Effect of Bacteriophages against Biofilms of *Escherichia coli* on Food Handling Surfaces

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Article

Effect of Bacteriophages against Biofilms of *Escherichia coli* on Food Handling Surfaces

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Abstract: The bacterial adhesion to food contact surfaces is a threat to human health, as these surfaces can serve as a reservoir of pathogenic bacteria. *Escherichia coli* is a biofilm-forming bacterium involved in surface contamination. Despite the application of disinfection protocols, contamination continues to occur. Hence, new effective and sustainable approaches are needed. Bacteriophages (or simply phages), viruses that only infect bacteria, have proven to be effective in reducing biofilms. Here, phage phT4A was applied to prevent and reduce *E. coli* biofilm on plastic and stainless steel. The assays were performed in Tryptic Soy Broth (TSB), at 25 °C. The biofilm formation capacity of phage-resistant and sensitive bacteria, after treatment, was also evaluated. Maximum reductions on *E. coli* biofilm of 5.5 log colony forming units (CFU)/cm² (after 6 h) and 4.1 log CFU/cm² (after 9 h), on plastic and stainless steel, respectively, were observed. In the prevention assays, phage prevented biofilm formation in 3.2 log CFU/cm², after 12 h. Although the emergence of phage-resistant bacteria has been observed during phage treatment, a lower biofilm formation capacity was observed by phage-resistant bacteria comparatively to phage-sensitive bacteria. Overall, phages have shown to be a promising approach to decontaminate food handling surfaces.

Keywords: *Escherichia coli*; bacterial biofilm; bacteriophages; surface decontamination; plastic; stainless steel; food safety

1. Introduction

The ingestion of contaminated food represents a global threat to public health [1]. *Escherichia coli* is known as one of the most common foodborne pathogens [2,3]. This bacterium can form biofilms on various matrices, including food and food handling surfaces [3]. The persistence of bacterial biofilms on food handling surfaces is a major concern, as these surfaces can serve as vehicles for food contamination with pathogenic bacteria [4,5].

According to the European Food Safety Authority (EFSA), Shiga toxin-producing *E. coli* (STEC) was the third most frequently detected pathogen in food companies in the European Union in 2021, with *E. coli* O157:H7 being one of the most problematic STEC serogroup [6]. From the 6,084 cases of human STEC infection, 901 hospitalizations and 18 deaths have been reported, mainly due to consumption of contaminated food and water, and contact with infected animals. Around 5 to 10% of people infected with *E. coli* O157 develop hemolytic-uremic syndrome and acute renal failure. In some cases, dialysis treatment and, in more severe cases, kidney transplantation may be required, reducing the quality of life [7,8] and leading to high treatment costs [3,9].

To eradicate biofilms in the food industry, aggressive chemical disinfectants such as chlorine and peracetic acid are commonly used in cleaning and decontamination procedures [1]. However, bacterial biofilms are more difficult to eradicate with disinfectants than bacteria in their planktonic state. In addition, the use of disinfectants can affect the quality of food products, have a negative impact on the environment, or damage or leave residues on treated surfaces [1,2,9,10]. It has also been observed that continuous exposure to sub-lethal doses of these disinfectants allows bacteria to adapt and survive, rendering them ineffective, thereby compromising food safety and, consequently, public

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health [11]. In this sense, it is crucial to implement an alternative and sustainable strategy to combat and prevent the formation of bacterial biofilms on food handling surfaces, namely by foodborne pathogenic bacteria.

Bacteriophages, also known as phages, are bacterial viruses that have the ability to infect and replicate only inside bacterial cells in a highly specific manner [12,13]. These viruses are considered the most abundant biological entities on the planet and can follow two types of cycles: lytic and lysogenic. Lytic phages are used as bactericidal agents for biocontrol purposes because of their ability to infect and lyse their bacterial host cells, killing them rapidly and releasing newly formed phage particles at the end of the cycle [3,9,12,14].

Several characteristics of phages, such as their high specificity, ubiquitous nature and no effect on the sensory properties of food compared to disinfectants, make them suitable candidates as antibacterial agents for biocontrol purposes in the food industry. It is also important to highlight the use of phages against bacterial biofilms [14–17].

Although the interaction between the biofilm and the lytic phage is somehow more complex than with planktonic cells, since the biofilm matrix acts as a protective barrier, phages are able to overcome this barrier and successfully penetrate. Some phages have specific hydrolytic enzymes that use polysaccharides or their derivatives as substrates, facilitating the process of bacterial infection by phages [9,10]. This ability makes phage treatment a promising alternative for the prevention and control of bacterial biofilms [18,19].

Currently, there are several available studies exploring the use of phages in biocontrol and thus food preservation, namely against multidrug resistant bacteria [12,17]. There are also some approved phage products for the biocontrol of the major foodborne pathogens, including *E. coli*, in food [1,12]. However, the number of studies on the use of phages in the disinfection of food handling surfaces is significantly lower. Further studies are needed to evaluate the potential of phages as antibacterial agents in the prevention of contamination throughout the food production and handling process. Therefore, the aim of this work was to evaluate the effect of phT4A phage on the prevention and reduction of *E. coli* biofilm formation on two different surfaces (plastic and stainless steel).

2. Materials and Methods

2.1. Bacterial Strain and Growth Conditions

In this work, the *E. coli* ATCC 13706 strain, the host of the used phage phT4A, was used to contaminate the different tested surfaces. Another *E. coli* strain, ATCC 25922, was used to compare biofilm formation with that of the test bacterium (*E. coli* ATCC 13706), as this strain is used as a model organism for biofilm formation since it is classified in the literature as a strong biofilm producer [20,21]. Both bacterial strains were purchased from the American Type Culture Collection (ATCC). Bacterial cultures were maintained in Tryptic Soy Agar (TSA) (Liofilchem, Roseto degli Abruzzi, Italy) at 4 °C. Prior to the assays, a colony was aseptically transferred to a flask containing 30 mL of Tryptic Soy Broth (TSB) (Liofilchem, Roseto degli Abruzzi, Italy) which was then placed at 37 °C, under stirring at 120 rpm for 24 h, until it reaches about 10^9 colony-forming units per milliliter (CFU/mL). This procedure was performed in all assays.

2.2. Phage Stock Preparation

The phage used in this work was phage phT4A, isolated in a previous work from wastewater from the Aveiro region (station EEIS9 of SIMRIA Multi Sanitation System of Ria de Aveiro), using *E. coli* ATCC 13706 as host [22]. The new phage stock was prepared in Saline Magnesium (SM) buffer [0.1 M NaCl (Sigma-Aldrich, St. Louis, MO, USA), 8 mM $MgSO_4$ (Sigma-Aldrich), 20 mM Tris-HCl (Sigma-Aldrich), pH 7.5], according to the method described by Costa et al. (2019) [23], with some modifications. Briefly, 200 µL of an *E. coli* culture in the exponential growth phase was added to 5 mL of 0.6% TSB [30 g/L TSB (Liofilchem), 6 g/L agar (Liofilchem), 0.05 g/L $CaCl_2$ (Sigma-Aldrich), 0.12 g/L $MgSO_4$ (Sigma-Aldrich), pH 7.4] and poured into a plate containing TSA. After solidification, 100 µL of a previously prepared phage stock was placed under the petri dishes and incubated for at

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least 8 h at 37 °C. This procedure was repeated on several plates. After incubation, the surface layer of the plates was removed and transferred to a flask containing SM buffer, which was incubated for 24 h at 60 rpm. Then, the entire content was distributed into falcon tubes and centrifuged at 10,000 rpm for 10 min, to remove intact bacteria or bacterial debris. The phage stocks were also subjected to a filtration process using a polyether sulfone membrane with 0.22 µm pore-size. Phage suspension was stored at 4 °C.

The titer of the new phage suspension was determined using the double-layer agar technique [24]. Successive dilutions of the phage suspension were performed in Phosphate Buffer Saline solution [PBS: 137 $mmol^{-1}$ NaCl (Sigma-Aldrich), 2.7 $mmol^{-1}$ KCl (Sigma-Aldrich), 8.1 $mmol^{-1}$ $Na_2HPO_4 \cdot 2H_2O$ (Sigma-Aldrich), 1.76 $mmol^{-1}$ KH_2PO_4 (Sigma-Aldrich), pH 7.4]. Five hundred microliters of each dilution (in duplicate) and 200 µL of fresh bacterial culture were mixed with 5 mL of 0.6% TSB and placed on TSA plates. The plates were incubated at 37 °C for approximately 8 h and the number of plaques was counted in the most appropriate dilution. The results were expressed in plaque forming units (PFU)/mL.

2.3. Formation of Biofilms

Biofilm formation was performed in 96-well microplates containing 200 µL of cell suspension corresponding to approximately 10^7 CFU/mL. Plates were incubated for 24 h at 37 °C, without agitation. After 24 h incubation, suspended cells were removed and the wells were washed three times with PBS to remove any planktonic or non-adherent bacteria.

2.4. Characterization of Bacterial Capacity Of biofilm Formation

Biofilm formation was performed in 96-well microplates as described in section 2.3. This procedure was carried out for *E. coli* ATCC 13706 and *E. coli* ATCC 25922. Biofilm formation was evaluated by crystal violet assay according to Mangieri et al. (2021) and Mukane et al. (2022), with some modifications [25,26]. Briefly, the wells were stained with 0.1% crystal violet for 15 min, washed three times with PBS and allowed to dry overnight at room temperature. After the 96-well plates were dry, 125 µL of 30% acetic acid were added to solubilize crystal violet for 15 min. The 125 µL of solubilized crystal violet were transferred to a new microtiter plate and the optical density (OD) was read at 540 nm using a plate reader (Thermo scientific, Multiskan FC). This procedure was repeated up to the stage of staining the wells with crystal violet in new 96-well plates to observe the differences in biofilm formation between the two strains under an inverted microscope (MOTIC AE31). The OD values allowed us to assess not only the ability to form biofilm formation capacity, but also the amount of biofilm produced by each bacterial strain. The biofilm formation capacity parameter was evaluated according the method by Mukane et al. (2022)[25]. This is based on comparing the absorbance value at $OD_{540\ nm}$ of the negative control (OD_C) with the absorbance value at $OD_{540\ nm}$ of the wells containing the bacteria (OD_B) (22). Thus, the ability of bacteria to form biofilm can be classified into four categories: non-former ($OD_B \leq OD_C$), weak former ($OD_C < OD_B \leq 2OD_C$), moderate former ($2OD_C < OD_B \leq 4OD_C$), strong former ($4OD_C < OD_C$). The parameter to evaluate the amount of biofilm formed (BF), by Mangieri et al. (2021) [26], was also applied, and is based on the equation: BF= BC/CW, where BC is the absorbance value at $OD_{540\ nm}$ of stained adherent bacteria while CW is the absorbance value at $OD_{540\ nm}$ of the control wells only stained and free of bacteria (21)BF values are categorized according to the amount of biofilm formation as strong: $BF \geq 6$, moderate: $5.99 \geq BF \geq 4$, weak: $3.99 \geq BF \geq 2$, and negative: $BF < 2$.

2.5. Formation and Quantification of the E. coli Biofilm on Plastic and Stainless Steel

The biofilm formation on plastic or stainless steel was performed in a 96-well plate or bars (2 cm x 1 cm), respectively, as described in section 2.3. For biofilm quantification, the plate (containing 200 µL of new TSB medium) or bars (in new sterilized tubes containing 5 mL of new TSB medium) were placed in the sonicator (BANDELIN, SONOREX SUPER RK 102 H) for 10 min. Aliquots were then collected, dilutions were made and seeded by the drop plate method (two drops of 10 µL each) on

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plates containing TSA. After the incubation period of 24 h at 37°C, the bacterial colonies were counted, and the results were expressed in CFU/cm^2.

2.6. Efficacy of the phT4A Phage in the Reduction of E. coli Biofilm on Plastic and Stainless Steel

The biofilm reduction assays were performed in 96-well plates based on the methods already described in the literature for this type of assay, by Park and Park (2021) and Zhu et al. (2022), with some modifications [27,28]. Biofilm formation was performed in 96-well microplates as described in section 2.3. Phage phT4A was added to the phage control and test sample (containing both bacterial biofilm and phage) at a final concentration of 10^9 PFU/mL to obtain a multiplicity of infection (MOI) of 10. Two controls were included: bacteria control and phage control. The bacteria control was inoculated with E. *coli* but not with phage, and the phage control was inoculated with phage phT4A but not with bacteria. The test sample and controls were incubated under the same conditions, at 25 °C, without agitation. After 0, 6, 12, 24, and 48 h, the 96-well plates were placed in the sonicator (BANDELIN, SONOREX SUPER RK 102 H) for 10 min and aliquots were collected and diluted in PBS. Phage titer was determined by the double-layer agar method at 37 °C for 12 h. Bacterial concentration was determined in triplicate in solid TSA medium after 24 h at 37 °C using the drop-plate method. Bacterial colonies or phage plaques were counted at the most appropriate dilution and the results were expressed as CFU/cm^2 or PFU/cm^2, respectively. Three independent experiments were performed in different dates, with three replicates in each condition.

Colonies of the test samples (BP) in plastic were picked and purified by successive sub-culturing in TSA in order to remove attached phage particles. Bacterial sensitivity of these colonies to phage, after phage exposure, was evaluated using the spot test procedure. Briefly, three hundred microliters of bacterial culture previously inoculated in TSB 0.6% agar were overlaid on solid TSA and spotted with 10 μL of the phage suspension. The plates were incubated at 37 °C for 8 h. Two resistant colonies (spot test negative) and two sensitive colonies (spot test positive) were selected for further experiments (as described in section 2.8).

2.7. Efficacy of phT4A Phage in the Prevention of E. coli Biofilm on Plastic

The ability of phT4A phage to prevent biofilm formation by *E. coli* was tested according to the protocol described by Zhu et al. (2022), with some modifications [27]. To obtain a MOI of 10, bacterial suspension at a final concentration of 10^8 CFU/mL and phage at a final concentration of 10^9 PFU/mL were added to the wells of a 96-well plate. Two controls were included: phage control and bacteria control. The test sample and controls were incubated at 25 °C, without agitation. After 0, 6, 12, 24, 30, 36 and 48 h of incubation, the plates were placed in the sonicator (BANDELIN, SONOREX SUPER RK 102 H) for 10 min. Bacterial concentration and phage titer were determined as described above (see section 2.6). This assay was repeated three times in different dates, with three replicates in each condition.

The formation of biofilm was evaluated according to crystal violet method as described in section 2.4. The plates were observed by inverted microscopy (MOTIC AE31).

2.8. Evaluation of the Biofilm Formation Ability of Resistant and Sensitive Bacteria to phT4A Phage

The biofilm formation capacity of mutant phage-resistant and sensitive bacteria was evaluated according to the method described by Filippov et al. (2011), with some alterations [29], using the viable cell counting and crystal violet staining method. First, two phage-resistant and sensitive colonies from an assay of biofilm reduction were isolated (see section 2.6). Then, resistant and sensitive bacteria, and a bacteria control (bacteria without contact with the phage phT4A) were allowed to grow as described in section 2.1. Then, the capacity of biofilm formation of these different cultures was evaluated by crystal violet assay as described in section 2.4. Also, the amount of biofilm formed was quantified as described in section 2.5 at time 0 and after 24 h. Three independent experiments were done in different dates, with three replicates in each condition.

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2.9. Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 8.4.3 program, San Diego, California, USA. Confirmation of the normal distribution of data was carried out using the Kolmogorov-Smirnov test and homogeneity of variances using the Welch test. To evaluate statistically significant differences between the bacteria control, phage control and the respective treatment groups (sections 2.5, 2.6 and 2.7) a two-way ANOVA complemented with the test Sidak (α=0.05), were performed. For the different treatments, the significance of the differences was assessed by comparing, at a given moment, the result obtained in the treatment group with the result obtained for the corresponding control group. Differences between phage-resistant bacteria, phage-sensitive bacteria and the control group were assessed for each sampling time using a two-way ANOVA (section 2.8). A value of $p < 0.05$ was considered statistically significant.

3. Results

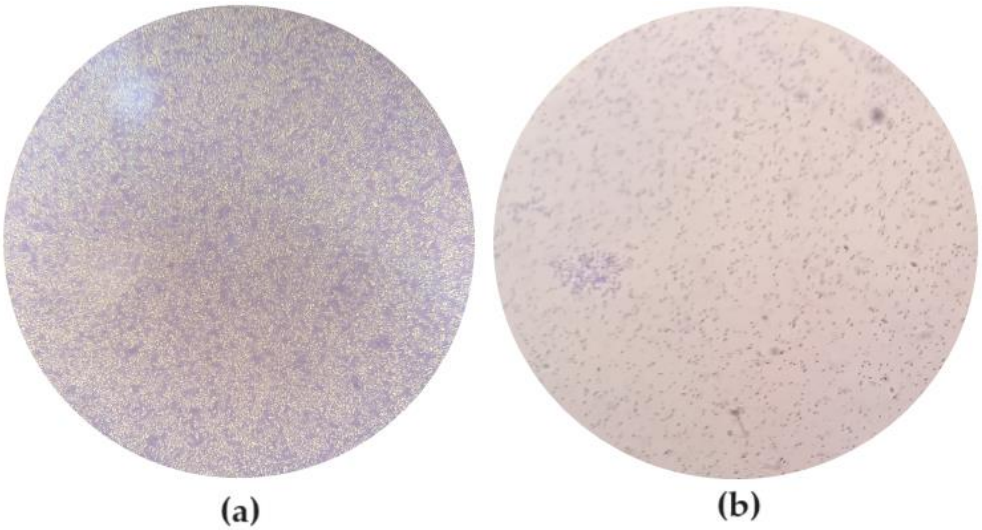
3.1. Characterization of Bacterial Capacity of Biofilm Formation

Table 1 includes the absorbance values at 540 nm necessary for further characterization of the bacterial ability to form biofilm.

Table 1. Optical density ($OD_{540\ nm}$) to characterize the intensity of biofilm formed.

	ATCC 13706	**ATCC 25922**
Stained adherent bacteria (BC or OD_B)	1.381	0.221
Acetic acid + Crystal violet (CW)	0.087	
TSB (OD_c)	0.040	

Considering the method that compares the OD obtained in the negative control OD_C to the one obtained in the wells containing the bacteria OD_B, *E. coli* ATCC 13706 has $4OD_C < OD_B$ and is classified as a strong biofilm-forming bacterium. On the contrary, *E. coli* ATCC 25922 has $OD_C < OD_B \leq 2OD_C$ and is therefore classified as a weak biofilm-forming bacterium. According to the equation BF = BC/CW and the values obtained from the averaged optical density of three assays (Table 1), *E. coli* ATCC 13706 presents $BF \geq 6$ and the biofilm formed by this bacterium is categorized as a strong biofilm. On the other hand, *E. coli* ATCC 25922 presents $2 \leq BF \leq 3.99$, so the biofilm formed by this bacterium is categorized as a weak biofilm.



(a) (b)

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Figure 1. Observation of the biofilms formed by the different strains after 24 h of incubation, under an inverted microscope (MOTIC AE31). (**a**) *E. coli* ATCC 13706, with 200x magnification; (**b**) *E. coli* ATCC 25922, with 100x magnification.

3.2. Efficacy of the phT4A Phage in the Reduction of E. coli Biofilm on Plastic and Stainless Steel

The effect of phT4A phage on reducing *E. coli* biofilm formed on plastic surfaces is shown in Figure 2. The treatment with phage phT4A significantly reduced biofilm formation by *E. coli* to 5.5 log CFU/cm² (Figure 2a, ANOVA, $p < 0.05$), after 6 h of incubation. The regrowth of biofilm cells on phage-treated plastic occurred between 6 and 48 h. Despite bacterial regrowth, the differences in bacterial density between the test and control groups (1.1 log CFU/cm²) remained statistically significant until the end of the experiment (ANOVA, $p < 0.05$, Figure 2a). In the bacteria control, a gradual increase was observed throughout the experiment with a maximum increase of 2.0 log CFU/cm² after 48 h (Figure 2a).

Phage concentration remained stable with no significant differences in the control group throughout the experiment (ANOVA, $p > 0.05$, Figure 2b). In the presence of the bacterial host, the phage titer increased significantly by 1.8 log PFU/cm² (ANOVA, $p < 0.05$, Figure 2b).

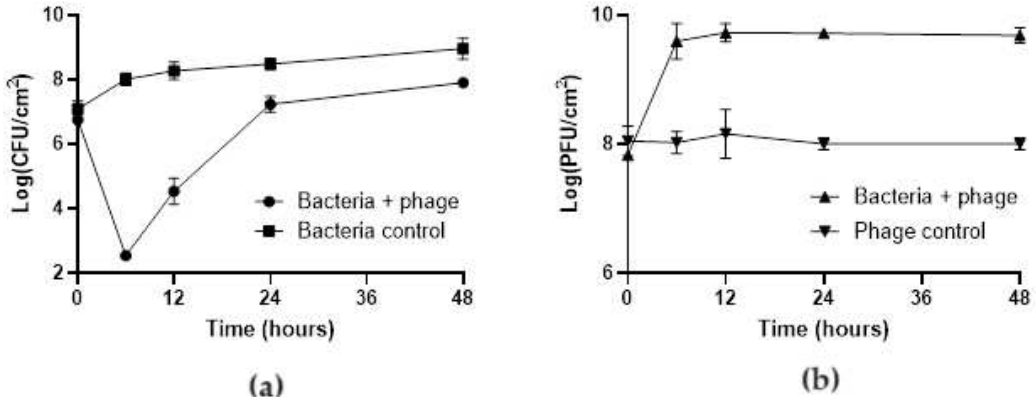

Figure 2. Effect of phT4A phage on the reduction of *E. coli* ATCC 13706 biofilms on plastic during 48 h at MOI 10. (**a**) bacterial concentration; (**b**) phage titer. The values shown in both graphs are the average of three independent assays and the error bars represent the standard deviation.

The effect of phT4A phage on the reduction of *E. coli* biofilm formed on stainless steel surfaces is shown in Figure 3. The maximum biofilm reduction was 4.1 log CFU/cm² (ANOVA, $p < 0.05$, Figure 3a), after 6 h of incubation. However, in the first 3 h of incubation, the mean viable biofilm in the untreated group (Bacteria control, Figure 3A) was similar (ANOVA, $p > 0.05$, Figure 3a) to that obtained in the treated group (Bacteria + phage, Figure 3a). The regrowth of biofilm cells on phage-treated stainless steel occurred between 6 and 24 h. At the end of the experiment, the density of biofilm cells in the treated group (Bacteria + phage, Figure 3a) and the untreated group (bacterial control, Figure 3a) (1.2 log CFU/cm²) was statistically significant (ANOVA, $p < 0.05$). In the bacteria control, the biofilm cell density increases by 4.0 log CFU/cm² (ANOVA, $p < 0.05$, Figure 3a) during the experiment.

Phage phT4A remained stable with no variation in phage titer during the assay period of 24 h in the control group (Figure 3b). When phage was incubated in the presence of *E. coli*, a significant increase (ANOVA, $p < 0.05$, Figure 3b) in phage titer of 3.2 log PFU/cm² was observed.

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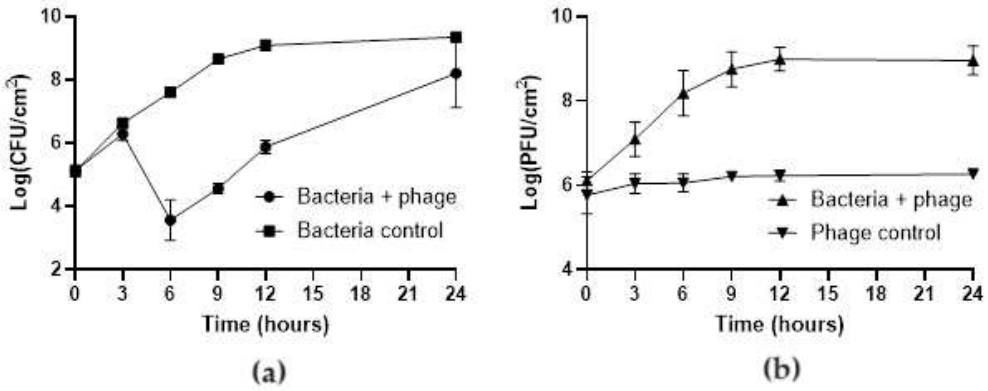

Figure 3. Effect of phT4A phage on the reduction of *E. coli* ATCC 13706 biofilms on stainless steel during 24 h at MOI 10. (**a**) bacterial concentration; (**b**) phage titer. The values shown in both graphs are the average of three independent assays and the error bars represent the standard deviation.

3.3. Efficacy of phT4A Phage in the Prevention of E. coli Biofilm on Plastic

The results of the prevention of biofilm formation on plastic are presented in Figure 4. In the bacteria control, the biofilm cell density increases by 3.8 log CFU/cm² (ANOVA, $p < 0.05$, Figure 4) during the experiment. The concentration of biofilm cells in the phage-treated group remained constant during the first 12 h of incubation and was similar to the initial concentration (4 log CFU/cm²). When compared the biofilm cell density in the phage-treated group with the untreated group, a decrease of 3.2 log CFU/cm² after 12 h of incubation (ANOVA, $p < 0.05$, Figure 4), was observed. The regrowth of biofilm cells on phage-treated group was observed after 12 h of incubation. However, at the end of the experiment, the biofilm cell density in the phage-treated group (Bacteria + phage, Figure 4) and untreated group (Bacteria control, Figure 4) was significantly different (decrease of 0.8 log CFU/cm², ANOVA, $p < 0.05$).

As observed in the previous experiments, phage control remained stable during the experiment and in the presence of *E. coli*, a significant increase in phage titer was observed (data not shown).

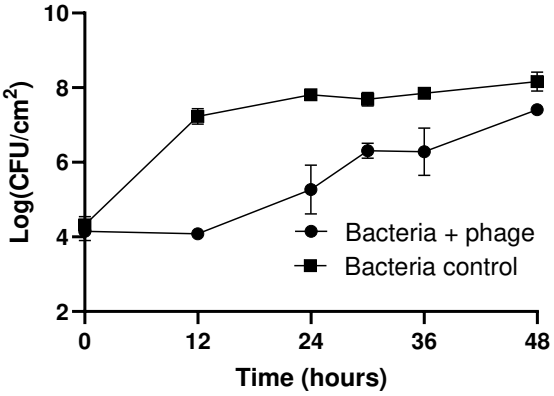

Figure 4. Evaluation of phage phT4A efficacy in preventing *E. coli* ATCC 13706 biofilm formation on plastic during 48 h. The values shown in the graph are the average of three independent assays and the error bars represent the standard deviation.

The ability of phT4A phage to prevent biofilm formation by *E. coli* was also evaluated using crystal violet staining. These results allowed us to observe the differences between the phage-treated group (Bacteria + phage) and the untreated group (Bacteria control) at time 0 and after 12, 24, 30, 36 and 48 h of treatment, by observation under inverted microscopy (Figure 5).

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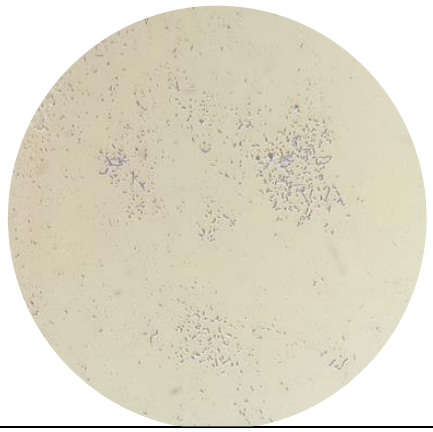
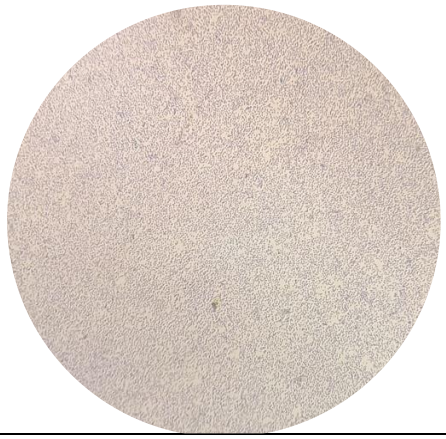
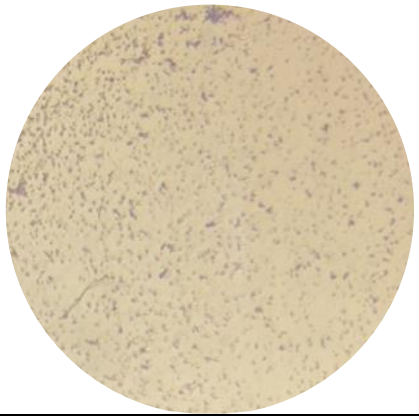
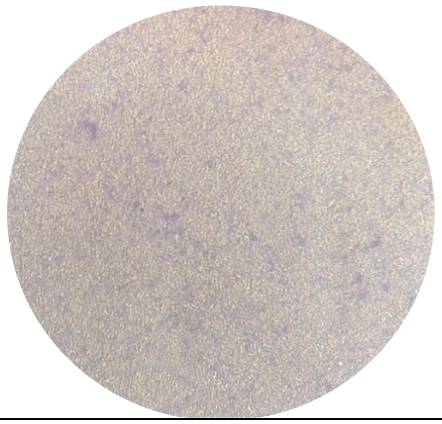
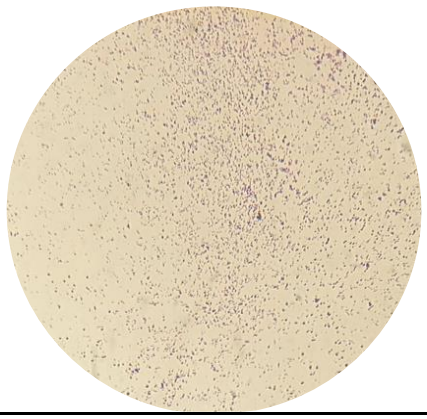
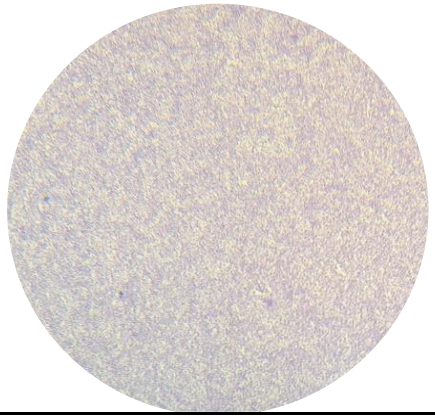
Time (h)	Bacteria + phage	Bacteria control
0		
12		
24		
30		

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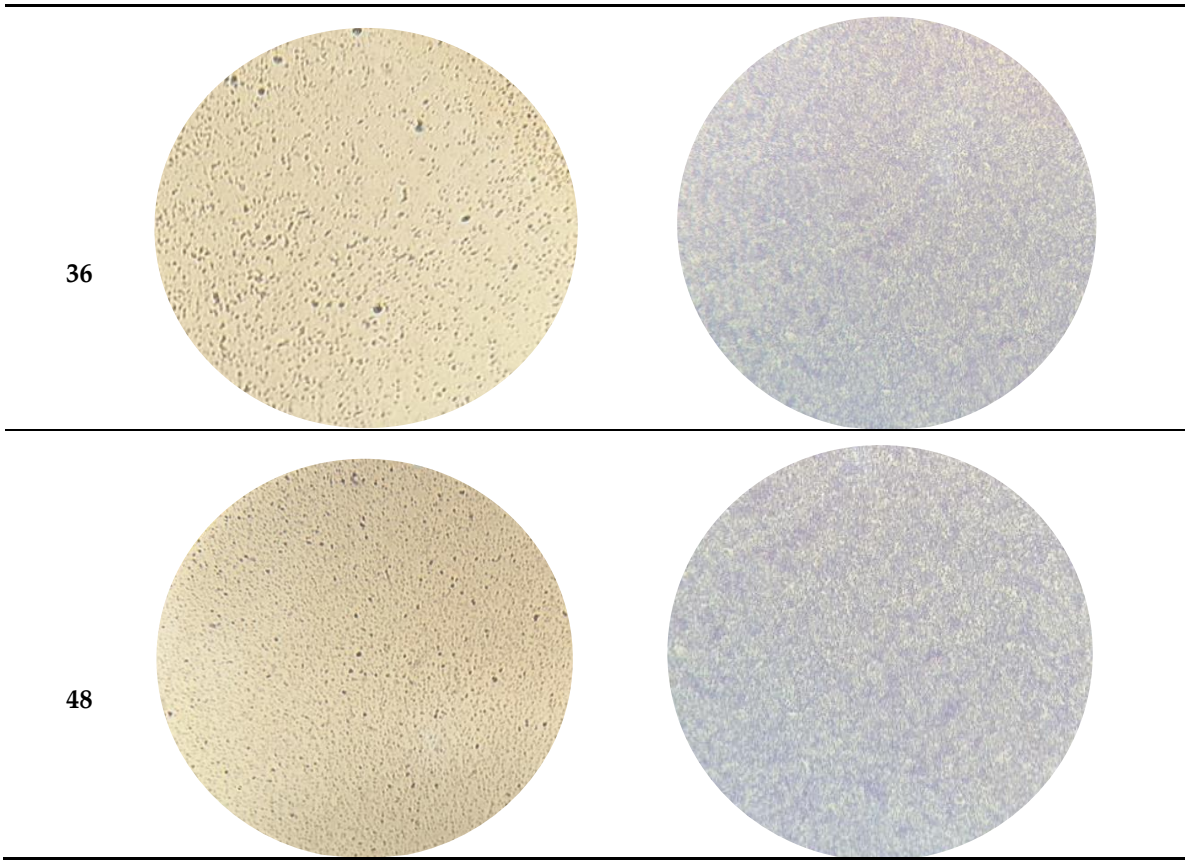

Figure 5. Observation of biofilm formation on plastic, at different incubation times, under an in-verted optical microscope (MOTIC AE31), with 200x magnification.

3.4. Evaluation of the Biofilm Formation Ability of Resistant and Sensitive Bacteria to phT4A Phage

The biofilm forming capacity of resistant and sensitive bacteria to phT4A phage, is shown in Figure 6. At the end of the experiment, the biofilm density in the group of resistant bacteria (R1 and R2) was significantly lower than in the bacterial control (ANOVA $p < 0.05$, Figure 6). However, no differences (ANOVA $p > 0.05$, Figure 6) were observed between the group of resistant bacteria (R1 and R2) and the group of sensitive bacteria (S1 and S2).

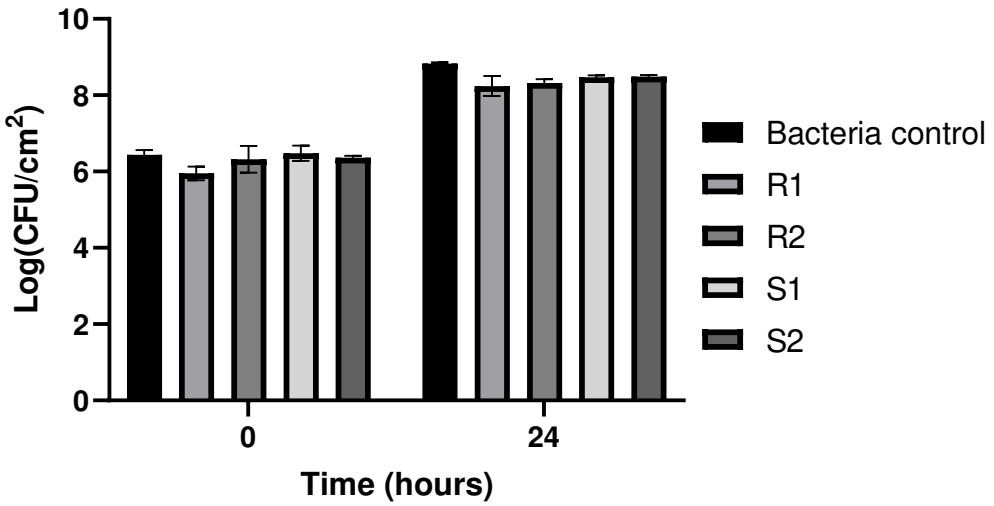

Figure 6. Assessment of the biofilm formation capacity of resistant and sensitive bacteria to phT4A phage, during 24 h of incubation. R1 and R2, bacteria resistant to phT4A phage after contact with

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phage; S1 and S2, bacteria sensitive to phT4A phage after contact with phage; Bacteria control, bacteria without contact with the phage phT4A. The values shown in the graph are the average of three independent tests and the error bars represent the standard deviation.

The ability to form biofilms by resistant and sensitive bacteria to phage phT4A was observed by inverted microscopy (Figure 7). The microscopy images showed that the group of bacteria resistant to phages phT4A (R1 and R2) had a lower bacterial cell density compared to the group of bacteria sensitive to phages phT4A (S1 and S2, Figure 7). However, the group of sensitive bacteria (S1 and S2) did not show any significant differences when compared to the control group (BC1 and BC2, Figure 7).

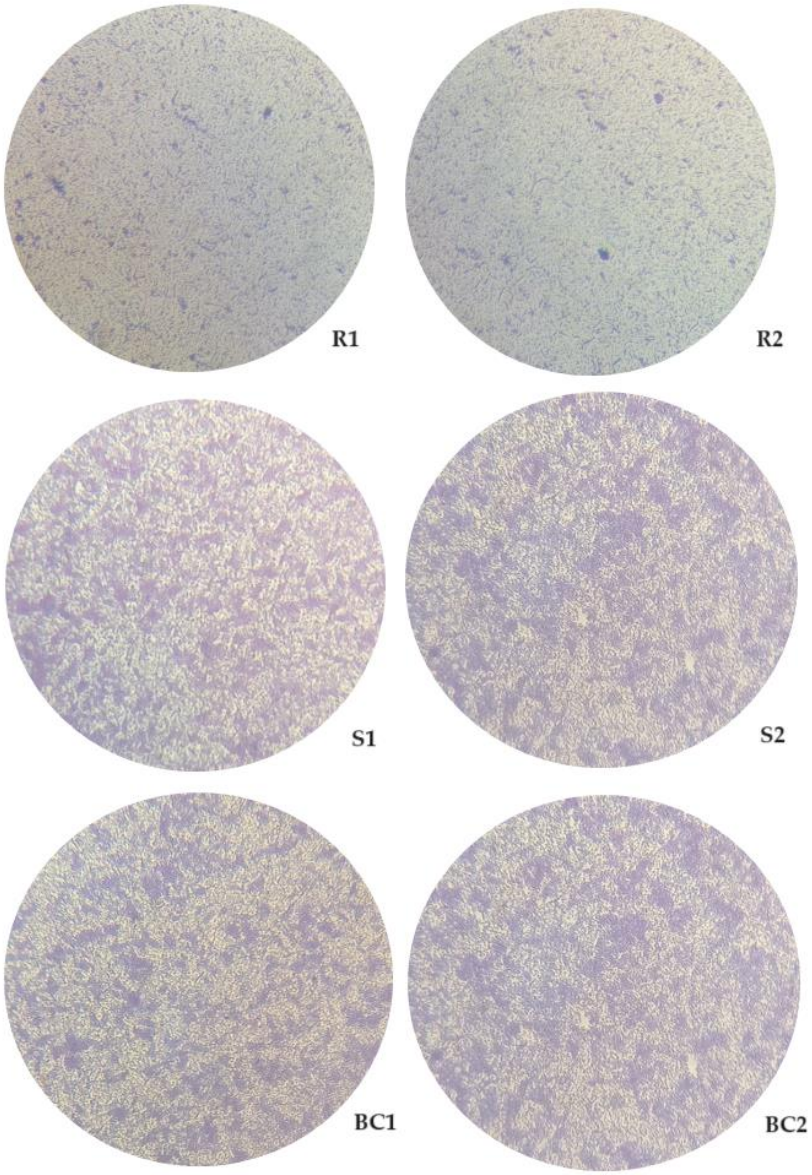

Figure 7. Observation of the biofilm formation on plastic by different groups of bacteria (phage-resistant, phage-sensitive and control), with 24 h of incubation, under an inverted microscope (MOTIC AE31) with 200x magnification. R1 and R2, Bacteria resistant to phT4A phage after contact with phage; S1 and S2, Bacteria sensitive to phT4A phage after contact with phage; BC1 and BC2, Bacteria without contact with the phage phT4A.

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4. Discussion

The bacterial strain used in this study (*E. coli* ATCC 13706) is a strong biofilm-forming bacteria, namely when compared with the *E. coli* ATCC 25922 strain, which is considered a model of biofilm-forming bacteria in the literature [20,21].

The phT4A phage, at MOI 10, had a significant impact on the reduction of biofilm formed in the plastic surface (5.5 Log CFU/cm^2), namely in the first 6 h of treatment. Similar results were obtained by Zhu et al. (2022)[27], with a reduction of approximately 6 log CFU/well, for a lower MOI of 0.1. However, this reduction occurred later, after 8 h, compared to our study (after 6 h). Additionally, the difference in the incubation temperature and the culture medium [37 °C for biofilm formation and 25 °C for phage treatment on TSB in our study vs. 37 °C for biofilm formation and phage treatment on Lysogeny Broth in Zhu et al. (2022)] [27], can significantly impact the results.

Considering that the optimal growth temperature for *E. coli* is around 37 °C, it would be expected that this temperature would result in a more mature and well-structured biofilm. This can lead to the reduced effectiveness of phages in eradicating this mature biofilm, since phages needs to bind to the host bacteria to infect them. However, when phages are able to penetrate the biofilm formed at 37 °C, due to the supposed higher bacterial density compared to the biofilm formed at 25 °C, there is a possible higher phage replication capacity, which could result in more effective inactivation. On the other hand, incubation at 25 °C may not provide optimal growth of the bacteria and thus negatively influence the cohesion strength of the biofilm. In the context of the food industry, which is our focus, food is often handled at room temperature closer to 25 °C. For our work, it is important that the choice of incubation temperature is as aligned as possible with the real one, since the objective is to replicate as much as possible the conditions of the food industry, hence the choice of an incubation temperature of 25 °C.

On stainless steel, our results highlight the effectiveness of phT4A phage, at a MOI of 10, in reducing the biofilm formed at 37 °C in 4.1 log CFU/cm^2, with greater evidence 9 h after the beginning of treatment. In another study of Wang et al. (2020), a similar protocol was carried out, however, biofilm with 24 h of maturation was formed at 24 °C instead of the 37 °C of our study [30]. The lower temperature of 24 °C was probably responsible for the lower reduction of 2.9 log CFU/bar even at a higher MOI of 100 [30].

Comparing the results obtained on both surfaces (plastic and stainless steel), it is possible to observe considerable differences: i) at the same MOI value, the reduction of biofilm on plastic surfaces is more pronounced than on stainless steel surfaces; ii) the maximum reduction in stainless steel occurs later (9 h after the start of treatment) than in plastic (6 h after the start of treatment). These differences suggest that bacteria possibly adhere differently to each type of surface. Phage can also behave differently on each surface. This indicates that in order to translate the phage application to the routine it is important to test the treatment in the different surfaces used in the industry.

In addition to biofilm reduction, the prevention of biofilm formation on food handling surfaces is also crucial to guarantee the safety and quality of food products. Our results indicated that phT4A phage prevented biofilm formation by *E. coli* ATCC 13706. Furthermore, the prevention was maintained up to 12 h of post-exposure to the phage. However, over time, a decrease in biofilm prevention capacity was observed. This may be due to the emergence of phage-resistant bacteria, limiting its effectiveness [31].

Our results revealed a significant difference in the biofilm formation capacity between phage-resistant and sensitive bacteria, after phage exposure. This suggests that although phage-resistant bacteria emerge during treatment, the phage-resistant bacteria showed slower growth and, consequently, a reduced ability to form biofilm. On the contrary, sensitive bacteria maintained the biofilm formation capacity similar to that of the control group.

Chemical disinfectants commonly used to disinfect surfaces achieve bacterial reductions of 4-5 log [1].Considering the results obtained in this work, the phT4A phage seems to be a promising alternative to disinfectants. An advantage of using phages on food handling equipment and surfaces, over traditional chemicals, is the fact that they do not affect the food properties [1].

In general, in both assays of biofilm reduction and prevention, there was a significant decrease in the bacterial concentration in the presence of phage, relatively to the bacteria control, within the first hours of treatment. However, after this period, bacterial regrowth was observed. This can be attributed to: (i) some bacterial cells located in the biofilm structure that can be inactive, hindering the phage multiplication [30,32](ii) the development of phage-resistance mechanisms by the bacteria, allowing them to survive and multiply again [32].The application of a phage cocktail containing several active phages for the same bacterial strain can prevent the development of bacterial resistance to phages [26,33]. Moreover, the application of several phage doses for sanitization purposes may increase the effectiveness of the disinfection.

Thinking about the concept of "One Health", the use of phages seems to be a promising approach to prevent and reduce biofilm formation in food handling surfaces, contributing to a better human, animal and environmental health. By reducing the need to use aggressive chemicals, the use of phages also reduces the impact associated with the disposal of these products. This leads the food industry to position itself as a leader in the search for more sustainable and safe solutions.

5. Conclusions

Phage phT4A was effective to reduce *E. coli* biofilm on plastic and stainless steel (reductions of 5.5 and 4.1 Log CFU/cm^2, respectively) and to prevent *E. coli* biofilm formation on plastic (prevention of 3.2 Log CFU/cm^2). Although the emergence of phage-resistant bacteria has been observed, these mutants were not as fit as their counterparts. These results suggest that phages may have applicability as surface disinfectants against pathogenic bacteria.

Author Contributions: A.B. performed the experiments. I.M. helped in some experiments. A.B., M.B. and J.D. participated in the conception and design of the experiments, and analysis of the resulting data. A.B. and M.B. wrote the paper and J.D. and C.P. also contributed to the writing. A.A. supervised the work, revised the paper, and contributed with reagents and analysis tools. All authors have read and agreed with the published version of the manuscript.

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