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Control of *Salmonella* Enteritidis on food contact surfaces with bacteriophage PVP-SE2

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ABSTRACT

Salmonella is one of the worldwide leading foodborne pathogens responsible for illnesses and hospitalizations, and its capacity to form biofilms is one of its many virulence factors. This work evaluated (bacterio)phage control of adhered and biofilm cells of *Salmonella* Enteritidis on three different substrata at refrigerated and room temperatures, and also a preventive approach in poultry skin. PVP-SE2 phage was efficient in reducing both 24- and 48-h old *Salmonella* biofilms from polystyrene and stainless steel causing 2 to 5 log CFU cm^{-2} reductions with a higher killing efficiency at room temperature. PVP-SE2 phage application on poultry skins reduced levels of *Salmonella*. Freezing phage-pretreated poultry skin samples had no influence on the viability of phage PVP-SE2 and their *in vitro* contamination with *S.* Enteritidis provided evidence that phages prevented their further growth. Although not all conditions favor phage treatment, this study endorses their use to prevent and control foodborne pathogen colonization of surfaces.

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Salmonella; bacteriophage; biofilm; control; prevention

Introduction

Foodborne bacteria continue to be a major cause of illnesses in humans around the world, causing severe threats to human health and safety. Two million deaths are estimated to occur annually due to illnesses related to contaminated food and water, according to the World Health Organization (WHO 2015). Hence, food safety constitutes an increasing worldwide public health concern, in which *Salmonella* remains one of the most common causes of reported food poisoning events (WHO 2015; European Food Safety Authority and European Centre for Disease Prevention and Control 2016). The disease caused by *Salmonella*, salmonellosis, is the result of ingestion of this bacterium, and it shows symptoms such as diarrhea, fever and abdominal pain that occur 12 to 72 h after consumption of contaminated food (CDC 2018a). The leading food sources identified for human salmonellosis are poultry products, in particular chicken products (Finstad et al. 2012; WHO 2018). During the different stages of food processing, from production to consumption, products are susceptible to cross-contamination, particularly in the case of poultry meat products (Grant et al. 2016). Improper handling by the consumer can also contribute to increased rates of infection (DuPont 2007).

The ecology and occurrence of *Salmonella* serovars in poultry compared to those directly associated with human salmonellosis remain difficult to quantify owing to serovar variability in culture media recovery (Mead et al. 2010). *Salmonella enterica* serovar Enteritidis is one of the most reported serovars related to salmonellosis outbreaks (CDC 2018b).

Problems related to *Salmonella* have significantly increased due to their growing antimicrobial resistance and also because of their inherent capacity to adhere to surfaces and consequently form biofilms (Joseph et al. 2001; Helke et al. 2017). Bacteria within biofilms have an increased resistance to antibiotics, disinfectants, surfactants, and other products with antimicrobial activity (Steenackers et al. 2012).

Phages are viruses that infect bacterial cells using the host's machinery to create new progeny. Because of their ability to kill bacteria, they appear to be a good alternative to other products usually used for this purpose (antimicrobials, disinfectants) (Gutiérrez et al. 2016). Phages present many advantages over traditional antibiotics, since they are specific and efficient against their target bacteria, thereby reducing

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the destruction of the host's normal flora (Clark and March 2006). They are also innocuous for humans, and they persist only as long as the target pathogen is present (Ashelford et al. 2003; Hanlon 2007).

In the food industry, the use of new phages and commercially available phage products has recently increased, especially owing to the good results for pathogen reduction reported by several authors (Atterbury et al. 2003; Goode et al. 2003; Carlton et al. 2005; Higgins et al. 2005). Also, the FDA's first approved phage product, Listex™ P100, to control *Listeria monocytogenes* in foods, was a strong incentive for the scientific community to start applying phages to food products (FDA and Department of Health and Human Services 2006). Currently, there are several phage-based products approved by the FDA including PhageGuard Listex (previously known as Listex™ P100), for *Listeria*, PhageGuard S (previously known as Salmonelex™), for *Salmonella*, ListShield™, EcoShield™, SalmoFresh™. In a previous study, several *Salmonella* phages were isolated and characterized into different groups. Some of these phages were verified to be good candidates for phage biocontrol of contaminated poultry products, including PVP-SE2 phage (previously known as φ38 (Sillankorva et al. 2010), which was used in the work described herein. The main goal of this work was to evaluate the *in vitro* efficacy of phage PVP-SE2 to infect adhered and biofilm cells of *Salmonella* Enteritidis on different surfaces, and minimize *S.* Enteritidis colonization of poultry skin surfaces.

Materials and methods

Bacteria and phages

Salmonella enterica serovar Enteritidis S1400 was used to propagate bacteriophage PVP-SE2 previously known as φ38 (Sillankorva et al. 2010). The bacterium was grown at 37 °C in liquid LB medium or in solid LB medium (LB +1.5% (w v^{-1}) of agar). The LPS mutants of *Salmonella* enterica serovar Typhimurium LT2 used in this study were obtained from the *Salmonella* Genetic Stock Centre (University of Calgary, Alberta, Canada).

Phage propagation and titration

Salmonella phage PVP-SE2 was amplified using the plate lysis and elution method (Sillankorva et al. 2008b). Titration of the phage was performed according to Adams (1959).

Transmission electron microscopy

The morphology of phage particles was observed by transmission electron microscopy (TEM), as previously described (Melo et al. 2014). Briefly, phage particles were collected after centrifugation (1 h, 25,000 × *g*, 4 °C). The pellet was washed twice in tap water using the same centrifugation conditions. Phages were deposited on copper grids with carbon-coated Formvar films, stained with 2% (w v^{-1}) uranyl acetate (pH 4.0) and imaged using a Philips EM 300 electron microscope, with magnification being monitored with T4 phage tails (Ackermann 2009).

Phage one-step growth characteristics

The one-step growth curve of phage PVP-SE2 was carried out as previously described (Sillankorva et al. 2008a). Briefly, 10 ml of mid-exponential phase *Salmonella* culture were harvested by centrifugation (7,000 × *g*, 5 min, 4 °C) and the pellet resuspended in 5 ml of fresh LB to obtain an OD_{620} of 1.0. To this suspension, 5 ml of phage were added to have a multiplicity of infection (MOI) of 0.001. Phage PVP-SE2 was allowed to adsorb for 5 min at room temperature. The mixture was centrifuged as above and the pellet resuspended in 10 ml of fresh LB. Samples were taken every 5 min, for 40 min, and immediately plated.

Phage DNA extraction, genome sequencing and annotation

Phage DNA was extracted essentially as described previously (Melo et al. 2014). Purified phages were treated with 0.016% (v v^{-1}) L1 buffer [300 mM NaCl, 100 mM Tris/HCl (pH 7.5), 10 mM EDTA, 0.2 mg ml^{-1} BSA, 20 mg ml^{-1} RNase A, 6 mg ml^{-1} DNase I] for 2 h at 37 °C. After thermal inactivation of the enzymes for 15 min at 70 °C, 50 µg ml^{-1} of proteinase K, 20 mM EDTA, and 1% (w v^{-1}) SDS were added and proteins were digested for 18 h at 56 °C. This was followed by phenol, phenol:chloroform:isoamyl alcohol (25:24:1, (v v^{-1})) and chloroform extractions, respectively. DNA was precipitated with isopropanol (100%) and 3 M sodium acetate (pH 4.6), centrifuged (15 min, 7,600 × *g*, 4 °C), and the pellet air-dried and further resuspended in nuclease-free water (Cleaver Scientific). Genome sequencing was performed on a 454 sequencing platform (Plate-forme d'Analyses Génomiques at Laval University, Québec city, QC, Canada) to 50-fold coverage. Sequence data were assembled using SeqMan NGen4 software (DNASTAR, Madison, WI, USA).

Phage genomes were autoannotated, using MyRAST (Aziz et al. 2008) and the presence of non-annotated CDSs, along with genes in which the initiation codon was miscalled, were checked manually using Geneious 6.1.6 (Biomatters, Newark, NJ, USA). Potential frameshifts were checked with BLASTX (Altschul et al. 1997), and BLASTP was used to check for homologous proteins (Altschul et al. 1990), with an E-value threshold of $<1 \times 10^{-5}$ and at least 80% query. Pfam (Finn et al. 2014) and InterProScan (Finn et al. 2017) were used for protein motif search, with the same cutoff parameters as used with BLASTP. Proteins molecular weight and isoelectric point were determined using ExPASy Compute pI/Mw (Wilkins et al. 1999). The presence of transmembrane domains was predicted operating TMHMM (Käll and Sonnhammer 2002) and Phobius (Käll et al. 2004), and membrane proteins were annotated when both tools were in agreement. The search of tRNA encoding genes was performed using tRNAscan-SE (Schattner et al. 2005). Putative promoter regions were checked using PromoterHunter from phiSITE (Klucar et al. 2009) and were further manually verified. ARNold (Naville et al. 2011) was used to predict rho-independent terminators and the energy was calculated using Mfold (Zuker 2003). The complete genome sequences of PVP-SE2 was submitted to GenBank under the accession number MF431252.

Stability of phage PVP-SE2 at refrigerated and frozen temperatures

Stability of PVP-SE2 on poultry skins was assessed at refrigerated (4 °C) and frozen (–18 °C) temperatures. Briefly, poultry skin samples (1 cm ×1 cm) were disinfected (Table S1), and to each skin square 100 µl of phage PVP-SE2 were added at different concentrations (10^4, 10^5 and 10^6 PFU ml^{-1}). Skins were dried for 30 min and after that they were transferred to the appropriate storage conditions (4 °C and –18 °C). To recover phage PVP-SE2 from the skin squares, samples were immerged in 1 ml of SM buffer (5.8 g l^{-1} NaCl, 2 g l^{-1} $MgSO_4.7\,H_2O$, 50 ml 1 M Tris, pH 7.5) and vortexed for 30 s. Serial dilutions were done in SM buffer for each phage PVP-SE2 concentration used and the plaque forming units (PFUs) determined. Samples were stored for 10 days with triplicate samples assessed every day.

Susceptibility of surviving cells and LT2 mutants to phages

Single colonies (n = 12) from each of the three independent phage treatments performed were randomly selected from the stainless steel (SS), polystyrene and poultry skin surfaces. The susceptibility of these colonies to four phages was tested according to a procedure previously described (Moons et al. 2013). The phages PVP-SE2, φ68, PVP-SE1, and φ135 have all been previously characterized (Sillankorva et al. 2010). PVP-SE2, φ68, PVP-SE1, and φ135 were also plated in *Salmonella* Typhimurium LT2 mutant strains that have different degrees of deletion in the lipopolysaccharide (LPS) chain (Figure S1). Briefly, 10 µl of 10-fold serial dilutions of phage starting at 10^9 PFU ml^{-1} were added to the bacterial lawns of the mutant strains. Plates were incubated overnight at 37 °C, and lytic activity was checked for the formation of lysis areas and phage plaque turbidity.

S. Enteritidis colonization of polystyrene and treatment with phage

Biofilms of *S.* Enteritidis S1400 were formed at 22 °C and 4 °C, in 24-well plates, under static conditions and without medium change to better mimic the handling/storage conditions of poultry products. For biofilm formation, 1 ml of *S.* Enteritidis at 1×10^4 CFU ml^{-1} prepared in LB was added to each well. Biofilms were formed for 1, 24, and 48 h at 22 °C, and for 4, 24, 48, and 72 h at 4 °C, washed once with saline solution (NaCl 0.9% (w v^{-1})) and treated with phage PVP-SE2 at multiplicities of infection (MOIs) of 0.1, 1 and 10. For this, 250 µl of LB and 750 µl of phage were added to each well in order to obtain the right MOI. In the negative control, instead of 750 µl of phage, 750 µl of SM buffer were added. At the end of the treatment, biofilms were washed twice and 1 ml of saline solution (NaCl 0.9% (w v^{-1})) with ferric ammonium sulfate (FAS) at 2 mM was added to each well. Microplates were sonicated (water bath sonicator, Sonic model SC-52, UK) for 6 min, all wells scraped and the number of viable cells in each well was counted.

S. Enteritidis colonization of SS and treatment with phage

SS coupons (stainless steel S30400) measuring 1 cm × 1 cm were disinfected by soaking in ethanol 96% (v v^{-1}) for 30 min, washed with distilled water, dried for 30 min at 60 °C and autoclaved at 121 °C for 15 min. The coupons were placed in 24-well plates and contaminated with *S.* Enteritidis S1400. For this, 1 ml of *S.* Enteritidis S1400 at 1×10^4 CFU ml^{-1} prepared in LB was added to each well. 24 and 48 h old biofilms were formed at 4 °C and 22 °C under static

conditions. After this, coupons were washed with 1 ml of saline solution (NaCl 0.9% (w v^{-1})), and to each well 250 µl of LB medium plus 750 µl of phage PVP-SE2 were added. Phage PVP-SE2 was added at different MOIs (0.1, 1 and 10). In the negative control, instead of 750 µl of phage, SM buffer was used. After incubation for 2, 5 or 24 h, the coupons were washed with saline solution (NaCl 0.9% (w v^{-1})), put in 1 ml of saline solution (NaCl 0.9% (w v^{-1})) with FAS at 2 mM, sonicated for 6 min (water bath sonicator, Sonic model SC-52, UK) and vortexed for 30 s. To determine the number of viable cells in each coupon, CFU counts were performed.

S. Enteritidis colonization of poultry skins and treatment with phage

Poultry skin samples were cut into small squares (1 cm ×1 cm) and immersed in different solutions and/or in an ultrasound bath for distinct periods of time (Table S1). This step was carried out to ensure that any effect observed was solely due to the added bacteria and not any other microorganisms, which could lead to variations in the interpretation of the results. After each treatment, viable cells were determined by CFU counts. Complete eradication of *Salmonella* from the skin samples was achieved after four distinct treatments: (1) acetic acid together with ultrasonic bath; (2) acetic acid (45 min, 4 °C); (3) lactic acid (2 min, 22 °C); and (4) ethanol 96% (30 s, 22 °C). Therefore, for all further experiments, the ethanol (96% (v v^{-1})) treatment was adopted since it was the fastest treatment tested and there was limited residual activity owing to subsequent evaporation. For this procedure, poultry skin squares (1 cm ×1 cm) were immersed in ethanol 96% (v v^{-1}), washed with sterile distilled water, and frozen until further use. Frozen skin squares were placed in 24-well microplates. Each square was inoculated on top with 100 µl of an overnight culture of *S.* Enteritidis S1400 diluted with fresh LB medium to a final concentration of 10^4 CFU m^{-1}. The plates were incubated at 4 °C for 4 and 24 h. After incubation, the skin samples were washed twice with saline solution (NaCl 0.9% (w v^{-1})), and PVP-SE2 (100 µl) was added to the skin squares at MOIs of 10 and 100. Negative control assays were performed with 100 µl of SM buffer instead of phage. Skin samples were incubated at 4 °C and phage infection was carried for 2, 5 and 24 h. After the treatment, skin samples were washed with 1 ml of saline solution (NaCl 0.9% (w v^{-1})), put in 1 ml of saline solution (NaCl 0.9% (w v^{-1})) with FAS at 2 mM, vortexed for 30 s and counting of CFUs was performed.

Phage PVP-SE2 pretreatment of poultry skin samples and post-contamination with S. Enteritidis

Phage PVP-SE2 solutions (100 µl) at concentrations of 10^4, 10^5, and 10^6 PFU ml^{-1} were added to disinfected poultry skin squares (1 cm ×1 cm) and incubated for 30 min at 4 °C. After that, *S.* Enteritidis S1400 (100 µl at 10^4 CFU ml^{-1}) was spread on top of the skin samples, which were then incubated at 4 °C for 5, 24, and 48 h. Negative controls were skin squares in which the 100 µl of phage solution were replaced by 100 µl of SM buffer. After incubation, skin samples were washed, vortexed for 30 s in 1 ml of saline solution (NaCl 0.9% (w v^{-1})) with FAS at 2 mM and CFU counts were performed.

Statistical analysis

Statistical analysis of the results was performed using GraphPad Prism 6 (GraphPad Software, La Jolla, California, USA, www.graphpad.com). Mean and standard deviations (SD) were determined for the independent experiments and the results were presented as mean ± SD. Results were compared using two-way ANOVA, with Tukey's multiple comparison statistical test. Differences were considered statistically different if $p \leq 0.05$ (95% confidence interval).

Results

Phage PVP-SE2

Phage PVP-SE2 was previously suggested as a good biocontrol agent due to its broad-host range (Sillankorva et al. 2010). The virion particle resembled phages belonging to the Jersey-like genus of the family *Siphoviridae*, having heads of 57 nm, tails of 125 nm in length and 8 nm wide, and a base plate with three or more spikes (Figure 1A). Furthermore, this phage formed clear plaques, 3 mm in diameter, without a halo, in bacterial lawns on its host (data not shown). The one-step growth characteristics revealed that the latent period was 15 min, with a rise period of 15 min, thereby giving an average of 240 progeny phages per infected cell (Figure 1B).

Genome analysis revealed that PVP-SE2 is a virulent phage that does not encode genes related with lysogeny nor known toxins of bacterial origin (Figure 2). PVP-SE2 has a linear dsDNA with 42,425 bp with a G + C

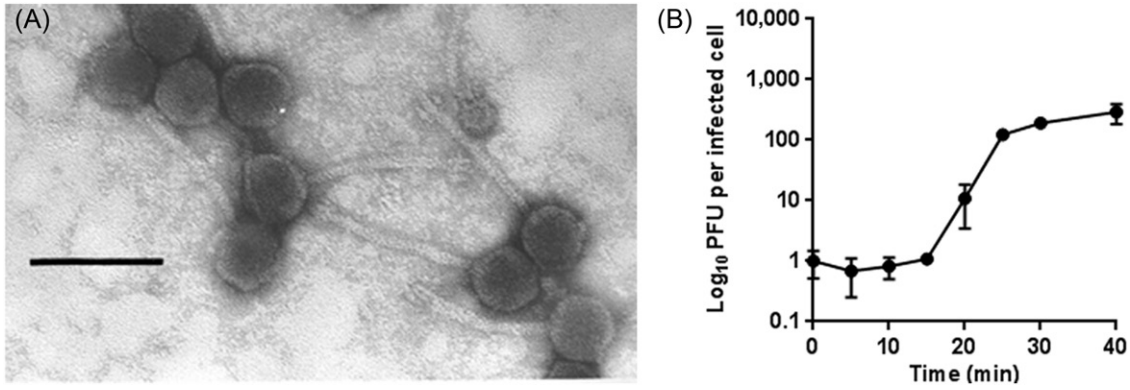

Figure 1. Phage PVP-SE2 characteristics. (A) TEM image of phage PVP-SE2, scale bar =100 nm; (B) one-step growth curve.

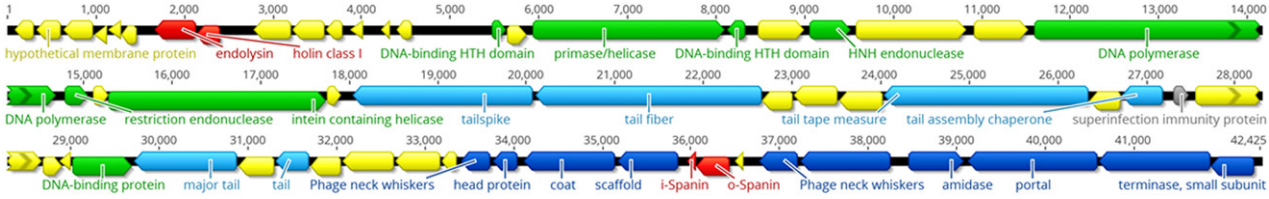

Figure 2. Linear map of phage PVP-SE2 genome sequence. The arrows represent the ORFs and point the direction of transcription.

content of 49.98%. This phage encodes 59 putative coding DNA sequences (CDSs), tightly packed, occupying 92% of its genome (Table S2). From the predicted CDSs, 29 had an assigned function, and no unique proteins nor tRNA genes were identified. Fifty-three CDSs possess methionine as a start codon, while four and three CDSs start with GTG and TTG, respectively. Seventeen of the genes were rightward oriented, while 43 were leftward oriented. Overall, 11 promoters and 14 rho-independent terminators were found throughout the genome. Based on the predictions, the genome appeared to be organized into four functional modules (packaging, structure/morphogenesis, host lysis and replication/regulation) which fits the typical structure of most dsDNA phages (Figure 2).

Whole-genome comparison using BLASTN algorithm revealed that PVP-SE2 is highly homologous (>90% coverage, >90% identity and an e-value of 0) to several *Salmonella* siphoviruses, namely of the genus Jerseyvirus, indicating that PVP-SE2 belongs to this genus. Overall, the analysis performed herein revealed that this phage is theoretically safe for *Salmonella* control on foods and surfaces.

PVP-SE2 viability at refrigerated and freezing temperature conditions

The viability and stability of phage PVP-SE2 on poultry skin samples were monitored over a 10-day period at 4 °C and −18 °C, after the disinfection with 96% ethanol, a treatment which had no effect on phage activity even after five days (data not shown). Test temperatures of 4 °C and −18 °C were chosen as they represent the most common temperatures used for the storage of poultry meat. The number of phage PVP-SE2 particles recovered from the samples stored at either 4 °C or −18 °C remained relatively stable after 10 days, and there was no statistically significant loss of activity (Figure 3A and B), which suggests that after defrosting the phages are still capable of actively targeting *Salmonella.*

Phage application to S. Enteritidis on different contact materials

In food industry environments, *Salmonella* can be found in processing areas, such as walls, floors, pipes and drains and on food contact surfaces such as SS, aluminum, rubber or polystyrene (Steenackers et al. 2012). To study adhesion and biofilm formation, three surfaces were chosen: polystyrene; SS; and poultry skin. Although the study was designed to evaluate phage control of biofilms, in many circumstances, after *in vitro* contamination of the surfaces with *Salmonella*, cells only adhered and did not increase in number. Therefore, the results of phage control of adhered and biofilm cells are presented herein. At 22 °C *S.* Enteritidis cells were allowed to adhere for 1 h to polystyrene after which phage treatment was immediately applied at varied time points

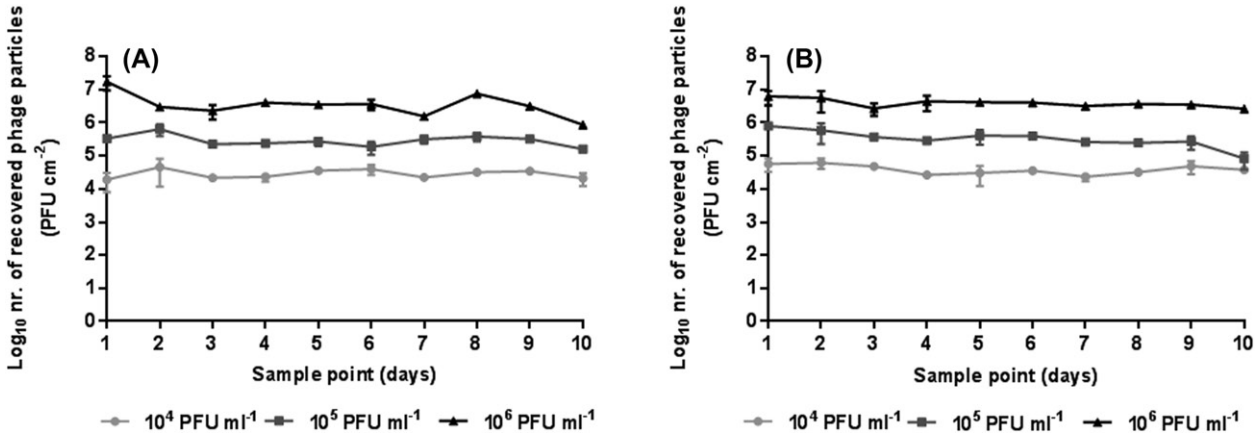

Figure 3. Phage PVP-SE2 viability following storage at (A) 4 °C and (B) −18 °C for 10 days.

(Figure 4A). Although phage application for 2 h had no significant effect on biofilms adhered for 1 h, treatment for 5 and 24 h significantly ($p < 0.05$) reduced the number of viable cells compared to the control samples that grew 10- to 100-fold in number. In general, these results show that higher reductions were obtained for lower MOIs (0.1 and 1), with the greatest decreases occurring when a MOI of 0.1 was used for treatment for 5 and 24 h, respectively. Phage control of biofilms formed on polystyrene was studied for 24 and 48 h-old *S.* Enteritidis biofilms grown at 22 °C (Figure 4B and C). *Salmonella* levels reached ~10^6 CFU cm^{-2} and 10^7 CFU cm^{-2} in 24 and 48 h-old biofilms, respectively. Treatment of both 24 h and 48 h old biofilms significantly ($p < 0.05$) decreased the number of cells compared to the control samples regardless of the phage:host ratios applied, with reductions ranging from 1.5 log to 3.4 log for 24 h biofilms (Figure 4B) and from 2.1 to 5.1 log CFU reductions in 48 h-old biofilms. Overall, the greatest reduction in bacterial numbers was obtained with the lowest MOI used of 0.1 (Figure 4C). While PVP-SE2 clearly showed promising results at room temperature (22 °C), the same was not evident at refrigerated temperatures. At 4 °C, phage control experiments were carried out between 4 h and 72 h. Even though *Salmonella* is able to grow at refrigerated temperatures, at 4 °C the levels of cells after 4 h were similar to those at 72 h (Figure 5) reaching ~2.1 log CFU. Phage treatment applied to either 48 h or 72 h adhered cells had only a minor effect (Figure 5C and D) which although statistically significant ($p < 0.05$) resulted only in a maximum of a 0.5 log reduction. SEM was used to assess biofilm formation at 22 °C on polystyrene prior to and after treatment with phage (Figure 6). After 24 h, biofilms were mainly a layer of individual cells dispersed on the surface (Figure 6E and F) while after 48 h the cells entirely covered the surface of the coupon (Figure 6G and H). Phage infection resulted in damaged cells, and a high amount of cell debris along with a few intact cells (Figure 7).

SS coupons were artificially contaminated with *S.* Enteritidis S1400 and held for 24 and 48 h before being challenged with phage PVP-SE2 (Figure 8). At 22 °C, phage treatment applied to 24 h biofilms caused reductions of up to 1.9 log CFU for all MOIs and periods of treatment (Figure 8A). Unlike the case with polystyrene, the main observation at 22 °C was that PVP-SE2 was more efficient at reducing the number of viable cells in younger (24 h) than in older biofilms (48 h), where only 1.4 log CFU was observed (Figure 8B). At 4 °C, treatment of the 24 h contaminated surfaces did not have a pronounced effect on reducing *Salmonella* numbers despite the numbers being statistically different compared to the control (Figure 8C). At this temperature, phages applied to 48 h contaminated surfaces only slightly limited the growth of cells when a MOI of 0.1 was used (Figure 8D). SEM images (Figure 6) of biofilms on SS showed that these biofilms were structurally different from those formed on polystyrene with microcolonies observed on 24 h biofilms (Figure 6A and B) and a dense biofilm after 48 h (Figure 6C and D). The same cell damage and debris observed in Figure 7 (polystyrene) was also observed on SS coupons (data not shown).

In this work the effect of phage control was further tested as well as *Salmonella* colonization inhibition on phage pretreated poultry skins as a potential strategy that could be used before the industrial packaging of poultry meat. However, before these experiments it was important to ensure that all skin surfaces were both *Salmonella*-free and free from other species that might be present in the skin since these could affect

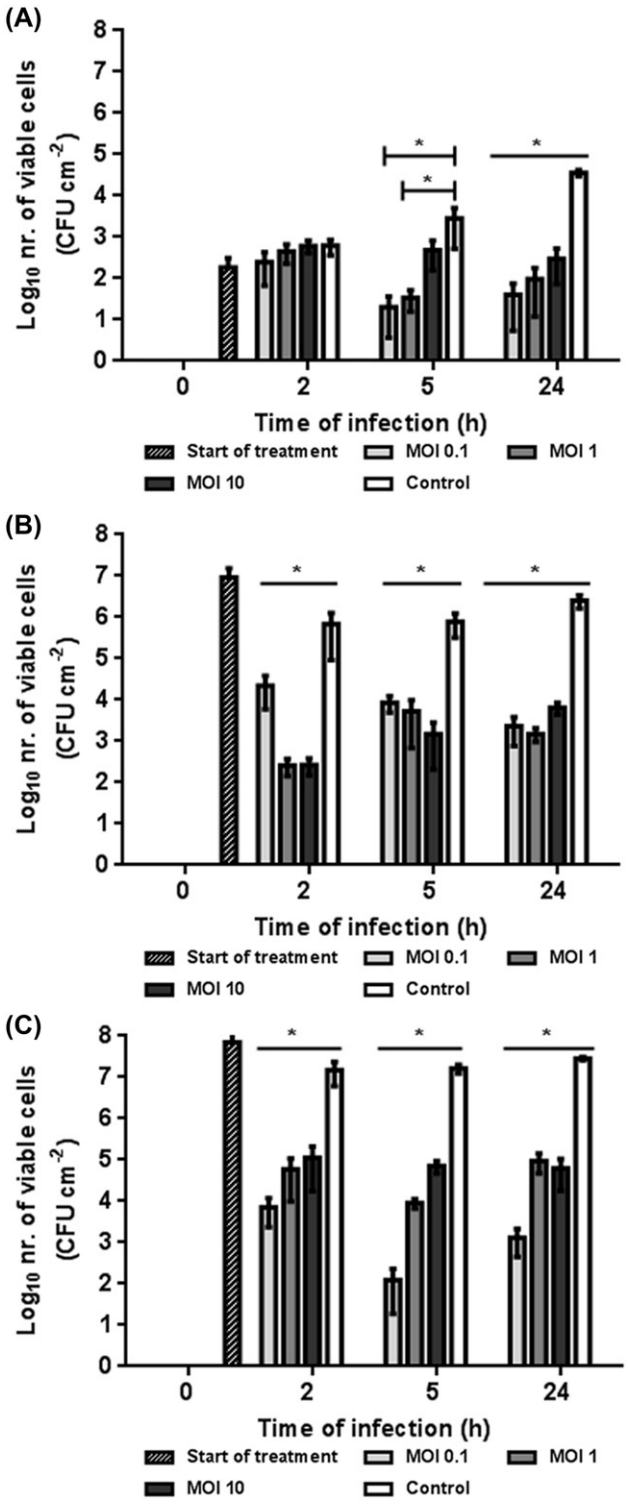

Figure 4. Phage PVP-SE2 control of *S.* Enteritidis S1400 colonizing 24-well polystyrene plates at 22 °C for (A) 1, (B) 24, and (C) 48 h and phage treatment for 5, 24 and 24 h. * indicate significant differences ($p < 0.05$) between PVP-SE2-treated and control samples.

the results obtained by changing the adhesion kinetics of the bacteria and biofilm formation due to competition from other microorganisms (Tsuno et al. 2002; Stubbendieck and Straight 2016). For instance, the presence of *Salmonella* or even other bacteria could affect the MOI constants used and affect the adsorption of PVP-SE2. Thus, a range of disinfection approaches were tested (Table S1) and the easiest, fastest and most efficient method to decontaminate the skin samples, when compared to the other tested methods (eg ultrasonic bath, 2% lactic acid), was adopted as the disinfection procedure. This consisted of applying 96% ethanol for 30 s at room temperature. For the phage control experiments on poultry skins, the tested MOIs were of 10 (for comparison purposes with the other two types of surface) and a higher one (MOI 100) (Figure 9A and B). Furthermore, besides this approach, phage pretreatment of skin surfaces to inhibit *Salmonella* colonization was also assessed (Figure 9C). For this latter experiment, skin samples were pretreated with PVP-SE2 at concentrations of 10^6, 10^7 and 10^8 PFU ml^{-1} before *in vitro* contaminating the skin samples with *Salmonella*. Experiments were only carried out at 4 °C since EFSA and USDA safety recommendations for poultry meat handling do not recommend any type of handling at temperatures above refrigerated temperatures before cooking (EFSA Panel on Biological Hazards (BIOHAZ) 2014; USDA and Food Safety and Inspection Service 2014). In the phage control strategy, *in vitro* contamination of skins was allowed to proceed for 4 and 24 h. The treatment showed that PVP-SE2 was always able to cause a minor but significant reduction in viable cells and maintained viable *Salmonella* numbers at steady and lower levels than in the control samples. Maximum reductions were obtained with a MOI of 100 (Figure 9A and B). This suggests that phage PVP-SE2 can be added to poultry meats to inhibit any further growth of *Salmonella*. In the prevention strategy phages were used to pretreat skin samples (treated using phage concentrations of 10^4, 10^5 and 10^6 PFU ml^{-1}) which were post-contaminated with *S.* Enteritidis at 4 °C (Figure 9C). The highest phage concentration provided the highest inhibition for *Salmonella* to colonize. Phage PVP-SE2 at 10^6 PFU ml^{-1} after 5, 24 and 48 h reduced the levels of *Salmonella* by 1.4, 1.1 and 1.2 log CFU, respectively.

These results suggest that phage pretreatment of skins, even using low phage concentrations, can decrease the colonization by *Salmonella*. Furthermore, it is once again important to refer that PVP-SE2 does not lose viability at refrigerated and frozen temperatures as assessed in this work (Figure 3). Thus, pretreatment of skins limits growth of *Salmonella* once it is taken from refrigerated or frozen temperatures.

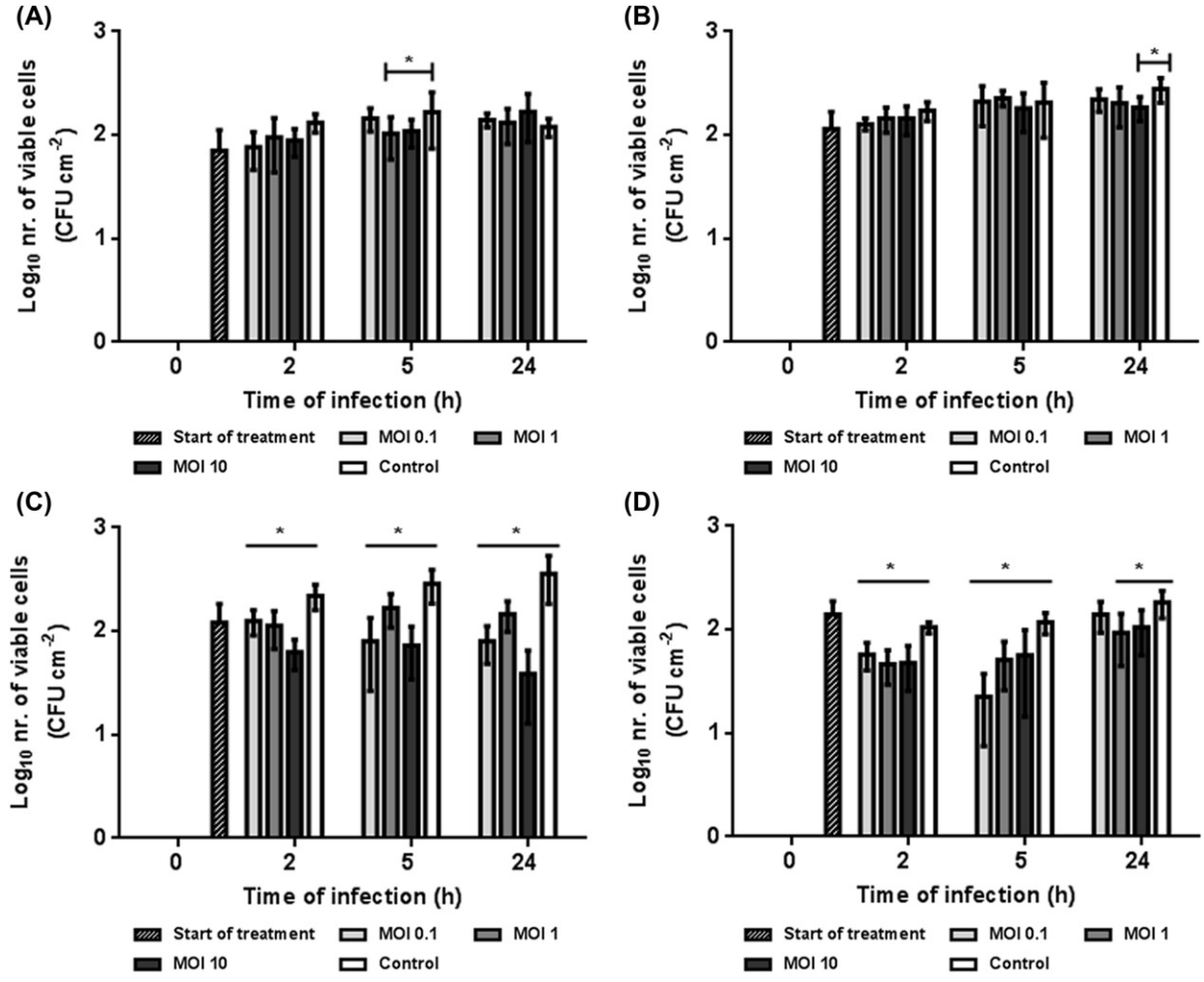

Figure 5. Phage PVP-SE2 control of *S.* Enteritidis S1400 colonizing 24-well polystyrene plates at 4 °C for (A) 4 h, (B) 24 h, (C) 48 and (D) 72 h and phage treatment for 5, 24 and 48 h. * indicate significant differences ($p < 0.05$) between PVP-SE2-treated and control samples.

Susceptibility of resistant colonies

The susceptibility of several colonies from the polystyrene surfaces that survived the phage treatment were tested against the stock PVP-SE2 solution, phages φ68 and φ135 (*Siphoviridae*), and PVP-SE1 (*Myoviridae*), respectively (Table 1). While the majority of cells remained susceptible to PVP-SE2, several colonies selected from the experiments performed at 22 °C were no longer susceptible to the other tested phages. At 4 °C, surviving cells remained mostly susceptible to three phages (PVP-SE2, φ68, and PVP-SE1), although a percentage of these cells had acquired resistance towards phage φ135.

Surviving colonies from assays on SS at 4 °C were susceptible to the phages tested. However, the surviving colonies from experiments at 22 °C were in general resistant to all the phages tested. The survival of colonies from both the control and the pretreatment experiments on poultry skins were susceptible to the phages tested, which is in accordance with the results presented above at 4 °C when SS and polystyrene were used. This suggests that there is no or very limited emergence of resistant phage phenotypes at low temperatures. To assess possible deletions occurring in *Salmonella*, all phages were plated on LT2 mutant strains, a panel of well-characterized *S.* Typhimurium strains (Figure S1). According to Table 1 and the results showing the ability of phages to form plaques (Figure S1) on these strains, it was hypothesized that the most probable mutation occurring in the majority of the surviving isolates was a deletion of the complete outer core region until the first or second heptose (hep) (Rd1 and Rd2 mutants) in the inner core region.

Discussion

The term foodborne disease, or more commonly food poisoning, is used to denote gastrointestinal

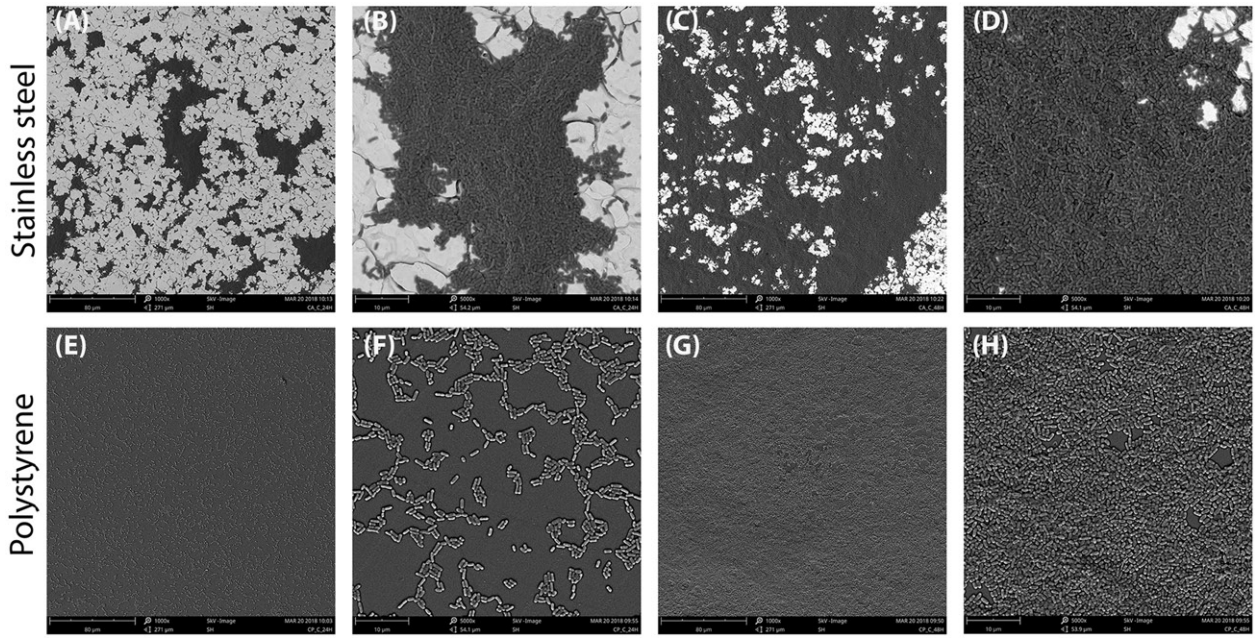

Figure 6. SEM micrographs of *S.* Enteritidis colonization before and after phage application to biofilms formed on SS (A–D) and polystyrene (E–H) at 22 °C, for 24 h (A, B, E and F) and 48 h (C, D, G and H).

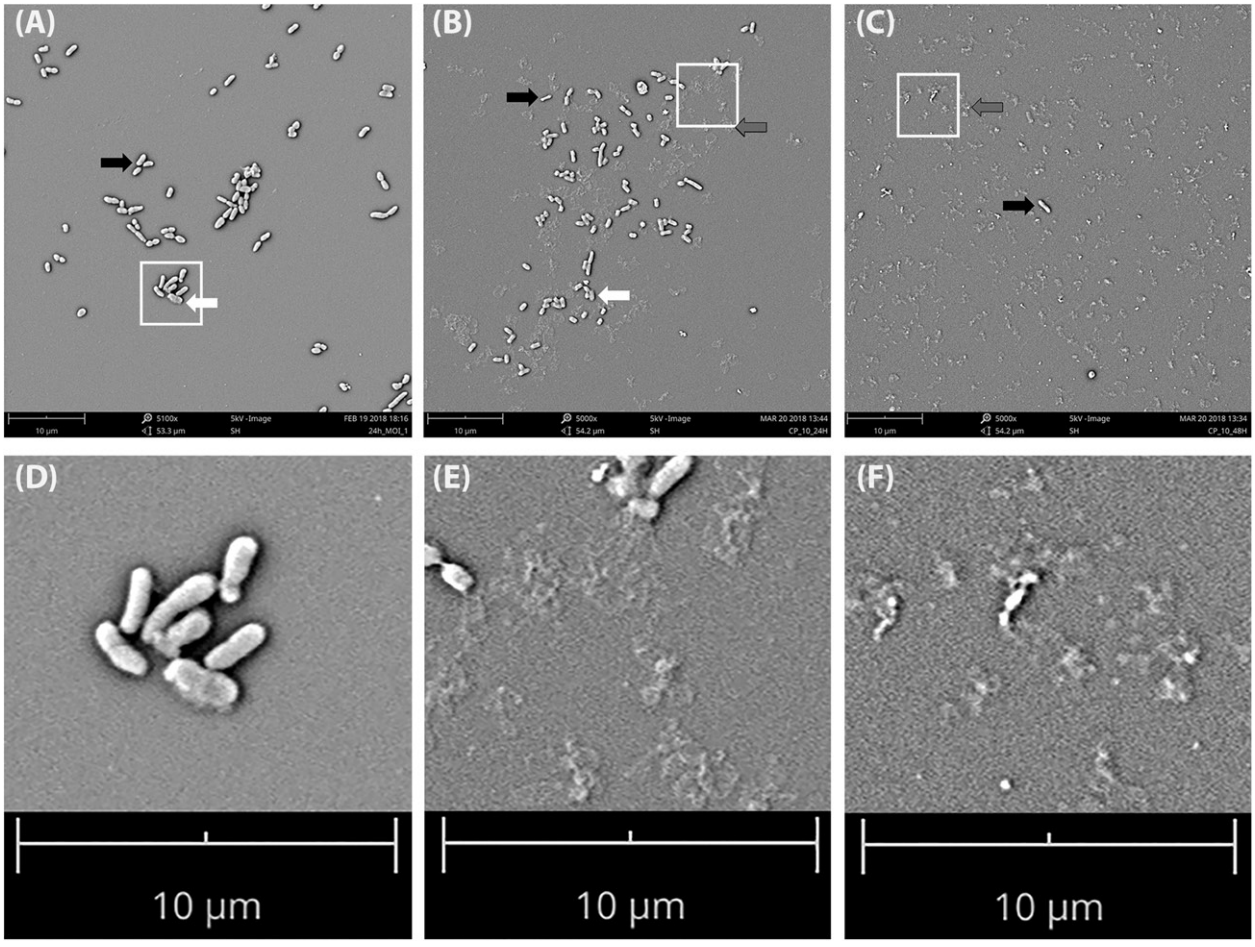

Figure 7. SEM micrographs (A, B and C) and their enlargements (D, E and F, respectively) after phage application to biofilms formed on polystyrene at 22 °C, showing intact cells (black arrows), damaged cells (white arrows), and cells debris (grey arrows). Magnified areas are indicated by white squares.

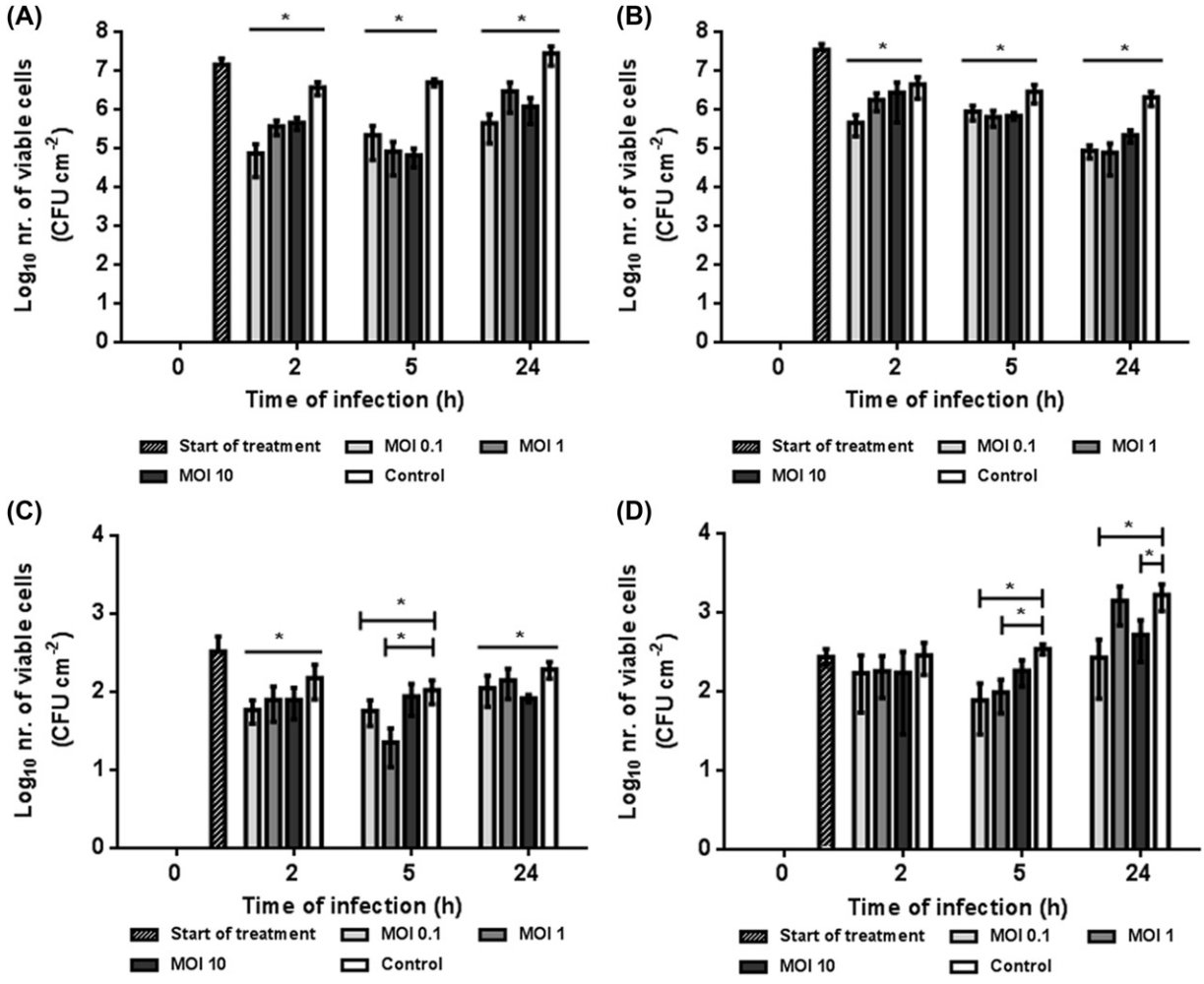

Figure 8. Phage PVP-SE2 control of *S.* Enteritidis S1400 colonizing SS coupons at 22 °C (A and B) and 4 °C (C and D). *S.* Enteritidis colonization for (A, C) 24 h, (B, D) 48 h and phage treatment for 5, 24 and 24 h. * indicate significant differences ($p < 0.05$) between PVP-SE2-treated and control samples.

complications that occur following the consumption of a particular contaminated food or drink. *Salmonella* is one of the microorganisms most commonly associated with this type of foodborne diseases (CDC 2018a), and its ability to form biofilms on different food working surfaces increases the risk of cross-contamination of food products, particularly poultry products (Grant et al. 2016), and the occurrence of food-outbreaks (Shi and Zhu 2009). A closer look at the surface colonization results obtained in this work show that *Salmonella* can either: (1) adhere to the different surfaces and not increase in number or (2) adhere and form biofilms. Cell adhesion is mostly observed at refrigerated temperatures and after surface incubation at room temperature for 1 h. This time point (1 h) at room temperature was chosen to mimic a processing surface that is not immediately disinfected and where, in a few hours, a significant increase in *Salmonella* numbers was observed (Figure 4). This leads to the need to develop new strategies to control this microorganism if present either in the food product itself or on food processing surfaces. The use of phages as substitutes to other antimicrobials appears to be a good alternative.

In this work, the *Salmonella* phage PVP-SE2, previously identified as a good candidate for phage biocontrol of *Salmonella* in poultry meat products (Sillankorva et al. 2010), was characterized and the genomic analysis revealed that the phage does not encode any genes associated with lysogeny or toxin proteins (Table S2). In theory, all virulent phages are able of carrying out generalized transduction, and this would also include phage PVP-SE2. However, this is a rare event since only a minority of new particles (1 in 10^4) contains bacterial DNA (Griffiths et al. 2000), and thus it is not considered a disadvantage of phage therapy. The potential of this phage to control and inhibit surface colonization by *S.* Enteritidis S1400 was investigated at refrigerated (4 °C) and room temperature (22 °C). The focus on these two temperatures is related to the presence of *Salmonella* in the slaughter and processing areas (room

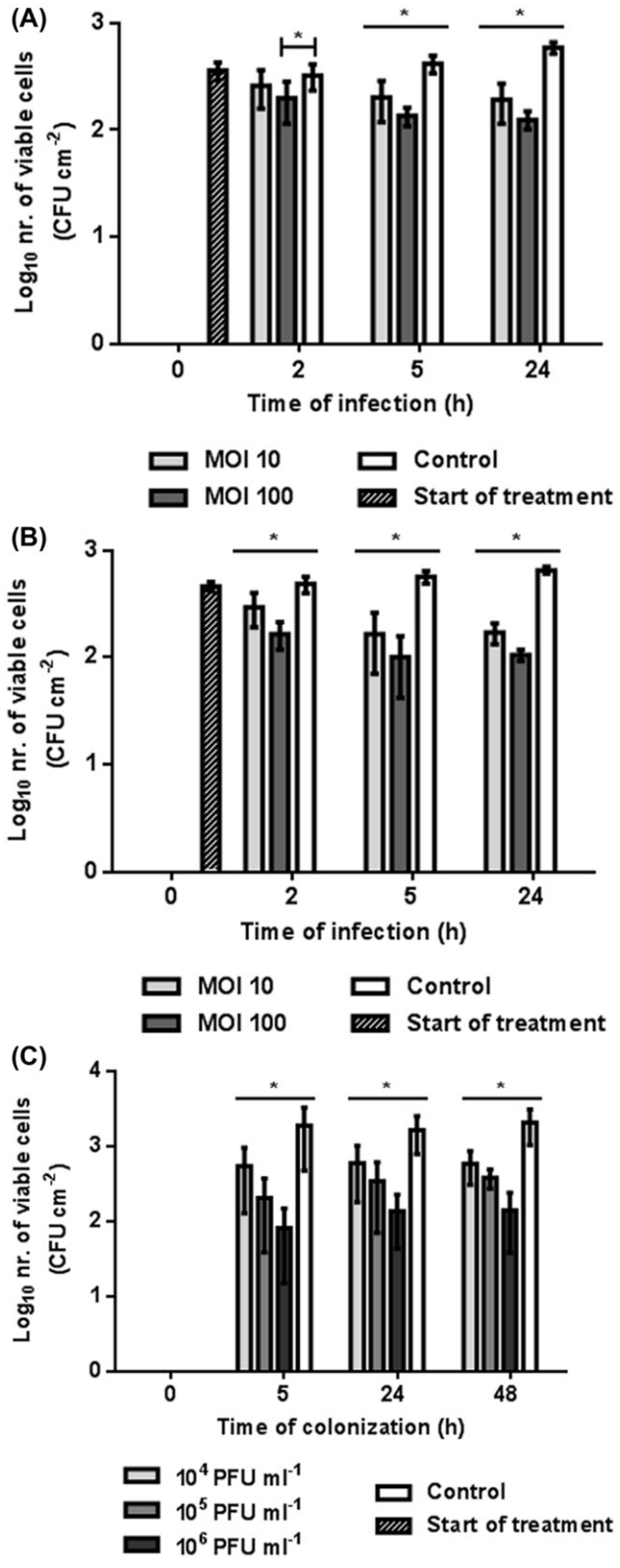

Figure 9. Phage PVP-SE2 control (A and B) and inhibition (C) of *S.* Enteritidis S1400 colonizing poultry skin samples at 4 °C. *S.* Enteritidis colonization for (A) 4 and (B) 24 h and phage treatment for 2, 5 and 24 h. (C) Phage pretreatment of skin samples was done with phage concentrations of 10^4, 10^5 and 10^6 PFU ml^{-1}. *S.* Enteritidis colonization for 5, 24 and 48 h. * indicate significant differences ($p < 0.05$) between PVP-SE2-treated and control samples.

Table 1. Susceptibility analysis of bacterial colonies that survived infection at 4 °C on all surfaces and at 22 °C on polystyrene and stainless steel to phages PVP-SE2, PVP-SE1, φ68 and φ135.

Surface	T (°C)	MOI or phage concentration (log PFU ml^{-1})	Phage resistance (%) PVP-SE2	φ68	PVP-SE1	φ135
Polystyrene	22	0.1	8.3	25.0	25.0	25.0
		10	0.0	8.3	33.3	0.0
	4	0.1	0.0	0.0	0.0	16.7
		10	25.0	0.0	0.0	33.3
Poultry control	4	10	8.3	0.0	0.0	0.0
		100	25.0	8.33	0.0	0.0
Poultry pretreatment	4	3	0.0	0.0	0.0	0.0
		5	0.0	0.0	0.0	0.0
Stainless steel	22	0.1	100.0	100.0	0.0	33.3
		10	100.0	100.0	100.0	100.0
	4	0.1	0.0	0.0	0.0	0.0
		10	0.0	0.0	0.0	0.0

temperature), as well as at lower temperatures (≈4 °C) where the products are stored. To test the ability of phage PVP-SE2 to control *Salmonella*, three surfaces were used: polystyrene and SS, since they are commonly used in food processing plants (Steenackers et al. 2012), and poultry skins.

The treatment with phage PVP-SE2 of adhered and biofilm cells grown in both polystyrene and SS was shown to be more effective at 22 °C than at refrigerated temperatures. This result was expected owing to the closer optimal growth conditions in which *Salmonella* maintains a quite active metabolism when compared to 4 °C. It is generally accepted that cell growth and temperature conditions have a major effect on phage replication. For instance, the replication of coliphage FRNA at lower than optimal cell growth temperature conditions is slower and can even cease because of the lower expression of F pili and adsorption inhibition (Knolle and Orskov 1967; Woody and Cliver 1995). Similar results have also been reported for phages of *Listeria* (Tokman et al. 2016) and *Pseudomonas fluorescens* (Sillankorva et al. 2004) where incubation at refrigeration temperatures resulted in an increase in the latent period, a reduction in phage burst size and overall lower killing efficacy.

Increasing the incubation period of *Salmonella* at 22 °C in polystyrene and SS led to approximately similar viable cell counts at the tested biofilm formation periods (~7 log CFU cm^{-2} after 24 h and closer to 8 log CFU cm^{-2} after 48 h) (Figures 4 and 7). Phage treatment of *Salmonella* was highly influenced by the type of surface the bacteria were attached to. Greater reductions were always observed for cells attached to polystyrene (3 to 5 log CFU cm^{-2}) rather than to SS (1 up to 2 log CFU cm^{-2}). In contrast, to

these results, it has been reported that biofilms grown on SS are more susceptible to sanitizers compared to those on Teflon surfaces (Pan et al. 2006; Poimenidou et al. 2016). According to these authors, polymers are more prone to chemical and mechanical damage and therefore provide increased shelter for bacteria. In the present study, the differences in phage efficacy might be explained by the different biofilm structures formed on both surfaces. Polystyrene is a hydrophobic smooth surface and therefore cells have more difficulty in attaching, forming thin and small clusters of cells spread on the surface (Figure 6E–H). Adhesion to SS is more favorable due to its roughness and therefore cells form thick layers covering all the surface leading to a denser structure which is more difficult to penetrate by phages (Figure 6A–D).

In the experiments there was never a straight correlation between the MOI applied and the cell lysis caused. For instance, in the experiments with polystyrene, the best cell lysis results were obtained with a MOI of 0.1, although it might be expected that the use of a MOI of 1.0 or higher would lead to higher viable cell reductions. For SS the results for the highest lysis efficiencies were far more inconsistent. This may be due to the roughness of the SS coupons since it potentiates the retention of the biofilm to the SS and also protects it from shear forces (Taylor et al. 1998; Nejadnik et al. 2008). Furthermore, deposition/adhesion of phages occurs to varied abiotic surfaces, such as SS (Sillankorva et al. 2008b), and has been shown to increase, for instance for F-specific RNA phages, with the increase in the degree of hydrophobicity and/or roughness (Dika et al. 2013). This non-specific binding may explain the lower effectiveness of phage on SS where, hypothetically, the phages deposit in higher amounts and consequently not all phages are free to complete infection cycles. Previous work suggests that higher phage doses lead to reduced lysing efficacy due to the mechanism of lysis inhibition, which happens when cell density reaches concentrations of 4×10^7 CFU ml^{-1} (Abedon 1990). This observation could explain the results obtained with polystyrene. However, this phenomenon should not be surface dependent, as observed in this experiment. Overall, these results suggest that there is not a universal phage MOI strategy for biofilm control, as it is dependent on the substrata chosen for the experiments, the 3-D biofilm architecture and the level of protection that the extracellular polymeric matrix confers.

In general, it was observed that phage application for 2 h was not enough to substantially reduce the number of viable cells, indicating that for a rapid surface sanitation stronger antimicrobials that act immediately are needed. Nevertheless, taking into account the results, phage PVP-SE2 seems to be a very good control agent of 24 and 48-h old biofilms of *S.* Enteritidis at 22 °C. Therefore, for a better antimicrobial effect desirable for short periods, phages could be mixed with other agents for a higher and possibly synergistic action against foodborne pathogens. For instance, phages added together with chlorine, a disinfectant commonly used in the food industry, acted synergistically in the control and removal of *Pseudomonas aeruginosa* biofilms (Zhang and Hu 2013). Chibeu et al. (2013) demonstrated that the combination of the bacteriophage preparation Listex^{TM} P100 with the chemical compounds potassium lactate and sodium diacetate resulted in an improvement in their action against bacteria present on the surface of Ready to eat (RTE) roast beef and cooked turkey when compared with each agent alone (Chibeu et al. 2013). Also, Sukumaran et al. (2015) showed that the combined or sequential application of the bacteriophage preparation SalmoFresh^{TM} with lauric arginate or cetylpyridinum chloride was beneficial in reducing *Salmonella* on chicken meat and chicken skin.

If the specific action of phage PVP-SE2 is compared with other phages, obviously tested using a different host, some conclusions can be drawn. For instance, in one study, *S.* Enteritidis biofilms grown for four days ($\approx 10^6$ CFU ml^{-1}) at 25 °C on SS coupons were challenged with a mixture of five phages at a MOI of 10 for 60 min (Ferreira et al. 2011). In this case, reductions lower than 0.4 logs were obtained. In another study, a group of *S. enterica* strains was grown on SS coupons and a pool of phages was applied for a period of eight days at room temperature (Gong and Jiang 2017). Although after phage treatment for seven days *S. enterica* biofilms suffered high inhibition, after treatment for one day the number of viable cells dropped less than 1 log. Both these results present similar or lower reduction values than those obtained with PVP-SE2.

The ability of PVP-SE2 as a control agent of *Salmonella* in artificially contaminated chicken skin samples was tested at 4 °C with MOIs of 10 and 100. The higher titer was more efficient with an increase in the number of *S.* Enteritidis cells killed if the treatment period was increased. Similar and higher reductions, at 4 °C, to those obtained with PVP-SE2 have been reported for *S.* Enteritidis on poultry skins (Goode et al. 2003; Hagens 2015).

The use of PVP-SE2 to prevent contamination of poultry meat showed that the PVP-SE2 phage

previously added to the chicken skin samples can decrease significantly the number of viable cells compared to the control samples. Higher phage concentrations used in the pretreatment of skins resulted in greater reductions in bacterial numbers for all time periods of skin colonization assessed (1.4, 1.1 and 1.2 log CFU cm^{-2} reductions at 5, 24 and 48 h, respectively). This result is a good indication that PVP-SE2 phage can be used as an agent to prevent contamination of poultry meat at refrigerated temperatures.

Even though the results at 4 °C from the SS and polystyrene experiments provided only a slight antimicrobial effect, if the samples are taken from refrigerated temperatures to room temperatures (eg periods from store to home) there is an evident advantage of phages to limit the growth of *Salmonella* present in foods. Also, strategically, phage survival at −18 °C was tested. The reason for this is that poultry meat is considered fresh when stored at 4 °C up to three days, otherwise it is stored at −18 °C (USDA and Food Safety and Inspection Service 2015). The results at this temperature show that phage PVP-SE2 maintained its viability on the surface of chicken skins for 10 days when kept either under fresh or frozen conditions. Previous studies have shown that tailed phages can remain viable at refrigerated temperatures even for 10–12 years (Ackermann et al. 2004). However, most phages tend not to remain stable at −20 °C and therefore lose viability, which is not the case for phage PVP-SE2. For instance, several *E. coli* phages were submitted to storage at freezing temperatures and were shown to be stable only for one day (Litt and Jaroni 2017). After this, the authors observed an accentuated reduction of phage titers (4.0–6.3 logs PFU ml^{-1}). This loss in titer is known to be due to the formation of ice crystals (Warren and Hatch 1969; Gould 1999). Although tailed phages tend to maintain viability at 4 °C, some phages are highly sensitive, such as phage Stx2, from *E. coli*, that was shown to substantially reduce in viability after only one day (Rode et al. 2011).

Bacteriophage hosts have developed several resistance mechanisms to survive these viruses (Labrie et al. 2010). In terms of the resistance of *S.* Enteritidis to phages, the main mechanism that this bacterium adopts is to lose the core O-polysaccharide (Santander and Robeson 2007). In this work the susceptibility of cells from the different experiments that had survived phage treatment were tested and four different phages (Table 1) were chosen. Three of these phages (PVP-SE2, φ68 and φ135) had been shown previously to be highly similar in genome size that varied between 32 and 38 kb (Sillankorva et al. 2010). As expected, higher numbers of resistant colonies emerged at 22 °C than at 4 °C, with percentages varying between 0 and 33.3% in cells recovered from biofilms formed on polystyrene and 0–100% in cells from SS surfaces, respectively. Since biofilms formed on SS were thicker compared to those formed on polystyrene, cells will be subjected to more stress, and the existence of cellular metabolites and oxygen reactive species surrounding biofilm cells may induce mutations at a higher rate due to the diffusional limitations (Pires et al. 2017). At 4 °C, most of the surviving cells recovered from polystyrene and SS surfaces as well as from poultry skins (control and pretreatment experiments) were susceptible to all four phages. Also, to assess the LPS chain mutations that led to negative results (no phage plaques formed), the four phages were plated on LT2 mutant strains, which have mutations in different regions of their LPS chain (Figure S1). All phages were infective towards the smooth wild type *S.* Typhimurium strain LT2 and the Re mutant strains. None of the tested phages produced plaques in LT2 mutants Rd1 and Rd2, which suggests that these surviving isolates are devoid of the necessary oligosaccharide component for phage adsorption. However, more surviving colonies should be assessed and their LPS characteristics analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis to confirm the suspected deletions.

In conclusion, it has been demonstrated that phage PVP-SE2 has the ability to act as a control agent of *S.* Enteritidis biofilm and adhered cells on different surfaces, *viz.* polystyrene, SS and poultry skin, at 4 and 22 °C. Furthermore, this phage showed promising results when used for the prevention of contamination of poultry skin at 4 °C. Taking all this information into account and the fact that the PVP-SE2 genome does not contain genes that encode for bacterial toxins nor lysogeny related genes, it can be concluded that phage PVP-SE2 is a good candidate for safe use in the control/prevention of *S.* Enteritidis contamination of food related surfaces.

Disclosure statement

No potential conflict of interest was reported by the authors.

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