



Application of UPWr_E124 phage cocktail for effective reduction of avian pathogenic *Escherichia coli* in mice and broiler chickens

David Sáez Moreno^a, Maciej Kuczkowski^b, Paweł Korzeniowski^c, Krzysztof Grzymajło^d, Anna Woźniak-Biel^b, Paulina Śliwka^c, Anita Rywińska^c, Marta Kuźmińska-Bajor^{c,*}

^a Centre of Biological Engineering, University of Minho, Braga 4710-057, Portugal

^b Department of Epizootiology and Clinic of Birds and Exotic Animals, Faculty of Veterinary Medicine, Wrocław University of Environmental and Life Sciences, Grunwaldzki Square 45, Wrocław 50-366, Poland

^c Department of Biotechnology and Food Microbiology, Faculty of Biotechnology and Food Sciences, Wrocław University of Environmental and Life Sciences, 37 Chelmońskiego St., Wrocław 51-630, Poland

^d Department of Biochemistry and Molecular Biology, Faculty of Veterinary Medicine, Wrocław University of Environmental and Life Sciences, 31 Norwida St., Wrocław, Poland

ARTICLE INFO

Keywords:

Bacteriophages
APEC
Phage therapy
Colibacillosis
Broilers

ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) is the main causative agent of colibacillosis, causing poultry respiratory infections, mortality and economic loss. APEC poses a serious threat to public health and food safety due to its multi-drug resistance and capacity to form biofilms. Bacteriophages (phages) have emerged as an alternative to antibiotics. To cure APEC-infected chickens, a cocktail consisting of UPWr_E1, UPWr_E2 and UPWr_E4 APEC-specific phages was developed and tested. In this study, we documented the maintenance of their activity in neutralized simulated gastric fluid (SGF) conditions and the efficiency of the UPWr_E124 phage cocktail in inhibiting APEC in biofilm structures on chicken breast meat surfaces. Further, we evaluated the efficacy of the UPWr_E124 phage cocktail against APEC *in vivo* in murine and chicken infection models. In experimentally infected mice, we evaluated the intraperitoneal and gastric gavage administrations of phages. The study revealed that gastric administration of phages reduced bacterial levels in the respiratory system. Moreover, we tested the UPWr_E124 phage cocktail in a chicken model of infection, where phages effectively reduced the number of APEC in the lungs, bursa of Fabricius and blood. These results suggest that the UPWr_E124 phage cocktail could be a potential treatment for colibacillosis in the poultry industry.

1. Introduction

One of the biggest challenges of our world today is to address the demand for animal protein without compromising animal welfare. According to an OECD-FAO report poultry meat is the most consumed meat worldwide and its consumption has increased substantially over the last decades (OECD, 2024). Market demands influence intensive and large-scale poultry farming. One of the main challenges in poultry production is diseases caused by bacterial pathogens. Avian colibacillosis, caused by avian pathogenic *Escherichia coli* (APEC), is considered to be the most common infectious bacterial disease that affects intensive poultry production processes (Fancher et al., 2021). APEC infection may occur in broiler chickens, turkeys, and egg-laying hens (Ewers et al., 2004; Kromann et al., 2024). In the broiler chickens, APEC infections are

considered to typically lead to colibacillosis; a syndrome that includes respiratory tract infection, airsacculitis, pericarditis, perihepatitis, splenomegaly, and swollen head syndrome. In mature laying hens, reproductive tract infection leading to salpingitis or salpingo-peritonitis syndrome is common (Ewers et al., 2004; Kromann et al., 2024; Collingwood et al., 2014). In all sectors, APEC infection is syndromic in nature, and ultimately, leads to reduced egg production and increased mortality rates in broilers and should be treated. Antibiotics are commonly used to treat poultry flocks affected with colibacillosis.

APEC has been identified to possess various virulence factors and resistance genes involved in pathogenesis and drug resistance (Xu et al., 2019; Oliveira et al., 2009a; Morrow, 2024), which contribute to its pathogenicity and make treatment challenging. Moreover, APEC is closely related to human pathogens collectively known as extraintestinal

* Corresponding author.

E-mail address: marta.kuzminska-bajor@upwr.edu.pl (M. Kuźmińska-Bajor).

<https://doi.org/10.1016/j.vetmic.2025.110398>

Received 12 June 2024; Received in revised form 15 January 2025; Accepted 18 January 2025

Available online 21 January 2025

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pathogenic *Escherichia coli* (ExPEC) (Hu et al., 2022). ExPEC has evolved mechanisms to cause infections beyond the gut, leading to a range of syndromes from mild gastroenteritis to severe extraintestinal infections including meningitis caused by newborn meningitis *E. coli* (NMEC), urinary tract infections affected by uropathogenic *E. coli* (UPEC), and sepsis originated by septicemic *E. coli* (SEPEC). Thus, APEC carries a potential risk of horizontal gene transfer and might be a reservoir of virulence genes and antibiotic-resistant genes for human ExPEC strains (Nawaz et al., 2024). One of the enablers of APEC introduced to the human food chain is contaminated food of poultry origin. Raw meat can serve as an abundant reservoir for APEC, leading to cross-contamination of other foods, surfaces, and utensils in the kitchen. The persistence of APEC in the food chain is further enabled due to its capacity to form biofilms on different surfaces such as chicken breast fillets (Anang et al., 2010; Possas et al., 2021). In fact, biofilms play a crucial role in the persistence and survival of APEC in adverse conditions due to its complex structure, containing a community of microorganisms adhering to surfaces, surrounded by a matrix of extracellular polymeric substances which they produce (Kromann et al., 2024). These biofilms provide protection and resilience to bacteria, allowing them to withstand various environmental stresses, including nutrient limitations and desiccation (Bridier et al., 2015; Sharma et al., 2016; Giaouris et al., 2014). This is an extra challenge for effective APEC treatment and contributes to its persistence on meat surfaces (Sharma et al., 2016; Goudartzalejerdi et al., 2022; Chen et al., 2010).

Thus, novel approaches for preventing and treating APEC and their biofilms, such as the development of vaccines or antimicrobial coatings, are under development (Soleymani et al., 2020). Among strategies against APEC strains, bacteriophages (phages) have emerged as a potential natural tool for biocontrol (Nicolas et al., 2023). Phages are viruses that can specifically target and kill bacteria and have demonstrated efficacy in the degradation of APEC in biofilm structures (Yao et al., 2023; Tang et al., 2023) and in preventing and treating APEC infections in broiler chicken (Oliveira et al., 2010; Jhandai et al., 2024; Sorour et al., 2020). Overall, phages represent an approach to combating APEC infections in poultry as part of the broader One Health strategy aimed at promoting animal health, reducing antimicrobial use, and safeguarding public health (Sinclair, 2019).

In the current study, we evaluated the potential of the bacteriophage cocktail UPWr_E124 for the treatment of APEC in an artificially contaminated chicken breast and in two different infection models *in vivo*. We compared intraperitoneal and gastric routes of administration in a murine model to examine the mode of action of the UPWr_E124 phage cocktail. Further, to evaluate the efficacy of the UPWr_E124 phage cocktail's antibacterial activity against APEC, we utilized a chicken infection model.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Escherichia coli strains 158B lux and NCTC 17848 were used in this study. The *E. coli* strain NCTC 17848 possesses a great ability to form biofilms and was used in anti-biofilm assays. *E. coli* strain 158B lux was obtained from the Strain Collection of the Department of Epizootiology and Clinic of Bird and Exotic Animals, Wrocław University of Environmental and Life Sciences. This strain was isolated from diseased chicken with colibacillosis, classified as avian pathogenic *E. coli* (APEC) and genetically modified as previously described by Riedel et al. (2007). Briefly, *E. coli* strain 158B was modified with the chromosomal integration vector *p16Slux* containing the *lux* operon from *Photobacterium luminescens* consisting of five genes, *luxCDABE* and the erythromycin resistance gene. *E. coli* 158B lux exhibits the capacity for bioluminescence, allowing it to be distinguished from commensal *E. coli*. These modifications in Gram-negative bacteria did not affect invasiveness in mice and chickens (Kuzmińska-Bajor et al., 2023). *E. coli* strains used in

this study were stored with 20 % (v/v) glycerol at -80°C . For propagation, strains were cultivated in Luria-Bertani (LB) broth (Sigma-Aldrich, Germany), under aerobic conditions at 37°C overnight with shaking at 150 rpm. *E. coli* 158B lux culture was supplemented with erythromycin (0.2 mg/ml).

2.2. Bacteriophages

The bacteriophages used in this study, UPWr_E1, UPWr_E2 and UPWr_E4, were isolated from samples of urban sewage samples from the Wrocław wastewater treatment plant and are described elsewhere (Śliwka et al., 2025). Taxonomically, UPWr_E1 belongs to *Krischvirus* within the *Straboviridae* family and UPWr_E2 and UPWr_E4 belong to the genus *Tequatrovirus* within the *Tevenvirinae* family. *In vitro* tests revealed lysis effectiveness rates of 64 % for UPWr_E1 and UPWr_S4 and 46 % for UPWr_E2 phages on 142 APEC strains. *In silico* analysis showed that their genomes are deprived of any known virulence, toxin, or pathogen-associated protein family or gene products of *E. coli* strains or any other pathogens. The complete genome sequences of phages UPWr_E1, UPWr_E2 and UPWr_S4 were deposited in GenBank under the accession numbers PP418985, PP418986, PP418987, respectively. Due to the characteristics of these phages, including their anti-APEC activity, they were considered a promising tool in combating APEC and hence were chosen to conduct *in vivo* phage efficacy experiments in mice and chickens.

2.3. Bacteriophage propagation and cocktail preparation

The amplification of UPWr_E1, UPWr_E2 and UPWr_E4 phages was performed on the APEC 158B lux host strain. Phages were amplified using a method described elsewhere (Kuzmińska-Bajor et al., 2021). Briefly, bacterial cultures were inoculated on 10 ml of LB broth with a single colony of APEC 158B lux, followed by overnight incubation at 37°C with shaking at 150 rpm. After incubation, 0.5 ml of overnight culture was inoculated into 10 ml of LB broth and incubated until the optical density ($\text{OD}_{600\text{ nm}}$) reached 0.2. In the next step, the bacterial culture was centrifuged for 10 min at $5000 \times g$ to remove any remaining cell debris and filtered through $0.22\text{ }\mu\text{m}$ pore size syringe filters. The 5 ml of resulting phage lysate, from the first step of propagation, was added to 150 ml of host culture ($\text{OD}_{600\text{ nm}} = 0.2$) and incubated overnight at 37°C . As a last step, the centrifugation and filtration steps were repeated. Bacteriophage titer was determined using the routine test dilution method (Adams, 1959). As a phage mixture, cocktail UPWr_E124 containing phages UPWr_E1, UPWr_E2, and UPWr_E4 was formulated by combining them in an equal ratio of 1:1:1.

2.4. UPWr_E124 survival in simulated gastric fluid conditions

In order to determine the stability of bacteriophages in conditions imitating chicken gastric juice, simulated gastric fluid (SGF) was used to test phage sensitivity to intragastric conditions according to a method described previously (Tang et al., 2013). The experiment was performed in SGF at pH 2 representing natural low pH stomach acidity and SGF neutralized by the addition of 14 % CaCO_3 as an antacid reflecting the neutralizing effect of the feed presence in the stomach. One milliliter of the 10^8 plaque-forming units (PFU) per 1 ml of UPWr_E124 phage cocktail was added to 9 ml of pre-warmed SGF and incubated at 42°C , representing the chicken's body temperature, for 2 h. Samples of 100 μl were taken every 15 minutes to measure the survival rate of phages. To measure the phage titer, samples were tenfold serially diluted in LB medium and spotted on the lawn of APEC 158B lux to determine the number of bacteriophages using the double-layer plate method. The plates were incubated at 37°C overnight and observed for the formation of plaques. This experiment was carried out in triplicate.

2.5. Biofilm-forming ability

To determine the ability of the APEC 158B strain to form biofilms, a method described elsewhere (Korzeniowski et al., 2022) was used and *E. coli* strain NCTC 17848 exhibiting high biofilm-forming capability was used as a control. For this purpose, overnight APEC 158B and NCTC 17848 cultures were diluted in LB broth to OD 0.2 (~2 × 10⁸ colony-forming units (CFU/ml) and 200 µl of this suspension was transferred to each well of 96-well polystyrene microtiter plates (Sarstedt, Germany). Plates were then 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). Cells growing in biofilms were quantified by staining with 0.5 % crystal violet (Merck, Germany) for 20 min followed by rinsing two times with PBS. Crystal violet 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.

2.6. Biofilm formation on chicken breast

The ability of APEC 158B lux and *E. coli* NCTC 17848 bacteria to form a biofilm on the surface of poultry meat was determined according to Vikram et al. 2020 with minor modifications. For this purpose, the chicken breast was sterilized with ultraviolet light, being turned twice during the sterilization and tested for sterility following the same procedure described below except for the immersion in bacterial cultures. Next, the chicken breast was cut into pieces measuring 1 cm³ with a sterile scalpel. Pieces of chicken breast were immersed for 120 seconds in 5 ml of the overnight cultures of APEC 158B lux and *E. coli* NCTC 17848, previously diluted with LB medium to obtain an optical density of 0.2 (~2 × 10⁸ CFU/ml) measured at a wavelength of 600 nm (OD_{600 nm}) in a 50 ml Falcon tube. Then, pieces of chicken breast were dried at room temperature for 20 minutes following immersion for 2 minutes in a suspension of UPWr_E124 phage cocktail with a titer of 1 × 10⁹ PFU/ml and dried for 20 minutes at room temperature. Pieces of meat were placed in a Petri dish containing sterile paper soaked in PBS buffer to maintain high humidity, protected with parafilm and incubated at 4°C until the 7th day after inoculation and placed at 37°C for 24 hours providing favorable conditions for bacterial growth. After inoculation, on days 1, 2, 5, 7 during incubation at 4°C and on day 8 after shifting to growth-permitting conditions, 4 pieces of chicken breast were placed in Falcon tubes containing 2 ml of PBS buffer and homogenized using a Qiagen TissueLyser II. After homogenization, the number of *E. coli* was determined by plating homogenates in tenfold serial dilutions on LB agar. Plates were incubated at 37°C overnight and the number of colonies was counted. The results are presented as the number of CFU per 1 cm³ of meat.

2.7. In vivo antibacterial activity assay of phage cocktail UPWr_E124 in the mouse model

The potential for reduction of APEC-targeting phage cocktail UPWr_E124 was examined in BALB/c female mice (Mossakowski Medical Research Centre Polish Academy of Sciences, Warsaw, Poland) experimentally infected with APEC 158B lux. All experimental work applying the mouse model of infection was approved by the Local Ethical Committee for Animal Experimentation (protocol code 115/2015; Wrocław, Poland) and performed according to the legal requirements. Mice at age 6–8 weeks were divided into 7 experimental groups, as shown in Table 1, and 10 mice were assigned to each group. In this study, 2 ways of APEC and phage cocktail application such as gastric gavage directly into the stomach with the gavage needle and intraperitoneal injection were examined. Mice in positive controls were challenged with APEC 158B lux in 100 µl of phosphate buffer saline (PBS) at doses of 10⁹ and 10⁷ CFU/mouse by gastric (group 1) and

Table 1

Groups of mice receiving UPWr_E124phage cocktail after being challenged with APEC 158B lux.

Group (n = 10)	Doses		Gavage
	UPWr_E124 [PFU]	APEC 158B lux [CFU]	
1 ^a	-	1 × 10 ⁹	gastric
2 ^b	1 × 10 ⁹	-	gastric
3 ^c	1 × 10 ⁹	1 × 10 ⁹	gastric
4 ^d	-	1 × 10 ⁹	intraperitoneal
5 ^e	1 × 10 ⁷	-	intraperitoneal
6 ^f	1 × 10 ⁷	1 × 10 ⁹	intraperitoneal
7 ^g	-	-	-

- ^a Positive control, gastrically infected with *E. coli* 158B lux
- ^b Negative control, gastrically bacteriophage-treated
- ^c Group gastrically infected with APEC 158B lux and treated with UPWr_E124 phage cocktail by gastric gavage
- ^d Positive control, infected with *E. coli* 158B lux by intraperitoneal injection
- ^e Negative control, bacteriophage-treated by intraperitoneal injection
- ^f Group infected and treated by intraperitoneal injection with APEC 158B lux with UPWr_E124 phage cocktail
- ^g Untreated group

intraperitoneal (group 4) gavage, respectively. Two groups were treated with the UPWr_E124 phage cocktail at a dose of 10⁹ PFU in 100 µl of PBS per animal by gastric (group 2) and intraperitoneal (group 5) gavage. Both groups 2 and 5 were negative controls without the bacteria. Mice in group 3 were infected with APEC 158B lux at a dose of 10⁹ CFU/mouse by gastric gavage and immediately gastrically treated with 10⁹ PFU of the UPWr_E124 phage cocktail. Similarly, mice in group 6 were infected by intraperitoneal injection with 10⁷ CFU of APEC 158B lux and 10⁹ PFU of the UPWr_E124 phage cocktail per mouse. Group 7 did not receive either APEC 158B lux or the UPWr_E124 phage cocktail. The summary of the challenge and treatment for each group is shown in Table 1. After 24 hours, all animals were weighed and humanely killed by cervical dislocation, and all test animals were subjected to gross necropsy and dissected. Their lungs, hearts, kidneys, livers, and spleens were analyzed for the presence of APEC 158B lux and phages. For bacterial number determination, the organs were homogenized in cold PBS, properly tenfold diluted, and plated on LB agar with 0.2 mg/ml erythromycin, and plates were incubated overnight at 37°C. The bioluminescent signals of APEC 158B lux CFU were counted using the NightOWL II LB 983 In Vivo imaging System (BERTHOLD TECHNOLOGIES, Germany). For phage enumeration tissue homogenates were centrifuged at 4 000 g to remove solid particles and then supernatants were filtered through 0.22 µm filters. The phage titration was estimated using a double agar layer plaque assay method. The mean CFU and PFU per 1 g of each tissue were calculated.

2.8. Bacteriophage therapy trials in the experimental chicken model

For the *in vivo* experiments on chickens, 7-day-old Ross 308 broilers obtained from a local farm were divided into four groups (10 birds/group) and housed separately in wire cages at an ambient temperature of 30°C. Sterile chicken feed (Broiler Grower II, Tasomix, Poland) and water were provided *ad libitum*. All experimental work involving birds was approved by the Local Ethics Committee for Animal Experimentation (protocol code 114/2015; Wrocław, Poland). Group 1 was a positive control for APEC 158B lux infection and birds were challenged intratracheally with 10⁸ CFU of APEC 158B lux in 1 ml of PBS per bird. Group 2 received only the UPWr_E124 phage cocktail at 1 and 5 days of the experiment. Group 3 was infected with 10⁸ CFU/ml of APEC 158B lux per bird and immediately inoculated with 10⁹ PFU of UPWr_E124 phage cocktail. At 5 days after infection birds from group 3 were treated with the second dose of UPWr_E124 phage cocktail. Group 4 was kept as an uninfected control (Table 2). On the 10th day after infection, all birds

Table 2
Chicken experimental design. UPWr_E124 phage cocktail and APEC 158B lux were administered directly to the crop.

Group (N = 10)	Doses		Treatment schedule of bacteriophages [dpi] ^e
	UPWr_E124	APEC 158B lux	
1 ^a	-	2 ml 1 × 10 ⁸ CFU/ml	-
2 ^b	1 ml 10 ⁹ PFU/ml	-	1, 5
3 ^c	2 ml 3 × 10 ¹⁰ PFU/ml	2 ml 1 × 10 ⁸ CFU/ml	1, 5
4 ^d	-	-	-

^a Positive control, infected with APEC 158B lux
^b Group infected with APEC 158B lux and bacteriophage treated
^c Negative control, bacteriophage treated
^d Untreated group
^e dpi – days post-infection

were necropsied and APEC 158B lux and bacteriophages in the internal tissues of every bird were counted. The lungs, spleen, liver, and bursa of Fabricius were removed aseptically and blood samples were taken. The bacterial and phage loads were assessed by estimating the number of CFU and PFU, respectively. The cecal tonsils and cecal contents were weighed and homogenized in 0.2 ml of PBS. The bursa of Fabricius, spleen, and liver were weighed after washing with PBS, and each organ was homogenized with 5, 5 and 50 ml of cold PBS, respectively. Ten-fold dilutions of homogenates were plated onto LB agar containing erythromycin (0.2 mg/ml) and plates were incubated overnight at 37°C. Identification of APEC 158B lux was carried out by bioluminescence imaging and luminescent colonies were counted using bioluminescence imaging. The titer of the UPWr_E124 phage cocktail was determined by counting plaques formed on the appropriate bacterial strain. The mean CFU and PFU per 1 g of each tissue and per 1 ml of blood were calculated.

2.9. Statistical analysis

All statistical tests were performed on log₁₀ transformed data using STATISTICA version 13 software (TIBCO Software Inc.). Student's *t*-test was employed for statistical analysis of data obtained from *in vitro* assay in simulated gastric juice. Data obtained from the chicken breast meat biofilm assay were analyzed by applying ANOVA along with the least significant difference (LSD) post-hoc test. Colonization of internal organs in both mouse and chicken models was analyzed using the Mann–Whitney *U* test with the non-Gaussian distribution. A *p*-value of < 0.05 was considered to be significant. All data were analyzed using two-tailed tests. To compare bacterial load (CFU) and phage load (PFU) recovered from chickens' internal organs the nonparametric Mann–Whitney *U*-test was employed.

3. Results

3.1. Degradation of *E. coli* biofilm formed on chicken meat

The effect of the UPWr_E124 phage cocktail in the degradation of biofilm formed on chicken breast was tested using the bacterial strains APEC 158B lux and *E. coli* NCTC 17848. These strains were classified as moderate and strong biofilm producers, respectively, according to the classification suggested by Stepanovic et al. (2004). *E. coli* NCTC 17848 was found to have approximately 2.8 times greater ability to form a biofilm than APEC 158B lux (Supplementary Material 1).

As depicted in Fig. 1, after treatment of the biofilms with the UPWr_E124 phage cocktail, the viability of both strains was significantly reduced relative to untreated controls (*p* < 0.01) at 1, 2, 5, 7 and 8 days. The untreated biofilm formed by APEC 158B lux was stable from day 1–7 and was estimated at around 5.1 and 5.2 log₁₀ CFU/cm³, respectively (Fig. 1). After treatment from the first day to the seventh day, the UPWr_E124 phage cocktail reduced the number of APEC 158B lux by 1.0–1.4 log₁₀ CFU/cm³. On the eighth day, the pieces of chicken breast were incubated at 37°C to allow APEC re-growth. On that day, untreated biofilm biomass increased to 5.9 log₁₀ CFU/cm³, while the phage-treated biofilm biomass was maintained at 4.2 log₁₀ CFU/cm³.

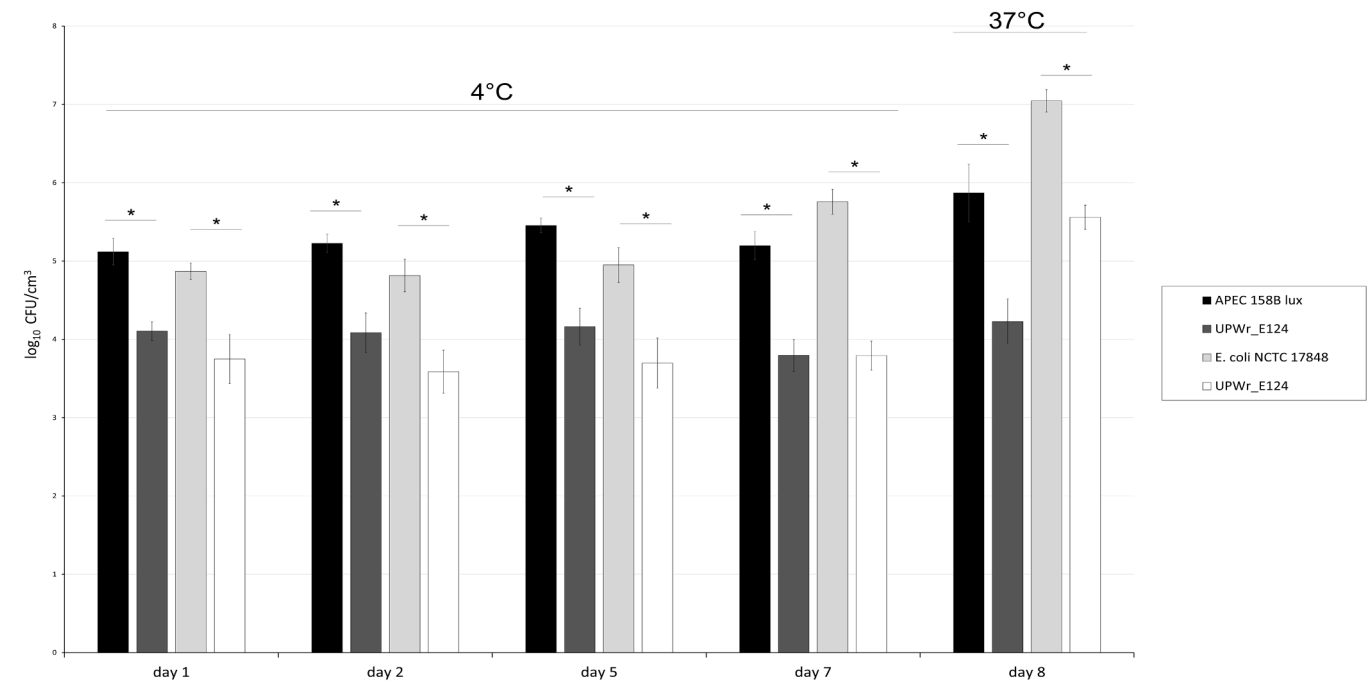


Fig. 1. Effect of UPWr_E124 phage cocktail on biofilm on chicken breast meat. Effect of UPWr_E124 phage cocktail on reduction of biofilm formed by APEC 158B, and effect of UPWr_E124 phage cocktail on reduction of biofilm formed by *E. coli* NCTC 17848. The breast meat was stored at 4°C for 7 days and then at 37°C for 24 hours. * represents *p* < 0.01 and indicates a significant difference. Values represent the mean with a standard deviation of three replicates.

($p < 0.01$). In the absence of the UPWr_E124 phage cocktail, the number of viable *E. coli* NCTC 17848 was stable for 5 days and was estimated to be between 4.9 and 5.0 $\log_{10}$ CFU/cm³ (Fig. 1). After 7 days of storage at 4°C the number of *E. coli* NCTC 17848 increased to 5.8 $\log_{10}$ CFU/cm³. In phage-treated *E. coli* NCTC 17848 biofilm, the number of bacteria was effectively maintained at a lower level between 3.7 (day 1) and 3.8 (day 7) $\log_{10}$ CFU/cm³ in comparison to untreated biofilm. On the eighth day, when pieces of untreated chicken breast were transferred to 37°C, the number of untreated bacteria rapidly increased by 1.8 $\log_{10}$ CFU/cm³. Under the same conditions, the number of *E. coli* NCTC 17848 on meat treated with the UPWr_E124 phage cocktail was significantly lower than that observed in the untreated control, with a difference of 1.3 $\log_{10}$ CFU/cm³. Sterility tests of chicken breast confirmed the sterility of the assay and the sterilization process. No bacterial growth was detected in chicken breasts that were not inoculated with bacteria.

3.2. Assessment of UPWr_E124 phage cocktail survival in the SGF model

The UPWr_E124 phage cocktail's ability to endure intragastric conditions was tested *in vitro* with and without the addition of an antacid, at 42°C, corresponding to the chicken's deep body temperature. The phages were affected by the low pH and the pepsin activity of SGF with the reduction of 3.6 $\log_{10}$ CFU after 15 minutes of incubation and were completely inactivated following 30 min of incubation (Supplementary material 2). However, the addition of the antacid 14 % CaCO₃ resulted in complete neutralization of the inactivation effect of SGF conditions on phages, which were stable over the 120 minutes tested ($p < 0.01$).

3.3. UPWr_E124 efficacy in the mouse model

To evaluate the *in vivo* effectiveness of the UPWr_E124 phage cocktail, a mouse model infected with APEC by gastric gavage and intraperitoneal injection was used. Regardless of the route of administration, for all euthanized mice infected with APEC 158B lux, no clinical symptoms of bacterial infection were detected. According to all the results presented in Fig. 2, in comparison to gastric gavage (groups 1 and 2), intraperitoneal injection of APEC 158B lux (groups 3 and 4) resulted in a significantly higher number of colonized organs such as lungs, heart, kidney, liver and spleen, accompanied by the large increased bacterial load of all tested organs ($p > 0.05$).

Using gastric gavage for the administration of the UPWr_E124 phage cocktail, we found a significant reduction in the colonization of lungs by

APEC in mice that received the UPWr_E124 phage cocktail (group 3) (Fig. 2A) compared to the untreated group 1. It also resulted in a reduction of the number of organs colonized by APEC 158B lux including a decreased mean number of bacteria isolated from organs such as the heart, kidney, liver and spleen by 0.6–1.1 $\log_{10}$ CFU/g. However, these differences were not statistically significant ($p > 0.05$). The highest organ colonization was recorded for spleens and the lowest in livers, as depicted in Fig. 2B.

Administering the UPWr_E124 phage cocktail intraperitoneally, the bacterial burden in all tested organs showed comparable levels of APEC 158B lux load and no statistically significant difference was detected in treated or untreated mice ($p > 0.05$) (Fig. 2B).

Phages composing the UPWr_E124 phage cocktail were detected only in organs taken from mice infected and phage-treated intraperitoneally (group 2) and the PFU were estimated to be $1.9 \pm 1.36 \log_{10}$ PFU in lungs, $1.8 \pm 1.67 \log_{10}$ PFU in hearts, $3.6 \pm 0.24 \log_{10}$ PFU in kidneys, $2.3 \pm 0.45 \log_{10}$ CFU in spleens and $48.1 \pm 1.97 \log_{10}$ CFU in livers with 4, 1, 4, 2 and 10 phage-positive organs, respectively.

3.4. UPWr_E124 phage cocktail effectiveness in experimental chicken model

To evaluate the effectiveness of the UPWr_E124 phage cocktail in poultry we used broiler chickens experimentally infected with APEC 158B lux. We examined the APEC 158B lux load in the lungs, liver and lymphoid organs such as the spleen, the bursa of Fabricius, and in the blood at 10 days after infection. In the phage-treated group, a significant reduction in APEC number was detected in the liver, bursa of Fabricius and blood ($p < 0.05$) when compared with untreated birds (Fig. 3A). The APEC numbers in the lungs and spleen of phage-treated chickens were lower by 3.04 and 2.88 $\log_{10}$ CFU/g, respectively, in comparison to the untreated group, but this difference was not statistically significant ($p > 0.05$).

In comparison to chickens from the uninfected group receiving only phages, the number of phages isolated from birds from the phage-treated group was significantly higher in the liver ($p < 0.05$), but not in the other organs ($p > 0.05$). In blood samples, we also detected the phage titer in samples taken from birds from the APEC-infected group and uninfected group receiving the UPWr_E124 phage cocktail, with mean values of 1.06 and 0.42 $\log_{10}$ PFU/g, respectively, but these differences were not statistically significant ($p > 0.05$) (Fig. 3B). Internal organ samples of control birds from uninfected and untreated groups were

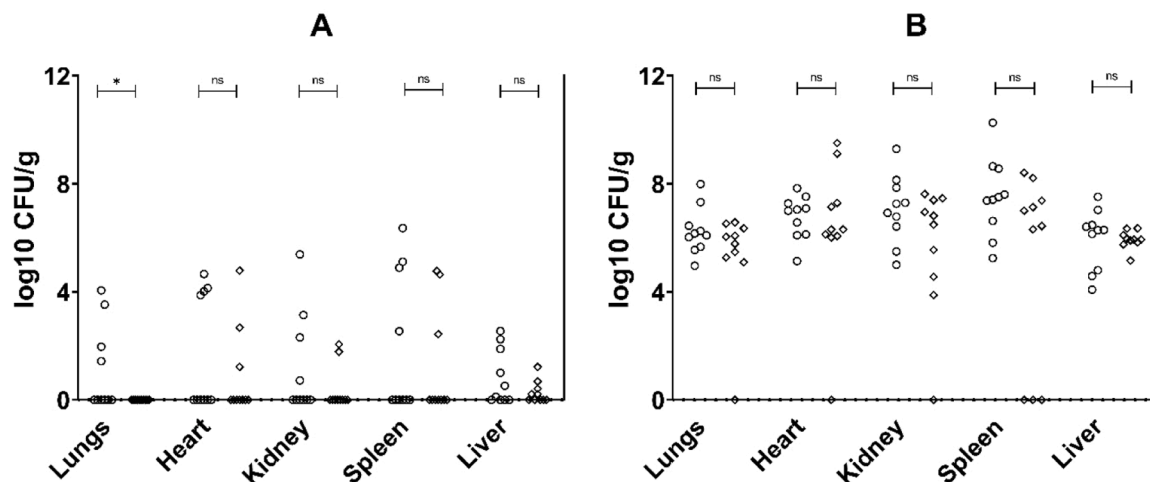


Fig. 2. Effect of phage cocktail UPWr_E124 on APEC 158B lux number in internal organs in an experimental murine model. Bacterial loads are shown as counts for individual animals treated by gastric gavage plus the mean ($n = 10$ per group) of control group 1 (circles) infected with APEC 158B lux and group 2 (diamonds) infected with APEC 158B lux treated with phage cocktail UPWr_E124 (A). Bacterial loads are shown as counts for individual animals treated by intraperitoneal injection plus the mean ($n = 10$ per group) for control group 3 (circles) treated with phage cocktail UPWr_E124 and group 4 (diamonds) infected with APEC 158B lux treated with phage cocktail UPWr_E124 (B). * represents $p < 0.05$ and indicates a significant difference between groups, n.s., not significant.

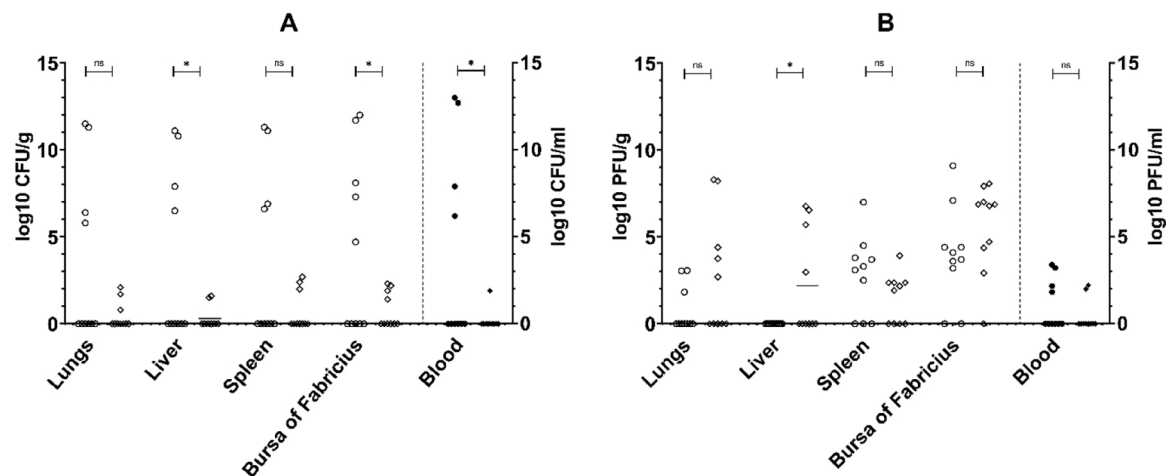


Fig. 3. Effect of phage cocktail UPWr_E124 on APEC 158B lux number in internal organs in an experimental chicken model. Results for bacterial load are shown as counts for individual animals plus the mean ($n = 10$ per group) of control group 1 in organs (empty circles) and blood (black circles) infected with APEC 158B lux and group 3 in organs (empty diamonds) and blood (black diamonds) infected with APEC 158B lux treated with phage cocktail UPWr_E124 (A). Results for phage load are shown as counts for individual animals plus the mean ($n = 10$ per group) for organs (empty circles) and blood (black circles) of control group 2 treated with phage cocktail UPWr_E124 and organs (empty diamonds) and blood (black diamonds) of group 3 infected with APEC 158B lux treated with phage cocktail UPWr_E124 (B). * represents $p < 0.05$ and indicates a significant difference between groups, n.s., not significant.

negative for both bacteriophages and APEC, indicating that no contamination occurred during the experimental set-up.

4. Discussion

Addressing the economic and public health impacts of APEC infections and their presence on poultry products requires a comprehensive approach that integrates preventive measures, surveillance, and targeted interventions such as the use of phages as a species-specific naturally existing antibacterial tool (Oliveira et al., 2010; Huff et al., 2004; Mosimann et al., 2021). In this study, we first investigated the ability of the UPWr_E124 phage cocktail to inhibit APEC growing in biofilm structures on the surface of chicken breast meat. We observed a significant reduction in the level of live APEC using the UPWr_E124 phage cocktail. This phage cocktail not only decreased the number of APEC 158B lux after phage application but also prevented further growth of bacteria, both in refrigerated conditions and growth-favoring temperatures. The UPWr_E124 phage cocktail exhibited higher effectiveness in the reduction of *E. coli* on the meat surface in comparison to the commercial EcoShield PX phage cocktail against the *E. coli* O157:H7 strain present on the surface of the chicken fillet by 0.7 log₁₀ CFU (Vikram et al., 2020). UPWr_E124 reduction is comparable to phages PBL66-CL1 and PBL116-CS6, which reduced the number of *E. coli* strain EBL116 on the surface of poultry meat by 2.02 and 1.67 log₁₀ CFU/4 cm² of meat pieces after 6 h at 25°C and 5°C, respectively (Hoang Minh et al., 2016). Importantly, there are results indicating that anti-*E. coli* phages effectively reduce the bacteria on meat. Phage CEH-162 was used against *E. coli* EH-162 on the surface of minced chicken meat, demonstrating no inhibition of bacterial growth at 4°C for 7 hours (Jassim et al., 2012). These results could be explained by the specific structure of minced meat that could favor the growth of bacteria, or by the lower phage effectiveness. In summary, we demonstrated the potential of the UPWr_E124 phage cocktail and the use of lytic phages as an effective bactericide for *E. coli* controlling on the surface of raw chicken meat.

For successful phage therapy treatment, phages need to reach bacteria at the infection site and establish a productive infection in sufficient numbers to reduce bacterial load. While many *in vivo* phage studies have shown great anti-APEC potential (Oliveira et al., 2010, 2009b; Huff et al., 2003), there have been some failed attempts (Tsonos et al., 2014) that highlight the importance of proper selection of APEC-targeting

phages that effectively combat these bacteria *in vivo*. Thus, it is extremely important to complete *in vitro* results with *in vivo* data.

In vivo, phages should withstand the proteolytic environment and low pH of the gastric juice (Colom et al., 2017). However, phages in the UPWr_E124 cocktail completely lose their ability to infect APEC 158B lux after incubation in simulated gastric conditions. Similar results were obtained by other phages recovered after exposure to SGF (Kuzmińska-Bajor et al., 2023; Mhone et al., 2022). To prevent their inactivation, we added calcium carbonate, which resulted in a protective effect on phage activity, confirming other studies (Koo et al., 2000; Yongsheng et al., 2008). In fact, we have previously reported that the presence of chicken feed in SGF had a comparable neutralizing effect on phage survivability to calcium carbonate (Kuzmińska-Bajor et al., 2023), suggesting that the presence of feed in the stomach reflecting natural conditions allows the maintenance of the UPWr_E124 phage cocktail's activity when administered orally.

To unequivocally establish the safety and suitability of using APEC-targeting phages in poultry-intensive production, comprehensive and multifaceted studies are essential. To assess the effectiveness of the phage cocktail *in vivo*, we sought to define the effectiveness of the method of administration performing *in vivo* studies on mice experimentally infected with APEC 158B lux and treated with the UPWr_E124 phage cocktail by intraperitoneal injection and by gastric gavage. The most important finding is that the UPWr_E124 phage cocktail administered by gastric gavage completely eradicated APEC 158B lux from the lungs, an APEC-targeted organ in colibacillosis. These results support our assumption regarding the ability of phages to overcome harsh gastric conditions. In organs such as the heart, spleen, liver, and kidney the impact of the UPWr_E 124 phage cocktail on bacterial load was insignificant in both models of phage administration tested. Mice infected with APEC 158B lux showed no clinical signs of infection regardless of the route of administration of the bacteria, with the bacterial load of internal organs estimated to be significantly higher for intraperitoneal infection compared to gastric gavage. The colonization that we found in internal organs of mice after intraperitoneal APEC administration is comparable to that found by Wang et al. (2021) and the APEC load in blood, brain, and lungs, at levels estimated to be 10⁸–10⁹ CFU/g at 12 hours after infection. Contrary to APEC 158B lux, in this study, such accumulation of APEC was accompanied by clinical signs of urinary tract infection confirming the close relatedness of APEC with UPEC. Yao et al. (2023) reported high mouse mortality (83.3 %)

within 24 hours after intraperitoneal injection of APEC strain AH50, suggesting a greater infection potential than that of APEC 158B lux. Mice mortality was significantly reduced by 66.7 and 83.3 % by treatment with 10^8 PFU/ml phage PEC9 at MOI= 1 at 6 and 12 hours after infection, respectively. Bacterial load in the spleens of infected and phage-treated mice was significantly lower by 1 log₁₀ CFU/g in comparison to untreated mice. These discrepancies in APEC's ability to invade mouse internal organs and induce clinical symptoms could be explained by the strong dependency of the infection on particular APEC strains, especially considering that APEC strains specifically infect poultry and cannot be expected to behave identically in mice and chickens.

Several studies have managed to translate their *in vitro* findings to *in vivo* conditions and have reported the successful treatment of APEC (Oliveira et al., 2010; Jhandai et al., 2024; Sorour et al., 2020). The most common modes of administration of phages against APEC have been spraying phages on surfaces, oral administration, and injection, including intravenous, intramuscular, and intraperitoneal routes. We further developed our research on application of the UPWr_E124 phage cocktail to assess various aspects of phage activity, particularly its effectiveness in targeting APEC in chickens. Our results from *in vivo* mouse studies indicated that the UPWr_E124 phage cocktail potentially can eliminate APEC from the respiratory system. In fact, we confirmed in chickens the results from studies on mice, where the bacterial load in the lungs of APEC-positive broilers was significantly reduced in the phage-treated group to 1.5 ± 0.7 CFU/g in comparison to 8.8 ± 3.0 CFU/g in the untreated group. The number of chickens positive for APEC was similar in treated and untreated groups, but still UPWr_E124 phage cocktail treatment of chickens resulted in a significant reduction of bacterial load in the liver, bursa of Fabricius and blood. These results show the efficient inhibition of APEC replication in lungs and other organs by the UPWr_E124 phage cocktail. This effect is comparable to other studies using phage cocktails administered through an intra-tracheal route that also significantly reduced APEC load in the liver, lungs, and heart in broilers (Oliveira et al., 2009b; Tawakol et al., 2019). Other authors have also reported effective APEC reduction by phages after application via intravenous (Huff et al., 2005) and intramuscular (Tang et al., 2023) injection. Local application of bacteriophages directly at the site of APEC infection, particularly through intra-air sac administration, could be an effective strategy for combating the disease. This approach allows a high concentration of bacteriophages to target the bacterial infection at the site of infection, in areas that may be relatively inaccessible via the circulatory system, increasing the effectiveness of treatment (Huff et al., 2006). This localized delivery method could potentially improve treatment outcomes and reduce the risk of systemic side effects and is applicable in large-scale poultry production. Nonetheless, there are several limitations of the study that could have biased our interpretation of the results. One key limitation is the use of a murine model for studying avian pathogens, which may not fully replicate the APEC infection and APEC-phage interactions seen in avian species. This might have affected the observed immune responses to APEC behavior and phage effectiveness, potentially leading to results that differ from those seen in a more suitable avian model. That is why we also tested the phages in chicken. Additionally, as a proof of concept in this study, UPWr_E124 phage cocktail was used to treat chickens infected with APEC 158B lux. However, APEC and phages were administered intratracheally to fully control the concentration of bacteria and phages. At the farm level, anti-colibacillosis phages should be administered by spray or with drinking water. Despite the limitations of the study, we believe that our animal trial data are important for understanding the potential for use of phages against colibacillosis in broiler chickens as an alternative to antibiotic treatment. Furthermore, investigating the impact of phage on preventing vertical transmission of APEC within commercial flocks is essential for assessing their utility in chicken breeding.

5. Conclusions

This study revealed the UPWr_E124 phage cocktail to be an effective tool in combating APEC in broiler chickens. The polyvalent UPWr_E124 phage cocktail can maintain activity after neutralization of the acid pH of SGF. We found that the phage cocktail UPWr_E124 was effective in combating APEC in chicken meat biofilms in storage conditions and at temperatures favoring bacterial growth. We proved the UPWr_E124 phage cocktail's efficacy in reducing the APEC burden in the lungs in the murine model of infection. High anti-APEC effectiveness was also corroborated in experimentally infected broiler chickens. Thus, these observations support the inclusion of the UPWr_E124 phage cocktail among effective biocontrol agents against APEC.

CRedit authorship contribution statement

David Sáez Moreno: Writing – original draft, Methodology, Investigation. **Kuczkowski Maciej:** Visualization, Software, Methodology, Investigation. **Korzeniowski Paweł:** Investigation. **Grzymajło Krzysztof:** Methodology, Investigation. **Woźniak-Biel Anna:** Investigation. **Śliwka Paulina:** Investigation. **Rywińska Anita:** Investigation. **Kuźmińska-Bajor Marta:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

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.

Acknowledgments

We thank Justyna Dubiel-Pieczynska for her technical assistance. This study was funded by the National Center for Research and Development, LIDER program no. LIDER/378/L-6/14/NCBR/2015. The APC/BPC is co-financed by Wrocław University of Environmental and Life Sciences.

Authors' contributions

DSM provided assistance throughout the study on the mouse model and conducted microbiological analysis of terminated mice and was a major contributor to writing the manuscript; MK performed and supervised *in vivo* trials in the chicken model and performed the statistical analysis; PK performed the analysis of phages in the SGF model on biofilm, and provided assistance throughout the study on the chicken model; KG performed and supervised the study on the mouse model; AW-B performed mouse necropsy and material sampling; PS assisted in the study on mice; AR conducted microbiological analysis of terminated chickens; 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.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.vetmic.2025.110398](https://doi.org/10.1016/j.vetmic.2025.110398).

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