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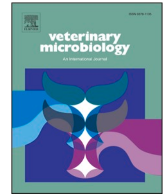
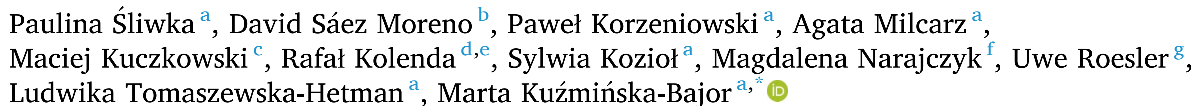


Avian pathogenic *Escherichia coli*-targeting phages for biofilm biocontrol in the poultry industry



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ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) is a principal etiologic agent of avian colibacillosis, responsible for significant economic losses in the poultry industry due to high mortality and disease treatment with antibiotics. APEC and its ability to form biofilms on food and processing surfaces contributes to its persistence within farms. Bacteriophages are promising antibacterial agents for combating APEC. This study focused on characterization of the newly isolated phages UPWr_E1, UPWr_E2, and UPWr_E4 as well as the UPWr_E124 phage cocktail containing these three phages. Methods included efficiency of plating assay, transmission electron microscopy, and characterization of their resistance to different pH values and temperatures. Moreover, phage genomes were sequenced, annotated and analyzed, and were compared with previously sequenced *E. coli* phages. All three phages are virulent and devoid of undesirable genes for therapy. Phage UPWr_E1 belongs to the genus *Krischvirus* within the order *Straboviridae* and both UPWr_E2 and UPWr_E4 belong to the genus *Tequatrovirus* within the subfamily *Tevenvirinae*, sharing over 95 % nucleotide identity between them. For their use on poultry farms, UPWr_E phages and the UPWr_E124 phage cocktail were tested for their anti-biofilm activity on two *E. coli* strains – 158B (APEC) and the strong biofilm producer NCTC 17848 – on two abiotic surfaces: a 96-well microplate, a stainless steel surface, and one biotic surface, represented by lettuce leaves. The reduction of biofilm formed by both strains in the 96-well microplate, on the stainless steel and lettuce leaf surface for bacteriophage treatment was very efficient, reducing biofilms by ranges of 50.2–83.6, 58.2–88.4 and 53–99.4 %, respectively. Therefore, we conclude that UPWr_E phages and the UPWr_E124 phage cocktail are promising candidates for APEC biocontrol.

1. Introduction

Poultry production plays a key role in addressing the escalating global demand for protein of animal origin (Hedman et al., 2020). In response, the industry has established large-scale poultry production systems capable of meeting this demand. This intensive and industrial-scale poultry production contributes to vertical transmission of pathogens within the flocks. Therefore, the establishment of a good disease prevention program is essential for poultry production.

One significant challenge faced by aviculture industries worldwide is the prevalence of avian pathogenic *Escherichia coli* (APEC) strains, the etiological agents of colibacillosis in poultry (Paudel et al., 2024). Colibacillosis is a multifaceted disease that affects various sectors of poultry production, including broiler chickens, turkeys, and egg-laying sectors. In broiler chickens, APEC infections typically manifest as respiratory tract infections, air sacculitis, pericarditis, perihepatitis, splenomegaly,

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and swollen head syndrome. In mature laying hens, it leads to reproductive tract infections, such as salpingitis or salpingo-peritonitis syndrome (Collingwood et al., 2014). These infections can cause reduced production, increased mortality rates and compromised animal welfare, resulting in severe economic losses for the poultry industry, and ultimately are treated with antibiotics (Nawaz et al., 2024).

The overuse and misuse of antibiotics in poultry production have raised concerns regarding the development of antibiotic resistance. This is confirmed by the fact that APEC strains show multi-drug resistance (MDR) including resistance to antibiotics such as ampicillin, tetracycline, trimethoprim, sulfamethoxazole, and streptomycin antibiotics and the "last chance" antibiotic colistin (Nawaz et al., 2024). The emergence of MDR APEC strains hampers the effective treatment and poses a potential risk for the horizontal transfer of antimicrobial resistance genes or virulence factors to related human pathogens. APEC strains serve as rich reservoirs for antibiotic resistance in the environment where they are transferred from farms as effluents or manure. The management and occurrence of colibacillosis are additionally complicated due to the ability of APEC to form biofilms. Although there is no direct impact of biofilm formation on the pathogenicity of APEC *in vivo*, it plays a significant role in the persistence and viability of APEC within the environment and serves as the reservoir of these bacteria on the farms (Ewers et al., 2008; Giaouris et al., 2014).

In fact, control of APEC is of utmost importance not only for poultry and the food industry but also for human health worldwide, since there is a substantial genetic similarity between APEC and other extra-intestinal pathogenic *E. coli* (ExPEC). ExPEC strains are associated with human diseases such as uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC), and sepsis-associated *E. coli* (SEPEC), which implies that APEC may pose a zoonotic risk for humans and could serve as a reservoir of virulence and resistance genes for other ExPEC (Collingwood et al., 2014). Therefore, strategies to tackle APEC and the development of antimicrobial resistance in APEC are essential to prevent infection and disease spread to humans considering the One Health approach (Sinclair, 2019).

In light of these challenges, there is a pressing need to explore alternative strategies for the treatment and prevention of APEC infections in poultry. One potential approach involves the use of bacteriophages, which are viruses that can specifically target and kill bacteria. As such, bacteriophage therapy has gained attention as a potential alternative to antibiotics in combating APEC infections. The administration of bacteriophages to poultry has demonstrated efficacy in preventing *E. coli* lethal respiratory infections in broiler chickens, as evidenced by successful outcomes reported in several studies (Huff et al., 2004; Oliveira et al., 2009a).

Considering the great potential of bacteriophages as antimicrobial agents in combating APEC and their biofilms, this study was undertaken to isolate and characterize phages against a wide spectrum of APEC. As a result, we characterized three novel bacteriophages, named UPWr_E1, UPWr_E2 and UPWr_E4. We proved that these phages were active against biofilms formed by APEC on biotic and abiotic surfaces, both as monophage preparations and combined in the UPWr_E124 phage cocktail containing all three phages.

2. Materials and methods

2.1. Bacterial strains and culture conditions

115 avian pathogenic *E. coli* (APEC) used in this study were obtained from the Strain Collection of the Department of Epizootiology and Clinic of Bird and Exotic Animals, Wrocław University of Environmental and Life Sciences, and 28 APEC were provided by the Institute of Animal Hygiene and Environmental Health, Freie University Berlin, Germany. *E. coli* strains were isolated from the internal organs of dead birds with colibacillosis and classified as APEC. In anti-biofilm assays, two *E. coli* strains were utilized: *E. coli* 158B, representing APEC, and NCTC 17848, exhibiting great ability to produce biofilm. All bacterial strains were cultivated in LB broth (A&A Biotechnology, Poland) under aerobic conditions at 37°C with agitation.

2.2. Bacteriophage isolation and purification

Forty-four urban sewage samples from the Wrocław wastewater treatment plant and samples including feces, litter, manure from poultry farms and drainage ditches located near poultry farms were collected from 2015 to 2018. For bacteriophage isolation, 5 ml of a sewage sample was mixed with 15 ml of LB broth, inoculated with five randomly chosen *E. coli* strains and incubated overnight at 37°C with agitation, as described previously (Kuźmińska-Bajor et al., 2021). Following centrifugation supernatants were filtered using a 0.22 μm filter (Merck Millipore, USA). The presence of phages was tested using a spot test. In this assay, overnight *E. coli* cultures were spread on LB agar plates and incubated for 40 minutes at room temperature. Serial dilutions of filtered supernatants were spotted onto the surface of the plates, left to dry and incubated at 37°C overnight. Plates were inspected for lysis zones or the presence of plaques. Clear, single and well-separated plaques were picked and eluted into 200 μl of LB broth culture, added to 5 ml of the fresh host culture and incubated overnight. To obtain a single phage, each bacteriophage was purified using five consecutive rounds of single-plaque picking and propagation.

2.3. Bacteriophage propagation

E. coli APEC strain no. 158B was used as a host strain for UPWr_E1, UPwr_E2, and UPWr_E4 phage propagation. Phages were propagated as previously described by (Oliveira et al., 2009). Briefly, 10 ml of LB broth (A&A Biotechnology, Poland) was inoculated with a single colony of host strain, following overnight incubation (37°C, 150 rpm). 0.5 ml of the overnight culture was transferred to 10 ml of fresh sterile LB broth and incubated (37°C, 150 rpm) until optical density ($OD_{600\,nm}$) reached 0.2. At that point in time, 5 ml of phage lysate was added and further incubated for 2 h. Next, the bacterial cultures were centrifuged (10 min, 5000 × g) and the supernatant was filtered through 0.22 μm pore size syringe filters (Merck Millipore, USA). The obtained phage lysate was added to 150 ml of the refreshed host culture ($OD_{600\,nm} = 0.2$) and incubated overnight at 37°C. Then, the centrifugation and filtration steps were repeated. To determine phage titer, the routine test dilution method was used (Adams, 1959). Phages UPWr_E1, UPWr_E2, and UPWr_E4 were mixed at equal ratios into a phage cocktail named UPWr_E124. Phage cocktail and phages alone were analyzed for their ability to reduce the number of *E. coli* in biofilms.

2.4. Bacteriophage DNA extraction, genome sequencing, assembly and annotation

Phages at titers of 10^{10}–10^{12} PFU/ml were used for DNA extraction. Prior to the extraction, sterile bacterial lysates containing phages obtained according to the phage propagation protocol were treated with DNase I (80 U/ml; Thermo Scientific, USA) and RNase I (80 μg/ml; Thermo Scientific, USA) at 37°C for 3 h to remove non-phage nucleic acids. Following phage capsid disruption by treatment with proteinase K and 0.5 % sodium dodecyl sulfate (Sigma-Aldrich) for 2 h at 56°C, phage DNA was extracted using a High Pure Viral Nucleic Acid Large Volume Kit (Roche, Mannheim, Germany). Phage genomes were sequenced with the Illumina MiSeq next-generation sequencing platform (Genomed SA, Poland) using MiSeq Reagent Kit v2 500-cycles (Illumina, USA). Sequencing quality was assessed based on average base quality, GC content and adapter contamination. Phage sequence assembly was conducted with the Shovill pipeline and assembly improvement pipeline (Kolenda et al., 2021) into one unique contig.

2.5. Analysis of phage genomes

Taxonomic classification was inferred from the closest sequenced relatives identified by BLAST analysis of phage sequences in NCBI's RefSeq database. Genes were annotated with Prokka (Seemann, 2014), the genome annotation services of BV-BRC (Seemann, 2014), with BLAST function of Geneious software and manually curated by protein family prediction (Uniprot, HHpred). The designation of genome modules was done according to annotations. Comparative genomics between phages was carried out with Geneious and with easyFig (BLASTn) (Brettin et al., 2015).

The lytic lifecycle of the 3 phages was confirmed computationally using PhageLeads and searching for lysogeny-related genes in the genome (Sullivan et al., 2011; Yukgehnaish et al., 2022). PhageLeads features integration of the ABRicate tool to determine the presence of antibiotic resistance genes and virulence genes. On top on that, antibiotic-resistance genes were analyzed by uploading the translated sequences to ResFinder 4.3.3 (Bortolaia et al., 2020), using parameters for "other" and for *E. coli*, targeting chromosomal point mutations and acquired antimicrobial resistance genes, with a threshold for ID of 90.0 % and a minimum length of 60.0 %. Absence of AMR (antimicrobial resistance) and virulence factor genes was confirmed by BLASTn using the Comprehensive Antibiotic Resistance Database (CARD, October 2023) (Alcock et al., 2023), and Virulence Finder 2.0 CGE, against *E. coli*, Software version: 2.0.3 (2020–05–21). Database version: (2022–12–02), with a threshold for ID of 90.0 % and a minimum length of 60.0 % (Camacho et al., 2009; Tetzschner et al., 2020; Joensen et al., 2014).

2.6. Morphology of phages by transmission electron microscopy

Electron microscopic analysis to determine phage morphology was conducted by adsorbing 5 µl of phage suspension onto 400 copper mesh grids (Sigma-Aldrich) coated with 2 % collodion solution (Sigma-Aldrich) and carbon (3 min), stained with 2 % uranyl acetate (pH 4.5) (BDH Chemicals, UK) (15 s) and air-dried. Electron microscopic analysis was performed at 120 kV using an FEI Tecnai G2 Spirit BioTWIN transmission electron microscope (TEM). Electron micrographs were taken at 250,000 × magnification, using Olympus Soft Imaging Solution software.

2.7. Bacteriophage host range

The ability of UPWr_E1, UPwr_E2 and UPWr_E4 to infect different bacterial strains was tested by employing the efficiency of plating (EOP) assay to determine the host range of the phages. All APEC strains were tested using the double agar method (Adams, 1959). An aliquot of 100 µl of overnight bacterial culture and 200 µl of each phage serially diluted ten-fold in SM buffer ranging from 10^4 to 10^9 PFU/ml was added to 4 ml of LB soft agar medium and immediately poured onto the LB agar plate. The plates were incubated overnight at 37°C. For each phage, the EOP was calculated as the ratio of the number of plaques that were formed by the phage on the appropriate strain and the number of plaques that were formed on the host strain. The EOP values were classified as high (EOP $\geq$ 0.5), moderate (0.01 $<$ EOP $<$ 0.5), and low (EOP $\leq$ 0.1) (Stepanovic et al., 2004). All the experiments were carried out in triplicate. A heatmap with EOP was generated with the package ggplot2 implemented in the R software as reported previously (Oliveira et al., 2009b).

2.8. Mitomycin C assay

The phage life cycle was determined in the mitomycin C assay (Oliveira et al., 2009b). Briefly, for prophage induction, the temperate phage λ with the host *E. coli* K12, the strictly lytic phage T4 and its host and ATCC 11303 were used as controls. Phages were spotted on the fresh bacterial lawns of respective hosts and incubated overnight at 37°C. After incubation, the presence of resistant bacterial clones was observed in lytic zones. At least 10 phage-resistant colonies were picked from one plate and purified by fivefold subculturing on MacConkey agar (Sigma-Aldrich, Germany) to remove attached phage particles. To confirm the phage resistance of isolated bacterial strains, a standard spot test was performed. For chemical induction of phages from phage-resistant strains, 100 ml of LB broth was inoculated with overnight bacterial cultures and cultivated until an optical density at 600 nm of 0.2 was reached. To stimulate prophage induction, mitomycin C (Sigma-Aldrich, Germany) was added to the final concentration of 1 µg/ml. Each of the analyzed bacterial cultures was grown in the absence of mitomycin C as a negative control. Overnight cultures were centrifuged at 4000 ×g, filtered through 0.22 µm filter and spotted on cultured Petri dishes with the appropriate *E. coli* host. After overnight incubation at 37°C, plates were analyzed for the presence of clear zones.

2.9. Bacteriophage growth parameters

To determine the latent time and the burst size of UPWr_E1, UPwr_E2 and UPWr_E4 bacteriophages, the one-step growth curve was determined according to Yu et al. (2013). Briefly, to determine optimal multiplicity of infection (MOI) the log phase cultures of host strains were infected with phage suspensions at MOIs of 0.001, 0.01, 0.1, 1, 10, and 100 PFU/CFU. After 3.5 h of incubation at 37°C, the samples were collected, and the titers of the phages were determined by serial dilution and the double-layer method. To calculate the one-step growth curve, 1 ml of bacteriophage was added to the host culture at MOI 1 and allowed to adsorb for 10 min at 37°C. The mixture was centrifuged at 10 000 × g for 10 min and the pellet was resuspended in 60 ml of fresh LB broth. The culture was incubated at 37°C for 2 hours. 100 µl of the samples were collected at 10-min intervals and bacteriophage titer was determined using the double-layer agar method (Adams, 1959). The burst sizes of phages were calculated by dividing the number of new phages released after the burst by the number of infecting phages (phage added-titer at T0).

2.10. pH and thermal tolerance

The stability of UPWr_E1, UPwr_E2 and UPWr_E4 phages at a titer of 10^8 PFU/ml was tested to determine phage survival at temperatures (4–42°C) representing temperatures of broiler handling, the chicken body and storage conditions, and over a wide pH (2–13). All stability experiments were performed in triplicate.

For thermal tolerance, 10 ml of bacteriophages at a titer of $\sim 10^8$ PFU/ml were incubated for 2 weeks at different temperatures corresponding to storage conditions (4°C), broiler handling at least half of the production cycle (20°C), post-hatch broiler handling (37°C), and broilers' deep body temperature (42°C). Phage titer at each temperature was determined after 7 and 14 days of incubation by using the double-layer agar method.

For pH tolerance, 10 ml of bacteriophage suspensions at a titer of $\sim 10^8$ PFU/ml were adjusted with 1 M NaOH or HCl to pH ranging from 2 to 13 and incubated for 1 h. After incubation, phage titer was determined using the double-layer agar method.

3. Effect of the phages on bacterial biofilm

3.1. Detection of antibiofilm activity of APEC-targeting phages against biofilms in a 96-well microplate assay

To quantitatively determine the phage efficacy in APEC biofilm reduction a method described elsewhere (Korzeniowski et al., 2022) was used. For this purpose, overnight APEC 158B and NCTC 17848 cultures were diluted in LB broth to OD 0.2 ($\sim 2 \times 10^8$ CFU/ml) and 200 µl of this suspension was transferred to each well of 96-well plates (Sarstedt, Germany). Polystyrene microtiter plates were incubated at 37°C for 72 h

to allow biofilm formation. To remove planktonic cells, wells were washed twice with sterile phosphate saline buffer (PBS; Sigma-Aldrich, Germany). For biofilm degradation, 200 μl of UPWr_E1, UPwr_E2 and UPWr_E4 monophage preparations and UPWr_E124 phage cocktail was added to each well at final titers of 10^6, 10^7, 10^8, and 10^9 PFU/ml. A positive control well was also included with a sterile LB. Plates were then incubated at 37°C for 4 h under static conditions, medium with planktonic cells was removed and wells were rinsed with PBS three times and allowed to air-dry. The remaining biofilms were quantified by staining with 0.5 % crystal violet solution in ddH_2O (CV) (Merck, Germany) for 20 min followed by rinsing two times with PBS. Crystal violet attached to the biofilm was dissolved using 96 % ethanol (Sigma-Aldrich, Germany). The absorbance of the released color was measured using an automated microtiter plate reader (Spark Tecan, Switzerland) at 570 nm. The experiments were performed independently three times.

3.2. Detection of antibiofilm activity of APEC-targeting phages against biofilms on stainless steel

To quantitatively determine the phage efficacy against APEC biofilm formed on stainless steel representing an abiotic surface, the method previously described by Korzeniowski et al. (2022) was applied. Overnight bacterial *E. coli* 158B and NCTC 17848 were suspended in 250 ml Erlenmeyer flasks with 100 ml of LB containing 20 sterile 10 mm diameter stainless steel washers to match the optical density (OD 600 nm) of 0.2 (~2×10^8 CFU/ml). The flasks were incubated for 72 h at 37°C to allow biofilm formation. Next, each washer was transferred using sterile forceps to a well of a 24-well plate (Sarstedt, Germany) containing UPWr_E1, UPwr_E2 and UPWr_E4 phages at different titers (10^6, 10^7, 10^8, and 10^9 PFU/ml) or sterile LB medium as a control and further incubated for 4 h at 37°C. The LB medium containing planktonic cells and phages was then removed, steel washers were rinsed with PBS three times, air dried, and the remaining bacteria were measured by CV staining for 20 min followed by washing with PBS twice. Next, the washers were transferred to clean 24-well plates containing 96 % ethanol to dissolve the biofilm-bound stain by incubating for 15 min at room temperature. Finally, washers were removed from wells and the absorbance of released color was measured at 570 nm using a microtiter plate reader. The experiment was repeated three times.

3.3. Detection of antibiofilm activity of APEC-targeting phages against biofilms on lettuce leaves

The effectiveness of UPWr_E1, UPwr_E2 and UPWr_E4 phages against biofilm formed by APEC on the biotic surface was measured on cut lettuce leaves according to (Kroupitski et al., 2009). To create a biofilm on such a surface, 4–6 inner leaves were taken from the lettuce head. The leaves were washed under running water, dried at room temperature for 10 minutes and cut with a sterile scalpel into 2 × 2 cm pieces. Then, leaf fragments were sterilized with 0.5-minute incubation in 70 % ethanol and dried. 25 ml of an overnight culture of *E. coli* 158B and NCTC 17848 was diluted with LB to obtain an optical density of 0.2 (~2×10^8 CFU/ml) measured at a wavelength of 600 nm. Cut lettuce leaves were incubated for 2 min in 1 ml of the *E. coli* 158B and NCTC 17848 suspension to adsorb bacterial cells to the plant surface. The pieces were transferred with sterile tweezers, 4 leaves per plate, to Petri dishes containing sterile paper moistened with sterile PBS, ensuring high humidity, then protected with parafilm and incubated at 37℃ for 72 h. Pieces of leaves with *E. coli* 158B or NCTC 17848 biofilm on their surface were immersed for 1.5 minutes in 1 ml phage suspension with titers of 10^6, 10^7, 10^8, and 10^9 PFU/ml or in PBS in the case of the control, and then incubated for 4 hours at 37℃. In order to determine the number of *E. coli* bacteria in the biofilm on the surface of lettuce, pieces of leaves were transferred with sterile tweezers to Falcon tubes containing 1 ml of PBS, intensively mixed to detach microbial cells from the biofilm structures, and then serial tenfold dilutions were prepared to estimate the number of *E. coli* in the obtained solution. Samples were plated on LB agar and incubated overnight at 37°C to determine the number of colony-forming units (CFUs). The experiments were performed in three repetitions.

3.4. Statistical analysis

Data were collected and analyzed using Statistica version 13 software (TIBCO Software Inc.). The Shapiro-Wilk test was performed to assess the normality of the data. The significance level of $p < 0.05$ was predetermined for all statistical tests. The results of thermal and pH stability were expressed as means and ± standard deviations and the significant differences were evaluated using one-way analysis of variance (ANOVA) and Tukey's HSD post-hoc test. Data obtained from the biofilm assays were analyzed by applying ANOVA along with the least significant difference (LSD) post-hoc test.

4. Results

4.1. Bacteriophage host range and EOP determination

The 3 selected phages were tested against 143 APEC strains isolated from poultry with colibacillosis. Phage UPWr_E1 was capable of lysing 91 of the APEC strains (64 %), including 22 strains being infected with moderate efficacy ($0.01 \leq EOP < 0.5$) and 11 strains being infected with high efficacy ($EOP \geq 0.5$). Phage UPWr_E2 showed the lowest host range and lysed 46 % of tested strains, including 15 strains infected with moderate and 3 with high efficacy ($EOP \geq 0.5$). Phage UPWr_E4 exhibited the highest host range and was able to infect 92 out of 142 (64 %) APEC strains. Sixteen of the APEC strains were moderately susceptible, and 19 of them were highly susceptible to phage UPWr_E4 (Fig. 1 **A).** Twenty-four percent of APEC strains (34 out of 143) were not lysed by UPWr_E1, UPwr_E2 or UPWr_E4 phages.

4.2. Characterization of bacteriophages

Based on the phage morphological analysis by TEM, phages UPWr_E1, UPWr_E2 and UPWr_E4 exhibited an icosahedral capsid morphology (Fig. 1 **B**), which is typical of the myovirus morphotype. Phage UPWr_E3 exhibited the siphovirus morphotype with isometric capsids. Morphological features are described in Table 1.

To assess the life cycle of the phages, induction with mitomycin C was performed on the *E. coli* host strain after phage treatment. All analyzed UPWr_E phages developed the lytic life cycle and did not contain any mitomycin C-inducible prophages. Moreover, all UPWr_E phages produce clear plaques on their *E. coli* host.

4.3. Genomic analysis of APEC phages

Bioinformatics analysis of the phages revealed that phage UPWr_E1 has a genome length of 167,943 bp, harboring 284 coding sequences (CDS) and a GC content of 40.6 %. Its closest homolog is phage RB49 (GCA_000840705.1) and it belongs to the genus *Krischvirus* within the *Straboviridae* family. Phage UPWr_E2 has a genome comprising 168,127 bps, harboring 297 CDS and 35.3 % of GC content in its genome. Its closest published homolog is phage vB_EcoM-G3F9 (MZ234037.1), with 99 % nucleotide identity. Phage UPWr_E4 genome comprises 168,295 bps, 298 CDS and a GC content of 35.3 %. Its closest published homolog is phage vB_EcoM-G3G7 (MZ234040.1). According to ICTV Taxonomy Release #38: 2022, based on 95 % DNA sequence identity, UPWr_E2 and UPWr_E4 belong to the genus *Tequatrovirus* within the *Tevenvirinae* family. It was found that UPWr_E2 and UPWr_E4 display a high level of homology to each other, with 98.78 % identity at the nucleotide level, and share 98 % of their sequence (Fig. 1 **C**). The main difference between these two phages lies in several mutations: four DNA insertions, with sizes ranging from 693 bp to 870 bp

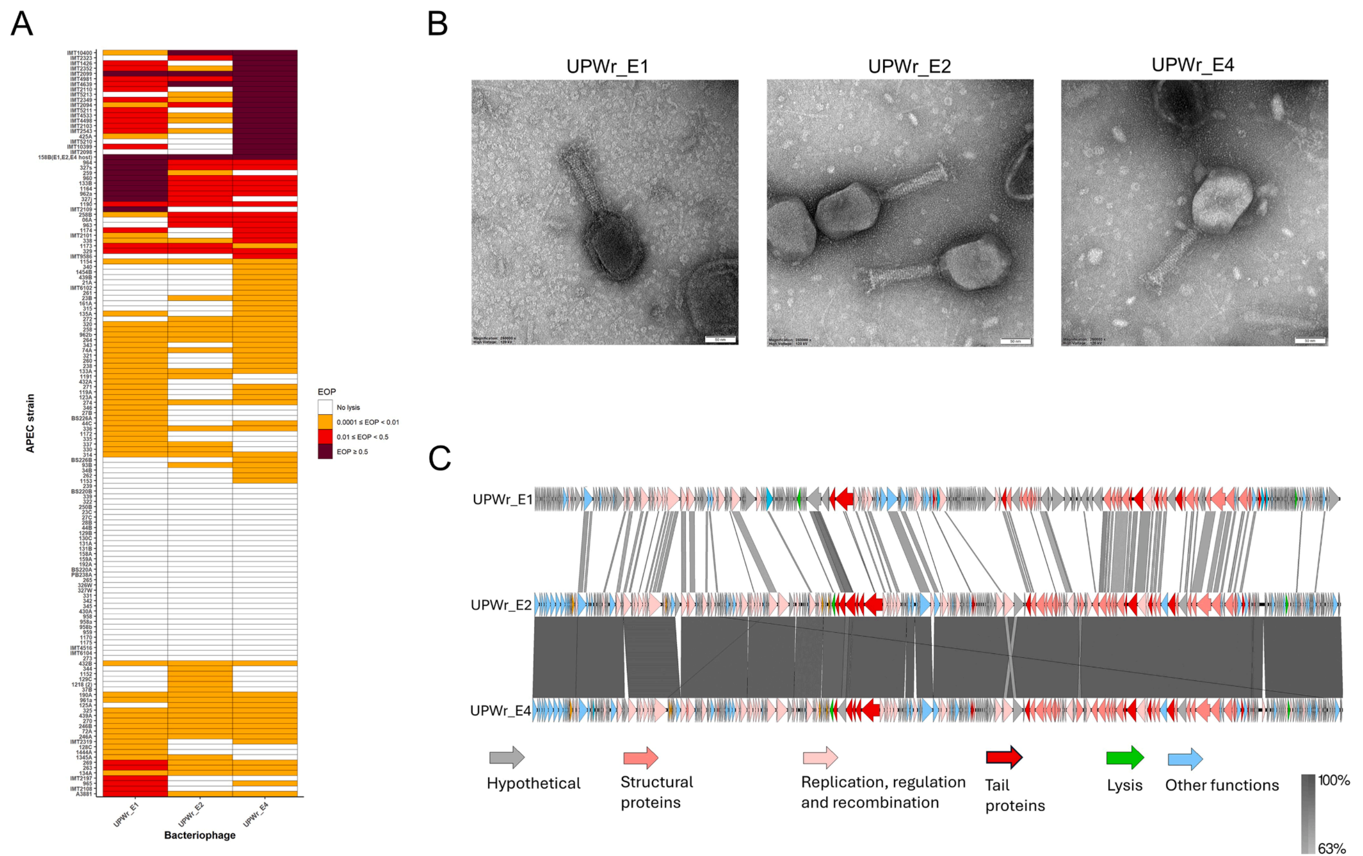

Fig. 1. (A) Bacteriophage host range and EOP. Bacteriophages were tested for host ranges and EOP against 143 APEC strains and the obtained results were plotted as a heatmap. Bacteriophages used in this analysis are presented on the X axis and the APEC strains are listed on the Y axis. Each rectangle shows the lytic effect of one bacteriophage to one host and the colors correspond to the lysis pattern: purple, EOP≥ 0.5; red, 0.01 ≤EOP$<$ 0.5; orange, 0.0001 $<$EOP$<$ 0.0; white, no lysis. **(B)** TEM image of UPWr_E1 (**a**), UPWr_E2 (**b**) and UPWr_E4 (**c**). The black bar represents 50 nm. **(C)** Genome comparison between UPWr_E1, UPWr_E2 and UPWr_E4 phages generated with BLASTn and visualized with EasyFig software. Relatedness between marked regions is presented by percent similarity (grayscale). Colors correspond to the functional protein group: dark pink – structural proteins; red – tail proteins; light pink – DNA replication and modification; green – cell lysis; blue other functions (thioredoxin, phosphatases, glutaredoxin, lipoprotein, thymidine related genes, tRNA synthetase, ribonucleotide reductases); gray – unknown function.

Table 1
UPWr_E phages' genome size, morphological features and biological characteristics.

	UPWr_E1	**UPWr_E2**	**UPWr_E4**
Genome size, bp	167,943	168,127	168,295
GenBank accession number	PP418985.1	PP418986.1	PP418987.1
GC content, %	40.6 %	35.3 %	35.3 %.
Capsid size, nm ± SE*,**	100 ± 3.2 × 78 ± 1.4	134 ± 4.6 × 77 ± .03	96 ± 3.2 × 77 ± 0.7
Tail size, nm ± SE	114 ± 0.2	144 ± 4	144 ± 2.9
The optimal MOI***	0.01	0.1	0.1
Latent period, min	40	20	20
Burst size, PFU/cell	300	23	195

* All measurements were made with the program ImageJ. 35 particles were measured for each phage and standard deviation was calculated (± SD).
** Capsid size was calculated for isometric capsid.
*** The optimal MOI refers to the bacteriophage/bacteria ratio producing the highest bacteriophage titer.

across the genome of UPWr_E4, that are not present in UPWr_E2. UPWr_E2 contains two regions of 1349 bp and 732 bp that are not present in UPWr_E4. These differences are in genes encoding hypothetical proteins inserted into genes encoding endonucleases or recombinases. Several SNPs (single nucleotide polymorphisms) were found to be distributed along the whole genome, including several genes encoding for tail fiber proteins. UPWr_E2 and UPWr_E4 phage genomes have sequences homologous to tail fiber genes in the regions from 64 379 bp to 72 548 bp, and from 62 702 bp to 72 384 bp, respectively. These regions are 96.3 % identical between both phages at the nucleotide level. In putative long tail fiber protein changes in 3 amino acid substitutions were found in a sequence of 372 amino acids. Nucleotide differences found in the sequence encoding for putative tail protein with peptidase activity correspond to 5 amino acid changes within 265 amino acids.

The phage genomes are linear, double-stranded and are organized in three differentiated modules, one comprising early genes with functions of replication, recombination and regulation functions, another comprising genes involved in lysis, and a late region containing structural genes and packaging functions (Fig. 1 **C**). The analysis of their lytic enzymes revealed that UPWr_E1 encodes for an endolysin with L-alanyl-D-glutamate peptidase activity and both UPWr_E2 and UPWr_E4 encode for an endolysin with lysozyme activity. No depolymerases were detected.

The genomic analysis demonstrated a lytic lifestyle of all characterized phages and their genomes were devoid of known genes or mutations mediating lysogeny, antibiotic resistance or virulence.

4.4. Phage stability at acidic and alkaline pH

The phages UPWr_E1, UPWr_E2 and UPWr_E4 were tested against a range of pH levels during 1-h incubation, to determine their stability under acidic and alkaline conditions (Fig. 2 **A, B and C**). All phages demonstrated high stability and remained active at pH values ranging

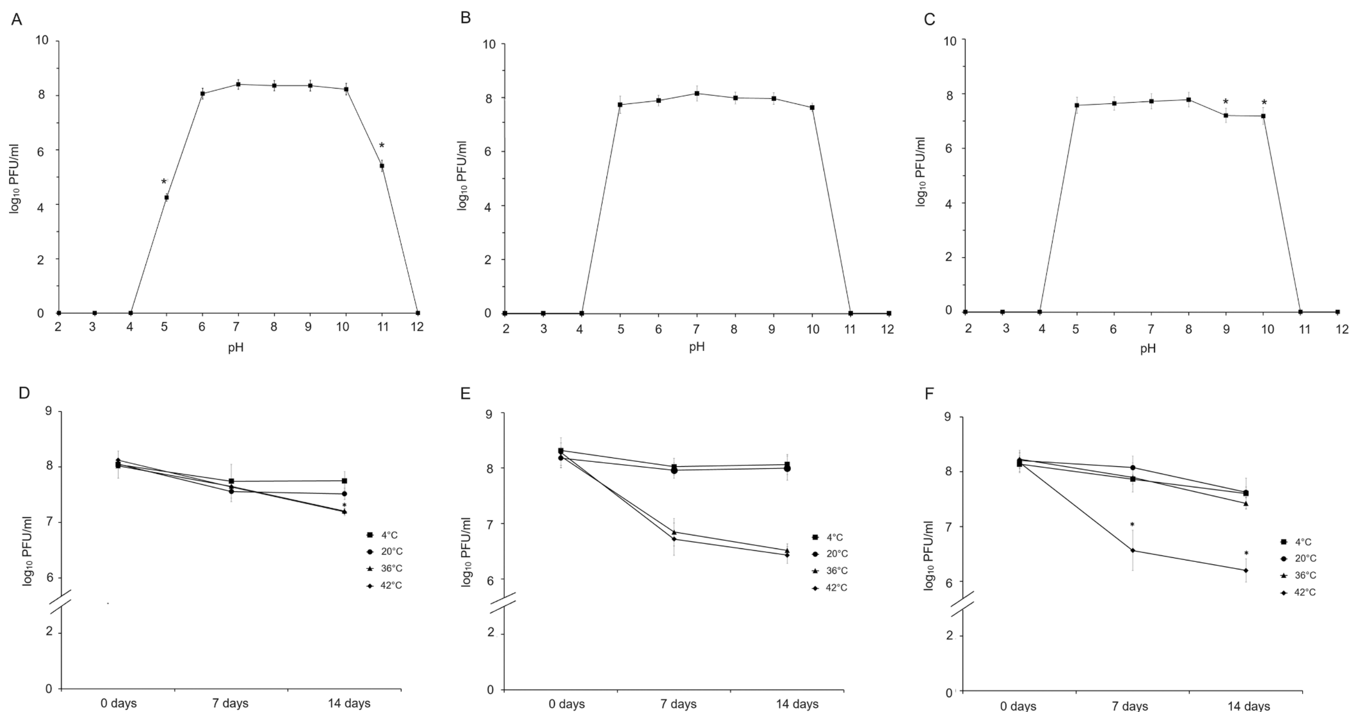

Fig. 2. pH stability of phages UPWr_E1 **(A)**, UPWr_E2 **(B)** and UPWr_E4 (C). Phage infectibility was estimated at a pH ranging from 2 to 12 after 1 hour of incubation. Values represent the mean with a standard deviation of three replicates. Effect of temperature on the stability of phages UPWr_E1 **(D)**, UPWr_E2 **(E)**, UPWr_E4 **(F)**. Phages were incubated for two weeks at four different temperatures. Each data point represents the mean of three independent experiments. * represents $p < 0.05$ and indicates a significant difference between experimental groups.

from 6 to 10.

The stability of phage UPWr_E1 significantly decreased to 1.9×10^4 PFU/ml (51 %) and 2.6×10^5 PFU/ml (62 %) at pH 5 and pH 11, respectively ($p < 0.01$). Phage UPWr_E2 showed significant stability between pH 5 and 10, but its lytic activity was completely lost at pH 4 and 11 ($p < 0.01$). Phage UPWr_E4 was stable at a pH range of 5–8, and a slight titer reduction to $1.5–1.6 \times 10^7$ PFU/ml (~86 %) ($p < 0.01$) was observed at pH 9 and pH 10.

4.5. Phage stability at different temperatures

The thermal tolerance of the phages UPWr_E1, UPWr_E2 and UPWr_E4 was examined at 4 temperatures, 4, 20, 36 and 42°C, for 2 weeks (Fig. 2 **D, E and F**). All phages remained thermally stable at 4 and 20°C during 2 weeks of incubation ($p < 0.01$). After 1 week of incubation at 36°C significant reduction in phage viability was observed for phage UPWr_E2 ($p < 0.01$). The second week of incubation at 36°C significantly decreased the titers of UPWr_E1 and UPWr_E2 ($p < 0.01$). UPWr_E1 was stable at 42°C for 1 week and decreased significantly when the phages were incubated at this temperature for 2 weeks ($p < 0.01$). At 42°C, the infection ability of phage UPWr_E2 was significantly reduced after 1 week and further at 2 weeks ($p < 0.01$). At 42°C viability of phage UPWr_E4 significantly decreased after 1 week of incubation ($p > 0.01$). In the second week of incubation, no significant reduction was observed ($p < 0.01$).

4.6. Phage activity against E. coli biofilms in vitro

Two APEC strains were evaluated for their capacity for biofilm formation. According to the classification suggested by Stepanović et al. (2004), the host strain *E. coli* 158B representing APEC and NCTC 17848 were classified as moderate and strong biofilm producers, respectively. The *E. coli* NCTC 17848 strain presented a ~2.2 times greater ability to form a biofilm than APEC 158B in 96-well plates (Fig. 3 **A and B**).

Then, the anti-biofilm activity of phages UPWr_E1, UPWr_E2 and UPWr_E4 and the phage cocktail UPWr_E124 was determined in 96-well plate assay with CV staining.

Biofilm biomass was reduced by the UPWr_E124 phage cocktail by 68.5–83.6 % for titers of 10^6–10^9 PFU/ml. All tested titers of monophages and UPWr_E124 phage cocktail exhibited a dose-dependent effect with significantly higher biofilm degradation for a titer of 10^9 PFU/ml in comparison to titers of 10^6–10^8 PFU/ml of both tested strains. *E. coli* 158B biofilm was lysed efficiently by the phage UPWr_E4 and phage cocktail UPWr_E124 at a titer of 10^6 PFU/ml. Phage UPWr_E1 exhibited the largest reduction of 82.5 % at a titer of 10^9 PFU/ml, while a significant decrease in biofilm degradation ability was observed for a titer of 10^6 PFU/ml of 52.9 % ($p < 0.05$). UPWr_E2 decreased biofilm biomass in the range 50.2–75.9 % for titers of 10^6–10^9 PFU/ml. The highest titer (10^9 PFU/ml) of phage UPWr_E4 reduced biofilm formation by 77.4 %, while the titer 10^6 PFU/ml decreased the biofilm level by 62.1 %. UPWr_1 reduced the *E. coli* NCTC 17848 biofilm biomass with activity by 52.9–82.5 % for titers of 10^6–10^9 PFU/ml (Fig. 3 **A**). Phages UPWr_E2 and UPWr_E4 exhibited ability to remove biofilm formed by *E. coli* NCTC 17848 by 57.2–64.8 % and 58.4–69.8 %, respectively. UPWr_E124 phage cocktail showed anti-biofilm activity in the range of 68.5–83.6 % (Fig. 3 **B**).

Overall, *E. coli* NCTC 17848, classified as a strong biofilm producer, exhibited higher sensitivity than *E. coli* 158B to treatment with monophage preparations and the phage cocktail UPWr_E124, at titer of 10^6 ($p < 0.05$). UPWr_E124 phage cocktail exhibited a comparable level to monophage preparations in biofilm reduction.

4.7. Degradation of E. coli biofilm on stainless steel

The activity of the UPWr_E1, UPWr_E2 and UPWr_E4 phages alone and UPWr_E124 phage cocktail was determined against *E. coli* 158B and NCTC 17848 biofilms on washers representing abiotic surface made of stainless steel used in the poultry industry. Microbial loads without the

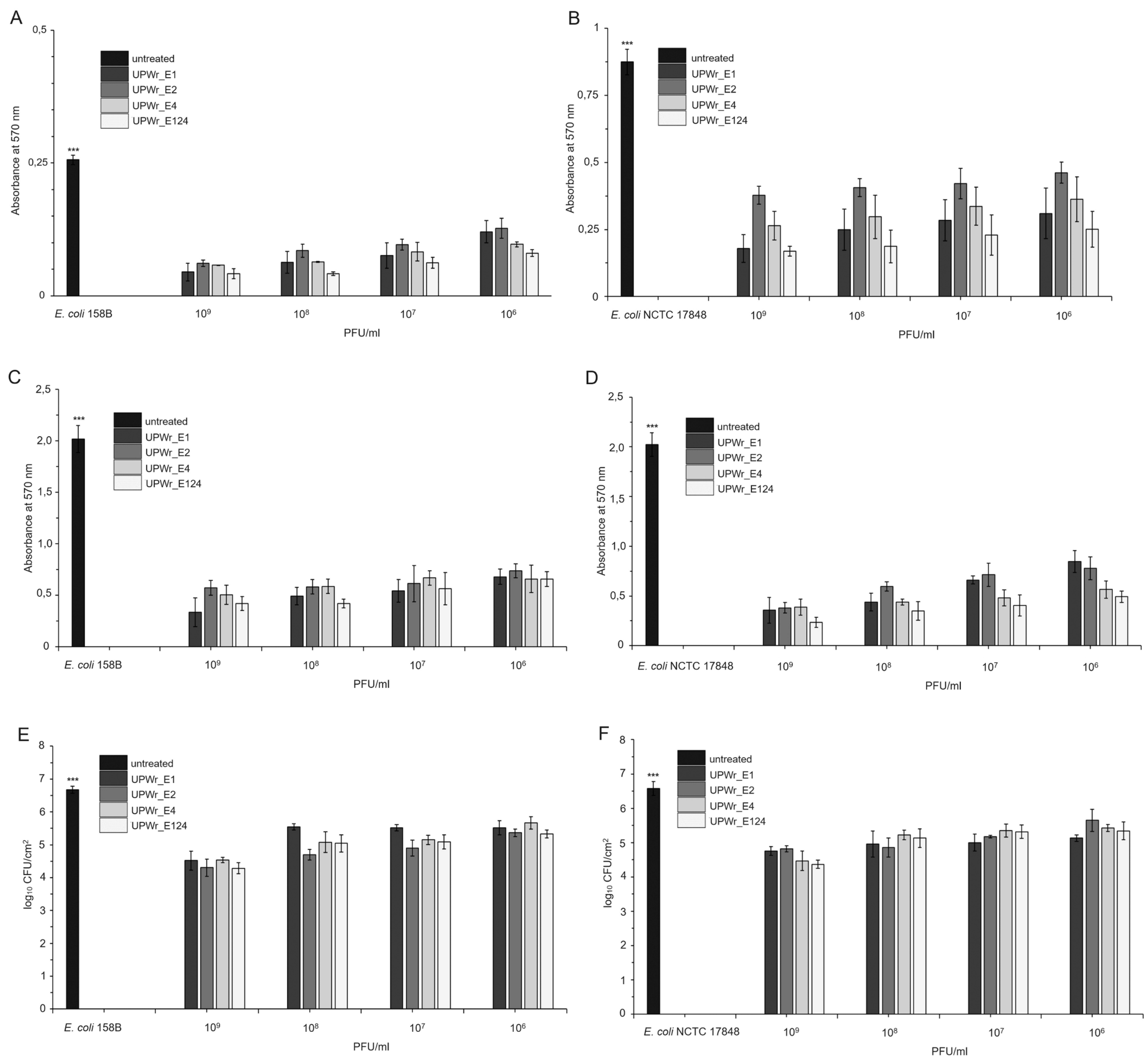

Fig. 3. Effect of UPWr_E1, UPWr_E2, UPWr_E3 and UPWr_E4 phages and phage cocktail UPWr_E124 on reduction of biofilm formed by APEC 158B in 96-well microplate (**A**) and, effect of UPWr_E1, UPWr_E2 and UPWr_E4 phages and phage cocktail UPWr_E124 on reduction of biofilm formed by *E. coli* NCTC 17848 in 96-well microplate (**B**). Effect of UPWr_E1, UPWr_E2and UPWr_E4 phages and phage cocktail UPWr_E124 on reduction of biofilm formed by APEC 158B on stainless steel washers (**C**), and effect of UPWr_E1, UPWr_E2 and UPWr_E4 phages and phage cocktail UPWr_E124 on reduction of biofilm formed by *E. coli* NCTC 17848 (**D**). Effect of UPWr_E1, UPWr_E2, UPWr_E3 and UPWr_E4 phages and phage cocktail UPWr_E124 on reduction of biofilm formed by APEC 158B on lettuce leaves (**E**), effect of UPWr_E1, UPWr_E2 and UPWr_E4 phages and phage cocktail UPWr_E124 on reduction of biofilm formed by *E. coli* NCTC 17848 on lettuce leaves (**F**). Values represent the mean with a standard deviation of three replicates. *** represents $p < 0.05$ and indicates a significant difference between untreated and phage-treated biofilms.

phage treatment on stainless steel washers were similar for both strains (Fig. 3 **C and D**). At a titer of 10^9 PFU/ml the UPWr_1 phage showed the highest ability to reduce biofilm biomass produced by APEC 158B by 83.4 % and significantly lower by 66.3 % at a titer of 10^6 PFU/ml ($p < 0.05$). UPWr_E2 decreased APEC 158B biofilm in the range 63.5–71.5 % for titers of 10^6–10^9 PFU/ml. Similarly, UPWr_E4 reduced biofilm biomass of this strain between 67.3 % and 75.0 % for titers of 10^6–10^9 PFU/ml. UPWr_E124 phage cocktail effectively lowered the biomass by 67.4–79.2 %. UPWr_E1 and UPWr_E2 decreased biofilm formed by the strain NCTC 17848 with a dose-dependent effect with the ranges 61.6–81.2 % for titers of 10^6–10^9 PFU/ml, respectively. UPWr_E4 phage exhibited ability to degrade biofilm produced by this strain by 72.1–80.8 % for titers of 10^6–10^9 PFU/ml. These results were similar for UPWr_E124 phage cocktail and were estimated to be 80.0–88.4 % for titers of 10^6–10^9 PFU/ml.

4.8. *Degradation of E. coli biofilm on lettuce leaves*

To demonstrate the anti-biofilm efficacy of the phages UPWr_E1, UPWr_E2 and UPWr_E4 and UPWr_E124 phage cocktail on a biotic surface commonly contaminated with *E. coli*, biofilms were formed on lettuce leaves and then subjected to phage treatment. After 72 hours of biofilm formation, the biofilm mass produced by APEC 158B and NCTC 17848 strains was similar ($p > 0.05$) and estimated to be 6.7 and

6.6 $\log_{10}$ CFU/cm^2, respectively (Fig. 3 **E and F).**

The viability of both strains during treatment was significantly reduced relative to untreated controls ($p < 0.05$) for all monophages and UPWr_E124 phage cocktail at titers of 10^6 to 10^9 PFU/ml. A dose-dependent effect on both strains was observed for all tested preparations apart from UPWr_E2 phage on APEC 158B. For biofilm formed by this strain on lettuce leaves, UPWr_E1, UPWr_E2 and UPWr_E4 phages exhibited a reduction by 0.9–1.9; 1.3–2.7; 1.3–2.0 log10 CFU/cm2 for titers of 10^6 to 10^9 PFU/ml, respectively. UPWr_E124 phage cocktail exhibited an ability to decrease bacterial load on lettuce leaves comparable to monophages in the range of 1.3–2.5 CFU/cm2. *E. coli* NCTC 17848 load on lettuce leaves was lowered by UPWr_E1 phage in the range of 1.4–1.7 CFU/cm2, UPWr_E2 by 0.7–1.7 CFU/cm2 and UPWr_E4 from 1.2 to 1.9 CFU/cm2 for titers of 10^6–10^9 PFU/ml. UPWr_E124 phage cocktail showed comparable capability to reduce biofilm by 0.9–2.0 CFU/cm2 as monophage preparations.

5. Discussion

APEC is responsible for colibacillosis outbreaks in the poultry industry leading to significant economic losses and contributing to increasing antimicrobial drug resistance (Sivaranjani et al., 2022). Phages are a promising bio-control tool against bacterial pathogens including APEC, which could be applied in the poultry industry. Phages intended for potential commercial application should exhibit a wide host range and be safe for therapeutic use. In this study, we selected 3 phages specific to APEC strains and capable of infecting 43–64 % of APEC poultry isolates. While the host range percentage is relative to the strain panel composition, we employed a bacterial panel comprising 143 strains originating from birds with colibacillosis. Only a limited number of studies have reported individual phage host ranges as wide as the ones reported in our study. Oliveira et al. (2009) tested phages against a panel of 148 APEC strains, finding that the phage with the broadest host range was phiF61E, targeting 48 % of the strains tested. Recently, Nicolas et al. (2023) reported their highest individual phage host range for 37 % of 30 APEC strains in the examined panel (Nicolas et al., 2023). These data are comparable for UPWr_E2 and lower than the host range of UPWr_E1 and UPWr_E4 phages, indicating their anti-APEC potential in comparison to other APEC-targeting phages. Some APEC strains exhibited resistance to all tested phages. Numerous studies have demonstrated that virulence factors and surface receptors are rarely all present in the same isolate, showing that APEC strains constitute a heterogeneous group (Schouler et al., 2012). These differences lead to divergent sensitivity to phages. Phage-host co-evolution has led to the development within bacterial populations of diverse and complex resistance mechanisms such as receptor adaptations, host defense systems, and phage-derived phage defense systems (Azam and Tanji, 2019; Egido et al., 2022). Determination of bacterial host receptors and anti-phage defense systems for bacteriophages included in the cocktails could provide an improved host range and delay the rise of resistance to phage cocktails (Acton et al., 2024).

All phages described here were shown to be deprived of known genes connected to lysogeny, AMR or virulence, thus being suitable for therapy. The closest homologue of UPWr_E1 is pseudo T-even bacteriophage RB46 specific to *E. coli*(Monod et al., 1997). Genome analysis of phages UPWr_E2 and UPWr_E4 revealed a 98 % sequence identity to each other; however, the closest homologue of UPWr_E2 is phage vB_EcoM-G3F9, while UPWr_E4's closest homologue is phage vB_EcoM-G3G7. Notably, both phages vB_EcoM-G3F9 and vB_EcoM-G3G7 are specific to UPEC clinical (Loose et al., 2021). This result suggests that not only closely related *E. coli* strains within the ExPEC group are similar, but also phages able to infect them. More studies are needed to expand knowledge on the relationship between anti-ExPEC phage relatedness and the common receptors that they target.

UPWr_E1, UPWr_E2 and UPWr_E4 phages were stable at pH 6 and pH 10, showing moderate tolerance ability to extreme environments. A previous study showed that an APEC O78-infecting phage, vB_Eco-M_APEC, can tolerate a pH range of 3.0–12 (Deng et al., 2021). Also APEC O78-infecting phages (ASO78A, ASO78B and AVIO78A) remained viable at a low pH of 2.5 even after 4 h of incubation (Smith et al., 2023). Phage CE1 was relatively stable within the pH range of 4–10 when incubated at different pH values (Tang et al., 2023). Compared to UPWr_E phages, APEC-targeting phages such as UPEC04 and UPEC1 are stable at pH close to neutral and exhibit significantly reduced viability at pH of 4 and 10 (Kazibwe et al., 2020). Another important factor for possible on-farm use is temperature stability. Our analysis showed that all UPWr_E phages remained active at 4 and 20°C, which reflect different farm conditions such as storage conditions and the temperature of broiler handling during at least half of the broiler production cycle, respectively. One-week incubation at 36 and 42°C, representing respectively slightly higher temperatures of post-hatch handling and broilers' deep body temperature, resulted in the decrease of phage titer. This result suggests that the application of phages should be repeated regularly to maintain the appropriate titer. Similar results were described for anti-Salmonella phages intended to be used in the poultry industry (Kuźmińska-Bajor et al., 2023).

To further confirm the anti-APEC efficacy of UPWr_E1, UPWr_E2 and UPWr_E 4 phages, they were mixed into the UPWr_E124 phage cocktail. Single phage preparations and UPWr_E124 phage cocktail were validated for their activity against biofilms on three surfaces directly related to poultry production. Previous studies show that biofilm reduction can depend on the properties of both the *E. coli* strain and the phage by presenting different biofilm reduction during short incubation lasting 3 and 6 hours (Jaroni et al., 2023). Ability of UPWr_E1, UPWr_E2 and UPWr_E 4 phages to destroy biofilm was tested in 4-hour incubation to study direct anti-biofilm activity of a bacteriophage to avoid the indirect effect of bacterial starvation and phage-resistance (Abedon et al., 2021). Two abiotic surfaces commonly found in farms – plastic and stainless steel – constitute a part of the equipment elements. The biotic surface was represented by lettuce leaves, food at high risk of contamination as a result of the application of organic fertilizers of poultry manure origin, likely being a source of virulence factors and resistance genes (Yang and Scharff, 2024).

It was found that phage treatments showed a dose-dependent response against biofilms formed by APEC and NCTC 17848 on all surfaces, contrary to the suggestion of (Amankwah et al., 2021), who stated that phage titer is not a main driver influencing anti-biofilm phage activity. Previous studies have already demonstrated significant APEC biofilm biomass reduction by phage cocktails measured by CV staining at titers between 1×10^6 and 1×10^8 PFU/ml. We found that reduction of the biofilm biomass by monophages and the UPWr_E124 phage cocktail was more effective than that shown by the phage *E. coli* O 78, able to reduce biofilm biomass by 32 % (Eid et al., 2022). Another anti-APEC phage, vB_EcoS_GN06, decreased biofilm biomass from 38 % to 42 % at MOI 0.1 and 1, respectively (Yao et al., 2023).

Levels of anti-biofilm activity of monophages and UPWr_E124 phage cocktail against biofilm formed on stainless steel correspond to the average reported reduction levels of other *E. coli* targeting phages and phage cocktails (Gildea et al., 2022). UPWr_E phages reduced *E. coli* on a stainless steel surface with a higher efficacy than phage DW-EC, suggested as an effective tool in destroying biofilms on industrial food processing equipment (Wiguna et al., 2022). This phage at a high MOI of 100 (titer of 10^7 PFU/ml), reduced EHEC, EPEC, and ETEC biofilm biomass measured by CV staining by 38.24 %, 38.46 % and 27.00 %, respectively. Our results suggest that even at a relatively low titer 10^6 PFU/ml all UPWr_E monophages and UPWr_E124 phage cocktail are able to significantly reduce biofilm biomass on stainless steel and could be considered as a biocontrol agent in poultry production. Other possibilities should also be considered in the future to further improve the biofilm biomass reduction of APEC biofilms. One of the promising approaches is artificially engineering biofilm-degrading bacteriophages. There are studies showing that the anti-biofilm effect could be enhanced

by bacteriophages expressing intracellularly bacterial dispersin B, responsible for biofilm matrix degradation (Lu and Collins, 2007).

Due to the consumption of raw lettuce leaves, this vegetable is associated with many foodborne disease outbreaks because of a risk of contamination from manure of livestock origin (Yang and Scharff, 2024). On lettuce leaves, the anti-biofilm activity assay is an established model for many bacterial pathogens such as *Salmonella* (Jahid et al., 2015), *E. coli* (Zhang et al., 2022), *Listeria monocytogenes* (Mizan et al., 2020)and multispecies biofilms (Jahid et al., 2015). Therefore, we investigated the anti-APEC activity of monophages and UPWr_E124 phage cocktail in biofilms on lettuce leaves. The decrease in *E. coli* numbers, by UPWr_E1, UPWr_E2 and UPWr_E4 phages and phage cocktail UPWr_E124, is comparable to the effect of other phages infecting *E. coli*. A similar reduction was recorded for phage FP43 at a titer of 10^9 PFU/ml after 24-hour treatment of mixed biofilms formed by *E. coli* O157:H7 and O91:H- strains on lettuce leaves by ~2.2 log10 CFU/cm2 (Zhang et al., 2022). The reduction of ~2.0 log CFU/cm2 was also observed for an *E. coli* O157:H7 population on lettuce treated with a phage cocktail (Sharma et al., 2009). Similarly, phage P762 decreased the number of *E. coli* serotypes O157 (EC201) and O145 (EC623) on lettuce leaves by 99.96 % and 99.99 %, respectively (Kong et al., 2022). In the study described by Litt and colleagues, phages P14-O111, P8-O121, and J29-O145 lowered STEC populations by 1.3–3.1 log10 CFU/cm2 on leafy greens on day 0, which is comparable to the results for UPWr_E monophages and UPWr_E124 phage cocktail. Still, the same authors presented evidence of phages exhibiting greater anti-biofilm potential against *E. coli*. Phages P9-O45, P13-O26, and P19-O103 reduced STEC populations to undetectable levels at day 0 of treatment (Litt et al., 2020). The authors suggested that phages exhibiting a narrow host range are more efficient in destroying biofilm.

Biofilms serve as APEC reservoirs and allow for its persistence in harsh conditions. Development of strategies to reduce biofilms are required, and bacteriophages are considered a promising option for biofilm control (Meneses et al., 2023). In this study, we performed characterization and analysis of the functional properties of APEC-targeting phages, demonstrating their lytic features and their biofilm activity in three different surfaces commonly found in the poultry industry. The results suggested that the phages UPWr_E1, E2 and E4, and the three-phage cocktail UPWr_E124 can be an effective tool in bacterial control applications against APEC and its biofilm.

6. Conclusions

In this study, we isolated and characterized three novel bacteriophages, UPWr_E1, UPWr_E2 and UPWr_E4, infecting the etiological factor of avian colibacillosis, APEC. These phages and their multiphage formulation effectively reduced biofilm formed by *E. coli* on biotic and abiotic structures. Therefore, because of their ability to infect various APEC strains, lytic life cycle and resistance to different pH and temperatures, they can be considered as useful tools in the biological control of colibacillosis.

Authors' contributions

PŚ isolated the phages and their DNA, performed phage characterization, drafted the figures and performed the statistical analysis; DM performed bioinformatic analysis and was a major contributor to writing the manuscript, PK performed assays on biofilms; AM performed EOP and calculations; MK provided APEC strains; RK performed phage genome assembly and was a contributor to writing the manuscript; SK (student) assisted with the research; MN performed transmission electron microscopy; UR provided APEC strains; LT-H verified the analytical methods; MK-B conceived and supervised the studies, had substantial inputs into the analysis and all drafts, obtained funding, and was a contributor to writing the manuscript. All authors contributed to the article and approved the submitted version.

CRediT authorship contribution statement

Rafał Kolenda: Methodology, Conceptualization. **Maciej Kuczkowski:** Resources. **Agata Milcarz:** Methodology. **Paweł Korzeniowski:** Methodology. **David Sáez Moreno:** Writing – original draft, Conceptualization. **Paulina Śliwka:** Visualization, Software, Methodology, Conceptualization. **Marta Kuźmińska-Bajor:** Writing – review & editing, Validation, Supervision, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Ludwika Tomaszewska-Hetman:** Methodology. **Uwe Roesler:** Resources. **Magdalena Narajczyk:** Methodology. **Sylwia Kozioł:** Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2024.110363.

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