Research Article

Application of Phages to Control *Escherichia coli* Infections in Native Noi Chickens

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Abstract | This study assessed the efficacy of two phages, MHH6 and PR2, against *Escherichia coli* serotype O6 infected in chickens. A total of 420 broilers were randomly assigned to seven treatment groups. The negative control (NC) birds received no *E. coli* or phages, whereas the positive control (PC) birds were infected with *E. coli* only. The NC+MHH6 and NC+PR2 treatments received 10^9 pfu/ml of phages MHH6 or PR2, respectively; whereas the PC+MHH6, PC+PR2, and PC+MHH6PR2 groups were infected with $10^{7.1}$ cfu/ml of *E. coli* strain O6 and treated with 10^9 pfu/ml of phages of MHH6, PR2, or both. *E. coli* infection was inoculated in two-day-old chicks, and phages were administered 24 hours later. The mortality rate of chickens in the PC+MHH6PR2 group was significantly lower (16.7%) than in the PC group (58.3%). The frequency of lesions and *E. coli* densities in the heart, liver, and spleen of chickens treated with MHH6 and PR2 phages were greatly decreased in infected chickens. After 98 days, the body weight of birds from the *E. coli*-infected groups treated with MHH6 and PR2 phages was lower than individuals in non-*E. coli*-infected groups (1,313-1,324 g/bird) but higher and significantly different from those in the PC group (1,172 g/bird). The majority of commercially important traits in chickens improved after phage treatment, proving that these phages are capable of controlling O6 *E. coli* infected in Noi chickens.

Keywords | Bacteriophage, Carcass characteristic, Chicken, *E. coli*, Growth performance

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INTRODUCTION

Increased consumption of livestock products such as meat, milk, and eggs necessitates an increase in production by animal producers to keep up with the demand. As a result, large-scale livestock production systems are continually developing, with high stocking densities facilitating disease transmission and economic losses (Nhung et al., 2017). *Escherichia coli* (*E. coli*) is regularly isolated from poultry intestines and other mucosal surfaces, and it is classed as a harmful bacterium for chicken, capable of producing colibacillosis, a gastrointestinal disease. Antibiotics are frequently used to treat infections caused by bacteria; however, their efficacy is being questioned due to a rising number of antibiotic-resistant bacteria. According to Nhung et al. (2017), the *E. coli* APEC strains were resistant to ampicillin, amoxicillin, and tetracycline at levels greater than 70% and ciprofloxacin, neomycin,

and chloramphenicol at rates between 50% and 70%. Alternative strategies are required in this situation, one of which is using bacteriophages (phages) to fight bacterial infections (Rios et al., 2016).

Numerous advantages make phage therapy an appealing option to antibiotics (Golkar et al., 2014). According to Domingo et al. (2016), phages are selective for certain bacteria, and phage therapy is deemed safe and effective compared to antibiotics. This mechanism of action does not affect the proliferation of gut flora (Wernicki et al., 2017; Cieplak et al., 2018). Additionally, because phages are abundant in nature, they can be isolated and selected quickly in opposition to antibiotic synthesis, which requires millions of dollars and years of research to generate an effective antibiotic (Golkar et al., 2014). Phage supplementation has been proven to increase feed efficiency, body weight gain, pathogen reduction, and egg production in broiler chickens and laying hens (Noor et al., 2020). Phage as a feed additive may be an effective way to regulate the gut microbiota of chickens by lowering particular pathogenic microbial populations and increasing beneficial bacteria, resulting in enhanced gut health (Clavijo and Florez, 2018).

Upadhaya et al. (2021) have established the efficacy of phage therapy at a range of doses, such as 10^8 pfu/ml and 10^6 pfu/ml. Their presence aided in decreasing harmful germs in poultry and animals before slaughter. Additionally, phage has been used to extend the life of food packaging materials and as a disinfectant on production lines (Lone et al., 2016). Oral administration of a bacteriophage mixture to rats significantly diminished *E. coli* O157:H7 colonization in the gastrointestinal tract (Dissanayake et al., 2019). The phage treatment also significantly reduced the number of *E. coli* O157:H7 bacteria on the meat surface (El-Shibiny et al., 2017). However, little study has been undertaken on the efficacy of phage therapy against *E. coli* infection in indigenous chickens. In a recent study, we isolated two phages, namely MHH6 and PR2, which were shown by the transmission electron microscopy to belong to the *Myoviridae* family based on their shape and the presence of the tail diameter (Ngu et al., 2020). These phages could survive at low pH levels and lyse *E. coli* strains of O1, O78, and O6. Therefore, the current study was undertaken to investigate the effects of MHH6 and PR2 phages on controlling *E. coli* in native Noi chickens, as measured by their growth performance, carcass characteristics, and the bacteria population in the internal organs.

MATERIALS AND METHODS

Ethical statement

All chickens were handled and cared for in line with Vietnam's Animal Husbandry Law, No. 32/2018/QH14 dated on December 22, 2018.

Bacteria and phages

In the present study, the *E. coli* strain of serotype O6 was purchased from ATCC (American Type Culture Collection) (ATCC® 25922TM), and two phages, MHH6 and PR2, were taken from our previous work (Ngu et al., 2020).

Chickens and experimental procedure

The study was conducted on the experimental farm of Can Tho University located at Campus IV, Phung Hiep district, Hau Giang province. Noi chickens were purchased from Soc Trang breeding company and kept in-house with four cages per treatment. Each cage was outfitted with two drinking troughs and two feeding troughs. Water for drinking was constantly accessible. Commercial feeds were fed *ad libitum* for two feeding periods from 1 to 28 days (16% crude protein, 4% crude fiber, metabolizable energy 2,800 Kcal/kg) and 29 to 98 days (14% crude protein, 5% crude fiber, metabolizable energy 2,800 Kcal/kg). Following the breeding company's directions, the vaccination schedule for chickens was thoroughly implemented during the rearing process.

The 98-day experiment included a total of 420 one-day-old Noi broiler chickens. The chicks were randomly assigned to one of seven experimental groups, each with four replicates and 15 broilers per replicate. The treatment groups were: Negative Control (NC) - treatment without *E. coli* challenge; NC+Bacteriophage 1 (NC+MHH6); NC+Bacteriophage 2 (NC+PR2); Positive Control (PC) - treatment with *E. coli* challenge; PC+Bacteriophage 1 (PC+MHH6); PC+Bacteriophage 2 (PC+PR2); PC+Bacteriophage 1 and Bacteriophage 2 (PC+MHH6PR2). Chicks were selected for oral infection with 1 ml of *E. coli* on day two at a dose of $10^{7.1}$ cfu/ml (based on lethal dose, LD_{50} results), and phages were administered orally at 10^9 pfu/ml starting 24 h after infection, followed for three consecutive days and repeated weekly until day 63.

Sampling and measurements

The chicken mortality rate was monitored during the trial. At 7, 21, 35, and 49 days after infection, one chick from each cage was randomly selected and euthanized by exsanguination to determine the density of *E. coli* (Gomes et al., 2014) and record the weights of internal organs such as heart, liver, and spleen (Lan et al., 2017).

Chicken body weight (BW) was determined weekly, and feed intake (FI) was recorded daily for each cage. These data were then utilized to compute body weight gain (BWG) and feed conversion ratio (FCR). At the end of the experiment, 56 chickens (8 per treatment, equal sex) were slaughtered to determine carcass characteristics and organ

weights. Breast, thigh, drumstick muscle, and wings were collected according to Faria et al. (2010). In addition, the liver, spleen, heart, and gizzard were removed and weighed, and data were expressed as percentages of body weight.

Statistical analysis

The data were analyzed using the General Linear Model procedure of the Minitab version 16 software (State College, PA, USA) (Minitab, 2010). Tukey's comparison test with $P \leq 0.05$ was performed to detect the mean difference between treatments.

RESULTS AND DISCUSSION

The mortality rate of chickens

Figure 1 shows the total mortality rate of the experimental chickens. In treatments without *E. coli* infection (NC) or only with phage treatment (NC+MHH6 and NC+PR2), no dead chickens were found. The highest mortality rate was found in chickens infected solely with *E. coli* (PC), at 58.3%, followed by PC+PR2 (25.0%) and PC+MHH6 (22.2%) treatments.

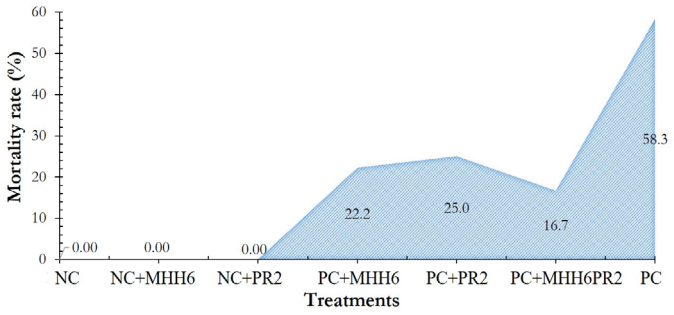

Figure 1: The efficacy of a bacteriophage on mortality rate of chicken *E. coli* challenged. NC: negative control, without *E. coli*, without phage; PC: positive control, *E. coli* challenged, without phages; NC+MHH6 and NC+PR2: negative control plus MHH6 or PR2 phage, respectively; PC+MHH6, PC+PR2: positive control plus MHH6 or PR2 phage, respectively; PC+MHH6PR2: positive control plus both MHH6 and PR2 phages.

The presence of *E. coli* in the internal organs of Noi chickens

During 49 days of infection, the lytic ability of phages and *E. coli* invasion were assessed by counting *E. coli* density in the heart, liver, and spleen of chickens (Figure 2A-C). During the experiment, *E. coli* were not found in birds' hearts, liver, and spleen under NC, NC+MHH6, and NC+PR2 treatments. The number of *E. coli* declined after the 7th day of phage application, and it was eliminated after 49 days in the therapy with both phages included (Figure 2B). Moreover, *E. coli* in the spleen was deficient and could not be detected in the PC+MHH6PR2 treatment at 35 days or the PR2 phage treatment at 49 days post-infection (Figure 2C). Moreover, the *E. coli* density in organs of chickens under MHH6 and PR2 treatments was relatively low (0.00-0.69 $\log_{10}$ cfu/ml) compared to the PC group with a higher *E. coli* population (1.37-2.36 $\log_{10}$ cfu/ml).

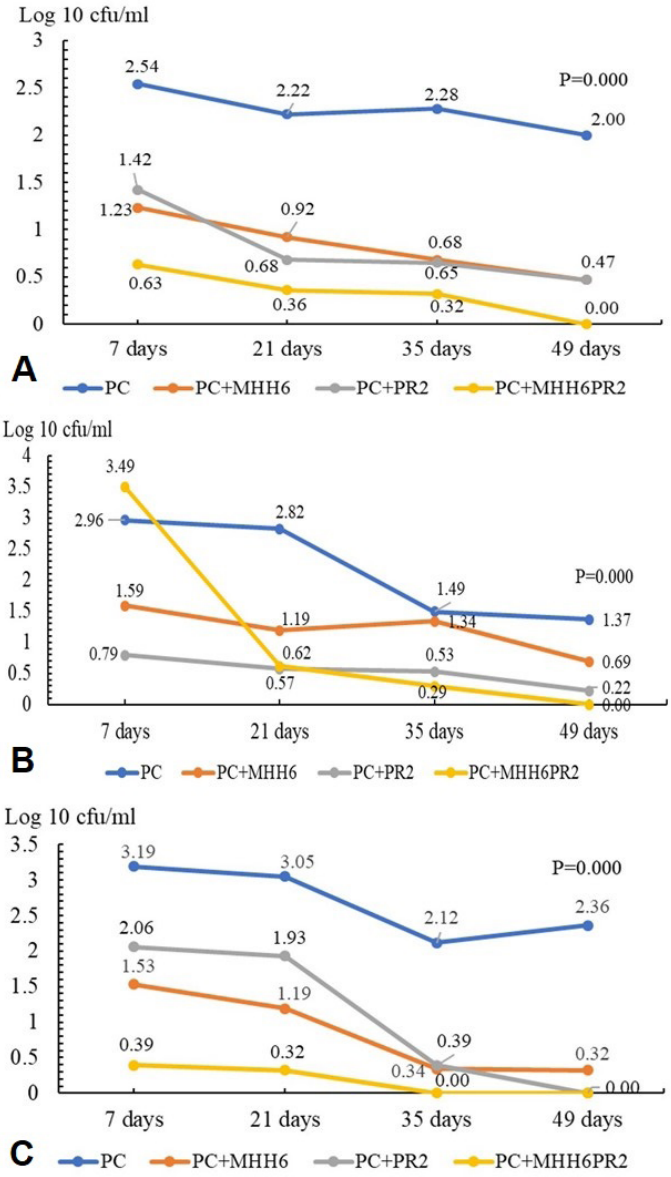

Figure 2: Number of *E. coli* bacteria in heart, liver, and spleen organs over 49 days of infection. (a) Heart; (b) Liver; (c) spleen; PC: positive control, *E. coli* challenged, without phages; PC+MHH6, PC+PR2: positive control plus MHH6 or PR2 phage, respectively; PC+MHH6PR2: positive control plus both MHH6 and PR2 phages.

Growth performance of chickens

The results in Table 1 indicate that BWG was higher in chickens treated with phages in the first 35 days (258 g/bird) and the whole period (1-98 days) (1,143 g/bird) compared with those in the PC treatment. There was no significant difference in bird growth between the *E. coli*-free treatments (NC and NC+MHH6; NC+PR2); however, differences were found when treatments with *E. coli* inoculation were accounted. The overall benefit of improved

BWG was also demonstrated in the treatment with both phages included (1,237 g/bird). In addition, higher feed consumption in the non-treated chickens, combined with lower BWG, were responsible for a higher FCR (4.09) in the PC treatment than in the others (3.32-3.71).

Carcass traits of chickens

The phage therapy had a considerable impact on the carcass and organ weight of infected chickens (Table 2). Chickens in the only-phage-treated group, as well as those in a co-inoculation group with *E. coli* and bacteriophages, had a higher carcass weight and carcass percentage than chickens in the only *E. coli*-infected group ($P<0.05$). Additionally, Table 2 demonstrates that the rate of organ weight treated with PC was generally more remarkable than that treated with bacteriophage, particularly the spleen ($P<0.01$).

Table 1: Effect of bacteriophage on on body weight gain (BWG, g), feed intake (FI, g) and feed converstion ratio (FCR, feed/gain).

Parameters	Treatments							SEM	P
	NC	NC+ MHH6	NC+ PR2	PC+ MHH6	PC+ PR2	PC+ MHH6PR2	PC		
Feed intake (g)									
1-35 d	834^{abc}	816^{abcd}	781^{cd}	798^{bcd}	768^{d}	851^{ab}	871^{a}	14.0	0.000
36-70 d	1,795^{bc}	1,804^{b}	1,776^{bc}	1,738^{bc}	1,679^{c}	1,970^{a}	2,003^{a}	26.4	0.000
71-98 d	1,682^{ab}	1,707^{ab}	1,700^{ab}	1,692^{ab}	1,601^{b}	1,773^{a}	1,805^{a}	29.8	0.003
1-98 d	4,312^{b}	4,328^{b}	4,259^{b}	4,228^{b}	4,049^{c}	4,595^{a}	4,680^{a}	38.6	0.000
Body weight gain (g)									
1-35 d	333^{a}	328^{a}	322^{a}	300^{c}	270^{bc}	302^{b}	258^{d}	8.96	0.000
36-70 d	694	698	689	645	687	663	536	40.2	0.105
71-98 d	396	438	439	353	387	396	478	27.2	0.068
1-98 d	1,285^{a}	1,296^{a}	1,284^{a}	1,194^{c}	1,218^{bc}	1,237^{b}	1,143^{d}	7.24	0.000
FCR (feed/ gain)									
1-35 d	2.50^{bc}	2.49^{bc}	2.43^{c}	2.66^{bc}	2.84^{b}	2.82^{b}	3.37^{a}	0.08	0.000
36-70 d	2.71^{b}	2.59^{b}	2.58^{b}	2.70^{b}	2.45^{b}	2.97^{b}	3.76^{a}	0.15	0.000
71-98 d	4.27	4.02	3.89	4.92	4.17	4.52	3.80	0.31	0.212
1-98 d	3.35^{d}	3.34^{d}	3.32^{d}	3.54^{c}	3.32^{d}	3.71^{b}	4.09^{a}	0.04	0.000

NC: negative control, without *E. coli*, without bacteriophage; PC: positive control, *E. coli* challenged, without bacteriophages; NC+MHH6 and NC+PR2: negative control plus MHH6 or PR2 bacteriophage, respectively; PC+MHH6, PC+PR2: positive control plus MHH6 or PR2 bacteriophage, respectively; PC+MHH6PR2: positive control plus both MHH6 and PR2 bacteriophages. ^{a,b,c,d} Within a row, values with different superscripts differ statistically at $P<0.05$.

Table 2: The effect of bacteriophage supplementation on carcass characterictis and organ weight in broilers.

Parameters	Treatments							SEM	P
	NC	NC+ MHH6	NC+ PR2	PC+ MHH6	PC+ PR2	PC+ MHH6PR2	PC		
Body weight (BW), g	1,314^{a}	1,324^{a}	1,313^{a}	1,222^{c}	1,247^{bc}	1,266^{b}	1,172^{d}	7.29	0.000
Carcass weight (CW), g	896^{b}	944^{a}	934^{ab}	845^{c}	818^{c}	900^{ab}	654^{d}	9.72	0.000
Carcass, % BW	68.2^{ab}	71.3^{a}	71.1^{a}	69.1^{ab}	65.6^{b}	71.1^{a}	55.8^{c}	0.96	0.000
Breast, % CW	15.7^{ab}	16.3^{ab}	17.2^{a}	15.9^{ab}	14.9^{b}	15.8^{ab}	15.1^{b}	0.38	0.009
Thigh + drumstick, % CW	24.5^{a}	24.7^{a}	24.5^{a}	23.4^{ab}	20.8^{c}	21.6^{bc}	20.2^{c}	0.53	0.000
Wing, % CW	14.0^{b}	14.9^{ab}	15.6^{a}	15.4^{ab}	15.5^{a}	15.1^{ab}	15.3^{ab}	0.31	0.031
Organ weight, % BW									
Liver	2.08^{b}	2.18^{ab}	2.13^{ab}	2.37^{a}	1.90^{b}	1.95^{b}	2.12^{ab}	0.06	0.001
Spleen	0.19^{b}	0.19^{b}	0.23^{ab}	0.19^{b}	0.19^{b}	0.21^{ab}	0.27^{a}	0.01	0.006
Gizzard	3.13^{a}	2.39^{cd}	2.67^{bcd}	2.76^{abc}	2.23^{d}	2.98^{ab}	2.77^{abc}	0.010	0.000
Heart	0.55^{a}	0.42^{b}	0.53^{ab}	0.51^{ab}	0.42^{b}	0.52^{ab}	0.52^{ab}	0.03	0.005

NC: negative control, without *E. coli*, without bacteriophage; PC: positive control, *E. coli* challenged, without bacteriophages; NC+MHH6 and NC+PR2: negative control plus MHH6 or PR2 bacteriophage, respectively; PC+MHH6, PC+PR2: positive control plus MHH6 or PR2 bacteriophage, respectively; PC+MHH6PR2: positive control plus both MHH6 and PR2 bacteriophages. ^{a,b,c,d} Within a row, values with different superscripts differ statistically at $P<0.05$.

According to Loc-Carrillo and Abedon (2011), a phage is an antibacterial, bactericidal agent that can increase in number during treatment and tends to disrupt the normal flora only to a minimum extent. The ability of phages to host diverse bacterial species reflects their specialization, and the host spectrum of a bacteriophage is defined as the number of bacteria employed by the phage. Therefore, when using phages with a broad host spectrum, it is possible to support the treatment of many strains of bacteria simultaneously. In the *in vitro* study (Ngu et al., 2020), MHH6 and PR2 phages demonstrated the wide host range and the ability to lyse O1, O78, and O6 *E. coli* strains; however, more testing *in vivo* was required. The data in this study showed that most commercially important traits in chickens improved after phage treatment (Tables 1 and 2). These phages could control O6 *E. coli* infected in native Noi chickens. At the same time, the mortality rate of the combined treatment of *E. coli* infected-birds and phage treated-birds was significantly reduced (Figure 1). These findings were consistent with some previous studies, where controlled *E. coli* bacteria in the intestinal tract of chickens significantly improved carcass quality (Isroli et al., 2018). Lau et al. (2010) also demonstrated that when phages were used, the mortality rate of chicken was dramatically decreased ($P<0.05$). At the end of the experiment, the total mortality rate of birds inoculated with 10^8 cfu of *E. coli* was 83.3%. When using a phage concentration of 1.5 x 10^9 pfu/ml, an average decrease of 25% in chicken mortality was noted (Oliveira et al., 2010). Additionally, Naghizadeh et al. (2019) reported that *E. coli* caused about 46.6% mortality in 15 days old chicks without phage treatment. Only 13.3% of birds died after being injected with 10^8 cfu *E. coli* and 10^{10} pfu of the comparable therapy phage. It can be inferred from these findings that phage therapy effectively inhibits the growth of *E. coli* and can be utilized to treat *E. coli* infection in broilers.

Regarding the frequency of lesions and *E. coli* densities in internal organs of chickens using phages to treat *E. coli*, Lau et al. (2010) reported that *E. coli* colonization was reduced in the liver, heart, and spleen and cleared from the blood samples during the experimental period. Utilizing a phage concentration of 1.5 x 10^9 pfu/ml resulted in a 41.7% reduction in infection incidence compared with the untreated group (Oliveira et al., 2010). Additionally, Lim et al. (2011) showed that after three weeks of follow-up, the rate of bacterial re-isolation in the liver and spleen of hens with just bacterial infections was 40-60%, compared to only 20-40% in the bacteriophage-treated group. It can be explained that phages can move through the mucosal surface and even the blood-brain barrier and effectively protect the host (Huh et al., 2019). These findings were consistent with the current study, according to which phage treatment could prevent and reduce the severity of infected chickens.

Colibacillosis is often lethal in chickens, particularly broilers and turkeys. *E. coli* enters the bloodstream from the site of infection, where it spreads to multiple internal organs, causing sepsis and bird death (Antao et al., 2008). Naghizadeh et al. (2019) also discovered a high presence of *E. coli* in the hearts, livers, and spleens of birds only infected with *E. coli* (3/8 birds) 10 days post-challenge. However, when using phage for treating, *E. coli* was reduced in the heart, liver, and spleen after ten days of the challenge. It can be stated that phage administration effectively inhibited *E. coli* growth in the internal organs of chickens in the present study. According to Bicalho et al. (2010), phages 1230-10 had the highest antibacterial activity and entirely stopped the growth of 71.7% of all isolates. Additionally, the combined action of phage preparations led to a broad spectrum of activity, completely inhibiting 80% of *E. coli* strains and significantly suppressing the growth of 90% of *E. coli* isolates. According to Dąbrowska (2019), phages are frequently found in the liver and spleen. The most effective phages are commonly found in these internal organs, even at concentrations more significant than those seen in the blood. Following systemic delivery, the phage can reach the spleen and liver in minutes and get relatively high titers in 1 to 3 hours (Tiwari et al., 2011). Typically, the spleen is the organ where live phages may be identified for the greatest period, even days after phage treatment (Trigo et al., 2013).

In contrast with the present study (Table 2), Kim et al. (2013) demonstrated that FI and FCR were not affected by phage treatment (0.05%, 0.1%, and 0.2%; 10^9 pfu/g) to broiler diets. The inclusion of phages at 0.05 and 0.1% in the feed did not affect the FI and FCR of broilers (Upadhaya et al., 2021). However, these authors showed a significant increase in BWG with an increase in bacteriophage supplement levels during the starter and slaughter phases. In contrast, Huff et al. (2004) suggested that BWG was not affected by the addition of either DAF6 and SPR02 phages via intramuscular injection (3.7 × 10^9 and 9.3 × 10^9 pfu/ml, respectively) in broiler chickens without *E. coli* infection. Moreover, Lau et al. (2010) discovered that chickens infected with *E. coli* but not treated with phage had the smallest weights at 14 and 21 days of age. The chicken weight was not significantly different between the *E. coli*-infected but bacteriophage-treated and the negative control groups.

In the context of the genomes, one may worry about the safety of using phages in chickens or other animal species. This issue is not covered in the present study; however, according to Summers (2001), the majority of phages identified to date are lytic and only a small proportion are capable of integrating their DNA into host chromosomes. Moreover, sequencing can support to eliminate the usage of phages whose genomes encode known harmful products,

such as toxins, transposases, or repressor proteins (Krylov et al., 2014). Previously, Krylov et al. (1993) also pointed out that large-scale manufacturing of phages results in a small proportion of mutated phages. The mutation process typically makes the phage inactive but not stronger, and the most commonly reported mutation renders phages incapable of infecting bacteria. Approximately 10% of phage particles undergo such mutations during large-scale production, but this process often does not compromise phage safety.

CONCLUSIONS AND RECOMMENDATIONS

The application of phage therapy in native Noi broilers considerably reduced mortality, as well as the severity of gross lesions in chicken infected with *E. coli* strain of serotype O6, and contributed to improving growth performance and carcass characteristics of infected birds. This study confirmed the beneficial effects of bacteriophages in controlling *E. coli* infection in native broilers, which have a slower growth rate but superior meat quality.

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NOVELTY STATEMENT

The isolated MHH6 and PR2 phages displayed a broad host range and the ability to lyse O1, O78, and O6 *E. coli* strains yet further *in vivo* testing still was required. The study revealed that phage treatment in native Noi broilers dramatically reduced mortality and the severity of gross lesions in chickens infected with the serotype O6 *E. coli* strain, and led to enhanced growth performance in infected birds.

AUTHOR'S CONTRIBUTION

LHA and NTN contributed to the study's design. The data collection and analysis were carried out by LHA, TTHM, LMT and LTTL. The first draft was prepared by LHA, HTL, and NHX, and the manuscript was corrected by NTN. The final manuscript has been read and approved by all authors.

Conflict of interest

The authors have declared no conflict of interest.

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