Characterization of a novel bacteriophage φCJ22 and its prophylactic and inhibitory effects on necrotic enteritis and *Clostridium perfringens* in broilers

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ABSTRACT High necrotic enteritis (**NE**) incidence and mortality rates in poultry can be caused by *Clostridium perfringens* (**CP**) coinfected with *Eimeria* spp., a causative agent of coccidiosis. Banning of prophylactic use of antibiotics in feed has been accompanied by increased NE outbreaks, resulting in economically devastating losses to the broiler industry. To determine alternatives for controlling NE, we isolated CP-specific bacteriophages (BP), characterized their properties, evaluated their inhibitory effects on pathogenic CP, selected a highly effective phage (φCJ22), and used φCJ22 as a dietary supplement in experimental NE-afflicted broiler chickens. Male broilers (n = 780) were randomly assigned to 60 pens (n = 13 broilers/pen) and into 5 groups [CP-uninfected negative control (**NC**), basal diet (**BD**) without CP and BP; CP-infected positive control (**PC**), BD + CP; and 3 BP groups receiving low- (**LP**; BD + CP+10^5 BP), medium- (**MP**; BD + CP+10^6 BP), and high-phage (**HP**; BD + CP+10^7 BP plaque-forming units/kg) concentrations]. The results showed that MP and HP groups presented an antimicrobial activity toward clinical CP isolate strains, and the groups decreased NE lesions and mortality rates without changes in chicken performance at the end of the experimental period. After CP-challenge body weight gain and feed efficiency were significantly lower in phage-fed groups than that in the PC group ($P < 0.05$), and NE-associated mortality was the lowest in the HP group ($P < 0.001$). Moreover, histopathology revealed lesser gastrointestinal mucosal damage in the NC and BP-treated (LP, MP, and HP) groups than that in the PC group, and MP and HP significantly lowered viable CP number in the cecum content by up to 1.24log10 relative to only CP-infected PC group ($P < 0.05$). These findings suggest that addition of φCJ22 to chicken feed might effectively ameliorate NE, which is accompanied by reduced CP strains in the gut and compensate the performance of NE-afflicted broilers.

Key words: *Clostridium perfringens*, *netB*, broiler chicken, necrotic enteritis, bacteriophage

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INTRODUCTION

Necrotic enteritis (**NE**) is a common and notorious disease resulting from intestinal mucosal damage caused by *Clostridium perfringens* (**CP**) coinfected with *Eimeria* spp., a causative agent of coccidiosis. The coinfection can lead to high NE incidence and mortality rates in poultry (Al-Sheikhly and Truscott, 1977; Jackson et al., 2003). C*lostridium perfringens* is a gram-positive, anaerobic, rod-shaped, spore-forming, nonmotile bacterium that mainly causes diarrhea and NE in poultry, leading to decreased rates of feed digestion, absorption, and efficiency as well as production (Elwinger et al., 1992; Kaldhusdal et al., 2001; Van Immerseel et al., 2004). Generally, CP induces high NE incidence and mortality after infection with *Eimeria* spp., which causes coccidiosis (a common protozoan disease) in broiler flocks (Jackson et al., 2003; Van Immerseel et al., 2004). The use of subtherapeutic concentrations of antimicrobials in food-producing animals has resulted in the animal food supply meeting the growing demand

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for animal protein via the prevention of infectious diseases, including NE (Hao et al., 2014). The per capita consumption based on retail weight and annual production of broiler meat in the United States during 2019 reached 95.1 pounds and 43,905 million pounds, respectively, which was the highest among meats (beef, 58.1 and 27,155 million pounds; pork, 52.4 and 27,638 million pounds; and turkey, 16.0 and 5,818 million pounds, respectively) (USDA-ERS, 2020).

Necrotic enteritis in chickens had previously been sustainably controlled with antimicrobials in recent decades; however, restrictions on the use of antimicrobials in animal feed have increased outbreaks of infectious diseases, including NE, resulting in tremendous economic losses to the broiler industry because of low meat production due to impaired feed conversion and reduced body weight (**BW**) (Elwinger et al., 1992; Wade and Keyburn, 2015). Moreover, the ban on antibiotic use in animal diets initiated by the European Union (EU Commission, 2005) has been expanded globally. Consequently, anticoccidial use in animal feed for prevention of disease and growth promotion can be restricted due to increased consumer and public health concerns (Van Immerseel et al., 2004). The ionophore coccidiostats, such as narasin, salinomycin, or monensin, inhibit pathogenic bacterial infection by disrupting the osmotic balance and remain in use by the chicken industry as growth promoters (VKM, 2015); however, the use of anticoccidial agents in animal diets might also be subsequently limited by global public concerns associated with increases in the number of antimicrobial-resistant bacteria (including nontyphoidal *Salmonella* spp., *Campylobacter* spp., *Staphylococcus aureus*, and *C. perfringens*) and coselection of antibiotic resistance by ionophores (Bae et al., 2015; VKM, 2015; Nhung et al., 2016; Aslam et al., 2018). Consequently, alternative methods (i.e., bacteriophages (**BP**), vaccines, or probiotics) for preventing NE are needed and require assessment (Hoang et al., 2008; Miller et al., 2010; Wang et al., 2017).

Phage therapy using BP was developed and used to treat pathogenic bacterial infections before the first discovery of antimicrobials in the 1910s (Salmond and Fineran, 2015). Antimicrobials exhibiting broad-spectrum antibacterial activity have suppressed the development of phage therapy, although recently, phage therapy has been renewed owing to increased antimicrobial resistance (Miller et al., 2010; Aslam et al., 2018). Bacteriophages are specialized viruses that infect and destroy targeted bacteria, which ensures the safety of phage treatment in the fields of human and veterinary medicine, agriculture, aquaculture, and the food industry (Salmond and Fineran, 2015). Phages can replicate inside host bacteria and ultimately destroy the host through a lytic infection cycle. Phages generally have a narrow host range; however, this range varies depending on the relationship between phage infectivity and the genetic diversity of hosts at the bacterial genus, species, or strain levels (De Jonge et al., 2019). Phage cocktails comprising multiple phages represent one method for broadening phage host range. There have been few studies focused on developing BP or their lysins against CP strains closely related to NE in broilers (Miller et al., 2010; Zimmer et al., 2002b). Miller et al. (2010) developed a BP cocktail ('INT-401') that reduced mortality rates and improved the body weight gain (**BWG**) and feed-conversion ratio (**FCR**) in CP-challenged broiler chickens (Miller et al., 2010).

To the best of our knowledge, no study has been performed to confirm the effects of a CP-specific BP on the reduction of the number of viable CP strains in the gut of NE-afflicted chickens. In the present study, we characterized a novel BP ('φCJ22') and evaluated the antibacterial effect of the phage against various CP isolates with production of NE-afflicted broiler chickens. In addition to broiler mortality and performance, we enumerated the challenge CP strain in the intestinal tissues using culture-based methods in the presence and absence of phage treatment.

MATERIALS AND METHODS

Isolation of the CP-Specific Phage φCJ22

Among the several BP isolated from various environmental niches, phage φCJ22 was isolated from a fecal sample at a chicken farm in South Korea. The procedures used for φCJ22 isolation are described in a patent application publication (Son et al., 2016). Briefly, ~7 g of chicken feces were diluted with 42 mL of saline-magnesium (**SM**) buffer (G-Biosciences, St. Louis, MO) and centrifuged at 4,000 × *g* for 10 min; the supernatant was filtered using a 0.45 μm filter (Millipore, Billerica, MA). An 18 mL mixture of the filtered sample solution and 150 μL of a clinical CP isolate [CP BCCP 17-1 (**CJ16**); obtained from the Korean Animal and Plant Quarantine Agency (Supplementary Table 1)] were anaerobically incubated at 37°C for 18 h. The optimal density of the clinical CP culture was 2 at 600 nm. The culture solution was then centrifuged at 4,000 × *g* for 10 min, and the supernatant was refiltered using a 0.45 μm filter. The CP plus fecal filtered supernatant (150 μL) was added to 5 mL of 0.7% agar (w/v), and the mixture was poured into a brain-heart infusion (**BHI**) agar (Difco Laboratories, Detroit, MI) plate with 0.2% sheep blood and allowed to harden, after which 10 μL of the filtered culture sample was dropped onto the soft agar and incubated at 30°C for 18 h. A single plaque was selected to isolate the single BP using a soft agar overlay assay as described previously (Guenther et al., 2009). The assay was repeated 3 times to purify the single phage. The purified lysate was stored in SM buffer containing 20% glycerol at −20°C until use. The viral titer of the phage was expressed as plaque-forming units per milliliter, gram, or kilogram (pfu/mL,/g, or/kg) of the mixture, intestinal digesta, or feed, respectively. The phage was deposited at the Korean Culture Center of Microorganisms under deposition number KCCM11364P.

Morphological, Genetic, and Protein-Pattern Analyses of Bacteriophage φCJ22

For the morphological observation of φCJ22, the purified phage was suspended in a 0.01% gelatin and fixed with a 2.5% glutaraldehyde. Five microliters of the fixed BP were then dropped on a carbon-coated mica plate, incubated for 10 min, and washed with sterile ddH_2O. The carbon film was placed on a copper grid, stained using 4% uranyl acetate for 30 s to 60 s, and dried. The morphology of φCJ22 was observed by transmission electron microscopy (JEM-1011; JEOL, Peabody, MA). The whole genome of φCJ22 was sequenced using a Roche GS-FLX 454 DNA sequencer (Roche Diagnostics, Basel, Switzerland), and sequence reads were assembled using GS *de novo* assembler software (Roche). Open reading frames were analyzed using GeneMark.hmm (http://exon.gatech.edu/GeneMark/), GLIMMER (v.3.02; http://ccb.jhu.edu/software/glimmer/index.shtml), and FGENESB (http://www.softberry.com/berry.phtml?topic=fgenesb&group=programs&subgroup=gfindb) software, and open reading frames were identified using BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) and InterProScan (https://omictools.com/interproscan-tool) to annotate protein sequences. For protein-pattern analysis, 15 μL of purified phage particles (10^{10} pfu/mL titer) was mixed with 3 μL of 5 × SDS sample loading buffer (Bio-Rad Laboratories Inc., Hercules, CA) and boiled for 5 min. Total protein of the phage was subjected to 15% SDS-PAGE, followed by staining of the gel with Coomassie Brilliant Blue (Bio-Rad Laboratories Inc.) for 1 h at room temperature.

Antibacterial Activity of CP-specific φCJ22

The antibacterial activity and specificity of BP φCJ22 against 45 wild-type CP strains (Supplementary Table 1) isolated from the gut material, feces, farm sewage, and drainage water of poultry processing facilities in South Korea were determined by plaque (spot) assay as previously published in our patent application (Son et al., 2016). Briefly, 10 μL of φCJ22 suspension (1.0×10^{10} pfu/mL) mixed with 150 μL of each CP culture was added to soft agar, which was poured onto a BHI agar plate and incubated at 30°C for 18 h, followed by observation of plaque(s) on the plates.

Bacteriophage φCJ22 Stability in Accordance With pH, Temperature, and Drying Conditions

We investigated the stability of φCJ22 at a wide range of pH, temperatures, and drying conditions. To confirm the stability at various pH values, 10 μL of the phage suspension (1.0×10^9 pfu/mL) were mixed with 90 μL of various pH solutions (0.2 mol sodium acetate buffer, pH 4.0, 5.5, and 6.4; 0.2 mol sodium phosphate buffer, pH 6.9 and 7.4; and 0.2 mol Tris-HCl, pH 8.2, 9.0, and 9.8) and incubated at room temperature for 30 min, 1 h, and 2 h. The reaction solution was serially diluted with the various pH solutions, 10 μL of the diluted solution at different time points was dropped onto BHI agar plate that was incubated for 18 h at 30°C, and phage activity was measured by counting the number of plaques formed using a soft agar overlay assay. To assess the temperature stability of the phage at 60°C, 200 μL of φCJ22 (1.0×10^8 pfu/mL) with SM buffer were incubated for 10, 30, 60, and 120 min, followed by determination of titer using a soft agar overlay assay with the same procedures used to evaluate pH effects. To assess phage stability during drying, we used a speed-vacuum concentrator 5301 (Eppendorf, Hamburg, Germany) to dry 100 μL of φCJ22 suspension (1.0×10^8 pfu/mL), with the dried pellet resuspended in a volume of SM buffer equal to that of the original phage suspension, followed by performance of a soft agar overlay assay using the previously described procedures.

Rates of φCJ22 Recovery From Chicken Feed

A prepared phage suspension was lyophilized, and phage powder was supplemented with excipient for direct application to animal feed (Son et al., 2016). The recovery rates of φCJ22 from feed during the entire study period were evaluated weekly in accordance with the same procedures described for phage isolation and purification. In addition, viral titer was determined using agar overlay plaque assay previously described. We used 100 g phage-treated diets [low-phage (**LP**), medium-phage (**MP**), and high-phage (**HP**) containing 10^5 pfu/kg, 10^6 pfu/kg, and 10^7 pfu/kg BP, respectively] collected from the final phage-mixed chicken diets. Three independent analyses of spiked-phage recovery from LP, MP, and HP diets were performed. For the spiked-phage recovery rates, BP were mixed in treatment diets (LP, MP, and HP containing 10^5 pfu/kg, 10^6 pfu/kg, and 10^7 pfu/kg BP, respectively]. BP titers from each chicken diet were determined weekly for 4 weeks using the agar overlay plaque assay.

Bacterial Strains and NE-inducing CP Selection

A total of 45 clinical strains (Supplementary Table 1) of CP isolated from chicken, cow, pig, and lamb were used to select a highly virulent CP strain capable of causing NE. The CP isolates were maintained in BHI broth with 20% glycerol and stored at −70°C until use. Isolates were grown in the Whitley DG250 Anaerobic Workstation (Don Whitley Scientific Ltd., Shipley, UK) at 37°C with a gas mixture of 90% N_2, 5% H_2, and 5% CO_2 as previously described (Chon et al., 2018). CP KJW-1 (**CJ17**) among the 45 clinical isolates (Supplementary Table 1) was selected as a challenge strain, and a small-scale pilot study confirmed its

pathogenicity for inducing NE in broilers (data not shown). The clinical isolate strain CJ17 harbored the *netB* gene. The gene was confirmed with *netB* primers (forward: 5′-CGCTTCACATAAAGGTTGGAAGGC-3′; reverse: 5′-TCCAGCACCAGCAGTTTTTCCT-3′). Strain CJ17 was maintained in fluid thioglycolate (**FTG**) broth (Sigma-Aldrich, St. Louis, MO) with 20% glycerol until use. Fifty microliters of the CJ17 frozen stocks were inoculated in 5 mL of FTG broth and anaerobically incubated at 37°C for 16 h. One milliliter of the culture grown in the FTG broth was inoculated in 25 mL of FTG broth for 24 h, after which 25 mL of the cultures grown in the FTG broth were poured into 700 mL of FTG broth, anaerobically incubated at 37°C for 10 h, and used for oral gavage to induce NE in broiler chicks. *Clostridium perfringens* number was expressed as the mean of cfu per gram or milliliter of intestinal digesta or bacterial culture, respectively.

Animals, Diets, and Experimental Design

A total of 780 one-day-old male broiler chicks (Ross 308) were obtained from a local hatchery (Samhwa Co., Hongseong, South Korea), individually weighed on arrival, and randomly assigned to one of 5 experimental groups [CP-uninfected negative control (NC), basal diet (BD) without CP and BP; CP-infected positive control (PC), BD + CP; and 3 BP groups receiving low- (LP; BD + CP+10^5 BP), medium- (MP; BD + CP+10^6 BP), and high-phage (HP; BD + CP+10^7 BP pfu/kg of diet) concentrations] with 12 replicates and housed in one of 60 floor pens (n = 13 chicks/pen). The experiment duration was 5 weeks, with the temperature in the broiler chicken house maintained at 30°C for the first week, followed by 3°C decreases weekly until reaching 24°C on day 21, after which the temperature was maintained until the end of the experiment (day 35). Chicks were housed in floor pens (2 × 1 m) and on a 23/1 h light/dark schedule throughout the experimental period, with feed and water provided *ad libitum*. Maize, wheat, and soybean meal–based grower and finisher diets were prepared as basal diets, with the composition and nutritional levels shown in Table 1. Antibiotics were not added to any of the diets. The basal diet and the phages were supplied weekly from CJ CheilJedang Corp. (Suwon, South Korea). The experimental diets were mixed weekly and then fed to the broiler chicks. Body weight per pen was determined weekly, and feed intake (**FI**) per pen was monitored during the postchallenge period (day 17 to 21) and used to calculate the FCR. The experimental scheme involving inoculation of the coccidia vaccine (day 9) and CP (day 14–16) and recording of mortality and lesion scores (day 17 and 18) is shown in Figure 1. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Konkuk University, Seoul, S. Korea (animal welfare assurance number: KU19009).

Table 1. Ingredients and composition of the basal diets.

Ingredients, %	Grower diet (0–21 d)	Finisher diet (22–35 d)
Corn	26.50	44.88
Wheat	30.05	15.00
De-fatted rice bran	—	3.00
Distiller's dried grains with solubles	5.00	1.10
Soybean meal 44% CP	22.80	15.00
Rapeseed meal	3.00	5.00
Corn gluten meal	—	3.50
Meat and bone meal	6.45	6.50
Tallow	3.70	3.70
Lysine, 55%	0.47	0.53
Methionine, 90%	0.30	0.25
Limestone	0.90	0.87
Monocalcium phosphate	0.33	0.20
Salt	0.28	0.25
Vitamin and mineral premix[1]	0.22	0.22
Total	100.00	100.00
Calculated nutrient composition, %		
AMEn (kcal/kg)[2]	2,829.02	2,996.82
Dry matter	89.21	89.00
Crude protein	22.56	21.17
Crude fat	6.92	7.40
Ash	5.36	4.91
Total phosphorus	0.63	0.64
Available phosphorus	0.21	0.21
Digestible lysine	1.15	1.05
Digestible methionine	0.56	0.53
Digestible Met + Cys	0.84	0.80

Vitamin A, 20,000 IU; vitamin D3, 5,000 IU; vitamin E, 30,000 ppm; vitamin K, 4,000 ppm; vitamin B1, 40,000 ppm; vitamin B2, 10,000 ppm; niacin, 70,000 ppm; pantothenic acid, 20,000 ppm; biotin 200 ppm; folic acid, 1,200 ppm, vitamin B12, 30,000 ug, Zn, 54,000 ppm; Fe, 54,000 ppm; Mn, 78,000 ppm; Cu, 8,000 ppm; Iodine, 1,200 ppm; Se, 180 ppm.

[1]Vitamin and mineral premix per kg.

[2]AMEn, nitrogen-corrected apparent metabolizable energy.

NE-Challenge Model

As shown in Figure 1, 1 mL of phosphate-buffered saline (**PBS**) containing 10 × live and nonattenuated anticoccidial vaccine (Coccivac-D; MSD Animal Health, Madison, NJ) with ∼20,000 sporulated oocysts was administered to broiler chicks at 9 d after hatching. Three mL of CJ17 (1.0×10^8 cfu/mL) culture were administered twice daily on day 14, 15, and 16 via oral gavage to induce subclinical NE. The *Eimeria* oocyst vaccine contains *E. acervulina*, *E. tenella*, *E. maxima*, *E. necatrix*, *E. brunetti*, *E. hagani*, *E. praecox*, and *E. mivati*. Chicks in non–CP-challenged (NC) group were inoculated with PBS and/or FTG broth. All (LP, MP, and HP) groups, except NC and PC, were treated with BP φCJ22 at different concentrations (10^5–10^7 pfu/kg) through their feed.

Sample Collection

A chick closest to the group average BW was randomly selected from each pen, euthanized by CO_2 asphyxiation on day 18. The jejunal, ileal, and cecal tissues were collected from the 5 groups to determine changes in the intestinal metabolites, lesion scores, CP numbers, and histopathology and stored at 4°C. The ileal and cecal digesta in the intestinal segments from 6 chickens from each group were immediately transferred from the KU poultry farm to analytical laboratory to determine the number of challenge CP strains. The ileal (approximately 5 cm after Meckel's diverticulum) segments and whole cecal tissues were collected from 6 chickens per treatment and fixed in 10% neutral-buffered formalin for histological analysis.

NE-Lesion Scoring and Mortality

Lesion scoring was conducted after oral gavage (day 14-16) of CP (Figure 1). On day 17 and 18, a chick from each pen was humanely euthanized. Jejunum tissues (approximately 15 cm before Meckel's diverticulum (middle part of the small intestine) were collected, gently washed with distilled water, and measured for lesion scoring (range: 0–4) (Xu et al., 2015) by 3 observers in a blinded fashion. After CP challenge, all dead chicks were necropsied to observe NE lesions to determine the cause of death. All NE-afflicted dead chicks exhibited the highest lesion scores but were not included in the results of the lesion scores as they were recorded in the NE-associated mortality.

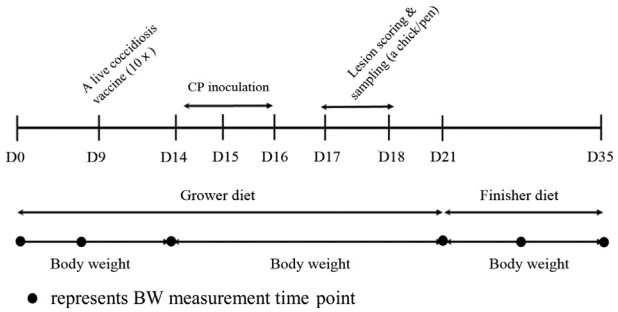

Figure 1. Experimental design and schedule. Broiler chickens were orally administered 10 × nonattenuated anticoccidial vaccine (20,000 sporulated oocysts) at 9-d after hatching, followed by *Clostridium perfringens* (CP) (1.0×10^8 cfu/mL) twice daily at day 14, 15, and 16 after hatching. Sixty broilers collected from 60 pens (5 groups with 12 replicates) were euthanized using CO_2 on day 17 and 18 for lesion scoring and collection of intestinal samples. Chicken were fed grower and finisher diets during the experimental period (day 0 to 21 and 22 to 35, respectively). The average BW of a broiler chick from each pen was recorded weekly until the end of the experiment.

Morphological Changes in the Intestinal Mucosa of Broiler Chicken

Morphological changes in the ileal and cecal tissues from 6 broilers of each group were analyzed. The tissues of ∼5 cm length were stained with hematoxylin and eosin (**H&E**). Images were taken using a microscope (Olympus BX43F, Japan). For determination of the villus length and crypt depth of the ileum and cecum in broilers, lengths were calculated using ImageJ software (NIH, Bethesda, MD). The average length of tissue samples was obtained from 3 different sections per each tissue sample ($n = 6$) and expressed as the mean ± SEM.

Clostridium perfringens *Counts in the Ileum and Cecum Samples*

Clostridium perfringens strains, including CJ17 used for CP challenge, were recovered (Chon et al., 2018) and counted using the culture-based method. On day 17 and 18, ∼3 cm to ∼5 cm of ileal and cecal tissues from 6 chicks of each group were collected, stored on ice, and moved to the laboratory within 4 h after collecting samples. Approximately 500 mg of ileal and cecal digesta were collected into 15 mL conical sterile polypropylene centrifuge tubes (SPL Life Sciences Co. Ltd., Pocheon-si, South Korea), diluted with 10-fold sterile PBS, and homogenized using a Vortex-Genie 2 vortex (Scientific Industries Inc., Bohemia, NY) at maximum speed for 15 s. The homogenates were serially diluted, and 100 μL of each homogenate was plated on tryptose-sulfite-cycloserine agar (Sigma) for CP selection in triplicate, followed by anaerobic incubation at 37°C for 48 h. The number of blackened CP colonies was determined by plate count (Chon et al., 2018). Two independent sets of samples from day 17 and 18 with 3 biological replicates ($n = 6$/group) were used.

Statistical Analysis

Pen was considered an experimental unit. All data were analyzed by analysis of variance, followed by Tukey's test using SAS (v.9.4; SAS Institute Inc., Cary, NC). Statistical significance was determined at $P < 0.05$. All data except recovery rates of φCJ22 from diets were expressed as the mean ± SEM.

RESULTS

Morphological, Biological, Genetic, and Protein-Pattern Characteristics of φCJ22

Transmission electron microscopy showed that the novel phage φCJ22 morphologically belongs to the family *Siphoviridae*, containing linear double-stranded DNA and having an isometric capsid and a long and noncontractile tail (Supplementary Figure 2). Phage evaluation under various stress conditions demonstrated its stability within a pH range of 4.0 to 9.8 for 2 h (Supplementary Figure 3A), and ∼10^8 pfu/mL of the phage remained stable during a 2 h incubation at 60°C, with a reduction of ∼1 log (pfu/mL) during incubation (Supplementary Figure 3B). In addition, phage titers decreased by ∼2 log (pfu/mL) after drying for 2 h (Supplementary Figure 3C). Analysis of the viral genome sequence and comparison with other BP revealed low levels of sequence homology, in agreement with a previous report (Son et al., 2016). Phage-protein analysis indicated the presence of proteins with molecular weights of ∼40, ∼51, ∼53, and ∼70 kDa (Supplementary Figure 4).

Antibacterial Activity of CP-specific φCJ22

The results indicated that 41 CP strains (91.1%) among 45 clinical isolate strains were infected and lysed by φCJ22. In addition, agar overlay plaque assays using soft agar showed clear plaques of φCJ22 in the lawn of one CP strain (ATCC 13124), and a spot assay demonstrated a φCJ22-dose-dependent increase in the size of clear spots (Supplementary Figure 5).

Recovery Rates of Phage φCJ22 From Experimental Diets

Figure 2 shows φCJ22 stability in treatment diets. Phage titers recovered from LP, MP, and HP diets were 5.3 log pfu/kg, 6.4 log pfu/kg, and 7.2 log pfu/kg, respectively. The mean viral titers of LP were 5.4, 5.2, 5.5, and 5.4 log pfu/kg at weeks 1, 2, 3, and 4, respectively, and those for MP and HP for the same time periods were 6.2, 6.0, 6.4, and 6.2 log pfu/kg and 7.3, 7.3, 7.0, and 7.1 log pfu/kg, respectively. Overall, phage-recovery rates from each of the diets throughout the experimental period did not differ significantly ($P > 0.05$).

Establishment of a Subclinical NE-Induced Animal Model

To establish an experimental model of subclinical NE in poultry, a CP strain harboring *netB* encoding a pore-forming toxin in the pathogen (Keyburn et al., 2008) was screened among the clinical isolates. PCR showed that the CJ17 strain harbored *netB* (data not shown). After administration of the coccidia vaccine and the CJ17 strain (1.0×10^8 cfu/mL), many focal NE and

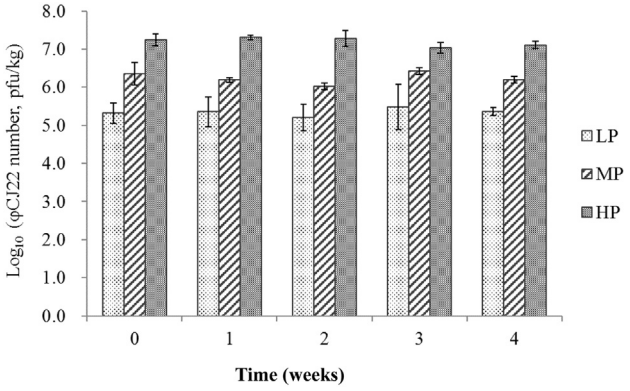

Figure 2. Recovery rates of φCJ22 from diets. Bacteriophage mixed in treatment diets [low-phage (LP), medium-phage (MP), and high-phage (HP) concentrations containing 10^5 pfu/kg, 10^6 pfu/kg, and 10^7 pfu/kg BP, respectively] were recovered from the diets throughout the experimental period. Scale bars represent the standard deviation of the mean.

hemorrhagic lesions in the gut of chicks were observed on day 17 and 18 (Figure 3), the small intestine was distended with gas (Figure 3A), hemorrhages were observed throughout the intestinal wall (Figure 3B), and most NE-afflicted mortalities occurred within 5 d after CP challenge.

Growth Performance

The growth performance of chicks in each group is shown in Table 2. Overall BW among groups did not differ significantly before CP challenge ($P > 0.05$). At day 21, the BW of broilers in the PC group was significantly reduced relative to that of the NC group. In addition, LP and HP showed an NE-induced growth inhibition ($P < 0.05$); however, there was no significant difference in growth between the NC and MP groups (Table 2). At day 35, BW in chicks from HP group were compensated, whereas chicks from the LP group have no compensation in BW up to the end of experimental period relative to the NC group ($P < 0.05$). A difference in BW was not significant among the BP-treated groups. On day 1 to 5 after CP challenge (day 17–21), BWG and FI (but not the FCR) between groups were significantly decreased in the NE-induced chickens relative to the NC group ($P < 0.05$). Moreover, the average BWG (g/d/bird) between groups (NC vs. PC/BP-treated groups and PC vs. BP-treated groups) differed significantly ($P < 0.05$), indicating that chicks in the NC, PC, LP, MP, and HP groups gained 53.84 g, 40.6 g, 44.96 g, 46.72 g, and 46.74 g daily, respectively (Table 2). Furthermore, the average BWG in chicks from the PC group relative to that in the NC group decreased significantly by up to 24.6% after CP infection, whereas the BWG of the BP-treated and CP-challenge groups increased by up to 15.1% relative to the PC group ($P < 0.05$).

From day 15 to 21, we observed a considerable difference in average FI between CP-challenged and -unchallenged groups ($P < 0.05$) but no significant difference

Figure 3. Focal necrotic enteritis and hemorrhagic lesions in broilers from the only *Clostridium perfringens*–challenged group. (A) Intestinal tissues appear glossy, gas-filled, and sloughed off. (B) Hemorrhagic lesions in the intestines.

between BP-treated (LP, MP, and HP) and -untreated (PC) groups ($P > 0.05$). In addition, FCR differed significantly between NC and PC groups ($P < 0.05$) at 1 wk after CP challenge (Table 2), and FCR in the BP-treated and CP-challenge groups was lower than that in the NC group ($P > 0.05$), although not significantly.

Mortality and NE-Lesion Scores

Table 3 shows mortality rates and NE-lesion scores in the jejunum of broilers in each group. Overall mortality rates were significantly decreased in the MP and HP groups relative to PC in accordance with the data presented in table 3. Data of the overall mortality rates in all groups showed similar patterns to those for NE-associated mortality ($P < 0.05$). The NE-related mortality rates in only CP-infected PC was significantly higher than those in the NC and BP-treated groups except LP, and chicks fed higher titers of φCJ22 in the HP group showed lower mortality rates than those in the LP group (Table 3). In addition, overall and NE-associated mortality rates in chicks were also significantly decreased in the MP and HP groups ($P < 0.05$) relative to the LP group, and the rates did not differ ($P < 0.05$) from those in the NC (Table 3). Although there was no significant difference in mortality rate between the PC group and that receiving the lowest phage concentration (LP), the mortality rate was the highest in PC chicks (6.41%), followed by LP (3.85%) and MP (2.56%). Moreover, there were no NE-related deaths in the NC and HP groups. Necropsy of chicks performed on day 18 revealed lesion scores in the PC group as being the highest (Table 3). On day 18, lesion scores in the PC group were significantly higher than those in the BP-treated groups ($P < 0.05$), although there was no significant difference between BP-treated groups (Table 3). Images of the NE lesions showed severe (multifocal) and mild lesions in the PC- and phage-treated groups, respectively (Figure 4).

Histopathology of Intestinal Mucosal Tissues

To evaluate intestinal integrity, the intestinal tissues from the ileum and cecum of broilers in each group were histologically examined, with the results of H&E staining shown in Figure 5. The data demonstrated less intestinal mucosal damage in the NC and BP-treated and CP-challenge groups relative to that in only the PC group. Moreover, the architectures of the

Table 2. Effect of dietary φCJ22 on the growth performance of NE-afflicted broiler chicken.[1]

			Bacteriophage (pfu/kg)				
			10^5	10^6	10^7		
Item	NC	PC	LP	MP	HP	SEM	*P*
BW, g/bird							
D1	41.05	41.14	40.92	40.87	40.99	0.142	0.691
D7	147.3	143.0	140.6	142.2	143.3	1.628	0.065
D14	312.0	308.5	319.5	328.0	315.5	6.504	0.281
D21	688.9^{a}	592.7^{c}	634.2^{b}	$655.0^{a,b}$	642.7^{b}	14.41	0.001
D35	$1,826^{a}$	$1,686^{b}$	$1,737^{b}$	$1,750^{a,b}$	$1,747^{a,b}$	27.75	0.021
At a week after CP challenge (days 17 to 21)							
BWG, g/d/bird	53.84^{a}	40.60^{c}	44.96^{b}	46.72^{b}	46.74^{b}	1.472	<0.0001
FI, g/d/bird	89.32^{a}	77.40^{b}	72.15^{b}	73.62^{b}	71.73^{b}	2.057	<0.0001
FCR, g/g	1.661^{b}	1.913^{a}	1.614^{b}	1.582^{b}	1.545^{b}	0.037	<0.0001

Abbreviations: BWG, body weight gain; FCR, feed conversion ratio; FI, feed intake; HP, high-phage concentration (10^7 pfu/kg); LP, low-phage concentration (10^5 pfu/kg); MP, medium-phage concentration (10^6 pfu/kg); NC, CP-uninfected negative control; PC, CP-infected positive control.

[1]Values are least square means of 12 replicates. Means within a row not sharing a common superscript are significantly different ($P < 0.05$).

Table 3. Effect of dietary bacteriophage on mortality and lesion scores of NE-afflicted broiler chicken.[1]

			Bacteriophage (pfu/kg)				
			10^5	10^6	10^7		
Item	NC	PC	LP	MP	HP	SEM	*P*-value
Mortality, %							
Overall mortality	8.97^{b}	17.95^{a}	15.38^{a,b}	8.33^{b}	8.33^{b}	2.747	0.038
NE-associated mortality	0.00^{c}	6.41^{a}	3.85^{a,b}	2.56^{b,c}	0.00^{c}	1.247	0.002
Lesion scores							
D17	0.00^{c}	1.00^{a}	0.69^{b}	0.97^{a}	1.00^{a}	0.080	<0.0001
D18	0.00^{c}	1.58^{a}	0.96^{b}	0.96^{b}	1.00^{b}	0.148	<0.0001

Abbreviations: BWG, body weight gain; FCR, feed conversion ratio; FI, feed intake; HP, high-phage concentration (10^7 pfu/kg); LP, low-phage concentration (10^5 pfu/kg); MP, medium-phage concentration (10^6 pfu/kg); NC, CP-uninfected negative control; PC, CP-infected positive control.

[1]Values are least square means of 12 replicates. Means within a row not sharing a common superscript are significantly different ($P < 0.05$).

ileal and cecal mucosal layers differed considerably between the NC and PC groups (Figure 5), with marked disruptions in the intestinal villi and crypt lamina propria, diffused necrotic debris from tissues, and collapsed mucosal architecture in the PC and LP groups. By contrast, the architecture of the intestinal mucosa of chicks fed with BP-added diets resembled those of chicks in the NC group, with the similarity increasing in accordance with the BP dose (Figure 5).

Intestinal Bacterial Count

The lytic effect of φCJ22 on clinical CP isolate strains in the ileum and cecum is shown in Figure 6. *Clostridium perfringens* counts between the PC and BP treatment groups in the ileum were not statistically different ($P > 0.05$), whereas the cell counts between the PC and BP treatment groups except LP in the cecum were significantly different ($P < 0.05$). In addition, the average viable CP numbers in the cecal digesta of broilers fed with relatively high concentration phage (MP and HP) diets was significantly decreased up to 1.24log, relative to that observed in the PC group ($P < 0.05$). Comparison of total viable CP number between PC groups from the ileum and cecum showed that the number in the cecum was 1.64log greater than that in the ileum (Figure 6).

DISCUSSION

Legislative or voluntary bans on the prophylactic use of antimicrobials in animal feed have resulted in the re-

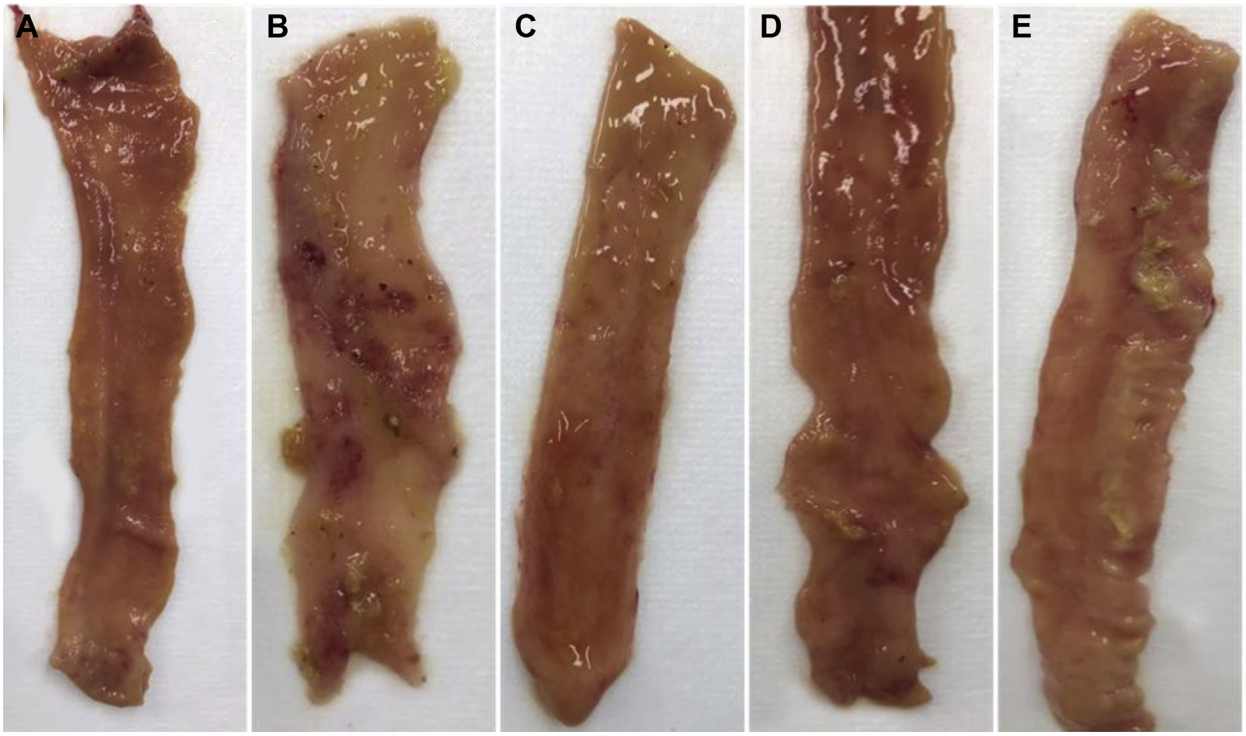

Figure 4. Effect of dietary φCJ22 on jejunal lesions in necrotic enteritis (NE)-afflicted broiler chicken. (A) CP-uninfected negative control (NC), (B) CP-infected positive control (PC), (C) low-phage concentration (10^5 pfu/kg), (D) medium-phage concentration (10^6 pfu/kg), and (E) high-phage concentration (10^7 pfu/kg).

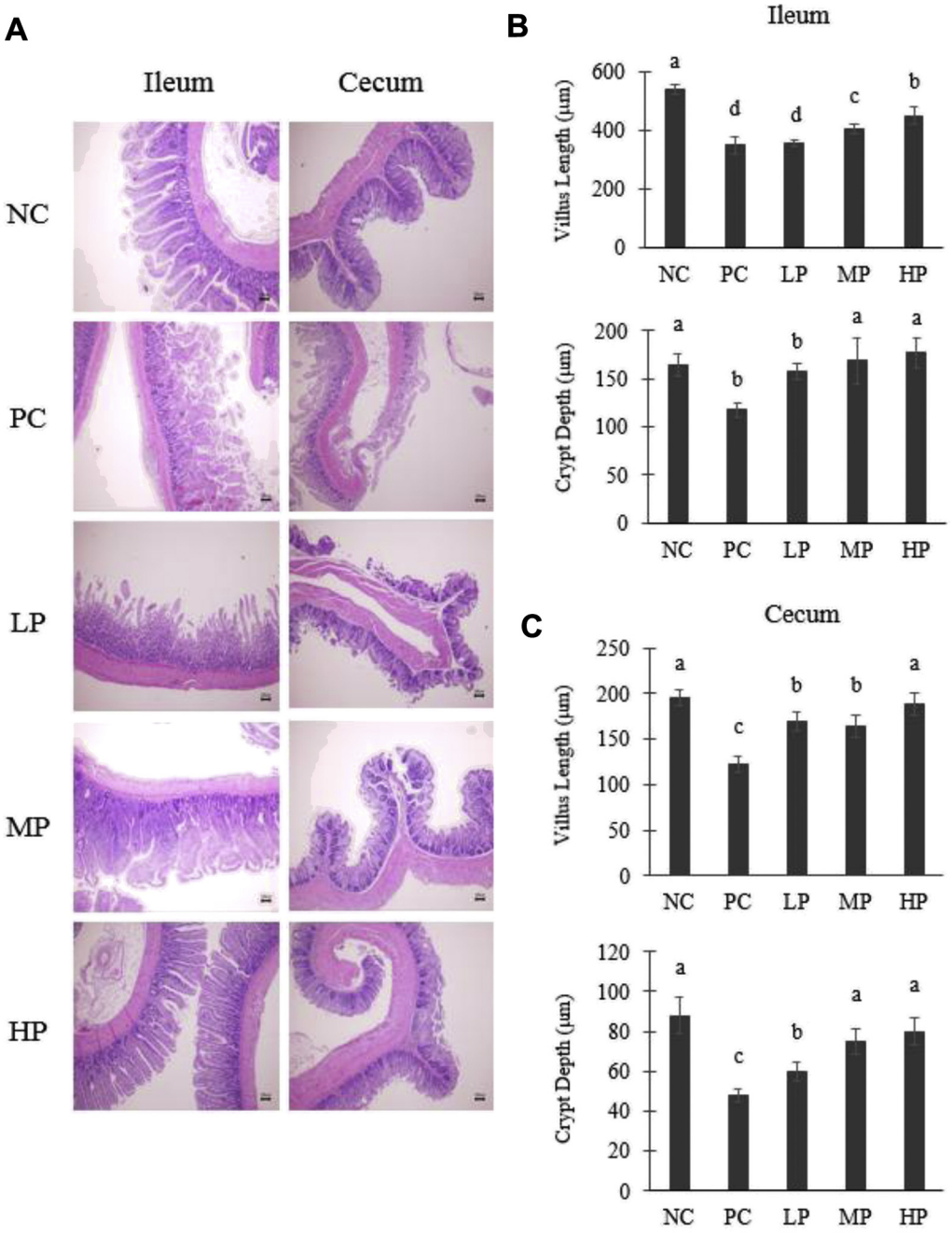

Figure 5. Changed architecture of the ileum and cecum of broiler chicken from each experimental group. (A) Histopathology of the intestine; CP-uninfected negative control (NC), CP-infected positive control (PC), low-phage (LP) concentration (10^5 pfu/kg), medium-phage (MP) concentration (10^6 pfu/kg), and high-phage (HP) concentration (10^7 pfu/kg) groups. Scale bar: 100 μmol. Magnification: 40 × . (B) Histopathological measurements in the intestine. Villus length and crypt depth in the ileum and cecum of broiler chickens from NC, PC, LO, MP, and HP groups. ImageJ software (http://imagej.nih.gov/ij/) was used to measure the villus length and crypt depth using the images of the ileum and cecum stained with H&E. Means within a panel with different letters in each of the lower and upper case letters differ significantly at $P < 0.05$.

emergence of NE accompanied by high mortality rates and increased global economic losses to the poultry industry (VKM, 2015; Wade and Keyburn, 2015). Coinfection with CP (Elwinger et al., 1992) and *Eimeria* spp. along with altered physicochemical properties of the diet, coccidiosis vaccine administration, immune status, farm management, and/or the presence of mycotoxins affect the risk of NE in chickens (Moran, 2014; Tsiouris, 2016; Rodrigues and Choct, 2018). Previous studies have evaluated the integrity of the intestinal mucosa and growth performance based on the feed efficiency in broiler chickens (Kaldhusdal et al., 2001; Miller et al., 2010; Gadde et al., 2017). The studies have examined alternative methods to replace antibiotics through the use of BP, competitive exclusion, synbiotics (probiotics and prebiotics), organic acids, antimicrobial peptides, and hyperimmune egg yolk IgY. We previously identified the novel CP-specific BP φCJ22 in accordance with DNA-sequence alignment and comparison of homologous genomic regions with

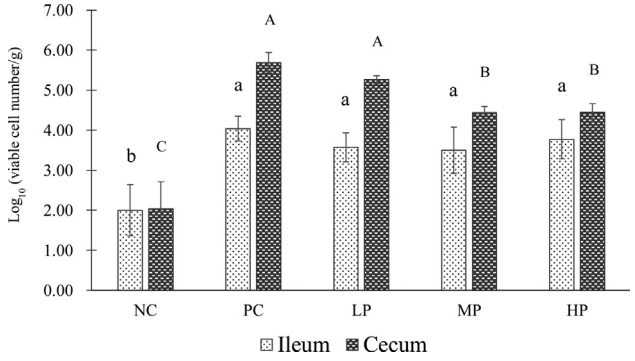

Figure 6. Comparison of the viable cell numbers of *Clostridium perfringens* (CP) strains for each experimental group in the ileum and cecum of broilers. CP strains collected from 1 g of the ileal and cecal digesta from CP-uninfected negative control (NC), CP-infected positive control (PC), low-phage (LP) concentration (10^5 pfu/g), medium-phage (MP) concentration (10^6 pfu/g), and high-phage (HP) concentration (10^7 pfu/g) groups. Two sets of tissue digesta samples with 3 replicates of chickens ($n = 6$) were used. Means within a panel with different letters differ significantly at $P < 0.05$. Error bars represent the standard error of the mean.

other phages (Son et al., 2016). In the present study, we evaluated its efficacy on NE control, growth performance, and reduction of CP strains in the gut of broilers.

Our results showed that CP-specific φCJ22 reduced CP counts or colonization of CP strains in the gut of broiler chicks. Few studies using BP have examined their efficacy at controlling CP strains in experimental models of NE-afflicted poultry (Miller et al., 2010; Zimmer et al., 2002b), with these reports indicating a narrow host range of the phages against CP strains (7.8–21.6%) (Zimmer et al., 2002a; Zimmer et al., 2002b). To extend their lytic activity against a wide range of pathogenic bacteria, the use of phage cocktails is necessary (Miller et al., 2010; Zimmer et al., 2002b). Our previous data demonstrated that φCJ22 exhibited broad antimicrobial activity against >90% of the CP strains evaluated (Son et al., 2016). It is possible that use of a variant with a wider host range might result in further decreases in the number of viable CP strains in the gut of broilers, on chicken farms, in raw and processed poultry products, and production-related environments, as well as possibly eliminating the pathogen from medical and veterinary environments. Furthermore, phage-directed CP knockdown in the gut could be a potential tool for altering the host health-related gut microbiota for the purposes of NE treatment. Identification of φCJ22 receptors on CP strains and their interaction with φCJ22 structural components associated with phage entry and/or lysin expression is required to elucidate the mechanisms associated with the broad host range of φCJ22.

Phage φCJ22 exhibited broad lytic activity against clinical CP isolates (Son et al., 2016) and reduced viable CP strains in the cecum of broiler chicks in the established NE experimental model (Figure 6), thereby confirming *in vitro* and *in vivo* inhibitory activity against pathogenic CP. Further studies on the effect of φCJ22 on the intestinal ecosystem that consists of a variety of bacteria would be needed because gut microbiota in chicks could be altered by a phage. Hsu et al. (2019) demonstrated that intestinal phages directly or indirectly influence the gut microbial structure and function. Consequently, the phages can modulate the gut metabolome (Hsu et al., 2019). It remains unknown how CP-induced NE alters the gut microbial structure; therefore, CP-specific phages might be useful for identifying specific bacterial species or protozoa associated with NE to characterize their function and to elucidate CP-mediated alterations to the microbiome.

In addition, phage φCJ22 might represent an alternative antimicrobial agent for NE treatment in chickens based on its high lytic activity against various wild-type CP strains. Moreover, we demonstrated the high level of stability of the phage under pH, temperature, and drying conditions (Supplementary Figure 3). Our results suggest that φCJ22 can be used to reduce CP contamination of poultry farms or food-processing plants as an alternative antimicrobial agent, disinfectant, or cleaner.

The integrity of the intestinal barrier is compromised by coccidial infection, which damages the intestinal mucosal layer, thereby enabling the penetration of pathogenic CP into the mucosal membrane to promote onset of severe NE lesions (Keyburn et al., 2008; Wu et al., 2016). Keyburn et al. (2010) reported *netB*-positivity in 70% (31/44) of CP strains from broilers afflicted with NE, whereas only ∼3.6% (2/55) of CP strains in healthy chickens harbored this gene. Through the present study, we used a live coccidia vaccine at 10 × dose, *netB*-positive CP, and a diet based on wheat and animal byproducts to induce subclinical NE in broiler chicks. Previous studies described NE-related mortality in established models ranging from 0% to 60% (Hofacre et al., 2003; Wu et al., 2010; Xue et al., 2018). Our study showed that CP-induced NE in experimental chickens severely disrupted the intestinal mucosa, depressed BWG and FI, and increased FCR, resulting in an NE-associated mortality rate of 6.41% and an average lesion score in the jejunum of 1.58 on a 4.0 scale (Figure 5 and Tables 2 and 3). These results suggested that the NE model established in this study might be suitable for the induction of subclinical NE.

We observed a decrease in BW of ∼7.7% and an increased FCR of ∼15.2% in our NE model after CP challenge (day 15 to 21) as compared with broiler chicks in the NC group (Table 2). Skinner et al. reported that subclinical NE was estimated to decrease BW by 12% and increase FCR by 10.9% (Skinner et al., 2010). Impaired absorption of available nutrients in the intestinal lumen can be directly caused by damage to the mucosal layer, goblet cells, and enterocytes in villi. The dual action of *Eimeria* spp. is to provide a suitable environment for CP infection and subsequent physical epithelial invasion by the virulence factors (Wu et al., 2010; Moran, 2014). In the present study, H&E staining revealed that small and large intestines exhibited a collapsed mucosal layer (including epithelial brush borders and enterocytes), which likely resulted in decreased BW and increased FCR following CP challenge and

relative to the NC group (Table 2). Interestingly, the degree of mucosal damage in BP-treated and CP-challenge groups decreased along with increases in the number of φCJ22 particles (Figure 5). The histological data suggested that phage φCJ22 was active on ingestion and demonstrated *in vivo* lytic activity against CP strains in the lumen, thereby positively affecting intestinal integrity and consequently leading to reduced mortality, lesion scores, and FCR. Feed intake in BP-treated and CP-challenge groups was significantly lower ($P < 0.05$) relative to the NC group. There was no significant difference in FCR between NC and BP-treated groups. It remains to be confirmed whether BP-mediated decreases in FI are BP-specific or a result of temporal BP intervention into the dynamics of the gut ecosystem. Previous studies reported no BP-specific effect on FI (Kim et al., 2013; Wang et al., 2013), and another study reported that BP supplementation of broiler diets increased BW in naïve chickens (Kim et al., 2014), chickens challenged with CP (Miller et al., 2010), and *Salmonella* Typhimurium (Toro et al., 2005). Based on our data, we found that φCJ22 can be suitable for use as a feed additive and exhibited activity in the lumen and against pathogenic CP, resulting in lower production of toxins, including those encoded by *netB*, to promote maintenance of normal gut integrity and gut morphology in BP-treated animals.

Clostridium perfringens strains were found in all groups, including NC (Figure 6). Although we found CP strains in the ileum and cecum digesta from the NC group, colony PCR data suggested that bacteria grown on tryptose-sulfite-cycloserine agar plates did not harbor *netB* (data not shown). Colony PCR using 5 colonies from 3 plates for each group verified colony origination from the challenge strain, revealing that all colonies from the NC group were *netB*-negative, whereas those from CP-challenged groups were confirmed as harboring up to 20% *netB*-negative CP strains (data not shown). Therefore, these *netB*-negative strains could have originated from the normal intestinal flora (Moran, 2014) or likely from the same clonal population of the challenged *netB*-positive strain (Keyburn et al., 2008). PCR is widely recognized as having sufficient sensitivity for bacterial quantification; however, it is limited in its ability to differentiate DNA from live or dead bacteria. Therefore, the enumeration of CP strains in the current NE-modeling study was performed using selective agar plates (culture-based methods) and confirmed with PCR to produce reliable results distinguishing CP-challenged from -unchallenged groups (data not shown). Further studies may be warranted to develop diagnostic tools capable of quantitatively and qualitatively measuring NetB toxin or *netB* in digesta samples for assessment of NE pathogenesis in chickens.

Our results suggest that the experimental model established in this study would be suitable for inducing subclinical NE, and that supplementation of chicken feed with the φCJ22 BP effectively reduced NE, compensated the performance in the NE-afflicted broilers, and decreased mortality without negatively affecting either the performance or intestinal integrity in broiler chicks. Additional data from field experiments are necessary to confirm the antieffect of the CP strains isolated from food-processing facilities or poultry farms.

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DISCLOSURES

The authors declare that they have no conflicts of interest.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at http://doi.org/10.1016/j.psj.2020.10.019.

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