Palmerston North; one strain from ESR, Christchurch; 38 strains from Plant and Food Research Limited (PFR), Auckland; and six strains from Cawthron Institute, Nelson. *L. monocytogenes* strains were stored in cryovials (Mast Diagnostics, 1EA, UK) at -80 °C. Monthly working cultures were recovered from the frozen cultures by streaking onto plates of trypticase soy agar (TSA) (Difco, MD, USA) with incubation at 30±1 °C for 18 h, and then stored at 4 °C. The characteristics of *L. monocytogenes* isolates (serotype and pulsotype) used in this study are described in Tables 3.1 and 3.2 (Cruz and Fletcher, 2011). Three listeriaphages, FWLLm1, FWLLm3 and FWLLm5 (Bigot et al., 2011; Lee, 2008), were kindly provided by ESR and filter-sterilized phage lysates were stored at 4 °C.

3.2.2 Preparation of phage stocks

Three phages FWLLm1, FWLLm3 and FWLLm5 were propagated using the double layer agar (DLA) plaque assay (Carlson, 2005) using an overlay of soft TSA agar prepared with trypticase soy broth (TSB; Difco) and 0.4% (w/v) agar (Difco) supplemented with 5 mM $CaCl_2$ (Scharlau Chemie S.A., Sentmenat, Spain). High titre phage stocks were prepared using the host strain *L. monocytogenes* 18GO1 which has a high efficiency of plating. Briefly, 5 ml saline-magnesium buffer (SM buffer) containing gelatin [100 mM NaCl (Merck, Darmstadt, Germany), 50 mM Tris-HCl (Sigma Aldrich, Steinem, Germany), 8 mM $MgSO_4$ (Merck), 0.001% (w/v) gelatin (LabChem, NSW, Australia) at pH 7.5] was added to each confluent-lysed DLA plate. The phages were extracted into the SM medium by agitating at 60 rpm (OM1 Orbital Mixer/Platform Mixer, Ratek Instruments Pty. Ltd, Victoria, Australia) for 5 h at 23±2 °C. The SM

medium containing phages and also the soft agar layer were collected into polypropylene conical centrifuge tubes and centrifuged at 3,000 *g* (Biofuge, Primo R, Heraeus, CA, USA) at 4 °C for 10 min and filtered through 0.2 µm cellulose acetate syringe filters (MSR, Shanghai, China). About 50 ml volumes of the resultant phage lysates were then purified by ultra-centrifugation (Ultracentrifuge Sorvall Discovery100SE, Inc, USA) in polypropylene oak-ridge bottles at 27,000 *g* for 17 h. The phages were eluted from the pellet in 1 ml of SM medium with incubation at 4 °C for 18 h followed by agitation at 40 rpm at 23±2 °C for 2 h. The phage suspensions were transferred into glass bottles wrapped with aluminium foil. Phage stocks were stored at 4 °C.

3.2.3 Host range of phages

The host range of phages FWLLm1, FWLLm3 and FWLLm5 was studied using the *Listeria monocytogenes* strains listed in Tables 3.1 and 3.2. All cultures were grown to exponential phase in 10 ml of TSB (pH 7.3) with incubation at either 30±1 °C for 18 h, 25±1 °C for 18-24 h or 15±1 °C for 48 h. Using the DLA method, 100 µl of each culture (≈10^5 CFU/ml) was overlaid onto TSA plates which were marked with grids (1.5 x 1.5 cm). Inoculated plates were allowed to solidify for about 1 h and then 20 µl drops of serial ten-fold dilutions of phages (≈10^5-10^8 PFU/ml) were pipetted onto labelled sections of the host inoculated plates. SM buffer drops without phage were also included on the host lawns as controls. Drops were allowed to absorb for about 1 h at 23±2 °C and then the plates were incubated at 15±1, 25±1 and 30±1 °C for 96, 72 and 48 h, respectively to determine the formation of lytic zones. Duplicate plates were prepared for each host strain.

The phage host pairs which resulted in lytic zones were further investigated by plaque assays using the DLA technique (Kutter, 2009) under similar incubation conditions. Control plates without inoculated phages were also included in the experiment. Host strains which permitted phage plaque formation (Table 3.2) were investigated for efficiency of plating (EOP) at 15±1 and 25±1 °C (Table 3.3) following the method described by Kutter (2009). *L. monocytogenes* 18GO1 was used as indicator host to determine the EOP. The EOP is defined as the % of phage plaque counts formed in a lawn of test host strain against the number of plaque counts of the lawn of the indicator host (Kutter, 2009). Additionally, three non-plaque forming host strains in which phages formed clear lytic zones only by the standard DLA method were further

assayed on agar supplemented with ampicillin (5 mg/l, ampicillin sodium salt; Sigma Aldrich) (Santos et al., 2009) in order to visualize plaques that were not visible under the normal plaque assay conditions. Each host was tested on duplicate plates in at least three separate experiments.

3.2.4 *In vitro* lysis of *L. monocytogenes* by phage FWLLm3

Exponential phase cultures of *L. monocytogenes* 19EO1, 19EO3 and 22BO7 grown at 25±1 °C for 18-24 h were centrifuged at 3,000 *g* for 10 min and then re-suspended in the same volume of fresh TSB medium. Cell concentrations of $\approx 10^3$-10^4 CFU/ml were prepared by serial 10-fold dilutions in TSB containing 5 mM $CaCl_2$ and an aliquot was stored on ice to determine initial cell counts. Each diluted host culture was infected with a series of different titres ($\approx 6x10^2$ to $6x10^8$ and $2x10^9$ PFU/ml) of phage FWLLm3 in TSB containing 5 mM $CaCl_2$, mixed and incubated at 25±1 °C for 30 min at 40 rpm. The sample aliquots (2 ml) were chilled immediately and plated in < 30min. The number of surviving host cells were enumerated by preparing serial ten-fold dilutions in phosphate buffered saline [PBS; 0.008 M Na_2HPO_4 (Merck), 0.001 M $NaH_2PO_4.H_2O$ (Merck), and 0.145 M NaCl (Merck); pH 7.5], spread-plating 0.1 ml samples on TSA plates in triplicate and incubating at 30±1 °C for 24-48 h. Cell counts of control cultures (without phage) of each host strain were also enumerated using the same technique and under identical incubation conditions. At least two independent experiments were conducted for each strain.

3.2.5 Inactivation of low counts of *L. monocytogenes* by FWLLm3 over 24 h

Exponential phase cell suspensions of *L. monocytogenes* strains 19EO1 and 18GO1 grown at 25±1 °C for 18-24 h were diluted to $\approx 10^3$ CFU/ml and mixed with listeriaphage FWLLm3 at $\approx 10^8$ PFU/ml following the same protocol described previously. A control culture in the absence of phage was also prepared using the same conditions. Surviving cells of the phage-treated and control cultures were enumerated at 0, 0.5, 1, 2, 2.5, 3, 6, 12 and 24 h at 25±1 °C using the spread plate technique previously described. Three independent experiments were performed.

3.2.6 Determination of adsorption rate constant (k value) of phage FWLLm3

Exponential cultures of *L. monocytogenes* 19EO1, 19EO3, 22BO7 and 18GO1 grown at 25±1 °C for 18-24 h containing $\approx 10^8$ CFU/ml were infected with phage at a titre of $\approx 10^5$ PFU/ml in TSB supplemented with 5 mM $CaCl_2$. The infected cultures

were mixed gently by vortexing for 30 s and incubated at 25±1 °C for 15 min. Sample aliquots (0.8 ml) were immediately filtered through 0.2 µm cellulose acetate syringe filters (Shao and Wang, 2008) at 2 to 3 min intervals for 15 min and stored in the dark at 4 °C. The sample filtrates were assayed for the un-absorbed phage titre by the DLA method. The listeriaphage k values were calculated using equation (1) of Hyman and Abedon (2009):

$$\ln P = -(kN)*t + \ln P_0 \quad (1)$$

Where N is bacterial density (CFU/ml); P_0 and P are the initial and final un-adsorbed phage titres (PFU/ml), respectively; k is the adsorption rate constant (ml/min); t is phage exposure time (min). The adsorption rate constant was calculated from the gradient of the graph using equation (1) which had > 0.9 correlation coefficient (Hyman and Abedon, 2009) with the assumption that bacterial growth was not significant over the 15 min adsorption assay (Shao and Wang, 2008). The k value of phage FWLLm3 with three individual host strains was investigated and the mean k value for each pair was calculated from three replicate experiments.

3.2.7 Estimation of MOI_{input} and minimum phage FWLLm3 titre

The MOI_{input} was calculated from equation (2) according to Kasman et al. (2002), with total lysis of a given host population as:

$$MOI_{actual} = (1-\exp^{-kct})\ MOI_{input} \quad (2)$$

Where MOI_{actual} is the ratio of titre of phage adsorbed onto cells (PFU) to number of host cells in a reaction chamber; MOI_{input} is the ratio of both phage titre (PFU) to host counts (CFU) added in a reaction chamber; k is the adsorption rate constant (ml/min) specific to a phage and host pair; c is the initial host cell count (CFU/ml); t is the phage exposure period in the infected culture (min).

Equation (3) Kasman et al. (2002) is a derivative of equation (2) which includes the volume of medium in which lysis of the host occurs. This equation was used to calculate the minimum input phage titre capable of lysing the total host cells.

$$P = \frac{MN}{\left[1-\frac{\exp(-ktN)}{V}\right]} \quad (3)$$

Where *P* is the minimum input titre of phage involved in total host cell lysis with respect to a MOI_{actual} selected; *M* is the value of MOI_{actual}; *N* is the host cell population (CFU/ml); *k* is the adsorption rate constant (ml/min); *t* is the phage exposure time (min); *V* is the volume of phage-infected culture (ml).

3.2.8 Data analysis

Descriptive statistical analysis was used to analyse bacterial cell counts, phage titres and k values using Microsoft Excel (Microsoft Office, Washington, USA). Multiple mean comparisons of k values ($p \leq 0.05$) were determined using the General Linear Model and *post-hoc* Duncan Test of SAS 9.1 version (SAS Institute, Cary, USA).

3.3 Results and discussion

3.3.1 Host range of three phages

Host ranges of the three phages against *L. monocytogenes* strains are presented in two groups according to the temperatures at which they were assayed. All strains were studied at 25 and 30 °C (Table 3.1) to represent food abuse temperatures while the other group (Table 3.2) was studied at 15 °C to replicate the ambient temperature of the specific area of the fish processing plant where the *L. monocytogenes* strains had been isolated. *L. monocytogenes* cultures were grown at the same temperatures (15, 25 and 30 °C) at which the host ranges were assessed to reduce the impact of culture growth temperature on phage sensitivity, a phenomenon previously demonstrated by Kim and Kathariou (200 9).

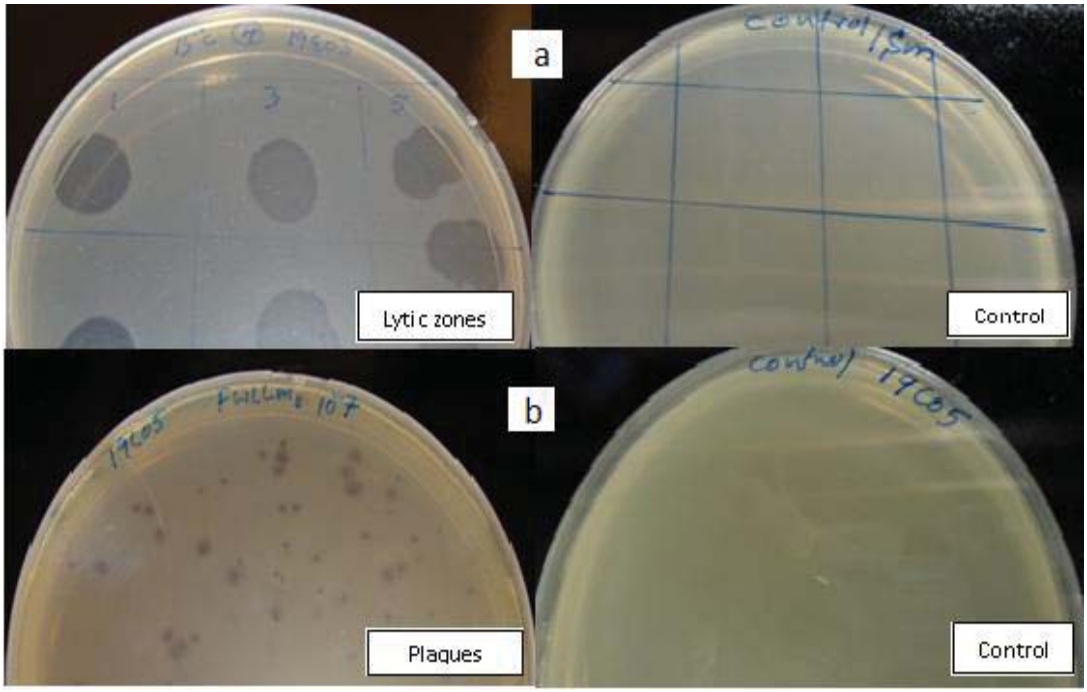

Figure 3.1 Host ranges of three listeriaphages were determined by the formation of either lytic zones or discreet plaques on host lawns: a, Lytic zone formed on a lawn of *L. monocytogenes* 19EO3 with 20 µl drops of listeriaphages (FWLLm1, FWLLm3 and FWLLm5) with respect to a control (20 µl drops of buffer) at 15±1 °C for 48 h; b, Plaques of listeriaphage FWLLm3 on a lawn of *L. monocytogenes* 19CO5 with respect to a control (host lawn only) at 25±1 °C after 48 h.

The host range of each phage was determined based on the formation of plaques or at least lytic zones on the lawn of the *L. monocytogenes* strain (Figures 3.1a and b). At 30 °C, all three phages produced clear plaques with 18 *L. monocytogenes* strains. Three phages also formed only lytic zones (> 10^7 PFU/ml) with a further seven strains. An additional five *L. monocytogenes* strains were infected by phages FWLLm1 and FWLLm5, forming lytic zones at 30 °C (Table 3.1). In the second group of *L. monocytogenes* strains, the three phages infected all strains at both 15 and 25 °C, resulting in plaque formation with 11 host strains and the formation of lytic zones with the remaining three host strains by the assay methods used (Figure 3.1, Table 3.2).

The diameter of phage plaques varied from about 0.5-3 mm across different host strains at 15, 25 and 30 °C. On lawns of *L. monocytogenes* 18GO1 (a phage-propagating host), three phages produced plaques with diameters of about 2.5, 2 and 1-2 mm at 15,

25 and 30 °C respectively. When plaques were aged on host lawns at least one day more than the set-incubation temperatures, narrow haloes (Figure 3.1 b) appeared around the plaques.

From the host range experiments, it was observed that six *L. monocytogenes* strains were not sensitive to any of the three phages tested at 25 and 30 °C (Table 3.1). These phage-insensitive strains belonged to serotype 1/2a or 3a, of which five strains belonged to PFGE subtype (AscI 51:ApaI 32) and the remaining strain (17KO9) belonged to pulsotype (AscI 74:ApaI 59) (Table 3.1). Pulsotype (AscI 51:ApaI 32) is persistent in some New Zealand seafood processing plants (Cruz and Fletcher, 2011). The insensitivity of these strains to the three phages may be due to lack of specific phage-receptors on the cell walls. Three *L. monocytogenes* strains belonging to serotype 1/2b, 3b or 7 were also included in the assessment of host range of the three phages. Two strains (18DO5 and 18DO7) of this serotype 1/2b, 3b or 7 groups were sensitive to all three phages, forming lytic zones (Table 3.2). The remaining strain (18GO5) belonging to serotype 1/2b, 3b or 7 formed lysis zones only with phages FWLLm1 and FWLLm5 (Table 3.1). All seven *L. monocytogenes* strains belonging to serotype 4b, 4d or 4e were infected productively by the three phages (Tables 3.1 and 3.2).

Varying EOP values were determined for three phages against eight selected *L. monocytogenes* strains at 15 and 25 °C (Table 3.3). The EOP values of the *L. monocytogenes* strains belonging to serotype 4b, 4d or 4e were found to be significantly lower than those determined for serotype 1/2a or 3a strains ($p > 0.05$) (Table 3.3). Likewise, some *L. monocytogenes* strains belonging to serotype 4b and 1/2c or 3c showed very low EOP (3.4×10^{-5}) for two listeriaphages isolated from a turkey processing plant (Kim and Kathariou, 2009; Kim et al., 2008). Los et al (2008) reported the absence of visible plaques on standard DLA plates even though productive infections had occurred. However, it was further demonstrated that productive infection in terms of triggering the formation of visible plaques was possible on the lawns of *E. coli* strains grown on agar supplemented with antibiotics such as kanamycin, amipicillin and tetracycline at sub-lethal concentrations. Using the same approach, *L. monocytogenes* strains 19DO3, 19EO1 and 19EO3, which did not form visible plaques with the standard DLA method in the current study, were assessed for the productive infections.

Table 3.1 Host ranges of phages FWLLm1, FWLLm3 and FWLLm5 across 36 *L. monocytogenes* strains isolated from fish, mussel and environmental swabs in seafood processing plants including two non-seafood strains.

L. monocytogenes strain[1]					Listeriaphage[2]					
Code of isolate	Serotype	PFGE subtype		Source	FWLLm1		FWLLm3		FWLLm5	
		AscI	ApaI		25°C	30°C	25°C	30°C	25°C	30°C
06BO3	1/2a	88	41	Raw Salmon	Pq	Pq	Pq	Pq	Pq	Pq
15EO3	1/2a or 3a	51	32	Raw mussel	-	-	-	-	-	-
15JO5	1/2a or 3a	38	02	Environmental	Pq	Pq	Pq	Pq	Pq	Pq
16EO8	1/2a or 3a	38	14	Environmental	LZ	LZ	LZ	LZ	LZ	LZ
15AO4	1/2a or 3a	38	14	Environmental	LZ	LZ	LZ	LZ	LZ	LZ
15IO6	1/2a or 3a	38	14	Environmental	LZ	LZ	LZ	LZ	LZ	LZ
15IO9	1/2a or 3a	38	14	Environmental	LZ	LZ	LZ	LZ	LZ	LZ
15JO8	1/2a or 3a	38	14	Environmental	LZ	LZ	LZ	LZ	LZ	LZ
15KO5	1/2a or 3a	38	14	Environmental	LZ	LZ	LZ	LZ	LZ	LZ
18CO9	1/2a or 3a	40	48	Environmental	Pq	Pq	Pq	Pq	Pq	Pq
15CO2	1/2a or 3a	51	32	Environmental	-	-	-	-	-	-
15DO1	1/2a or 3a	51	32	Environmental	-	-	-	-	-	-
15GO1	1/2a or 3a	51	32	Environmental	-	-	-	-	-	-
16I1O	1/2a or 3a	51	32	Environmental	-	-	-	-	-	-
15EO6	1/2a or 3a	51	32	Environmental	LZ	LZ	-	-	LZ	LZ
15GO4	1/2a	51	32	Environmental	LZ	LZ	-	-	LZ	LZ
16E10	1/2a or 3a	51	32	Environmental	LZ	LZ	-	-	LZ	LZ
15FO5	1/2a or 3a	51	82	Environmental	LZ	LZ	-	-	LZ	LZ
15AO7	1/2a or 3a	65	02	Environmental	Pq	Pq	Pq	Pq	Pq	Pq
17AO2	1/2a or 3a	70	02	Environmental	Pq	Pq	Pq	Pq	Pq	Pq
17KO9	1/2a or 3a	74	59	Environmental	-	-	-	-	-	-
16JO8	1/2a or 3a	89	81	Environmental	LZ	LZ	LZ	LZ	LZ	LZ
2000/47	1/2a or 3a	20	15	Environmental	Pq	Pq	Pq	Pq	Pq	Pq
CW1	1/2a or 3a	08	72	Environmental	Pq	Pq	Pq	Pq	Pq	Pq
CW2	1/2a or 3a	08	72	Environmental	Pq	Pq	Pq	Pq	Pq	Pq
SSM91	1/2b	35	27	Smoked mussel	Pq	Pq	Pq	Pq	Pq	Pq
SMAC91	1/2a	23	42	Smoked mackerel	Pq	Pq	Pq	Pq	Pq	Pq
KM92	1/2a	38	60	Smoked mussel	Pq	Pq	Pq	Pq	Pq	Pq
CW4	1/2a or 3a	08	72	Environmental	Pq	Pq	Pq	Pq	Pq	Pq
NCTC7973	1/2a or 3a	43	26	Guinea pig lymph	Pq	Pq	Pq	Pq	Pq	Pq
18AO1	1/2a or 3a	02	02	Environmental	Pq	Pq	Pq	Pq	Pq	Pq
18GO5	1/2b, 3b	86	75	Environmental	LZ	LZ	-	-	LZ	LZ
CW3	4b,4d or	63	31	Environmental	Pq	Pq	Pq	Pq	Pq	Pq
CW5	4b,4d or	63	31	Environmental	Pq	Pq	Pq	Pq	Pq	Pq
CW6	4b,4d or	23	12	Environmental	Pq	Pq	Pq	Pq	Pq	Pq
SMAL91	4b	23	12	Chilled Salmon	Pq	Pq	Pq	Pq	Pq	Pq

[1]Typing of *L. monocytogenes* isolates were performed by Plant and Food Research Limited (Cruz and Fletcher, 2011). The isolates with indistinguishable PFGE subtypes have been isolated from different samples and sampling periods from a same processing plant or from different processing plants. These isolates may represent recurrent strains. The strains isolated from environment (Environmental sources) including food contact and non-contact surfaces. Strain 200/47, a meat- borne *L. monocytogenes* strain linked to listeriosis (Sim et al., 2002); Strain KM92, isolated from smoked mussel linked to listeriosis (Brett et al.,1998) and also coded as NZ isolate SB92/870 (Bremer et al., 2002).

[2] Pq, Plaques; LZ, Lytic zone; -, Not sensitive to phage.

Table 3.2 Host ranges of listeriaphages FWLLm1, FWLLm3 and FWLLm5 using 14 *L. monocytogenes* strains isolated from one salmon processing plant.

L. monocytogenes strain[1]				Listeriaphage[2]					
Strain Code	Serotype	PFGE subtype		FWLLm1		FWLLm3		FWLLm5	
		AscI	ApaI	15 °C	25 °C	15 °C	25 °C	15 °C	25 °C
22BO5	1/2a or 3a	65	02	Pq	Pq	Pq	Pq	Pq	Pq
22BO7	1/2a or 3a	08	72	Pq	Pq	Pq	Pq	Pq	Pq
18DO5	1/2b,3b or 7	35	27	LZ	LZ	LZ	LZ	LZ	LZ
18DO7	1/2b,3b or 7	35	27	LZ	LZ	LZ	LZ	LZ	LZ
19DO3[3]	4b,4d or 4e	23	12	Pq	Pq	Pq	Pq	Pq	Pq
19EO5	4b,4d or 4e	63	31	Pq	Pq	Pq	Pq	Pq	Pq
19CO7	4b,4d or 4e	63	31	Pq	Pq	Pq	Pq	Pq	Pq
19EO3[3]	1/2a or 3a	51	32	Pq	Pq	Pq	Pq	Pq	Pq
19DO9	1/2a or 3a	59	42	LZ	LZ	LZ	LZ	LZ	LZ
18GO1	1/2a or 3a	83	42	Pq	Pq	Pq	Pq	Pq	Pq
19CO5	1/2a or 3a	08	46	Pq	Pq	Pq	Pq	Pq	Pq
19EO1[3]	1/2a or 3a	59	46	Pq	Pq	Pq	Pq	Pq	Pq
18FO9	1/2a or 3a	59	46	Pq	Pq	Pq	Pq	Pq	Pq
19CO9	1/2a or 3a	08	72	Pq	Pq	Pq	Pq	Pq	Pq

[1] Environmental strains isolated from one fish processing plant. Serotyping and Pulsed Field Gel Electrophoresis (PFGE) subtyping of *L. monocytogenes* isolates were performed by Plant and Food Research Limited (Cruz and Fletcher, 2011). The isolates with same PFGE typing characteristics have been isolated from different samples and sampling periods from one processing plant. Therefore, these isolates may be more related strains or represent same strain.

[2] Pq, Plaques; LZ, Lytic zone; -, Not sensitive to phage.

[3]Formed minute plaques when soft trypticase soy agar contained 5 mg/l ampicillin.

These *L. monocytogenes* strains produced visible plaques with the three phages on agar containing 5 mg/l of ampicillin (Table 3.2). Similarly, using suitable antibiotic supplemented agar (Comeau et al., 2007), host strains which have very low infection vigour (Hyman and Abedon, 2010) can be preliminarily identified whether they are subject to productive infections by a particular phage.

Table 3.3 Relative efficiency of plating (%EOP) of listeriaphages FWLLm1, FWLLm3 and FWLLm5 on exponential phase *L. monocytogenes* strains on trypticase soy agar containing 5 mM $CaCl_2$ at 15±1 °C for 48-72 h and 25±1 °C for 24-48 h.

L. monocytogenes		Relative EOP[1,2] of listeriaphage					
Strain code	Serotype	FWLLm1		FWLLm3		FWLLm5	
		15 °C	25 °C	15 °C	25 °C	15 °C	25 °C
19CO7	4b,4d or 4e	19.6^{g}	34.9^{f}	33.7^{e}	38.0^{c}	40.3^{f}	43.8^{e}
19EO5	4b,4d or 4e	27.0^{f}	34.2^{f}	34.7^{e}	37.5^{c}	41.4^{e}	43.2^{e}
22BO5	1/2a or 3a	79.7^{b}	92.5^{b}	92.7^{ab}	85.3^{ab}	100.0^{a}	100.0^{a}
19CO9	1/2a or 3a	67.5^{c}	66.4^{d}	100.0^{a}	97.4^{a}	76.4^{c}	85.9^{b}
19CO5	1/2a or 3a	100.0^{a}	76.0^{c}	86.5^{bc}	85.3^{ab}	94.2^{b}	96.7^{a}
18GO1	1/2a or 3a	95.3^{a}	100.0^{a}	81.3^{c}	100.0^{a}	73.8^{dc}	85.4^{b}
18FO9	1/2a or 3a	53.4^{d}	64.4^{d}	56.5^{d}	89.1^{ab}	71.7^{d}	68.1^{c}
2000/47	1/2a or 3a	42.5^{e}	55.5^{d}	60.6^{d}	76.0^{b}	57.6^{e}	61.1^{d}

[1]EOP was the ratio of plaque counts of a strain to plaque counts of reference strain on which the highest plaque counts formed at each temperature. The relative EOP value of the reference strain was 100.

[2] Lower case superscripts with relative EOP values of each phage represent significance ($p < 0.05$) among strains within a column at each temperature (n=2).

In this study, the three phages demonstrated broad host ranges similar to those of other well-characterized lytic listeriaphages such as A511, P100, the cocktail of six phages (ListShield™) (Carlton et al., 2005; Klumpp et al., 2008; Loessner and Busse, 1990; Pasternack and Alexander, 2009) and the turkey processing plant phages previously mentioned (Kim et al., 2008). Based on the sensitivity of seafood-borne *L. monocytogenes* strains, phages FWLLm1 and FWLLm5 had host ranges of about 87% (42/48 strains) and phage FWLLm3 infected 81% (39/48 strains) of the strains tested at 25 °C (Tables 3.1 and 3.2). In the present study, host ranges were investigated based on the formation of plaques or lytic zones. However, there are other criteria which could be used to evaluate host ranges such as adsorption assays and efficiency of centre of infection (ECOI) productive infection assays (Hyman and Abedon, 2010).

3.3.2 *In vitro* lysis of *L. monocytogenes* cells by phage FWLLm3

In this study, the lysis of low counts of *L. monocytogenes* was assessed to simulate the likely levels of the bacteria in food processing plant environments. For the phage-lysis kinetic experiments, *L. monocytogenes* 18GO1, 19EO1, 19EO3 and 22BO7 strains were selected based on their varying sensitivities demonstrated in the host range experiment (Tables 3.2 and 3.3). Initial cell suspensions of ≈3.5, 3.8 and 3.5 $\log_{10}$

PFU/ml pertaining to *L. monocytogenes* 19EO1, 19EO3 and 22BO7 respectively, were infected individually with a series of phage FWLLm3 dilutions (Figure 3.2). The surviving cells of the cultures infected with high phage titres of ≈8.8 and 9.8 $\log_{10}$ PFU/ml dropped to non-detectable levels (< 1 $\log_{10}$ CFU/ml) within about 30 min (Figure 3.2). However, surviving cells were detected in the cultures infected with lower phage titres (≈2.8-7.8 $\log_{10}$ CFU/ml) (Figure 3.2).

In another experiment, *L. monocytogenes* 19EO1 and 18GO1 cultures containing ≈3.8 and 4.9 $\log_{10}$ CFU/ml, respectively were infected with phage FWLLm3 at ≈8.7 $\log_{10}$ PFU/ml (Figure 3.3). At time zero, immediately after being mixed with phages, initial viable counts of *L. monocytogenes* 19EO1 and 18GO1 were reduced from 3.8 to 1.8 $\log_{10}$ CFU/ml and from 4.9 to 1.5 $\log_{10}$ CFU/ml, respectively. Therefore, the percentage of the immediately infected cells accounted for ≈99.0% and 99.9% in the two cultures, respectively. After about 30 min, any surviving cells were not detected (< 1 $\log_{10}$ CFU/ml) and re-growth of phage-infected cultures was not detected at 25 °C during a 24 h period (Figure 3.3). Observations on the lysis of host cells suggest that total infection of cells may have occurred in the first rounds of phage-infections (Figure 3.2). Similar results have been reported for lysis of low count cultures of *L. monocytogenes* strains by phages at high titres *in vitro* (Bigot et al., 2011; Soni and Nannapaneni, 2010a). In previous similar experiments, the samples containing high phage titres were centrifuged to remove the free phage before plating the samples in order to enumerate surviving cell counts (Bigwood et al., 2008, Soni and Nannapaneni, 2010a). In present study, the un-adsorbed phage fraction was not separated in samples before plating the samples for enumerating the surviving cells. Therefore, phage-infection of cells could be expected to occur during the plating of samples. Nevertheless, samples were chilled on ice immediately after the required exposure time in order to retard the phage adsorption beyond the sampling time and plated within about 30 min in present study. Moldovan et al. (2007) reported a sharp reduction of the adsorption rate of a lambda phage at < 10 °C. Results of the present study suggest that phage-lysis of cells might not have occurred in plates during incubation because colonies (1.8 and 1.5 $\log_{10}$ CFU/ml) were detected on the agar plates of the time-zero samples of 19EO1 and 18GO1, respectively, which contained high phage titres of ≈8.7 $\log_{10}$ PFU/ml (Figure 3.3).This may be due to the immobilization of phages on the 1.5% agar plates. Supporting this view, Heringa et al. (2010) have shown that plate counts of

samples from which a free high phage titre is removed by centrifugation and samples containing free phages are not significantly different.

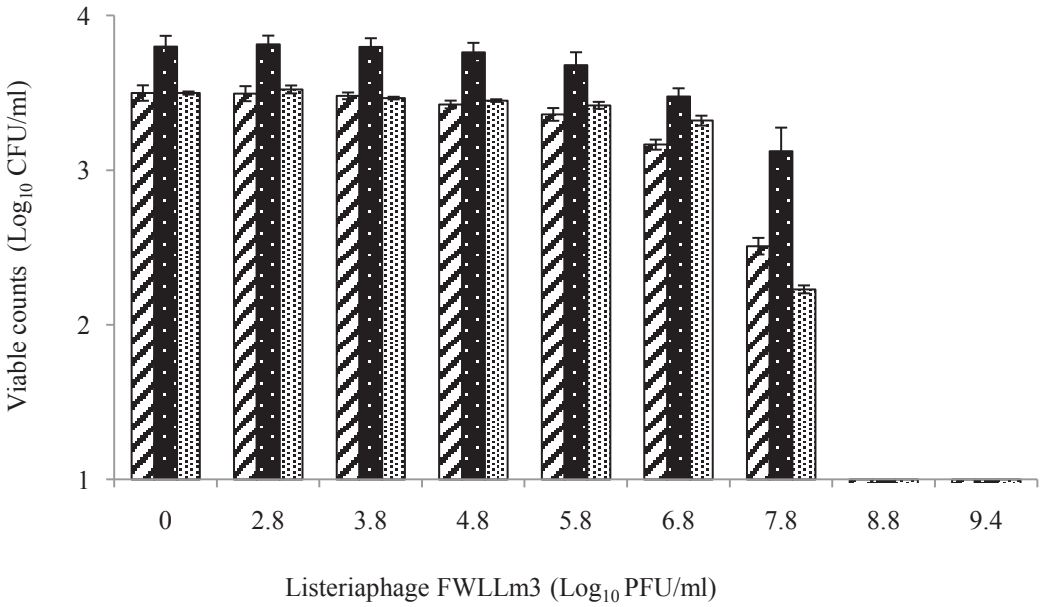

Figure 3.2 Viable cell counts of *L. monocytogenes* after infection with a series of listeriaphage FWLLm3 titres (0 (control), 2.8, 3.8, 4.8, 5.8, 6.8, 7.8, 8.8 and 9.4 $\log_{10}$ PFU/ml) in trypticase soy broth containing 5 mM $CaCl_2$ at 25±1 °C for 30 min. Phage infected cultures of *L. monocytogenes* strains: , 19EO1; , 19EO3; , 22BO7. Mean surviving host cell counts of three determinants of one experiment. Error bars represent standard deviation of mean. Detection limit = 1 $\log_{10}$ CFU/ml.

Results of the present study suggest that phage-lysis of cells might not have occurred in plates during incubation because colonies (1.8 and 1.5 $\log_{10}$ CFU/ml) were detected on the agar plates of the time-zero samples of 19EO1 and 18GO1, respectively, which contained high phage titres of ≈8.7 $\log_{10}$ PFU/ml (Figure 3.3).This may be due to the immobilization of phages on the 1.5% agar plates.

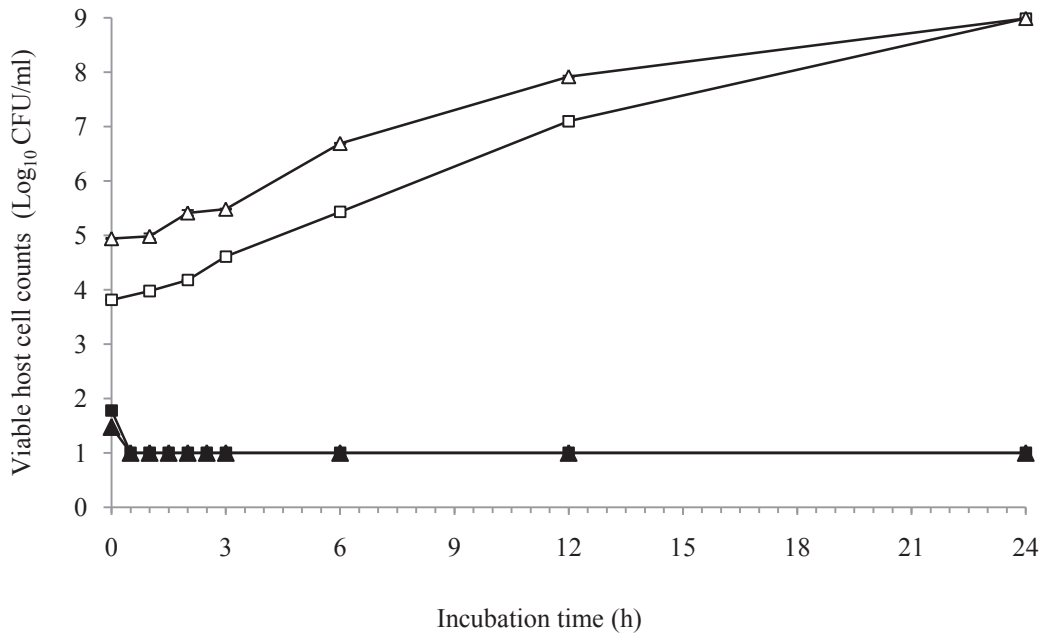

Figure 3.3 Viable cell counts of *L. monocytogenes* after infection with ~8.7 $\log_{10}$ PFU/ml of listeriaphage FWLLm3 in trypticase soy broth containing 5 mM $CaCl_2$ for 24 h at 25±1 °C. Cultures of *L. monocytogenes* strain 19EO1: ▭, Control; ■, Phage FWLLm3; 18GO1: △, Control; ▲, Phage FWLLm3. Mean surviving host cell counts ($\log_{10}$ CFU/ml) on triplicate plates of one experiment. Error bars represent standard deviation of mean. Detection limit = 1 $\log_{10}$ CFU/ml.

3.3.3 The k value of phage FWLLm3

The k value is influenced by the morphology and dimension of a phage; the nature and density of phage receptors on host cell wall; environmental factors such as viscosity of medium, cofactors and temperature (Moldovan et al., 2007; Gallet et al., 2009; Delbruck, 1940). Landry and Zsigray (1980) reported the k value of bacteriophage 41c with its host *Bacillus subtilis* at $1.1x10^{-9}$ and $3.7x10^{-9}$ ml/min in growth media containing 0.1 and 10 mM Ca^{2+}, respectively, at 30 °C. Schlesinger (1932) reported the average k values of coliphage when adsorbed onto heat-killed and live host cells at $1x10^{-11}$ and 2.7 $x10^{-11}$ ml/min, respectively. Gill (2008) reported the estimates of k values of different bacteriophages specific for host genera ranging from $1x10^{-7}$ to $2x10^{-11}$ ml/min. The k value is also an important parameter in models of phage-based lysis kinetics (Kasman et al., 2002; Abedon, 2009; Bigwood et al., 2009; Hagens and Loessner, 2010).

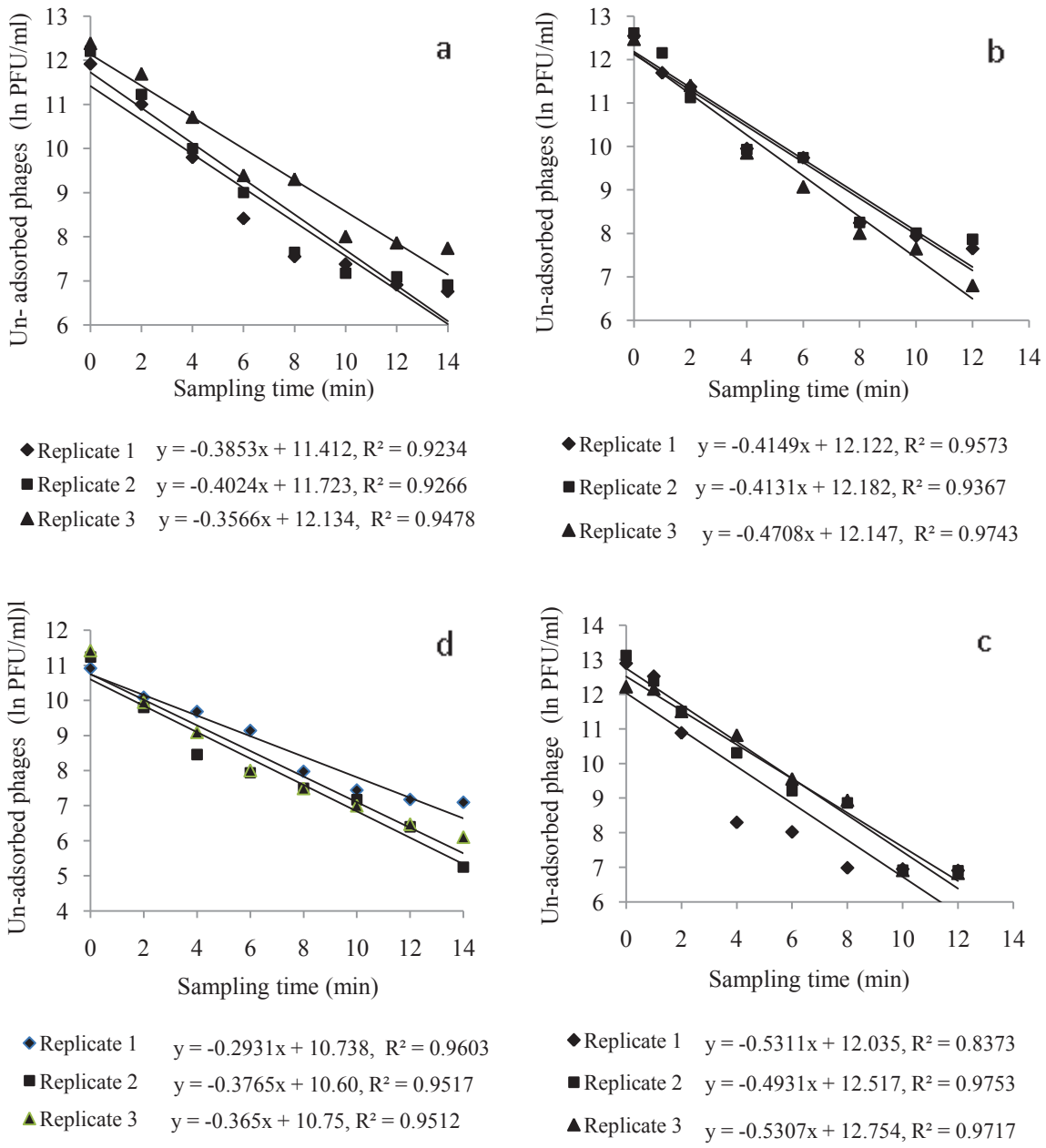

Figure 3.4 The adsorption kinetics of phage FWLLm3 on *L. monocytogenes* 18GO1, 19EO1, 19EO3 and 22BO7. The Phage (≈10^5 PFU/ml) was adsorbed on exponential phase cells (≈10^8 CFU/ml) of each strain in triptricase soy broth containing 5 mM Ca^{2+} at 25°C. Un-adsorbed phages were determined. The samples (0.8 ml) at 2-3 min intervals were filtered through 0.2 µm cellulose acetate syringe filters. The un-adsorbed phages were determined by the DLA method. The natural logarithm (ln) value of un-adsorbed phage titres were plotted against the sampling time. The adsorption rate constant (k) was calculated by equation 1 of Hyman and Abedon (2009).

Table 3.4 Estimates of input multiplicity of infection (MOI_{input}) and minimum practical (input) listeriaphage FWLLm3 titres (doses) which accomplish total lysis of *L. monocytogenes* cultures.

L. monocytogenes		k (ml/min)	t (min)	Estimates relevant to lysis of total cells [1]	
Strain	Experimental (CFU/ml)			MOI_{input}	Minimum input phage (PFU/ml)
19EO1	3.3×10^3	1.4×10^{-9}	30	7.1×10^4	2.4×10^8
19EO3	6.5×10^3	1.2×10^{-9}	30	4.2×10^4	2.8×10^8
22BO7	3.1×10^3	1.3×10^{-9}	30	8.1×10^4	2.5×10^8
18GO1	8.7×10^4	1.6×10^{-9}	30	2.3×10^3	2.0×10^8

[1]Value of MOI_{actual} was assumed as 10 (Abedon, 2009) and the estimates were based on equation 1 given by Kasman et al (2002).

Therefore, this study calculated the k value of phage FWLLm3 in order to use it in the phage titre estimation experiments. The k values were calculated from gradients of the phage adsorption kinetic graphs (Figure 3.4). The k values of phage FWLLm3 pertaining to *L. monocytogenes* 19EO1, 19EO3, 22BO7 and 18GO1 were $1.4\pm0.36\times10^{-9}$, $1.2\pm0.34\times10^{-9}$, $1.3\pm0.48\times10^{-9}$ and $1.6\pm0.91\times10^{-9}$ ml/min, respectively (Table 3.4). These k values were not significantly different ($p > 0.05$). A phage which has k values in the range of 10^8-10^9 ml/min is considered to have fast-adsorbing capacity (Gallet et al., 2009; Kasman et al., 2002). Therefore, the k value of phage FWLLm3 can be attributed to fast-adsorbing capabilities. This is the first study to that the k value of a listeriaphage.

3.3.4 Estimation of MOI_{input} and minimum dose of phage FWLLm3

At an MOI of 10, phage infection is expected to occur at about 99.99% assuming that phage adsorption onto host cells follows the Poisson distribution (Abedon, 2009). However, practically the MOI_{input} value of 10 (10 times more phage than the host cell count) may not be effective to lyse total cells at every cell concentration. This is especially so where a low count of a bacterial culture is subject to phage infection since it has been shown that a high titre phage input is required. These parameters have been accounted for by the model in equation (2) which explains MOI_{input} and MOI_{actual} (Kasman et al. 2002). The value of MOI_{input} was estimated using equation (2) in which the value of MOI_{actual} was assumed as 10.

By substituting initial host cell counts, k values, phage exposure time of 30 min and the assumed value of 10 for the MOI_{actual} in equation (2), values for the MOI_{input} were estimated for each host strain. These estimated values ranged from $2.3x10^3$ to $8.1\ x10^4$ for *L. monocytogenes* strains 19EO1, 19EO3, 22BO7 and 18GO1 (Table 3.4). Similarly, by substituting the above experimental values in equation (3), estimates of the minimum phage titres required for total infection of *L. monocytogenes* 19EO1, 19EO3, 22BO7 and 18GO1 were in the range of $2x10^8$-$2.8x10^8$ PFU/ml (Table 3.4).

The estimated phage titres (Table 3.4) determined by the model were within the phage titres used to effect total infection of the experimental cell cultures (Figures 3.2 and 3.3). Phage titres $\leq 7.8\ \log_{10}$ PFU/ml were not effective in achieving complete lysis of the host cell cultures (Figure 3.2). According to the model, incomplete phage infection of host cells might have resulted from the low phage densities and/or the insufficient phage exposure time used.

In phage challenge experiments, results showed that phage resistant cells were not detected (Figure 3.3). The opportunity for the development of phage resistance among low count bacterial populations remains very low (Hagens and Loessner, 2010).

3.3.5 Conclusion

Listeriaphage FWLLm1, FWLLm3 and FWLLm5 demonstrated broad host ranges against *L. monocytogenes* strains isolated from seafood and seafood processing environments at 15 and 25 °C. The phage FWLLm3 was effective in the lysis of low count cells *in vitro*. The study has provided useful base line information for the three phages as potential candidates for further investigations to control *L. monocytogenes* in seafood processing and handling systems.

Authors' contributions to the manuscript

The experiments were planned and conducted, data were analyzed and the first draft of the manuscript was prepared by GJGA. CDC, BMDM, LM, and SHF and ANM supervised and provided advice on interpretation of results and discussion and improving the writing of the overall manuscript. CB and AH reviewed the manuscript.

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Chapter 4: Characteristics of three listeriaphages isolated from New Zealand seafood environment

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Abstract

Aim: To isolate and characterize listeriaphages from seafood environments.

Methods and Results: Listeriaphages (phages) isolated from seafood environments were distinguished by physical, biological and restriction digestion of phage DNA. Three phages belonged to the order *Caudovirales* and showed psychrotrophic characteristics. The phages had broad host ranges against 23 *Listeria* strains by productive infection or at least by adsorption. At 15 ± 1°C, the adsorption rate constants of the three phages ranged from 8.93 x 10^{-9} to 3.24 x 10^{-11} ml min^{-1} across different *L. monocytogenes* strains. In an indicator host, the mean burst sizes of phages LiMN4L, LiMN4p and LiMN17 were ≈17, 17 and 11 PFU per cell, respectively. The latent period was ≈270 min for phages LiMN4p and LiMN17 at 15 ± 1°C. The latent period was ≈270 min for phages LiMN4p and LiMN17 while it was ≈240 min for phage LiMN4L at 15 ± 1°C.

Conclusions: The three virulent psychrotrophic phages isolated from seafood processing environments had broad host ranges and low productive replication. The characteristics suggested that these phages may be suitable as passive biocontrol agents for seafood-borne *L. monocytogenes.*

Significance and Impact of Study: This is the first report on the isolation of autochthonous virulent listeriaphages from seafood processing environments and information on single-step replication and adsorption characteristics of such listeriaphages.

Keywords: Psychrotrophic *Listeria* phage, seafood processing environment, single-step replication, host range, adsorption rate

4.1 Introduction

Virulent listeriaphages isolated from different environments are now gaining acceptability in the control of *Listeria monocytogenes* in food systems. These include commercial phage products such as Listex™ containing phage P100 (Anon 2006; Carlton *et al*. 2005) and Listshield™, a six-phage cocktail (Pasternack and Sulakvelidze 2009). The virulent phages for these two products were isolated from a milk processing plant in Germany (Carlton *et al*. 2005) and from the *Baltimore* inner harbour water in USA (Pasternack and Sulakvelidze 2009), respectively. Loessner and Busse (1990) isolated a group of phages, which included phage A511 from a wastewater treatment plant. Listeriaphages have also been isolated from human wastewater effluents (Dykes and Moorhead 2002), sheep feces (Bigot e*t al*. 2011; Lee 2008), silage (Hodgson 2000; Schmuki et al. 2012) and turkey processing plants (Kim *et al*. 2008).

The information on host ranges of some of these phages has shown that they could be a useful tool to increase food safety in processing plants (Carlton *et al*. 2005; Kim *et al*. 2008; Loessner and Busse 1990; Pasternack and Sulakvelidze 2009). Efficacy of phage-lysis of *L. monocytogenes* has been investigated in broth cultures and food models (Bigot e*t al*. 2011; Carlton *et al*. 2005; Dykes and Moorhead 2002; Guenther *et al*. 2009; Guenther and Loessner 2011; Leverentz *et al.* 2003; Leverentz *et al.* 2004; Soni and Nannapaneni 2010a; Soni *et al.* 2010). Moreover, some phages have been shown to be effective in dealing with *Listeria* cells adhered to surfaces and as biofilms formed on hard surfaces that simulate food contact surfaces (Montanez-Izquierdo *et al*. 2011; Roy *et al*. 1993; Soni and Nannapaneni 2010b). The phages, however, need to be characterized including the single-step replication and adsorption capabilities in conditions under which phages are intended to be used (Gill 2010).

This study reports the characteristics of three virulent phages isolated from seafood processing environments with the potential to control *L. monocytogenes* in similar environments at low temperatures.

4.2 Materials and methods

4.2.1 *Listeria* strains and reference bacteriophage

Twenty one *L. monocytogenes* strains isolated from seafood or seafood processing environments were obtained from different laboratories in New Zealand comprising two strains (SMAC91 and SSM91) from smoked mackerel and smoked mussel respectively (Bremer *et al*. 2002). Other strains from environmental samples (Cruz and Fletcher 2011) (Table 4.2) were kindly provided by Plant and Food Research Limited (PFR), Auckland, and Cawthron Institute, Nelson in New Zealand. Two other *Listeria* strains (*L. ivanovii* and *L. innocua*) obtained from in-house laboratory culture collection of Massey University were also used in this study. The typing characteristics of *L. monocytogenes* strains (serotype and subtype) used in this study are summarized in Table 4.2. *L. monocytogenes* strains were stored in cryovials (Cryobank-CRYO/M, Mast Diagnostics, Merseyside, UK) at -80°C. Monthly working cultures were recovered by streaking the frozen cultures on trypticase soy agar (TSA; Difco, Sparks, MD, USA) plates, incubating at 30 ± 1°C for 18 h. The culture plates were stored at 4°C. The broth cultures were prepared by transferring inocula of monthly cultures of *Listeria* strains into trypticase soy broth (TSB; Difco) and incubating at selected temperatures described below. A suspension of virulent phage A511 (Loessner and Busse 1990) which was used as reference phage was kindly provided for this study by the Institute of Food, Nutrition and Health, Zurich, Switzerland.

4.2.2 Isolation and purification of phages from environmental samples

Field samples were collected from different locations (interior wall of draining receptacle of the milli-screen in the final dewatering section, floor, floor-drains and interior wall of final sump prior to the discharge of screened fisheries effluent) of a fish waste treatment unit of a fish processing plant in Nelson in the South Island of New Zealand in March 2011. The samples were collected using 25 cm^2 swabs (BSN code 71786-05, Propax brand, BSN Medical, Hamburg, Germany) pre-moistened with 5 ml of transport medium [1 g of peptone (Merck KGaA, Darmstadt, Germany), 5 ml of 10% (w/v) sodium thiosulphate (Scharlau Chemie S.A., Barcelona, Spain), 10 g of Tween 20 (Ajax Finechem Pty Ltd, Sydney, Australia), 1 l of ultra pure water] over 50 cm^2 on surfaces of sampling locations.

The sample swabs in plastic bags were transported in ice to the laboratory at the Cawthron Institute (Nelson, New Zealand) within 2 h. Each swab sample was placed in glass bottle containing 10 ml of 10% (w/v) Meat Extract (ME) (Merck) vortex-mixed

(WISE Mix VM-10, Daihaw Scientific company Ltd, Seoul, Korea) for 30 s to expel gas bubbles and incubated at 4 ± 1°C for ≈18 h (Hurst *et al.* 1991; Khan *et al.* 2002; Mark R Liles, personal communication). Ten percent (w/v) ME facilitates desorption of phages by intercalating the small protein molecules between phage particles and hard surfaces thereby weakening the mutual attractive electrostatic forces (Wait and Sobsey 1983). The samples were sonicated (70 W, 43 kHz and 80% power; Ultrasonic power for 160-HT, Soniclean Pty Ltd, Thebarton, Australia) for 2 min, incubated at room temperature (21 ± 1°C) for 1 h, mixed by vortex for 30 s and the liquid squeezed out from the swab using forceps, centrifuged at 3000 *g* for 10 min, filtered [0.45 µm cellulose acetate syringe filter (MSR, Shanghai, China)]. In the next analytical step, the sample extract was diluted with TSB to ≈0.6% (w/v) ME in order to avoid any potential concentration effect by 10% (w/v) ME. Some phages are dependent on cofactors such as divalent cations (≈1-10 mmol l^{-1}) for their attachment to the host or intracellular growth (Twest and Kropinski 2009). In isolation protocols for other listeriaphages, ≈1.25-10 mmol l^{-1} Ca^{2+} were used (Hodgson 2000; Kim *et al*. 2008; Lee 2008). Therefore, the diluted sample enrichment was supplemented with Ca^{2+} (2 mmol l^{-1}).

and then diluted with TSB supplemented with 2 mmol l^{-1} of calcium chloride (Ca_2Cl_2) (Scharlau Chemie) to give a final concentration of 0.6% (w/v) ME. The sample filtrates (10 ml) were inoculated with 100 µl of exponential phase (≈5 x 10^8 CFU/ml) *L. monocytogenes* strains 19CO9 and 18AO1 (indicator hosts) separately, incubated at 25 ± 1°C for 48 h, centrifuged at 3000 *g* at 4°C for 20 min and the supernatants were treated with chloroform ($CHCl_3$) (Analytical Grade, Merck) at 1% (v/v) for ≈1 h in the dark, to lyse the bacteria (Walakira *et al.* 2008). The bacteria-free sample enrichment lysate was assayed by the double layer agar (DLA) plate method (Kropinski *et al*. 2009) using a TSAYE [TSB, 0.6% (w/v) yeast extract (Scharlau Chemie)] overlay containing 0.4% (w/v) agar (Difco) plus $CaCl_2$ (2 mmol l^{-1}) and TSAYE base layer [1% (w/v) agar]. The plates were examined for phage plaques formed at 25 ± 1°C after 18-48 h.

From phage positive plates, individual plaques with a clear centre were transferred using a pipette tip into 1 ml saline-magnesium buffer containing gelatine (SM) [100 mmol l^{-1} NaCl (Merck), 50 mmol l^{-1} Tris-HCl (Sigma-Aldrich, Steinheim, Germany), 8 mmol l^{-1} of $MgSO_4$ (Merck), 0.001% (w/v) of gelatine solution (LabChem, NSW, Australia) at pH 7.5] and stored at 4°C. The phage isolates were streaked individually using a tooth pick on base agar plates (Serwer *et al*. 2004) and overlay containing *L. monocytogenes* 19CO9 was poured on the phage streaks and plates (DLA

method) were incubated at 25 ± 1°C for 24 h. The selected phage plaques were purified over four cycles by directly streaking the phages from a well separated plaque on fresh agar plate as above. Purified phage plaques were picked by boring the agar using a filter tip (Labcon filter tips, Interlab, Wellington, New Zealand) of ≈1.5 mm diameter. The agar plug was transfered into a labeled glass bottle containing 2 ml of SM medium and 50 µl of $CHCl_3$ and stored in the dark at 4°C.

4.2.3 Plaque morphology at different gel concentrations

A DLA plaque assay was performed for each phage isolate on two types of overlays [0.4% (w/v) agar and 0.1% (w/v) agarose (Sigma-Aldrich)] with a base layer of TSA containing 1% (w/v) agar (Serwer *et al.* 2004) using *L. monocytogenes* 19CO9 as an indicator host. Plaque morphology (appearance and diameter) was observed on both overlay types after incubation at 25 ± 1°C for 24-48 h.

4.2.4 Preparation of purified high titre phage stocks

Phages (new isolates and A511) were propagated separately in *L. monocytogenes* 19CO9 using DLA in a 2 l flask (Sillankorva *et al.* 2004) at 25 ± 1°C. The phgae lysate was centrifuged at 8000 *g* for 30 min at 4°C (Biofuge, Primo R, Heraeus, Conquer scientific, CA, USA). The supernatant was treated with $CHCl_3$ (≈200 ml lysate/0.5 ml $CHCl_3$) for ≈18 h and then centrifuged at 8000 *g* for 30 min. About 50 ml of clear phage lysate was ultracentrifuged (SS-34 rotor, Sorvall RC6+ Centrifuge, Thermoscientific Sorvall, Frankfurt, Germany) at 25 000 *g* for 6 h (Ackermann 2009a). The phage pellet was suspended in 1 ml of SM for 18 h at 4°C followed by agitaion at 22±1°C with shaking (60 rev min^{-1}) for 1-2 h in dark. The concentrated phage suspension was transfered into glass bottles and stored with a few drops of $CHCl_3$ at 4°C. The titre of the phage stock was assayed over 10-fold dilution series in SM using the DLA plate method as described above.

4.2.5 Efficiency of plating (EOP) of phages in citrate agar

The phages were discriminated based on their dependency on free Ca^{2+} in growth medium by the DLA plaque assay using both agar layers supplemented with same level of citrate (Tri-sodium citrate; Biolab Ltd, Victoria, Australia) either at 0.5 or 1 mol l^{-1} (Olsen *et al*. 1968). Control DLA plates containing agar without citrate supplements, were also inoculated with phages. Triplicate plates were inoculated for each treatment

and plates were incubated at 25 ± 1°C for 24-48 h. The EOP of phage isolates was calculated with respect to plaque counts on non-citrate plates (Kutter 2009).

4.2.6 Heat tolerance of phages at 50 and 60°C

Phages can be discriminated by heat tolerance profiles. Volumes (2 ml) of phage lysates containing ≈10^6 PFU ml^{-1} were heated at 50 ± 1 and 60 ± 1°C in thin-walled narrow glass vials (Ø, 1.5 cm and height 6 cm) for 60 min using the method described by Ackermann *et al.* (1978). Viable phage titre was assessed by DLA plaque assay (Detection limit = 1 $\log_{10}$ PFU ml^{-1}). Three independent experiments were carried out.

4.2.7 Electron microscopy of phages

Phage lysate (≈10^9 PFU ml^{-1}) was purified in 0.1 mol l^{-1} of ammonium acetate (Scharlau Chemie) by ultracentrifugation (25 000 *g*) for 2 h (Ackermann 2009a) and the phage pellet was suspended in ≈0.5 ml of SM as previously described. The purified phage suspension (>10^{10} PFU ml^{-1}) was adsorbed onto the carbon-coated copper grids stained with 2% (w/v) aqueous uranyl acetate solution. The stained phages were then observed by transmission electron microscopy (TEM) (Philips CM12; FEI Company, Eindhoven, Netherlands) (Zink and Loessner 1992) and the images were photographed (Model 792 Bioscan, CA, USA). Dimensions of the phage images with scale bars were measured using Image J 1.45s software (National Institute of Health, Maryland, USA).

4.2.8 Restriction enzyme digestion of phage DNA

The DNA of phages, including A511 as a control (Loessner, Krause, Henle, & Scherer, 1994), was extracted from high titre phage lysates (>10^9 PFU ml^{-1}) as described by Pickard (2009) and individually digested with ClaI and SacI (NEB, New England Biolabs, Massachusetts, USA) restriction enzymes at 37 ± 1°C for 1.5 h. Genomic profiles of digested DNA phage were separated in a 0.7% (w/v) agarose gel (Sigma-Aldrich) followed by staining with 1 µg ml^{-1} ethidium bromide (Sigma-Aldrich) for 30 min. HindIII digest of phage lambda (λ) DNA (NEB) was used as a molecular ladder. Images were taken using Fluorescence Imaging System (Gel Doc™ 1000 BioRad, Inc., California, USA). Three replicates were performed for each restriction enzyme.

4.2.9 Host range of phages

Using the DLA method (TSA of 0.4% agar overlay and 1% agar base), the host range of each phage strain was determined by the formation lysis zones on the spots of 20 µl

phage drops (drop test) or plaques (plaque assay) in host lawns of 23 *Listeria* strains (Carlson 2005; Kutter 2009) at 15 ± 1 and 25 ± 1°C. The EOP of a phage in a test *Listeria* strain was calculated with respect to plaque counts formed by *L. monocytogenes* 19CO9 (Kutter 2009). The test host strains in which phages did not form visible plaques in plaque assays, were also assessed in overlays supplemented with 5 mg l^{-1} of ampicillin (ampicillin sodium salt; Sigma-Aldrich) which increases infection vigor in the host causing visible plaques on host lawn (Santos *et al*. 2009). Each host was tested in duplicate in three independent experiments.

4.2.10 Adsorption rate constant (k) of phages

Exponential phase cultures of *L. monocytogenes* 19CO9, 19DO3, 18DO5 and 18DO7 strains (≈10^7-10^8 CFU ml^{-1}) were centrifuged at 8000 *g* for 10 min, re-suspended in phosphate buffered saline (PBS) [0.008 mol l^{-1} Na_2HPO_4 (Merck), 0.001 mol l^{-1} $NaH_2PO_4.H_2O$ (Merck), and 0.145 mol l^{-1} NaCl; pH 7.5] and then mixed with each phage (≈10^4-10^5 PFU ml^{-1}) suspended in PBS (Gallet *et al.* 2009). The co-culture was then incubated at 15 ± 1°C and 1 ml volumes were withdrawn into chilled plastic tubes containing $CHCl_3$ (100 µl) at 1-2 min intervals, mixed by vortexing for 20 s, incubated at room temperature in the dark for at least 1 h and then centrifuged at 8000 *g* for 10 min. The un-adsorbed phage titres of samples were determined by the DLA method using *L. monocytogenes* 19CO9. Initial counts of each host strain cultures were enumerated using the standard spread plate technique and then incubating the plates at 30 ± 1°C for 48 h. The k value of phage was calculated based on the equation given by Hyman and Abedon (2009).

4.2.11 Plaque formation temperature range of phages

Three phages were assessed for plaque formation using the DLA method as in above host range experiment between 4-37°C using exponential phase broth cultures of an indicator strain (*L. monocytogenes* 19CO9) grown at 4 ± 1°C for 20 d and at 15 ± 1, 25 ± 1 and 35 ± 1 or 37 ± 1°C for 48, 24 and 18 h, respectively. The plaque counts were obtained by incubating the inoculated plates at each temperature for further 2-3 d more than the incubation time used to grow the exponential phase broth cultures at respective temperatures. Six replicate plates were prepared at each temperature. The EOP of phage at each temperature was calculated with respect to plaque counts at 25 ± 1°C.

4.2.12 Single-step replication (growth) of phages

Single step replication of three phages was determined at 15 ± 1, 25 ± 1 and 35 ± 1°C. Briefly, 10 ml exponential cultures of *L. monocytogenes* 19CO9 (≈10^8 CFU ml^{-1}) was infected with each phage to reach an input-multiplicity of infection value of 0.1 and mixed well. The phage adsorption (initiation of the infection) in a co-culture was allowed at 15 ± 1°C for 10 min and at 25 ± 1 or 35 ± 1°C for 5 min with shaking (60 rev min^{-1}). The co-culture was immediately diluted 10-fold with pre-incubated TSB at 15 ± 1, 25 ± 1 or 35 ± 1°C and mixed well to prepare 300 ml volumes of 10^{-4} and 10^{-5} dilutions. The diluted co-culture was swirled (60 rev min^{-1}) throughout the sampling period at 25 ± 1 and 35 ± 1°C, while the co-cultures incubated at 15 ± 1°C statically and were shaken well before each sampling. Two series of samples were withdrawn from 10^{-4} and 10^{-5} dilutions of a co-culture at 25 ± 1 or 35 ± 1°C every 10 min and at 15 ± 1°C every 30 min. One series of samples was assayed for plaques directly by the DLA method, while the other set was immediately treated with $CHCl_3$ (1% v/v) and then assayed for plaques (Carlson and Miller 1994). Single-step growth parameters were calculated following the method described by Carlson and Miller (1994) and Hyman and Abedon (2009).

4.2.13 Statistical analysis

Descriptive statistical analyses were performed using Microsoft Office Excel 2007 (Microsoft Office, Washington, USA) and SAS univariate procedure (SAS 9.1 version, SAS Institute, North Carolina, USA). One way ANOVA was performed on the different characteristics or treatments to determine significance ($p < 0.05$) and significant treatments were separated using *post-hoc* Duncan's Multiple Range Test (SAS 9.1).

4.3 Results

4.3.1 Isolation of listeriaphages based on plaque characteristics

The field samples were screened on host lawns for the presence of plaques formed by any phages present. When field samples were phage-positive, plaques were visible in lawns of both indicator host strains (*L. monocytogenes* 19CO9 and 18AO1). The phage plaques were consistently larger on lawns of *L. monocytogenes* 19CO9 than on lawns of 18AO1 and the plaques on the former lawn were selected for further studies. Based on

different plaque characteristics (diameter and appearance), two types of phage isolates were identified on a same sample-plate originating from the milli-screen drain receptacle. A phage from a plaque (Ø ≈1.0-1.5 mm) was designated as LiMN4L while the other phage from another plaque (Ø ≈0.7-1.0 mm) was designated as LiMN4p (Supporting Information, Table 4.S1). Another phage, named LiMN17, was derived from a plaque (Ø ≈0.5-1.0 mm) picked from a sample plate originating from the final sump (Supporting Information, Table 4.S1). Plaques were transferred separately into SM and purified by re-streaking.

The three phages formed plaques with narrow haloes, which looked similar for all three phages on a 0.4% (w/v) agar overlay after incubation at 25 ± 1°C for 48 h (Supporting Information, Table 4.S1). The three phages produced comparatively larger plaques with haloes of different appearance on 0.1% (w/v) agarose overlay plus 1% (w/v) agar base (Fig. 4.1). With these double layers, phage LiMN4p produced plaques consisting of one halo (Fig. 4.1B), while phages LiMN4L and LiMN17 formed plaques with additional secondary haloes (Fig. 4.1A and C). However, all three phages produced secondary haloes in the 0.1% (w/v) agarose overlay when a 1.5% (w/v) agar base was used (data not shown). Phage A511 also showed plaque morphology similar to LiMN4p on 0.1% (w/v) agarose overlay (data not shown). Considering the plaque characteristics and different locations of the field samples, the three phages LiMN4L, LiMN4p and LiMN17 were characterized further.

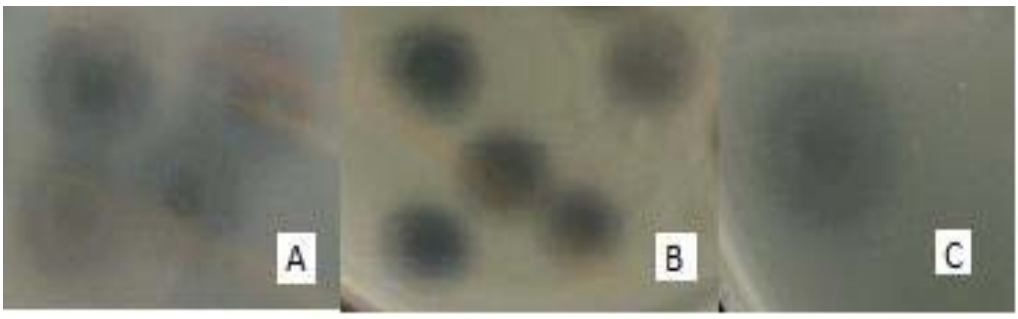

Figure 4.1 Listeriaphage plaques on trypticase soy yeast extract double layer agar (0.1% agarose overlay and 1% agar base) at 25 ± 1°C after 48 h. Plaque diameter: A, LiMN4L (≈3-4 mm); B, LiMN4p (≈3-4 mm); C, LiMN17 (≈3.0-3.5 mm).

4.3.2 EOP of phages in citrate agar

Different phages depend on free Ca^{+2} at varying degrees when adsorbed on cell surfaces and on replication. Three phages were discriminated at the highest level in 1 mol l^{-1} citrate agar compared in 0.5 mol l^{-1} agar (Fig. 4.2). The difference of % EOP of LiMN4L at both citrate levels was not significant ($p > 0.05$) (Fig. 4.2). Phages LiMN4p had the lowest EOP (≈54%) at 1 mol l^{-1} of citrate concentration (Fig. 4.2). Phage LiMN17 showed the lowest dependency on Ca^{+2} by giving the highest EOP (85%) at 1 mol $l^{-1}$of citrate.

4.3.3 Heat treatment of phages at 50 and 60°C

Phages can be discriminated based on the sensitivity of phages at different temperatures. At 50 ± 1°C, phage LiMN17 was undetectable (<1 $\log_{10}$ PFU ml^{-1}) after 10 min of incubation, while phage LiMN4p was reduced by ≈3 log units. Comparatively, ≈17, 12 and 7% of phage LiMN4L survived at 50 ± 1°C for 10, 30 and 60 min, respectively. Phage LiMN4L was recovered at levels of approx. 9, 0.04 and <0.0001% at 60 ± 1°C after 10, 30 and 60 min, respectively. Phages LiMN4p and LiMN17 were more heat labile and did not survive (<1 $\log_{10}$ PFU ml^{-1}) after 10 min at 60 ± 1°C (Supporting Information, Table 4.S2).

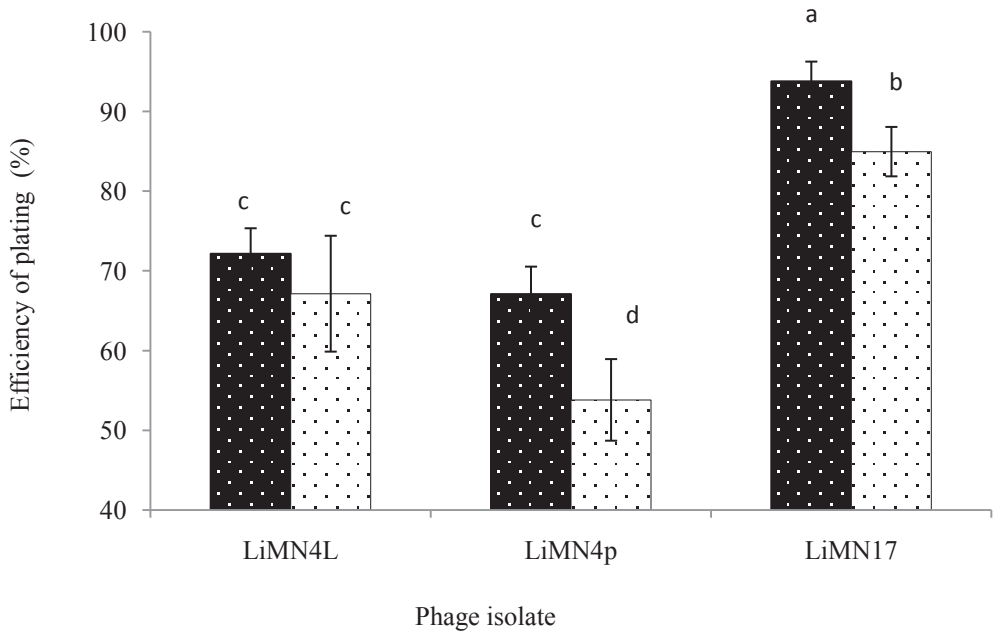

Figure 4.2 Efficiency of plating of listeriaphages LiMN4L, LiMN4p and LiMN17 in trypticase soy yeast extract double layer agar (DLA) containing citrate (two levels) with respect to DLA without containing citrate at 25 ± 1°C after 48 h. Citrate: , 0.5 mol l^{-1}; , 1 mol l^{-1}. Bars represent standard error in three determinants (n = 3). Different letters (a, b, c, d) indicate significant difference of mean, ($p < 0.05$).

4.3.4 Electron microscope images of phages

According to the TEM images, each phage comprised of an icosahedral head and a long, rigid, contractile tail with tail fibres and all three therefore, belonged to the order *Caudovirales* and family *Myoviridae* (Ackermann 2009b) (Fig. 4.3). Dimensions of the phage were calculated over at least 20 phage particles which were within the inter-quartile range (between Q1 and Q3) and three phages were different in dimensions. The mean head diameter of phage LiMN4L was significantly larger ($p < 0.05$) than phages LiMN4p and LiMN17 (Table 4.1). Phage LiMN4p had a significantly longer tail than the other two phages ($p < 0.05$). The tail width of phage LiMN4L was narrower ($p < 0.05$) than phages LiMN4p and LiMN17 (Table 4.1).

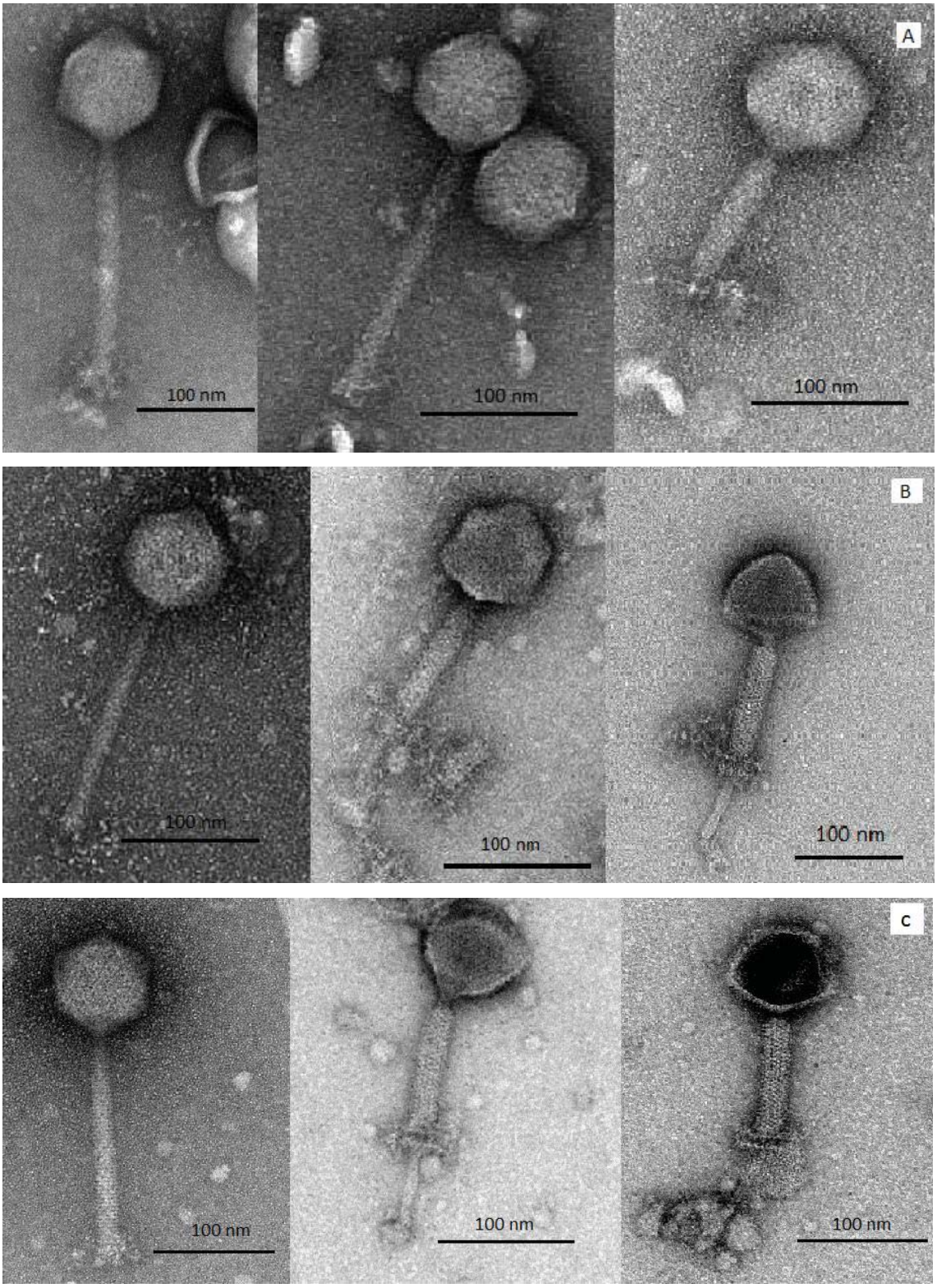

Figure 4.3 Transmission Electron Microscope images of listeriaphages [negatively stained with 2% (w/v) uranyl acetate]. A, LiMN4L, B, LiMN4p, C, LiMN17.

Table 4.1 Dimensions of listeriaphage LiMN4L, LiMN4p and LiMN17 determined on TEM images

Listeriaphage	Dimensions (nm)[a] of images of negatively stained listeriaphage (Mean[b] ± Standard deviation)[c]			
	Number[d]	Head diameter	Tail length	Tail width
LiMN4L	20	$86.49^{A} \pm 2.6$	$185.38^{A} \pm 4.47$	$17.83^{B} \pm 1.39$
LiMN4p	22	$82.29^{B} \pm 2.95$	$192.71^{B} \pm 5.64$	$19.86^{A} \pm 1.19$
LiMN17	20	$81.45^{B} \pm 3.41$	$186^{A} \pm 6.21$	$19.74^{A} \pm 1.5$

[a] Dimensions of phage images were measured using Image J 1.45s software (NIH, USA)

[b] The upper case letters within each column indicate the significant difference at 0.05% level among three phages for each dimension parameter separately

[c] Mean and standard deviation values of dimensions within Q1 and Q2 percentiles

[d] Number of phage

4.3.5 Phage DNA restriction analysis

Restriction digestion profiles of phage DNA were used to distinguish three phages. Restriction digest profiles of phages LiMN4L, LiMN4p and LiMN17 with ClaI (Lanes 2-4) and SacI (Lanes 2-4) were compared with phage A511 in Lane 5 (Fig. 4.4 and 4.5 respectively). LiMN4p showed a different profile with ClaI and SacI (Fig. 4.4 and 4.5). Therefore, phage LiMN4p was different from the other two phages and from phage A511. But, phage LiMN4p showed some relatedness to phage A511 (Fig. 4.5).

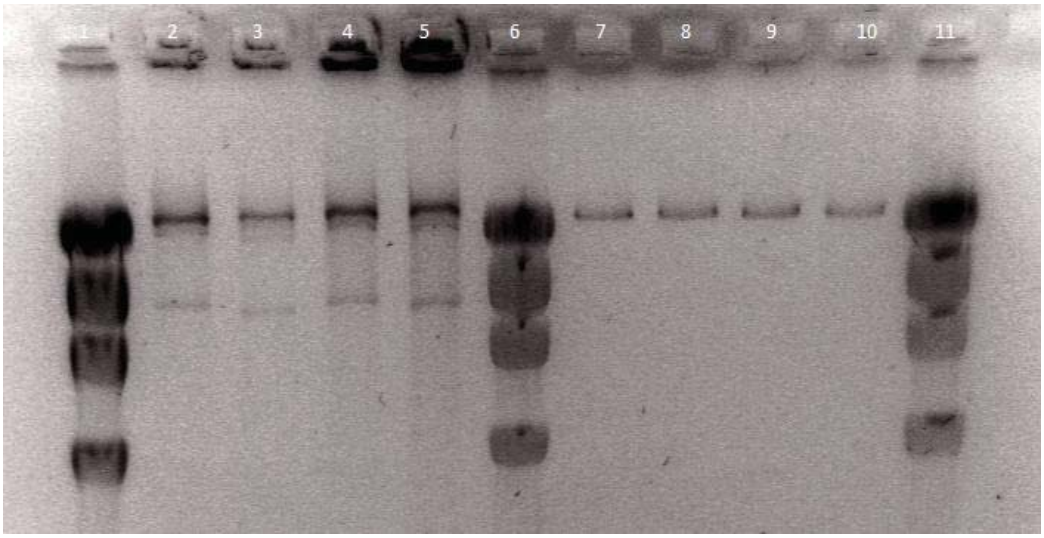

Figure 4.4 Listeriaphage DNA profiles on 0.7% agarose gel. Enzyme-digested (ClaI) profiles of phage DNA with λ HindIII marker (Lanes 1-5): 1, λ HindIII marker; 2, LiMN4L; 3, LiMN4p, 4, LiMN17; 5, A511. Undigested phage DNA with λ HindIII marker (Lanes 6-11): 6, λ HindIII marker, 7, LiMN4L; 8, LiMN4p; 9, LiMN17; 10, A511; 11, λ (Hind 111 digest).

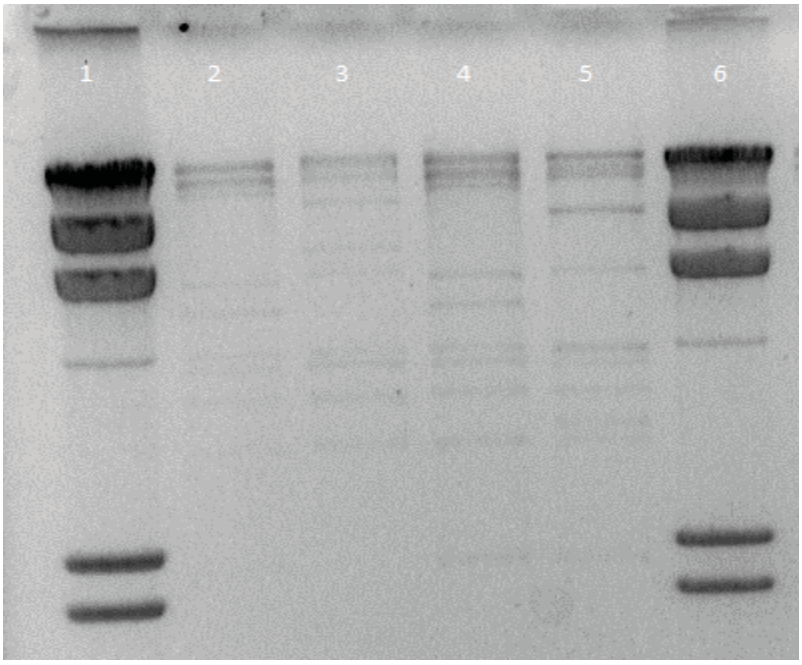

Figure 4.5 Enzyme-digested (SacI) profiles of phage DNA with λ HindIII marker on 0.7% agarose gel. Lanes 1 to 6: 1, λ HindIII marker; 2, LiMN4L; 3, LiMN4p; 4, LiMN17; 5, A511; 6, λ HindIII marker.

4.3.6 Host ranges of the phages

Three phages infected many of the *Listeria* strains (Table 4.2). Three phages did not produce plaques in lawns of strains 19EO1, 19EO3 and 19DO9 using the standard DLA method. However, at 10^4-10^8 PFU ml^{-1}, they formed pin-point sized plaques on these strains in agar overlays supplemented with ampicillin (0.05 mg l^{-1}). The minute plaques were difficult to enumerate exactly, therefore the EOP of the three phages was estimated to be in the range of $\approx 10^{-2}$-10^{-6} (Table 4.2). Phage LiMN17 did not make plaques by the plaque assay, but phage drops (>10^9 PFU ml^{-1}) resulted in clear lytic zones on the spots of original phage drops on lawns of *L. monocytogenes* SMAC, 19DO9, 19DO3 and *L. ivanovii* strain. Three phages did not form plaques on the lawns of 18DO5 and 18DO7 strains, but formed hazy lytic zones ("host kill zones") which were not completely clear lytic zones and contained turbidity throughout the zones on the spots of original phage drops (>10^9 PFU ml^{-1}) on the lawns of 18DO5 and 18DO7 strains (Carlson 2005, Kutter 2009) compared with blank control drops containing SM only (Table 4.2).

Table 4.2 Percentage efficiency of plating (% EOP) of listeriaphages LiMN4L, LiMN4p and LiMN17 on trypticase soy yeast extract double agar layers (0.4% agar overlay and 1% agar base) at 15 ± 1 and 25 ± 1°C

Listeria strains*			% EOP of listeriaphage					
Strain code	Typing characteristics		LiMN4L		LiMN4p		LiMN17	
	Serotype	Pulsotype	15°C	25°C	15°C	25°C	15°C	25°C
L. monocytogenes strains								
15JO5	1/2a or 3a	382	79	96	114	89	96	103
CW2	1/2a or 3a	872	93	96	88.	104	84	87
SMAC91	1/2a	2342	78	90	94	90	$<10^{-7}$	$<10^{-7}$
18AO1	1/2a or 3a	22	91	95	89	96.	72	87.3
22BO5	1/2a or 3a	652	77	91	92	97	45	67
22BO7	1/2a or 3a	872	78	91	98	93	45	67
18GO1	1/2a or 3a	8342	57	87	101	94	65	77
19CO5	1/2a or 3a	846	89	100	76	79	75	80
17AO2	1/2a or 3a	702	76	91	91	102	54	94
15AO7	1/2a or 3a	652	41	89	101	55	81	119
19EO1 †¶	1/2a or 3a	5946	$<10^{-3}$	$<10^{-3}$	$<10^{-2}$	$<10^{-2}$	$<10^{-3}$	$<10^{-3}$
18FO9	1/2a or 3a	5946	$<10^{-3}$	$<10^{-3}$	85	94	$<10^{-3}$	$<10^{-3}$
19CO9	1/2a or 3a	872	100	100	100	100	100	100
19EO3†¶	1/2a or 3a	5132	$<10^{-2}$	$<10^{-2}$	$<10^{-2}$	$<10^{-2}$	$<10^{-2}$	$<10^{-2}$
19DO9 †¶	1/2a or 3a	5942	$<10^{-5}$	$<10^{-5}$	$<10^{-3}$	$<10^{-3}$	$<10^{-7}$	$<10^{-7}$
19DO3¶	4b,4d or 4e	2312	$<10^{-3}$	$<10^{-3}$	$<10^{-1}$	$<10^{-1}$	$<10^{-7}$	$<10^{-7}$
19EO5	4b,4d or 4e	6331	67	72	77	79	46	68
19CO7	4b,4d or 4e	6331	66	74	78	80	46	65
SSM91	1/2b	3527	48	93	72	98	89	92
18DO7	1/2b,3b or	3527	$<10^{-7}$	$<10^{-7}$	$<10^{-7}$	$<10^{-7}$	$<10^{-7}$	$<10^{-7}$
18D05	1/2b,3b or	3527	$<10^{-7}$	$<10^{-7}$	$<10^{-7}$	$<10^{-7}$	$<10^{-7}$	$<10^{-7}$
L. ivanovii¶	-	-	$<10^{-3}$	$<10^{-3}$	$<10^{-2}$	$<10^{-2}$	$<10^{-7}$	$<10^{-7}$
L. innocua¶	-	-	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$

**L. monocytogenes* strains were isolated from seafood processing environments except two strains (SMAC91 and SSM91) isolated from smoked mackerel and smoked mussel, respectively. Serotyping and pulsotyping (Pulsed Field Gel Electrophoresis based subtyping) of strains were performed by Plant and Food Research Limited and pulsotype codes are based on a unique numbering system (Bremer *et al*. 2002; Cruz and Fletcher 2011). Sources of isolation and typing characteristics of *L. ivanovii* and *L. innocua* are not recorded.

† Visible plaques on agar overlay supplemented with 0.05 mg l^{-1} ampicillin (Santos *et al*. 2009).

¶ Pin-point size plaques were difficult to enumerate.

Phage LiMN4L, however, at a titre of $>10^9$ PFU ml^{-1}, formed 1-2 plaques per plate on the lawns of strains 18DO5 and 18DO7. Phages LiMN4L and LiMN4p infected *L. ivanovii* and *L. innocua*, while phage LiMN17 was found to infect only *L. innocua* (Table 4.2). Phages LiMN4L, LiMN4p and LiMN17 were distinguished from each other by the EOP with *L. monocytogenes* SMAC91 and 18FO9. Phage LiMN4p showed ≈94% and 85% EOP with both strains respectively, at 15°C. The EOPs of phage LiMN4L with two host strains (SMAC91 and 18FO9) were ≈78% and <0.001%, respectively, at 15°C. Phage LiMN17 was unable to make plaques with *L. monocytogenes* SMAC91 even with titres $>10^9$ PFU ml^{-1} at both 15 and 25°C (Table 4.2). Based on the EOP range of 10^{-5}-100%, host range of phages LiMN4L, LiMN4p and LiMN17 were 21/23 (≈91%), 21/23 (≈91%) and 17/23 (≈74%), respectively strains (Table 4.2) at 15°C.

4.3.7 The k values of phages

The k values of the three phages (LiMN4L, LiMN4p and LiMN17) were assessed on four *L. monocytogenes* strains (19CO9, 19DO3, 18DO5 and 18DO7). The k values of three phages on *L. monocytogenes* 19CO9, 19DO3, 18DO5 and 18DO7 ranged between ≈1 x 10^{-9} and 3 x 10^{-11} ml min^{-1} (Table 4.3). The three phages showed varying % EOP values between $<10^{-7}$and 100% with the four strains (Table 4.2). All three phages had k values $>10^{-9}$ ml min^{-1} with the indicator strain 19CO9 giving an EOP of 100% (Table 4.2 and 4.3). The phage LiMN17 did not infect ($<10^{-7}$ % EOP) strain 19DO3 (Table 4.2). However, LiMN17 showed a high adsorption rate (10^{-9} ml min^{-1}) on 19DO3, similar to 19CO9 (Table 4.3). Therefore, for the EOP-based host range (74%) of phage LiMN17 was broadened to 18/23 (78%) by the high adsorption rates on an additional strain (*L. monocytogenes* 19DO3). The k values of the three phages were also low (10^{-10}-10^{-11} min ml^{-1}) on 18DO5 and 18DO7 in which EOP values were $<10^{-7}$ (Table 4.3).

Table 4.3 Adsorption rate constants (k) for phages LiMN4L, LiMN4p and LiMN17 with different *L. monocytogenes* strains in phosphate buffered saline at 15 ± 1°C

L. monocytogenes strain	k (ml min^{-1}) of listeriaphage (Mean[#] ± SEM*)		
	LiMN4L	LiMN4p	LiMN17
19CO9[§]	$(8.93^{a} \pm 0.46) \times 10^{-9}$	$(3.16^{c} \pm 0.97) \times 10^{-9}$	$(2.42^{d} \pm 0.27) \times 10^{-9}$
19DO3	$(1.44^{e} \pm 0.27) \times 10^{-9}$	$(7.35^{b} \pm 0.18) \times 10^{-9}$	$(1.56^{e} \pm 0.43) \times 10^{-9}$
18DO5	$(5.26^{f} \pm 0.70) \times 10^{-11}$	$(4.10^{f} \pm 0.06) \times 10^{-10}$	$(1.17^{f} \pm 0.15) \times 10^{-10}$
18D07	$(7.54^{f} \pm 2.12) \times 10^{-11}$	$(4.82^{f} \pm 1.16) \times 10^{-11}$	$(3.24^{f} \pm 0.63) \times 10^{-11}$

[§] Indicator *L. monocytogenes* strain for plaque assay.

[#]Superscript lowercase letters (a, b, c, d, e, f) indicate significant differences among mean k values (n = 3, $p < 0.5$).

*SEM indicates standard error of the mean.

4.3.8 Plaque formation temperature range of phages

Phages LiMN4L and LiMN4p produced plaques at 4, 15, 25, 30 and 35°C and the % EOPs of both phages, however, remained <50 and $<4 \times 10^{-4}$ at 30 and 35°C, respectively (Supporting Information, Table 4.S3). Both phages did not form plaques but formed lytic zones on host lawns at 37°C. Phage LiMN17 formed plaques between 4 and 30°C, and formed lytic zones with phage drops ($\geq 4 \times 10^{6}$ PFU ml^{-1}) on host lawns at 35 and 37°C. Phages LiMN4L and LiMN4p adsorbed to the host at 37°C while phage LiMN17 adsorbed at 35 and 37°C even though they did not make productive infections in preliminary trials in single-step replication experiments.

4.3.9 Single-step replication of phages

The replication parameters of three phages were calculated using single–step replication curves (Fig 4.S1-3) and are summarized in Table 4.4. Three phages showed optimum burst size at 25°C within a comparatively short time at 15 and 35°C (Table 4.4).

Table 4.4 Single-step replication kinetics of listeriaphages LiMN4L, LiMN4p and LiMN17 grown with *L. monocytogenes* 19CO9 in trypticase soy broth at 15 ± 1, 25 ± 1 and 35 ± 1°C

Listeriaphage	Temperature (°C)	Single-step growth parameters			
		Latent period (min)	Rise period (min)	Eclipse period (min)	Burst size (PFU per cell)
LiMN4L	15	240	150	150	17 ± 5
	25	90	40	60	30 ± 4
	35	130	30	NI*	5 ± 2
LiMN4p	15	270	90	150	17 ± 4
	25	90	35	60	37 ± 3
	35	130	30	NI*	4 ± 2
LiMN17	15	270	120	180	11 ± 1
	25	90	40	60	20 ± 1
	35	-	-	-	NP§

4.4 Discussion

Phage isolates were examined for various physical properties to obtain more distinguishing characteristics. Large plaques of phages can be obtained by using low concentration (0.3%) of agar (Kropinski *et al*. 2009). *Pseudomonas chlororaphis* -specific phages (201Φ2-1 and 201Φ2-2) which had different dimensions of about two-fold in both head diameter and tail length were discriminated based on plaque size in low strength gel (0.1% agarose) (Serwer *et al.* 2004). However, by following the same method, the three phages of present study could not be distinguished based on plaque size in 0.1% agarose (Fig. 4.1 and Supporting Information, Table 4.S1). The TEM images of the three phages showed significant ($p < 0.05$) differences in the dimensions although they were less than the two-fold differences reported by Serwer *et al.* (2004). Nevertheless, use of agarose at low concentration permitted discrimination of phages by the appearance of plaque haloes (Fig. 4.1). Loessner and Busse (1990) also reported large plaques of *Listeria*-specific phage A513 containing one or more zones of secondary lysis. In phage infections, lysis of the host cell wall is mediated by phage endolysins. Endolysins are produced in excess during phage replication (Wang 2006) and degrade the cell wall of Gram-positive bacteria, inclusive from the exterior of the cell wall since they lack an outer-envelope (Schmelcher et al., 2012). Therefore, free

endolysins may have diffused out of the plaque and lysed cell walls of neighbouring uninfected cells resulting in haloes around the plaques in the soft agar overlay in the present experiments. Similarly, listeriaphage-endolysin (ply 118) expressed in *E. coli* 109(DE3), diffused from the dead *E. coli* colonies and lysed the adjacent cells of *L. monocytogenes* 1001-lawn giving haloes in the agar overlay (Loessner *et al*. 1995). The difference in the appearance of haloes between phage LiMN4p and the other two phages might be due to differences in heterogeneity of deploymerases (Fig. 4.1).

A group of *Pseudomonas* phages were distinguished in agars containing citrate (different concentrations), which are capable of depleting free Ca^{2+} ions in the growth medium (Olsen *et al.* 1968). By using this methodology, the three phages had significantly different EOP values (Fig. 4.2). Phage LiMN17 had high EOPs at both 0.5 and 1 mol l^{-1} citrate levels indicating it's reduced dependency on free Ca^{+2} compared to the other two phages. Alternatively, in adsorption assays, the adsorption rate (k) of phage LiMN17 was lower in the presence of Ca^{2+} while the k values of phages LiMN4L and LiMN4p were increased at 5 and 10 mmol l^{-1} levels of Ca^{2+}, respectively (data not shown). Based on the percentage survival of phage at 60°C, a group of phage isolates of *Pseudomonas* were distinguished into two groups. One group contained the psychrotrophic phages which seemed to diminish to non-detectable levels at 60°C after 10 min. The other contained mesophilic phages which survived over 40% at 60°C after 30 min (Olsen *et al.* 1968). Three phages in the present study were more heat-labile since only LiMN4L survived up to ≈10% at 60°C for 10 min and the other two phages reduced to non-detectable levels in 10 min.

Similar to the phages discussed here, the listeriaphages A511, P100 (Klumpp *et al.* 2008), FWLLm1, FWLLm3 and FWLLm5 (Lee, 2008) also belong to the family *Myoviridae*. The ClaI and SacI DNA restriction digestion profiles of listeriaphage A511 (Loessner *et al*. 1994), P35 (Dorscht *et al*. 2009), P100, 20422-1 and 805405-1 (Kim *et al*. 2008) were different from the phages studied here. From restriction enzyme digestion profiles, phage LIMN4p was distinguishable from phages LiMN4L and LiMN17 and these latter two may be closely related.

Genome size of phages LiMN4L, LiMN4p and LiMN17 were 146544, 140663 and 144992 base pairs (bp), respectively and unique sequence lengths (informational unit) of the genomes were 135993, 134719 and 136039 bp, respectively. The remaining sequences of three phage genomes (10551, 5601 and 8953 bp, respectively) included

the linear redundant terminals (unpublished data). Genomes of three phages showed more similarities to the genome of broad host range virulent phage A511 (Klumpp *et al.* 2008). Based on PHACTS analysis (McNair *et al.* 2012; Volozhantsev *et al*. 2012), all three phages were predicted to have a strictly virulent life style (unpublished data). Toxin encoding bacteria genes (Pasternack and Sulakvelidze 2009) were also not detected in the genomes of three phages at a cut off level of E value at $>5x10^{-4}$ (unpublished data). The phage genome sequencing information indicates that three phages were acceptable to be used as biocontrol agents. Characteristics of the three phage genomes details will be found in another report.

The host ranges of phages are suggested as a medium level criterion to distinguish new phage isolates (Ackermann *et al.* 1978). It was demonstrated that the three phages were different to each other. Santos *et al.* (2009) demonstrated productive infections by triggering the formation of visible plaques on host lawns using agar supplemented with different antibiotics at sub-optimal levels. In this study, three phages formed tiny visible plaques on lawns of some host strains in agar containing ampicillin (Table 4.2). The adsorption of a phage to host strains may be used to define its host range (Hyman and Abedon 2010). The results of the adsorption assay, therefore, indicated that the low levels ($<10^{-7}$%) of EOP of phages in different host strains did not directly correlate with the adsorption rates and that they might be influenced by other phenomenon such as blocking of the uptake of the phage genome, restriction modification, abortive infection, reduced infection vigor and interference of phage dissemination (Hyman and Abedon 2010). However, further studies are required to investigate the viable counts of cultures immediately after phage-adsorption.

The magnitude of k value is a function of the structure of adsorption appendages of both phage and bacteria in addition to the extrinsic parameters such as cofactors, temperature and viscosity of medium etc, (Gallet *et al,.* 2009; Hyman and Abedon, 2009). Phages with side tail fibres showed high k values in the range of $\approx 10^{-8}$-10^{-9} ml min^{-1} (Gallet *et al.* 2009). The three novel phages of this study also had side tail fibres (Fig. 4.3) and showed high k values on some host strains while low k values (10^{-10}-10^{-11} ml min^{-1}) were observed on other strains (Table 4.3). Therefore, significantly low k values ($\approx p < 0.05$) of the same phages may be attributed to the cell wall characteristics of particular host strains. The low adsorption rates of *E. coli* infecting phages maybe related to the presence of phage receptors in low densities on the host cell wall (Kasman *et al*. 2002; Schwartz 1976).

The phages used in this trial were isolated at 25°C to target phages favouring growth at low temperatures (Olsen *et al.*1968), since the phages were intended to kill *Listeria* at low temperatures in seafood processing plants. Similarly, listeriaphage P35 (ΦLMUP35) is reported to have been isolated and propagated at room temperature (Dorscht *et al.* 2009; Hodgson 2000). There are reports of other psychrotrophic non-listeriaphages such as those that infect *Pseudomonas* spp. and *Brochothrix thermosphacta* which have been isolated using similar low temperature protocols (Greer 1983; Olsen 1967; Olsen *et al.* 1968). Not surprisingly, the three phage isolates investigated in this study showed productive infections from 4 to 30°C while phages had low productivity or were non-productive at 35°C (Supporting Information, Table 4.S3). Phage LiMN17 did not infect host cells productively at 35°C, unlike the other two phages (Supporting Information, Table 4.S3**).** Contrary to the results of present study, Kim and Kathariou (2009) reported that listeriaphages isolated by analyzing the samples at 37°C had the highest host ranges and high EOPs at temperatures above 30°C.

Three phages replicated optimally at 25°C. At 15°C, three phages replicated at low threshold in longer latent periods (240-270 min) than latent time (90 min) at 25°C (Table 4.4). Loessner *et al*. (1995) reported that the latent time of phages A511 and A118 were 55 and 65 min respectively at 30°C. Nevertheless, currently, the detailed information on the single step replication of listeriaphages is not abundant. Phage LiMN17 may be more psychrotrophic, as demonstrated by its inability to make productive infections at 35°C, unlike the other two phages. The least survival at 60°C, high EOP value at low temperatures and single step replication <35°C may be attributed to psychrotrophic characteristics of the three phages adapoted to low temperature habitats.

4.5 Conclusion

The three psychrotrophic phages isolated from seafood processing premises had broad host ranges against seafood-borne *L. monocytogenes* strains at low temperature and showed low levels of burst size. The results suggest that these three virulent phages may be suitable decontaminating agents under the ambient conditions of seafood processing plants using passive biocontrol strategies which will be investigated in future studies.

Authors' contributions to the manuscript

The experiments were planned and conducted, data were analyzed and the first draft of the manuscript was prepared by GJGA. JY assisted in obtaining photographs of phage DNA analysis gels. ANM assisted the planning of experiments and analysis of data. ANM, BMDM, CDC, LM and SHF supervised and provided advice on interpretation of results and discussion and improving the writing of the overall manuscript. CB and AH reviewed the manuscript.

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Supporting Information

Table 4.S1 Listeriaphage (LiMN4L, LiMN4p and LiMN17) plaques on lawn of *L. monocytogenes* 19CO9 on trypticase soy yeast extract double layer agar [0.4% (w/v) agar or 0.1% (w/v) agarose overlay and 1% (w/v) agar base] at 25 ± 1°C after 48 h

Phage isolate	Plaque characteristics			
	0.4% (w/v) agar		0.1% (w/v) agarose	
	Appearance	Diameter (mm)	Appearance (mm)	Diameter (mm)
LiMN4L	Clear large centre with a narrow halo	1-1.5	Large clear center (1.5-2) with secondary haloes	3-4
LiMN4p	Small clear center with a narrow halo	0.7-1	Large clear center (2-2.5) with an one distinct halo	3-4
LiMN17	Small clear center with a narrow halo	0.5-1	Large clear center (1-2) with secondary haloes	3-3.5

Table 4.S2 Percentage survival of listeriaphages LiMN4L, LiMN4p and LiMN17 in lysates containing initial titre of ≈10^6 PFU ml^{-1} after exposure to different temperatures for 10, 30 and 60 min

Phage isolate	60 ± 1°C			50 ± 1°C		
	10 min	30 min	60 min	10 min	30 min	60 min
LiMN4L	8.7 ± 2	0.04 ± 0.01	(3.8 ± 2.3)10^{-5}	17.4 ± 1.2	11.7 ± 0.2	7.4 ± 0.47
LiMN4p	<DL	<DL	<DL	0.13 ± 0.02	<DL	<DL
LiMN17	<DL	<DL	<DL	<DL	<DL	<DL

DL indicates detection limit (=10 PFU/ml) per plate

Table 4.S3 Percentage efficiency of plating (%EOP) and formation of lytic zones (LZ) by listeriaphages LiMN4L, LiMN4p and LiMN17 on *L. monocytogenes* 19CO9 lawn at 4 ± 1, 15± 1, 25± 1, 30± 1, 35± 1 and 37 ± 1°C

Listeriaphage	Incubation temperature (°C)					
	% EOP[a] or LZ[b]					
	4	15	25	30	35	37
LiMN4L	71	96	100	48	$< 4 \times 10^{-4}$	LZ
LiMN4p	90	102	100	43	$< 4 \times 10^{-4}$	LZ
LiMN17	92	104	100	38	LZ	LZ

[a] EOP of a listeriaphage isolate is the ratio of phage titre at a test temperate and phage titre at 25 ± 1°C.

[b] Visible plaques could not be found but lytic zones were formed on the spots of original phage drops of $\geq 4 \times 10^{6}$ PFU ml^{-1}.

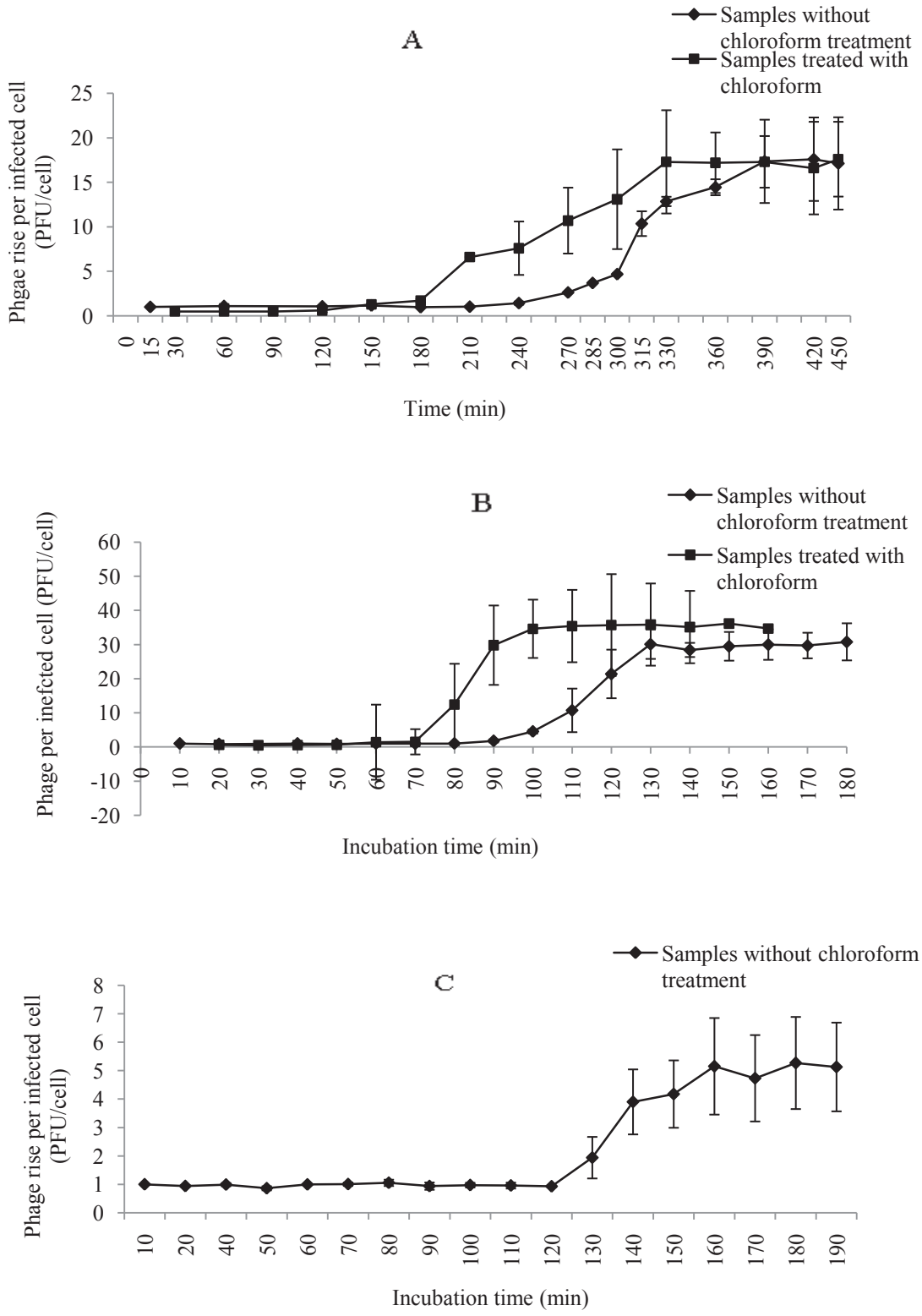

Figure 4.S1 Single-step growth curves of phages LiMN4L at 15, 25 and 35°C. A, 15°C; B, 25°C; C, 35°C.

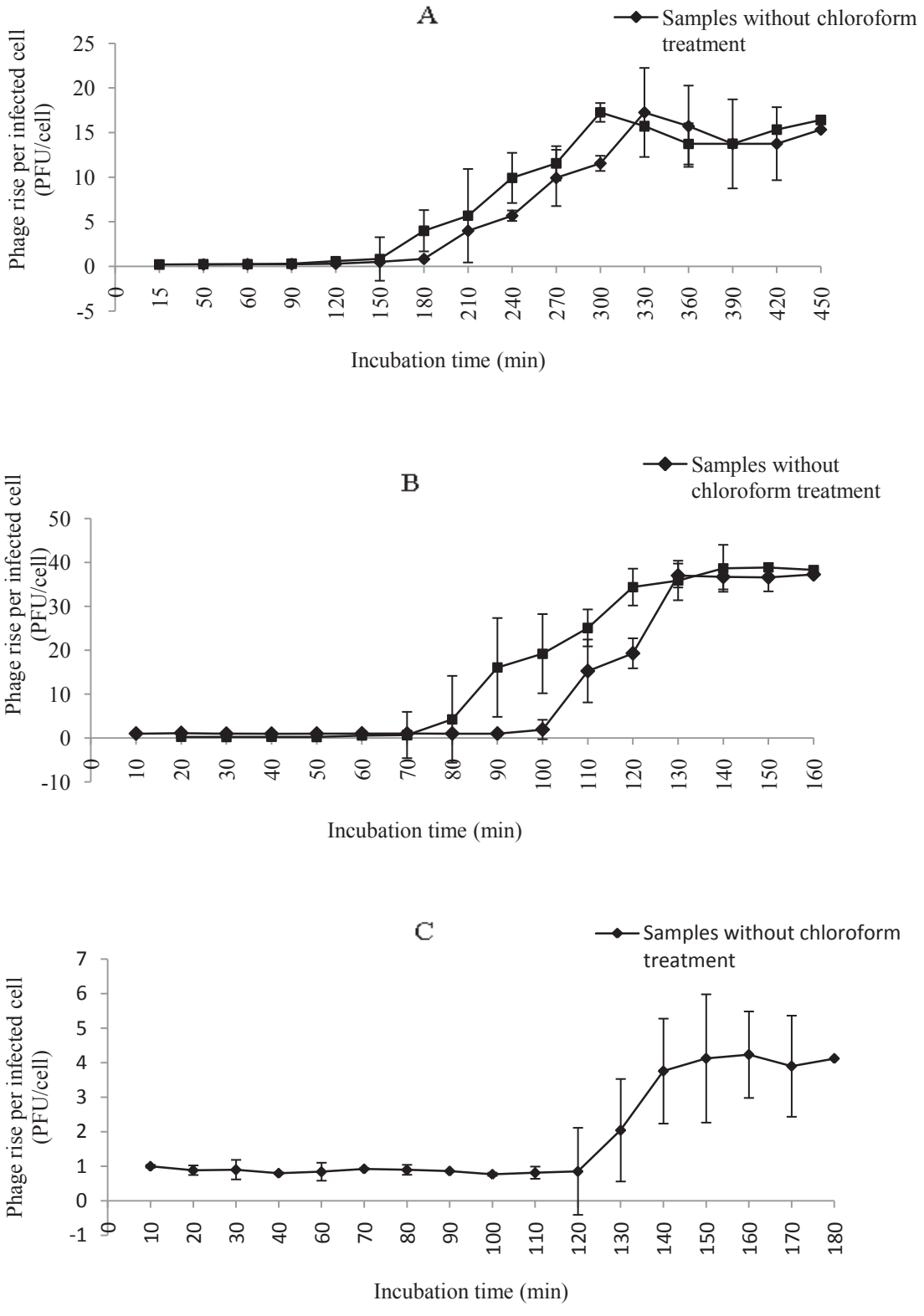

Figure 4.S2 Single-step growth curves of phages LiMN4p at 15, 25 and 35°C. A, 15°C; B, 25°C; C, 35°C.

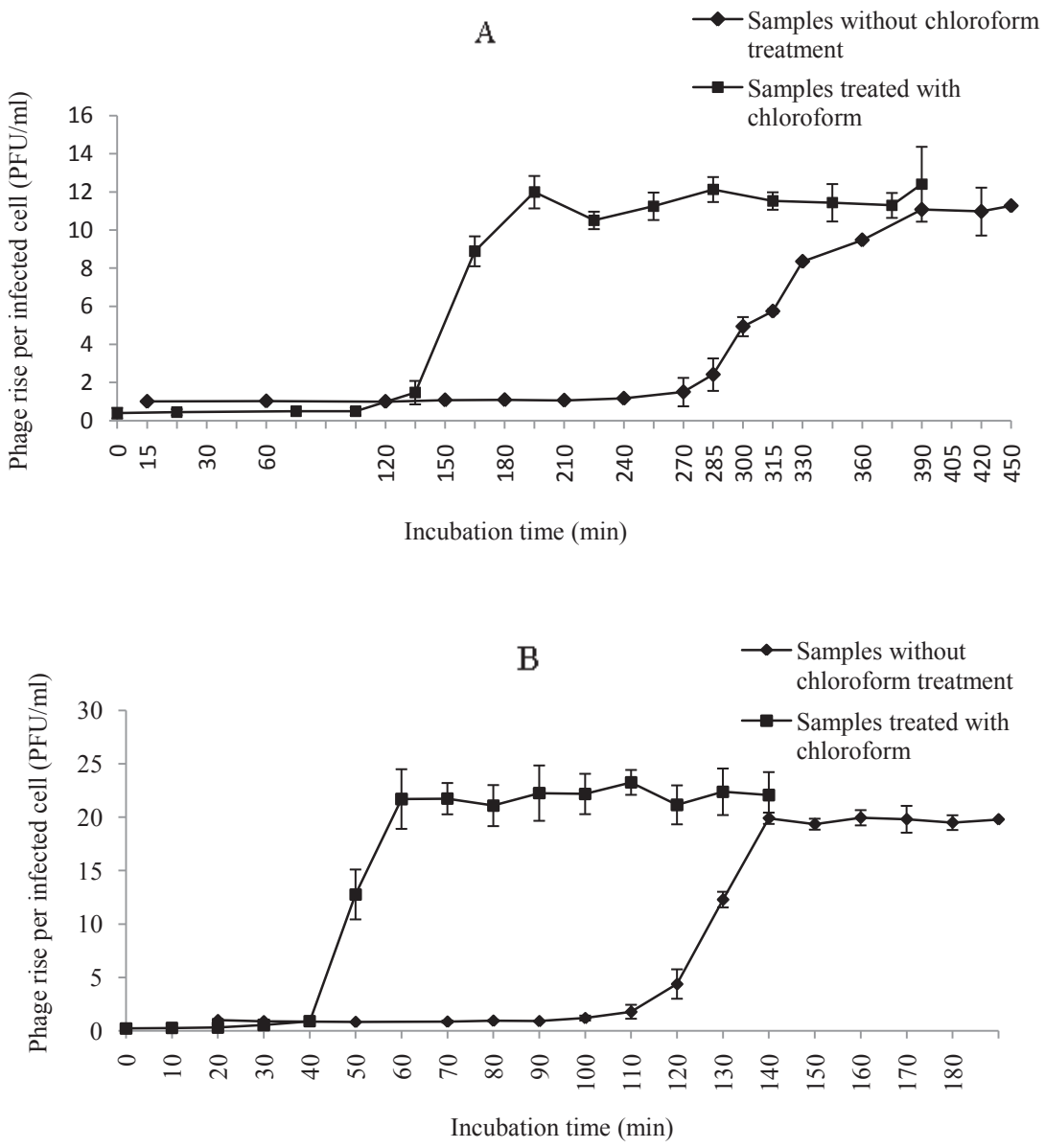

Figure 4.S3 Single-step growth curves of phages LiMN17 at 15 and 25°C. A, 15°C; B, 25°C.

Chapter 5: Preliminary investigation of bacteriophage-lysis of physiologically stressed *L. monocytogenes* in seafood processing environments

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Abstract

In food processing plants, contaminating bacteria may undergo different stresses due to exposure to nutrient deficient harsh environment that are not ideal for microbial growth. *L. monocytogenes* has the potential to adapt and develop resistance to environmental conditions including food processing and preservation operations and decontamination treatments commonly used in seafood processing plants. Bacteriophages (phages) may be used as alternative biocidal agents for this resistant subpopulation. Three strains of *L. monocytogenes* (19O9, 19DO3 and 19EO3) which were in late exponential phase (15 °C for 72 h), metabolically injured by heat (54-55 °C for 40-60 min) and stressed by starvation (25 °C for 3 weeks) and 9% (w/v) sodium chloride (15 °C for 72 h) were used. Physiologically affected low count cultures were challenged *in vitro* with three phages (LiMN4L, LiMN4p and LiMN17) individually at 15 ^{o}C for 30 min. Three phages (≈9 $\log_{10}$ PFU/ml) reduced late exponential phase *L. monocytogenes* strains (≈2-3 $\log_{10}$ CFU/ml) in a fish protein broth [10% (v/v)] to non-detectable level (< 1 $\log_{10}$ CFU/ml) in 30 min and re-growth of infected cultures did not occur over 4 days at 15 ^{o}C compared to increased cell counts of uninfected control cultures to ≈5-7 $\log_{10}$ CFU/ml. Each phage (≈8.5 $\log_{10}$ PFU/ml) lysed the heat-injured cells (2.5-3.6 $\log_{10}$ CFU/ml) of three *L. monocytogenes* strains to < 1 $\log_{10}$ CFU/ml. Individual phage decreased low counts (≈2-3 $\log_{10}$ CFU/ml) of starved *L. monocytogenes* strains which had coccoid cell shape to < 1 $\log_{10}$ CFU/ml. Each phage also reduced salt-stressed cells of three *L. monocytogenes* strains (≈3 $\log_{10}$ CFU/ml) by > 2 $\log_{10}$ units. The results demonstrated that the three phages may have potential to control physiologically affected *L. monocytogenes* cells commonly found in seafood processing environments.

5.1 Introduction

Pathogenic bacteria are most likely to exist in a metabolically stressed or injured state in natural environments, especially in food processing environments and processed foods (Gill, 2010). This is because bacteria are subject to sublethal injuries due to unit operations such as heating, freezing, drying, curing and exposure to chemical detergents and disinfectants in food processing plants as well as intrinsic properties (physical and chemical factors) of the food (Bunduki et al., 1995; Busch and Donnelly, 1992; Wesche et al., 2009). *L. monocytogenes* is a cause of food-borne illness, listeriosis due to contamination of ready-to-eat foods including seafoods (Brett et al., 1998; Donnelly, 2001; Gudbjornsdottir et al., 2004; Klontz et al., 2008). Stressed *L. monocytogenes* develop adaptive tolerance and resistance to subsequent antibacterial treatments (Lou and Yousef, 1997; Mendonca et al., 2004; Wesche et al., 2009). The virulence potential of stressed cells of some *L. monocytogenes* strains also remains similar to healthy cells (Mendonca et al., 2004; O'Driscoll et al., 1996; Wesche et al., 2009). Therefore, contamination of foods with injured cells found in food processing plant environments is a potential food safety hazard (Bunduki et al., 1995; Mendonca et al., 2004). The development of a subpopulation of bacteria resistant to sanitizers can be prevented by using alternative antibacterial agents on a rotational basis (Hagens and Loessner, 2010; Roy et al., 1993). Lytic natural phages are an alternate class of antibacterial agents effective against food-borne pathogens including *L. monocytogenes* (Carlton et al., 2005; Goodridge and Bisha, 2011; Guenther et al., 2009; Soni and Nannapaneni, 2010; Soni et al., 2010). However, the efficacy of phages in lysing physiologically damaged *L. monocytogenes* is currently unknown.

High count bacteria populations show heterogeneity on phage-sensitivity and a small fraction of cells may remain insensitive for lytic phages (Chapman-McQuiston and Wu, 2008). Most reported studies have focused on *E. coli* strains while Gram positive strains have been studied least in this context. The total cells of particular *L. monocytogenes* strains targeted for control should be lysed by phage applications as decontaminating agents. Culture heterogeneity of seafood environmental strains of *L. monocytogenes* for phage-sensitivity is not known.

N-acetylglucosamine (GLcNAc) present in the cell wall of *Listeria* strains belonging to serovars 1/2, 3, 4, 5, 6 and 7, acts as a binding site for some *Listeria* infecting phages (Eugster et al., 2011). In *Listeria* strains belonging to serovars 1/2, 3 and 7, the GLcNAc exists as free moieties anchored onto teichoic acid chains of the cell wall. The GLcNAc of *Listeria* belonging to serovars 4, 5 and 6 remains cross-linked within the teichoic acid chains. Additionally, the cross-linked GLcNAc of some strains of latter group are decorated further with galactose and glucose molecules (Eugster et al., 2011; Nir-Paz et al., 2012).

This study becomes the first study on phage-lysis of *L. monocytogenes* cells injured by heat and stressed by starvation or 9% (w/v) salt at 15 °C. We also studied the existence of phage-sensitivity of high count cell cultures (≈6-8 log CFU/ml) of *L. monocytogenes* strain isolated from seafood processing environment and the emergence of any phage resistant cells over 48 h at 15 °C. Furthermore, the role of the host cell wall GLcNAc of *L. monocytogenes* strains as receptors for three *Listeria*-infecting phages was also assessed.

5.2 Materials and methods

5.2.1 *L. monocytogenes* strains and phages

This study used *L. monocytogenes* 19CO9 (serotype 1/2a or 3a), 19DO3 (serotype 1/2a or 3a), and 19EO3 (serotype 4b, 4d or 4e) strains isolated from seafood processing environments (Cruz and Fletcher, 2011). Experimental broth cultures were prepared using inocula obtained from monthly working cultures of *L. monocytogenes* strains maintained on trypticase soy agar (TSA, Difco, Sparks, MD) plates stored at 4 ^{o}C.

Listeria phages LiMN4L, LiMN4p and LiMN17 isolated from a waste water treatment unit of a fish processing plant in New Zealand were selected for this study. Individual phage lysates were prepared by propagating at 25 °C using *L. monocytogenes* 19CO9 (indicator host) on double layer agar (DLA) prepared with TSA [overlay of 0.4% (w/v) agar and base of 1% (w/v) agar] in flasks (2 l) by scaling up the DLA protocol given by Sillankorva et al (2004). The phage lysates were sterilized with chloroform ($CHCl_3$) (Analytical Grade, Merck) [≈0.25% (v/v)], cleared by centrifuging

at 8,000 g for 20 min, purified by centrifuging at 25,000 g (SS-34 rotor, Sorvall RC6+ Centrifuge, Sorvall, Germany) at 4 ^{o}C (Ackermann, 2009) for 6 h and the pellets were suspended in phosphate buffered saline [PBS; 0.008 M Na_2HPO_4 (Merck, Darmstadt, Germany), 0.001 M $NaH_2PO_4.H_2O$ (Merck) and 0.145 M NaCl (Merck); pH 7.5] for 18 h at 4 °C in the dark. The phage stocks were stored in the dark at 4 °C. The phage titre was assessed over 10-fold dilutions prepared using PBS by DLA method in plates (Kropinski et al., 2009).

5.2.2 Phage-based lysis of low count of *L. monocytogenes*

Late exponential phase cultures of *L. monocytogenes* strains (19CO9, 19DO3 and 19EO3) were obtained by growing the cells in trypticase soy broth (TSB, Difco) at 15±1 °C for 72 h, centrifuged at 8,000 g for 10 min and suspended in PBS. The 10-fold serial dilution of each strain was prepared to obtain culture containing ≈3 $\log_{10}$ CFU/ml. the cell dilution was centrifuged, the pellet was re-suspended in 10% (v/v) aqueous fish extract (Bernbom et al., 2006) and then cells were infected with three phages LiMN4L, LiMN4p and LiMN17 (≈9 $\log_{10}$ PFU/ml) separately. An uninfected control host cell culture was also prepared in parallel. Both infected and control cultures were incubated at 15±1 °C for 96 h. At 0, 0.5, 1, 4, 24, 48, 72 and 96 h intervals, 1 ml aliquots were removed and centrifuged at 8,000 g for 10 min. The cell pellets were re-suspended in 1 ml of PBS and 0.1 ml spread plated on PALCAM agar (Difco) using the standard spread plate technique (detection limit = 1 $\log_{10}$ CFU/ml). Triplicate sample plates were incubated at 35±1 °C for 48 h.

5.2.3 Preparation of heat-injured *L. monocytogenes*

Exponential phase cultures of *L. monocytogenes* strains (19CO9, 19DO3 and 19EO3) grown in TSB at 15±1 °C for 48 h were diluted to contain ≈8.7 $\log_{10}$ CFU/ml (A_{600nm}, ≈0.5) using TSB. The cultures were heat-treated at different temperature and time combinations (55±1 °C for 50 min, 54±1 °C for 60 min and 54±1 °C for 40 min, respectively) which were determined in preliminary trials to obtain the highest fraction (> 95%) of injured cells. Two ml of each culture was mixed with pre-heated 28 ml volume of TSB at respective temperatures for ≈30 min in a 250 ml conical flask with shaking (50 rpm) in a water bath (Busch and Donnelly, 1992). Heat-treated cultures were chilled immediately to 4 °C in an ice slurry, centrifuged (8000 x g) at 4 °C for 10 min and re-suspended in PBS.

The counts of total (undamaged and heat-injured) and undamaged cells were enumerated on trypticase soy agar containing yeast extract (Difco) (TSAYE) and TSAYE agar containing 4% (w/v) NaCl (TSAYEN), respectively by plating 10-fold serial dilutions of heated-treated culture using standard spread plate technique (detection limit =1 log CFU/ml). Inoculated plates were incubated at 30±1 ^{o}C for 72 h. Heat-damaged cells were calculated using equation 1 (Busch and Donnelly, 1992).

$$\%\ \text{Injured cells by heat} = \left[1 - \frac{(\text{Counts on TSAYE} - \text{Counts on TSAYEN})}{\text{Counts on TSAYE}}\right] \text{x} 100 \qquad (1)$$

5.2.4 Phage-lysis of heat-injured *L. monocytogenes*

The cells of heat-treated culture (≈4 log CFU/ml) and two subsequent 10-fold dilutions (≈3 and 2 $\log_{10}$ CFU/ml, respectively) were infected with phage LiMN4L (≈8.5 $\log_{10}$ PFU/ml) separately in PBS. The first dilution of injured culture of each strain (≈3 $\log_{10}$ CFU/ml) was infected with phages LiMN4p and LiMN17 (≈8.5 $\log_{10}$ PFU/ml), separately. The uninfected control cultures were also prepared in parallel to each infected culture in PBS. The cultures were incubated at 15±1 °C for 30 min. One ml aliquots were centrifuged at 8,000 g for 10 min, the cell pellets were re-suspended in 1 ml of PBS and viable counts were enumerated using TSAYE and TSAYEN plates as above (detection limit = 1 $\log_{10}$ CFU/ml).

5.2.5 Preparation and phage-lysis of starved *L. monocytogenes*

Exponential phase cultures (≈8 $\log_{10}$ CFU/ml) of *L. monocytogenes* strains (19CO9, 19DO3 and 19EO3) grown in TSB at 15±1 °C for 48 h were rinsed with MilliQ water (pH 6.0) twice by centrifuging at 8,000 g for 10 min and re-suspending in MilliQ water in equal volumes. The cultures in MilliQ water were incubated at 25±1°C in darkness for three weeks (Besnard et al., 2000). Cultures containing ≈3 $\log_{10}$ CFU/ml were infected with phages LiMN4L, LiMN4p and LiMN17 (≈8.5 $\log_{10}$ PFU/ml) and control cultures were also set up without phage. The infected and control cultures were incubated for 30 min and viable counts were enumerated on TSAYE following the spread plate method described for heat injured cells (detection limit = 1 $\log_{10}$ CFU/ml).

5.2.6 Preparation and phage-based lysis of salt-stressed *L. monocytogenes*

Exponential phase cell suspensions (≈8 $\log_{10}$ CFU/ml) of *L. monocytogenes* strains (19CO9, 19DO3 and 19EO3) grown in TSB at 15 °C for 48 h were rinsed twice in MilliQ water, re-suspended in 9% (w/v) sodium chloride (NaCl) (Jorgensen et al., 1994) and incubated at 15±1 °C for 72 h. Cultures containing ≈3 $\log_{10}$ CFU/ml were infected with three phages for 30 min and viable counts were enumerated on TSAYE following the protocol above for heat-injured cells (detection limit = 1 $\log_{10}$ CFU/ml).

5.2.7 Fluorescence microscopy of stressed *L. monocytogenes* cells stained with Acridine Orange

L. monocytogenes cultures (10 µl) were spread on glass slides and allowed to attach on the slide for 5 min, stained with 20 µl of sterile 0.01% (w/v) Acridine Orange (BDH, Chemical Ltd, Poole, England) at room temperature (22±1 °C) for 5 min in the dark (Carpentier and Chassaing, 2004), rinsed off excess stains with sterile MilliQ water and blotted by touching the edge of the stained cell smear with a Whatman paper™. The stained cells were observed under fluorescence microscopy (Axio Star Plus, Transmitted Light Microscope Carl Zeiss, Germany) using excitation (450-490 nm) and emission (515 nm) filters (Filter set 9, Carl Zeiss).

5.2.8 Phage- insensitive cells of high count *L. monocytogenes* culture

Exponential phase cultures of *L. monocytogenes* 19CO9 grown in TSB at 15±1 °C for 48 h were diluted to contain approximately 8 $\log_{10}$ CFU/ml (A_{600nm}, ≈0.1) using TSB. The culture was infected with three phages LiMN4L, LiMN4p and LiMN17 (≈9 $\log_{10}$ PFU/ml) separately at 15±1 ^{o}C and continued the incubation at 15±1 ^{o}C. A control culture was also set up. The surviving cells of the same culture were re-infected at 3, 5.5, 7.5 and 8.5 h. The cells of both phage-infected and control cultures were pelleted by centrifuging at 8000 g for 10 min, re-suspended in TSB and then, re-infected with phages. At each time point, 1 ml samples were removed from re-suspended culture and viable counts were enumerated on duplicate plates by pour plate method using TSA and incubating the inoculated duplicate plates at 30±1 ^{o}C for 72 h (detection limit = 1 CFU/ml).

Another experiment was carried out to investigate the emergence of phage-insensitive *L. monocytogenes* cells over 48 h at 15±1 °C as described later. A host cell

culture containing approximately 6 $\log_{10}$ CFU/ml was infected with three phages LiMN4L, LiMN4p and LiMN17 (≈9 $\log_{10}$ PFU/ml) in TSB and incubated at 15±1 ^{o}C for 48 h. A longer exposure time was given than that used in the previous experiment to ensure phage attack of the lower number of cells. The 48 h phage-infected culture was re-infected to determine if the surviving cells were sensitive or resistant to phage infection. The surviving counts of the infected cultures were compared with the counts of uninfected cultures at each time point. Surviving cells were enumerated using the pour plate method as above (detection limit = 1 CFU/ml).

5.2.9 Determination of N-acetylglucosamine (GLcNAc) in cell wall of *L. monocytogenes*

A fluorescently labeled wheat germ agglutinin (WGA) binding assay was performed to demonstrate the presence of GLcNAc in the cell walls of *Listeria* strains (Eugster and Loessner, 2011). The seven strains used in this study belonged to serovars 1/2, 3 and 7 (Table 1). *Listeria* strain culture grown to late exponential phase (15±1 ^{o}C for 72 h) was washed in PBS twice and re-suspended in half the original volume using PBS containing Tween (PBST) [120 mM NaCl, 50 mM phosphate and 0.1% (v/v) Tween 20 (Ajax Finechem Pty Ltd, Sydney, Australia), pH 8.0]. A volume of 100 µl of cells in PBST and 50 µl of WGA reagent [0.1 mg WGA (Alexa Fluor 488 Dye, Invitrogen, Life Technologies, CA, USA) and 1 ml PBST] were mixed gently by pipetting and incubated at 25±1 ^{o}C for 1 h. They were then washed three times in PBS by centrifuging at 12,000 g for 1 min and re-suspending in PBS. The labelled cells were observed under fluorescence microscopy as previously described with Acridine Orange. The unlabeled strain cell samples were also observed under the light-microscope to ascertain the presence of cells on the slides. Each strain was assayed at least three times.

5.2.10 Statistical analysis

Descriptive statistical analyses were performed using Microsoft Office Excel 2007 (Microsoft Office, Washington, USA).

5.3 Results and discussion

5.3.1 Phage-lysis of low count cells of *L. monocytogenes* in fish broth

Late exponential phase cells of *L. monocytogenes* were suspended in a fish protein solution to mimic the potential contamination of planktonic *L. monocytogenes* in stagnant water smears on fish processing surfaces. The low count cells of ≈2-3 $\log_{10}$ CFU/ml of each strain (*L. monocytogenes* 19CO9, 19DO3 and 19EO3) were reduced to non-detectable level (< 1 $\log_{10}$ CFU/ml) after exposure to each phage for ≈30 min. Thereafter the re-growth of cells did not occur for at least over 4 days compared with the control cultures (Figs. 5.1a-c). Similarly, P100 (≈7.8 $\log_{10}$ PFU/cm^2) eradicated low counts (≈1.3 $\log_{10}$ CFU/cm^2) of *L. monocytogenes* LmC contaminated *in vitro* on red smear cheese stored for 10 days at 14 °C followed by packed storage for 5 days at 6 °C (Carlton et al., 2005).

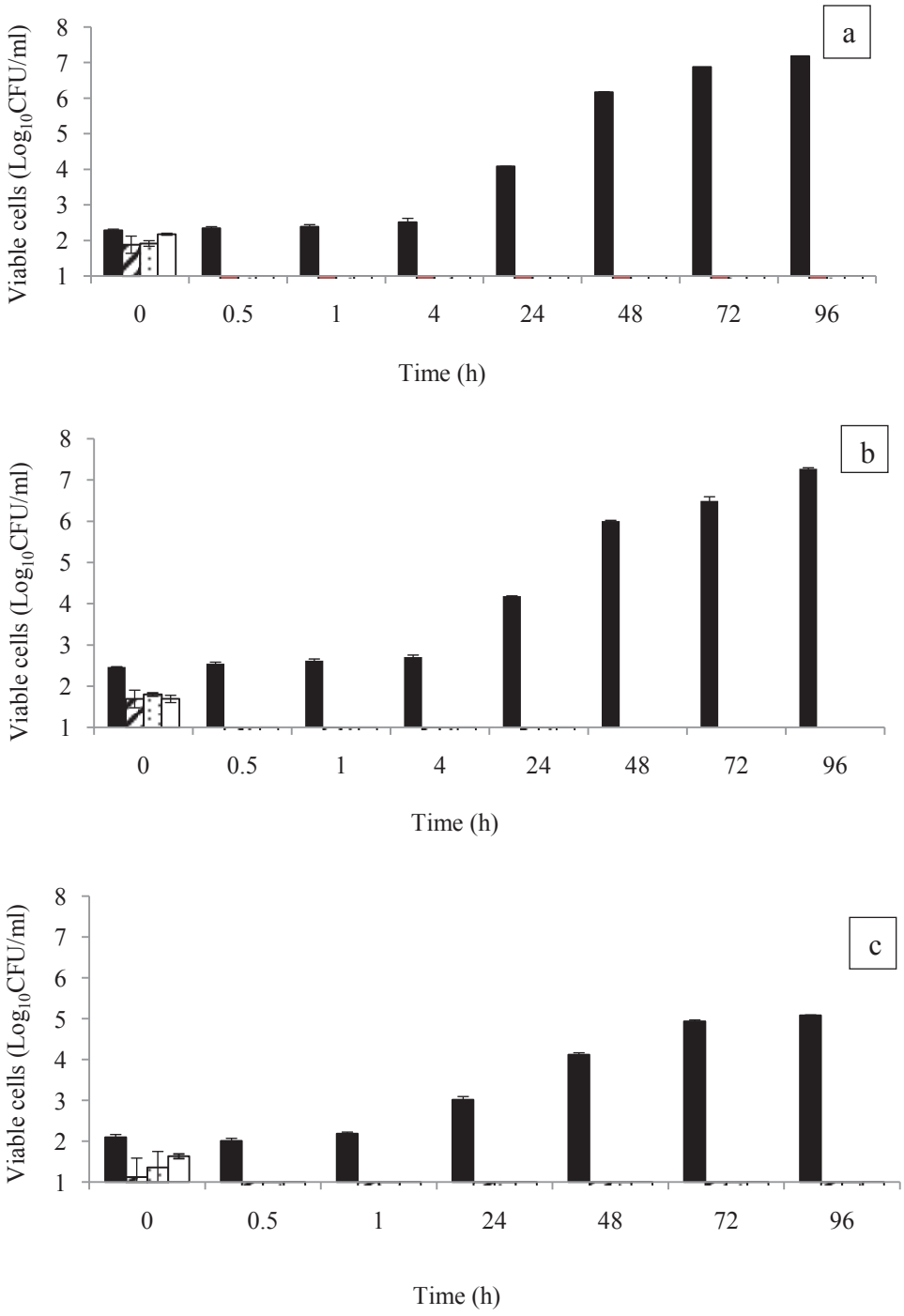

Figure 5.1 Viable counts of phage infected and control cultures of late exponential phase three *L. monocytogenes* strains: a, 19CO9; b, 19DO3 and c, 19EO3 in half strength fish broth (Bernbom et al., 2006) at 15±1 °C. Cultures: ■, Control; ▨, Infected with phage LiMN4L; ⁘, with LiMN4p; □, with LiMN17. Vertical bars indicate standard deviation of three determinations in one experiment. Detection limit = 1 CFU/ml.

5.3.2 Phage-mediated lysis of heat-injured *L. monocytogenes*

Heat-injured *L. monocytogenes* cells may enter into a processing environment along with steamed fish or mussel. Heat-injured cells can repair the injury in yeast extract containing medium (TSAYE) while they do not resuscitate in TSAYE plus 4% (w/v) salt (TSAYEN) (Busch and Donnelly, 1992). Therefore, a fraction of injured cells was determined using these two media (Busch and Donnelly, 1992). The grown colonies of heat-injured cultures of *L monocytogenes* 19CO9 were only detected on TSAYE (≈4.2 $\log_{10}$ CFU/ml) and were not found on TSAYEN. Based on equation 1, all the cells, therefore were assumed to be heat-injured (data not shown). The phage LiMN4L reduced the cells of the high count culture (≈4.2 $\log_{10}$ CFU/ml) by ≈0.5 log units (Fig. 5.2a) and reduced the initial counts of the other two diluted cultures (3.2 and 2.6 log units) to <1 $\log_{10}$ CFU/ml (Fig. 5.2a). Phages LiMN4p and LiMN17 also reduced the initial cell counts of ≈3.2 log units to < 1 $\log_{10}$ CFU/ml.

In the heat-treated culture of *L monocytogenes* 19DO3, total viable cells and undamaged cells were ≈4 and 2.5 $\log_{10}$ CFU /ml, respectively. From equation 1, total cells injured by heat treatment were ≈96% cells. Phage LiMN4L decreased cells (≈4 $\log_{10}$ CFU/ml) of heat-treated culture by ≈1.6 log units while LiMN4L reduced the cells of other two dilutions (≈3 and 1.3 $\log_{10}$ CFU/ml) to < 1 $\log_{10}$ CFU/ml (Fig. 5.2b). Phage LiMN4p and LiMN17 also reduced initial counts (≈3 log units) to < 1 $\log_{10}$ CFU/ml (data not shown). The heat-treated culture of *L. monocytogenes* 19EO3 contained total viable cells and undamaged cells of ≈4.5 and 1.8 $\log_{10}$ CFU /ml, respectively. By substituting theses data in equation 1, > 99% of cells were injured by heat treatment. Phage LiMN4L lysed the initial counts of heat-treated cultures (≈4.5 $\log_{10}$ CFU/ml of initial cells) by ≈1.5 log units and the cell counts of the other two cultures (≈3.6 and 2.5 $\log_{10}$ CFU /ml) to < 1 $\log_{10}$ CFU/ml (Fig. 5.2c). Phage LiMN4p and LiMN17 also lysed cultures (≈3.6 $\log_{10}$ CFU /ml) to < 1 $\log_{10}$ CFU/ml (data not shown).

Using cycloserine D, Bunduki et al (1995) showed that the *Listeria* cell wall was not affected by heat treatment. This observation was supported by our present finding that heat- injured cells were susceptible to phage infection, because phage-infection of a cell is initiated after adsorption of phages on cell wall (Abedon, 2009; Gill, 2010). Bacteriophages have been reported to adsorb on heat-killed *E coli* cells (Schlesinger, 1964). The bacteria will then be killed by phage infection depending on the physiological state of the cell (Abedon, 2009). Bunduki et al (1995) demonstrated that heat treatments affect the cell metabolism such as synthesis of proteins, mRNA and

oxidative phosphorylation by using three specific inhibitory antibiotics (chloramphenicol, rifampicin and carbonylcyanide m-chlorophenyl-hydrazone (CCCP), respectively). Nevertheless, intracellular contents (proteins and nucleic acids) did not leak in heat-damaged cells (Bunduki et al., 1995). The results of the current study showed that injured cells were susceptible to phages. Therefore, the level of *L. monocytogenes* injury caused by heat treatment might not have affected phage infection process, at least in initial stages after which host cells were subject to subsequent death regardless the completion of a phage infection cycle (Abedon, 2009; Abuladze et al., 2008).

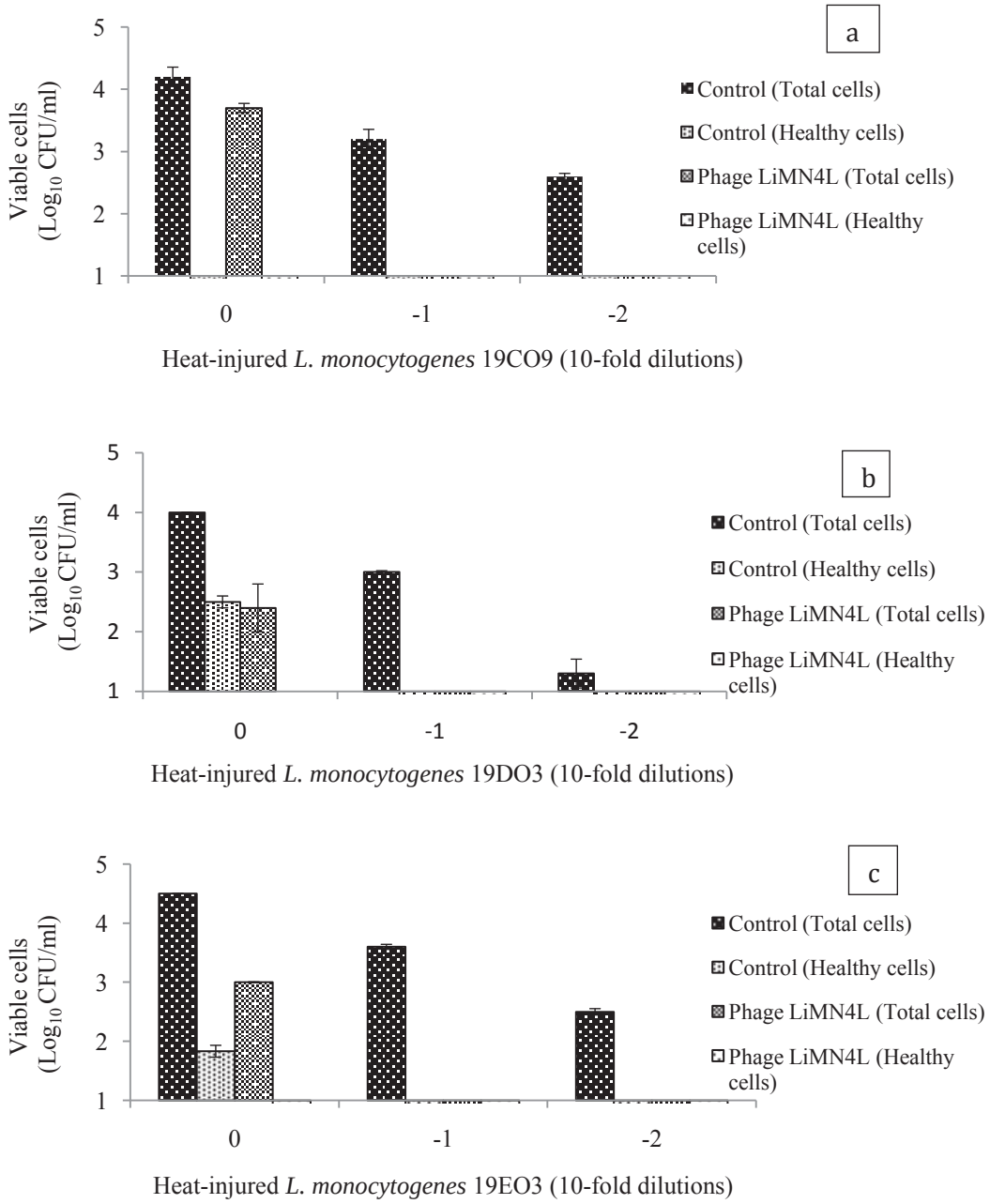

Figure 5.2 Viable counts of control and phage-infected cultures of heat-treated *L. monocytogenes* strains at 15±1 °C after 30 min. Each strain culture was heat-treated with different temperature/time combinations. Strain: a, 19CO9 (55±1 °C, 50 min); b, 19DO3 (54±1 °C, 60 min); c, 19EO3 (54±1 °C, 40 min). Cultures were infected with phage LiMN4L (≈8.5 $\log_{10}$ PFU/ml). Total cells (healthy and injured cells): Counts on trypticase soy yeast extract agar, Healthy cells: Counts on trypticase soy yeast extract agar plus 4% (w/v) NaCl. Data represent means of triplicate from one experiment. Detection limit = < 1 $\log_{10}$ CFU/ml.

5.3.3 Phage-based lysis of starved *L. monocytogenes* cells

The contaminant *L. monocytogenes* is more likely to undergo starvation under nutrient deficient conditions such as in cleaner environments of food processing plants. In this study, cells were starved *in vitro* in MilliQ water. The rod shaped *L. monocytogenes* (19CO9, 19DO3 and 19EO3) strains were transformed to coccoid shape during starvation (Fig. 5.3). The transformation of the typical rod shaped (bacillus cell shaped) *L. monocytogenes* could have been considered a sign of starvation, as reported with *L. monocytogenes*, *Pseudomonas fluorescens* and *Campylobacter jejuni* cultures starved in microcosms (Jorgensen et al., 1994; Rollins and Colwell, 1986; Wen et al., 2009). The low counts (≈2-3 log CFU/ml) of starved *L. monocytogenes* (19CO9, 19DO3 and 19EO3) were lysed to < 1 log CFU/ml by three phages LiMN4L, LiMN4p and LiMN17 (Fig. 5.4). In a simulated study, *P. aeruginosa* cells which are starved in lake water for up to 5 years are reported to be susceptible to *Pseudomonas* specific bacteriophages ACQ and UT1 by resulting in productive infections (Schrader et al., 1997). In the present study, the three phages lysed cells of three *L. monocytogenes* strains starved *in vitro* for three weeks. Starved *Listeria* cells are able to maintain viability by nutrients released from lysis of a fraction of cells in the population (Lindback et al., 2010). Microscopic observations showed that starved *L. monocytogenes* 19CO9 cultures formed larger cell clumps (aggregates) compared to the other two strains under static conditions (Fig. 5.3) and formation of clumps also may be a surviving strategy attributed to this strain. In phage challenge experiments, uniformly distributed low count cultures prepared by vigorous agitation and pipetting of starved culture over serial dilution were used. Therefore, the effectiveness of phages *in vitro* experiments may not represent the lytic efficacy of phages against cell clumps, which are more likely to form in stagnant natural environments. Therefore, efficacy of phage lysis of cell clumps ought to be studied *in situ* using suitable techniques such as microscopic methods. Starvation or the long-term-survival of *Listeria* cells has been reported where cells acquired resistance to lethal treatments such as high-pressure processing, irradiation, heat and H_2O_2 (Lou and Yousef, 1997; Mendonca et al., 2004; Wen et al., 2009). The study demonstrated that phages may provide an alternative to control *L. monocytogenes* cells stressed by starvation.

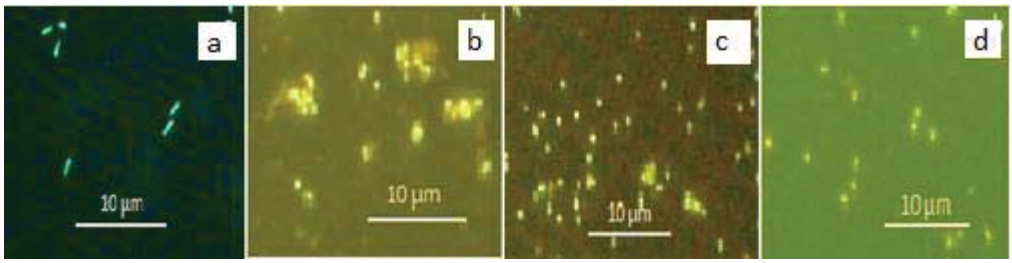

Figure 5.3 Coccoid-form cells of *L. monocytogenes* strains starved in MilliQ water at 25±1 °C for 3 weeks. a, Initial rod shape of strain 19CO9; Starved cells of strain: b, 19CO9; c, 19DO3; d, 19EO3.

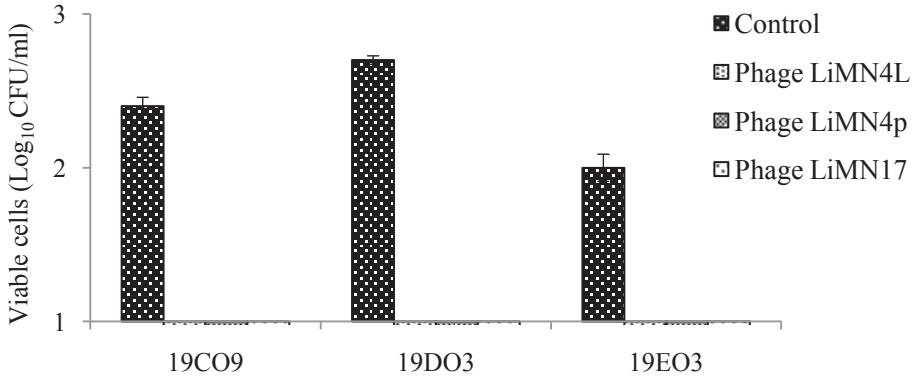

Figure 5.4 Viable counts of control and phage-infected cultures of starved *L. monocytogenes* strains (19CO9, 19DO3, 19EO3) at 15±1 °C after 30 min. Cells were starved in MilliQ water (pH 6.0) at 25±1 °C for 3 weeks. The phage titre used was ≈8.5 $\log_{10}$ PFU/ml. Data represent means of triplicate from one experiment. Detection limit = 1 $\log_{10}$ CFU/ml.

5.3.4 Phage-based lysis of salt-stressed *L. monocytogenes*

Salt is a common preservative in many food products (Gnanou et al., 2000). *Listeria* cells tolerate up to 10% (w/v) salt (Farber et al., 2008). *L. monocytogenes* 19CO9 became a diplobacillus form while *L. monocytogenes* 19DO3 and 19EO3 changed into coccoid shape following salt stress (Fig. 5.5). Phages LiMN4L, LiMN4p and LiMN17 lysed low count cells (≈3 log CFU/ml) of salt-stressed *L. monocytogenes* strains (19CO9, 19DO3 and 19EO3) by > 2 log units or reduced to < 1 $\log_{10}$ CFU/ml

(Fig. 5.6). *L. monocytogenes* has been recovered from smoked fish containing high salt concentrations (Fonnesbech et al., 2001). Salt shock does not damage the cell wall, cell membrane or the synthesis of nucleic acids (Wesche et al., 2009). *L. monocytogenes* stressed in 7% (w/v) NaCl for 1 h increased the resistance of cells when exposed to 1% (v/v) H_2O_2 (Lou and Yousef, 1997). The rod shaped *L. monocytogenes* 19CO9 was not completely transformed to a coccid shape after 72 h in salt compared to the other two strains (Fig. 5.5) and therefore, this strain may be more resistant to salt stress.

Besnard et al (2000) demonstrated that high numbers of cells close to initial counts of *L. monocytogenes* culture remain viable but non culturable (VBNC) in 7% (w/v) salt at 20 °C for up to about 10 weeks. However, Lindback et al (2010) demonstrated that VBNC of *in vitro* starved cultures of *L. monocytogenes* strains isolated from seafood processing environments were avirulent by a morbidity assay of immune-deficient mice and plaque assay on human cell monolayers. However, VBNC cells could be resuscitated under some favourable conditions (Cappeliera et al., 2007). Therefore, these cells also should be targeted in lethal treatments. The use of phage to lyse these cells should be studied using suitable methods such as LIVE/DEAD (Invitrogen) cell viability assays and changes in ATP levels. The present study demonstrated that three phages were lethal for *L. monocytogenes* stressed by 9% (w/v) salt for about 3 days. Present results suggest the need for further studies in phage-susceptibility of *L. monocytogenes* implicated in high salt concentrations together with different exposure times which are more likely to be encountered in seafood processing environments.

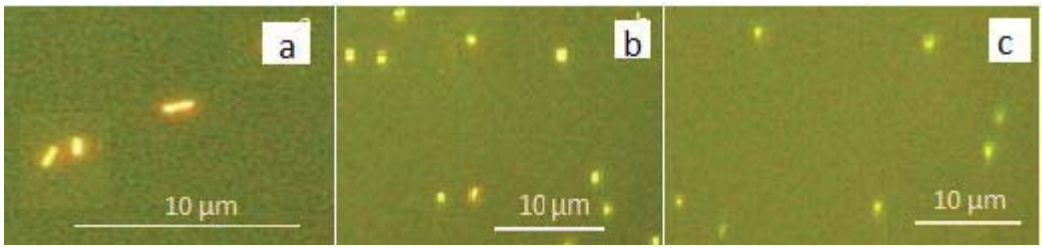

Figure 5.5 Stressed cells of *L monocytogenes* strains in 9% (w/v) NaCl at 15±1 °C for 72 h. a, 19CO9 (diplobacillus-form); b, 19DO3 (coccoid-form); c, 19EO3 (coccoid-form).

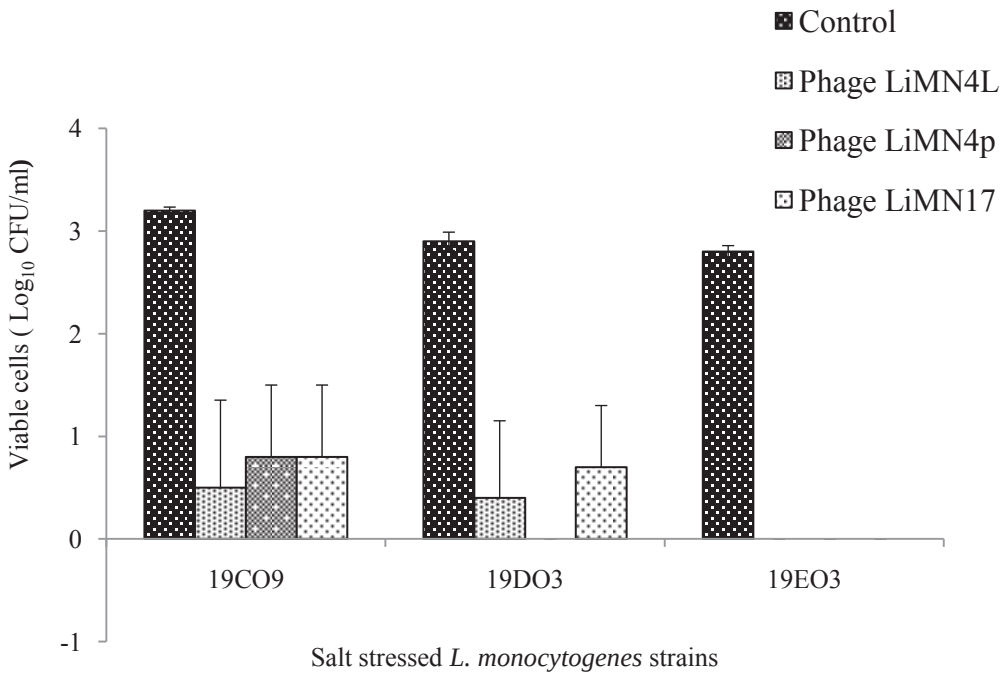

Figure 5.6 Viable counts of control and phage-infected cultures of salt-stressed *L. monocytogenes* strains (19CO9, 19DO3, 19EO3) at 15±1 °C after 30 min. Cells were stressed in 9% (w/v) NaCl at 15±1 °C for 72 h. The phage titre used was ≈8.5 $\log_{10}$ PFU/ml. Data represent means of triplicate from one experiment. Detection limit = 1 $\log_{10}$ CFU/ml.

5.3.5 Phage-insensitive cells of exponential phase *L. monocytogenes* 19CO9 culture

Viable counts (8 $\log_{10}$ CFU/ml) of exponential phase *L. monocytogenes* 19CO9 were reduced to <1 CFU/ml after infection of cultures with each phage (LiMN4L, LiMN4p and LiMN17) repeatedly at 3 h (first infection), 5 h (first re-infection) and 7.5 h (second re-infection) (Fig. 5.7). All cells of high count culture of exponential phase *L. monocytogenes* 19CO9 were sensitive to three phages following re-infection of the initial high count cultures with each phage, resulting in < 1 CFU/ml survivors. Therefore, all cells of a high count culture were phage sensitive or cells remained below < 10^{-8} CFU/ml. In another previous experiment, total cells of late exponential phase low count culture were lysed by phage in 30 min. Phage sensitivity of cells is a useful parameter in modeling of the cell lysis kinetics.

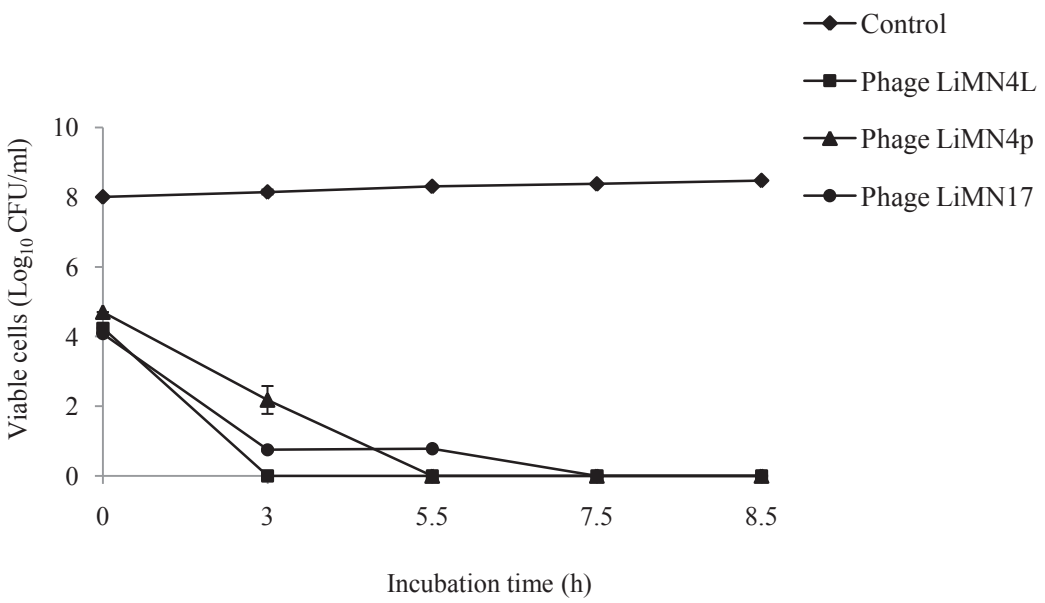

Figure 5.7 Viable counts of exponential phase *L. monocytogenes* 19CO9 during re-infection with phages LiMN4L, LiMN4p and LiMN17 (≈9 $\log_{10}$ PFU/ml) separately compared to uninfected control culture trypticase soy broth at 15±1 °C. Data represent means of duplicate from one experiment. Detection limit = 1 CFU/ml.

5.3.6 Emergence of phage-resistant *L. monocytogenes* 19CO9 cells over 48 h

The initial viable counts (6.8 log units) of *L. monocytogenes* 19CO9 deceased to ≈1-2 log units after exposure to phages for 48 h (Fig. 5.8). The viable counts of phage infected cultures reduced further to a non-detectable level (< 1 $\log_{10}$ CFU/ml) when re-infected with each phage separately for 2 h exposure time (Fig. 5.8). The results showed that resistant cells did not emerge within 48 h or occurred at a very low level (< 10^{-6} CFU/ml) compared to the initial host cell counts. Similarly, emergence of phage-insensitive cells of *L. monocytogenes* has not been reported in many phage challenge experiments (Carlton et al., 2005; Guenther et al., 2009). Nevertheless, phage insensitive colonies of *L. monocytogenes* 103/2005 were recovered in cheese treated with lytic phage A511 (≈3x10^8 PFU/cm^2) and ripened for 22 days (Guenther and Loessner, 2011). The emergence of bacteriophage insensitive mutants (BIMs) may be due to adaptations including a mutation of the binding cell wall receptors and restriction modification of bacteria (Enikeeva et al., 2010; Guenther and Loessner, 2011). The use of different biocidal agents on rotational basis or use of a phage cocktail can avoid or

minimize the emergence of BIMs. (Abuladze, et al., 2008; Carlton, et al., 2005; Hagens and Loessner, 2010; Roy, et al., 1993). Each phage of a certain cocktail should have different receptor-specificities for cell wall receptors of targeted bacteria (Tanji et al., 2004). Three phages should also be investigated whether cell wall binding legends of phages are different.

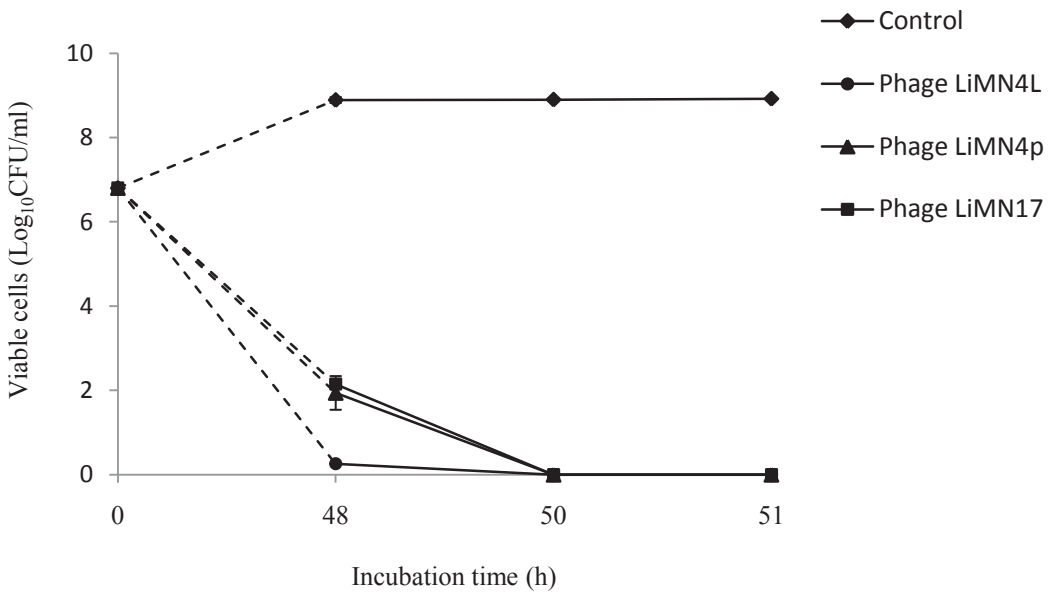

Figure 5.8 Viable counts of *L. monocytogenes* 19CO9 after exposure to phages LiMN4L, LiMN4p and LiMN17 (≈9 $\log_{10}$ PFU/ml) separately in trypticase soy broth at 15±1 ^{c}o for 48 h and after re-infection with three phages. Data represent means of triplicate from one experiment. Detection limit = 1 CFU/ml.

5.3.7 Presence of GLcNAc in the cell wall of *L. monocytogenes*

The cell wall GLcNAc, which serve as binding sites for some listeriaphages, can be stained with WGA. Seven *L. monocytogenes* strains were investigated with a WGA binding assay (Table 5.1). *L. monocytogenes* 19CO9 cells stained with fluorescently labeled WGA are shown in Figure 5.9. The WGA was bound to the cell wall of *L. monocytogenes* 19CO9, 19EO3, 18DO5, 19DO9 and 18AO1 strains belonging to 1/2a,

3a, 3b and 7. Therefore, these strains contained cell wall GLcNAc moieties. The GLcNAc has been shown to serve as binding receptors for phages A511 and P35.

The cell wall of remaining the three strains (19DO3, SMAC91 and 18DO7) did not stain with GLcNAc moieties (Table 5.1). Inability to bind WGA on theses strains maybe due to following reasons. Cell wall GLcNAc of *L. monocytogenes* 19DO3 strain may not be accessible to binding with extraneous WGA as GLcNAc may be integrated and stereoscopically hindered as has been reported for some strains of serotype 4b, 4d or 4e (Eugster, et al., 2011). Strains SMAC91 and 18DO7 which belong to 1/2a and 1/2b, 3b or 7 may not contain cell wall GLcNAc. However, the three phages were able to adsorb on all seven *Listeria* strains (unpublished data). Therefore, the present results suggest that in addition to the GLcNAc, other carbohydrate moieties such as rhamnose present on cell wall teichoic acid chains or the cell wall polypeptidoglycan layer itself may act as binding receptors for phages phages LiMN4L, LiMN4p and LiMN17 (Wendlinger et al., 1996). Therefore, the exact cell wall receptors types for these three phages need to be investigated in further studies.

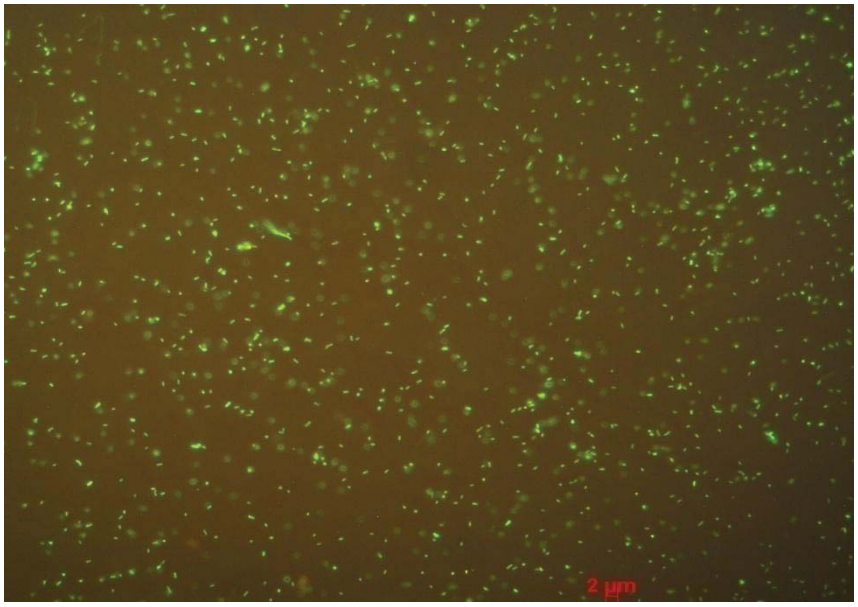

Figure 5.9 *L. monocytogenes* 19CO9 cells stained with fluorescently labelled wheat germ agglutinin (Alexa Fluor 488 WGA).

Table 5.1 *L. monocytogenes* strains assayed with fluorescently labelled wheat germ agglutinin

L. monocytogenes strain	Serotype	Phage adsorption[¶]			GLcNAc on cell wall[‡]
		LiMN4L	LiMN4p	LiMN17	
19CO9	1/2a or 3a	+	+	+	+
19DO3	4b,4d or 4e	+	+	+	–
19EO3	1/2a or 3a	+	+	+	+
18DO5[§]	1/2b,3b or 7	+	+	+	+
18DO7[§]	1/2b,3b or 7	+	+	+	–
SMAC91	1/2a	+	+	+	–
19DO9	1/2a or 3a	+	+	+	+
18AO1	1/2a or 3a	+	+	+	+

[‡], Fluorescently labeled Wheat germ agglutinin (WGA, Alexa Fluor 488 WGA) binds on cell wall (Eugster *et al*. 2011). (+), Assay positive; (–), assay negative

[¶], High adsorption rate constants (≈10^9 per ml/min, unpublished data)

[§], Low adsorption rate constants (10^{-11} - 10^{-10} per ml/min, unpublished data)

5.3.8 Conclusion

Three phages (LiMN4L, LiMN4p and LiMN17) could be used to control a contamination of *L. monocytogenes* strains injured by heat or stressed by starvation and salt which are common stress factors likely to be encountered in seafood processing plant environments. The results suggest the need for further investigation in phage-susceptibility of *L. monocytogenes* affected by the stress factors studied and other stresses that may be encountered in processing plants. *L. monocytogenes* strain 19CO9 did not show phage-resistance in exponential phase high count populations and when exposed to phage for 48 h. The present investigations speculate that the three phages may bind to undefined cell wall receptors of *L. monocytogenes* and that needs to be confirmed by further studies.

Authors' contributions to the manuscript

The experiments were planned and conducted, data were analyzed and the first draft of the manuscript was prepared by GJGA. SHF, LM, CDC, BMDM and ANM supervised and provided advice on interpretation of results and discussion and improving the writing of overall manuscript.

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Chapter 6: Effectiveness of phages in the decontamination of *Listeria monocytogenes* adhered to clean stainless steel, stainless steel coated with fish protein, and as a biofilm

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Abstract

L. monocytogenes is a food-borne pathogen which causes listeriosis and is difficult to eradicate from seafood processing environments and therefore, more effective control methods need to be developed. This study investigated the effectiveness of three bacteriophages (LiMN4L, LiMN4p and LiMN17), individually or as a three-phage cocktail at ≈9 $\log_{10}$ PFU/ml, in the lysis of three seafood-borne *L. monocytogenes* strains (19CO9, 19DO3 and 19EO3) that were adhered on a conditioned layer formed by soluble fish protein broth on stainless steel coupons (FBSSC), on a clean stainless steel coupons (SSC), in seven-day biofilm, and dislodged biofilm cells at 15±1 °C. The phage cocktail reduced the cells adhered on FBSSC and SSC (≈3.8-4.5 and 4.6-5.4 $\log_{10}$ CFU/cm^2, respectively), to less than detectable levels after ≈75 min (detection limit <0.9 $\log_{10}$ CFU/cm^2). Single phage treatments (LiMN4L, LiMN4p or LiMN17) decreased cells on both surfaces by ≈3-4.5 log units, respectively. One-hour phage treatments (LiMN4p, LiMN4L and a cocktail) in three consecutive repeat applications resulted in decrease of seven-day biofilms (≈4 $\log_{10}$ CFU/cm^2) of *L. monocytogenes* by ≈2-3 log units. Single phage treatments reduced dislodged biofilm cells of each *L. monocytogenes* strain by ≈5 $\log_{10}$ CFU/ml in 1 h. The three phages were effective in controlling *L. monocytogenes* strains on stainless either clean or soiled with fish proteins as is likely to occur in seafood processing environments. Phages were more effective on biofilm cells dislodged from the surface compared with undisturbed biofilm cells. Therefore, for short-term phage treatments of biofilm it should be considered some disruption of the biofilm cells from the surface are recommended prior phage treatment.

Key words: *Listeria* phages, biofilms, low count cells, stainless steel, low temperature

6.1 Introduction

The biocidal effectiveness of lytic natural bacteriophages (phages) against food-borne pathogenic bacteria has been widely reported on various foods and food contact surfaces [29, 31, 34, 38, 47]. The use of phages for the control of pathogens in the food industry has advantages as the phages kill only the target pathogen, do not alter sensory characteristics of food and are non-toxic and biodegradable [11, 12]. Commercial phage products intended for use in the food industry [40, 49] have been accepted by international regulatory organizations such as the Food and Drug Administration (FDA) and Environmental Protection Agency (EPA) and by food standard agencies in the United States of America, Switzerland, Australia and New Zealand [24, 26, 31, 49, 62].

L. monocytogenes is a food-borne pathogen adapted to survive and grow in a variety of environments, including the seafood processing industry, where food is harvested, processed and stored [3, 4, 17, 28, 32, 50, 65, 68]. Contaminated seafood has been linked to several outbreaks of listeriosis [9, 22, 32, 64] resulting in a zero tolerance policy for *L. monocytogenes* in ready-to-eat (RTE) foods including processed mussel products [27, 41]. Raw seafood can be a source of *L. monocytogenes* contamination of processing environments [21, 39]. *L. monocytogenes* colonizes and becomes persistent due to the ability to grow at low temperatures and form biofilms [15, 52, 54, 55]. This pathogen is difficult to eliminate from processing environments due to the development of stress hardening [45, 60], resistance against regular chemical disinfectants and protection from the biofilm matrix [25] contributing to its establishment in food processing lines, which may lead to the contamination of final products [17, 65].

Investigations on alternate decontaminating agents have demonstrated that phages control *L. monocytogenes* by destroying biofilm cells or preventing cell attachment on abiotic surfaces [36, 56]. ListShield^{TM}, a cocktail of six *Listeria* phages, has been approved by the EPA to control *L. monocytogenes* on food processing contact surfaces [62]. However, the efficacy of ListShield^{TM} in the decontamination of abiotic surfaces has not yet been reported. Phage P100 of Micreos Food Safety [49] has also been shown to decrease biofilms [51, 61]. Despite many reports on the effectiveness of phages, phage-based control of biofilms has not yet been fully developed [63]. As the efficacy of disinfectants is affected by the presence of soils, hard water and temperature [17, 30], the lytic activity of phages varies depending upon the conditions of the environment [33, 34]. This study investigated the biocidal effect of three novel phages

at low temperature against three *L. monocytogenes* strains adhered on stainless steel surfaces (clean and conditioned with fish proteins) [7]. Dislodged *L. monocytogenes* biofilm cells were also challenged with the phages to compare efficacy with treatment of undisturbed biofilms.

6.2 Materials and Methods

6.2.1 *L. monocytogenes* strains and *Listeria* phages

L. monocytogenes strains 19CO9 (serotype 1/2a or 3a), 19DO3 (serotype 1/2a or 3a) and 19EO3 (serotype 4b, 4d or 4e) [18] were used in this study. *L. monocytogenes* strains were maintained and sub-cultured monthly on trypticase soy agar (TSA, Difco, Sparks, MD, USA) at 4 °C and used to prepare broth cultures for the experiments. *Listeria* phages LiMN4L, LiMN4p and LiMN17, isolated from seafood processing environments in New Zealand, were selected for this study. Using *L. monocytogenes* 19CO9, three phages were propagated individually at 25±1 °C. Phage lysates were prepared using the TSA double layer agar (DLA) method [59]. The phage lysate was centrifuged at 8,000 x *g* for 20 min, bacteria lysed with $CHCl_3$ (Analytical Grade, Merck, Darmstadt, Germany) overnight, centrifuged at 8,000 x *g* for 20 min, and ultracentrifuged at 25,000 x *g* (SS-34 rotor, Sorvall RC6+ Centrifuge, Sorvall, Frankfurt, Germany) at 4 °C for 6 h [2]. The phage pellet was re-suspended in 1 ml of phosphate buffered saline (PBS) [8 mM Na_2HPO_4 (Merck), 1 mM $NaH_2PO_4.H_2O$ (Merck), and 145 mM NaCl (Merck); pH 7.5], stored at 4 °C in the dark. The phage titre was assayed by the DLA method [42] using saline-magnesium buffer (SM) [100 mM NaCl, 50 mM Tris-HCl (Sigma-Aldrich, Steinem, Germany), 8 mM of $MgSO_4$ (Merck), 0.001% (w/v) of gelatin solution (LabChem, NSW, Australia) at pH 7.5] as diluents and TSA. The phages were used in experiments either individually or as a cocktail (three phages). Each phage treatment suspension contained approximately 9 $\log_{10}$ PFU/ml.

6.2.2 Test surfaces

Stainless steel coupons: Stainless steel (Grade 304-2B) coupons (SSC) measuring 1 x 1 x 0.09 cm^3 were cleaned by soaking in acetone for 1 h to dissolve any greasy substances, air dried in a fume hood for 30 min, soaked in neutral detergent (Sunlight,

Pental products Pty Ltd, Australia) for 30 min, washed five times with potable water (100 coupons in 1 l), followed by sonication (70 W, 43 kHz) in MilliQ water (100 coupons in 1 l) for 30 min, then rinsed in MilliQ water (100 coupons in 1 l). The clean and dried coupons were sterilized by autoclaving at 121 °C for 15 min.

Fish broth conditioning layer: A fish protein broth was prepared using the method given by Bernbom *et al.* [7]. Clear fish extract was obtained by boiling fresh cod fish cubes of ≈1 cm^3 in water (1 kg fish/500 ml MilliQ water) for 5 min, passing fish liquor through a strainer and then filtering the liquor through a standard coffee filter (Melitta Aroma Coffee Filter, Melitta Group, Germany). Fish broth was prepared by dissolving salts in the boiled clear fish extract to contain 0.056 M KH_2PO_4 (Merck) and 0.44 M HK_2PO_4 (Merck) and adjusting the pH to 6.5 with 0.1 M NaOH and HCl. The fish broth was sterilized at 100 °C (boiled) for 30 min. The batch of fish broth was used in experiments within one month of preparation while stored at 4 °C in the dark [7]. The fish broth based conditioned layer on stainless steel coupons (FBSSC) was formed by dipping the coupons in the fish broth [7] at 15±1 °C for 1.5 h, followed by rinsing three times in 20 ml sterilized potable water. The FBSSC were prepared immediately before exposing to *L. monocytogenes* for cell attachment.

6.2.3 Attachment of *L. monocytogenes* on surfaces

Exponential phase cultures of *L. monocytogenes* strains 19CO9, 19DO3 and 19EO3, grown individually in trypticase soy broth (TSB; Difco) at 15±1 °C for 48 h were centrifuged at 8,000 x *g* (4 °C) for 10 min and re-suspended in 1/10 (v/v) TSB (pH 7.3) separately to contain ≈8 $\log_{10}$ CFU/ml (A_{600nm}, ≈0.10). A suspension containing all three strains was also prepared by mixing equal volumes of each strain. Initial cell counts of all four cell suspensions were enumerated by the pour plate method using TSA. Two batches of FBSSC (n=28) and SSC (n=28) were used for each strain. The coupons were immersed in the cell suspension at a ratio of one coupon to 1 ml of cell suspension in Petri dishes (cell adhesion bath) and incubated at 15±1 °C for 1.5 h. After incubation, the treated coupons were removed from the bacterial suspensions by carefully holding near the edge of coupons using sterile forceps, draining by touching the edge of a coupon on sterile blotting paper and then rinsing in 20 ml sterile potable water (three times) to remove any unattached cells from the test surfaces. A batch of coupons (n=4) was analyzed using the method described below to enumerate the cells adhered to the coupons. The remaining coupons (n=24) were placed in closed plastic tubes positioned

on a ≈30 °angle containing ≈0.5 ml sterile water to keep moist. The tubes were incubated at 15±1 °C for 5 h to stabilize the adhered cells. After incubation, the coupons were rinsed with sterile tap water to remove loosely attached cells. A batch of coupons (n=4) was analyzed at this step for the surviving cells after incubation for 5 h as described below and the remaining coupons were used for the next stages of the experiment.

6.2.4 Phage-lysis of *L. monocytogenes* cells adhered on surfaces

The coupons with adhered cells (n=20) pertaining to one strain were subject to four phage treatments (LiMN4L, LiMN4p, LiMN17 and cocktail). Each treatment consisted of four coupons (n=4) and each test coupon was immersed in 1 ml of phage suspension in a Bijou bottle and incubated at 15±1 °C for 15 min. The phage suspension was then removed by pipetting and coupons with cells were incubated for a further 1 h at 15±1 °C. Four coupons (n=4), which served as controls for each treatment, were immersed in PBS and incubated under the same conditions. Viable cell counts on both control and phage-infected surfaces were enumerated after dislodgment from the surface (Table 6.2).

6.2.5 Enumeration of cells adhered to surfaces

Each phage treated or control coupon was rinsed in 20 ml of sterile water three times and placed in a plastic tube containing 20 ml of chilled PBS and 10 g of glass beads (Ø =2 mm) and agitated on a vortex mixer (WISE Mix VM-10, Daihaw Scientific Company Ltd, Korea) at maximum speed for 2 min. Triplicate 1 ml aliquots of the dislodged cell culture were mixed with melted TSA (47±1 °C) and plated using the standard pour plate technique. Plates were incubated at 30±1 °C for 72 h and the colonies enumerated. The detection limit of the method was 0.9 $\log_{10}$ CFU/cm^2. The initial level of host cells adhered on the surfaces was expressed in terms of the specific adhesion (SA) value (Table 6.1).

6.2.6 Preparation of seven-day old *L. monocytogenes* biofilm on stainless steel coupon

Cultures of *L. monocytogenes* 19CO9, 19DO3 and 19EO3 were grown separately in TSB at 15±1 °C for 48 h, centrifuged at 8,000 x *g* at 4 °C for 10 min and re-suspended

in TSB. The cell densities were adjusted to ≈8 $\log_{10}$ CFU/ml level (A_{600nm}, ≈0.10) using TSB. A mixed cell suspension was prepared by mixing equal volumes of three *L. monocytogenes* strains to give a total concentration of ≈8 $\log_{10}$ CFU/ml level (A_{600nm}, ≈0.10). The SSC were immersed in the cell suspension at a ratio of one SSC to 1 ml culture in a Petri dish and incubated at 15±1 °C for 24 h. The coupons contaminated with *L. monocytogenes* for 24 h were then incubated for a further seven days, while replacing the liquid phase culture with 1/10 strength TSB every two days [61]. The loosely attached cells on the seven-day old biofilm were dislodged by rinsing the coupons in 3 x 20 ml aliquots of sterile potable water.

6.2.7 Infection of seven-day old biofilm with *Listeria* phages

Seven-day old biofilms were re-infected with phages (LiMN4L, LiMN4p or cocktail) at 15±1 °C over three consecutive 1 h cycles. A batch of four coupons was analyzed separately for both control and phage-infected biofilms at the end of each cycle. Briefly, in the first round of infection, 20 coupons were treated with phage by immersing in phage suspension (one coupon/1 ml) while four coupons were treated with PBS as controls. The infected and control coupons were incubated for 1 h, after which the coupons were rinsed and blotted as described in the cell adhesion experiment. At the end of the first round of infection, four treated coupons and the four control coupons were analyzed for viable counts of *L. monocytogenes*. In the second cycle, 12 out of the 16 remaining coupons from the first infection, were re-infected with phages and the other four (control) coupons were immersed in PBS. Four of the infected and the control coupons were assessed for viable *L. monocytogenes* at the end of the second cycle. In the third cycle, four of the eight remaining coupons from the second cycle were re-infected with phages and the other four (control) were immersed PBS. The viable biofilm cells were enumerated at the end of the third infection also. The coupons were analyzed for the viable *L. monocytogenes* following the same protocol described for the cell adhesion experiment except the coupons with biofilm were agitated for 4 min on the vortex mixer. Each coupon represented one replicate.

6.2.8 Preparation of dislodged seven-day old biofilm cells

About 5 g of stainless steel fibres (Product code 53A, Grade 434, kindly provided by SIFA Pty Ltd, Sydney, Australia) cleaned and sterilized as above for SSC, were immersed in 50 ml broth cultures of each *L. monocytogenes* strain (19CO9, 19DO3 and

19EO3) prepared as per the previous biofilm experiment. The cells were allowed to attach and form biofilm on steel fibres (Ben Somerton and Steve Flint, personal communication) at 15±1 °C for 24 h followed by incubation for seven days statically, while replacing medium with 1/10 TSB every two days. The biofilms grown on the stainless steel fibers were rinsed with 100 ml sterile tap water five times to remove loosely attached cells. The biofilms cells were dislodged from the stainless steel fibres by shaking by hand for five minutes in tubes containing 20 ml of PBS and 30 g of glass beads (Ø = 2 mm). Suspended biofilm cells in PBS were investigated for phage-lysis.

6.2.9 Phage-lysis of dislodged seven-day old biofilm cells

The dislodged biofilm cells dislodged were washed twice in PBS by centrifuging at 5,000 x *g* at 4 °C for 10 min and re-suspending in equal volumes of PBS. The dislodged biofilm cells of each *L. monocytogenes* strain were infected with three phages (LiMN4L, LiMN4p and LiMN17) separately at a titre of ≈9 $\log_{10}$ PFU/ml in PBS at 15±1 °C for 1 h. The control biofilm cells were incubated in PBS under the same conditions. One ml aliquots of phage-infected or control biofilm cell cultures were pour plated by mixing with melted TSA (47±1 °C) (detection limit = 1 CFU/ml). Triplicate plates were prepared per treatment and incubated at 30±1 °C for 72 h prior to colony enumeration.

6.2.10 Fluorescence microscopy of cells on surfaces

The coupons with adhered cells and biofilms were stained with sterile 0.01% (w/v) Acridine Orange (BDH, Chemical Ltd, Poole, England) at room temperature (22±1 °C) for 5-10 min in the dark [13], rinsed in sterile MilliQ water and blotted on Whitman paper ™. The stained coupons were observed under fluorescence microscopy (Axiom Star Plus, Transmitted Light Microscope, Carl Zeiss, and Deutschland, Germany) using excitation (450-490 nm) and emission (515 nm) filters (Filter set 9, Carl Zeiss).

6.2.11 Data analysis

The means and standard errors of viable cell counts were calculated using Microsoft Office Excel 2007 (Microsoft Office, Washington, and USA). Mean cell counts of treatments were compared by General Linear Model and any significant differences between the treatment means were separated by the Least Significant Difference (LSD) at $p < 0.05$ using SAS version 9.1 for Windows (SAS Institute, Inc., Cary, North Carolina, USA).

6.3 Results

6.3.1 Adhesion of *L. monocytogenes* strains to FBSSC and SSC

L. monocytogenes cells adhered on fish protein-conditioned and clean stainless steel surfaces were evaluated. Fewer cells adhered onto the FBSSC ($p < 0.05$) than the SSC with a difference of ≈1.4, 1.7, 0.8 and 1.4 $\log_{10}$ CFU/cm^2 for *L. monocytogenes* 19CO9, 19DO3, 19EO3 and mix of the three *Listeria* strains, respectively (Table 6.1). From the SA values, strain 19DO3 showed a significantly ($p < 0.05$) lower adhesion affinity (SA of -5.0) on the FBSSC compared with the adhesion of strains 19CO9 and 19EO3 (SA of -4.7 and -4.8, respectively) (Table 6.1). *L. monocytogenes* 19EO3 had the lowest adhesion affinity ($p < 0.05$) on SSC (-4.0) compared with strains 19CO9 and 19DO3 which showed high SA (-3.3) on SSC (Table 6.1). Fluorescence microscopy also showed adhesion of fewer cells of the three strain mix on the FBSSC compared to the high cell counts adhered on the SSC (Figs. 6.1a-b). Similar observations were found for the attachment of the individual *L. monocytogenes* cultures (results not shown).

Table 6.1 Initial counts (mean ± standard error) of *L. monocytogenes* strains in 1/10 (v/v) trypticase soy broth (cell adhesion bath), on the surfaces of fish broth conditioned stainless steel coupon (FBSSC) and clean stainless steel coupon (SSC) after immersion in cell bath at 15±1 °C for 1.5 h

Strain cultures	Initial counts of cell adhesion bath (Log_{10} CFU/ml)	FBSSC		SSC	
		Counts adhered Log_{10} (CFU/ml)	Specific adhesion	Counts adhered Log_{10} (CFU/ml)	Specific adhesion
19CO9	8.5 ± 0.00	3.8 ± 0.03	-4.7 ^{cod} ± 0.03	5.2 ± 0.02	-3.3^{a} ± 0.03
19DO3	8.9 ± 0.01	3.8 ± 0.01	-5.0 ^{e} ± 0.02	5.5 ± 0.05	-3.3^{a} ± 0.05
19EO3	8.6 ± 0.01	3.8 ± 0.04	-4.8 ^{d} ± 0.05	4.6 ± 0.03	-4.0^{b} ± 0.04
Three strain mix	8.5 ± 0.03	3.9 ± 0.05	-4.6 ^{c} ± 0.06	5.3 ± 0.10	-3.2^{a}± 0.11

Different superscript letters (a-e) indicate significance differences of cell attachments across different host strains and between two surface types (n = 4, $p < 0.05$).

SA Specific adhesion-the ratio of cells adhered on surface (CFU/cm^2) to initial count of cell adhesion bath (CFU/ml) [7].

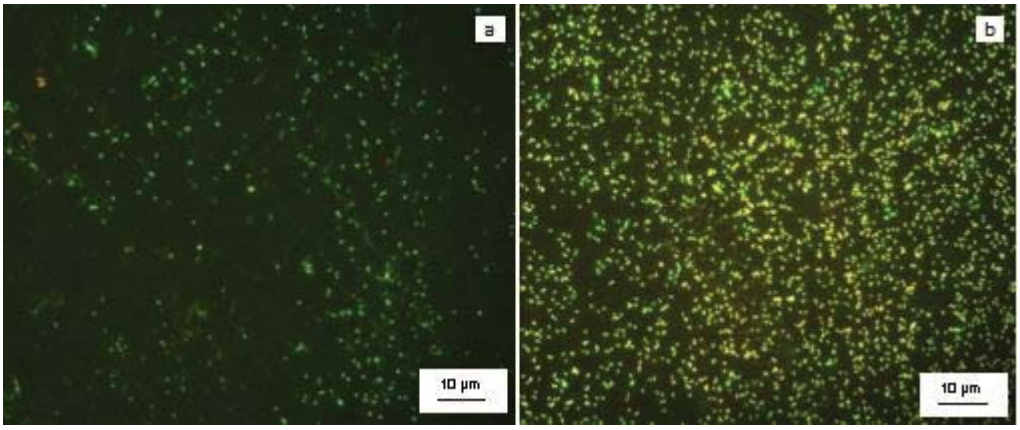

Figure 6.1 Fluorescent microscopy images of a cell-mix of three *L. monocytogenes* strains (19CO9, 19DO3 and 19EO3) cocktail adhered to stainless steel coupons after immersion in cell suspension containing ≈8 log_{10} CFU/ml in 1/10 (v/v) trypticase soy broth at 15±1 °C for 1.5 h. a: Fish broth conditioned surface on stainless steel, b: Clean stainless steel surface.

6.3.2 Phage-lysis of *L. monocytogenes* adhered to surfaces

The viable cells adhered to the coupons immediately after removing from cell adhesion bath, after 5 h incubation following adhesion and the controls of the phage infection experiment were not significantly different ($p > 0.05$) on either FBSSC or clean SSC (Table 6.2). The three-phage cocktail reduced the adhered cells on FBSSC and SSC, (≈3.8-4.5 and 4.6-5.4 $\log_{10}$ CFU/cm^2, respectively) to a non-detectable level in ≈75 min (Table 6.2). Phage LiMN4p also decreased the initial counts of *L. monocytogenes* 19EO3 on SSC to a non-detectable level (>4.5 log units). Other single-phage treatments decreased the cell counts on both FBSSC and SSC surfaces by ≈3-3.5 and 3.5-4.5 log units, respectively (Table 6.2).

Table 6.2 Cells adhered on fish broth based conditioned coupons (FBSSC) and stainless steel coupons (SSC) with respect to the mean cell counts on the control coupons after infection with phages LiMN4L, LiMN4p, LiMN17 and three-phage cocktail (9 $\log_{10}$ PFU/ml) by dipping in the phage suspensions for 15 min followed by incubation in air for 60 min at 15 °C

L. monocytogenes strain	Surface	Viable cell counts (mean ± standard error) on surfaces (Log_{10} CFU/cm^2)[a]					
		Adhesion level after 5 h at 15 °C	Control	LiMN4L	LiMN4p	LiMN17	Three-phage cocktail
19CO9	FBSSC	3.8 ± 0.02	3.8 ± 0.03	0.6 ± 0.35	0.6 ± 0.21	1.0 ± 0.13	<Dl[b]
	SSC	5.2 ± 0.03	5.2 ± 0.02	1.1 ± 0.11	0.5 ± 0.00	1.0 ± 0.39	<Dl[b]
19DO3	FBSSC	4.5 ± 0.05	4.5 ± 0.04	0.6 ± 0.17	0.7 ± 0.20	0.9 ± 0.00	<Dl[b]
	SSC	5.4 ± 0.06	5.4 ± 0.02	0.8 ± 0.26	1.0 ± 0.13	1.2 ± 0.15	<Dl[b]
19EO3	FBSSC	3.8 ± 0.04	3.8 ± 0.06	0.5 ± 0.00	0.8 ± 0	0.7 ± 0.17	<Dl[b]
	SSC	4.6 ± 0.06	4.6 ± 0.11	0.5 ± 0.00	<Dl[b]	0.6 ± 0.21	<Dl[b]
Three strain mixture	FBSSC	3.9 ± 0.07	3.8 ± 0.06	0.6 ± 0.17	0.5 ± 0.00	0.8 ± 0.00	<Dl[b]
	SSC	5.3 ± 0.10	5.4 ± 0.14	0.8 ± 0.28	0.5 ± 0.00	0.8 ± 0.12	<Dl[b]

[a] Number of coupons = 4;

[b] Detection limit (0.9 $\log_{10}$ CFU/cm^2) for enumeration of viable counts

6.3.3 Phage-lysis of seven-day old biofilms on stainless steel

The counts of biofilms that were re-infected with three different phage applications (phage LiMN4L, LiMN4p and cocktail) separately compared to the initial control biofilms (≈4 $\log_{10}$ CFU/cm^2) are given in Figures 6.2a-c, respectively. The phage LiMN4L reduced cells by ≈0.5, 0.7 and 0.3 log units in the first, second and third cycles, respectively compared with the control biofilms decreasing the total biofilm cell count by ≈2 log units with most lysis occurring in the first and second phage applications ($p < 0.05$) (Fig. 6.2a). Phage LiMN4p reduced the cells by 1.8, 0.2 and 1 log units in first, second and third cycles, respectively with most lysis occurring in the first and third cycles ($p < 0.05$) resulting in a total cell reduction of ≈3 log units (Fig. 6.2b). The phage cocktail decreased cells by ≈1.1, 0.2 and 0.9 log units in first, second and third cycles, respectively with most reduction in the first and third cycles ($p < 0.05$) representing ≈2.4 log units reduction of total cells (Fig. 6.2c). The cells of control biofilms during the second and third stages also decreased ($p < 0.05$) (Fig. 6.2a-c). Three phage applications caused the gradual thinning of biofilms and representative images of biofilms treated with the phage cocktail over three treatment cycles are shown in Online Resource (ESM Fig. 6.1a-d).

6.3.4 Phage-lysis of dislodged seven-day old biofilm cells

The initial counts of the dislodged seven-day old biofilm cells of *L. monocytogenes* strains 19CO9, 19DO3 and 19EO3 were ≈5.3-5.5 $\log_{10}$ CFU/ml (Fig. 6.3). These counts were not significantly different ($p > 0.05$) from the counts of control biofilm cells (Fig. 6.3). Treatment with individual phages LiMN4L, LiMN4p and LiMN17 decreased the dislodged biofilm cell concentrations of *L. monocytogenes* 19DO3 and 19EO3 by >5 log units reaching a non-detectable level. Each of the three single phage treatments reduced the dislodged biofilm cells of 19CO9 strain by >4.5 log units (Fig. 6.3).

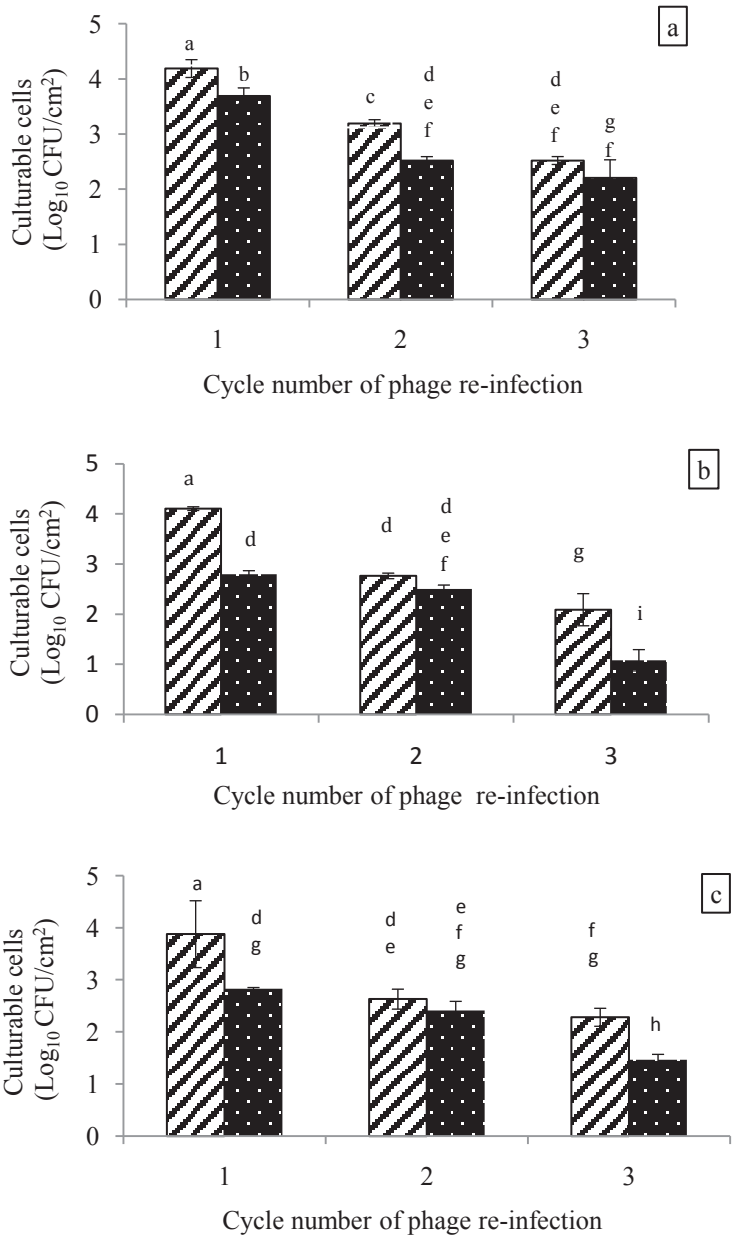

Figure 6.2 Counts of a three strain mix of a seven-day old *L. monocytogenes* (19CO9, 19DO3 and 19EO3) biofilm on stainless steel coupon after three phage applications: a, Phage LiMN4L; b, Phage LiMN4p; c, Cocktail (phages LiMN4L, LiMN4p and LiMN17). The phage treatment consisted of three consecutive repeat infection cycles on biofilm compared with the uninfected control. One infection cycle consisted of 1 h phage (≈9 $\log_{10}$ PFU/ml) exposure period at 15±1 °C followed a rinsing step. Biofilm: ▨, Control; ▩, Phage infected. Bars indicate standard error of mean counts (CFU/cm^2) of coupons (n=4). Different lowercase letters (a-i) on bars report significance across the treatments and controls during re-infection cycles (1-3) with three phage applications (a-c) at $p < 0.05$

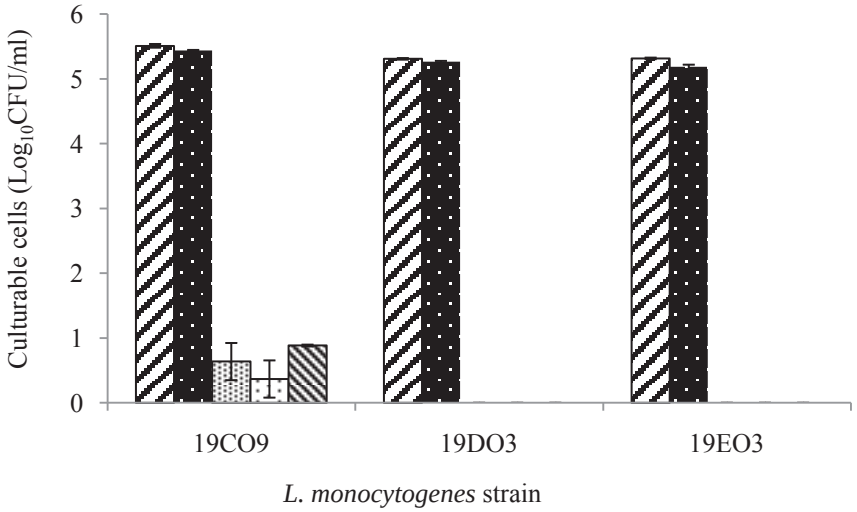

Figure 6.3 Counts of dislodged seven-day old biofilm cell cultures of individual *L. monocytogenes* strains (19CO9, 19DO3, 19EO3) after infection with individual phages (LiMN4L, LiMN4p, LiMN17) at ≈9 $\log_{10}$ PFU/ml in phosphate buffered saline at the 15±1 °C for 1 h. Dislodged biofilm counts: ▨, Initial; ▩, Control parallel to phage infected culture; ▩, LiMN4L; ▩, LiMN4p; ▩, LiMN17. Detection limit = 1 CFU/ml.

6.4 Discussion

In this experiment, the use of clean and fish protein conditioned stainless steel coupons at 15 °C represented clean and dirty surfaces likely to be found in seafood processing plants. The variations in the adhesion affinities of the strains could be assessed based on the SA values [7] even though cell adhesion levels were similar in FBSSC (Table 6.1). The reduced attachment to fish protein coated surfaces seen in this study has been previously reported for *L. monocytogenes* N53-1, *Vibrio anguillarum*, *Pseudomonas aeruginosa* and *Aeromonas salmonicida* [7, 8]. The factors that are intrinsic to the three *Listeria* strains may have resulted in varied adhesion affinities. The cell adhesion is determined by the net effect of the interactions between the bacteria cell wall and the abiotic surface [14, 23, 46, 52, 66], adhesion medium [19, 37], temperature and pH [6, 20, 35, 43, 66]. Alpha-tropomyosin (35 kDa) of cod fish broth (extract) which makes the surfaces more wettable (hydrophilic) has been attributed to the repulsion of bacteria adhesion [7, 8, 48, 67]. The cells adhered on surfaces were incubated for 5 h to

represent a possible delay in factory cleaning, however, this delay also might have resulted in a more permanent attachment from the initial transient attachment to the substrate [13, 66]. The cell counts did not vary significantly over 5 h and this may be attributed to the slow cell growth under the conditions used in the experiment. Therefore, the soluble fish proteins spilled over the working surfaces may reduce initial *Listeria* adhesion and colonization on processing surfaces for at least 5 h.

Similar to the highest efficacy achieved by the phage cocktail in this study, Roy et al. [56] reported a higher efficacy of a three-phage cocktail compared to monophage applications. Three phage strains individually and in a cocktail (8.5 $\log_{10}$ PFU/ml) decreased the initial counts (≈4-5 $\log_{10}$ CFU/ml) of two *L. monocytogenes* strains separately adhered onto stainless steel and polypropylene by 99.0-99.9%, when the *Listeria* contaminated surfaces were dipped into phage suspensions at 26 °C for 1 h [56]. Hibma et al. [36] showed that the attachment of L-form cells on SSC was prevented at 30 °C for 6 h using a suspension of ≈5 $\log_{10}$ CFU/ml treated with the specific phage (≈9 $\log_{10}$ PFU/ml). The ability of phages to decontaminate cells recently attached to clean and protein-contaminated surfaces (FBSSC), as demonstrated in this study, is important at an early stage to prevent colonization and the formation of biofilm [14, 53]. The treatment with bacteriophage should be relatively short for industrial applications. A study by Abuladze et al. [1] using a three-phage cocktail containing 10 $\log_{10}$ PFU/ml after about a 5 min phage exposure time, produced close to a 100% reduction of initial *E. coli* O157:H7 cells (3.5 x 10^3-10^4 CFU/6.25 cm^2) attached on gypsum surfaces that were pre-conditioned with 5% skim milk. The results from the present study suggest further investigation of phages for bacterial decontamination using phages at minimal exposure times and low doses.

Three *Listeria* strains in a mixed biofilm grown statically at 15 °C for seven days aimed to replicate the stagnant environment of seafood processing surfaces. The three phage treatments (Fig. 6.2a-c) significantly reduced the intact biofilm cells ($p < 0.05$) in the first cycle. This may be due to the presence of high numbers of viable cells near the surface of the biofilm. All three phage applications resulted in low cell reductions in the second cycle (Fig. 6.2a-c) and these may be explained by the protection of or low numbers of cells located deep within the biofilm matrix. During the third cycle, with phage LiMN4p and the three-phage cocktail, the reduction of cells ($p < 0.05$) (Fig. 6.2b-c) may be due to the infection of cells located deep in the biofilm due to the penetration of phages thorough voids in the biofilms [16] as well as lesions

formed by the lysis of cells in the biofilm in the preceding phage treatments. The number of cells infected in the preceding infections, decreasing in the control biofilms during the second and third cycles may be caused by the activity of residual phage trapped in the biofilm (Fig. 6.2a-c). The results suggest that phage LiMN4p alone and the three-phage cocktail may be more effective than phage LiMN4L in degrading the biofilm. Montanez-Izquierdo et al. [51] reported that treatment of a three-day biofilm of *L. monocytogenes* on stainless steel (initial counts of ≈4.3 $\log_{10}$ CFU/cm^2) with phage P100 at both 7 and 8 $\log_{10}$ PFU/ml, resulted in ≈3.4 log units reduction of biofilm cells in 8 h with no detectable viable cells after 48 h at 22 °C. These results suggest that prolonged exposure to phage is required to eradicate the biofilm. Sillankorva et al. [57] investigated phage (ΦIBB-PF7A) lysis of a seven-day old biofilm of *Pseudomonas fluorescens* formed on 1x1 cm^2 stainless steel under static conditions, replacing the medium at two day intervals. In this study [57], biofilms were infected using 3 ml phage (7 $\log_{10}$ PFU/ml) at 30 °C resulting in ≈2.5 and 3 log units of cell reduction after 2 and 4 h, respectively [57].

In our work the phages showed fewer efficacies in the lysis of intact biofilm cells compared with recently attached exponential phase planktonic cells (Fig 6.2 and Table 6.2). This may be attributed to the shielding effect of the biofilm matrix and/or phage resistance of biofilm cells deep in the biofilm matrix [23, 54]. Soni and Nannapaneni [61] reported that 24 h intervention using phage P100 (9 $\log_{10}$ PFU/ml) in two- and seven-day biofilms formed from a mix of five strains of *L. monocytogenes* on SSC (initial count of ≈7 and 6.6 $\log_{10}$ CFU/cm^2, respectively) resulted in the decrease of the biofilm cells by ≈5.4 and 3.5 units, respectively at 22 °C. The results suggest that the efficacy of phage-lysis is affected by the age of the biofilm. Pan et al. [54] elaborated that 50 ppm of H_2O_2 decreased the dislodged cells of seven-day old *L. monocytogenes* biofilm efficiently by ≈2.1-2.9 $\log_{10}$ CFU/cm^2 in 60 sec compared with insignificant cell lysis (<0.3 $\log_{10}$ CFU/cm^2) of the undisturbed biofilm treated with 100 ppm H_2O_2. These findings indicate that biofilm matrix limits the treatment of bioflm with both chemical and phage treatments [51, 54, 57, 61]. The findings of the present study are also in agreement with previous reports since both dislodged biofilm cells and recently attached planktonic cells adhered on SSC (Table 6.2) were reduced similarly (>5 log units) by the phage treatments. In addition to cell lysis by phage infection, the biofilm matrix also can be degraded using engineered phages which bear exopolysaccharide (EPS) depolymerases [44, 63]. Lu and Collins [44] reported those phages reduced *E.*

coli biofilm by ≈0.82 log units more in 5 h than the level reduced by the control phage which lacks depolymerase enzyme. The level of biofilm degradation was assessed using culture methods and fluorescence microscopy.

The presence of viable but non-culturable cells in a biofilm has been reported [51] however; results of this study were based only on viable and cutlurable cells. Other techniques that have been used to determine the efficacy of phages on biofilms are absorbance assays which measure the level of reduction of total biofilm in plastics (polystyrene wells) [61], differentiation of biofilm cells *in situ* by using live and dead staining [51] and scanning electron microscopy [10]. Sillankivora et al. [58] studied the effectiveness of a phage (ΦIBB-PF7A) in controlling *P. fluorescens* in a seven-day old heterogeneous biofilm which contained *Staphylococcus lentus* as the non-specific host. The team reported an efficient decrease in the target bacterium along with a sloughing effect on a non-specific host. A reduction of both strains occurred using a two-phage cocktail (ΦIBB-PF7A and ΦIBB-SL58B) which infected both strains [58]. Heterogeneous biofilms are likely to exist in a seafood processing plant [5, 13]. Therefore, the three *Listeria* phages used in the present study also need to be investigated in the presence of other bacterial genera.

6.5 Conclusion

The three *Listeria* phages, used individually or as a cocktail, were effective in decontaminating stainless steel surfaces contaminated with seafood-borne strains of *L. monocytogenes* with or without fish broth coatings. Further research is needed to determine the minimum effective treatment time and phage concentration. It is noteworthy that fish proteins impaired the initial attachment of *L. monocytogenes* to stainless steel. Phages were less effective against intact biofilm cells than recently attached exponential phase cells of *L. monocytogenes* however, once dislodged from the matrix; the biofilm cells were sensitive to phage treatment. Additional treatment of biofilms may be needed to maximize the effect of phage on biofilms of *L. monocytogenes.*

Authors' contributions to the manuscript

The experiments were planned and conducted, data were analyzed and the first draft of the manuscript was prepared by GJGA. LR assisted in obtaining microscopic images. ANM, BMDM, CDC, LM, SHF, and supervised and provided advice on interpretation of results and discussion and improve the writing of overall manuscript. AGC assisted in ultracentrifuging of phage lysates and reviewed the manuscript.

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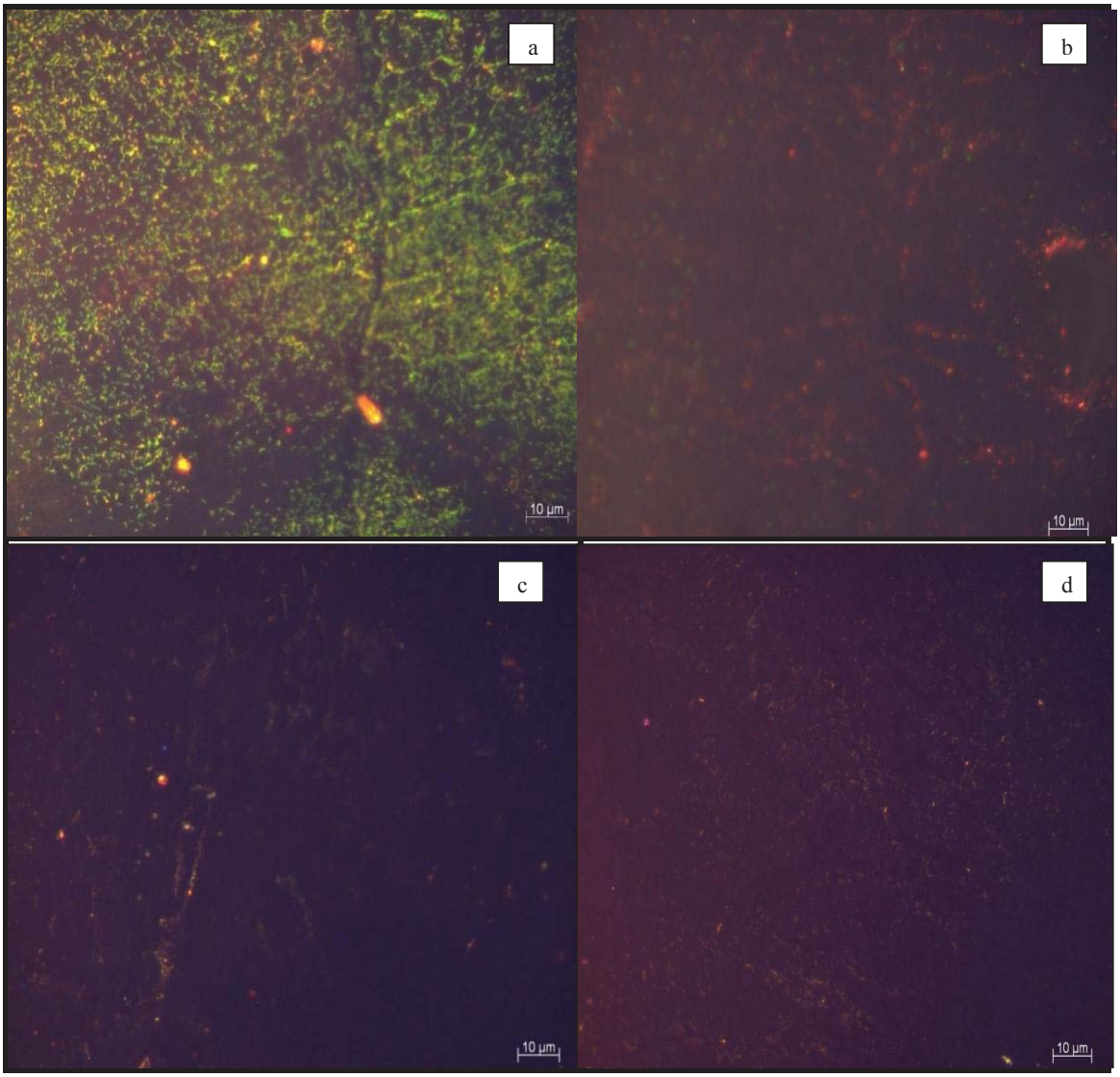
Electronic supplementary material (ESM)



ESM Figure 6.1. Degradation of seven-day biofilm of mixed cells of *L. monocytogenes* strains 19CO9, 19DO3 and 19EO3 followed application of phage cocktail (LiMN4L, LiMN4p, LiMN17) at 9 $\log_{10}$ PFU/ml repeatedly three times at 15±1 °C. a, Initial control biofilm; b, After first infection cycle; c, After second infection cycle; d, After third infection cycle.

Chapter 7: Bacteriophages for the control of low levels of *Listeria monocytogenes* in a simulated fish processing environment

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Abstract

L. monocytogenes causes listeriosis, a potentially fatal illness, and is capable of adapting to harsh environments and developing resistance against the disinfectants used in food processing plant. Natural lytic bacteriophages are an alternative to chemical disinfectants for the control of food-borne pathogens. This study investigated the efficacy of three *Listeria* phages (LiMN4L, LiMN4p and LiMN17) to control low counts of *L. monocytogenes* strains (19CO9, 19DO3 and 19EO3) on stainless steel coupons (SSC) and in a fish protein solution. The stability of phages was studied under the conditions likely to be found in fish processing plants. Cocktail of three phages at ≈5.8, 6.5 and 7.5 $\log_{10}$ PFU/cm^2 eliminated *Listeria* contamination (≈1.5-1.7 CFU/cm^2) in ≈15 min. Phages LiMN4L and LiMN4p survived at pH 4-10 while phage LiMN17 survived at pH 4-9 for 1 h. The three phages were stable under fluorescent light (487±30 lux) for 6 h, in a fish protein solution at 15 °C for 24 h, and in phosphate buffered saline at 4 °C for 12 months. Phages LiMN4L and LiMN17 decreased by ≈1 log unit while LiMN4p reduced by ≈3 log units in 100 ppm Quat (benzalkonium chloride) after 5 min. The infectivity of phages LiMN4L, LiMN4p and LiMN17 desiccated on SSC remained stable at 4 °C for 2 h and decreased by ≈2.0, 1.2 and 0.3 log units respectively at 15 °C after 24 h. The results indicated that three phages were efficient as decontaminating agent to control low counts of *L. monocytogenes* contamination under conditions similar to those found in fish processing plants.

Keywords: *Listeria monocytogenes*, phage, decontamination, desiccated, fluorescent light, stainless steel

7.1 Introduction

Food contact surfaces contaminated with pathogens are potential sources of cross contamination during food processing (Chmielewski and Frank, 2006; Rorvik et al., 2000; Tompkin, 2002). *L. monocytogenes* is a food-borne pathogen prevalent in seafood processing plants (Bremer et al., 2002; Cruz and Fletcher, 2011; Rorvik et al., 2000). This bacterium causes listeriosis, a severe human disease with about 20-30% mortality reported among pregnant women and infants, the elderly, and those with impaired immune systems (Abram et al., 2006; Donnelly, 2001; Mook et al., 2011; Schuchat et al., 1991; Swaminathan and Gerner-Smidt, 2007). In order to control food-borne listeriosis incidences, zero tolerance limits have been specified for all ready-to-eat (RTE) foods by the United States Food and Drug Administration (USFDA) agency (Klontz et al., 2008). A tolerance limit of <100 CFU/g of *L. monocytogenes* for RTE foods which do not support the growth of *L. monocytogenes* during storage is applicable in the European Union (Koutsoumanis and Angelidis, 2007).

L. monocytogenes is difficult to control due to its ability to grow at low temperatures, tolerance under extreme ambient conditions, persistence as biofilms and resistant to disinfectants, (Chan and Wiedmann, 2008; Folsom and Frank, 2006; Møretrø and Langsrud, 2004; Pan et al., 2006; Purkrtova et al., 2010). This is evident from the number of food recalls worldwide reported by all types of foods contaminated with *L. monocytogenes* including New Zealand (Crerar et al., 2011; Klontz et al., 2008; Wong et al., 2000). Contaminant bacteria are expected to be present at low levels on food contact surfaces in processing plants operating under good manufacturing practices (Hagens and Loessner, 2010). Low levels of pathogens such as *L. monocytogenes* are a concern due to their possible low infective doses and its ability to grow at refrigeration temperatures. Chemical disinfectants are corrosive at the most effective levels and their residues on contact surfaces may be toxic to consumers (Coffey et al., 2010; Sulakvelidze and Pasternack, 2010; Wirtanen and Salo, 2003). Therefore, there is interest in the development of alternative bacteriocidal methods (Goodridge and Bisha, 2011; Roy et al., 1993) including natural lytic bacteriophages (Coffey et al., 2010; Hagens and Loessner, 2010). These are attractive to consumers as natural control measures (Goodridge and Bisha, 2011; Leonard et al., 2010). The virulent bacteriophages are biologically safe to use, non-toxic and biodegradable (Carlton et al.,

2005; Hagens and Loessner, 2010; Sulakvelidze and Pasternack, 2010). Natural lytic bacteriophages that infect *L. monocytogenes*, *Salmonella* serotypes and *Escherichia coli* O157:H7 have been developed into commercial products, and some of these products have been approved for use in the food industry by the USFDA or are awaiting approval (FDA, 2006; Intralytix, 2013; Micreos Food Safety, 2013). Listex^{TM} containing *Listeria* phage P100 has been granted GRAS (generally recognized as safe) status by the USFDA (FDA, 2007; Micreos Food Safety, 2013).

Phage P100 at both 10^7 and 10^8 PFU/ml decreased three-day old *L. monocytogenes* biofilm containing initial counts of ≈4.3 $\log_{10}$ CFU/cm^2) on stainless steel by ≈3.4 log units at 22 °C in 8 h (Montanez-Izquierdo et al., 2010). Twenty-four hour infection of phage P100 with an initial dose of 9 $\log_{10}$ PFU/ml resulted in ≈5.4 and 3.5 log reductions of two- and seven-day old *L. monocytogenes* biofilms, respectively, that had initial count of ≈7 and 6.6 $\log_{10}$ CFU/cm^2, respectively, on stainless steel at 22 °C (Soni and Nannapaneni, 2010). A single phage or cocktail of three phages at ≈8.5 $\log_{10}$ PFU/ml reduced the *L. monocytogenes* cells (≈4-5 $\log_{10}$ CFU/ml) adhered on stainless steel and polypropylene surfaces by 99-99.9% at 26 °C in 1 h (Roy et al., 1993). *L. monocytogenes* cells (≈10^5 CFU/ml) were prevented from adhering on SSC for 6 h at 30 °C when a BRED phage (≈10^9 PFU/ml) was initially mixed with the cell suspension (Hibma et al., 1997).

In order to become effective decontaminating agents, a given phage needs to be investigated with respect to the environment in which it is to be used (Abad et al., 1994; Guenther et al., 2009; Hagens and Loessner, 2010). Stability of *Listeria* phages in different ambient conditions commonly found in seafood processing environments are not abundant. Bacteriophages and certain pathogenic virus are more resistant to quaternary ammonium compounds (QAC) compared with other anti-microbial chemicals such as sodium hypochlorite, ethanol, peracetic acid acid, glutaraldehyde, virkon (Solomon et al., 2009; Mercanti et al., 2011; Rode et al., 2011). Titres of bacteriophage MS2, Hepatitis A virus (HAV) and Feline calcivirus (FCV) were reduced by 0.63, 0.65, and 1.9 $\log_{10}$ PFU/ml respectively in 848 ppm quaternary ammonium salt after 10 min (Solomon et al., 2009). Rode et al. (2011) report that infectivity of phage Stx2 is reduced by about < 0.5 $\log_{10}$ PFU in 1% (w/v) QAC after 5 min indicating resistance to the QAC. Mercanti et al. (2011) compared the susceptibility of two phages of *Lactobacillus paracasei* against 0.25% (w/v) quaternary ammonium chloride.

Particles of Φ iLp84 were reduced by 99% in < 5 min while the titre of Φ iLp1308 decreased only 1 $\log_{10}$ PFU after 45 min of incubation at 25 °C.

Phage Stx2, bacteriophage carrying a Shiga toxin, is reported to withstand a range of pH 4-10 with a variation in titre of <1 $\log_{10}$ PFU for 120 min at room temperature (Rode et al., 2011). The stability of desiccated bacteriophage Φ*Xacm* 2004-16 that infect *Xanthomonas axonopodis* was investigated under the fluorescent light (Iriarte et al., 2007). These trials examined sensitivity to light with and without organic material, using a cycle consisting of 16 h exposure to fluorescent light and 8 h of darkness for 15 days. The investigators found that initial titre of ≈6 $\log_{10}$ PFU per micro well was completely diminished after 15 days where no organic material was present, whereas in the presence of organic material, the ≈2 log units reduction occurred after 60 days indicating a strong protective effect of the organic material. Phage Stx desiccated on stainless is susceptible to the desiccation reducing by ≈7 $\log_{10}$ PFU 20 °C after 24 h (Rode et al., 2011). Abad et al. (1994) demonstrated that varying survival profiles of different enteric virus desiccated on different hard surfaces (Aluminium, polystyrene, glazed tiles, latex paper, and cotton cloths) at 4 and 20 °C. Enteric adenovirus (ADV) and poliovirus (PV) showed the highest susceptibility (decrease of ≈1.5-4.3 $\log_{10}$PFU/cm^2) during drying on hard surfaces for 3-5 h. Hepatitis A virus (HAV) and human rotavirus (HRV) reduced by ≈0.1-1.6 $\log_{10}$ PFU/cm^2, ADV and PV which were desiccated on aluminum reduced by 3.5-4 $\log_{10}$ PFU/cm^2 after 24 h. HAV and HRV reduced by 0.5-2 $\log_{10}$ PFU/cm^2 at 20 °C after 24 h. All desiccated viruses survived better at 4 °C than at 20 °C. This study showed survival of desiccated phages was also affected by the relative humidity (Abad et al., 1994). These findings suggest that phage survival is varied across different phage strains with respect to ambient conditions.

The present study aimed to investigate the lysis efficacy of three *Listeria* phages against a low number (≈1.5 $\log_{10}$ CFU/ml) of *L. monocytogenes* cells adhered to stainless steel in the presence of fish proteins. The survival level of three novel phages was also evaluated under the conditions such as QAC-based disinfectant solutions, fish protein medium, different pH levels, artificial light and desiccation on stainless steel which are most likely encountered in its decontaminating efficacy and stability in storage of stocks.

7.2 Materials and methods

7.2.1 *L. monocytogenes* strains and *Listeria* phages

L. monocytogenes 19CO9 (serotype 1/2a or 3a), 19DO3 (serotype 1/2a or 3a) and 19EO3 (serotype 4b, 4d or 4e) isolated from seafood plant environments (Cruz and Fletcher, 2011) were used in this study. The experimental broth cultures were prepared using inocula obtained from monthly working cultures of *L. monocytogenes* maintained on trypticase soy agar (TSA, Difco, Sparks, MD) plates at 4 ^{o}C.

Listeria phages LiMN4L, LiMN4p and LiMN17 isolated from seafood processing environments in New Zealand were selected for this study. The stocks of phage lysate were prepared by individually propagating in *L. monocytogenes* 19CO9 (an indicator host) at 25±1 °C on double agar layer (DAL) in flasks (2 l) by scaling up the DLA protocol given by Sillankorva et al. (2004) using TSA [overlay of 0.4% (w/v) agar and base of 1% (w/v) agar]. The phage lysate was centrifuged at 8,000 x g for 20 min, bacteria lysed with $CHCl_3$ (Analytical Grade, Merck, Darmstadt, Germany) (≈0.25% v/v $CHCl_3$) by mixing well and incubating for overnight, and then centrifuged at 8,000 x g for 20 min. The clear lysate (50 ml portion) was ultra-centrifuged at 25,000 x g (SS-34 rotor, Sorvall RC6+ Centrifuge, Sorvall, Germany) at 4 ^{o}C (Ackermann, 2009) for 6 h and the pellet was suspended in 1 ml of phosphate buffered saline [PBS; 8 mM Na_2HPO_4 (Merck), 1 mM $NaH_2PO_4.H_2O$ (Merck), and 145 mM NaCl (Merck); pH 7.5] over 18 h in the dark at 4 °C. The phage stocks were stored in the dark at 4 °C. The phage titre was assessed by DLA method on agar plates (Kropinski et al., 2009) using saline magnesium medium (SM) [100 mM NaCl, 50 mM Tris-HCl (Sigma Aldrich, Steinem, Germany), 8 mM $MgSO_4$ (Merck), 0.001% (w/v) gelatin (LabChem, NSW, Australia) at pH 7.5] as the diluents, TSA [overlay of 0.4% (w/v) agar and base layer of 1.5% (w/v) agar] and *L. monocytogenes* 19CO9 (the indicator strain). The inoculated plates were incubated at 25±1 °C for 24 h and plaques were enumerated.

7.2.2 Preparation of stainless steel coupon (SSC)

Stainless steel (Grade 304-2B) coupons (1x1x 0.09 cm^3) (SSC) were cleaned by soaking in acetone (Analytical Grade, Merck) for 1 h to dissolve any greasy substances present, air dried in a fume hood for 30 min, soaked in neutral detergent (Sunlight, Pental products Pty Ltd, Australia) for 30 min, washed five times with potable water

(100 coupons per l), followed by sonication (35 kHz, Bandelin Sonorex Super RK 510 H ultrasonic bath, Berlin, Germnay) in MilliQ water (100 coupons per l) for 30 min, then rinsed two times in MilliQ water (100 coupons per l). The clean and dried coupons were sterilized by autoclaving at 121 °C for 15 min.

7.2.3 Fish broth (FB)

Fish broth was prepared as described by Bernbom et al. (2006). Fresh cod fish (*Gadus morhua*) cut into cubes (≈1 cm^3) were boiled in MilliQ water (1 kg fish/500 ml water) for 5 min and the fish extract was filtered using a strainer. The fish extract was boiled for another 5 min, cooled for 5 min at room temperature and filtered through a standard coffee filter (Melitta Aroma Coffee Filter, Melitta Group, Germany). The clear fish extract was buffered using dissolved KH_2PO_4 (Merck) and HK_2PO_4 (Merck) solutions to obtain a final molarity of 0.056 M and 0.44 M, respectively in the final FB mixture and then adjusted to pH 6.5 with 0.1 M NaOH and HCl. The FB was then sterilized by boiling (100 °C) for 30 min.

7.2.4 Phage-based lysis of low count cells on SSC

Late exponential phase *L. monocytogenes* 19CO9, 19DO3 and 19EO3 cultures grown in trypticase soy broth (TSB, Difco) at 15±1 °C for 72 h were centrifuged at 8,000 x g for 10 min, re-suspended in PBS. *L. monocytogenes* 19CO9 strain alone or three strain mix cells of ≈10^4-10^5 CFU/ml were suspended in 10% (v/v) FB solution and 2 µl of cell suspension was spread on SSC using a short bar (≈0.5 cm) glass spreader made up with a Pasteur pipette. The cells spread on the SSC were dried for 5-10 min in biohazard safety cabinet (Hera Safe 18, Heraeus safety cabinet-Class 2, Hanau, Germany). An aliquot (3 µl) of phage LiMN4p or a cocktail of three phages (LiMN4L, LiMN4p and LiMN17) at titres of ≈7.3, 8.3, 9.0 and 10.0 $\log_{10}$ PFU/ml were spread over the host cell smears on the SSC using a clean glass spreader while 3 µl of PBS was spread over the control surfaces. The coupons were incubated in a closed Petri dish at 15±1 °C for 0, 10 or 15 min and then the viable cells were dislodged by shaking each coupon in 5 ml of chilled PBS on a vortex mixer (WISE Mix VM-10, Daihaw Scientific company Ltd, Seoul, Korea) for 2 min. Sample aliquots (2 ml) were centrifuged at 8,000 x g for 10 min, the cell pellet was re-suspended in 1 ml of PBS and then 1 ml of suspension was pour plated using PALCAM agar (Oxoid Limited, Hampshire, United Kingdom) melted at 47±1 °C (detection limit = 0.39 $\log_{10}$ CFU/cm^2). Two plates were

inoculated per SSC. The plates were incubated at 35±1 °C for 48 h. Four replicate coupons were analysed per treatment. The treatments which resulted in reduction of cells to non-detectable levels were repeated and a total 5 ml volume of dislodged cell suspension filtered through sterile 0.45 µm filter (detection limit = 1 CFU/cm^2) in <2 min. The sample-filter was placed up-side down on PALCAM agar (Difco) plates and incubated at 35 °C for 72 h. Additionally, phage-treated and control coupons were incubated in *Listeria* Enrichment Broth (LEB) (Difco) at 30 °C for 72 h. The incubated enrichments were streaked on PALCAM agar plates and incubated at 35 °C for 72 h in order to observe growth of typical *L. monocytogenes* colonies.

7.2.5 Stability of *Listeria* phages under different ambient conditions

7.2.5.1 Stability of phages in QAC solution

The stability of phages LiMN4L, LiMN4p and LiMN17 was evaluated with two concentrations of commercial QAC disinfectant (Jasol Powerquat Blue, Auckland, New Zealand) which contains benzalkonium chloride as the active ingredient. Following the manufacturer's instructions, QAC solutions (100 and 200 ppm) were prepared using sterile potable water. Aliquots (100 µl) of individual phage suspension (≈9-10 $\log_{10}$ PFU/ml) were added to 10 ml of QAC disinfectant solutions pre-incubated at 15±1 ^{o}C, mixed well on a vortex mixer at maximum speed and incubated at 15±1 ^{o}C. At 5, 10 and 30 min intervals, 1 ml sample aliquots were removed and neutralized immediately by mixing with 9 ml neutralizing broth (Pan et al. 2006) containing 0.53% (w/v) Lecithin (ULTRALEC^{R} P Deoiled Lecithin, Product code 700851, Archer Daniels Midland Company, Illinois, USA), 3.75% (w/v) Tween 80 (Ajax Finechem Pty Ltd, Sydney, Australia), and 0.05% (w/v) KH_2PO_4 (Merck). The samples were further diluted with SM medium and surviving phage titres enumerated. One hundred µl aliquot samples were assayed for the phage titre by the DLA plaque assay on triplicate plates. Detection limit = 2 $\log_{10}$ PFU/ml. Two samples were assessed for each treatment.

7.2.5.2 Stability of phages in FB and PBS

Phages LiMN4L, LiMN4p and LiMN17 were assessed for their stability in FB and PBS at 15±1 °C for 24 h. Aliquots (100 µl) of phage suspension (≈9-10 $\log_{10}$ PFU/ml) were mixed well with 10 ml of FB and PBS that were pre-incubated at 15±1 ^{o}C. The phage suspensions were incubated at 15±1 °C and at 0, 0.5, 1, 2, 4, 6, and 24 h intervals, 100 µl sample aliquots were assayed for the phage titre by the DLA plaque

assay on triplicate plates. Detection limit = 1 $\log_{10}$ PFU/ml. Two samples were assessed in two separate experiments.

7.2.5.3 Stability of phages in culture media at different pH

Phages LiMN4L, LiMN4p and LiMN17 were investigated for pH stability in TSB. Aliquots (100 µl) of phage suspension (≈9-10 $\log_{10}$ PFU/ml in PBS) was mixed with 10 ml of TSB which had been adjusted to pH values of 3±0.1, 4±0.1, 5±0.1, 6±0.1, 7±0.1, 8±0.1, 9±0.1, 10±0.1, 11±0.1, and 12±0.1 with 0.1 M NaOH and HCl using a pH meter (Sartorius Basic pH Meter PB-20, Gottingen, Germany) and vortex mixer. Each phage suspension was incubated at room temperature (22±1 °C) with swirling at 50 rpm for 1 h, and then 100 µl samples were immediately diluted 1:100 using 10 ml of TSB (pH 7.3) in order to neutralize the pH. The surviving phage titres of the samples (100 µl) were assessed by the DLA plaque assay using ten-fold serial dilutions prepared using the SM medium on triplicate plates (detection limit = 3 $\log_{10}$ PFU/ml). Additionally, the surviving phages in the pH 3, 11, and 12 treatments were assayed by performing the DLA with undiluted samples immediately after the pH treatment. Two samples were assessed in two separate experiments.

7.2.5.4 Stability of phages in artificial light

Phages LiMN4L, LiMN4p and LiMN17 were assessed under two fluorescent light intensities: low intensity light (487±30 lux) that is normal light in working area of a laboratory and high intensity light (1710±30 lux) of a biohazard safety cabinet at 24±1 °C. The light intensity was measured using a light meter (Lutron LM-8000, 4-In-1 Professional Instrument, LT Lutron, Jakarta Barat, Indonesia). Two millilitres of phages suspended in PBS were transferred into thin-walled narrow glass vials (Ø, 1.5 cm and height 6 cm) and capped tightly. The vials containing phage were placed at ≈30° angle under the light. Samples (100 µl) were taken at 1 h intervals for 6 h and the phage titres were determined by the DLA agar plaque assay on triplicate plates. Two samples were assessed in one experiment and two independent experiments were performed for each phage.

7.2.5.5 Survival of phages desiccated on SSC

Phages LiMN4L, LiMN4p and LiMN17 were investigated in this experiment. Twenty microlitres of phage suspension (≈9 $\log_{10}$ PFU/ml) in PBS were spread on a SSC (1 cm^2) using a 10 µl tungsten inoculating loop and the SSC was dried at 24±1 °C for ≈2.5 h in the dark in a biohazard safety cabinet. The SSCs with phage adhered were

incubated at 15±1 ^{o}C in the dark in a closed Petri dish containing a wet cotton swab to maintain saturated humidity. The SSCs were sampled at 0, 2, and 24 h and the surviving phages on the SSC determined using the protocol of Abad et al. (1994) with some modifications. This involved immersing each coupon in 980 µl of 10% (w/v) meat extract (ME) (Merck) at pH 7.5, agitating (40 rpm) for 30 min at 22±1 ^{o}C and then mixing at maximum speed on a vortex mixer for 1 min. The titre of initial phage suspension at 15±1 ^{o}C served as the control and at each time point, 20 µl of phage suspension were mixed with 980 µl of ME extract containing fresh SSC and analyzed as described for the test coupons. The eluted phage in ME was further diluted with SM medium and the surviving phage titres were determined by the DLA plaque assay on duplicate plates. Four replicate test coupons were assessed at each time point for each phage per experiment.

7.2.5.6 Stability of phages during refrigerated storage

LiMN4L, LiMN4p and LiMN17 phage suspensions in PBS (pH 7.5) were separately stored in the dark at 4±2 °C. The surviving titres of each phage were assessed by analyzing four sample units (2 ml sample vials) from storage at least every three months for one year. The infectivity of phages was assayed using DLA method on duplicate plates.

7.2.6 Statistical analysis

The mean and standard error values of viable counts and plaque counts of treatments were calculated using MS Office Excel 2007 (MS Office, Washington, USA). The mean values of treatments were analyzed by one way ANOVA and significant differences between the treatment means were separated using the Least Significant Difference (LSD) at $P < 0.05$ using SAS version 9.1 for Windows (SAS Institute, Inc., Cary, N.C.).

7.3 Results

7.3.1 Phage-lysis of low counts of *L monocytogenes* adhered on SSC

Decontamination of low count *L. monocytogenes* on stainless steel surfaces by phage treatments at 15 °C was investigated. The SSC contaminated with *L.*

monocytogenes cells were treated with ≈4.8, 5.8, 6.5 and 7.5 $\log_{10}$ PFU/cm^2, respectively. At levels of 5.8, 6.5 and 7.5 $\log_{10}$ PFU/cm^2, phage LiMN4p alone and the phage cocktail lysed ≈1.5 $\log_{10}$ CFU/cm^2 of *L. monocytogenes* 19CO9 attached on the SSC to a non-detectable level in 15 min. Similarly, the phage cocktail at these three levels reduced the initial cell contamination (≈1.5-1.7 $\log_{10}$ CFU/cm^2) of a mixed culture of the three *L. monocytogenes* strains (19CO9, 19DO3 and 19EO3) to a non-detectable level (detection limit = 0.39 $\log_{10}$ CFU/cm^2) while individual phages at 4.5 PFU/cm^2 resulted in a 0.5 log unit decrease in 15 min (Fig. 7.1). The minimum exposure time of phage cocktail at 6.5 $\log_{10}$ PFU/cm^2 which resulted in complete decontamination of the three strain mixed culture was 15 min (Fig. 7.2). The complete decontamination of the surfaces by all phage treatments was confirmed since colonies were not formed from filtered dislodged cell suspensions and growth did not occur in LEB.

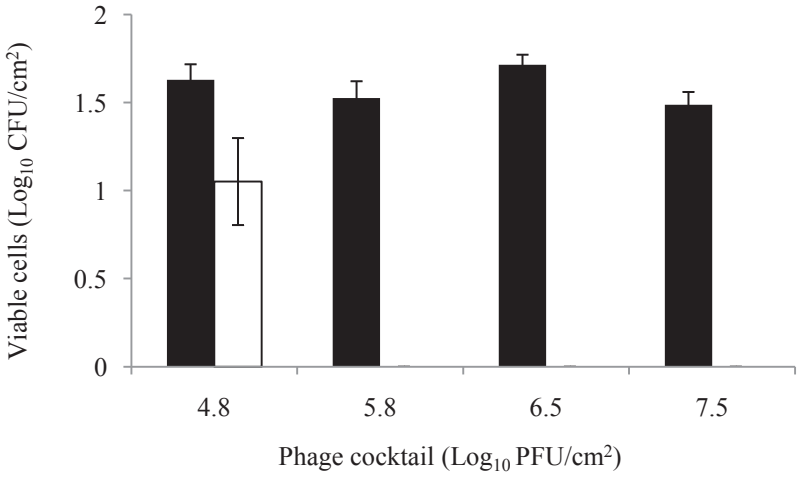

Figure 7.1 Viable counts of a mixture of three *L. monocytogenes* strains (19CO9, 19DO3 and 19EO3) adhered with 10% fish proteins broth on stainless steel at 15 °C for 15 min. ■, Control culture; □, Three-phage cocktail (LiMN4L, LiMN4p and LiMN17) at ≈4.8, 5.8, 6.5 and 7.5 $\log_{10}$ PFU/cm^2. Error bars indicate standard error of mean CFU/cm^2, n=4. Detection limit = 0.39 $\log_{10}$ CFU/cm^2.

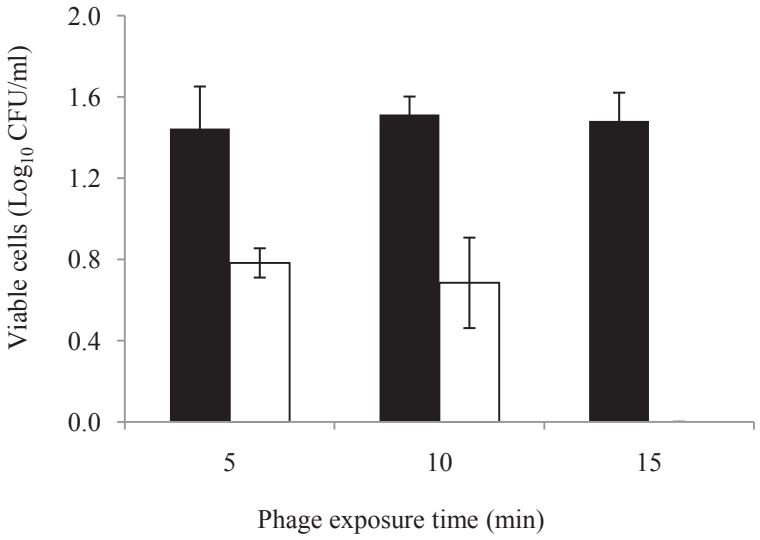

Figure 7.2 Viable counts of a mixture of three *L. monocytogenes* strains (19CO9, 19DO3 and 19EO3) adhered with 10% fish protein broth on stainless steel at 15 °C after 5, 10 and 15 min. ■ ,Control culture; □ , Phage cocktail (LiMN4L, LiMN4p and LiMN17) at ≈6.5 $\log_{10}$ PFU/cm^2. Error bars indicate standard error of mean CFU/cm^2, n=4. Detection limit = 0.39 $\log_{10}$ CFU/cm^2.

7. 3.2 Stability of *Listeria* phages under different conditions

7.3.2.1 Stability of phages in QAC solution

The infectivity of three phages in QAC for 30 min at 15 °C was assessed. The titre of phage LiMN4L was reduced significantly at 5, 10 and 30 min ($P < 0.05$) in both 100 and 200 ppm QAC. The initial titre (≈9.2 $\log_{10}$ PFU/ml) of phage LiMN4L decreased by ≈1.4, 1.8 and 2.2 log units after 5, 10 and 30 min, respectively in 100 ppm QAC. In 200 ppm, phage LiMN4L was reduced by ≈3.6, 4.5 and 6.2 log units after 5, 10 and 30 min, respectively (Fig. 7.3).

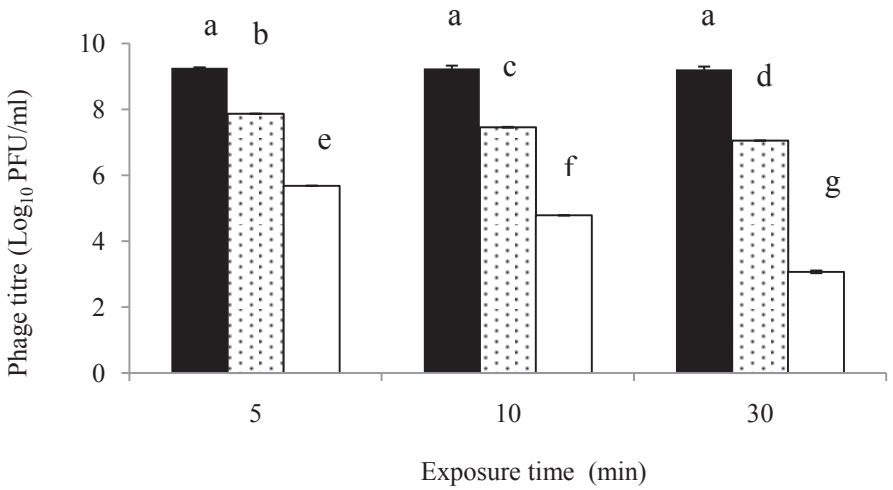

Figure 7.3 Titre of *Listeria* phage LiMN4L in QAC exposed at 15±1 °C for 5, 10 and 30 min. Phage titre: ■, Control without QAC; ▩, 100 ppm QAC; □, 200 ppm QAC. The bars indicate standard deviation of the mean of two samples. Different lowercase letters above the bars indicate the significance among the treatments ($P < 0.05$). Error bars represent standard error of two samples. Detection limit = 2 $\log_{10}$ PFU/ml.

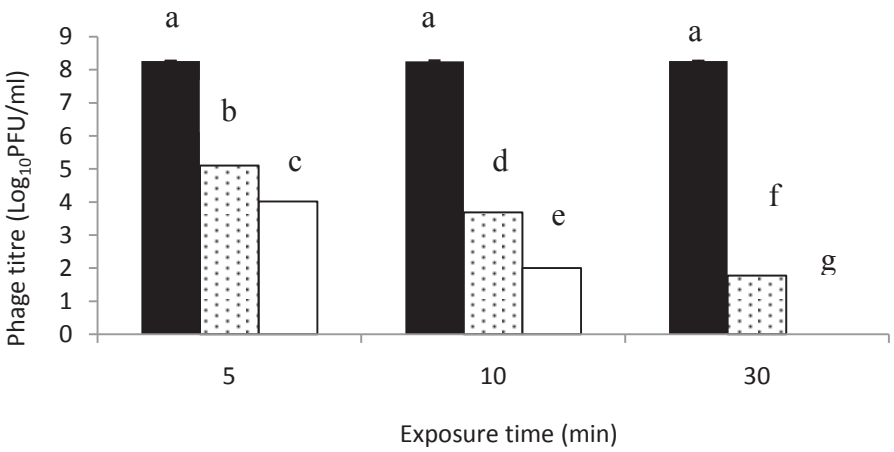

Figure 7.4 Titre of listeriaphage LiMN4p in QAC exposed at 15 ^{o}C for 5, 10, and 30 min. ■, Control phage suspension without QAC; ▩, Phage titre in 100 ppm of QAC; □, Phage titre in 200 ppm of QAC. Different lowercase letters above the bars indicate the significance among the treatments ($P < 0.05$). Error bars represent standard error of two samples. Detection limit = 2 $\log_{10}$ PFU/ml.

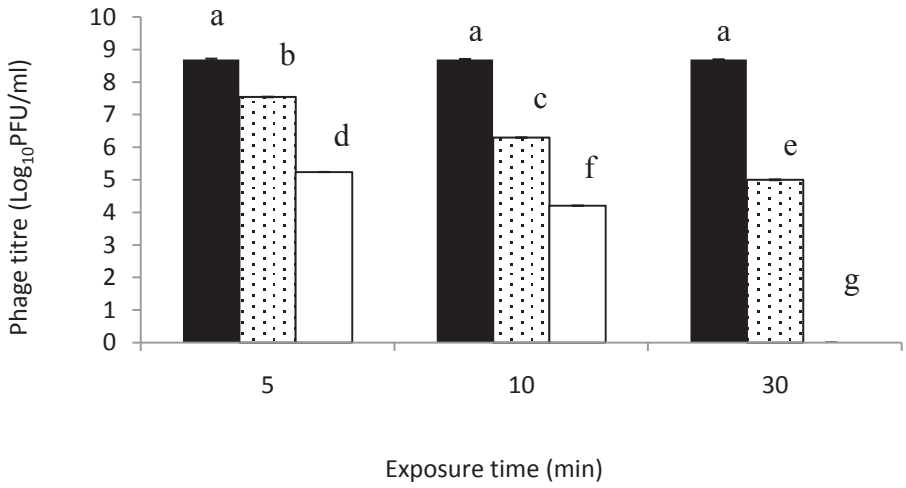

Figure 7.5 Titre of listeriaphage LiMN17 in QAC exposed at 15 ^{o}C for 5, 10, and 30 min. ■, Control phage suspension without QAC; ▩, Phage titre in 100 ppm of QAC; □, Phage titre in 200 ppm of QAC. Different lowercase letters above the bars indicate the significance among the treatments ($P < 0.05$). Error bars represent standard error of two samples. Detection limit = 2 $\log_{10}$ PFU/ml.

Phage LiMN4p also significantly ($P < 0.05$) decreased at 5, 10 and 30 min during incubation in both 100 and 200 ppm QAC solutions (Fig. 7.4). Compared to the initial phage content (≈8 $\log_{10}$ PFU/ml), ≈3, 4.5 and 6.5 log units of phages were destroyed in 100 ppm QAC in 5, 10 and 30 min, respectively. In 200 ppm, phage LiMN4p was reduced by ≈4 and 6 $\log_{10}$ PFU/ml after 5 and 10 min, respectively, and reached a non-detectable level after 30 min (Fig. 7.4). The susceptibility profiles of phage LiMN17 (Fig. 7.5) were similar ($P > 0.05$) to that of phage LiMN4L in QAC (Fig. 7.3). A significant decrease of ≈1, 2 and 3.5 log units ($P <0.05$) occurred after exposure to 100 ppm for 5, 10 and 30 min, respectively. The initial amount of phage LiMN17 (≈8.7 $\log_{10}$ PFU/ml) was also reduced significantly by ≈3, 4.5 and 6 log units ($P < 0.05$) after 5, 10 and 30 min, respectively in 200 ppm QAC (Fig 7.5).

7.3.2.2 Stability of phages PBS and FB at 15 °C

Phages LiMN4L, LiMN4p and LiMN17 were stable in PBS at 15 °C for 24 h (Fig. 7.6). The phage titres also remained stable for about 24 h in FB solution. However,

titre levels of phages LiMN4L and LiMN4p showed a slight decrease ($P > 0.05$) in FB (≈0.1 and 0.2 log units, respectively) after 24 h.

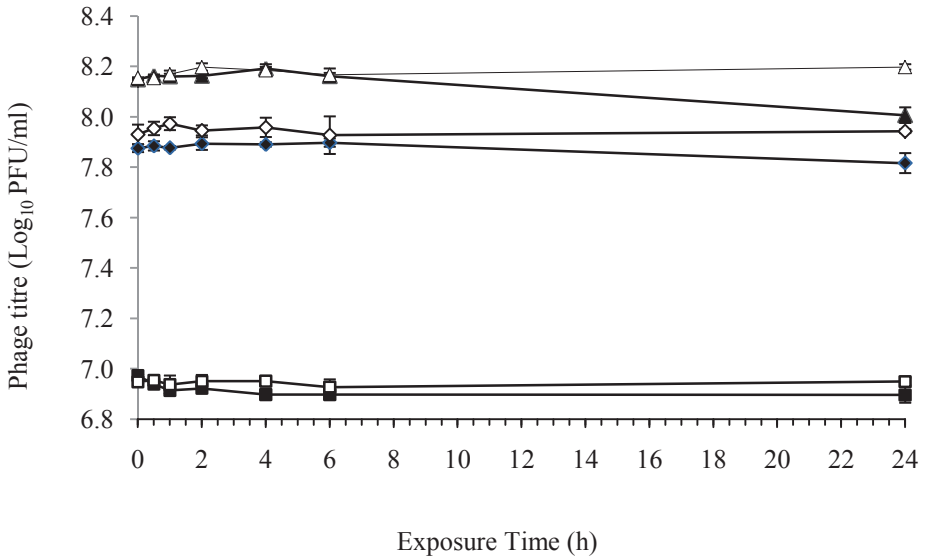

Figure 7.6 Titre of three *Listeria* phages in fish broth (FB) and phosphate buffered saline (PBS) at 15±1 °C for 24 h. Phage LiMN4L: ◆, FB; ◇, PBS; Phage LiMN4p: ▲, FB; △, PBS; Phage LiMN17: ■, FB; □, PBS. Error bars represent standard error of the mean of two independent samples. Detection limit = 1 $\log_{10}$ PFU/ml.

7.3.2.3 Survival of phages at different pH levels

Phages LiMN4L and LiMN4p were stable at pH 4-10 at room temperature for 1 h with slightly varying titres ($P > 0.05$) while the titre of phage LiMN17 was stable at pH 3-9 for 1 h (Fig. 7.7). At pH 10, LiMN17 decreased by ≈0.5 log units compared to pH 7 (Fig. 7.7). None of the phages survived at pH 3, 11 and 12.

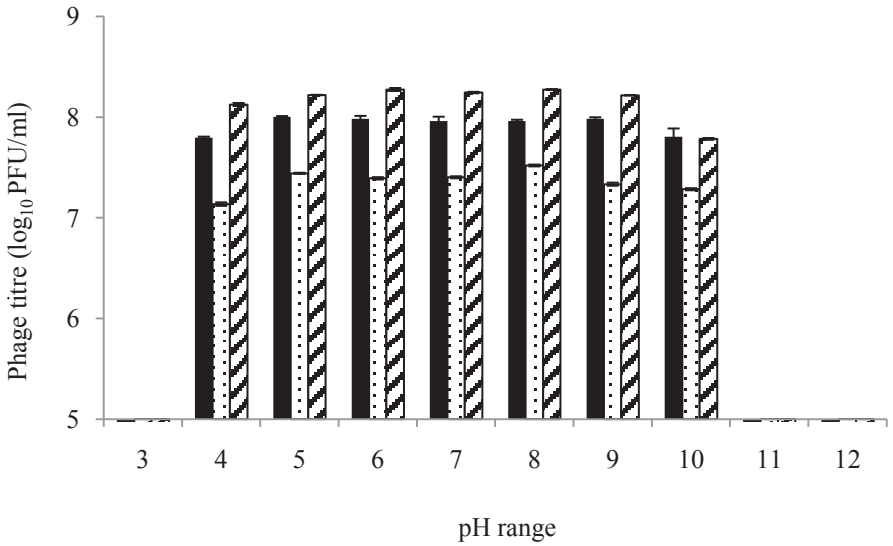

Figure 7.7 Titre of three *Listeria* phages in trypticase soy broth at pH 3-12 at 24±1 °C for 1 h. ■, phage LiMN4L; ⁞⁞⁞, phage LiMN4p; ▨, phage LiMN17. Error bars indicate standard error of mean of two independent samples. Detection limit = 3 $\log_{10}$ PFU/ml.

7.3.2.4 Survival of phages under artificial light

The surviving numbers of phages LiMN4L, LiMN4p and LiMN17 under low and high fluorescent light at 24±1 °C for 6 h is shown in Figure 7.8. There was no significant decrease ($p > 0.05$) in the titres of each phage under both light intensities. However, a general trend in the reduction of phage numbers towards the end of the 6 h incubation period was observed (Fig. 7.8).

7.3.2.5 Longevity of phages on stainless steel coupons

The concentration of phages LiMN4L and LiMN4p placed on SSC was decreased by ≈0.4 and 1.0 $\log_{10}$ PFU/cm^2 while drying for ≈2.5 h at 24±1 °C and then remained stable for 2 h at 15 °C (Fig. 7.9). During further storage at 15 °C for 24 h, the titres of phages LiMN4L and LiMN4p on SSC decreased by ≈2.0 and 1.2 log units, respectively. The LiMN17 decreased by ≈2.0 log units during drying on SSC and then remained stable on the dried smear for 2 h followed by a reduction of ≈0.3 log units over 24 h (Fig. 7.9).

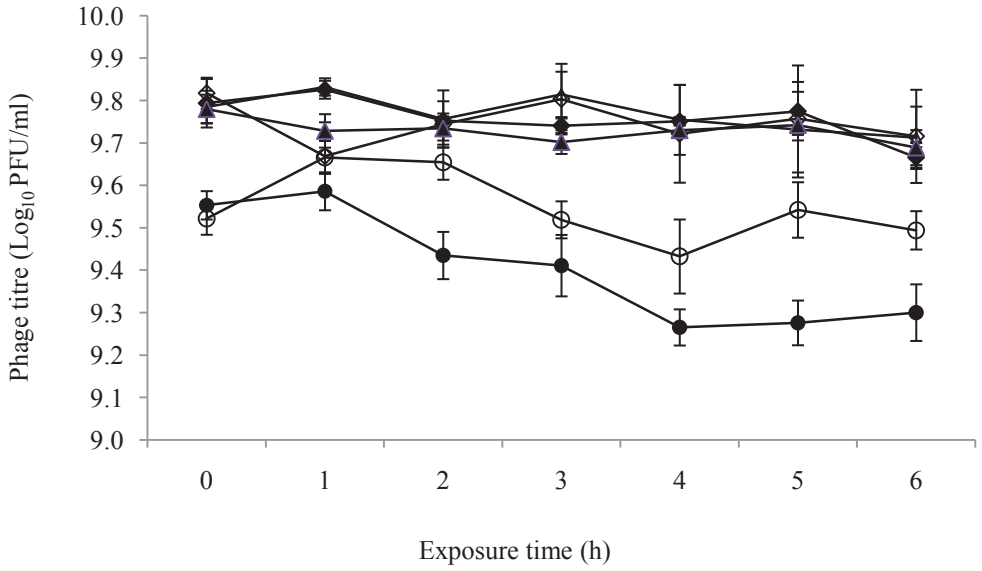

Figure 7.8 Surviving titres of three *Listeria* phages in low (487±30 lux) and high (1710±130 lux) intensity light at 24±1 °C. LiMN4L: ◇, low light; ◆, high light; LiMN4p: △, low light; ▲, high light; LiMN17: ○, low light; ●, high light. Error bars represent standard errors of mean of four independent samples. Detection limit = 1 $\log_{10}$ PFU/ml.

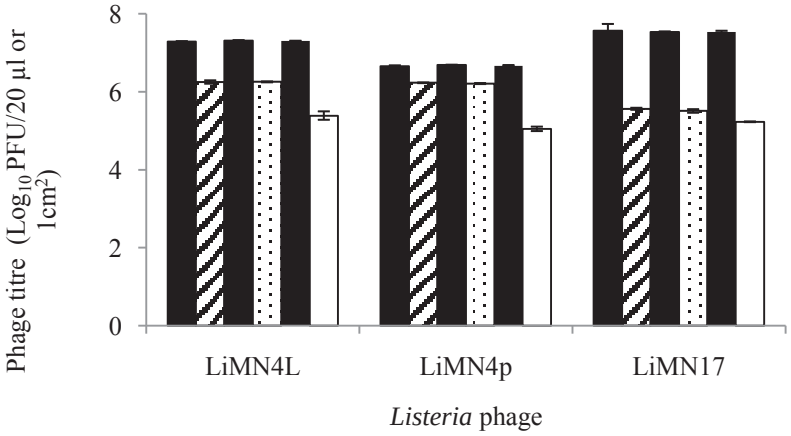

Figure 7.9 Surviving titres of three *Listeria* phages in the control (suspension) and attached on the stainless steel coupon (SSC). ■, Phage titre of control (Log_{10} PFU/20 µl) at 0, 2 and 24 h, respectively at 15±1 °C; Phage titre adhered on SSC (Log_{10} PFU/cm^2): ▨, 0 h (immediately after drying for 2.5 h at 24±1°C); ⁘, 2 h (15±1°C); ▭, 24 h (15±1°C). Error bars indicate standard error of mean of four samples (n=4).

7.3.2.6 Refrigerated storage

The viable numbers of phages LiMN4L, LiMN4p and LiMN17 in PBS at 4±2 °C storage in the dark is shown in Figure 7.10. The infectivity of the three phages remained stable for one year (Fig. 7.10).

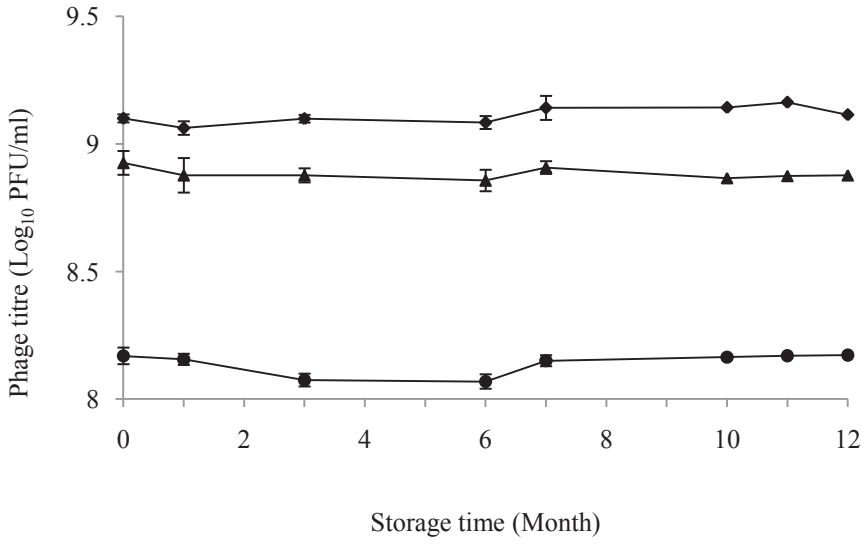

Figure 7.10 Surviving titres of three *Listeria* phages in phosphate buffered saline at 4±2 °C. ◆, LiMN4L; ▲, LiMN4p; ●, LiMN17. Error bars represent standard errors of mean of two independent samples.

7.4 Discussion

The assessment of phage-mediated decontamination of low levels of late exponential *L. monocytogenes* strains in the presence of fish proteins on SSC has not been previously reported. The inoculation of low count cells on SSC was followed by ≈5-10 min of drying time in order to enhance the establishment of cells and fish proteins to simulate conditions of a fish processing plant environment. The minimum volume that could be spread quickly and uniformly over the bacterial contamination (smear) on a 1 cm^2 SSC was 3 µl. The use of a phage cocktail reduces likelihood of selecting phage resistant mutants (Roy et al., 1993; Sulakvelidze and Pasternack, 2010; Tanji et al., 2004) and such cocktail should consist of phages that bind specifically to different receptors on the target bacterium (Tanji et al., 2004). The efficacy of 3 µl of phage suspension per 1 cm^2 shows the application potential of a 3 ml phage preparation over

approximately 1000 cm^2 of stainless steel surface. The results indicate that a phage cocktail (≈8.3 log PFU/ml) could be used to decontaminate low counts of *L. monocytogenes* cells. In comparison to commercial phages, ListShield™ is recommended for use of one millilitre (≈9 log PFU/ml) over 500 cm^2 for treating food or processing surfaces against *L. monocytogenes* (Bren, 2007; Intralytix, 2013).

Bacteriophages and certain pathogenic viruses are reported to be more resistant to QAC compared to other anti-microbial chemicals such as sodium hypochlorite, a formulation of Virkon (Antec Inc., Sudbury, United Kingdom), ethanol, peracetic acid, and glutaraldehyde (Mercanti et al., 2012; Rode et al., 2011; Solomon et al., 2009). The stability of *Listeria* phages was investigated in QUATAL [10.5% N-alkyldimethyl-benzylammonium-HCI (40% C-12, 50% C-14, and 10% C-16) and 5.5% glutaraldehyde] (Roy et al., 1993). The authors reported that titres of three *Listeria* phages (2671, H387, and H387-A) were stable in <50 ppm of QUATAL for 4 h, while titres were reduced by ≈3-4 log_{10} PFU in 75 and 100 ppm of QUATAL in 1 h, respectively. These results suggest that the susceptibility of this particular phage depends on the concentration of QAC. In our study, the formula of QAC is different from the formula in other studies. This product is currently used by several New Zealand food industries as part of their sanitation regimes, with 200 ppm of QAC as the recommended concentration for disinfecting fish processing plant. The three phages used in this study were susceptible ($p < 0.05$) to 100 and 200 ppm QAC levels in 5 min. Therefore, if these phages are to be used with QAC disinfectants, lower concentrations of QAC would need to be tested to determine if the phage would survive.

The survival profiles of phages at 15 °C for 24 h suggest that PBS is a suitable medium for the delivery of phages in practical applications. It is important to note that during cleaning procedures in the industries, detergents and disinfectants may alter the pH of food contact surfaces, thereby affecting survival of phage. The pH range for phage P100 stability is 5.5-9.5 with an optimum of 7.7 (Micreos Food Safety, 2013). Phages LiMN4L and LiMN4p survived well between pH 4 and 10 while phage LiMN17 showed good survival between pH 4 and 9 at room temperature for 1 h. The stability of three phages over a broad pH range indicates the potential for application in the presence of acidic and caustic decontaminating agents. A *Listeria* phage cocktail (LMP102) diminished rapidly in apple tissues in 30 min at 10 °C due to the acidic

nature (pH 4.37) of the tissues (Leverentz et al., 2003). Another point to highlight is that in a fish processing environment, it is likely that phage would dry onto surfaces.

In the present study, the phages LiMN4L, LiMN4p and LiMN17 were stable in the dried form on the stainless steel at 15 °C for 2 h and then phage titre was decreased by ≈1, 0.4 and 2 log units, respectively over 24 h. This loss of viability was similar to results for enteric virus desiccated on hard surfaces (Abad, et al., 1994). Exposure to artificial (fluorescent) light for longer period has been shown to inactivate phytopathogenic bacteriophage (Iriarte et al., 2007). If this were to occur in fish processing environment treated with *Listeria* phages, this would limit the usefulness of phage treatment. Thus the stability of the three phages used in this study was evaluated under low fluorescent lighting simulating a typical fish processing plant as well as high intensity lighting exposure. The titres of the three phages decreased only by ≈0.2 log units ($p > 0.05$) after 6 h exposure, providing some confidence that these phages are likely to be effective under industrial lighting conditions. The stability of any *Listeria* phage at refrigeration temperatures is also an important parameter to be evaluated in terms of keeping effective phage stocks in storage and also applying in seafood processing plants where temperatures are low. Listex^{TM} (P100) suspended in saline is stable at 2-8 °C for >6 months (Micreos Food Safety, 2013), while ListShield^{TM} phage cocktail is contained in phosphate buffered saline at pH 7.0-7.5 and is stable at 2-6 °C in the dark (Intralytix, 2013). Similarly, in the present trial the three phages suspended in PBS showed good stability in the dark at refrigeration for 1 year.

7.5 Conclusion

Based on the results obtained in this study, a *Listeria* phage cocktail containing phages LiMN4L, LiMN4p and LiMN17 suspended in PBS (3 ml of ≈8.3 $\log_{10}$ PFU/ml) could be used as an effective treatment to eliminate low concentrations of *L. monocytogenes* over an about 1,000 cm^2 stainless steel surface. The phages cannot be used in the presence of QAC sanitizers at the concentrations used in fish processing plants. They may be however, used in acidic or alkaline environments following cleaning and still remain effective under fluorescent lighting, refrigeration temperatures and dry conditions common in fish processing environments.

Authors' contributions to the manuscript

The experiments were planned and conducted, data were analyzed and the first draft of the manuscript was prepared by GJGA. SHF, CDC, LM, BMDM and ANM supervised and provided advice on interpretation of results and discussion and improving the writing of overall manuscript.

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Highlights

- Phage cocktail eliminated low count *L. monocytogenes* on stainless steel in ≈15 min
- Phages were stable under fluorescent light for 6 h and desiccation for 2 h
- Three phages survived pH 4-9 for 1 h and were unstable in 100 ppm QAC after 5 min

Chapter 8: Summary and future directions of study

8.1 Summary

This study investigated the lytic efficacy of phages against *L. monocytogenes* strains under the conditions commonly found in seafood processing environments. Initial experiments (chapter three) were carried out using three phages FWLLm1, FWLLm3 and FWLLm5 isolated from sheep feaces from a previous project. These phages showed broad host ranges at 25 °C across *L. monocytogenes* strains isolated from the seafood industry. Therefore, these three phages may have potential in controlling *L. monocytogenes* strains in seafood processing environments. Using phage FWLLm3, this study demonstrated that the minimum phage doses required to kill total cells of a low count of *L. monocytogenes* could be estimated by fitting the experimental parameters in a mathematical model and these estimates will be useful in planning and assessing decontamination regimes.

Chapter four described characteristics of three novel phages (LiMN4L, LiMN4p and LiMN17) isolated from seafood processing environments. The characteristics of different phages such as host range, adsorption rates, and burst size and temperature range of replication are important factors to determine their applications. Three virulent phages belonging to the family *Myoviridae*, had broad host ranges and high adsorption rate constants at 15 °C. The mean burst sizes of phages LiMN4L, LiMN4p and LiMN17 were about 17, 17 and 11 PFU per cell respectively, in an indicator with a greater than 240 min (4 h) latent period at 15 °C. These characteristics suggested that LiMN4L, LiMN4p and LiMN17 phages were good candidates against *L. monocytogenes* contamination through passive biocontrol approaches at low temperatures as found in the seafood industry.

Chapters five, six and seven covered the biocidal efficacy of three phages (LiMN4L, LiMN4p and LiMN17) against physiologically stressed *L. monocytogenes* by aging (late exponential phase), heat, starvation and salt. These phages were also tested against *L. monocytogenes* attached on stainless steel either clean or soiled with fish proteins and in biofilms at low temperature under conditions representing fish processing plant environments. The three phages lysed the low count cells of *L.*

monocytogenes affected by aging, heat, starvation and salt to non-detectable levels (by more than 2 log units) *in vitro*.

Surfaces in a seafood processing plant are likely to be contaminated with fish proteins. Three phages lysed *Listeria* cells adhered on clean or fish protein conditioned stainless steel surfaces to less than detectable levels or by ≈3-4.5 log units respectively at 15 °C in about 75 min. Pathogens are most likely to exist in food processing premises in low numbers. Phage LiMN4p and a cocktail of three phages (LiMN4L, LiMN4p and LiMN17) at ≈5.48, 6.48 and 7.48 $\log_{10}$ PFU/cm^2 eliminated low counts (≈1.5-1.7 $\log_{10}$ CFU/cm^2) of a three *L. monocytogenes* strain mix in ≈15 min. The cells in intact biofilms were reduced by ≈2-3 log units over three consecutive phage applications at 1 h exposure times at 15°C. However, dislodged biofilm cells of each *L. monocytogenes* strains were reduced by mono-phage treatments by ≈5 $\log_{10}$ CFU/ml in 1 h 15 °C. The results infer that the biofilm matrix may limit the access of phages to cells within undisturbed biofilm.

The phages LiMN4L, LiMN4p and LiMN17 were stable for at least 6 h under the ambient conditions of light, temperature and protein residues commonly found in fish processing premises. Phages survived in broth at pH 4-10 for 1 h and desiccation on stainless steel at 15 °C for 2 h. These results indicate that the three phages are stable in conditions similar to those found in seafood processing plants. Preliminary investigations showed that cell wall GLcNAc, a common phage receptor did not need to be present for phage adsorption. This suggests that other cell wall constituents may also serve as receptors for phages LiMN4L, LiMN4p and LiMN17 studied.

High count bacteria populations show heterogeneity on phage-sensitivity and a small fraction of cells may be insensitive to lytic phages (Chapman-McQuiston and Wu, 2008). The total cells of particular *L. monocytogenes* strains targeted for control should be lysed by the phage. Culture heterogeneity of environmental strains of *L. monocytogenes* for phage-sensitivity is not known. Guenther and Loessner (2011) recovered phage insensitive colonies of *L. monocytogenes* 103/2005 from cheese that was treated with lytic phage A511 (≈3 x 10^8 PFU/cm^2) and ripened for 22 days. In the present trial, phage insensitive cells in exponential phase high count cultures (10^8 CFU/ml) of *L. monocytogenes* 19CO9 were $<10^{-8}$ CFU ml and phage insensitive cells did not emerge in cultures (10^6 CFU/ml) at 15 °C for 2 days.

The three phages isolated in this project, as individual phages or as a phage cocktail, have potential to be used as decontaminating agents in seafood processing plants once investigations on the emergence of phage resistance *L. monocytogenes* mutants, heterogeneity of cell wall receptors of the phage cocktail and field trials are completed.

8.2 Future directions of study

Next stage of this research project should be a pilot study on the decontaminating properties of phages in real seafood processing environments. Suitable protocols need to be adopted such as ensuring surfaces are devoid of cells after the phage treatment. The lytic-activity of phages should be extended beyond the predetermined phage-intervention time used in earlier protocols. Therefore, virucidal agents should be used *in situ* at the end of the phage intervention time in order to determine the efficacy of phages and/or the optimum time for phage treatment of hard surfaces. The seafood-borne *L. monocytogenes* strains which formed cell clumps under static conditions during the present study are also more likely to be remain as cell clumps in real contamination environments. Therefore, the lysis efficacy of such cell clumps using phage needs to be investigated *in situ* microscopically using techniques such as staining of live and dead cells.

Another aspect to be studied is the potential emergence of phage resistant *L. monocytogenes* mutants including the development of cross-resistance when a phage cocktail is used as a regular decontaminating agent. The resistance mechanism should be investigated phenotypically and at the molecular level, including a study of the modification of cell wall receptor sites and restriction modification systems. An ideal phage cocktail should consist of phages which have different cell wall-binding ligands. Therefore, host binding ligands of the present phages need to be determined (e.g. tail appendages). The *L. monocytogenes* strains which showed resistance to phage-lysis in the present study should also to be investigated for the phage-resistant mechanism involved. This study has found some *L. monocytogenes* strains that do not produce plaques and form only host-kill zones with phage drops. These phage and host pairs will be useful in detailed studies on the lethal mechanism, such as abortive infections or lysis

from without which have potential as biocontrol strategies. Currently, information on these mechanisms relevant to *L. monocytogenes* is not available.

The different types of lysin are potentially encoded by the three phages used in this study since secondary lytic zones (haloes) of the three phages showed differences in appearance. Therefore, it is would be interesting to characterize the lysins of these three phages as *Listeria* potential decontaminating agents. An investigation of the lytic spectrum against *L. monocytogenes* strains and minimum effective concentration of lysins would be a useful initial study.

The present study on lysis of biofilms formed by *L. monocytogenes* needs to be extend for heterogeneous biofilms to represent natural biofilms. It would be advantageous to assess the efficacy of phage treatments as well as phage-lysins in the degradation of both biofilm cells and matrix using different approaches including scanning microscopic techniques in addition to enumeration of differential cell counts such as viable counts and viable-but-non-culturable cells.

Identification of non pathogenic *Listeria* species such as *L. innocua* strain as propagating-hosts for the three phages will help ensure the safety of phage-cocktail.