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

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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 common manifestation of listeriosis and although the occurrence in healthy individuals is relatively rare, the disease can progress to septicemia and death in immunocompromised individuals, pregnant women, neonates and the elderly (Vázquez-Boland et al., 2001; Smith et al., 2018).

Issues of food contamination with *L. monocytogenes* are primarily associated with ready-to-eat (RTE) foods, which are foods intended by the producer or the manufacturer for direct human consumption without the need for cooking or other processing effective to eliminate or reduce to an acceptable level micro-organisms of concern [Regulation (EC) No. 2073/2005]. *L. monocytogenes* is found ubiquitously in the environment, present in soil, water, animal feed, vegetation, industrial plants, and on farms. It can enter the RTE food chain via a number of routes, soil and water being the most common, and can persist along the food chain (Ferreira et al., 2014). The control of *L. monocytogenes* in the food processing setting has proven difficult due to its ability to adapt to and survive in a wide range of environmental conditions. To minimize the risk associated with *L. monocytogenes*, processing of RTE foods may include the addition of preservatives (sodium nitrite, sorbic acid), decontamination (water, acid), curing, smoking, fermentation, heating (pasteurizing, cooking, baking, boiling, and steaming), and drying (Ricci et al., 2018). For RTE foods that undergo mild processing treatments (e.g., washing), such as fresh fruit and vegetables, the pathogen may still survive. Therefore, antimicrobials or bacteriostatic agents may be applied to minimize the growth. An antimicrobial is defined by the USDA Food Safety and Inspection Service as a substance in or added to an RTE product that has the effect of suppressing or limiting the growth of *L. monocytogenes* throughout the shelf life of a product (Savage, 2004). Recent reports on bacteriophages (phages) as natural and environmentally friendly antimicrobial agents have shown effective, specific targeting of bacterial pathogens of interest in different food matrices (Taylor, 2018). The specificity of phage-host interactions ensures the targeting of pathogenic bacteria in food without disturbing the normal microbiota of the food. There is no risk of the formation of secondary components upon their application; a reported disadvantage of using chemical agents in the decontamination process of foods (Shen et al., 2016). Commercially available phage products are usually water-based solutions consisting of purified phages, with low levels of salt and little or no preservatives or additives, making them an attractive alternative to chemicals for producers of "clean label" foods and consumers (Moye et al., 2018). Research also suggests that the application of phages does not affect the organoleptic properties of foods. In comparison to other antimicrobials and food safety interventions, the cost of application of phage preparations is relatively low, reportedly costing 1–4 cents per

pound of food treated (Moye et al., 2018). These biological properties and qualities of commercially available phages make them an attractive proposition for improving the safety of RTE foods (Moye et al., 2018).

To date, over 500 Listeria phages have been isolated from environmental sources adding to the knowledge of the phenotypes and genotypes of these phages (Lee et al., 2017; Vongkamjan et al., 2017; Mutlu and Şahin, 2018; Song et al., 2019). All of these phages belong to the order *Caudovirales*, either featuring the long non-contractile tail of the *Siphoviridae* family or the contractile tail of the *Herelleviridae* family (Klumpp and Loessner, 2013; Adriaenssens et al., 2020). Commercial listeriophage 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 CaCl$_2$ (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

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

Strain	Serogroup	Country of origin	Source[†]	EOP[‡]
3053	1/2a, 3a	Ireland	Mushrooms	1
3076	1/2a,3a	Ireland	Floor swab of mushroom plant	0.97
2956	1/2a,3a	Ireland	Cheese	0.96
3025	1/2a,3a	Ireland	Mushroom	0.85
3048	1/2b,3b,7	Ireland	Mushroom Compost	0.83
3026	1/2b,3b,7	Ireland	Mushroom	1
702	1/2c	Ireland	Cheese	0.97
3027	4b,4d,4e	Ireland	Mushroom	0
RM3388	4e	USA	USDA-ARS	0.03
RM3820	4e	USA	USDA-ARS	0.19
RM2218	4e	USA	USDA	0.27
AUS5098	4a/4c	Austria	UVM, Austria	0
3023	4b,4d,4e	Ireland	Mushroom	0
3049	4b,4d,4e	Ireland	Mushroom Peat	0.71
FSL-S4378	4ab (*Listeria innocua*)	USA	Cornell University	0
CDL231	4e	Austria	UVM, Austria	0
FSL-S4120	6a (*Listeria marthii*)	USA	Cornell University	0.56
FSL-R9915	7	USA	Cornell University	0

[†] *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).*

to the 1 ml of the bacterial host-cell (strain 3053). The volume was increased to 10 ml with TSB containing 18.5 mM $CaCl_2$ and 10% inoculum of strain 3053. The mixture was incubated for 18 h at 25°C. Following overnight incubation, the sample was filtered using a 0.45 µm pore filter (Sarstedt, Wexford, Ireland). Phage titer (Plaque forming unit /ml) (PFU/ml) was determined using the host sensitive strain 3053, 1 ml of phage lysate was diluted 1:10 in maximum recovery diluent (MRD) (Oxoid Ltd. Basingstoke, UK) to dilution 10^{-8}. A plaque assay was then performed using each dilution whereby a 5 ml sloppy agar tube (TSA, 0.4% agarose) contained 2% of strain 3053 and 20% of the phage lysate dilution to be tested and 3.7 mM $CaCl_2$. The mixture was overlaid onto TSA plates (Becton Dickinson and Company, Le pont de Claix, France) and incubated at 25°C for 18 h.

Bacteriophage Lytic Spectrum

The lytic spectrum of phage P61 was determined by plaque assays against a range of *L. 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).

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 *L. monocytogenes* was grown in TSB to a concentration of ~1 × 10^9 Colony forming unit/ml (CFU/ml) and 20 mM of $CaCl_2$ was added. P61 (1 × 10^8 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 × 10^9 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 × 10^{10} PFU/ml. The lysate was filtered through a 0.45 µm filter (Starstedt) and centrifuged at 6,000 × 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 10 ml of 0.1 M ammonium acetate and centrifuged at 25,000 × g for 1.5 h at 4°C. The resulting pellet was then 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 $MgSO_4$). After

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, Leicestershire, 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 (1×10^9 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 choloroform-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 dsH₂O 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 NGen 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 10^3 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 2×10^9 PFU/ml. The phage was added to the test samples at a concentration of 2×10^6 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. Technical and biological replicates were performed in duplicate for this experiment.

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 2×10^3 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. *L. monocytogenes* was allowed to acclimatize to the spinach samples for ∼1 h prior to the addition of phages. To the samples to be treated with phages, 500 µl aliquots of P61 were added to achieve a concentration of ∼2×10^8 PFU/g of spinach. Samples were then stored at 25, 12, and 8°C for a total of 6 days (shelf life of baby spinach). Samples were diluted 1:10 in full Fraser broth (Sigma-Aldrich, St Louis, USA) and homogenized at 230 rpm for 1 min and plated as described above. 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.

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 (S4120) 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 collasped 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 collasped 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
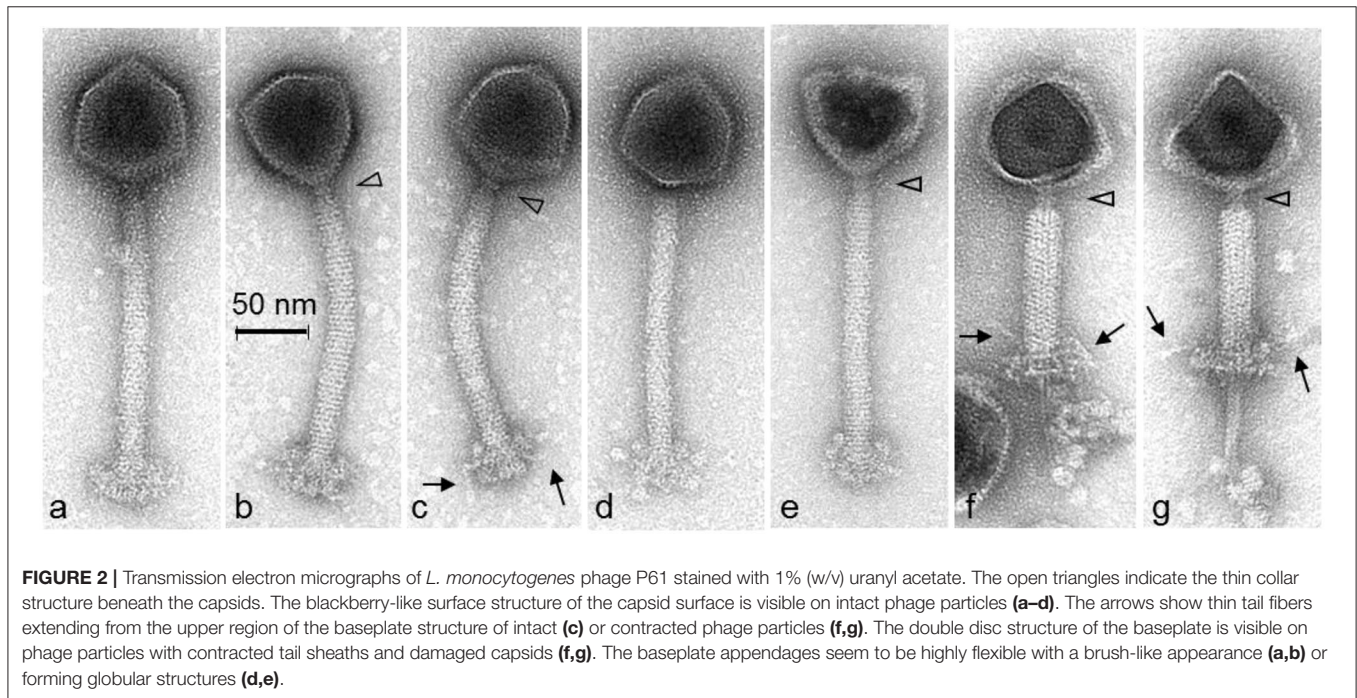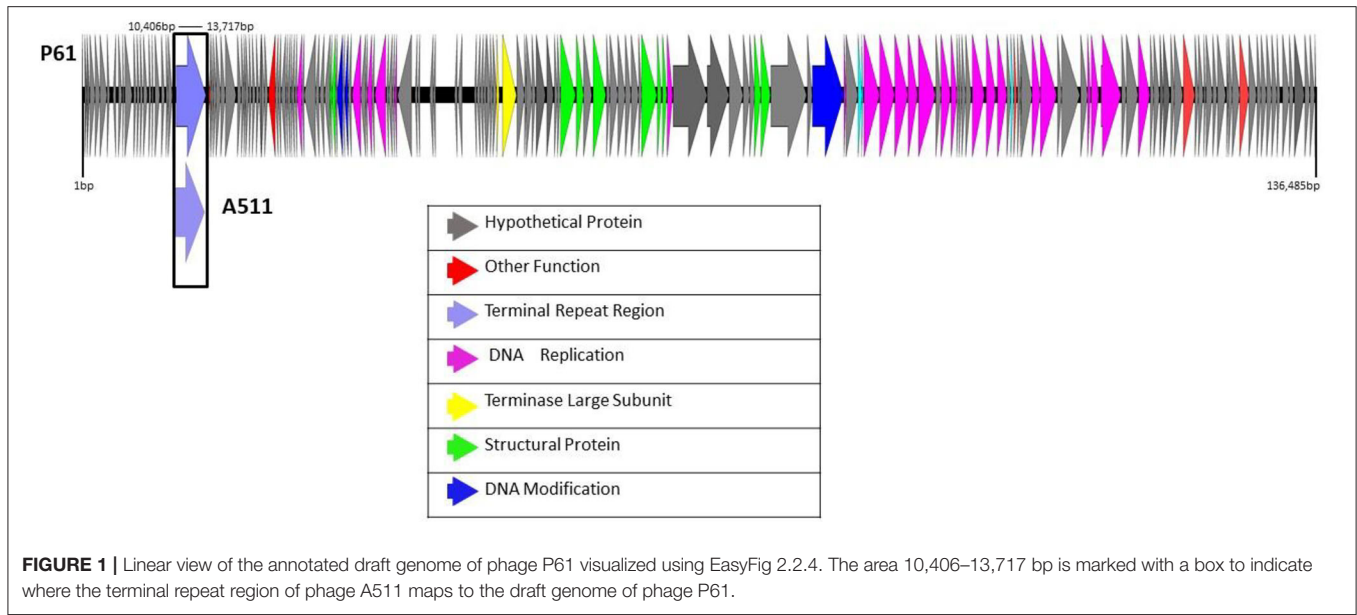
P61 (**Supplementary Table 1**). Blastp and Blastn analysis of the nucleotide sequences and amino acid sequences revealed the genome of phage P61 is similar in size and sequence to that of the *Listeria* phage P100 (131,384 bp) which encodes 174 gene products and 18 tRNAs (Carlton et al., 2005) and that 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 occuring 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^{-5} ($n = 24$).

FIGURE 1 | Linear view of the annotated draft genome of phage P61 visualized using EasyFig 2.2.4. The area 10,406–13,717 bp is marked with a box to indicate where the terminal repeat region of phage A511 maps to the draft genome of phage P61.

FIGURE 2 | Transmission electron micrographs of *L. monocytogenes* phage P61 stained with 1% (w/v) uranyl acetate. The open triangles indicate the thin collar structure beneath the capsids. The blackberry-like surface structure of the capsid surface is visible on intact phage particles **(a–d)**. The arrows show thin tail fibers extending from the upper region of the baseplate structure of intact **(c)** or contracted phage particles **(f,g)**. The double disc structure of the baseplate is visible on phage particles with contracted tail sheaths and damaged capsids **(f,g)**. The baseplate appendages seem to be highly flexible with a brush-like appearance **(a,b)** or forming globular structures **(d,e)**.

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 2×10^8 CFU/ml in untreated samples vs. 1×10^5 CFU/ml in phage-treated samples at 8°C, and $2 \times$

10^9 CFU/ml in untreated samples vs. 3×10^8 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 1×10^3 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
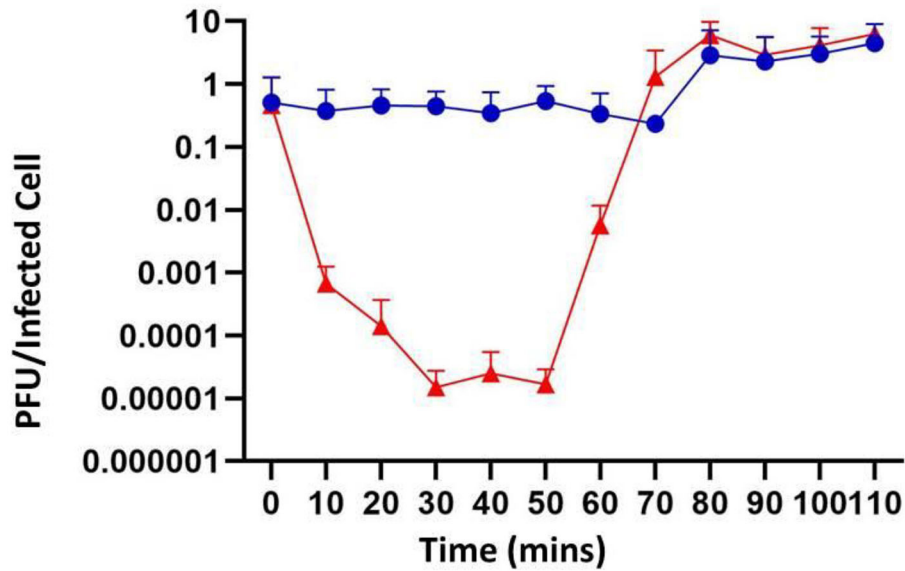
FIGURE 3 | One-step growth curve of phage P61. Blue circles represent samples that were directly plated, representing the cumulative concentration of absorbed phages and unabsorbed phages. Red triangles represent samples that were treated with chloroform prior to plating, representing the total concentration of phages including intracellular phage. All values are means of three independent experiments.
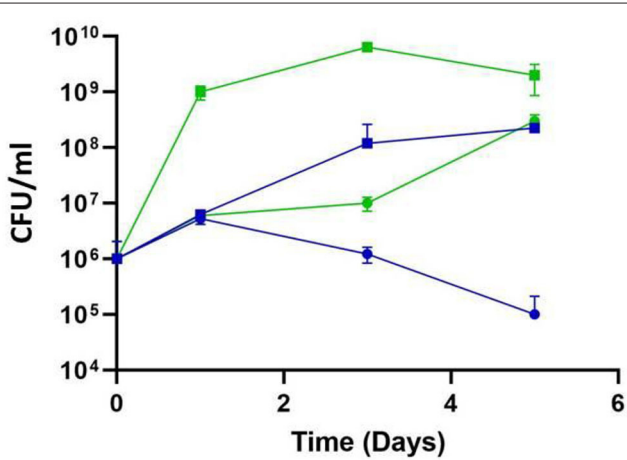
FIGURE 4 | Impact of temperature on the efficacy of phage P61 infection in TSB. *L. monocytogenes* cells were added to TSB broth at a final concentration of 10^6 CFU/ml and phage added at 10^8 PFU/ml (MOI = 100). Samples were incubated at 8°C (blue symbols) and 12°C (green symbols) and *L. monocytogenes* enumerated daily. Squares represent untreated samples; circles represent phage-treated samples.
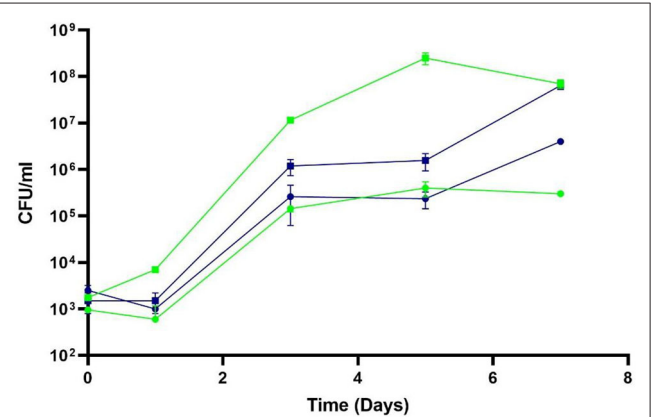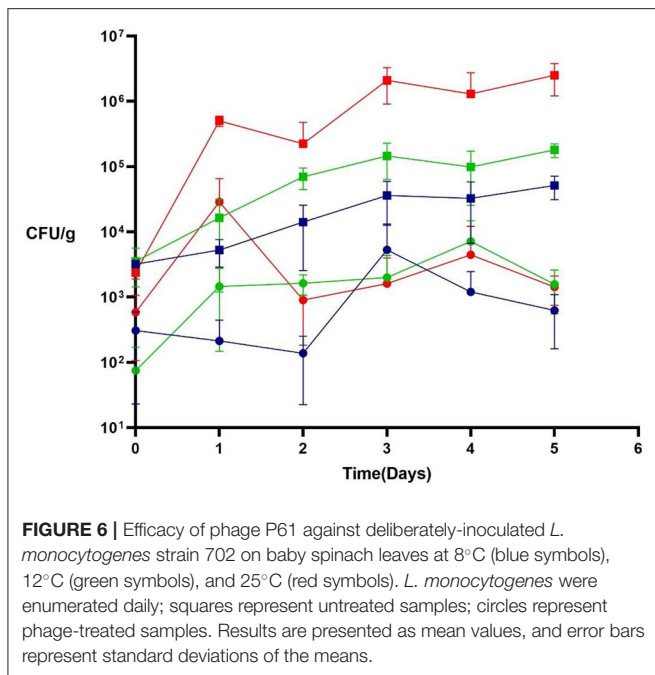

FIGURE 5 | Efficacy of phage P61 against deliberately-inoculated *L. monocytogenes* strain 3,053 in pasteurized liquid milk at 8 and 12°C. Samples were incubated at 8°C (blue symbols) and 12°C (green symbols) and *L. monocytogenes* enumerated daily. Squares represent untreated samples; circles represent phage-treated samples. Results are presented as mean values, and error bars represent standard deviations of the means.

7. When stored at 8°C a reduction of 1.2 log (93.75% reduction) was observed at day 7 (shelf-life of milk) ($p = 0.0173$, **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

FIGURE 6 | Efficacy of phage P61 against deliberately-inoculated *L. monocytogenes* strain 702 on baby spinach leaves at 8°C (blue symbols), 12°C (green symbols), and 25°C (red symbols). *L. monocytogenes* were enumerated daily; squares represent untreated samples; circles represent phage-treated samples. Results are presented as mean values, and error bars represent standard deviations of the means.

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 *L. monocytogenes* was left to acclimatize 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 *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 *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, *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 *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 *L. monocytogenes* to phages. The team highlight that *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, *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 *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 *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 *L. monocytogenes* used influences the ability of phage P100 to reduce the growth of *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 *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 *Listeria* phage 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 *Listeria* phage A511 to hot dogs, chocolate milk, and cabbage, these authors noted that lower doses of phages resulted in a less significant growth suppression of *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 *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 *L. monocytogenes* than when lower

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*.

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.

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

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