For: Food Control

Isolation, characterization and evaluation of virulent bacteriophages against *Listeria monocytogenes*

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Abstract

Bacteriophages are considered a potential antimicrobial food intervention due to their ability to inactivate target bacteria in different environments. This study was undertaken to isolate, characterize, and identify a mixture of phages that could be used for control of *Listeria monocytogenes*. A total of 37 phages were isolated from raw sewage sludge using *L. monocytogenes* serotypes 4b, 1/2a and 1/2b strains. The host range of these phages was determined by spot-testing. Ten lytic phages were selected for further evaluation with efficiency of plating (EOP) analysis and characterization by pulsed field gel electrophoresis (PFGE). According to the spot tests and EOP results, the isolated phages against *L. monocytogenes* 4b strains were found to be capable of lysing a wider range of ribotypes. Also, three phages specific for 1/2a and 1/2b serotypes had similar lysis capabilities over a range of target *Listeria* with high EOP values between 0.5 and 1.0. PFGE analysis identified 5 distinct restriction profiles of the 10 selected phages whose DNA was digested with either *EcoRI* or *HinDIII*. Transmission electron microscopy (TEM) pictures revealed that five selected phages belonged to the Myoviridae and one phage to the Podoviridae family. Based on screening results, a phage cocktail (LP1) comprised of six lytic phages was used to inactivate a mixed culture of five *L. monocytogenes* strains. LP1 was able to lyse bacterial cells within 30 to 60 min at multiplicity of infection values (MOI, PFU/CFU) of 1,000 and maintained the target cells under detection levels for 24 to 48 h at 4, 10, and 30°C. These results suggested the LP1 phage collection contained effective and strongly lytic phages that may serve as antibacterial preparation to control *L. monocytogenes* of different serotypes.

Keywords: *Listeria* phages, lysis, *Listeria* control, antibacterial, bacteriophages, food safety

47 1. Introduction

48 *Listeria monocytogenes* represents a serious foodborne bacterial pathogen responsible for
49 an estimated 255 deaths annually in the U. S. (Scallan et al., 2011). This bacterium causes
50 listeriosis, a severe illness that involves diarrhea, stillbirths, meningitis, and encephalitis, and it is
51 associated with high mortality rates (20-30%). Most human listeriosis cases are linked to the
52 serotypes 1/2a, 1/2b, and 4b and associated with contaminated ready to eat (RTE) foods (Chan
53 and Wiedmann, 2009; Kim et al., 2008). One of the particular concerns of *Listeria* contamination
54 is RTE food that can sustain bacterial growth, even under refrigeration conditions. The infectious
55 dose determined in foods that have been related with listeriosis, has typically been greater than
56 1,000 CFU/g (ICMSF, 1994; ICMSF, 1996; Tompkin, 2002). In 2011, a multistate *L.*
57 *monocytogenes* outbreak was linked to cantaloupe and poor sanitization conditions of the
58 relevant facilities which emphasizes the need to minimize the risk of *L. monocytogenes*
59 contamination in RTE foods as well as in produce facilities (CDC, 2011).

60 *L. monocytogenes* is a pathogen that poses a risk due to its unique characteristics:
61 ubiquitous presence in the environment, psychrotrophic growth at refrigeration temperatures or
62 lower (Membré et al., 1999; Walker et al., 1990), and tolerance to high salt concentrations
63 (Guenther et al., 2009; Seeliger and Jones, 1986). These unique properties allow *L.*
64 *monocytogenes* to resist harsh environments, reproduce, and become a food contaminant. This
65 microorganism is capable of colonize and develop biofilms in food processing facilities and even
66 become established in equipment surfaces.

67 Bacteriophages are the largest and most ubiquitous biological entities on earth. Phages
68 might coexist in all the habitats of their host bacteria (Coffey et al., 2010; Hendrix, 2003).

Virulent phages infect and complete their life cycle by lysing specific bacterial cells, produce and release progeny that lyse more bacteria in that environment. In the context of finding a biocontrol agent with desirable characteristics such as natural, safe and specific towards the target pathogen, virulent bacteriophages offer a viable option as antibacterial agents (Sulakvelidze et al., 2001).

The Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA) Food Safety Inspection Service have implemented a zero tolerance policy for this pathogen in RTE foods. Several countries also follow this same regulatory policy. Food companies have adopted a variety of interventions to be able to meet this regulation. In 2006, the FDA and USDA approved the use of two phage preparations to control *L. monocytogenes* in food, LMP-102, an additive for RTE foods such as meat and poultry products, and Listex P100, as GRAS ingredient for all food products (Carlton et al., 2005; FDA., 2006; Monk et al., 2010). The approval of anti-*Listeria* phages by government agencies suggested a formal recognition of promising and realistic antibacterial agents for food and food production facilities.

Several studies have been conducted to determine the phage efficacy to biocontrol *L. monocytogenes* in foods and processing plants (Hudson et al., 2005; Klumpp et al., 2008; Leverentz et al., 2003). However, limited availability of broad-spectrum phages and the lack of information regarding their degree of infection to control *L. monocytogenes* in different environments, stress the need for an effective and novel approach to reduce the persistence and severity of this particular pathogen in food and food production facilities. In search for an effective, alternate intervention, the long-term goal of this study was to develop a collection of highly lytic phages with wide host ranges, which would be able to infect *L. monocytogenes*. In this study, the specific objectives were to isolate and characterize lytic bacteriophages against *L.*

monocytogenes, and to develop and evaluate the efficiency of the anti-*Listeria* phage cocktail, LP1 as a potential antibacterial.

2. Materials and Methods

2.1. Sample collection

Samples were collected from waste sources to isolate phages against different strains of *Listeria monocytogenes*. Raw sewage samples were collected from the city sewer, Rice Lake City, WI, the wastewater treatment plant in Saint Paul, MN, and the Seneca wastewater treatment plant in Eagan, MN.

2.2. Bacterial strains

L. monocytogenes isolates used in this study are listed in Table 1. These strains were used as host strains to screen phages from sources samples. All of these strains were associated with human sporadic cases or outbreaks. The strains of *L. monocytogenes* that were used were representative of the wide range of serotypes and ribotypes that have been associated with food borne illness and are frequently present in food. Most bacterial strains were obtained from the International Life Sciences Institute Culture Collection at Cornell University and stored at -80° C. These strains were first streaked from the frozen cultures onto tryptic soy agar (TSA; Neogen Corp., East Lansing, MI) and polymyxin-acriflavine-LiCl-ceftazidime-aesculin-mannitol (PALCAM) agar plates (Neogen Corp.). Individual colonies were inoculated into tryptic soy broth (TSB, Neogen Corp.) and incubated at 37°C for 18 h.

2.3. Isolation, purification and preparation of bacteriophages

The isolation, purification, and preparation of the bacteriophages were based on methods developed and used by Klieve, and by Twest, and Kropinski (Klieve, 2005; Twest and Kropinski, 2009) with modifications. After collection, effluent samples were first filtered, centrifuged at $4,650 \times g$ for 10 min and supernatants were incubated overnight at 30°C. Overnight TSB cultures of single *L. monocytogenes* strains (Table -1) were diluted 100-fold into brain heart infusion (BHI; Neogen Corp.) or Luria Bertani (LB) broth (Neogen Corp.) and were incubated for 18 to 24 h at 30°C to serve as host strains. Filtered and centrifuged samples were again centrifuged at $8,944 \times g$ for 10 min and supernatants were filtered through 0.25 or 0.45 μm filter syringes. Filtrates from this last step were used for spot testing.

Spot tests were conducted as a first step to isolate phages from samples. For isolation purpose, we used individual *L. monocytogenes* strains, no mixed bacterial cultures were used. Fresh overnight cultures of *L. monocytogenes* were mixed with a modified tryptone top agar (TTA, 0.5% agar) (Akhtar et al., 2014) and poured on a Luria Bertani bottom agar (LBBA, Luria Bertani broth (Neogen Corp.) with 1.5% agar) to create a bacterial lawn. Filtrates were spotted (1 μL :10 μL , filtrate:host) onto the lawns of freshly prepared hosts in a row and incubated at 30°C for 18 to 24 h. Clear spots of lysed agar were cut and suspended into phosphate buffer saline (PBS; Neogen Corp.), centrifuged, filtered, and stored as "spot filtrate" at 4°C.

For isolation and purification of individual phages from "spot filtrate", plaque assays were conducted. Spot filtrates were diluted in PBS and mixed with exponential phase *L. monocytogenes* cultures (host:spot filtrate; 1:3) and incubated at 30°C for 15 min. After incubation, this host-spot filtrate mixture was suspended into TTA agar overlay and poured into BHI or LBBA bottom agar. Plates were incubated overnight at 30°C. After incubation, clear, individual plaques were picked up from agar plates, suspended in PBS, centrifuged, and the

supernatants were filtered to obtain a single bacteriophage stock. For a purified phage stock, the plaque assay was repeated 3-4 times with each individual phage.

Purified phages were used to prepare high titer phage stocks for further analyses. Purified phages were diluted into PBS and plaque assays were conducted as described above with overnight host bacterial cultures. Briefly, purified phage stock (10^4 - 10^6 PFU/mL) were propagated with host strains, mixed with TTA and poured into LBBA plates, and incubated at 30°C for 18 to 24 h. After lysis, agar plates with dense plaques were selected. Five mL aliquots of phage storage buffer (PSB) were poured onto the agar and the top agar layer was scraped and collected with the buffer into a centrifuge tube. The agar phage suspensions were centrifuged at $8944 \times g$ for 10 min to pellet debris and loose agar, and supernatants were filtered to remove bacterial contaminants. The filtrates were used as high titer (10^8 - 10^{10} PFU/ mL) phage stocks and stored at 4°C for further analysis.

2.4. Determination of host range

Spot tests were conducted to identify the lytic capability of individual phages against different *L. monocytogenes* strains. As described above, target bacterial lawns were prepared with overnight cultures on BHI agar plates and purified phages were spotted in a row as a 1:10 phage to host ratio on each plate with individual *Listeria* strains. Plates were incubated at 30°C overnight and clear spots were measured for positive lytic spots against *L. monocytogenes* strains.

Following spot testing, 10 phages were selected, based on their lytic efficiency against a wide range of *L. monocytogenes* serotypes, for efficiency of plaquing (EOP) analysis. EOP testing was conducted as a secondary analysis to measure the magnitude and efficiency of the

infection ability of the lytic phages in comparison with relative host strains. Ten-fold serial dilutions of phage suspensions (100 µL) were mixed with 200 µL of the target or host bacterium and incubated for 5 min at room temperature (24°C) before being mixed with 400 µL of molten TTA and plated as double-layers on TSA. Phage activity was examined by a spot test assay that entailed placing 20 µL of each phage (10⁵ PFU/mL) on TSA seeded with each strain of *L. monocytogenes*. The plates were checked for clearance zones after incubating for 18-24 h at 37°C. The presence of lytic zones was considered evidence of phage susceptibility and no lysis was considered evidence of phage resistance.

2.5. Bacterial challenge study in liquid broth

Based on the EOP and spot test data, a phage cocktail, named LP1 was created by combining 6 of the isolated phages (LMA4, LMA8, LMA9, LMB3, LMD3, and LME3) and a bacterial challenge study was performed to evaluate their combined lytic ability against *L. monocytogenes* strains. The phage cocktail contained phages specific for *L. monocytogenes* serotypes 4b, 1/2a, and 1/2b. Five strains of *L. monocytogenes* were randomly selected from different serotypes for the bacterial challenge study. The bacterial mixture included strains Scott A (4b), C1-056 (1/2a), J1-094 (1/2a), ATCC 51775 (1/2c) and C1-115 (3a). The strains were freshly grown from overnight cultures in TSB at 37°C for 18 h. The cells were harvested by centrifugation at 8944 × *g* for 10 min and washed once with PBS (pH 7.2). The pellets were resuspended in PBS and diluted to a concentration of approximately 10⁶ CFU/mL. Then, 1 mL aliquots of each of the five strains of *L. monocytogenes* were mixed together in equal volumes to prepare the inocula. The exact concentration of *L. monocytogenes* inocula determined by plate counts on PALCAM and TSA plates at 37°C for 24 h.

To test the lytic ability of LP1, ten mL volumes of TSB were inoculated with *L. monocytogenes* mixed cultures to achieve a final concentration of approx. 10^4 CFU/mL. The TSB tubes were incubated at 4, 12, 23 or 30°C for 1 h. LP1 was then added to TSB tubes at different concentrations of 1, 10, 100, or 1000 multiplicity of infection (MOI; ratio of PFU/CFU). Control tubes were treated with PSB buffer (Carlson, 2005) without any phage treatment. All tubes were incubated at 4, 10, 23 or 30° C for 24 to 48 h. Each treatment was performed in duplicates. Samples were collected after 30 min, 1, 2, 24 and 48 h of incubation from control and treatment broths and plated in duplicate on TSA plates. All plates were incubated at 37°C for 24 h and bacterial colonies were counted.

2.6. Characterization

To obtain images of the morphology of six purified lytic phages that were used to create the phage cocktail, LP1, transmission electron microscopy (TEM) was used. Phages were diluted into PBS to avoid the gelatin microstructures and negatively stained with either ammonium molybdate or 0.5% phosphotungstic acid or 3% uranyl acetate (O'Flynn et al., 2004; Sillankorva et al., 2008) . The phages were first fixed on copper grids, and samples were imaged and analyzed by a JEOL 1200 EXII TEM (JEOL USA, Inc., Peabody, MA 01960), with accelerating voltage from 40 to 120 kV, at the Characterization Facility, University of Minnesota, Minneapolis, MN. Genome sizes of 10 selected phages were measured by pulsed field gel electrophoresis (PFGE). Purified phage stocks were used (1:1) to make DNA plugs with 1.4% (w/v) molten PFGE grade agarose (Ultra Pure DNA Grade Agarose: BioRad, Hercules, CA). All DNA plugs were lysed with proteinase K (0.1 mg/ mL) and lysis buffer (10% w/v Sarcosyl, 10% w/v SDS) for phage DNA extraction and stored in 0.5× Tris EDTA (TE) buffer at 4°C. Phage DNA plugs that were already treated by proteinase K were digested with *EcoRI* and *HinDIII*

(New England Biolabs, Boston, MA) for digesting the DNAs for further characterization. High titer phage stocks were used to make these DNA plugs than the genome size to obtain the clear multiple bands of digested DNA. Plugs were then digested with restriction enzymes by following the manufacturer's protocol and were run on a PFGE gel in a CHEF-DR II PFGE, BioRad for 18 h and imaged on an ethidium bromide-stained agarose gel (1%, W/V) by a BioRad gel imaging system. The RE digested DNA images were analyzed to identify the distinct DNA features among ten purified phages.

2.7. Data analysis

Bacterial counts per mL were expressed as log CFU/mL. The detection limit for bacterial counts was <1 log CFU/ mL for all experiments. When the bacterial level was not detected, 0.9 log CFU/ mL was used for calculation in the analysis. All experiments were carried out in duplicates and were repeated. Mean and standard deviations were determined using Microsoft Excel (Version 14.0; Microsoft, Corp; Redmond, WA). Statistical analyses were performed by analysis of variance (ANOVA) and Duncan's multiple range test for differences ($P < 0.05$) between control and treatment groups using SAS software (9.2; Statistical Analysis systems, Cary, N.C., U.S.A.).

3. Results

3.1. Sample collection and isolation of bacteriophages

Initially, waste effluents samples were collected in different seasons from water treatment plants. In this study, sewage samples were tested with different serotypes of *L. monocytogenes* host strains (Table 1) to increase the potential for a versatile phage collection. All samples underwent an overnight enrichment procedure and a total of 37 bacteriophages were isolated

from sewage samples against eight strains of *L. monocytogenes* (Table 2). The majority (N=17) of phages were isolated against the 4b strains. A third of the phages were isolated against one single strain, *L. monocytogenes* Scott A, a 4b serotype. Twelve phages were isolated against 1/2a serotypes and 5 phages against 1/2b strains. Only 3 phages were isolated using 3b strains. No phages were isolated when 3a, 3c and 1/2c serotypes were used as hosts.

3.2. Host range of phages

Spot testing was conducted to determine the host range for each lytic phage against *L. monocytogenes* strains. All of the isolated phages formed clear plaques with the host strains, but 23 phages were chosen for spot analysis based on their plaque morphology (clearest) and host serotypes. The selected phages were representative of 4b, 1/2a, 1/2b and 3b serotype phages. The susceptibility of 22 strains of *L. monocytogenes* was tested; those included (number of phages in parenthesis): 4a (2), 4b (9), 3a (1), 3b (1), 3c (1), 1/2a (4), 1/2b (3) and 1/2c (N=1). Spot test results (Table 3) revealed that the lytic potential of phages were well distributed throughout different serotypes of *L. monocytogenes*.

Phages isolated against Scott A, serotype 4b were able to infect the majority of tested strains from all serotypes except 3c. Among all phages, LMA6, LMA8 and LMA9 were found to be the most virulent phages infecting 100% of the tested strains of 4b, 3a, 3b, 1/2a and 1/2c serotypes. Phages isolated against the strains of 1/2a serotype were able to infect a wide range of *L. monocytogenes* (50-100%) towards their specific host serotype. LMB4, the phage isolated specifically against 1/2a *L. monocytogenes*, lysed all tested strains of 1/2a, 1/2b and 1/2c serotypes. Only two phages against 3b were analyzed by spot testing, both LMF1 and LMF2 were lytic against 3a, 3b, 1/2a, 1/2b and 1/2c serotype strains with very poor to no lysis against 4b serotypes.

For further characterization for host ranges, EOP was used to test the 10 most lytic bacteriophages. These phages were selected based on the spot test results with the efficient lysis (clear plaques) against a wide range of *L. monocytogenes* strains. Also, a total of ten *L. monocytogenes* strains were chosen from different serotypes to test their degree of susceptibility against these broad specific phages. These serotypes were 4b (2), 4a (2), 1/2a (4), 1/2b (1) and 1/2c (1). With very few discrepancies, all phages were highly efficient (EOP value, 0.5-1) in making plaques against wide range of tested *Listeria* (Table 4).

Six phages isolated against 4b serotypes were investigated for efficient plaque forming. The majority of them were able to lyse 4b, 1/2a 1/2b and 3a strains with medium to high efficiency (EOP, 0.2-1). However, LMA4, LMA5, LMA6 and LMA7 showed little or no plaque formation with the 4a strain, J1-031. LMB3, a phage with a 1/2a host, was more potent (EOP, 0.5-1; against 80% of the tested *Listeria*) than LME3 phage which was low to medium efficient in making plaques (EOP, 0.001-0.5) with all tested strains. LMD3 and LMD4, the 1/2b phages were the most virulent to form plaques on a wide variety of hosts. All *L. monocytogenes* strains were susceptible to LMD3 with a record of high EOP values (0.5-1). Similar lysis data were observed with LMD4 except there was no plaque formation (EOP, 0) against strain C1-056. Overall, EOP results showed the virulent features of the selected phages with an ability of plaque formation over 90% of the tested strains.

3.3. Characterization of phages

Six virulent phages were selected for morphological analysis with TEM. Phages were negatively stained and imaged to determine the classification (Figure 1). All the isolated phages were observed as tailed ones and belonged to the order Caudovirales. LMA4, LMA9, LMA8, LMD3 and LME3 were Myoviridae phages while LMB3 showed the features of Podoviridae

phages. LMA4 and LMA9 resembled the morphotype of T4 phages with a contractile tail surrounded by a contractile sheath and icosahedral head, while LMA8, LMD3 had non-contractile tails. LMD3 was found to have the longest tail among these with a length of approx. 220 nm. Also, LMA8 and LMD3 had a base plate with tail fibers attached to their tails.

The molecular sizes of ten selected phage genomes were determined by PFGE to ensure the purity of phage stock. All ten phages were found to be pure stocks and only one DNA band was produced for each phage. All of the phages had a genome size of approx. 130-140 kb. The genome of phages LMB3, LMD3 and LMD4 was 130 kb, those of LMA4, LMA5, LMA6, and LMA7 were 135 kb, and the remainder LME3, LMA8 and LMA9 had a size of 140 kb. After determining the molecular size, phage DNAs of ten selected phages were digested with two RE enzymes *EcoRI* and *HinDIII*. When compared, LMA7, LMA9 and LME3 showed similar patterns, while ~5 visible bands were produced for each phage when using *HinDIII*. LMB3 showed approx. 4 bands with *HinDIII*. LMA6 and LMA8 DNAs were cut into 2 larger pieces by *EcoRI* digestion. LMD3 and LMD4 produced approx. 5 fragments and LMA4 was uncut with *EcoRI* enzyme.

3.4. Bacterial challenge study in liquid broth

L. monocytogenes mixed cultures were infected with LP1 at different MOI's *in vitro* (Figure 2). A total of four MOI's were evaluated to measure the lysis in TSB for 30 min to 48 h depending on the incubation temperatures (4, 10, and 30°C). At 30°C, when the bacterial population had optimum growth conditions, lower MOI (1, 10 and 100) treatments did not show drastic decreases of *L. monocytogenes*. Bacterial inactivation was observed more than 2 log CFU/mL when compared with controls, but treatment tubes showed turbidity after 24 h incubation period. Treatments with 1000 MOI totally lysed the bacterial cells within 30 min of

the incubation period and no cells were detected until 24 h later. When the test performed at 23°C with 1000 MOI, bacterial counts were below the detection level within 30 min and both 100 and 1000 MOI treatments achieved at least 8.5 log CFU/mL reduction by 48 h.

Results at 10°C and 4°C were quite similar. Small (0.5- 2 log CFU/ mL) reductions were achieved at lower MOI's (1 and 10), but phage cocktail with higher MOI's (1000) were highly effective against *Listeria* cells which reached undetectable levels in 1 h at 1000 MOI and no viable counts were observed at 100 and 1000 MOI after 48 h of incubation.

4. Discussion

This study conducted the isolation and characterization of lytic, wide-host range phages as a potent antibacterial against an important human pathogen, *L. monocytogenes*. These findings were an initial step for developing an antibacterial preparation by using a broad spectrum phage cocktail to control *L. monocytogenes* in foods and food processing environments. It had been suggested that isolation of *Listeria* phages from different locations and habitats can lead to the isolation of genetically diverse phages (Kim et al., 2008). Previously reported virulent *Listeria* phages, P100 and A511 were isolated from dairy plant effluents (Carlton et al., 2005), but in this study, sewage samples yielded a diversity of lytic phages and all 37 phages were isolated against *L. monocytogenes* from that source.

In the process of developing the LP1 phage cocktail, host ranges were determined to identify the specificity of isolated phages which provided a background to segregate the lytic phages that were able to infect a wide range of *L. monocytogenes* strains. The majority of the isolated phages were active against serotype 4b and a good number of them versus serotypes 1/2a, 1/2b and 3b. Regarding the serotypes associated with human listeriosis such as 4b, 1/2a and

1/2b (Kim et al., 2008), it was important to include virulent phages that were particularly lytic against these serotypes. Interestingly, strains of 4b serotypes were found as the most susceptible followed by 1/2a then 1/2b against phage infections. Some 3b strains were also susceptible, but a significant proportion of these strains were resistant. Serotype-associated resistance was also described in food processing environments in other studies. Kim et al. isolated *Listeria* strains from turkey processing environments and 42% of 1/2a and 63% of 1/2b or 3b serotype were resistant to phage infections (Kim et al., 2008). The authors speculated that the most prevalent serotypes could be resistant to a broad range of phages increasing their adaptive capacity and chance of survival, as well as becoming the predominating flora in that specific environment.

Spot test results were also correlated with the EOP data during isolation. In spot testing, when the isolated phages were screened against more strains from different serotypes, phages were specific for the host serotypes and a proportion of phages were highly virulent and able to infect cross serotypes. The majority of 4b-specific phages were most virulent and capable of lysing different serotypes. Phages were also evaluated for their lytic ability against serotypes that were not used as host to isolate phages. Only two phages were more virulent against other serotypes than the strains of their host serotypes. None of the phages were able to infect a strain of serotype 3c. As reported previously, strains of serovar 3 are the most resistant ones in comparison to serovar 4 and 1/2 strains against phage infection (Loessner and Rees, 2005). In another study, environmental strains of 3c serotype of *L. monocytogenes* was completely resistant to the infection by all tested isolated phages (Kim et al., 2008) .

Although the tested strains were few in numbers, the susceptibility to different serotypes followed a pattern against phage infections throughout these experiments. According to the EOP results most 4b-specific phages were capable of infecting a wide range of *L. monocytogenes*

serotypes among the ten selected phages. The 1/2b-specific phage, LMD3, was the most virulent phage among 10 phages with a high EOP value of 0.75-1 against the tested bacterial strains. Serotype susceptibility to phage infection might differ due to the non-specific binding receptors on host cell wall or other resistant mechanisms such as defective adsorption, abortive infection, restriction/modification systems, inhibition of phage DNA in case of phage infection (Duckworth et al., 1981; Walker and Klaenhammer, 2003). Also, phages might contain tail fiber proteins which help to recognize the receptor molecules on bacterial cell wall, and restrict to bind to non-specific bacterial cells (Guttman et al., 2005; Singh et al., 2010).

Although distinct restriction profiles were noted, the *EcoRI* and *HinDIII* digestion patterns were similar in few isolated phage DNA. The images of two Myoviridae phages, LMA9 and LMA4, also displayed similar morphotype by TEM. Interestingly, phages with similar digestion profiles and matching appearance were differed in their lytic ability to infect target bacteria. It could be explained by the fact that virulent phages have highly similar genome sizes sharing homology over >90% of their length (Chopin et al., 2001). Spot test and EOP data (Tables 3 and 4) indicated that these phages exhibited differences in infecting the *L. monocytogenes* strains. The morphological features of the phages that comprised LP1 resembled previously identified lytic, potent phages which rapidly lysed *L. monocytogenes* cells without any DNA integration. (Ackermann, 2009; Carlton et al., 2005; Zink and Loessner, 1992). However, unlike other myoviridae phages, LMA8 and LMD3 had non-contractile tails similarly as A511 which was reported earlier as a new phage species (Zink and Loessner, 1992).

Host species specificity is desirable for potent phage application, but sometimes phages are not only serotype specific but also strain specific. This could become a liability with single dose of one phage cocktail if the target population contains mixed strains (Denou et al., 2009).

To overcome this limitation as well as strain resistance, the LP1 cocktail covers lytic effectiveness against multiple serotypes of *L. monocytogenes*. In bacterial challenge tests, the treatment with a single dose of LP1 was highly effective inactivating the mixture of target cells in TSB by 8.5 log CFU/mL at higher temperatures (23 and 30°C), and by 3.6 and 4.6 log CFU/mL at 4 and 10°C, respectively. These results suggested that this treatment could be implemented in a wide range of temperatures. However, the optimum conditions to achieve the maximum reduction by LP1 to lyse *L. monocytogenes* cells were varied by time, temperature and dose. Studies with the bacteriophage cocktail LMP-102 indicated that phages were mostly effective at pH 7-8, and achieved an more than 2 log (CFU/mL) reduction against *L. monocytogenes* strain LCDC 81-861 after 2 h of incubation at 30°C in liquid broth (Leverentz et al., 2003). That study is one of the few reports that have tested a mix of phages against *Listeria*, similar to this work, but it should be noted that activity was only reported after 2 h with an MOI of 1:1. Our phage cocktail required larger MOI (1:100 and 1:1000) to be effective against a mixture of 5 *Listeria* strains within the first 30 min. The use of a single *Listeria* strain may have resulted in a smaller number of particles of LMP-102 needed to cause a significant reduction in 2 h.

As suggested previously, use of phage cocktail cannot be successful if bacterial strains develop resistance against the specific phage treatment. The emergence of bacterial resistance can be handled mainly by use of a mixture of virulent phages of wide host range (Guenther et al., 2009). We have not identified any emergence of bacterial resistance with LP1 cocktail treatments in this study. However, the effectiveness of LP1 as an antibacterial was greatly dose-dependent.

5. Conclusion

L. monocytogenes is a serious foodborne pathogen of concern for public health. With the currently available methods to control pathogen contamination, lytic phages could be used as a very useful tool to control a wide range of pathogenic bacteria. The data presented in this study demonstrated the lytic efficacy of this phage cocktail to control *L. monocytogenes* multiple strains at different temperatures. This study was the initial step to identify a potent phage cocktail to control *L. monocytogenes*, the suitability of LP1 as a safe and effective *L. monocytogenes* decontaminate on food matrices and processing equipment needs to be further explored.

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505 *Figure captions*

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507 **Figure 1** Morphological features of selected bacteriophages against *Listeria monocytogenes* by
508 transmission electron microscopy (TEM)

509 **Figure 2** Lysis of a mixture of five *Listeria monocytogenes* strains by the lytic phage cocktail,
510 LP1 at different levels of MOI (host:phage), time of incubation and temperature in tryptic soy
511 broth (TSB). Values within the 0.5/1 h time point and different types of LP1 treatment doses followed by
512 the same capital letter are not significantly different and values of the 24/48 h time point with different
513 types of LP1 treatment doses followed by the same lowercase letter are not significantly different ($p >$
514 0.05)

515

516

517 **Table 1**

518 List of *Listeria monocytogenes* strains with serotypes and isolation sources used for this study

Serotypes	Strain ID	No. of strains	Source
4b (I)	FSL J1-110	6	Mexican style cheese, epidemic, LA 1985
	FSL J1-225		Human epidemic (MA, USA), 1983
	FSL N1-227		Food (hot dog), epidemic (USA), 1998-99
	FSL R2-500		Mexican style cheese, epidemic (NC, USA), 2000
	FSL C1-122		Human sporadic case, 1988
	FSL J1-119		Human sporadic case
4b (III)/ 4b	Scott A	3	Food outbreak, Massachusetts, 1983
	7776		Food (Frankfurter)
	FSL J1-158		Animal goat, 1997
4a (III)	FSL J1-031	2	Human sporadic case
	FSL J1-168		Human sporadic case, 1996
3c (I)	FSL J1-049	1	Human sporadic case;
3b (I)	FSL J1-169	1	Human sporadic case, 1996
3a (II)	FSL C1-115	1	Human sporadic, 1998
1/2c (II)	FSL J1-094	1	Human sporadic, WHO strain collection
1/2b (I)	FSL J2-064	3	Animal, cow, 1989
	FSL R2-502		Chocolate milk, epidemic (IL, USA), 1994
	FSL J1-177		Human sporadic case, 1997
1/2a (II)	FSL J2-031	4	Animal, bovine, 1996
	FSL N3-031		Food (hotdog), sporadic
	FSL C1-056		Human sporadic case
	ATCC 51775		Cheese
N/A	ATCC 15313	1	Rabbit

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Table 2 List of isolated bacteriophages against *Listeria monocytogenes* from swage samples

List of isolated phages	<i>L. monocytogenes</i> host strain	No. of phages
LMA1, LMA2, LMA3, LMA4, LMA5, LMA6, LMA7, LMA8, LMA9, LMA10, LMA11, LMA12	Scott A	12
LMB1, LMB2, LMB3, LMB4	FSL N3-013	4
LMC1, LMC2, LMC3, LMC4	FSL R-2500	4
LMD1, LMD2, LMD3, LMD4, LMD5	FSL J2-064	5
LMG1, LMG2, LMG3, LMG4	FSL J2-031	4
LME1, LME2, LME3, LME4	FSL C1-056	4
LMF1, LMF2, LMF3	FSL J1-169	3
LMH1	FSL J1-110	1

Table 3 Determination of susceptibility of different serotypes of *Listeria monocytogenes* to phage infection by spot testing

Phages	% of positive spots against different serotypes of <i>Listeria monocytogenes</i>								
	4a (III) (n=2)	4b (I) (N=6)	4b (III) (N=3)	3a (II) (N=1)	3b (I) (N=1)	3c (I) (N=1)	1/2a (II) (N=4)	1/2b (I) (N=3)	1/2c (II) (N=1)
LMA1	100	67	67	100	100	0	100	33.3	100
LMA2	100	50	66.7	100	100	0	100	33.3	100
LMA3	100	50	66.7	100	100	0	100	33.3	0
LMA4	100	100	33.3	100	100	0	100	33.3	100
LMA5	100	100	33.3	100	0	0	75	33.3	100
LMA6	100	100	100	100	100	0	100	33.3	100
LMA7	100	100	100	100	100	0	75	33.3	100
LMA8	100	100	100	100	100	0	100	33.3	100
LMA9	100	100	100	100	100	0	100	33.3	100
LMA10	100	66.7	33.3	100	0	0	75	33.3	100
LMB2	0	66.7	33.3	100	100	0	100	33.3	100
LMB3	50	66.7	33.3	100	100	0	100	33.3	100
LMB4	0	0	0	100	100	0	100	100	100
LMD1	50	0	0	100	0	0	75	33.3	0
LMD3	100	16.7	66.7	100	0	0	75	33.3	100
LMD4	100	66.7	66.7	100	0	0	75	33.3	100
LME2	0	16.7	0	100	100	0	50	33.3	100
LME3	50	83.3	66.7	100	100	0	100	33.3	100
LMF1	0	0	0	100	100	0	75	33.3	100
LMF2	0	16.7	33.3	100	100	0	100	33.3	0
LMG1	0	67.7	33.3	100	100	0	100	33.3	100
LMG2	0	50	33.3	100	100	0	100	33.3	100
LMG3	100	16.7	33.3	100	0	0	75	66.7	0

538 **Table 4** Efficiency of plaquing (EOP) of bacteriophages against different *Listeria monocytogenes* strains

<i>L. monocytogenes</i> Strains/ Serotype	Phage isolates (EOP) Efficiency of plaquing									
	LMB3	LMD3	LMD4	LMA4	LMA5	LMA6	LMA7	LMA8	LMA9	LME3
J1-031/ 4a(III)	+	+++	+++	-	-	-	+	+++	+++	+
C1-056/ 1/2a(II)	+++	+++	-	+++	+++	+++	++	+++	+++	Host
J2-031/ 1/2a(II)	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
Scott A/ 4b	+++	+++	+++	Host	Host	Host	Host	Host	Host	+
J1-094/ 1/2c(II)	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
J2-064/ 1/2b(I)	+++	Host	Host	+++	+++	+++	++	+++	++	+
J1-168/ 4b	+	+++	+++	+++	+++	+++	+++	++	++	+
C1-115/ 3a(II)	+++	+++	+++	+++	++	++	+++	+++	++	+
R2-500/ 4b	+++	+++	+++	+++	+++	+++	+++	+++	++	++
51775/ 1/2a (II)	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
N3-031/ 1/2a (II)	Host	-	-	-	-	-	-	-	-	-

539 *+++; EOP 1 to 0.5; ++, EOP 0.5 to 0.2; +, EOP 0.2 to 0.001; -, (< 0.001) bacterial strain was not susceptible to phage attack

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541

542 **Figure 1**

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Figure 2