Isolation and Characterization of Listeria monocytogenes Phage vB_LmoH_P61, a Phage With Biocontrol Potential on Different Food Matrices

Edel Stone 1,2, Antoine Lhomet 1,3, Horst Neve 4, Irene R. Grant 2, Katrina Campbell 2 and Olivia McAuliffe 1,5

1 Teagasc Food Research Centre, Moorepark, Cork, Ireland, 2 Institute for Global Food Security, School of Biological Sciences, Queen’s University Belfast, Belfast, United Kingdom, 3 IUT Dijon-Auxerre, University of Burgundy, Dijon, France, 4 Department of Microbiology and Biotechnology, Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Kiel, Germany, 5 VistaMilk SFI Research Centre, Cork, Ireland

The high mortality rate associated with Listeria monocytogenes as well as its ability to adapt to the harsh conditions employed in food processing have ensured that this pathogen has become a significant concern in the ready-to-eat food industry. Lytic bacteriophages are viruses that hijack the metabolic mechanisms of their bacterial host as a means to grow and replicate, subsequently leading to host cell death due to lysis. This study reports the biological and genomic characterization of L. monocytogenes phage vB_LmoH_P61 (P61) and its potential application for the reduction of L. monocytogenes in artificially contaminated foods. Phage P61 is a virulent bacteriophage belonging to the family Herelleviridae and has a genome size of 136,485bp. The lytic spectrum of phage P61 was investigated and it was shown to infect serotypes 1/2a, 1/2b, 1/2c, 4b, 4e, and 6a. Treatment of artificially contaminated milk stored at 8 and 12°C with phage P61 resulted in a significant reduction in L. monocytogenes numbers over the product shelf life. Similarly, phage P61 reduced L. monocytogenes numbers on artificially contaminated baby spinach stored at 8, 12, and 25°C. The research findings indicate that biocontrol of L. monocytogenes with phage P61 may offer a safe and environmentally friendly approach for the reduction of L. monocytogenes numbers in certain ready-to-eat foods.

Keywords: bacteriophage, Listeria monocytogenes, biocontrol, broad-host range, food safety

INTRODUCTION

On an annual basis, it is estimated that 600 million people globally (~1 in 10) fall ill following consumption of contaminated food, and of these, 420,000 die (World Health Organization, 2020). Contamination of food with pathogenic bacteria poses a severe threat to our socio-economic balance and healthcare systems. Bacteria such as Campylobacter, Salmonella, Shiga toxin-producing Escherichia coli (STEC), and Listeria monocytogenes are responsible for the majority of outbreaks related to bacterial foodborne illness occurring in the EU (EFSA, 2018). Compared to the other bacterial species, the numbers of cases of illness caused by L. monocytogenes is low. In 2018, there...
were 2,549 cases of listeriosis in the EU compared to 246,571 cases of campylobacteriosis, 92,857 cases of salmonellosis and 8,161 cases of STEC (EFSA, 2018). However, listeriosis accounts for the highest proportion of hospitalized cases (97%) and the highest number of deaths (229) in Europe, making it one of the most serious foodborne diseases (EFSA, 2018). In addition, the trend in case numbers in Europe is continuing upward over the past 10 years (EFSA, 2018). Gastroenteritis is the most serious foodborne disease (EFSA, 2018). In addition, the trend in case numbers in Europe is continuing upward over the past 10 years (EFSA, 2018). Commercial listerio phage preparations have been available to the food industry for some time, comprising either single broad-host range phages or a cocktail of phages for application either on food matrices or on food processing plant surfaces (Golkar et al., 2014). In a research setting, the efficacy of phages as biocontrol agents in various food matrices have been tested including lettuce, cheese, smoked salmon, and frozen entrees (Perera et al., 2015), and melon, pear, and apple products (juices and slices) (Oliveira et al., 2014). The aim of this study was to phenotypically and genotypically characterize a L. monocytogenes phage, vB_LmoH_P61 (P61), isolated from grass silage. In addition, we evaluated the potential of P61 as a biocontrol agent against L. monocytogenes in both liquid and solid food matrices. In particular, given the increase in the consumption of fresh and minimally processed vegetables in recent years, we chose to evaluate the efficacy of phage P61 on baby spinach, a product on which the occurrence of L. monocytogenes has been reported.

Materials and Methods

Bacterial Strains and Culture Conditions

Listeria monocytogenes strains used in this study were obtained from a collection of isolates housed at the Teagasc Food Research Center, Moorepark, Cork, Ireland, unless otherwise indicated (Table 1). Overnight cultures of each strain were prepared following 18 h of incubation in tryptic soy broth (TSB) (Oxoid Ltd., Basingstoke, UK) at 25°C under aerobic conditions. Solid agar and soft agar TSB overlays contained 1.5% agar and 0.4% agarose (both Sigma-Aldrich, St. Louis, USA), respectively.

Bacteriophage Isolation and Propagation

Phage P61 was isolated from grass silage sourced from the Teagasc Moorepark farm, Fermoy, Cork following the protocol outlined by Alemayehu et al. (2009). Subsequently, individual phage plaques were subjected to four successive rounds of purification. To propagate the phage, 1 ml of TSB (Becton Dickinson and Company, Le Pont de Claix, France) contained 10% inoculum of an overnight culture of the host organism (L. monocytogenes strain 3053) and 18.5 mM CaCl2 (Merck, Darmstadt, Germany) and was incubated for 3 h at 25°C. Following this incubation, a single plaque was aseptically removed from an overlay plate using a 5 ml pipette and added...
One Step Growth Curve to Determine the Growth Kinetics of P61

To determine the growth kinetics of phage P61, a one-step growth curve was conducted according to Denes et al. (2015). Briefly, a 5 ml culture of *Listeria monocytogenes* was grown in TSB to a concentration of ~1 × 10⁸ Colony forming unit/ml (CFU/ml) and 20 mM of CaCl₂ was added. P61 (1 × 10⁸ PFU/ml) was then added to the solution [multiplicity of infection (MOI) of 0.1]. The solution was incubated at 30°C while shaking (120 rpm). Two samples were taken every 10 min, one 100 µl was transferred into a tube containing 4 drops of chloroform, and the other sample was immediately diluted and enumerated using strain 3053 as the host bacterial cell. At the end of the timeline, chloroform-treated phages were enumerated yielding the total concentration of viable phage particles in the sample, including intracellular phages. The average burst size was calculated by dividing the average concentration of infected cells and free viable phages by three timepoints following the first step of lysis (time point 80, 90, and 100 min) by the average concentration of infected cells and free viable phage from the first free time points post-infection (Hyman and Abedon, 2009; Denes et al., 2015). The experiment was independently repeated three times.

Determination of Bacteriophage-Insensitive Mutant Frequency

To determine the frequency of cases of bacteriophage-insensitive mutants (BIMs), a protocol was adapted from Filippov et al. (2011). A total of 24 colonies were selected to test the frequency of BIMs. A single colony of strain 3053 was inoculated into TSB and incubated overnight at 25°C. Following the overnight incubation, the sample was serially diluted in MRD and 100 µl spread onto *Listeria* Chromogenic agar (Neogen) with or without a double agarose overlay (0.4%) containing 1 × 10⁸ PFU/ml of phage P61. Plates were incubated for 48 h at 37°C. Resulting colonies were counted and BIM frequency was determined using the formula (surviving viable counts divided by initial viable counts).

Ammonium Acetate Precipitation of Phage Lysate

Phage P61 was concentrated using a protocol adapted from Casey et al. (2015). A volume of 1.5 L of P61 was propagated to a titer of ~1 × 10¹⁰ PFU/ml. The lysate was filtered through a 0.45 µm pore filter (Sarstedt, Wexford, Ireland) and diluted 1:10 in maximum recovery diluent (MRD) (Oxoid Ltd., USA). The solution was incubated at 30°C for 1.5 h and the resulting pellet was resuspended in 10 ml of 0.1 M ammonium acetate. The lysate was centrifuged at 25,000 × g at 4°C to remove any cell debris. Filtrates were then centrifuged at 25,000 × g at 4°C for 1.5 h and the resulting pellet was resuspended in 1 ml of 0.1 M ammonium acetate. The 1 ml solution was centrifuged again at 25,000 × g for another 1.5 h at 4°C and pellet resuspended in 1 ml of 0.1 M of ammonium acetate.

Transmission Electron Microscopy

Phage lysates were dialyzed for 20 min against SM-buffer buffer (20 mM Tris-HCl [pH 7.2], 10 mM NaCl, 20 mM MgSO₄). After

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**TABLE 1** | Origin and serogroup of *Listeria monocytogenes* strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serogroup</th>
<th>Country of origin</th>
<th>Source†</th>
<th>EOP‡</th>
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<tr>
<td>3053</td>
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<td>Mushrooms</td>
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<tr>
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<tr>
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</tr>
<tr>
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<td>Cheese</td>
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<tr>
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<td>4e</td>
<td>USA USDA-ARS</td>
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<td></td>
</tr>
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<td></td>
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<td>UVM, Austria</td>
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<tr>
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<td>4e</td>
<td>Austria</td>
<td>UVM, Austria</td>
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† USDA-ARS, US Department of Agriculture-Agricultural Research Service, Albany, CA, USA; UVM, University of Veterinary Medicine, Vienna, Austria; Cornell University, Ithaca, NY, USA. ‡ EOP or efficiency of plaquing, is calculated by dividing the phage titer (PFU/ml) of a given test strain, by the phage titer (PFU/ml) of the host sensitive strain (strain 3053).

Bacteriophage Lytic Spectrum

The lytic spectrum of phage P61 was determined by plaque assays against a range of *Listeria monocytogenes* strains (listed in Table 1) using the protocol outlined by Casey et al. (2015). Efficiency of plaquing (EOP) figures were calculated by dividing the phage titer (PFU/ml) of a given test strain, by the phage titer (PFU/ml) of the host sensitive strain (strain 3053).
15-min adsorption to ultrathin carbon films, 10-min fixation with 1% glutaraldehyde and negative staining with 1% (wt/vol) uranyl acetate, transmission electron microscopy was performed at an accelerating voltage of 80 kV (Tecnai 10; FEI Thermo Fisher Scientific, Eindhoven, The Netherlands). Micrographs were captured with a MegaView G2 CCD camera (Emsis, Muenster, Germany).

**Phage DNA Extraction**

DNA was extracted from phage P61 using the method adapted from Casey et al. (2015). Briefly, 1 µl of RNase (Thermo Fisher Scientific, Leicester, UK) (1 mg/ml) and 12 U/µl of DNase I (Roche Diagnostics, Mannheim, Germany) (6 µl of 2,000 U/ml) were added to 1 ml of P61 lysate (1 × 10⁹ PFU/ml) and incubated at 37°C for 30 min. The sample was then centrifuged at 18,407 g for 5 min and the supernatant was transferred to a fresh 1.5 ml sterile Eppendorf tube. Lysis buffer (100 µl) (0.5 M Tris-HCl pH9, 0.25 M EDTA, 2.5% SDS) was then added to the sample, vortexed and incubated at 65°C for 5 min. One hundred twenty-five microliter of 8 M potassium acetate (Sigma-Aldrich, Poole, UK) was then added and mixed by inverting the tube 4–6 times. The sample was placed at −20°C for 15 min. Following this, the samples were centrifuged at 18,407 g for 5 min and the supernatant was aliquoted equally into two fresh Eppendorf tubes. The sample was extracted twice with phenol chloroform-isoamyl alcohol (Sigma-Aldrich). The top layer was again placed into a fresh Eppendorf tube where an equal volume of isopropanol (Sigma-Aldrich) was added and mixed by inverting. The sample was placed at −20°C for 40 min. The sample was centrifuged at 18,407 g for 5 min and the isopropanol was removed. The pellet was washed twice with 70% ethanol (Scharlau, Barcelona, Spain) and left to dry at 37°C. Twenty microliter of ddH₂O was added to the pellet and was incubated for 30 min at 37°C. DNA was quantified using the Invitrogen Qubit 4 fluorometer (Thermo Fisher Scientific, Renfrew, UK) using the protocol outlined by the manufacturer, and visualized on a 0.8% agarose gel.

**Genome Sequencing and Comparative Genomics**

The whole genome sequence of phage P61 was elucidated using the illumina MiSeq Next Generation Sequencing platform at Microbes NG (Birmingham Research Park, UK). Prior to assembly, the trimmed reads were passed through FLASH (Fast Length Adjustment of Short reads) to eliminate overlapping paired end reads. The genome of P61 was assembled using the SeqMan NG application of DNAStar Lasergene Genomics Suite (DNAStar, Inc., USA). Once assembled, the genome was uploaded onto the RAST server for annotation. Annotations were confirmed through analysis using BLASTp (https://blast.ncbi.nlm.nih.gov/), HMMER (http://hmmer.org/), and Artemis (https://www.sanger.ac.uk/science/tools/artemis). The genome sequence of P61 has been uploaded to the NCBI database with the accession no: MT438761 and the illumina paired reads have been uploaded onto SRA NCBI database reference PRJNA599330.

**Determination of the Efficacy of Phage P61 for the Reduction of L. monocytogenes in Milk**

Pasteurized milk (3.5% fat) was purchased from a local retail outlet and was screened for the presence of *L. monocytogenes* and *Listeria* phages prior to commencing the trial. An overnight culture of *L. monocytogenes* strain 3053 was diluted 1:5 in TSB and incubated for 3 h at 25°C. Cells were then diluted in maximum recovery diluent (Oxoid Ltd.) to the desired cell numbers. Target cell numbers in the milk were 10⁹ CFU/ml. 18.5 mM CaCl₂ (Merck) was also added to the milk. Phage P61 was propagated using the protocol outlined above to a final concentration of 2 × 10⁹ PFU/ml. The phage was added to the test samples at a concentration of 2 × 10⁶ PFU/ml. Samples were then stored at 12 and 8°C for a total of 7 days (shelf-life of milk). Bacterial viable counts (CFU/ml) and phage counts (PFU/ml) were determined immediately after phage addition (T0) and subsequently at days 1, 3, 5, and 7. For enumeration of *L. monocytogenes*, samples were serially diluted and plated onto *Listeria* Chromogenic Agar. Plates were incubated at 37°C for 48 h. For enumeration of P61, samples were diluted, and plaque assays performed using the protocol outlined above.

**Determination of the Efficacy of Phage P61 for the Reduction of L. monocytogenes in Baby Spinach**

*L. monocytogenes* strain 3053 and phage P61 were prepared following the protocol described above. An overnight culture of strain 3053 which had been grown at 25°C was diluted 1:5 in TSB at 25°C for 3 h and the optical density determined at 600 nm and cells were diluted to desired cell numbers. Target cell numbers in spinach were 2 × 10³ CFU/g and the inoculum volume was 500 µl. Baby spinach was purchased from a local retail outlet and weighed out into 10 g portions and placed into sterile bags. Bacteria and phages were then separately applied by pipetting onto the surface of the baby spinach and shaking the bag vigorously for 30 s. For enumeration of *L. monocytogenes* and *Listeria* spp.

Technical and biological replicates were performed in duplicate for this experiment. For enumeration of P61, samples were serially diluted to 10⁻⁷ and plaque assays performed using the protocol outlined above. Technical and biological replicates were performed in duplicate for this experiment.

**Isolation and Characterization of Listeria monocytogenes Phage vB_LmoM_P61**
RESULTS AND DISCUSSION

Phage P61 Infects Six Serotypes of L. monocytogenes

The host range of phage P61 was determined by performing efficiency of plaquing assays against a series of L. monocytogenes strains. The strains selected for analysis represent isolates from a range of environmental niches, sample types and were of nine different serogroups (Table 1). P61 was shown to be capable of infecting 6 of the 9 serogroups of L. monocytogenes tested (1/2a, 1/2b, 1/2c, 4b, 4e, and 6a). An EOP value of 0.56 was obtained for a single strain of L. marthii that was tested (54120) indicating that phage P61 can infect another species of Listeria. Phage P61 was unable to infect strains of serotype 4ab (Listeria innocua), 4a/c and 7. Further testing revealed that P61 infected all three L. monocytogenes strains tested belonging to the serotypes 1/2a and 1/2b, but only infected one out of two strains of the serotype 1/2c and 4b, and three out of four strains of serotype 4e. Strains tested are representatives from a given serotype, there is considerable variation between strains within a serotype, accounting for the differences shown in susceptibility to phage P61 here. Vongkamjan et al. (2012), isolated 114 Listeria phages from two dairy farms and tested the host range of these phages against 13 strains representing 9 major serotypes. This revealed that 12.3% of phage isolates showed a narrow host range and 28.9% of phages were broad host range phages (Vongkamjan et al., 2012). Vongkamjan showed that the broad host range phages in this study infected 84.6% of strains tested while the narrow host range phages infected between 7.7% and 38.5% of strains tested. In this study 17 strains representing 9 major serotypes were used to test the host range of P61 and 64.5% of strains tested were susceptible to phage infection. P61 has a limited host range in comparison to broad host range Listeria phages that have been characterized (Loessner et al., 1994; Carlton et al., 2005; Vongkamjan et al., 2017) and can infect a wider range of serotypes than narrow host range Listeria phages that have been characterized (Casey et al., 2015; Sumrall et al., 2019). Thus, P61 cannot be defined as a narrow or broad host range phage but has a moderate host range.

General Characteristics of the Genome of Phage P61

The assembled genome of P61 presented here is a draft assembly with collapsed terminal repeat regions positioned approximately at 10,000–13,700 bp. Figure 1 shows the terminal repeat regions of phage A511 mapped to the draft assembly of phage P61 indicating the position of the collapsed terminal repeats in the genome of phage P61. This draft assembly of P61 has a genome size of 136,485 bp and a G +C content of 35.92%, which correlates well with values for other Listeria phages, e.g., the Herelleviridae LP-125 (135,281 bp) with a GC content of 35.9%, the Herelleviridae LP-124 (135,817 bp) with a GC content of 35.9%, and the Herelleviridae LP-048 (133,096 bp) with a GC content of 36.0% (Denes et al., 2014). The total number of reads generated was 291,863 and mean coverage was 880.637x. A total of 192 open reading frames (ORFs) and a cluster of 17 tRNA genes were identified in the draft assembly of phage P61 (Supplementary Table 1). Blastp and Blastn analysis of the nucleotide sequences and amino acid sequences revealed the genome of phage P61 is similar in size and sequence to that of the Listeria phage P100 (131,384 bp) which encodes 174 gene products and 18 tRNAs (Carlton et al., 2005) and that of A511 (134,494 bp), encoding 190 gene products and 16 tRNAs. The absence of genes or homologs related to the lysogenic life cycle, e.g., integrases, excisionases, the two repressor proteins Cro repressor and cI repressor (Ohlendorf et al., 1998), attachment sites, in the genome of phage P61 suggests that it follows a strictly lytic lifecycle, a vital characteristic if phages are to be exploited as a biocontrol agent to avoid integration of phage DNA in the host cell genome (Casjens and Hendrix, 2015). Genes playing a role in host cell lysis were also identified. ORF 61 appears to encode the endolysin of phage P61, an N-acetylmuramoyl-L-alanine amidase which shares 100% sequence similarity with the N-acetylmuramoyl-L-alanine amidase of Listeria phage LP-125 (Denes et al., 2014).

Transmission EM Confirms Phage P61 as a Member of the Family Herelleviridae

Electron microscopy analysis was performed to further characterize phage P61 in terms of morphology (Figure 2). Measurements of 12 phage P61 particles was shown to possess an isometric capsid of 85.5 ± 2.5 nm in diameter, with a thin collar structure evident beneath the capsid and blackberry-like surface structures on the capsid surface. A tail length of 218.5 ± 2.7 nm and width 22.4 ± 1.0 nm was measured. Thin tail fibers extending from the upper region of the baseplate structure were observed. A double disc baseplate structure was observed, with the baseplate height measured at 30.8 ± 2.3 nm and width of 50.7 ± 3.7 nm, with the collar shown to be 6.4 ± 0.4 nm (n = 7) in height and 13.8 ± 0.8 nm (n = 7) in width. The baseplate of phage P61 is complex with conformational changes occurring when the tail contracts. The baseplate appendages seem to be highly flexible, either with a brush-like appearance or forming globular structures. Morphologically, phage P61 is similar to A511 and P100 (Klumpp et al., 2008).

One-Step Growth Curve and Bacteriophage Insensitive Mutant Frequency

To determine the infection kinetics of phage P61, a one-step growth curve was performed on the host strain 3053 (Figure 3). Following 30 min of incubation, 99.99% of phage particles adsorbed to the bacterial host cell. Having a high percentage of adsorption suggests that there may be a strong concentration of available host bacterial cell receptors and phage P61 has a strong affinity for these receptors. The burst size of phage P61 was calculated to be 11.03. The eclipse period, defined as the time interval between viral penetration and the production of progeny virions, was calculated as 40–50 min, and the latent period, defined as the time taken for the infected cell to lyse post-infection, was calculated as 60–70 min. The frequency of BIMs against host strain 3053 was calculated to be 4.36 × 10⁻³ (n = 24).
Phage P61 Reduces *L. monocytogenes* Numbers in Liquid Milk and Baby Spinach

Phage P61 was evaluated as a potential biocontrol agent for the reduction of *L. monocytogenes* in both liquid (pasteurized milk) and solid food matrices (baby spinach). Preliminary experiments in TSB at 8 and 12°C showed that the application of phage P61 at an MOI of 100 resulted in inhibition of the growth of *L. monocytogenes* strain 3053 at both temperatures, with counts of *L. monocytogenes* reaching $2 \times 10^8$ CFU/ml in untreated samples vs. $1 \times 10^5$ CFU/ml in phage-treated samples at 8°C, and $2 \times 10^9$ CFU/ml in untreated samples vs. $3 \times 10^8$ CFU/ml in phage-treated samples at 12°C (Figure 4). Experiments at 8 and 12°C were the conducted using pasteurized milk (purchased at a local retail outlet) where samples were spiked with *L. monocytogenes* strain 3053 at a concentration of $1 \times 10^3$ CFU/ml, following which phage P61 was added at an MOI of 10,000. At 12°C, a significant decrease ($p = 0.05$) in CFU/ml of *L. monocytogenes* strain 3053 was observed compared to the phage free control (Figure 5), with a reduction in *L. monocytogenes* counts of log 2.36 (99.57% reduction) in treated vs. untreated samples at day
7. When stored at 8°C a reduction of 1.2 log (93.75% reduction) was observed at day 7 (shelf-life of milk) \((p = 0.0173, \text{ Figure 5})\). Prior to these experiments it was shown that P61 survives well in the absence of \(L.\) monocytogenes with the inoculated concentration of P61 staying consistent throughout the 7 days.

A similar experiment was conducted with baby spinach artificially inoculated with \(L.\) monocytogenes strain 3053 and treated with phage P61 at an MOI of 10,000. However, the results were not promising, with no significant difference observed between the untreated and phage-treated samples during the trial, other than at a single time point on day 3 where a reduction of 0.8 log \((p = 0.0034)\) was observed (data not shown). Further investigation revealed that \(L.\) monocytogenes strain 3053 was not actively growing on the surface of the spinach, thus potentially hindering the phage action. A number of strains were tested on the spinach surface and \(L.\) monocytogenes strain 702 was...
chosen for further analysis as this strain was found to grow well on spinach and was also shown to be susceptible to phage P61 (Table 1). The experiment on spinach was repeated with strain 702 and on this occasion, the \textit{L. monocytogenes} was left to aclimatize to the surface of the spinach prior to the application of phage P61 (MOI of 100,000). A reduction of 1.93 log (98.78\%) was seen on day 5 at 8°C (\(p = 0.0024\)), 2.06 log (99.12\%) at 12°C (\(p = 0.002\)) and 3.3 log (99.95\%) at 25°C (\(p = 0.0083\); Figure 6).

Our results suggest that there are a number of determining factors as to whether the application of phage P61 will successfully reduce or inhibit the growth of \textit{L. monocytogenes}. These include the food matrix, the storage temperature, the stage of growth of the bacterial pathogen and the MOI. In addition to liquid milk and baby spinach, we had previously tested a number of other semi-solid and solid food products, including smoked salmon, hummus and cream cheese. Phage P61 did not inhibit or reduce the growth of \textit{L. monocytogenes} in these matrices, despite the fact that the organism grew well and the phage survived well in these environments (data not shown). Marcó et al. (2010) suggest that the diffusion of phages could be hindered or favored depending on the structure and composition of the matrix and the environmental conditions. To exploit phages as a biocontrol agent on different food matrices, the application must be specifically optimized for individual food systems. The storage temperature is also a key determinant of phage activity. In our study, notable differences were seen when phage P61 was applied at 8 and 12°C in pasteurized milk, and 8, 12, and 25°C on baby spinach. In the case of pasteurized milk, \textit{L. monocytogenes} counts decreased by 1.20 log in the phage-treated samples stored at 8°C, in comparison to the 2.36 log reduction in the phage-treated samples stored at 12°C after 7 days. There was little difference between the growth of \textit{L. monocytogenes} at the two temperatures (0.03 log difference), and thus, this may indicate that strain 3053 used in this experiment is less susceptible to phage P61 at lower temperatures in a liquid medium. Tokman et al. (2016), discuss the effect of temperature on the susceptibility of \textit{L. monocytogenes} to phages. The team highlight that \textit{L. monocytogenes} can gain physiological refuge from phage infection indicating that adsorption rates of phage can be affected by the physiological state of the host (Tokman et al., 2016). In the case of baby spinach, a reduction of 1.93 logs, 2.06 log, and 3.3 log was seen in phage treated samples at the end of the storage period at 8, 12, and 25°C, respectively. As expected, in samples that were not treated with phages, \textit{L. monocytogenes} grew best at 25°C and it was at this temperature where the highest reduction in CFU/ml was observed in the phage-treated samples. The increase in the concentration of actively growing host cells may increase the likelihood of phages to come into contact with these cells and begin the infection cycle. Henderson et al. (2019) recently discussed the effect of temperature and surrounding environmental conditions on the efficiency of phage application to reduce the growth of \textit{L. monocytogenes} in a laboratory cheese model. These authors reported that treatment with phage P100 is more effective when the food is stored at higher temperatures, showing that the average \textit{L. monocytogenes} counts on phage-treated samples were significantly lower when samples were stored at 22°C as opposed to samples stored at 6 or 14°C (Henderson et al., 2019). This correlates with what we see for the solid matrix tested in our study, baby spinach. Henderson et al. (2019) also concluded that the pH of the food and serotype of \textit{L. monocytogenes} used influences the ability of phage P100 to reduce the growth of \textit{L. monocytogenes}. This has also been observed for a number of other foodborne pathogens and their phages in food system experiments (O’Flynn et al., 2004; Hong et al., 2016). In the O’Flynn et al. (2004) study, the authors noted that significant reductions in \textit{E. coli} cell numbers were obtained at 30 and 37°C; however, the lytic ability of the phage cocktail was greatly reduced at 12°C. Radford et al. (2016) described the efficient production of a high concentration of \textit{Listeria} under refrigeration conditions, a method that may be adapted to optimize the application of phages as a biocontrol on food matrices in future experiments (Radford et al., 2016).

The MOI is also a determining factor as to whether the application of phages as a biocontrol agent will be successful. Tomat et al. (2013) suggested that the application of higher numbers of phages and higher MOI values will result in a greater reduction of the numbers of the foodborne pathogen. Guenther et al. (2009) also report the significance of phage concentration. When applying \textit{Listeria} phage AS11 to hot dogs, chocolate milk, and cabbage, these authors noted that lower doses of phages resulted in a less significant growth suppression of \textit{L. monocytogenes}, with the higher titer of phage increasing the likelihood of phage making contact with its bacterial host cell (Guenther et al., 2009). Seo et al. (2016) used varying MOI values (1,000–100,000) for the inhibition of \textit{E. coli} O157:H7 in beef, pork and chicken meat and indicated using a higher MOI value of 100,000 had a greater inhibitory effect compared to lower values used. In these set of experiments using a MOI of 100,000 resulted in greater log reductions of \textit{L. monocytogenes} than when lower

![Figure 6](image-url)
MOIs were used in preliminary studies in broth and pasteurized milk (Seo et al., 2016).

Finally, the presence of actively growing bacterial host cells vs. stationary phase cells seems vital for the application of phage as a biocontrol agent. Abedon (2017) distinguishes between an “active treatment” and a “passive treatment” for phage biocontrol. In an active treatment, phages should actively produce newly formed virions in situ to create sufficient titers to eradicate the bacterial pathogen over reasonable timeframes. By contrast, passive treatment uses phages that are bactericidal but incapable of generating new phage virions during their interactions with the bacterial host cell. It could be argued that having actively growing host cells allows the phage to hijack its host’s metabolic mechanisms allowing for successful completion of its lifecycle and release of newly formed phages into the surrounding environment (Abedon, 2017). *L. monocytogenes* strain 702, used in the second set of experiments, actively grew on the baby spinach leaves in comparison to strain 3053 used in the initial study, which did not grow during the course of the experiment. Phage P61 coming into contact with a growing host cell (strain 702) may have increased the release of newly formed progeny into the environment, thus increasing the infection rate and subsequent reduction in *L. monocytogenes* numbers.

**CONCLUDING REMARKS**

The work outlined in this study indicates that phage P61 could potentially be exploited for use as a biocontrol agent, particularly in liquid foods, for the inhibition of *L. monocytogenes*. Findings from this study indicate that the application of phages as a biocontrol agent against foodborne pathogens needs to be optimized for each food matrix used and the environment it is applied to. Temperature, food matrix, environment, and the growth phase of the bacterial host cell are all factors contributing to the success of application. The inhibition may also be affected by the type of application used (spraying, spreading, and pipetting). However, the positive results observed on the spinach matrix show promise for further study on this and other horticultural products, where reports of the occurrence of *L. monocytogenes* are increasing and where limited options are available for safe, natural antimicrobials for inhibition of *L. monocytogenes*.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

**AUTHOR CONTRIBUTIONS**

ES: original research and writing. AL and HN: research. OM: conceptualization, resourcing, writing, and editing. IG and KC: editing. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2020.521645/full#supplementary-material

**REFERENCES**


