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Effectiveness of Bacteriophages Against Biofilm-forming Shiga-toxigenic *Escherichia coli In-vitro* and on Food-Contact Surfaces

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Research Article

Effectiveness of Bacteriophages Against Biofilm-forming Shiga-toxigenic *Escherichia coli In-vitro* and on Food-Contact Surfaces

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Abstract: (1) Background: Formation of biofilms on food-contact surfaces by Shiga-toxigenic *Escherichia coli* (STEC) can pose a significant challenge to the food industry, making conventional control-methods insufficient. Targeted use of bacteriophages to disrupt these biofilms could reduce this problem. Previously isolated and characterized bacteriophages (n=52) were evaluated against STEC biofilms *in-vitro* and on food-contact surfaces. (2) Methods: Phage-treatments (9 logs PFU/ml), in phosphate-buffered-saline, were used individually or as cocktails. Biofilms of STEC (O157, O26, O45, O103, O111, O121, and O145) were formed in 96-well micro-titer plates (7 logs CFU/ml; 24 h) or on stainless-steel (SS) and high-density-polyethylene (HDPE) coupons (9 logs CFU/cm^2; 7 h), followed by phage-treatment. Biofilm-disruption was measured *in-vitro* at 0, 3, and 6 h as a change in optical-density (A_{595}). Coupons were treated with STEC-serotype-specific phage-cocktails or a 21-phage cocktail (3 phages/serotype) for 0, 3, 6 and 16 h and surviving STEC populations enumerated. (3) Results: Of the 52 pages, 77% showed STEC-biofilm disruption *in-vitro*. Serotype-specific phage-treatments reduced pathogen population within the biofilms by 1.9-4.1 and 2.3-5.6 logs CFU/cm^2, while the 21-phage cocktail reduced it by 4.0 and 4.8 logs CFU/cm^2 on SS and HDPE, respectively. (4) Conclusions: Bacteriophages can be used to reduce STEC and their biofilms.

Keywords: bacteriophages; Shiga-toxigenic *E. coli*; biocontrol; biofilms; food-contact surfaces

1. Introduction

Shiga-toxigenic *Escherichia coli* (STEC) are human pathogens responsible for multiple foodborne disease outbreaks. Their infections can range from mild to severe bloody diarrhea and hemorrhagic colitis (HC), leading to life-threatening complications, such as hemolytic-uremic syndrome (HUS) and thrombotic thrombocytopenic purpura [1]. While the STEC serotype, O157:H7, is frequently associated with HC and HUS, other non-O157 serotypes, reported to cause similar infections, have also emerged. These include the top 6 serotypes, O26, O45, O103, O111, O121, and O145, that have been responsible for multiple outbreaks and infections in the last two decades [2–4]. According to the Centers for Disease Control and Prevention (CDC) estimates, STEC O157 and non-O157 together cause more than 265,000 illnesses each year in the United States [5]. These infections could be due to the consumption of contaminated fresh produce, undercooked meat, unpasteurized milk, or drinking water [6]. Other factors include, person to person contact, or livestock handling and contact with their environment on the farm, livestock events or through petting zoos [7].

These pathogens can also adapt to adverse environmental conditions by forming biofilms on a wide variety of food-contact surfaces, as well as on fresh produce and meat products [8–10]. Biofilms consist of a network of adhesive carbohydrates, exopolysaccharides (EPS), that are difficult to penetrate and can protect the bacteria from stressful conditions and antimicrobials [11,12]. It has been reported that STEC biofilms have an increased tolerance to common sanitizers such as chlorine and quaternary ammonium compounds [10,13–15]. This could be due to a combination of bacterial resistance mechanisms such as, diffusional resistance of the EPS matrix, chemical and enzymatic inactivation of sanitizers and disinfectants, physiological changes in the cell, and induction of stress

responses in the cell [16]. The ability of these pathogens to form strong biofilms poses a significant threat of cross-contamination to the food-processing industry. It is therefore critical to develop effective strategies to prevent, remove or control biofilms in the food industry for improved food safety.

Bacteriophages are viruses that infect and kill bacteria and have garnered significant attention as antibacterial agents, primarily due to their target-specificity towards the host bacteria [17–19]. They are present as commensal microflora in the gastrointestinal tract of animals and have also been isolated from various food and water sources [20,21]. The host-specificity and ubiquitous nature of lytic bacteriophages makes them highly desirable as antibacterial agents [18]. Under *in-vitro* conditions, virulent bacteriophages have shown the potential for selective elimination of foodborne pathogens [22,23]. Attention has also been given to the use of bacteriophages for removal of biofilms formed by foodborne pathogens on various food-contact surfaces [24–27]. Viazis et al. [27] tested a phage cocktail against *E. coli* O157:H7 biofilms on stainless steel (SS), ceramic tile, and high-density polyethylene (HDPE) coupons. The phage cocktail effectively reduced pathogen populations in biofilms on all three surfaces, within an hour of the treatment. In another study, phage treatment on spinach-harvesting blade reduced *E. coli* O157:H7 in biofilms by 4.5 logs CFU/blade [25]. The majority of these studies have been limited to evaluating bacteriophage potential against *E. coli* O157:H7 and its biofilms. Very little is known about the effectiveness and applicability of phages against non-O157 STEC and their biofilms. The objective of the present study was to evaluate the efficacy of previously isolated and characterized bacteriophages [28,29] against O157 and non-O157 STEC biofilms *in-vitro* and on food-contact surfaces.

2. Materials and Methods

2.1. Bacterial cultures

The STEC isolates used in the study included, *E. coli* O157:H7 (ATCC 43895, wild type (WT): LF4, KF10); O26 (CDC 2003-3014, WT: QF6, BF8); O45 (CDC 2000-3039, WT: EF2, AF1); O103 (CDC 2006-3008, WT: GF6, AF10); O111 (CDC 2010C-3114, ATCC: 2440, 2180), O121 (CDC 2002-3211, ATCC: 2219, 2203); and O145 (CDC 99-3311, ATCC: 2208, 1652). The WT isolates were retrieved from the Jaroni laboratory culture collection, originally isolated from bovine feces or cattle farm environment [30]. Biofilm forming capability of these isolates was previously tested *in-vitro* [31].

For *in-vitro* studies, overnight cultures of STEC isolates were prepared in Luria Broth (LB; MP Biomedicals, CA) and incubated while shaking (180 rpm; V.390W, Fisher Scientific, NJ) at 37°C for 16 h. For phage propagation and food-contact surface studies, overnight cultures were prepared in tryptic soy broth (TSB; Bacto™, Difco, BD, Sparks, MD) and incubated statically at 37°C for 18 h. Bacterial cocktail suspensions were prepared by mixing (1:1) isolates of STEC serotypes, at a population of 10^9 CFU/ml (Table 1-3).

Table 1. Bacterial and phage cocktails used in *in-vitro* Experiment-II.

STEC	Bacterial Cocktail	Phage Cocktail Treatment (CT)	
O157	ATCC 43895, WT: LF4, KF10	CT1-O157	P1, P2, P4, P6
		CT2-O157	P1, P2, P5, P7
		CT3-O157	P3, P5, P7
		CT4-O157	P2, P3, P4, P7
O26	CDC 2003-3014, WT: BF8, QF6	CT5-O26	P10, P11, P12, P13
O45	CDC 2000-3039, WT: AF1, EF2	CT6-O45	P9, J12, J13, J15
O103	CDC 2006-3008, WT: AF10, GF6	CT7-O103	P19, P20, P21
O111	CDC 2010-3114, ATCC: 2440, 2180	CT8-O111	P14, P15, P16, P17
O121	CDC 2002-3211, ATCC 2219, 2203	CT9-O121	P8, J1, J4, J7
		CT10-O121	P8, J3, J6, J9
O145	CDC 99-3311, ATCC 1652, 2208	CT11-O145	J21, J24, J26, J27
		CT12-O145	J25, J28, J29, J30

Table 2. Multi-serotype bacterial (14 strains) and phage (21 serotype-specific phages) cocktails used in *in-vitro* Experiment-III and in food-contact surface Experiment-II.

STEC	Bacterial Cocktail	Phage Cocktail
O157	ATCC 43895, WT LF4	P2, P6, P7
O26	CDC 2003-3014, WT QF6	P11, P12, P13
O45	CDC 2000-3039, WT AF1	P9, J12, J15
O103	CDC 2006-3008, WT AF10	P19, P20, P21
O111	CDC 2010-3114, ATCC 2180	P14, P15, P17
O121	CDC 2002-3211, ATCC 2219	P8, J3, J7
O145	CDC 99-3311, ATCC 1652	J18, J21, J29

Table 3. Selected phage cocktails used in food-contact surface study, in Experiment I.

STEC	Bacterial Cocktail	Phage Cocktail
O157	ATCC 43895, WT: LF4, KF10	P3, P5, P7
O26	CDC 2003-3014, WT: BF8, QF6	P10, P11, P12, P13
O45	CDC 2000-3039, WT: AF1, EF2	P9, J12, J13, J15
O103	CDC 2006-3008, WT: AF10, GF6	P19, P20, P21
O111	CDC 2010-3114, ATCC: 2440, 2180	P14, P15, P16, P17
O121	CDC 2002-3211, ATCC: 2219, 2203	P8, J3, J6, J9
O145	CDC 99-3311, ATCC: 2208, 1652	J21, J24, J26, J27

2.2. Phage preparation

Phages were propagated by suspending 100 µl overnight culture of host bacterium in molten (0.75%) NZCYM agar (RPI Corp, IL; Fisher Scientific, NJ) and plating via double layer agar method [28]. Phage working-stock solutions were prepared as previously described [28] and stored at 4°C, until further use. Prior to an experiment, phage titers were determined as plaque forming units per ml (PFU/ml), by serially diluting the phage working-stock in phosphate buffered saline (PBS: pH 7.4: NaCl, KCl, NaH2PO4 and KH2PO4, Sigma-Aldrich, MO, USA) and performing a plaque assay [28]. All phage treatments were prepared at a population of 10^9 PFU/ml.

2.3. STEC biofilm disruption

2.3.1. In vitro biofilm disruption

In-vitro STEC biofilm disruption was determined as previously described [32], in the following three experiments (I, II, III). In Experiment-I, previously isolated and characterized phages (n=52) were used individually to test their efficacy against their host STEC biofilms. Based on the results from Experiment-I, phages were selected to prepare host-specific cocktails (Table 1), which were then tested in Experiment-II. In Experiment-III, a multi-phage cocktail, containing 21-phages (3 phages per STEC serotype), was tested against biofilms of multi-serotype STEC cocktail (Table 2).

Overnight pathogen cultures were diluted (1:100) in M9 medium (MP Biomedicals, CA), supplemented with 0.4% (wt/vol) glucose (Fisher Scientific, NJ) and minerals (1.16 mM MgSO4, 2 µM FeCl3, 8 µM CaCl2, and 16 µM MnCl2; Fisher Scientific, NJ), and incubated with shaking (180 rpm) for 24 h at 37°C. Following incubation, bacterial cultures were further diluted (1:100) in M9 medium (containing glucose and minerals) and allowed to form biofilms in 96-well microtiter plates (Thermo Scientific, Denmark) by aliquoting 150 µl in each well (in triplicates) and incubating the plates at 37°C for 24 h. Wells filled with sterile M9 were used as the negative control. After incubation, the liquid culture was carefully removed using a micropipette and the wells washed three times with PBS (150 µl), without disturbing any biofilm formed at the bottom of the wells. Plates were dried at 37°C for 15 min and 150 µl of the respective bacteriophage treatment (individual or cocktail) or PBS (positive control) was added to the wells. Plates were incubated further at 37°C for 0, 3, and 6 h. After each incubation period, the treatment solution was removed and the wells washed with PBS, as described

above. After drying at 37°C for 15 min, biofilms were stained by placing crystal violet (CV; Fisher Scientific, NJ) solution (0.1% in distilled water; wt/vol) in the wells for 2 min, washing thrice with PBS, and drying (37°C for 15 min). The stain was released with 150 μl of ethanol:acetone solution (80:20; vol/vol; Fisher Scientific, NJ) and biofilm disruption quantified by measuring the optical density (OD) at 595 nm (SpectroMax M3; Molecular Devices LLC., CA). Wells filled with ethanol:acetone solution were used as the blank.

2.3.2. STEC biofilm disruption on food-contact surfaces

The efficacy of bacteriophages to disrupt STEC biofilms on food-contact surfaces was studied in two experiments (I and II). In Experiment-I, a cocktail of each STEC serotype (described in 2.1) was used to form biofilms on stainless steel (SS) and high-density polyethylene (HDPE) coupons and treated with their respective phage cocktail for 16 h (Table 3). In Experiment-II, a 21-phage cocktail was evaluated against biofilms formed by a cocktail of 14 STEC isolates (2 isolates per serotype), on SS and HDPE coupons, for 0, 3, 6 and 16 h (Table 2).

2.3.2.1. Preparation of coupons

Stainless steel (304 finish, type 4; Stillwater Steels, OK) and HDPE (1/8"x 24"x 48"; Polymersan, Hialeah, FL) coupons (2 x 5 cm^2) were used for the study and prepared as described by Hood and Zottola [33]. Coupons were cleaned by soaking in acetone (30 min), followed by distilled water rinse (5 min), soaking (1 h) in 1 N NaOH (Fisher Scientific, NJ) and sonicating (40KHz; Branson, CT) in distilled water (1 h). Following sonication, coupons were rinsed in distilled water, air-dried and sterilized prior to use.

2.3.2.2. Phage treatment of STEC biofilms

For each STEC serotype, 3 coupons were inoculated, where one coupon was designated as the inoculated, untreated control, one was treated with the respective phage cocktail, and one treated with PBS (control). In each experiment (I and II), coupons were first immersed in 30 ml suspension of bacterial cocktail (10^9 CFU/ml) in a 50 ml centrifuge tube (Fisher Scientific, GA) and incubated at 25°C for 2.5 h, to facilitate bacterial attachment to the surface. Coupons were gently removed from the suspension using sterile forceps and placed in a sterile 50 ml tube for 5 h in a biosafety hood, allowing further bacterial attachment and biofilm formation. After incubation, coupons were rinsed in 30 ml sterile distilled water to remove unattached bacterial cells from the surface. One coupon of each surface material (SS or HDPE) was sampled to determine the initial pathogen population within the biofilm (inoculated, untreated control). The remaining inoculated coupons were then suspended in 30 ml bacteriophage (10^9 PFU/ml) treatment (Table 3) or PBS at 37°C for 16 h in Experiment-I, and 0, 3, 6 and 16 h in Experiment-II. After incubation, coupons were sonicated for 5 min at 40 KHz to dislodge bacterial cells from the coupon surface. Immediately following, 3 g of glass beads (Genlantis, CA) were added to the tube and agitated for 1 min, using a vortex (Fisher Scientific, NJ), to remove any remaining attached cells from the coupon [26]. Surviving pathogen populations in the solution were enumerated (CFU/cm^2) on TSA or STEC CHROM agar (CHROMagar, Paris, France). Any injured cells were recovered by 24 h enrichment in TSB at 37°C.

2.4. Statistical analysis

All the experiments were repeated 3 times. Surviving STEC populations, recovered after treatments, were converted to log$_{10}$ CFU/cm^2 and the mean values of the three replicates obtained. Data were analyzed using General Linear Model (SAS v.9.3 software; SAS Inst., Cary, NC, USA) to determine analysis of variance (ANOVA) for phage treatment effects. Significant differences between the treatment means were separated by the least significant difference (LSD) at $P<0.10$ for *in-vitro* studies and $P<0.05$ for food-contact surface studies.

3. Results and Discussion

3.1. STEC biofilm disruption

Effectiveness of individual and cocktails of phages to disrupt biofilms of 7 STEC serotypes, *in-vitro* and on food-contact surfaces (SS and HDPE), was determined. Results demonstrated that the phages were more effective in reducing biofilms on food-contact surfaces than *in-vitro*.

3.1.1. In vitro STEC biofilm disruption

Biofilm disruption by individual, serotype-specific bacteriophages, was determined in-vitro by measuring OD (A_{595}) at 0, 3 and 6 h. STEC-biofilm disruption was observed with a reduction in OD from 2.262 nm (0 h) to 0.808 nm (6 h) in phage-treated wells. All phages (n=52) reduced biofilm formation by their respective host bacteria, with 77% showing significant ($P<0.10$) reductions at 3 and 6 h (Figure 1A-G).
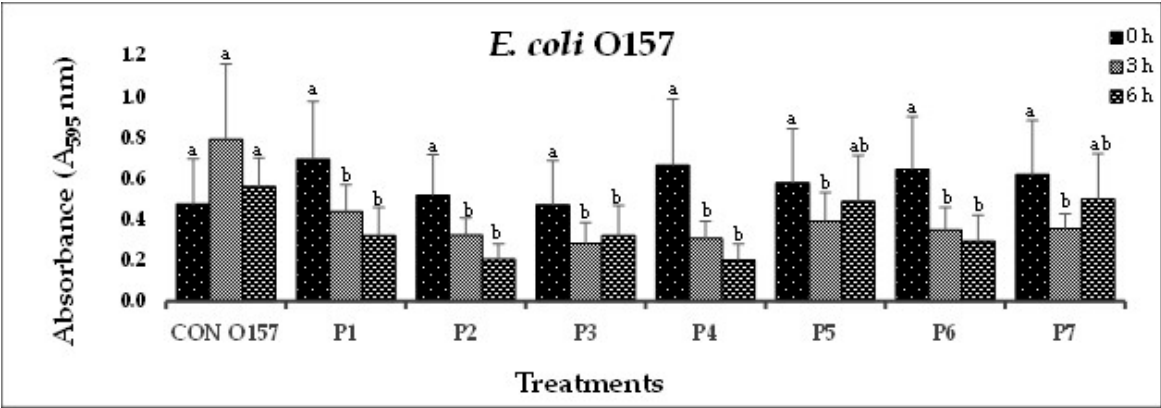
Figure 1. (A). *In-vitro* biofilm disruption by individual bacteriophages specific to *E. coli* O157:H7 after treatment for 0, 3, and 6 h.
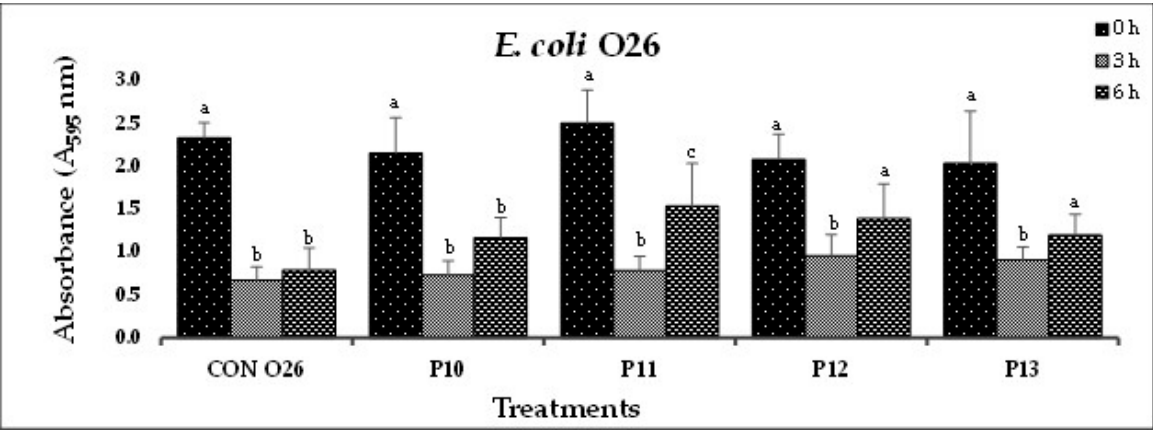

Figure 1. (B). *In-vitro* biofilm disruption by individual bacteriophages specific to *E. coli* O26 after treatment for 0, 3, and 6 h.
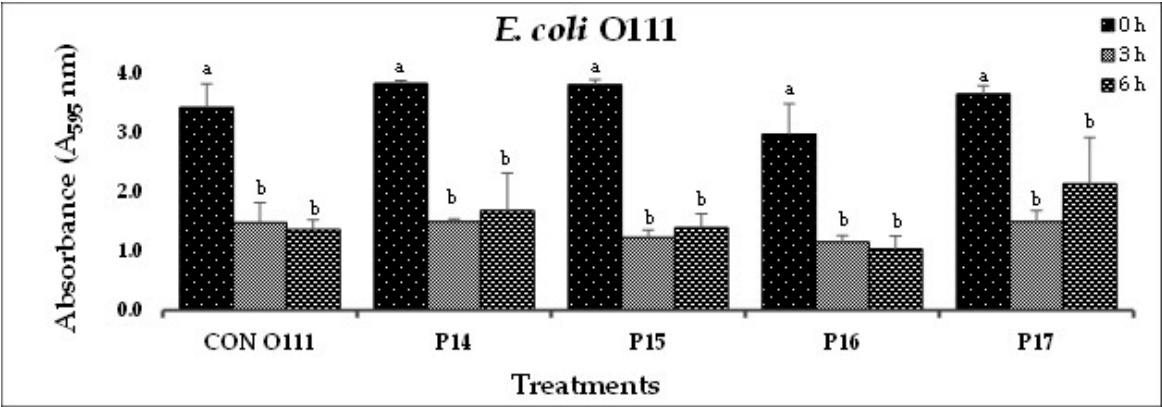
Figure 1. (C). *In-vitro* biofilm disruption by individual bacteriophages specific to *E. coli* O111 after treatment for 0, 3, and 6 h. .
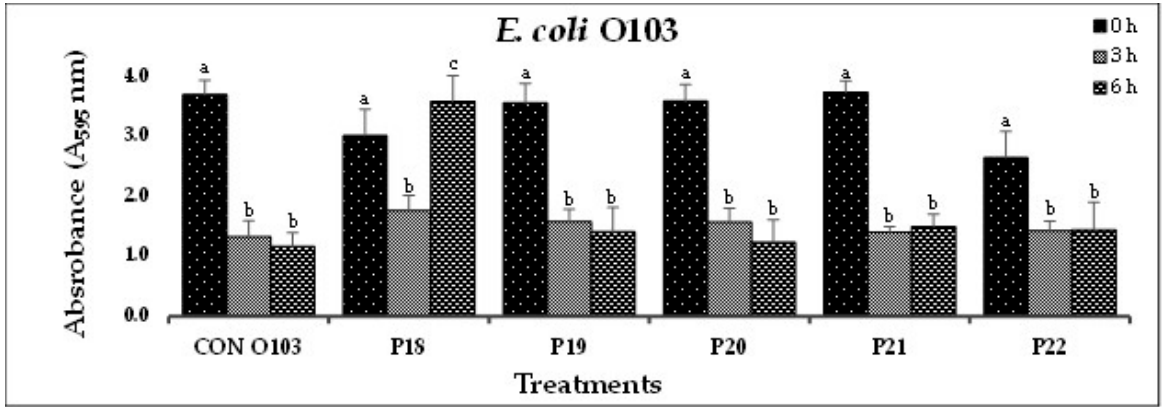

Figure 1. (D). *In-vitro* biofilm disruption by individual bacteriophages specific to *E. coli* O103 after treatment for 0, 3, and 6 h.
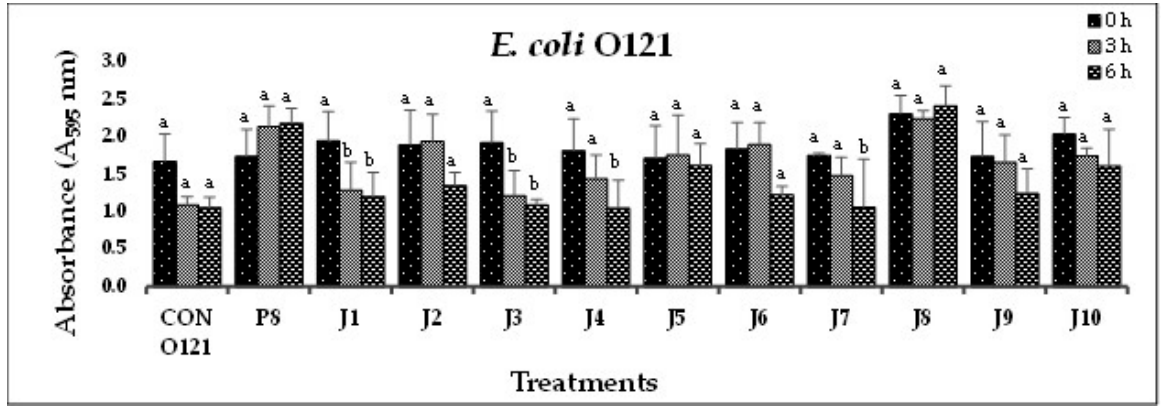

Figure 1. (E). *In-vitro* biofilm disruption by individual bacteriophages specific to *E. coli* O121 after treatment for 0, 3, and 6 h.
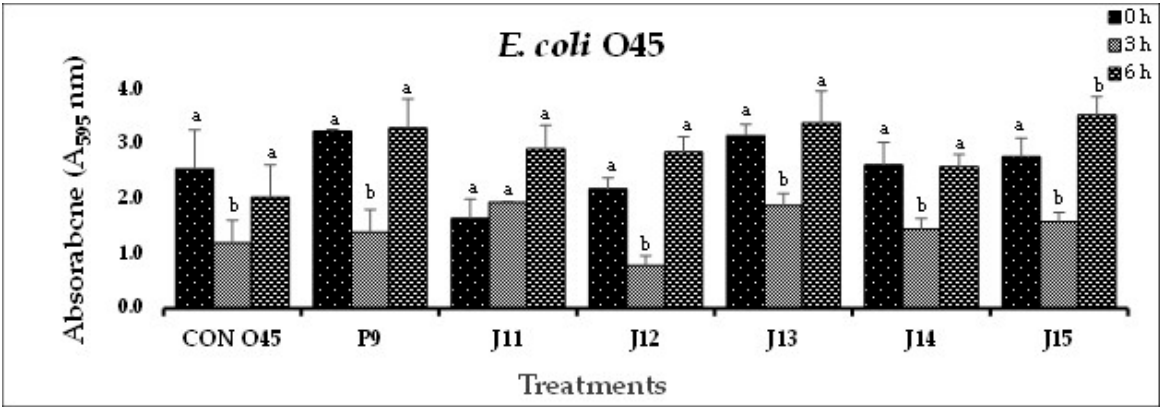
Figure 1. (F). *In-vitro* biofilm disruption by individual bacteriophages specific to *E. coli* O45 after treatment for 0, 3, and 6 h. .
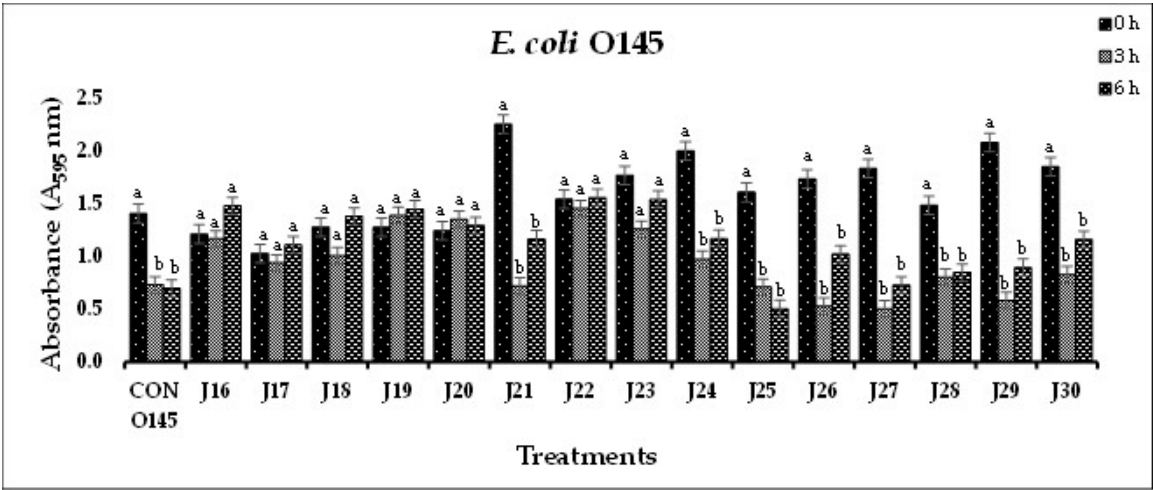

Figure 1. (G). *In-vitro* biofilm disruption by individual bacteriophages specific to *E. coli* O145 after treatment for 0, 3, and 6 h. .

In Experiment-I, where individual phages were tested against their host pathogens, varying results were obtained. All the O157-phages (P1-O157 to P7-O157) showed a reduction in pathogen biofilms at 0 h (A_{595} = 0.472 – 0.696), 3 h (A_{595} = 0.284 – 0.441) and 6 h (A_{595} = 0.202 – 0.321), except phages P3, P5 and P7, where an increase in absorbance was observed at 6 h (Figure 1A). At 3 and 6 h, phage P4-O157 showed the highest reduction compared to the rest of the phages. Non-O157 STEC biofilms, treated with phages, also showed significant ($P<0.10$) reductions at 0 h (A_{595} =1.034 – 3.853), 3 h (A_{595} = 0.506 – 2.631) and 6 h (A_{595} = 0.202 – 3.577) (Figure 1B-G). However, STEC O45-specific phages were more effective after 3 h than after 6 h treatment. A list of the most effective serotype-specific phages is provided in Table 4.

Table 4. Most effective serotype-specific individual phages and phage cocktail treatments *in-vitro*.

STEC	Individual Phage Treatment	Phage Cocktail Treatment (CT)
O157	P4	CT-2, CT-3
O26	P11	CT-5
O45	P9	CT-6
O103	P21	CT-7
O111	P15	CT-8
O121	J3	CT-10
O145	J29	CT-12

Based on the results from Experiment-I, selected phage cocktails were tested against their respective STEC-serotype biofilms (Experiment-II). A list of the most effective serotype-specific phage cocktails is provided in Table 4. Results revealed that all O157-phage cocktails (CT-1 to CT-4) were able to disrupt *E. coli* O157:H7 biofilms, reducing OD from 1.990 – 2.329 at 0 h to 0.576 – 0.636 at 6 h (Figure 2A). The CT-3 cocktail showed the highest reduction from 2.250 at 0 h to 0.748 at 3 h and CT-2 showed the highest reduction at 6 h from 2.319 to 0.576. Among the STEC nonO157 phage cocktails, CT5-O26, CT7-O103 and CT12-O145 performed the best by continuing to reduce biofilms at 6 h (Figure 2B,C). Phages from Experiment-II were selected to make a 21-phage cocktail (3 phages per serotype) to treat multi-serotype STEC biofilms (2 bacterial isolates per serotype). Results showed that phage cocktail reduced biofilm after 3 h of treatment, where OD decreased from 2.561 at 0 h to 1.321 at 3 h (Figure 2D). However, a slight increase in absorbance (2.186) was recorded after 6 h of phage-cocktail treatment (Figure 2D).
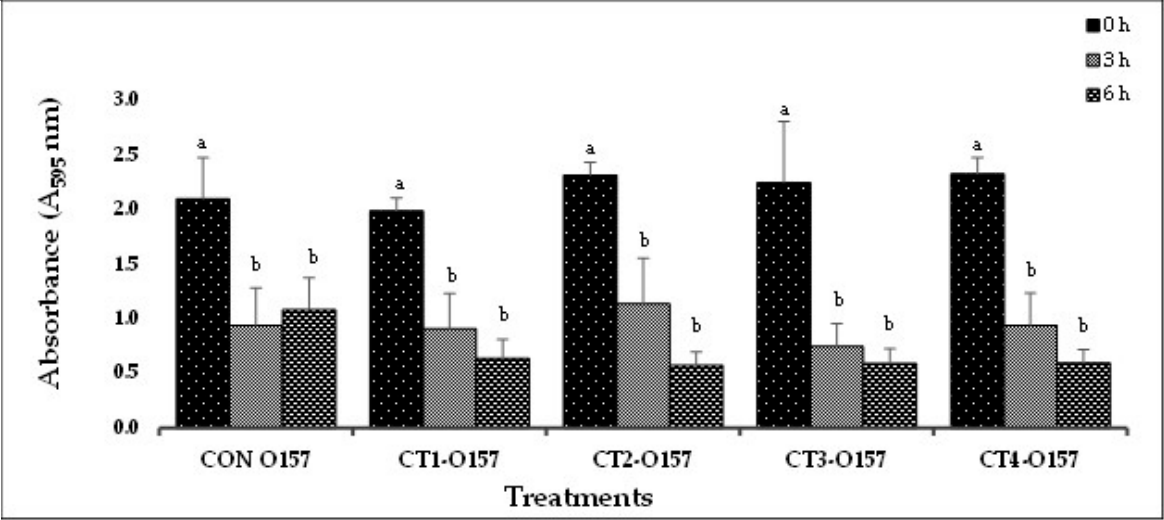

Figure 2. (A). *In-vitro* biofilm disruption by bacteriophage cocktails specific to *E. coli* O157 after treatment for 0, 3, and 6 h.
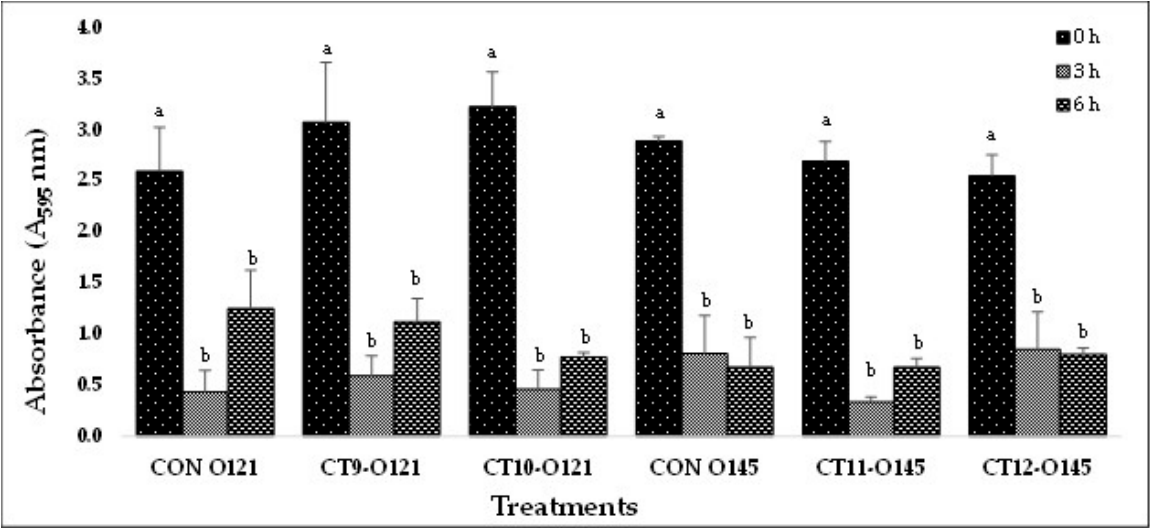

Figure 2. (B). *In-vitro* biofilm disruption by bacteriophage cocktails specific to *E. coli* O121 and O145 after treatment for 0, 3, and 6 h.
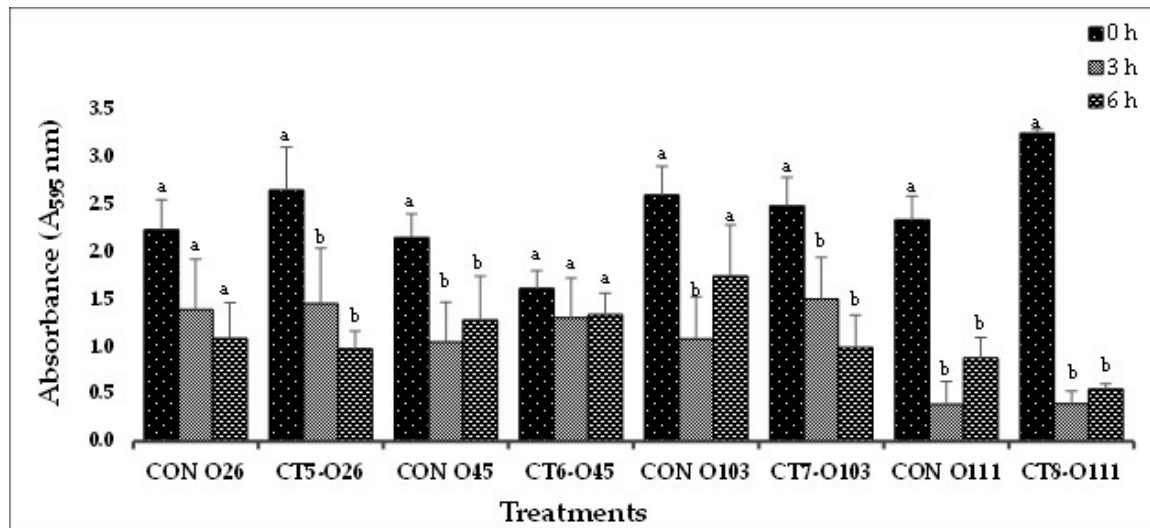
Figure 2. (C). *In-vitro* biofilm disruption by bacteriophage cocktails specific to *E. coli* O26, O45, O103 and O111 after treatment for 0, 3, and 6 h.
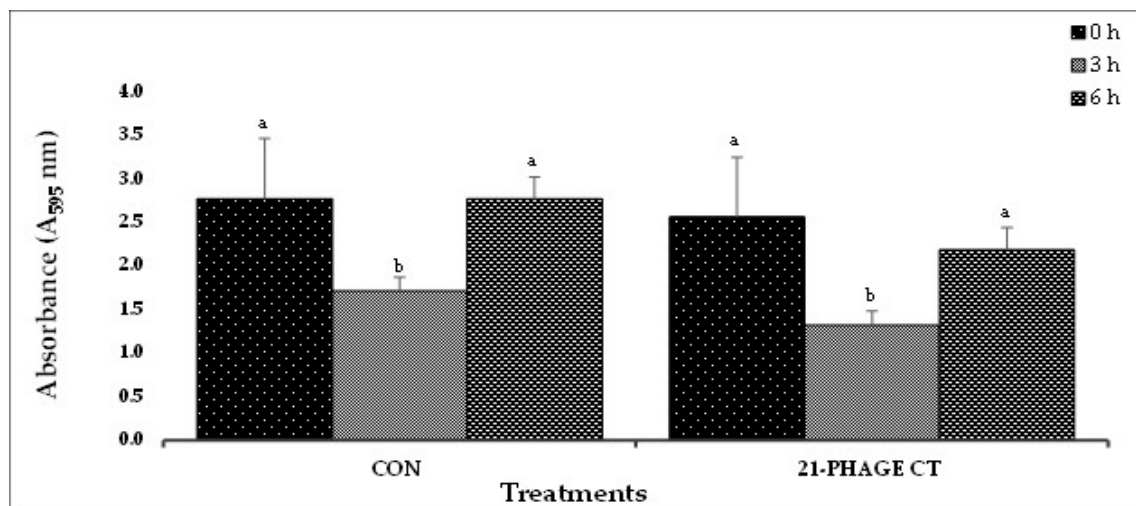

Figure 2. (D). *In-vitro* STEC biofilm disruption by 21-phage cocktail after treatment for 0, 3, and 6 h. Values represent the average of three replications. Different letters a, b etc. represent significant difference (P<0.10) between incubation times (0, 3, 6 h) for the control or phage treatment. The bars with the same letters indicate that there is no significance difference (P<0.10) between incubation times (0, 3, 6 h) for the control or phage treatment..

In the current study, a slight increase in OD was observed with some phages and phage cocktails at 6 h treatment. Results of the current study agree with other studies assessing phage treatment effects on bacterial biofilms [34,35]. Chan et al. [34] observed an increase in *E. coli* biofilms treated with T-4 phage on day 4, after an initial decrease (30%) on day 3. Similarly, Hughes et al. [36] obtained a maximum reduction in biofilm within 2 h or 5 h, depending on the biofilm studied. Factors such as bacterial appendages, used by the bacteria for biofilm attachment, could decrease the efficacy of phages in the biofilm [36,37]. Additionally, changes in bacterial biofilm profile over time, such as inconsistent expression and accumulation of protein through biofilm development stages, could affect phage efficacy [38,39]. Higher protein production at later stages of biofilm formation could impair phage movement within the medium and interfere with phage efficacy against bacterial biofilm [34,40]. Furthermore, static biofilms are known to produce higher polysaccharide and protein content, which could interfere with phage infection efficacies [41]. Vogeleer et al. [32] showed variation in biofilm matrices of individual non-O157 STEC serotypes under static and dynamic

conditions. This variation in biofilm matrix could minimize phage effectiveness against biofilm, due to the highly specific nature of its depolymerase enzyme [24,36]. This enzyme is highly specific to EPS produced by host-bacteria, and even a minor change in the EPS composition could prohibit its activity, leading to reduced phage activity against biofilm.

Loss in phage effectiveness after 6 h could also be due to generation of phage resistant mutants [42]. Incubation of biofilms at optimum temperatures (30-37°C) could trigger the rapid growth of bacteria in the biofilm, including phage-resistant bacteria, resulting in decreased efficacy of the phage treatment [37,43]. It has also been shown that STEC serotypes, grown together to form biofilms, could generate an abundance of morphological variants, which could exhibit varying susceptibility to treatments [32,44]. Some newly generated mutants could lose the receptor responsible for bacteriophage susceptibility and become resistant to phages. The use of more diverse phages with the ability to bind to different bacterial receptors could help in controlling the emergence of bacterial mutants by exerting selective pressure on bacterial population in the biofilm and increasing the effectiveness of phage cocktail treatment [45]. At the same time, phage resistance has been shown to be transient in bacterial cells and they could revert to phage susceptible state [46]. Additionally, this decrease in phage effectiveness can be overcome by application of a cocktail of multiple phages [46,47]. Multi-phage cocktails were therefore evaluated in the current study for their effectiveness against STEC biofilms.

3.2. STEC biofilm disruption on food-contact surfaces

Based on the *in-vitro* experiments, the most effective phage cocktails were selected to conduct application studies on SS and HDPE coupons. In Experiment-I, where STEC serotype-specific cocktails were used to form biofilms on SS and HDPE coupons, phage cocktails significantly ($P < 0.05$) reduced bacterial population in the biofilm when compared to the control (Figure 3A,B). Populations of *E. coli* O45, O111, O121, and O145 on SS were reduced to undetectable levels after treatment with respective phage cocktails (Figure 3A). A reduction of 3.1 and 3.7 logs was observed in *E. coli* O26 and O103 populations on SS coupons treated with phages, compared to the control (Figure 4A). On HDPE surface, *E. coli* O121 population was reduced to undetectable levels after respective phage cocktail treatment (Figure 3B). A reduction between 2.3-5.3 logs was observed in *E. coli* O26, O45, O103, O111 and O145 populations on HDPE coupons treated with respective phage cocktails, compared to the control (Figure 4B). Phage cocktails for specific STEC serotypes were very effective in reducing pathogen populations on both, SS (1.9-4.1 logs CFU/cm^2 reduction) and HDPE (2.3-5.6 logs CFU/cm^2 reduction) surfaces. These cocktails were more effective on SS than on HDPE, with the phage cocktails for *E. coli* O45, O145, O111, and O121 reducing pathogen populations to undetectable levels. These differences could be due to the surface type or difference in the biofilm composition, where different types of EPS were produced, which could provide increased resistance to bacteria against phage [47–49].
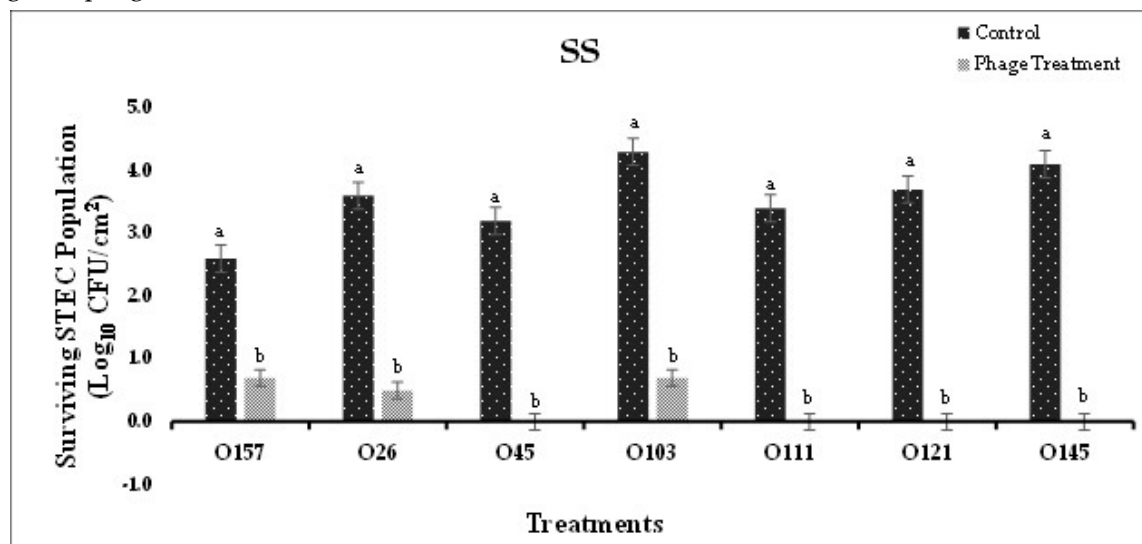
Figure 3. (A). Biofilm disruption on SS surface by bacteriophage cocktails specific to STEC after treatment for 16 h.
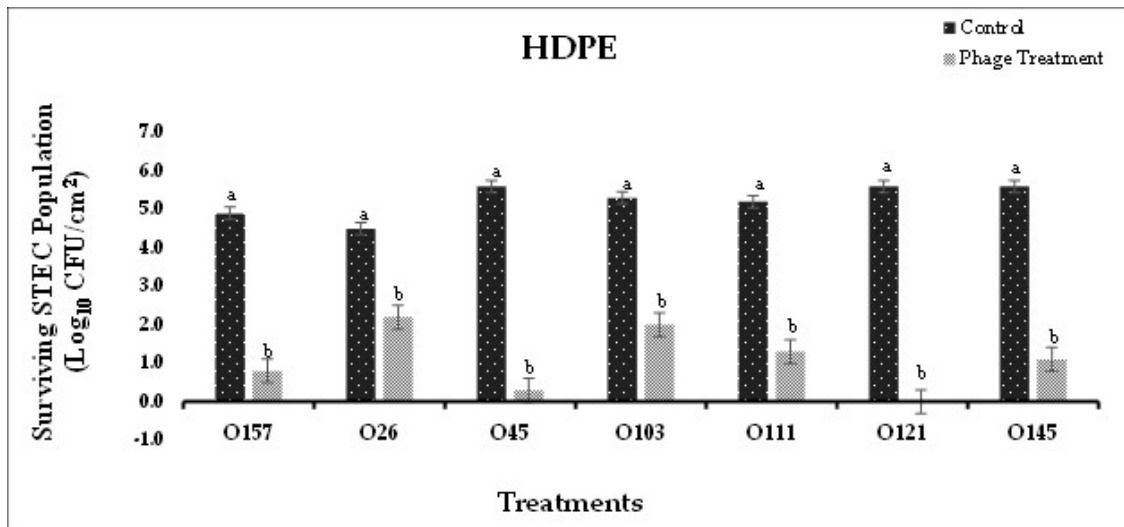

Figure 3. (B). Biofilm disruption on HDPE surface by bacteriophage cocktails specific to STEC after treatment for 16 h.

In Experiment-II, biofilms of a cocktail of 14 STEC isolates (two isolates per serotype), on SS and HDPE coupons, were treated with a 21-phage cocktail (three phages per serotype). The cocktail showed an immediate (0 h) reduction in STEC populations on HDPE (1.5 logs CFU/cm^2) and SS (1.0 log CFU/cm^2) (Figure 4A,B). At 3, 6 and 16 h, the 21-phage cocktail showed significant reductions ($P<0.05$) in STEC populations on both the surfaces when compared to the control (Figure 4A,B). At 3 h, pathogen populations were reduced by 2.8 and 1.7 logs CFU/cm^2 on SS and HDPE, respectively. At 6 h, pathogen populations were reduced to undetectable levels on SS and by 1.7 logs CFU/cm^2 on HDPE. At 16 h, STEC populations were reduced to undetectable levels on both surfaces. These results suggest that the tested bacteriophage treatments effectively reduced STEC populations in the biofilms on SS and HDPE surfaces.
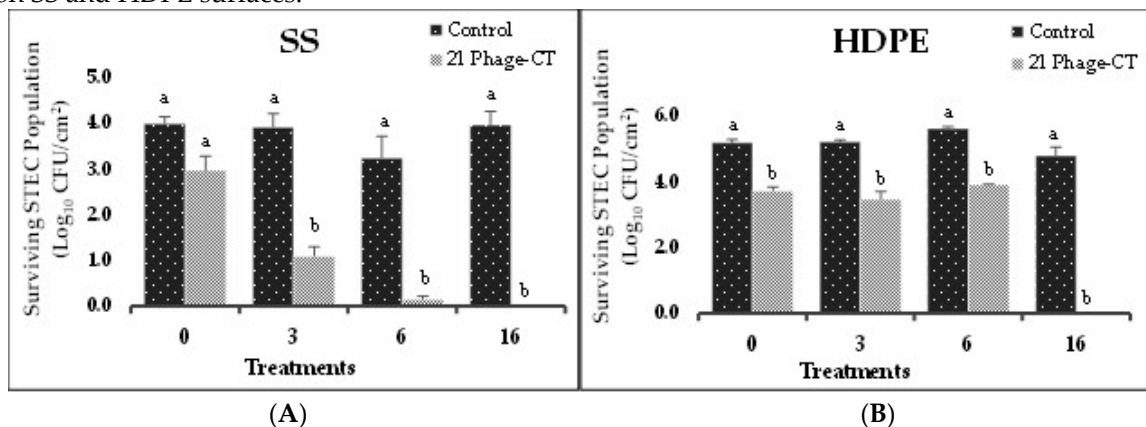

(A)   (B)

Figure 4. Biofilm disruption on food contact surfaces by 21-phage cocktail after treatment for 0, 3, 6 and 16 h on (A) SS and (B) HDPE surfaces. Values represent the average of three replications ± standard deviation. Different letters a, b etc. represent significant difference ($P < 0.05$) between control (0 h and PBS) and treatments. The bars with the same letters indicate that there is no significance difference ($P<0.05$) between control (0 h and PBS) and treatments..

Studies have previously shown that bacteriophages can effectively reduce pathogens attached to hard surfaces found in food processing environments [26,50,51]. Reductions between 3.5 to 5.4 logs CFU, in attached *Listeria monocytogenes* population, were observed on SS after 24 h of phage P100

treatment [52]. In a study by Sharma et al. [26], lytic bacteriophages reduced *E. coli* O157:H7 populations by 1.2 logs CFU on SS coupon. In the current study, phage treatments reduced *E. coli* O157:H7 by 1.9 logs CFU/cm² and non-O157 STEC by 3.1-4.1 logs CFU/cm² on SS surface. Studies have shown that phages can diffuse through the biofilm formed by bacteria [53] and that the formation of biofilms does not provide additional protection to bacteria against phage attack [26]. Biofilm break-down mechanism of phage is speculated to be associated with enzymatic means. Studies have shown that phages produce enzymes that can degrade the EPS layer, the major component of a biofilm matrix [24,36]. Hughes et al. [36] showed that bacteriophage specific for *Enterobacter agglomerans* disrupted biofilm through a combination of lytic activity against bacterial cells and degradation of EPS through phage-associated polysaccharide depolymerase enzyme. The presence of phage-depolymerase in an O45-phage (P-9), used in the current study, has been confirmed (data not shown), suggesting that other phages in the cocktail tested may also produce EPS-degrading enzyme, resulting in reduction of bacteria embedded in the biofilm matrix.

Variations in pathogen attachment and biofilm formation were observed on the food- contact surfaces tested in the present study. Higher STEC attachment was observed on HDPE (up to 5.6 logs CFU/cm²) compared to SS (up to 4.3 logs CFU/cm²) surface. A similar trend has been observed in previous studies. Higher bacterial attachment and biofilm formation has been observed on plastic surfaces (polyethylene, polypropylene, polyvinyl chloride) than on stainless steel or glass surfaces [48]. Variations found in polymer surfaces, such as smoothness, charge, Zeta-potential and active chemical groups, could influence bacterial attachment to the surface [54]. Studies have also shown that a variety of active chemical groups released by HDPE surfaces, such as phenols, quinones, aromatic hydrocarbons, aldehydes, and ketones, could be utilized by bacteria as carbon sources and result into higher bacterial attachment [55–57].

5. Conclusions

This study has shown the potential for use of lytic bacteriophages, against 7 STEC serotypes as a treatment to control biofilm formation. Future studies need to be conducted to understand phage-bacteria interactions in biofilm using kinetic modeling. The preliminary results from the current study show promise, due to the fact that phages within the biofilms effectively targeted and lysed STEC and were also able to disperse the extracellular matrix forming the biofilm.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, D.J. and P.K.L.; methodology, D.J. and P.K.L.; validation, D.J. and P.K.L.; formal analysis, D.J. and P.K.L.; investigation, D.J. and P.K.L.; resources, D.J.; data curation, D.J., P.K.L., K.R., and P.B; writing—original draft preparation, D.J. and P.K.L.; writing—review and editing, D.J., K.R. and P.B.; visualization, D.J.; supervision, D.J.; project administration, D.J.; funding acquisition, D.J. All authors have read and agreed to the published version of the manuscript."

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References

1. Scallan, E.; Hoekstra, R. M.; Angulo, F. J.; Tauxe, R. V.; Widdowson, M.-A.; Roy, S. L.; et al. Foodborne illness acquired in the United States-major pathogens. *Emerg. Infect. Dis*. 2011, *17(1)*.
2. Brooks, J. T.; Sowers, E. G.; Wells, J. G.; Greene, K. D.; Griffin, P. M.; Hoekstra, R. M.; et al. Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983-2002. *J. Infect. Dis*. 2005, *192(8)*, 1422-1429.
3. Dewey-Mattia, D.; Manikonda, K.; Hall, A. J.; Wise, M. E.; & Crowe, S. J. Surveillance for foodborne disease outbreaks-United States, 2009-2015. *MMWR Surveill. Summ*. 2018, *67(10)*, 1.

4. Tack, D. M.; Marder, E. P.; Griffin, P. M.; Cieslak, P. R.; Dunn, J.; Hurd, S.; et al. Preliminary incidence and trends of infections with pathogens transmitted commonly through food-Foodborne Diseases Active Surveillance Network, 10 US sites, 2015-2018. *Morb. Mortal. Wkly. Rep.* 2019, *68(16)*, 369.

5. Centers for Disease Control and Prevention (CDC). *National Shiga toxin-producing Escherichia coli (STEC) Surveillance Annual Report*, 2020. Available at: https://www.cdc.gov/ecoli/surv2020/, Accessed date: May 2023.

6. Ferens, W. A.; & Hovde, C. J. *Escherichia coli* O157: H7: animal reservoir and sources of human infection. *Foodborne Pathog. and Dis.* 2011, *8(4)*, 465-487.

7. Kintz, E., Brainard, J.; Hooper, L.; & Hunter, P. Transmission pathways for sporadic Shiga-toxin producing *E. coli* infections: A systematic review and meta-analysis. *Int. J. Hyg. and Environ. Health.* 2017, *220(1)*, 57-67.

8. Marouani-Gadri, N.; Augier, G.; & Carpentier, B. Characterization of bacterial strains isolated from a beef-processing plant following cleaning and disinfection-influence of isolated strains on biofilm formation by Sakaï and EDL 933 *E. coli* O157: H7. *Int. J. Food Microbiol.* 2009, *133(1)*, 62-67.

9. Silagyi, K.; Kim, S.-H.; Lo, Y. M.; & Wei, C.-i. Production of biofilm and quorum sensing by *Escherichia coli* O157: H7 and its transfer from contact surfaces to meat, poultry, ready-to-eat deli, and produce products. *Food Microbiol.* 2009, *26(5)*, 514-519.

10. Wang, R.; Bono, J. L.; Kalchayanand, N.; Shackelford, S.; & Harhay, D. M.Biofilm formation by Shiga toxin-producing *Escherichia coli* O157: H7 and Non-O157 strains and their tolerance to sanitizers commonly used in the food processing environment. *J. Food Prot.* 2012, *75(8)*, 1418-1428.

11. Gilbert, P.; & McBain, A. J. Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. *Clin. Microbiol. Rev.* 2003, *16(2)*, 189-208.

12. Weerarathne, P.; Payne, J.; Saha, J.; Kountoupis, T.; Jadeja, R.; & Jaroni, D. Evaluating the efficacy of sodium acid sulfate to reduce *Escherichia coli* O157:H7 and its biofilms on food-contact surfaces. *LWT- Food Sci. Technol.* 2021, *139*, 110501.

13. Fouladkhah, A.; Geornaras, I.; & Sofos, J. N. Biofilm Formation of O157 and Non-O157 Shiga toxin-producing *Escherichia coli* and multidrug-resistant and susceptible *Salmonella* Typhimurium and Newport and their inactivation by sanitizers. *J. Food Sci.* 2013, *78(6)*.

14. Houari, A.; & Di Martino, P. Effect of chlorhexidine and benzalkonium chloride on bacterial biofilm formation. *Lett. Appl. Microbiol.* 2007, *45(6)*, 652-656.

15. Uhlich, G. A.; Rogers, D. P.; & Mosier, D. A. *Escherichia coli* serotype O157: H7 retention on solid surfaces and peroxide resistance is enhanced by dual-strain biofilm formation. *Foodborne Pathog. and Dis.* 2010, *7(8)*, 935-943.

16. Gilbert, P.; Allison, D.; & McBain, A. Biofilms *in vitro* and *in vivo*: do singular mechanisms imply cross-resistance. *J. Appl. Microbiol.* 2002, *92(s1)*.

17. Coates, A.; & Hu, Y. Novel approaches to developing new antibiotics for bacterial infections. *Br. J. Pharmacol.* 2007, *152(8)*, 1147-1154.

18. Sulakvelidze, A.; Alavidze, Z.; & Morris, J. G. Bacteriophage therapy. *Antimicrob. Agents and Chemother.* 2001, *45(3)*, 649-659.

19. Summers, W. C. Bacteriophage therapy. *Annu. Rev. Microbiol.* 2001, *55(1)*, 437-451.

20. Ashelford, K. E.; Day, M. J.; & Fry, J. C. Elevated abundance of bacteriophage infecting bacteria in soil. *Appl. Environ. Microbiol.* 2003, *69(1)*, 285-289.

21. Synnott, A. J.; Kuang, Y.; Kurimoto, M., Yamamichi, K., Iwano, H., & Tanji, Y. Isolation from sewage influent and characterization of novel *Staphylococcus aureus* bacteriophages with wide host ranges and potent lytic capabilities. *Appl. Environ. Microbiol.* 2009, *75(13)*, 4483-4490.

22. Raya, R. R.; Varey, P.; Oot, R. A.; Dyen, M. R.; Callaway, T. R.; Edrington, T. S.; et al. Isolation and characterization of a new T-even bacteriophage, CEV1, and determination of its potential to reduce *Escherichia coli* O157: H7 levels in sheep. *Appl. Environ. Microbiol.* 2006, *72(9)*, 6405-6410.

23. Tanji, Y.; Shimada, T.; Fukudomi, H.; Miyanaga, K.; Nakai, Y.; & Unno, H. Therapeutic use of phage cocktail for controlling *Escherichia coli* O157: H7 in gastrointestinal tract of mice. *J. Biosci. and Bioeng.* 2005, *100(3)*, 280-287.

24. Hughes, K. A.; Sutherland, I. W.; & Jones, M. V. Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase. *Microbiology* 1998, *144(11)*, 3039-3047.

25. Patel, J.; Sharma, M.; Millner, P.; Calaway, T.; & Singh, M. Inactivation of *Escherichia coli* O157: H7 attached to spinach harvester blade using bacteriophage. *Foodborne Pathog. Dis.* 2011, *8(4)*, 541-546.

26. Sharma, M.; Ryu, J. H.; & Beuchat, L. Inactivation of *Escherichia coli* O157: H7 in biofilm on stainless steel by treatment with an alkaline cleaner and a bacteriophage. *J. Appl. Microbiol.* 2005, *99(3)*, 449-459.

27. Viazis, S.; Akhtar, M.; Feirtag, J.; & Diez-Gonzalez, F. Reduction of *Escherichia coli* O157: H7 viability on hard surfaces by treatment with a bacteriophage mixture. *Int. J. Food Microbiol.* 2011, *145(1)*, 37-42.

28. Litt, P. K.; & Jaroni, D. Isolation and physio-morphological characterization of *Escherichia coli* O157:H7-infecting bacteriophages recovered from beef cattle operations. *Int. J. Microbiol.* 2017, 7013236.

29. Litt, P. K.; Saha, J.; and Jaroni, D. Characterization of bacteriophages targeting non-O157 Shiga toxigenic *Escherichia coli*. *J. Food Prot.* 2018, *81*:785-794.

30. Jayasundera, B. P. K. Identification of contamination sources and occurrence of shigatoxigenic *E. coli* on small-scale cow/calf operations in Oklahoma and Louisiana (Masters Thesis). Oklahoma State University, 2015.

31. Kountoupis, T.; Litt, P. K.; Kakani, R.; & Jaroni, D. Biofilm forming capabilities of Shiga-toxigenic *Escherichia coli* recovered from cow calf operations in Oklahoma and Louisiana. Poster session presentation at the annual meeting of International Association for Food Protection, Tampa, FL,USA, 2017.

32. Vogeleer, P.; Tremblay, Y. D.; Jubelin, G.; Jacques, M.; & Harel, J. Biofilm-forming abilities of Shiga toxin-producing *Escherichia coli* isolates associated with human infections. *Appl. Environ. Microbiol.* 2016, *82(5)*, 1448-1458.

33. Hood, S. K.; & Zottola, E. A. Adherence to stainless steel by foodborne microorganisms during growth in model food systems. *Internat. J. Food Microbiol.* 1997, *37(2)*, 145-153.

34. Chan, T.; Lee, K.; & Roulin, P. *Escherichia coli* biofilm development and bacteriophage T4 susceptibility. *J. of Expt. Microbiol. Immunol.* 2007, *11*, 73-80.

35. Lu, T. K.; & Collins, J. J. Dispersing biofilms with engineered enzymatic bacteriophage. *Proc. Natl. Acad. Sci.* 2007, *104(27)*, 11197-11202.

36. Hughes, K. A.; Sutherland, I. W.; Clark, J.; & Jones, M. V. Bacteriophage and associated polysaccharide depolymerases-novel tools for study of bacterial biofilms. *J. Appl. Microbiol.* 1998, *85(3)*, 583-590.

37. Gong, C.; & Jiang, X. Application of bacteriophages to reduce biofilms formed by hydrogen sulfide producing bacteria on surfaces in a rendering plant. *Can. J. Microbiol.* 2015, *61(8)*, 539-544.

38. Domka, J.; Lee, J.; Bansal, T.; & Wood, T. K. Temporal gene-expression in *Escherichia coli* K-12 biofilms. *Environ. Microbiol.* 2007, *9(2)*, 332-346.

39. Schembri, M. A.; Kjaergaard, K.; & Klemm, P. Global gene expression in *Escherichia coli* biofilms. *Mol. Microbio*. 2003, *48(1)*, 253-267.

40. Rabinovitch, A.; Aviram, I.; & Zaritsky, A. Bacterial debris-an ecological mechanism for coexistence of bacteria and their viruses. *J. Theor. Biol.* 2003, *224(3)*, 377-383.

41. Sillankorva, S.; Neubauer, P.; & Azeredo, J. Phage control of dual species biofilms of *Pseudomonas fluorescens* and *Staphylococcus lentus*. *Biofouling* 2010, *26(5)*, 567-575.

42. Meng, X.; Shi, Y.; Ji, W.; Meng, X.; Zhang, J.; Wang, H.; et al. Application of a bacteriophage lysin to disrupt biofilms formed by the animal pathogen *Streptococcus suis*. *Appl. Environ. Microbiol.* 2011, *77(23)*, 8272-8279.

43. Gong, C.; Heringa, S.; Singh, R.; Kim, J.; & Jiang, X. Isolation and characterization of bacteriophages specific to hydrogen-sulfide-producing bacteria. *Can. J. Microbiol.* 2012, *59(1)*, 39-45.

44. Tremblay, Y.D.; Vogeleer, P.; Jacques, M.; & Harel, J. High-throughput microfluidic method to study biofilm formation and host-pathogen interactions in pathogenic *Escherichia coli*. *Appl. Environ. Microbiol.* 2015, *81(8)*, 2827-2840.

45. Goodridge, L. D.; & Bisha, B. Phage-based biocontrol strategies to reduce foodborne pathogens in foods. *Bacteriophage* 2011, *1(3)*, 130-137.

46. O'Flynn, G.; Ross, R.; Fitzgerald, G.; & Coffey, A. Evaluation of a cocktail of three bacteriophages for biocontrol of *Escherichia coli* O157: H7. *Appl.Environmen. Microbiol.* 2004, *70(6)*, 3417-3424.

47. Kudva, I. T.; Hatfield, P. G.; & Hovde, C. J. Characterization of *Escherichia coli* O157: H7 and other Shiga toxin-producing *E. coli* serotypes isolated from sheep. *J. Clin. Microbiol.* 1997, *35(4)*, 892-899.

48. Rogers, J.; Dowsett, A.; Dennis, P.; Lee, J.; & Keevil, C. Influence of plumbing materials on biofilm formation and growth of *Legionella pneumophila* in potable water systems. *Appl. Environ. Microbiol.* 1994, *60(6)*, 1842-1851.

49. Sutherland, I. W. Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology* 2001, *147(1)*, 3-9.

50. Abuladze, T.; Li, M.; Menetrez, M. Y.; Dean, T.; Senecal, A.; & Sulakvelidze, A. Bacteriophages reduce experimental contamination of hard surfaces, tomato, spinach, broccoli, and ground beef by *Escherichia coli* O157: H7. *Appl. Environ. Microbiol.* 2008, *74(20)*, 6230-6238.

51. Hibma, A. M.; Jassim, S. A.; & Griffiths, M. W. Infection and removal of L-forms of *Listeria monocytogenes* with bred bacteriophage. *Int. J. Food Microbiol.* 1997, *34(3)*, 197-207.

52. Soni, K. A.; & Nannapaneni, R. Removal of *Listeria monocytogenes* biofilms with bacteriophage P100. *J. Food Protect.* 2010, *73(8)*, 1519-1524.

53. Briandet, R.; Lacroix-Gueu, P.; Renault, M.; Lecart, S.; Meylheuc, T.; Bidnenko, E.; et al. Fluorescence correlation spectroscopy to study diffusion and reaction of bacteriophages inside biofilms. *Appl. Environ. Microbiol.* 2008, *74(7)*, 2135-2143.

54. Triandafillu, K.; Balazs, D.; Aronsson, B.-O.; Descouts, P.; Quoc, P. T.; van Delden, C.; et al. Adhesion of *Pseudomonas aeruginosa* strains to untreated and oxygen-plasma treated poly (vinyl chloride)(PVC) from endotracheal intubation devices. *Biomaterials* 2003, *24(8)*, 1507-1518.

15

55. Brocca, D.; Arvin, E.; & Mosbaek, H. Identification of organic compounds migrating from polyethylene pipelines into drinking water. *Water Res.* 2002, *36(15)*, 3675-3680.

56. Kowalska, B.; Kowalski, D.; & Rożej, A. Organic compounds migrating from plastic pipes into water. *J. Water Supply: Res. Technol.-AQUA* 2011, *60(3)*, 137-146.

57. Skjevrak, I.; Lund, V.; Ormerod, K.; & Herikstad, H. Volatile organic compounds in natural biofilm in polyethylene pipes supplied with lake water and treated water from the distribution network. *Water Res.* 2005, *39(17)*, 4133-4141.