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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 *Listeria monocytogenes* is a food-borne pathogen which causes listeriosis and is difficult to eradicate from seafood processing environments; 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 $\approx$9 $\log_{10}$ PFU/ml, in the lysis of three seafood-borne *L. monocytogenes* strains (19CO9, 19DO3 and 19EO3) adhered to a fish broth layer on stainless steel coupon (FBSSC) and clean stainless steel coupon (SSC), in 7-day biofilm, and dislodged biofilm cells at 15 $\pm$ 1 °C. Single phage treatments (LiMN4L, LiMN4p or LiMN17) decreased bacterial cells adhered to FBSSC and SSC by $\approx$3–4.5 log units. Phage cocktail reduced the cells on both surfaces ($\approx$3.8–4.5 and 4.6–5.4 $\log_{10}$ CFU/cm^2, respectively), to less than detectable levels after $\approx$75 min (detection limit = 0.9 $\log_{10}$ CFU/cm^2). The phage cocktail at $\approx$5.8, 6.5 and 7.5 $\log_{10}$ PFU/cm^2 eliminated *Listeria* contamination ($\approx$1.5–1.7 $\log_{10}$ CFU/cm^2) on SSC in $\approx$15 min. One-hour phage treatments (LiMN4p, LiMN4L and cocktail) in three consecutive applications resulted in a decrease of 7-day *L. monocytogenes* biofilms ($\approx$4 $\log_{10}$ CFU/cm^2) by $\approx$2–3 log units. Single phage treatments reduced dislodged biofilm cells of each *L. monocytogenes* strain by $\approx$5 $\log_{10}$ CFU/ml in 1 h. The three phages were effective in controlling *L. monocytogenes* on stainless steel either clean or soiled with fish proteins which 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 that some disruption of the biofilm cells from the surface prior to phage application will be required.

Keywords *Listeria* phages · Biofilms · Low count cells · Stainless steel · Low temperature

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Introduction

Food products including ready-to-eat seafood contaminated with *Listeria monocytogenes* have caused highly devastating listeriosis outbreaks [4, 9, 21, 26, 65]. In addition to the high mortality rates of up to 30 % among the cases affected, the management of listeriosis outbreaks and clinical cases is costly [39, 53]. Foods

contaminated with *L. monocytogenes* also result in costly food recalls globally [15, 70]. The bacterium is ubiquitous in the environment and, therefore, has become a major pathogen in the seafood processing industry, where food is harvested, processed and stored [20, 21, 26, 66]. *L. monocytogenes* colonizes and persists in processing environments owing to its ability to form biofilms, grow at low temperatures, and its tolerance to up to 10 % (w/v) sodium chloride [16, 26, 66]. The *Listeria* biofilms shield cells from desiccation and decontaminating treatments on food contact surfaces [14, 49, 50, 68]. This biofilm contaminant also develops stress hardening [43, 57] and resistance against regular preservatives and chemical disinfectants [49, 50]. These factors make this pathogen difficult to eliminate from processing environments and, therefore, poses a significant risk of cross-contamination of foods during processing [14, 66].

Currently, consumers prefer the use of natural antimicrobial agents which are generally recognized as safe (GRAS) and eco-friendly alternatives in food processing environments because of potential health hazards of synthetic chemical preservatives [31, 40]. Wirtanen and Salo [69] have reported the potential to detect residual chemicals on food contact surfaces after the sanitation procedures.

Some essential oils of plant extracts, nisin and lactic acid bacteria (LAB) cultures have been reported to significantly inactivate *L. monocytogenes* biofilm cells [8, 40, 41]. Lytic bacteriophages (phages) and phage endolysins are also biodegradable non-toxic food grade bactericidal agents that specifically lyse particular bacteria [33, 58]. The products containing phages specific for *Listeria*, *Salmonella* and *Escherichia coli* have been approved as food additives, decontaminating agents on food processing surfaces and awarded GRAS status by the US Food and Drug Administration (FDA) and Environmental Protection Agency (EPA) [22, 37, 46, 62]. Some of these *Listeria*-phage-containing products have also been approved for use in food industries by respective regulatory bodies in Switzerland, Australia and New Zealand [24, 31, 46, 62].

Listeria phages control *L. monocytogenes* contamination in a variety of food matrices including seafood [31, 32, 59, 61]. Phage P100, at a dose of 10^8 PFU/g, decreased a mixed culture of *L. monocytogenes* EGD and Scott A strains at initial concentrations of $\approx$2, 3 and 4.5 $\log_{10}$ CFU/g on salmon fillets by 1.8, 2.5 and 3.5 log units CFU/g, respectively, in 30 min, at either 4 or 22 °C [59]. In another study, phage P100 was assessed for the control of a two-strain mixture of *L. monocytogenes* [EGD (1/2a) and Scott A (4b)] in catfish tissues [61]. When using the phage P100 at 2×10^7 PFU/g, the authors reported that the initial *L. monocytogenes* contamination level ($\approx$4.3 $\log_{10}$ CFU/g) in catfish tissues was reduced by $\approx$0.8 and 1.3 $\log_{10}$ CFU/g after 15 and 30 min, respectively, at 22 °C, with no further significant reduction for $\approx$1.5 h ($p > 0.05$). Therefore, the results suggested that the catfish tissues should be treated with phages for at least 30 min for the best control of *L. monocytogenes* [61]. However, the efficacy of these phages on seafood cannot be extrapolated to commercial seafood processing environments because *L. monocytogenes* strains used in these experiments were of non-seafood origin [19]. Guenther et al. found that phage A511 was less effective than P100, being reduced by about 0.5–1 log units from an initial *L. monocytogenes* concentration of 3×10^8 PFU/g on smoked fish over 1 day at 6 °C compared to complete lysis of the same initial level of *Listeria* contamination on other high-moisture food surfaces. Therefore, the report suggested using high phage doses and larger liquid volumes to overcome the problem thought to be due to the dry and uneven surface of smoked fish [32].

The phage cocktail ListShield™ has been approved as a decontaminating agent (microbial pesticide) by the EPA to control *L. monocytogenes* on food contact surfaces [62]. However, the decontamination efficacy of ListShield™ on abiotic surfaces has not yet been reported to our knowledge. Different *Listeria* phages are reported to reduce bacteria contaminations on surfaces such as stainless steel and polypropylene commonly used in food processing plants under different ambient conditions. The lysis of two strains of *L. monocytogenes* adhered to stainless steel and polypropylene was investigated with three *Listeria* phage strains (2671, H38 and H387-A) at 26 °C in 1 h [52]. Hibma et al. [35] investigated the control of cell-wall-deficient *L. monocytogenes* (L-form) adhered to stainless steel using a modified phage (BRED) at 30 °C over 6 h. Soni and Nannapaneni [60] examined the efficiency of P100 in the degradation of 2- and 7-day-old biofilms formed by five *L. monocytogenes* strains under static conditions on stainless steel at 22 °C. Montanez-Izquierdo et al. [47] evaluated the effect of P100 on a 3-day biofilm formed by *L. monocytogenes* CCUG 15526 on stainless steel at 22 °C. Despite the existence of several reports on the effectiveness of phages, the technologies of phage-based control of biofilms have not yet been fully developed [63]. We compared the surface decontamination efficacies of the *Listeria* phages in the aforementioned studies with the data of this study. According to the above investigations, the lytic effectiveness of *Listeria* phages

against seafood-borne *L. monocytogenes* strains at high and low counts on abiotic surfaces and in biofilms at low temperature (15 °C) has not been reported elsewhere.

The biocidal activity of phages needs to be investigated in the environmental conditions relevant to their potential applications. The efficacy of disinfectants is affected by the presence of soil, hard water and temperature [14, 29]; the lytic activity of phages is also variable depending upon the conditions of the environment [32, 33]. This study investigated the biocidal effect of three novel phages against three *L. monocytogenes* strains adhered to stainless steel surfaces (clean and conditioned with fish proteins) and in a biofilm at low temperature. Dislodged *L. monocytogenes* biofilm cells were also challenged with each phage to compare the resultant efficacy with that on treatment of undisturbed biofilms.

Materials and methods

Listeria monocytogenes strains and *Listeria* phages

L. monocytogenes strains 19CO9 (1/2a or 3a), 19DO3 (1/2a or 3a) and 19EO3 (4b, 4d or 4e) isolated from seafood processing environments [16] were used in this study. *L. monocytogenes* strains were maintained and subcultured 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, which were isolated from a seafood waste water treatment unit of a seafood processing plant in New Zealand, used in this research project in 2011 are stored in the Culture Collection of Massey University [27, 28]. Using *L. monocytogenes* 19CO9, we propagated three phages individually at 25 ± 1 °C. Phage lysates were prepared using the TSA double layer agar (DLA) method [56]. The phage lysate was centrifuged at 8,000×*g* for 20 min, *L. monocytogenes* culture cells were lysed with $CHCl_3$ (Analytical Grade, Merck, Darmstadt, Germany) overnight, centrifuged at 8,000×*g* for 20 min, and ultracentrifuged at 25,000×*g* (SS-34 rotor, Sorvall RC6 + Centrifuge, Sorvall, Frankfurt, Germany) at 4 °C for 6 h [3]. The phage pellet was re-suspended in 1 ml phosphate buffered saline (PBS) [8 mM Na_2HPO_4 (Merck), 1 mM $NaH_2PO_4{\cdot}H_2O$ (Merck) and 145 mM NaCl (Merck); pH 7.5] and stored at 4 °C in the dark. The phage titre was assayed by the DLA method [38] using saline–magnesium buffer (SM) [100 mM NaCl, 50 mM Tris–HCl (Sigma-Aldrich, Steinem, Germany), 8 mM of $MgSO_4$ (Merck), 0.001 % of gelatine solution (LabChem, NSW, Australia) at pH 7.5] as diluent and TSA. The phages were used in experiments either individually or as a three-phage cocktail. Each phage treatment suspension contained approximately 9 $\log_{10}$ PFU/ml.

Test surfaces

Stainless steel coupons

Stainless steel (grade 304-2B) coupons (SSC) measuring 1 × 1 × 0.09 cm^3 were cleaned by soaking in acetone (Analytical Grade, Merck) 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 (5 × 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 (2 × 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 with fresh cod fillets using the method given by Bernbom et al. [6]. The fish broth based conditioned layer on stainless steel coupons (FBSSC) was formed by dipping the coupons in the prepared fish broth 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.

Attachment of high count cells 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×*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 to give a final concentration of ≈8 $\log_{10}$ CFU/ml (A_{600nm}, ≈0.10). 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, drained by touching the edge of a coupon on sterile blotting paper and then rinsed in 20 ml sterile potable water (three times) to remove any unattached cells from the test surfaces. A batch of coupons ($n = 4$) was analysed 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 which represents a possible delay in factory cleaning. After incubation, the coupons were rinsed with sterile tap water to remove loosely attached cells. A batch of coupons ($n = 4$) was also analysed, as described below, to enumerate the surviving cells after incubation for 5 h and the remaining coupons were used for the next stages of the experiment.

Phage lysis of high count cells of *L. monocytogenes* adhered to surfaces

The coupons with adhered cells ($n = 20$) pertaining to one strain were subjected 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-treated surfaces were enumerated after dislodgment from the surface (Table 2).

Briefly, 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 ($\phi = 2$ mm) and agitated on a vortex mixer (WISE Mix VM-10, Daihaw Scientific Company Ltd, Korea) at maximum speed for 2 min [71]. 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 to the surfaces was expressed in terms of the specific adhesion (SA) value [6] (Table 1).

Phage lysis of low count cells of *L. monocytogenes* in fish proteins adhered to 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 diluted to contain ≈8.6 $\log_{10}$ CFU/ml (A_{600nm}, ≈0.25) centrifuged at 8,000×g for 10 min and then re-suspended in 10 % (v/v) fish broth (FB) to give cell suspension containing ≈4.6 $\log_{10}$ CFU/ml. A total of 2 µl of a cell suspension of *L. monocytogenes* 19CO9 strain, either alone or in a three-strain mix (≈2 $\log_{10}$ CFU/2 µl), was spread on SSC using a short bar (≈0.5 cm) glass spreader prepared from a Pasteur pipette and allowed to dry for 5–10 min in a biohazard safety cabinet (Hera Safe 18, Heraeus Safety Cabinet-Class 2, Hanau, Germany). Aliquots (3 µl) of a cocktail of three phages (LiMN4L, LiMN4p and LiMN17) at four concentrations (≈7.3, 8.3, 9.0 or 10.0 $\log_{10}$ PFU/ml) were spread over the host cell smears on the SSC using a fresh glass spreader while 3 µl of PBS were spread over the control surfaces. The inoculated coupons were incubated in a closed Petri dish at 15 ± 1 °C for ≈5, 10 and 15 min. The viable cells were dislodged from coupons by agitating each coupon without glass beads in 5 ml of chilled PBS on a vortex mixer for 2 min at high speed. Two-millilitre aliquots of dislodged cell suspension were centrifuged at 8,000×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, UK) (detection limit = 0.39 $\log_{10}$ CFU/cm^2). Duplicate plates inoculated for each SSC and plates were incubated at 35 ± 1 °C for 48 h. The phage treatments, which reduced adhered cells to undetectable levels, were further investigated by adopting a filter technique in the

Table 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

L. monocytogenes strain	Initial counts of cell adhesion bath ($\log_{10}$ CFU/ml)	FBSSC		SSC	
		Cell adhesion level ($\log_{10}$ CFU/cm^2)	SA	Cell adhesion level ($\log_{10}$ CFU/cm^2)	SA
19CO9	8.5 ± 0.00	3.8 ± 0.03	-4.7^{cd} ± 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 mixture	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 significant 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 to surface (CFU/cm^2) to initial count of cell adhesion bath (CFU/ml) [7]

enumeration of the dislodged viable cells in order to increase the detection limit.

Briefly, the total 5-ml volume of dislodged cell suspension obtained as described above was filtered through sterile a 0.45-μm cellulose membrane filter ($\phi = 47$ mm; Advantec MFS, Inc, CA, USA) in less than 2 min using a sterile suction filtration apparatus. The test filter was placed with the cell-retaining face down on a PALCAM agar (Difco) plate and incubated in a plastic bag at 35 °C for 72 h (detection limit = 1 CFU/cm^2). Additionally, the adhered cells of phage-treated and control coupons were enriched individually in 10 ml of *Listeria* Enrichment Broth (LEB) (Difco) at 30 °C for 72 h. Enrichments of each coupon were streaked on PALCAM agar plates separately and incubated at 35 °C for 72 h in order to observe growth of typical *L. monocytogenes* colonies [36]. Four replicate coupons were analysed for each treatment and control.

Preparation of 7-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×*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 7 days, while replacing the liquid phase culture with 1/10 strength TSB every 2 days [60]. The loosely attached cells on the 7-day-old biofilm were dislodged by rinsing the coupons in 3 × 20-ml aliquots of sterile potable water.

Phage lysis of 7-day-old biofilm

Seven-day-old biofilms were investigated with three different phage applications (LiMN4L, LiMN4p or cocktail). Each application consisted of three 1-h consecutive infection cycles at 15 ± 1 °C. In each cycle of a phage application, a batch of four coupons was analysed 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 analysed 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 viable biofilm cells on coupons were enumerated following the same protocol used for enumeration of cells adhered to coupons. Cells were dislodged using glass beads, as described above, except the biofilm coupons were agitated on the vortex mixer for 4 min. From prior trials an agitation time of 4 min was determined to be the optimum time required to dislodge the greatest number of cells into PBS. Each coupon represented one replicate.

Preparation of dislodged 7-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* strains (19CO9, 19DO3 and 19EO3) prepared as per the previous biofilm experiment. The cells were allowed to attach and form biofilms on steel fibres (Ben Somerton and Steve Flint, personal communication, 11 May 2012) at 15 ± 1 °C for 24 h followed by incubation for 7 days statically, while replacing medium with 1/10 TSB every 2 days. The biofilms grown on the stainless steel fibres were rinsed with 100 ml sterile tap water five times to remove loosely attached cells. The biofilm cells were dislodged from the stainless steel fibres by hand-shaking tubes containing 20 ml of PBS and 30 g of glass beads ($\phi = 2$ mm) for 5 min. Suspended biofilm cells in PBS were investigated for phage sensitivity.

Phage lysis of dislodged 7-day-old biofilm cells in suspension

The biofilm cells dislodged were washed twice in PBS by centrifuging at 5,000×*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-millilitre 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 for each treatment and incubated at 30 ± 1 °C for 72 h prior to colony enumeration.

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 [11], rinsed in sterile MilliQ water and blotted on Whatman™ paper. The stained coupons were observed under epifluorescence 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).

Data analysis

The means and standard errors of viable cell counts were calculated using Microsoft Office Excel 2007 (Microsoft Office, Washington, DC, USA). Mean cell counts of treatments were compared by analysis of variance of the 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., NC, USA).

Results

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

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 the mixture of the three *Listeria* strains, respectively (Table 1). From the SA values, strain 19DO3 showed a significantly lower ($p < 0.05$) 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 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 1). Epifluorescence microscopy also indicated adhesion of fewer cells of the three-strain mix on the FBSSC compared to the high cell counts adhered to the SSC (Supplemental Fig. 1a, b). Similar observations were found for the attachment of the individual *L. monocytogenes* cultures (results not shown).

Phage lysis of high count cells of *L. monocytogenes* adhered to FBSSC and SSC surfaces

The viable counts adhered to the coupons immediately after removing from the cell adhesion bath, after 5 h incubation following adhesion, and the controls were not significantly different ($p > 0.05$) for the phage-infection experiment on either FBSSC or clean SSC (Tables 1, 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 an undetectable level in ≈75 min (Table 2). Phage LiMN4p also decreased the initial counts of *L. monocytogenes* 19EO3 on SSC by more than 4.5 log units reaching an undetectable level. All 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 2).

Phage lysis of low count cells of *L. monocytogenes* adhered to SSC surfaces

The SSC contaminated with *L. monocytogenes* cells were treated with phages by manually spreading ≈4.8, 5.8, 6.5 and 7.5 $\log_{10}$ PFU/cm^2, respectively. At the higher levels of 5.8, 6.5 and 7.5 $\log_{10}$ PFU/cm^2, the phage cocktail lysed the initial counts (≈1.5 $\log_{10}$ CFU/cm^2) of *L. monocytogenes* 19CO9 attached to the SSC to an undetectable level in 15 min (data not shown). At these three dose levels, the phage cocktail also reduced the initial contamination level (≈1.5–1.7 $\log_{10}$ CFU/cm^2) of a three *L. monocytogenes* strain mix on SSC to an undetectable level (Fig. 1a). However, the lower level of phage cocktail at 4.8 $\log_{10}$ PFU/cm^2 decreased the three-strain mixture of *L. monocytogenes* by only ≈0.5 log units in 15 min (Fig. 1a). In an independent experiment, the three-strain mix (≈1.5 $\log_{10}$ CFU/cm^2) on SSC was decreased by a phage cocktail (dose level of 6.5 $\log_{10}$ PFU/cm^2) to 0.78 and 0.68 $\log_{10}$ CFU/cm^2 after 5 and 10 min, respectively, and complete decontamination occurred after 15 min (Fig. 1b). The complete decontamination of the surfaces by the respective phage treatments was confirmed as colonies were not formed on filters retaining the dislodged cell suspensions (<1 CFU/cm^2) and growth did not occur in LEB.

Phage lysis of 7-day-old biofilms on stainless steel surfaces

The counts of biofilms that were re-infected with three different phage applications (LiMN4L, LiMN4p and cocktail) separately compared to the initial control biofilms (≈4 $\log_{10}$ CFU/cm^2) are given in Fig. 2a–c, respectively. The phage LiMN4L reduced cells by ≈0.5, 0.7 and 0.3 log

Table 2 Cells adhered to 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 by dipping in the phage suspensions for 15 min followed by incubation in air for 1 h 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
	SSC	5.2 ± 0.03	5.2 ± 0.02	1.1 ± 0.11	0.5 ± 0.00	1.0 ± 0.39	<DL
19DO3	FBSSC	4.5 ± 0.05	4.5 ± 0.04	0.6 ± 0.17	0.7 ± 0.20	0.9 ± 0.00	<DL
	SSC	5.4 ± 0.06	5.4 ± 0.02	0.8 ± 0.26	1.0 ± 0.13	1.2 ± 0.15	<DL
19EO3	FBSSC	3.8 ± 0.04	3.8 ± 0.06	0.5 ± 0.00	0.8 ± 0.00	0.7 ± 0.17	<DL
	SSC	4.6 ± 0.06	4.6 ± 0.11	0.5 ± 0.00	<DL	0.6 ± 0.21	<DL
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
	SSC	5.3 ± 0.10	5.4 ± 0.14	0.8 ± 0.28	0.5 ± 0.00	0.8 ± 0.12	<DL

DL detection limit (0.9 $\log_{10}$ CFU/cm^2) for enumeration of viable counts

[a] Number of coupons = 4

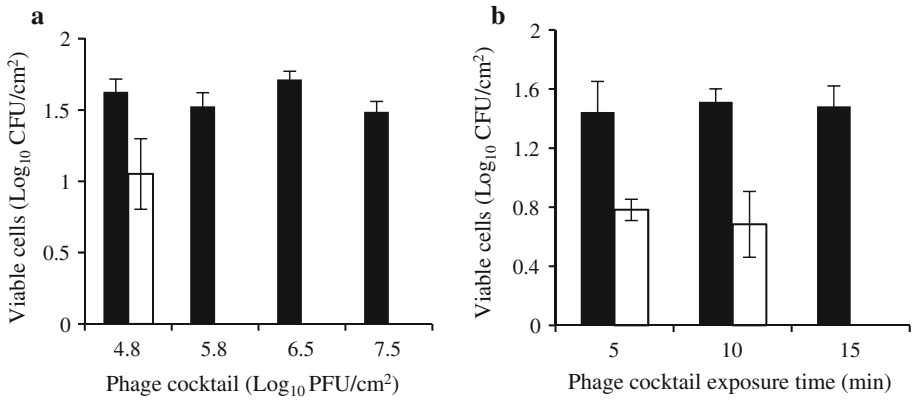

Fig. 1 Viable counts of three *L. monocytogenes* strains (19CO9, 19DO3 and 19EO3) adhered to 10 % fish proteins broth on stainless steel at 15 °C. Counts were enumerated on PALCAM agar (detection limit = 0.39 $\log_{10}$ CFU/cm^2). **a** Culture infected with three-phage cocktail (LiMN4L, LiMN4p, LiMN17) at ≈4.8, 5.8, 6.5 and 7.5 $\log_{10}$ PFU/cm^2 for 15 min (*white bar*); control culture (*black bars*). **b** Three-phage cocktail at ≈6.5 $\log_{10}$ PFU/cm^2 for 5, 10 and 15 min (*white bars*); control culture (*black bars*). *Error bars* indicate standard error of mean CFU/cm^2, $n = 4$

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. 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. 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. 2c). The cells of control biofilms during the second and third stages also decreased in all three phage applications ($p < 0.05$) (Fig. 2a–c). Three phage applications caused the gradual thinning of biofilms and images relevant to phage cocktail are given in Supplemental Fig. 2a–d.

Phage lysis of dislodged 7-day-old biofilm cells

The initial counts of the dislodged 7-day-old biofilm cells of *L. monocytogenes* strains 19CO9, 19DO3 and 19EO3 were ≈5.3–5.5 $\log_{10}$ CFU/ml (Fig. 3). These counts were not significantly different ($p > 0.05$) from the counts of control biofilm cells (Fig. 3). Treatment with individual phages LiMN4L, LiMN4p and LiMN17 decreased the dislodged biofilm cell concentrations of *L. monocytogenes* 19DO3 and 19EO3 by more than 5 log units reaching an undetectable level, showing a similar lytic efficacy. Each of

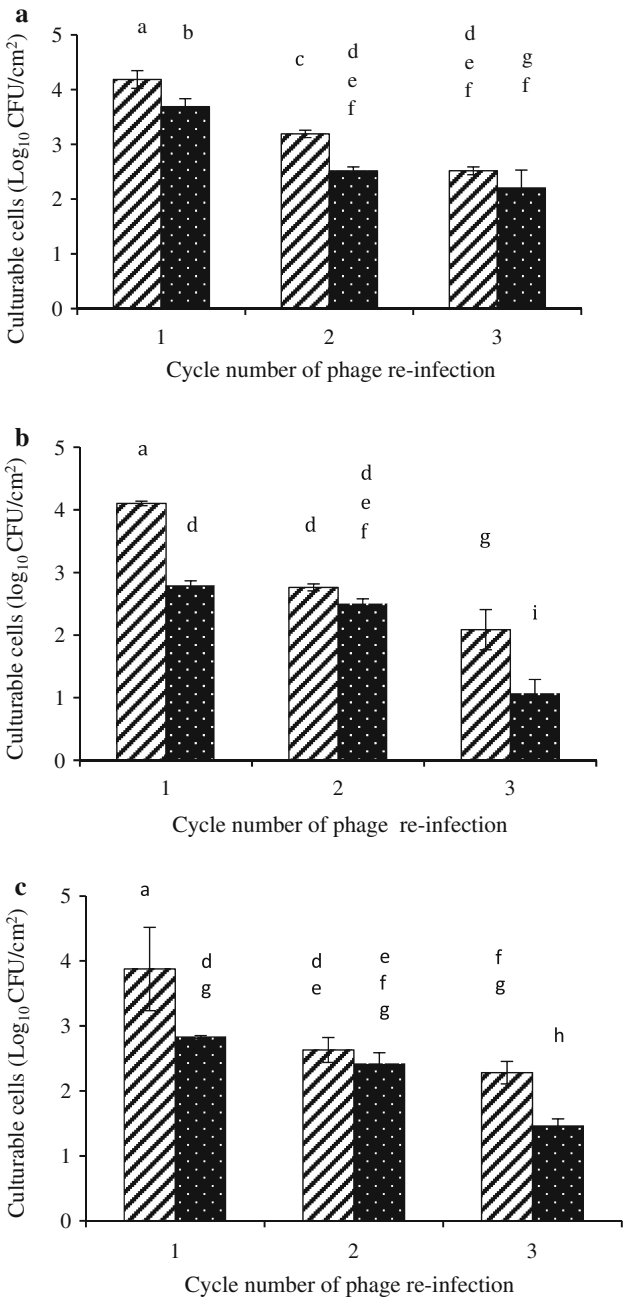

Fig. 2 Counts of three-strain mix 7-day-old *L. monocytogenes* (19CO9, 19DO3, 19EO3) biofilm on stainless steel coupon after three phage applications: **a** phage LiMN4L, **b** phage LiMN4p, **c** cocktail (LiMN4L, LiMN4p, LiMN17). Phage treatment consisted of three repeat-infection cycles on biofilm against the 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*) report significance difference across treatments and controls of re-infection cycles (1–3) and three phage applications (**a–c**) at $p < 0.05$

the three single-phage treatments reduced the dislodged biofilm cells of 19CO9 strain by more than 4.5 log units (Fig. 3).

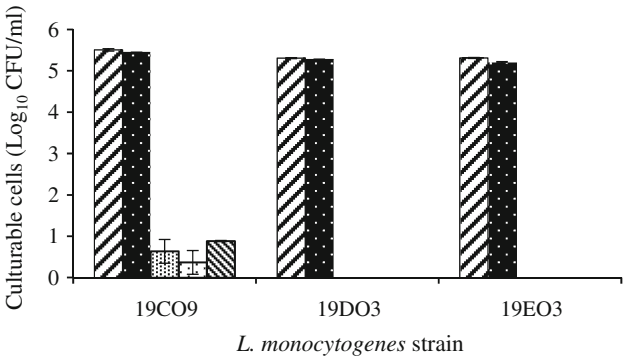

Fig. 3 Counts of dislodged 7-day-old biofilm cell cultures of individual *L. monocytogenes* strains (19CO9, 19DO3, 19EO3) after infection with individual phages LiMN4L, LiMN4p, LiMN17 at ≈ 9 $\log_{10}$ PFU/ml in phosphate buffered saline at the 15 ± 1 °C for 1 h. Dislodged biofilm counts: ▨, initial; ▩, control parallel to phage infected culture; ▩, LiMN4L; ▩, LiMN4p; ▩, LiMN17

Discussion

In this experiment the use of stainless steel and fish protein conditioned stainless steel coupons at 15 °C with seafood-borne *L. monocytogenes* strains represents the natural contamination occurring in seafood processing plants. The variations in adhesion affinities of the strains could be assessed on the basis of the SA values [6] even though cell adhesion levels were similar in FBSSC (Table 1). Similar to the present result, an adhesion repulsion effect on surfaces coated in fish protein has been reported with different seafood-borne bacterial strains including *L. monocytogenes* N53-1, *Vibrio anguillarum*, *Pseudomonas aeruginosa* and *Aeromonas salmonicida* [6, 7]. 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 [12, 23, 44, 67], adhesion medium [17], temperature and pH [18, 34, 67]. Alpha-tropomyocin (35 kDa) of cod fish broth (extract) makes the surfaces more wettable (hydrophilic) and leads to repulsion of the bacteria adhesion [6, 7, 45]. The cells adhered to 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 [11, 67]. The cell counts did not vary significantly over 5 h and this may be attributed to 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. also reported a higher efficacy of a three-phage cocktail compared to monophage

applications. Three phage strains tested individually and in a cocktail (8.5 $\log_{10}$ PFU/ml) decreased the initial counts ($\approx$4–5 $\log_{10}$ CFU/ml) of two *L. monocytogenes* strains separately adhered to 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 [52]. Hibma et al. [35] showed that the attachment of L-form *L. monocytogenes* cells on SSC was prevented at 30 °C for 6 h using a cell suspension of $\approx$5 $\log_{10}$ CFU/ml treated with the specific phage ($\approx 10^9$ PFU/ml). The ability of phages to decontaminate cells recently attached to clean and protein-contaminated surfaces (FBSSC), as demonstrated in the present study, is important at an early stage to prevent colonization and the formation of biofilm [12, 48]. These investigations all report lytic efficacy of *Listeria* phages against comparatively high count surface contaminations. Ideally, in industrial applications treatment with bacteriophage should be relatively short and be effective in the conditions found in an industrial environment. Therefore, this study investigated phage-based decontaminations of low counts of cells adhered to stainless steel in the presence of fish proteins at low temperature, as would be expected in a seafood processing plant.

The inoculation of low counts of cells on SSC in a fish broth was followed by $\approx$5–10 min of drying to simulate conditions of a fish processing 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. A phage cocktail at greater than 5.8 $\log_{10}$ PFU/cm^2 with a 15-min intervention time eliminated the contamination on the SSC. The rapid surface decontamination of *Listeria* cells may result from a passive bicontrol strategy [30]. The latent time (lysis time) of the three phages is 240–270 min at 15 °C (unpublished results). The intervention time for a passive bicontrol strategy is shorter than for an active control approach which relies on phage replication [1]. Therefore, in this trial, the phages may have been sufficiently uniformly spread to allow infection of all contaminating cells without the need for phage replication. Phage infection following irreversible adsorption on a bacterium results in the infected bacterium losing its own synthetic pathways [1, 2] and the cell dies. The high adsorption capabilities of phages will be advantageous in ensuring fast cell lysis [1, 25]. The three phages (LiMN4L, LiMN4p, LiMN17) consisted of numerous side tail fibres (unpublished results) which indicated high adsorption capacities [25] and had high adsorption rate constants [$(1.5–2) \times 10^{-9}$ ml/min] on the *L. monocytogenes* strains used in this study (unpublished results). In another study, initial counts ($3.5 \times 10^3 - 10^4$ CFU/6.25 cm^2) of *E. coli* O157:H7 attached to gypsum surfaces pre-conditioned with 5 % skim milk were lysed by approximately 100 % by a three phage cocktail (10 $\log_{10}$ PFU/ml) after 5 min exposure [2]. Another study reported complete elimination of a *Yersinia* strain cell contamination (2×10^4 CFU/6.25 cm^2) on stainless steel by applying 100 µl of suspensions containing sufficiently high titres (10^9, 10^8, and 10^7 PFU/ml) for $\approx$5 min [51]. However, the decontamination time of 5 min in these two experiments cannot be compared with results from the present study because of differences in experimental protocols and intrinsic factors of the phage strains and bacterial genera used in the different experiments.

The efficacy of 3 µl ($\approx$8.3 $\log_{10}$ PFU/ml) of phage suspension per 1 cm^2 can be extrapolated to indicate the potential for 3 ml of phage to treat approximately 1,000 cm^2 of stainless steel surface. For the commercial phage preparation, ListShield™, the recommendation is to use 1 ml ($\approx$9 $\log_{10}$ PFU/ml) to treat over 500 cm^2 of food or processing surfaces against *L. monocytogenes* [37]. However, intervention time relevant to the decontaminating action of this product is not specified. The efficacy of the phage cocktail used as a decontaminating agent in this study will need to be investigated on other types of contact surfaces such as polypropylene in order to broaden potential areas of application. There are concerns over the emergence of phage-resistant variants with regular use of phages as decontaminating agents [19] which will need to be investigated further. However, the use of a phage cocktail reduces likelihood of selecting phage-resistant mutants [52, 62, 64], especially if such a cocktail consists of phages that bind specifically to different receptors on the target bacterium [64].

Three *Listeria* strains in a mixed biofilm grown statically at 15 °C for 7 days aimed to replicate the stagnant environment of seafood processing surfaces. All three phage applications (Fig. 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. 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. 2b, c) may be due to the infection of cells located deep in the biofilm owing to the penetration of phages through voids in the biofilms [13] 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. 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. [47] reported that treatment of a 3-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. investigated phage (ΦIBB-PF7A) lysis of a 7-day-old biofilm of *Pseudomonas fluorescens* formed on 1 × 1 cm^2 stainless steel under static conditions, replacing the medium at 2-day intervals. In this study [54], 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 [54].

In the present work, the phages showed reduced efficacy in the lysis of intact biofilm cells compared with recently attached exponential phase planktonic cells (Fig. 2; Table 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, 49]. Soni and Nannapaneni [60] reported that 24-h intervention of phage P100 (9 $\log_{10}$ PFU/ml) in 2- and 7-day biofilms formed from a mix of five strains of *L. monocytogenes* on SSC (initial cell counts 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 log 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. [49] reported that 50 ppm of H_2O_2 decreased the dislodged cells of 7-day-old *L. monocytogenes* biofilm efficiently by ≈2.1–2.9 $\log_{10}$ CFU/cm^2 in 60 s compared with insignificant cell lysis (<0.3 $\log_{10}$ CFU/cm^2) of the undisturbed biofilm treated with 100 ppm of H_2O_2. These findings indicate that biofilm matrix limits the biofilm cell lysis efficacy of both chemical and phage treatments [47, 49, 54, 60]. The findings of the present study are also in agreement with previous reports because both dislodged biofilm cells and recently attached planktonic cells on SSC were reduced similarly (>5 log units) by the phage treatments. In addition to cell lysis by phage-infection, biofilm matrix can also be degraded using the engineered phages which bear exopolysaccharide (EPS) depolymerases [42, 63]. Lu and Collins [42] reported that 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 [47]. However, results of this study were based only on viable and culturable state 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) [60], differentiation of biofilm cells in situ by using live and dead staining [47] and scanning electron microscopy [10]. Sillankorva et al. studied the effectiveness of a phage (ΦIBB-PF7A) in controlling *P. fluorescens* in a 7-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 non-specific host. A reduction of both strains occurred by using a two-phage cocktail (ΦIBB-PF7A and ΦIBB-SL58B) which infected both strains [55]. Heterogeneous biofilms are likely to exist in a seafood processing plant [5, 11]. Therefore, the three *Listeria* phages used in the present study also need to be investigated in the presence of other bacterial genera.

Conclusions

The three *Listeria* phages used individually or in combination were effective in decontaminating stainless steel surfaces contaminated with seafood-borne strains of *L. monocytogenes* with or without fish protein coatings. The three-phage cocktail decontaminated the low count cells of seafood-borne *L. monocytogenes* strains on stainless steel at low temperature in 15 min. Phages were less effective, with a 1-h intervention time, against intact biofilm cells than attached exponential phase cells of *L. monocytogenes*. However, once dislodged from the matrix, the biofilm cells were sensitive to phage treatment. Therefore, our results indicate the need for other additional agitation treatments to maximize the effect of these phages on mature biofilms of *L. monocytogenes* at low temperature.

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References

1. Abedon ST (2009) Kinetics of phage-mediated biocontrol of bacteria. Foodborne Pathog Dis 6:807–815
2. Abuladze T, Li M, Menetrez MY, Dean T, Senecal A, Sulakvelidze A (2008) Bacteriophages reduce experimental contamination of hard surfaces, tomato, spinach, broccoli, and ground beef by *Escherichia coli* O157: H7. Appl Environ Microbiol 74:6230–6238
3. Ackermann H-W (2009) Basic phage electron microscopy. In: Clokie MRJ, Kropinski AM (eds) Bacteriophages methods and protocols. Volume 1: isolation, characterization, and interaction. Humana, Hertford, pp 113–126
4. Allerberger F, Wagner M (2009) Listeriosis: a resurgent foodborne infection. Clin Microbiol Infect 16:16–23
5. Bagge-Ravn D, Ng Y, Hjelm M, Christiansen JN, Johansen C, Gram L (2003) The microbial ecology of processing equipment

in different fish industries-analysis of the microflora during processing and following cleaning and disinfection. Int J Food Microbiol 87:239–250
6. Bernbom N, Jorgensen RL, Ng Y, Meyer R, Kingshott P, Vejborg RM, Klemm P, Besenbacher F, Gram L (2006) Bacterial adhesion to stainless steel is reduced by aqueous fish extract coatings. Biofilms 3:25–36
7. Bernbom N, Ng Y, Jorgensen RL, Arpanaei A, Meyer RL, Kingshott P, Vejborg RM, Klemm P, Gram L (2009) Adhesion of food-borne bacteria to stainless steel is reduced by food conditioning films. J Appl Microbiol 106:1268–1279
8. Bower C, McGuire J, Daeschel M (1995) Suppression of *Listeria monocytogenes* colonization following adsorption of nisin onto silica surfaces. Appl Environ Microbiol 61:992–997
9. Brett MSY, Short P, McLauchlin J (1998) A small outbreak of listeriosis associated with smoked mussels. Int J Food Microbiol 43:223–229
10. Briandet R, Lacroix-Gueu P, Renault M, Lecart S, Meylheuc T, Bidnenko E, Steenkeste K, Bellon-Fontaine MN, Fontaine-Aupart MP (2008) Fluorescence correlation spectroscopy to study diffusion and reaction of bacteriophages inside biofilms. Appl Environ Microbiol 74:2135–2143
11. Carpentier B, Chassaing D (2004) Interactions in biofilms between *Listeria monocytogenes* and resident microorganisms from food industry premises. Int J Food Microbiol 97:111–122
12. Chae MS, Schraft H (2000) Comparative evaluation of adhesion and biofilm formation of different *Listeria monocytogenes* strains. Int J Food Microbiol 62:103–111
13. Chavant P, Gaillard Martinie B, Hébraud M (2004) Antimicrobial effects of sanitizers against planktonic and sessile *Listeria monocytogenes* cells according to the growth phase. FEMS Microbiol Lett 236:241–248
14. Chmielewski R, Frank J (2003) Biofilm formation and control in food processing facilities. Compr Rev Food Sci Food Saf 2:22–32
15. Crerar SK, Castle M, Hassel S, Schumacher D (2011) Recent experiences with *Listeria monocytogenes* in New Zealand and development of a food control risk-based strategy. Food Control 22:1510–1512
16. Cruz CD, Fletcher GC (2011) Prevalence and biofilm-forming ability of *Listeria monocytogenes* in New Zealand mussel (*Perna canaliculus*) processing plants. Food Microbiol 28:1387–1393
17. Dat NM, Hamanaka D, Tanaka F, Uchino T (2010) Surface conditioning of stainless steel coupons with skim milk solutions at different pH values and its effect on bacterial adherence. Food Control 21:1769–1773
18. Di Bonaventura G, Piccolomini R, Paludi D, D'orio V, Vergara A, Conter M, Ianieri A (2008) Influence of temperature on biofilm formation by *Listeria monocytogenes* on various food-contact surfaces: relationship with motility and cell surface hydrophobicity. J Appl Microbiol 104:1552–1561
19. EFSA Panel on Biological Hazards (BIOHAZ) (2012) Scientific opinion on the evaluation of the safety and efficacy of ListexTM P100 for the removal of *Listeria monocytogenes* surface contamination of raw fish. EFSA J 10(3):2615
20. Eklund MW, Poysky FT, Paranjpye RN, Lashbrook LC, Peterson ME, Pelroy GA (1995) Incidence and sources of *Listeria monocytogenes* in cold-smoked fishery products and processing plants. J Food Prot 58:502–508
21. Ericsson H, Eklöw A, Danielsson-Tham M, Loncarevic S, Mentzing L, Persson I, Unnerstad H, Tham W (1997) An outbreak of listeriosis suspected to have been caused by rainbow trout. J Clin Microbiol 35:2904–2907
22. FDA (2006) Food additives permitted for direct addition to food for human consumption, bacteriophage preparation. 21 CFR Part 172 Fed Regis 71:47729–47732
23. Flint S, Brooks J, Bremer P (1997) The influence of cell surface properties of thermophilic *Streptococci* on attachment to stainless steel. J Appl Microbiol 83:508–517
24. Food Standards Australia New Zealand (2013) Application A1045—bacteriophage preparation as a processing aid. Food Standards Australia New Zealand. http://www.foodstandards.gov.au/foodstandards/applications/applicationa1045bact4797.cfm. Accessed 15 Jan 2013
25. Gallet R, Lenormand T, Wang IN (2012) Phenotypic stochasticity prevents lytic bacteriophage population from extinction during bacterial stationary phase. Evolution 66:3485–3494
26. Gandhi M, Chikindas ML (2007) *Listeria*: a foodborne pathogen that knows how to survive. Int J Food Microbiol 113:1–15
27. Ganegama Arachchi GJ (2013) A study of natural lytic *Listeria* phages with decontaminating properties for use in seafood processing plants. Unpublished doctoral dissertation, Massey University, New Zealand
28. Ganegama Arachchi GJ, Mutukumira AN, Dias-Wanigasekera BM, Cruz CD, McIntyre L, Young J, Hudson A, Flint SH (2012) Characterisation of *Listeria*-infecting bacteriophages isolated from seafood environments. In: Poster session presented at the 57th annual scientific meeting of the New Zealand Microbiological Society Inc, Dunedin, New Zealand
29. Gibson H, Taylor J, Hall K, Holah J (2001) Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms. J Appl Microbiol 87:41–48
30. Gill JJ (2010) Practical and theoretical considerations for the use of bacteriophages in food systems. In: Sabour PM, Griffiths MW (eds) Bacteriophages in the control of food- and waterborne pathogens. ASM, Washington, DC, pp 217–235
31. Goodridge LD, Bisha B (2011) Phage-based biocontrol strategies to reduce foodborne pathogens in foods. Bacteriophage 1:130–137
32. Guenther S, Huwyler D, Richard S, Loessner MJ (2009) Virulent bacteriophage for efficient biocontrol of *Listeria monocytogenes* in ready-to-eat foods. Appl Environ Microbiol 75:93–100
33. Hagens S, Loessner MJ (2010) Bacteriophage for biocontrol of foodborne pathogens: calculations and considerations. Curr Pharm Biotechnol 11:58–68
34. Herald PJ, Zottola EA (2006) Attachment of *Listeria monocytogenes* to stainless steel surfaces at various temperatures and pH values. J Food Sci 53:1549–1562
35. Hibma AM, Jassim SAA, Griffiths MW (1997) Infection and removal of L-forms of *Listeria monocytogenes* with bred bacteriophage. Int J Food Microbiol 34:197–207
36. Hitchins AD, Jinneman K (1998) Detection and enumeration of *Listeria monocytogenes* in foods. In: Bacteriological Analytical Manual, 8th edn, Revision A. http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm2006949.htm. Accessed 21 July 2013
37. Intralytix (2013) Food safety products. ListShield™. http://www.intralytix.com/Intral_products.htm. Accessed 30 July 2012
38. Kropinski AM, Mazzocco A, Waddell TE, Lingohr E, Johnson RP (2009) Enumeration of bacteriophages by double agar overlay plaque assay. In: Clokie MRJ, Kropinski AM (eds) Bacteriophages methods and protocols. Volume 1: isolation, characterization, and interaction. Humana, Hertford, pp 69–76
39. Lake RJ, Cressey PJ, Campbell DM, Oakley E (2009) Risk ranking for foodborne microbial hazards in New Zealand: burden of disease estimates. Risk Anal 30:743–752
40. Leonard C, Virijevic S, Regnier T, Combrinck S (2010) Bioactivity of selected essential oils and some components on *Listeria monocytogenes* biofilms. S Afr J Bot 76:676–680
41. Leriche V, Chassaing D, Carpentier B (1999) Behaviour of *L. monocytogenes* in an artificially made biofilm of a nisin-

producing strain of *Lactococcus lactis*. Int J Food Microbiol 51:169–182
42. Lu TK, Collins JJ (2007) Dispersing biofilms with engineered enzymatic bacteriophage. Proc Natl Acad Sci 104:11197–11202
43. Lundén J, Autio T, Markkula A, Hellström S, Korkeala H (2003) Adaptive and cross-adaptive responses of persistent and non-persistent *Listeria monocytogenes* strains to disinfectants. Int J Food Microbiol 82:265–272
44. Lunden JM, Miettinen MK, Autio TJ, Korkeala HJ (2000) Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact times. J Food Prot 63:1204–1207
45. Meyer R, Arpanaei A, Pillai S, Bernbom N, Enghild J, Ng Y, Gram L, Besenbacher F, Kingshott P (2013) Physicochemical characterization of fish protein adlayers with bacteria repelling properties. Colloid Surf B Biointerfaces 102:504–510. doi:10.1016/j.colsurfb.2012.08.044
46. Micreos Food Safety (2009) Micreos food safety-LISTEX™. http://www.micreosfoodsafety.com/en/profile-mission.aspx. Accessed 30 July 2012
47. Montanez-Izquierdo VY, Salas-Vazquez DI, Rodriguez-Jerez JJ (2012) Use of epifluorescence microscopy to assess the effectiveness of phage P100 in controlling *Listeria monocytogenes* biofilms on stainless steel surfaces. Food Control 23:470–477
48. Palmer J, Flint S, Brooks J (2007) Bacterial cell attachment, the beginning of a biofilm. J Ind Microbiol Biotechnol 34:577–588
49. Pan Y, Breidt F Jr, Kathariou S (2006) Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. Appl Environ Microbiol 72:7711–7717
50. Purkrtová S, Turoňová H, Pilchová T, Demnerová K, Pazlarová J (2010) Resistance of *Listeria monocytogenes* biofilms to disinfectants. Czech J Food Sci 28:326–332
51. Rashid MH, Revazishvili T, Dean T, Butani A, Verratti K, Bishop-Lilly KA, Sozhamannan S, Sulakvelidze A, Rajanna C (2012) A *Yersinia pestis*-specific, lytic phage preparation significantly reduces viable *Y. pestis* on various hard surfaces experimentally contaminated with the bacterium. Bacteriophage 2:168–177
52. Roy B, Ackermann HW, Pandian S, Picard G, Goulet J (1993) Biological inactivation of adhering *Listeria monocytogenes* by listeriaphages and a quaternary ammonium compound. Appl Environ Microbiol 59:2914–2917
53. Scharff RL (2012) Economic burden from health losses due to foodborne illness in the United States. J Food Prot 75:123–131
54. Sillankorva S, Neubauer P, Azeredo J (2008) *Pseudomonas fluorescens* biofilms subjected to phage phiIBB-PF7A. BMC Biotechnol 8:79. doi:10.1186/1472-6750-8-79
55. Sillankorva S, Neubauer P, Azeredo J (2010) Phage control of dual species biofilms of *Pseudomonas fluorescens* and *Staphylococcus lentus*. Biofouling 26:567–575
56. Sillankorva S, Oliveira R, Vieira MJ, Sutherland I, Azeredo J (2004) Bacteriophage Φ S1 infection of *Pseudomonas fluorescens* planktonic cells versus biofilms. Biofouling 20:133–138
57. Skandamis PN, Yoon Y, Stopforth JD, Kendall PA, Sofos JN (2008) Heat and acid tolerance of *Listeria monocytogenes* after exposure to single and multiple sublethal stresses. Food Microbiol 25:294–303
58. Solanki K, Grover N, Downs P, Paskaleva EE, Mehta KK, Lee L, Schadler LS, Kane RS, Dordick JS (2013) Enzyme-based listericidal nanocomposites. Sci Rep 3:1584. doi:10.1038/srep01584
59. Soni KA, Nannapaneni R (2010) Bacteriophage significantly reduces *Listeria monocytogenes* on raw salmon fillet tissue. J Food Prot 73:32–38
60. Soni KA, Nannapaneni R (2010) Removal of *Listeria monocytogenes* biofilms with bacteriophage P100. J Food Prot 73:1519–1524
61. Soni KA, Nannapaneni R, Hagens S (2010) Reduction of *Listeria monocytogenes* on the surface of fresh channel catfish fillets by bacteriophage Listex P100. Foodborne Pathog Dis 7:427–434. doi:10.1089/fpd.2009.0432
62. Sulakvelidze A, Pasternack GR (2010) Industrial and regularity issues in bacteriophage applications in food production and processing. In: Sabour PM, Griffiths MW (eds) Bacteriophages in the control of food- and waterborne pathogens. ASM, Washington, DC, pp 297–326
63. Sutherland IW, Hughes KA, Skillman LC, Tait K (2006) The interaction of phage and biofilms. FEMS Microbiol Lett 232(1):1–6
64. Tanji Y, Shimada T, Yoichi M, Miyanaga K, Hori K, Unno H (2004) Toward rational control of *Escherichia coli* O157:H7 by a phage cocktail. Appl Microbiol Biotechnol 64:270–274
65. Tham W, Ericsson H, Loncarevic S, Unnerstad H, Danielsson-Tham ML (2000) Lessons from an outbreak of listeriosis related to vacuum-packed gravad and cold-smoked fish. Int J Food Microbiol 62:173–175
66. Tompkin R (2002) Control of *Listeria monocytogenes* in the food-processing environment. J Food Prot 65:709–725
67. Tresse O, Lebret V, Benezech T, Faille C (2006) Comparative evaluation of adhesion, surface properties, and surface protein composition of *Listeria monocytogenes* strains after cultivation at constant pH of 5 and 7. J Appl Microbiol 101:53–62
68. Truelstrup Hansen L, Vogel BF (2011) Desiccation of adhering and biofilm *Listeria monocytogenes* on stainless steel: survival and transfer to salmon products. Int J Food Microbiol 146:88–93
69. Wirtanen G, Salo S (2003) Disinfection in food processing-efficacy testing of disinfectants. Rev Environ Sci Biotechnol 2:293–306
70. Wong S, Street D, Delgado SI (2000) Recalls of foods and cosmetics due to microbial contamination reported to the US Food and Drug Administration. J Food Prot 63:1113–1116
71. Zhao T, Doyle MP, Zhao P (2004) Control of *Listeria monocytogenes* in a biofilm by competitive-exclusion microorganisms. Appl Environ Microbiol 70:3996–4003