International Journal of Veterinary Sciences and Animal Husbandry

ISSN: 2456-2912
NAAS Rating (2025): 4.61
VET 2025; 10(10): 187-192
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www.veterinarypaper.com
Received: 01-09-2025
Accepted: 04-10-2025

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Adding of bacteriophage in diet to treatment of pullorum infection induced experimentally in broiler chicken (Immunological and Histopathological study)

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DOI: https://www.doi.org/10.22271/veterinary.2025.v10.i10c.2631

Abstract
This study investigated the potential of dietary bacteriophage supplementation as an alternative to antibiotics in broiler chickens experimentally challenged with *Salmonella pullorum*. A total of 250 one-day-old Ross 308 chicks were divided into five groups: G1 (vaccination plus bacteriophage), G2 (vaccination plus florfenicol), G3 (bacteriophage post-challenge), G4 (challenged control), and G5 (negative control). Performance parameters, mortality rates, antibody titers, and Histopathological changes were assessed. Results showed that bacteriophage-treated groups (G1 and G3) achieved significantly higher body weights and lower mortality compared to antibiotic-treated and challenged controls. Moreover, bacteriophage supplementation markedly enhanced antibody responses against Newcastle disease and infectious bronchitis. Histopathological findings further revealed improved intestinal architecture in phage-treated groups relative to florfenicol and challenged controls. Overall, the results highlight that bacteriophage supplementation, either prophylactically or therapeutically, offers effective protection against S. pullorum while improving immunity and gut health. These findings support bacteriophages as a sustainable alternative to antibiotics for controlling bacterial infections in poultry production.

Keywords: *Salmonella spp*., Pullorum disease, bacteriophage, broilers

Introduction

Pullorum disease is a significant global poultry affliction in avian species because to its substantial economic repercussions, widespread prevalence, and challenges associated with disease management (Endris *et al*., 2013; Agapé *et al*., 2025) [8, 2]. Significant economic detriment resulting from elevated death rates that may attain 100%, reduction in production (eggs and chicks), and expenses associated with medicine for both humans and animals (Netsanet *et al*., 2012) [21]. Including Iraq and other Asian nations, it also incurs significant economic losses in the poultry business. Antimicrobial medications are regrettably employed not only for therapeutic purposes but also to enhance animal production, growth rate, and feed conversion efficiency in food-producing animals across numerous developing nations (Snary *et al*., 2004) [25]. Numerous studies have demonstrated that incorrect antibiotic use enhances productivity while also elevating the selection pressure for antimicrobial-resistant microorganisms (Levy, 2014) [15]. Antibiotic resistance has no boundaries, as germs that have acquired resistance to certain antibiotics can swiftly disseminate from countries with robust surveillance systems to those lacking such measures. Consequently, a collaborative strategy among developing nations is essential (Galperina & Kyian, 2021) [9]. The prominent emergence of multidrug-resistant bacterial illnesses in poultry farms renders treatment with conventional antibiotics impractical, hence demanding the adoption of alternative therapies (Abreu *et al*., 2023) [1]. Phage treatment is an efficacious method for addressing drug-resistant bacterial infections in animals while preserving their normal gut microbiota (Mohsen *et al*., 2024) [18].

Bacteriophages possess several attributes that render them potentially advantageous as therapeutic agents for bacterial infections. One of them is the remarkable specificity and efficiency in lysing particular pathogenic microorganisms (Nabil *et al*., 2018) [20]. Furthermore, several investigations have documented effective decrease in *Salmonella* spp. (Wernicki *et al*., 2022; Zeng *et al*., 2024; García *et al*., 2024) [26, 29, 10]. Rydman and Bamford (2002) [22] elucidated the strategy employed during phage-bacterium interactions, which involves the degradation of extracellular polymeric substances in the biofilm through the production of polysaccharide depolymerases, thereby facilitating phage access to encased bacterial cells and inducing their lysis. The objective of this study was to evaluate the beneficial effects of bacteriophage on bodily performance and the gut microbiome in the context of *S. pullorum* challenge.

Materials and Methods

Drugs and vaccines

Florfenicol 100 mg/ml was purchased from (Cenavisa-Spain).

Bacteriophage

E. coli Bacteriophage 3.2×10^8 PFU/g was procured from EASY BIO-Korea and used in the current study as described by (Aljuhaishi and Albawi, 2024) [4].

Birds

The chicks in this study were weighed at one day of age (mean weight 43 g±2) and subsequently brought to the poultry hall. The chicks are housed in enclosures separated by wooden barriers, each measuring 1 x 1.5 m. The hen coop bedding consisted of sawdust and measured 7 cm in thickness. Access to potable water and sustenance is provided gratuitously and facilitated by a continuous illumination system. No clinical manifestations of illness were observed prior to the commencement of the investigation. This study involved 250 one-day-old Ross308 broilers, which were randomly assigned to five treatments, with two replication pens containing 25 birds each. This research was conducted at the broiler farm of the College of Veterinary Medicine, Al-Qasim Green University, located in Babylon City, Iraq.

Experimental design

250 broiler chicks divided into 5 groups as follow:

- **G1:** Using vaccine program and bacteriophage as feed additive from one day to the end of experiment as (dosage 3.2* 10^10 PFU/g)
- **G2:** Same vaccination but without use bacteriophage as feed additive with treated at one day to five days by using Florfenicol and after challenge
- **G3:** Using bacteriophage as feed additive after challenge (from 14 days until the end of experiment).
- **G4:** Challenged 14 days without any treatment (control positive)
- **G5:** Control group without challenge and without using any treatment.

All groups vaccinated with Traditional Vaccines against viral diseases like (ND, AI, IB, and IBD).

Vaccination of chicks

The birds received vaccinations against infectious bronchitis at one day old with the IB QX strain administered via eye drop, and at ten days old with the IB H120 strain provided through drinking water. Newcastle disease was inoculated at one day old with the LaSota attenuated strain via eye drop, at ten days old with the Clone attenuated strain through drinking water, and at twenty days old with the LaSota attenuated strain via drinking water. Additionally, avian influenza was administered on day one and infectious bursal disease on day fourteen.

Statistical analysis

The analysis was conducted using the pre-existing statistical software SAS. The data were presented as mean±standard deviation (STD) unless specified otherwise. SAS (2018) was utilized for data analysis using the Randomized Completely Block Design (RCBD). The disparities among the coefficients of the initial experiment were evaluated utilizing Duncan's multilevel test, with a significance threshold of 0.05 (Duncan, 1955; Dirwal *et al*., 2020) [6, 7].

Results and Discussion

A. Live body weight in experimental study

Table (1) shows the live body weight in experimental groups. At 7 days, group G1 (bacteriophage-treated) had significantly higher body weight (205.3 g) compared to G2 (201.4 g), G3 (201.9 g), and G4 (203.1 g), while G5 (208.2 g, negative control) recorded the highest weight ($p<0.05$). At 14 days, G1 remained the highest (517.8 g) compared to other groups, with G5 (505.2 g) having the lowest. At 21 days, G1 and G3 showed higher body weights compared to G2, G4, and G5, with significant differences ($p<0.05$). At 28 days, G3 (1631.2 g) showed the best growth, followed closely by G1 (1629 g), whereas G4 (1429 g, positive control) had the lowest weight, indicating significant improvement by bacteriophage and combined treatments.

Table 1: Live body weight in experimental study

Age Groups	G1	G2	G3	G4	G5
7 days	205.3±0.565 D a	201.4±1.55 D b	201.9±0.28 D a	203.1±0.42 D d	208.2±0 D c
14 days	517.8±1.6 C a	513.1±1.55 C b	510.6±0 C a	508.2±1.131 C d	505.2±1.4 C c
21 days	955.6±3.95 B a	943.8±1.13 B b	949.9±0.707 B a	932.3±4.10 B d	933.7±4.38 B c
28 days	1629±0.28 A a	1608.2±5.091 A b	1631.2±2.26 A a	1429±4.525 A d	1533.1±3.25 A c

* Means±Std Error

*Means with the same Capital letters in the same column are not significantly different

*Means with the same Small letters in the same Row are not significantly different

B. Mortality rates

The results of mortality rate illustrated in Table 2, demonstrated a clear group difference. G1 (bacteriophage-treated) and G5 (negative control) recorded 0% mortality, indicating full protection. G2 (antibiotic-treated) showed 32% mortality, while G3 (combined treatment) recorded only 10%, significantly lower than G2 ($p<0.05$). The highest mortality was observed in G4 (positive control), reaching 70%, which was significantly higher than all other groups.

Table 2: Mortality rate of different treatment groups

Groups	No of chicks in group	No chicken Died/day/post received *S. Pullorum*					Mortality rate	Mortality percentage
		1	2	3	4	5		
G1	50	0	0	0	0	0	0/50	0%
G2	50	0	2	6	3	5	16/50	32%
G3	50	0	0	3	2	0	5/50	10%
G4	50	4	7	9	5	10	35/50	70%
G5	50	0	0	0	0	0	0/50	0%

C. Antibody titer against Newcastle diseases

Table 3 presents antibody titers against Newcastle disease. At day 7, G1 showed the highest antibody level (2751) compared to other groups ($p<0.05$). By day 14, G1 (3442) and G3 (3129) were significantly higher than G2 and G4, while G5 had the lowest (1983). At day 21, G1 remained superior (2589) with significant differences, while G4 and G5 were markedly reduced. At day 28, G1 showed the highest antibody response (6591), significantly higher than G2 (4411) and G3 (5269), while G4 (437) and G5 (212) had the lowest responses ($p<0.05$).

Table 3: Results of antibody titer against Newcastle disease

Age Groups	Day 7	Day 14	Day 21	Day 28
G 1	2751±7.26 Ac	3442±7.17 A b	2589±5.59 Ac	6591±9.67 A a
G 2	2099±5.77 B c	2820±5.35 B b	1512±4.23 B c	4411±7.51 C a
G 3	2339±7.82 B c	3129±5.32 A b	2087±5.65 B c	5269±8.72 B a
G 4	1949±5.75 C b	2453±5.38 B a	1191±6.24 C c	437±1.3 D d
G 5	1657±8.13 C b	1983±4.15 C a	426±0.9 D c	212±0.8 Dc

* Means±Std Error
*Means with the same Capital letters in the same column are not significantly different
*Means with the same Small letters in the same Row are not significantly different

D. Antibody titers against Infectious Bronchitis disease

Table (4) shows antibody titers against infectious bronchitis. At day 7, G1 (3209) and G3 (2875) were significantly higher than G2 (2377), with G4 and G5 having the lowest titers. At day 14, G1 (3950) and G3 (3659) maintained higher antibody responses compared to G2, G4, and G5 ($p<0.05$). By day 21, G1 (2957) and G3 (2093) were significantly better than G2, while G4 and G5 showed very low responses (1221 and 731, respectively). At day 28, G1 (6763) and G3 (5342) remained significantly higher than G2 (3424), while G4 (1353) and G5 (789) recorded the lowest antibody titers, confirming the superior immune response in bacteriophage and combined treatments.

Table 4: Results of antibody titer against infectious bronchitis disease (M±Std) in different days by ELISA test of the experiment

Age Groups	Day 7	Day 14	Day 21	Day 28
G 1	3209±9.23 A	3950±7.67 A	2957±3.34 A	6763±7.89 A
G 2	2377±4.22 B	3385±8.79 B	1780±2.56 C	3424±4.55 B
G 3	2875±5.13 A	3659±7.56 A	2093±4.22 B	5342±8.91 A
G 4	1970±4.57 C	2011±3.77 C	1221±3.59 C	1353±3.34 C
G 5	1591±5.33 C	1994±3.54 C	731±1.5 D	789±1.7 D

* Means±Std Error
*Means with the same Capital letters in the same column are not significantly different
*Means with the same small letters in the same Row are not significantly different

E. Histopathological study of intestine

- **Control negative:** Photomicrograph of small intestine of Control Negative group broiler chicken. Normal histological architecture of the small intestinal mucosa showing the crypts of Lieberkühn (black arrow) with villi (yellow arrow) projecting above the crypt layer (Figure 1A).

- **Control positive:** The Histopathological findings showed severe necrosis of epithelial cells in the crypts of Lieberkühn and villi is observed, with infiltration of inflammatory cells and epithelial cells hyperplasia (black arrow) occupying the necrotic areas, leading to fusion of villi and loss of normal intestinal architecture. Focal necrotic areas (yellow arrow) are also present within the affected regions. Destruction of villi (blue arrow) is noted as adjacent to the necrotic zones (Figure 1B).
- **Florfenicol group:** Photomicrograph of small intestine of Florfenicol group broiler chicken. A and B/ Mild to moderate necrosis was observed in the villi, accompanied by infiltration of inflammatory cells in the villus core, leading to increased villus thickness. In addition, destruction of villus tips (yellow arrow) was noted in the affected areas (Figure 1C).
- **14 days bacteriophage group:** Photomicrograph of small intestine of day 14 bacteriophage group broiler chicken. A and B/ Marked thickening of the affected villi is observed due to infiltration of inflammatory cells (black arrow) in the villus core, with significant hyperplasia of epithelial cells (yellow arrow). In addition, destruction of villus tips is noted in the affected areas, with necrotic cell debris (blue arrow) present in the intestinal lumen (Figure 1D).
- **1 day bacteriophage group**: Photomicrograph of small intestine of day 1 bacteriophage group broiler chicken. A and B/ Moderate thickening of the affected villi is observed due to infiltration of inflammatory cells (black arrow) in the villus core, with moderate hyperplasia of epithelial cells (yellow arrow), (Figure 1D).

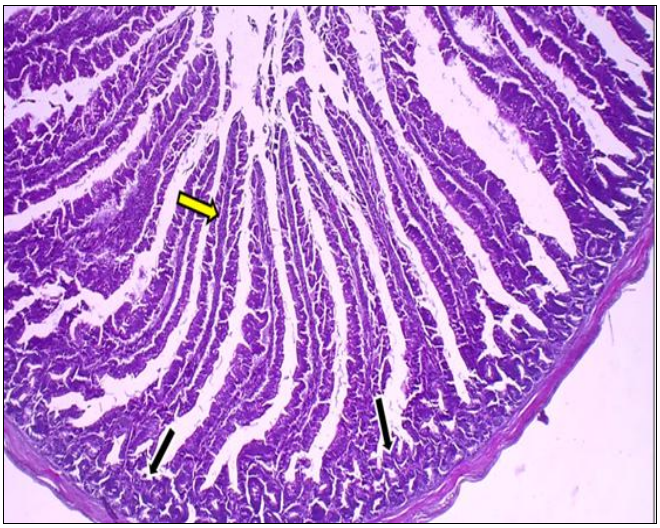

Fig 1: Photomicrograph of small intestine of Control Negative group broiler chicken. Normal histological architecture of the small intestinal mucosa showing the crypts of Lieberkühn (black arrow) with villi (yellow arrow) projecting above the crypt layer. H&E. 40x

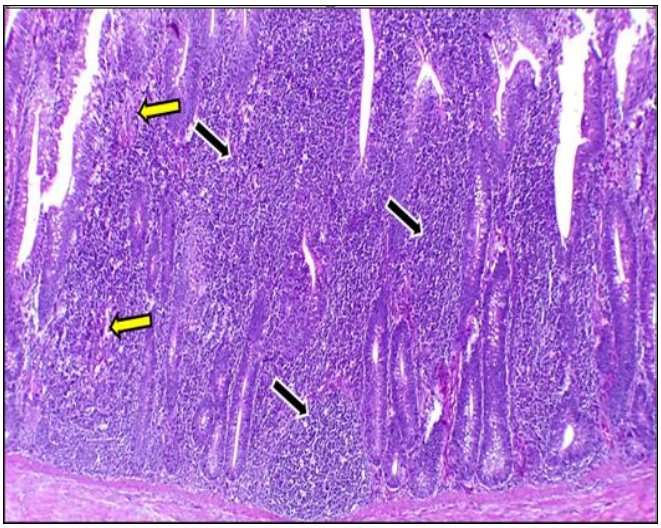

Fig 2: Photomicrograph of small intestine of Control Positive group broiler chicken. Severe necrosis of epithelial cells in the crypts of Lieberkühn and villi is observed, with infiltration of inflammatory cells and epithelial cells hyperplasia (black arrow) occupying the necrotic areas, leading to fusion of villi and loss of normal intestinal architecture. Focal necrotic areas (yellow arrow) are also present within the affected regions. H&E. 100x

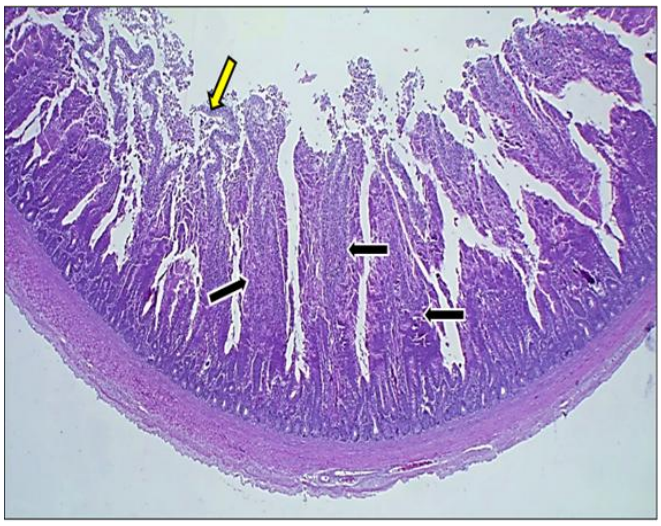

Fig 3: Photomicrograph of small intestine of Florfenicol group broiler chicken. Mild to moderate necrosis was observed in the villi, accompanied by infiltration of inflammatory cells in the villus core, leading to increased villus thickness. In addition, destruction of villus tips (yellow arrow) was noted in the affected areas. H&E. A: 40x

Fig 4: Photomicrograph of small intestine of day 14 bacteriophage group broiler chicken. Marked thickening of the affected villi is observed due to infiltration of inflammatory cells (black arrow) in the villus core, with significant hyperplasia of epithelial cells (yellow arrow). In addition, destruction of villus tips is noted in the affected areas, with necrotic cell debris (blue arrow) present in the intestinal lumen. H&E. A: 40x

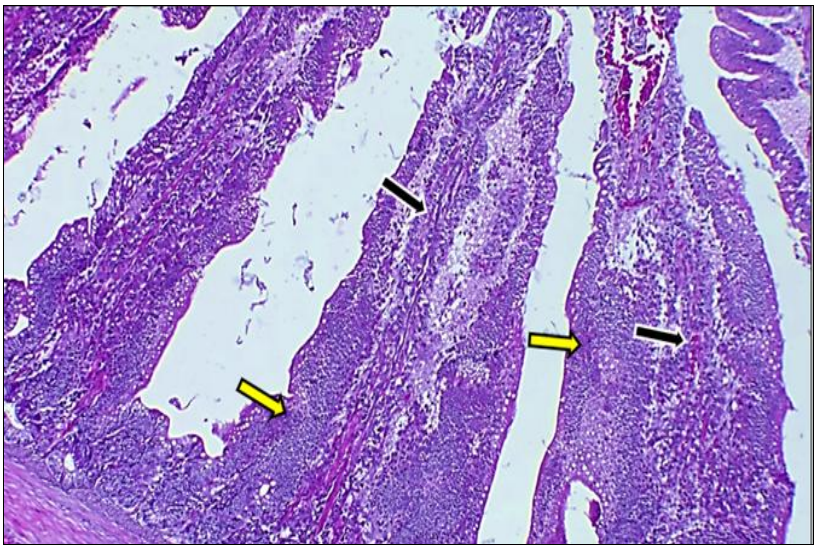

Fig 5: Photomicrograph of small intestine of day 1 bacteriophage group broiler chicken. Moderate thickening of the affected villi is observed due to infiltration of inflammatory cells (black arrow) in the villus core, with moderate hyperplasia of epithelial cells (yellow arrow). H&E. A: 40x and B: 100x

Discussion

The findings of this study demonstrated that dietary supplementation with bacteriophages significantly improved growth performance, immune responses, and intestinal health in broiler chickens experimentally challenged with *Salmonella pullorum*. The results align with previous research, highlighting the potential of phage therapy as an alternative to antibiotics in poultry production (Clavijo *et al*., 2023; Kosznik-Kwaśnicka *et al*., 2022) [5, 13]. Bacteriophage-treated groups (G1 and G3) exhibited superior body weight gain compared to antibiotic-treated (G2) and untreated challenged groups (G4), indicating that phages enhanced feed efficiency and nutrient utilization. This outcome is consistent with reports that phages restore gut microbial balance and reduce pathogenic colonization (Kakasis & Panitsa, 2019; Seo *et al*., 2025) [12, 24]. Interestingly, G3 showed comparable growth at 28 days to G1, suggesting that even post-infection phage application can mitigate disease severity. Mortality rates further support the therapeutic value of phages, with G1 and the negative control showing 0% mortality, while the antibiotic group had 32% and the positive control reached 70%. These results emphasize that florfenicol alone may not provide full protection under severe infection pressure, corroborating earlier findings that antibiotics have limited efficacy against resistant *Salmonella* strains (Lee *et al*., 2021) [14]. The immunological assessments revealed that bacteriophage groups had significantly higher antibody titers against Newcastle disease and infectious bronchitis, particularly at later stages of the experiment. This suggests that phages not only reduce pathogen load but may also enhance systemic immunity, potentially through preservation of gut integrity and modulation of host immune signaling (Lopez-Colom *et al*., 2020; Nabil *et al*., 2021; Aljuhaishi DA & Albawi, 2024) [17, 19, 4]. Histopathological analysis confirmed these outcomes, as phage-treated bird's maintained better villus architecture compared to antibiotic and positive control groups, where necrosis, villus fusion, and epithelial hyperplasia were evident. These findings underscore the gut-protective effect of phages, consistent with recent reports describing their role in maintaining intestinal barrier function and reducing inflammatory responses. Overall, the results provide strong evidence that bacteriophage supplementation, either prophylactically or therapeutically, offers superior protection against S. pullorum compared to conventional antibiotic treatment. This supports growing international efforts to reduce antibiotic use in animal production and adopt sustainable alternatives to combat antimicrobial resistance (WHO, 2022) [27].

Conclusion

Bacteriophage supplementation effectively protected broiler chickens against *Salmonella pullorum*, improving growth, survival, immunity, and gut health compared to antibiotics. Florfenicol offered limited protection, while phages provided strong prophylactic and therapeutic benefits. These results support bacteriophages as a sustainable alternative to antibiotics in poultry production and a valuable tool against antimicrobial resistance.

Conflict of interest

Not available

Financial Support

Not available

Reference

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How to Cite This Article
Hassan MJ, Al-Bawi FH. Adding of bacteriophage in diet to treatment of pullorum infection induced experimentally in broiler chicken (Immunological and Histopathological study). International Journal of Veterinary Sciences and Animal Husbandry. 2025;10(10):187-192.

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