


***Salmonella* phage CKT1 significantly relieves the body weight loss of chicks by normalizing the abnormal intestinal microbiome caused by hypervirulent *Salmonella* Pullorum**

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ABSTRACT Pullorum disease caused by *Salmonella* Pullorum remains an important disease for the poultry industry due to high morbidity and mortality in many countries. Phage therapy is becoming an alternative strategy to control multidrug-resistant *Salmonella* infections in young chicks. However, how bacteriophages affect the growth performance of chicks infected with *S. Pullorum* remains poorly understood. Herein, we assessed the therapeutic efficacy of *Salmonella* phage CKT1 against hypervirulent arthritis-causing *S. Pullorum*. The results showed that single phage treatment after hypervirulent *S. Pullorum* infection significantly improved body weight loss of chicks. Compared with enlarged liver and spleen in only *Salmonella* challenged group, phage administration substantially reduced the liver/body and spleen/body weight ratios, bacterial loads in organs and the degree of hepatic sinusoidal dilatation and congestion. Moreover, phage CKT1 can enter the organs of chicks and stay for at least 3 d in liver and spleen, and promote higher serum levels of IL-6 produc-

tion within 6 d postinfection, indicating phage-induced bacterial lysis may be involved in inflammatory immune response to *S. Pullorum* infection. Analysis of the microbiome of gastrointestinal tract of chicks demonstrated that *Salmonella* challenge significantly reduced the relative abundances of *Lachnoclostridium* and *Blautia*, resulting in remarkably increased *Escherichia-Shigella* and *Klebsiella* becoming the predominant bacterial taxa. In contrast, the use of phage CKT1 significantly reduced *Escherichia-Shigella* and *Klebsiella* populations in intestine, permitting the proliferation of beneficial microbiota in *Firmicutes* including *Lachnoclostridium*, *Ruminococcus*, *Lactobacillus*, and *Pseudoflavonifractor*. In addition, phage alone treatments did not affect the normal gut microbiota structure of chicks, and phage therapy on *Salmonella* infected chicks increased bacteria species richness in the cecum. These results suggest that *Salmonella* phage CKT1 could improve growth performance of chicks challenged with *S. Pullorum* by normalizing the abnormal intestinal microbiome.

Key words: *Salmonella* Pullorum, bacteriophage, chick, growth performance, intestinal microbiome

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INTRODUCTION

Poultry, the largest livestock population in the world, represents important sources of food and income in the world in general and in China in particular. However, many bacterial diseases (Salmonellosis, Colibacillosis, Mycoplasmosis, etc.) comprise approximately half of the non-outbreak-related mortality in broiler breeders and commercial layers, causing high economic losses in poultry production

(Thøfner and Christensen, 2021). Salmonellosis particularly pullorum disease (PD) and fowl typhoid are the most common diseases in poultry (Barrow and Freitas Neto, 2011). There are more than 2,600 serovars of *Salmonella enterica*; *Salmonella enterica* serovar Gallinarum biovar Pullorum (*S. Pullorum*), the causative agent of pullorum disease, could result in a high mortality rate among embryos and chicks, as well as weakness and white diarrhea in many developing countries (Barrow and Freitas Neto, 2011). Moreover, arthritis associated with *S. Pullorum* infection has become increasingly frequent in chicken flocks, especially in Chinese native chicken breeds (Guo et al., 2019). The hypervirulent arthritis-causing *S. Pullorum* isolated from Chinese native Qingjiaoma chicken breeds had greater negative effect on growth performance of chicks than white diarrhea-

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causing strain (Li et al., 2021). Due to the increased complexity and difficulty of salmonellosis control, new ways should be explored to reduce *Salmonella* contamination besides extensive testing and eradication programs in poultry farms.

For the increasing risk of antibiotic-resistant bacteria to public health, many countries have imposed a ban on the use of antibiotics as growth promoters in food-producing animals, resulting in more severe infections and great economic loss (Cui et al., 2021). Phage therapy, the use of bacteriophages for the treatment of bacterial infections, has gained a renewed interest as increased antibiotic resistance in bacteria (Young and Gill, 2015). Bacteriophages (phages) are viruses that are specific obligate bacterial parasites and usually possess high specificity for one bacterial species; thus, their use in treatment offers the specific targeting of a group of bacteria without affecting the normal microbiota (Kortright et al., 2019). Phages multiply inside the infected host cell in a so-called lytic infection cycle, and are released from host cells by bacteriolysis. It has been shown that phage has a profound impact on the dynamics of the gut microbiome, not only affecting certain species directly but also having a cascading effect on others (Hsu et al., 2019). In addition, phage- and bacterial-derived pathogen-associated molecular patterns released after bacterial lysis could stimulate local innate immune responses, thus promoting bacterial clearance (Roach et al., 2017; Krut and Bekeredjian-Ding, 2018).

Many trials with phages, including several commercially available *Salmonella* bacteriophages, have been conducted in poultry, and the positive effects of phage therapy have been observed in reducing mortality rate and *Salmonella* population in the intestinal tract of chicks (Nabil et al., 2018; Tie et al., 2018), combating horizontal infections induced by *Salmonella* in layer and broiler chickens (Lim et al., 2011, 2012; Hong et al., 2013), and reducing the contamination level of *Salmonella* in the environment and poultry carcasses (Henriques et al., 2013; Atterbury et al., 2020). It was also showed that phage treatment by coarse spray could significantly reduce the disease symptoms in the chicks and the incidence of *Salmonella* in the ceca (Borie et al., 2009; Henriques et al., 2013). However, how *Salmonella* phages shape the gut microbiome and the persistence of phages in organs of chicks remain poorly understood.

We previously showed that a hypervirulent arthritis-causing *S. Pullorum* 20JS04 isolated from Chinese native Qingjiaoma chicken breeds had greater negative impact on the growth performance of chicks compared with the white diarrhoea-causing *S. Pullorum* standard strain (Li et al., 2021). The current study aims to assess the therapeutic efficacy of *Salmonella* phage CKT1 against the hypervirulent *S. Pullorum* infection in young SPF White Leghorn chicks, and investigate the effects of *Salmonella* phage particles on gut microbiota.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Hypervirulent *S. Pullorum* strain 20JS04 was isolated from Chinese native Qingjiaoma chicken breeders with arthritis symptoms. The standard strain of *S. Pullorum* CVCC526 used as *Salmonella* phage CKT1 host for phage propagation was purchased from the China Veterinary Culture Collection Center (Beijing, China). All *Salmonella* strains stored at -70°C were firstly streaked onto xylose lysine desoxycholate (XLD) agar plate and incubated at 37°C for 24 h for subsequent tests.

Preparation of the Bacteriophage

Salmonella phage CKT1 (GenBank accession OK143508) was isolated from poultry sewage in Shandong province, China and exhibited a clear plaquing phenotype on both *S. Pullorum* host strain 20JS04 and CVCC526. To prepare *Salmonella* phage CKT1, fresh overnight culture of the host strain CVCC526 was inoculated at 1:10 into 3 mL of new LB media, then 0.1 mL of a 10^6 PFU/mL phage suspension was added to the tube. The culture was incubated at 37°C , 180 rpm for 4 h to allow phage propagation. Phage lysate was centrifuged at 10,000 g for 5 min, and the supernatant was filtered through a $0.22\text{-}\mu\text{m}$ filter. Phage titer was determined by decimally diluting the phage suspension in SM buffer (10 mM MgSO_4 , 100 mM NaCl, 0.01% w/v gelatin, 50 mM Tris-HCl, pH 7.5) and plating on the seeded top agar plates. Phage stocks can be stored at 4°C for 72 h before application.

Table 1. Experimental design of bacteriophage treatment in SPF chicks challenged with *S. Pullorum*.

Group	Treatment	Doses		Age of <i>Salmonella</i> infection	Treatment schedule of phage	Time of euthanasia
		<i>Salmonella</i>	phage			
1	Nonchallenged and nontreated control negative	-	-	-	-	d 3 and 6 postinfection
2	Only <i>Salmonella</i> challenged	$0.5\text{ mL } 2 \times 10^6$ CFU/chick	-	2nd day	-	
3	Only phage treated	-	$0.5\text{ mL } 2 \times 10^7$ PFU/chick	-	8 h postinfection	
4	<i>Salmonella</i> challenged and phage treated	$0.5\text{ mL } 2 \times 10^6$ CFU/chick	$0.5\text{ mL } 2 \times 10^7$ PFU/chick	2nd day	8 h postinfection	

Experimental Design of the SPF Chick Model

All animal experiments were carried out in strict accordance with animal protocols that were approved by the Ethical Committee of Animal Experiments of Shandong Agricultural University (permit number SDAUA-2018-027). As shown in Table 1, the SPF chicks under experiment were divided into 4 groups (20 chicks/group), and each group was housed in a separate air-filtered isolation cabinet in a well-ventilated room with 4 compartments of about 10 square meters. Chicks were provided with sterile feed and water ad libitum. The chicks in group 2 (only *Salmonella* challenged) and 4 (*Salmonella* challenged and phage treated) were challenged orally at d 2 with a single dose of 2×10^6 CFUs *S. Pullorum* in a total volume of 0.5 mL. The control group (Group 1) and group 3 (only phage treated) were orally inoculated with the same volume of bacteria free-buffered PBS. At 8 h postinfection, both group 3 and group 4 were orally treated with 0.5 mL of phage suspension at 10^7 PFU/chick, and the other 2 groups (Groups 1 and 2) were orally inoculated with the same volume of SM buffer. Chicks were maintained at an age-appropriate temperature for 7 d and monitored daily for clinical signs and mortalities.

Bacterial Burden, Phage Titer, and Histopathology

At d 3 and 6 postinfection, 6 randomly selected chicks from each group were weighed, and blood was collected from jugular vein. Then chicks were sacrificed by cervical dislocation, and subjected to gross lesions investigation. Samples of the liver and spleen of the chicks were taken to determine the number of *Salmonella* bacteria and bacteriophage titer. Briefly, the tissue samples were weighed and homogenized in 1 mL of PBS, and serial dilutions of the homogenates were plated onto XLD plates for counting of bacteria. Three suspected *S. Pullorum* colonies for each sample were identified by PCR assays using a specific target gene ipaJ (Xu et al., 2018). To determine phage titer, the homogenates were centrifuged at 10,000 rpm for 10 min and the supernatant was serially diluted and quantified by double agar overlay assay. The livers from each group at d 3 postinfection were fixed by 4% paraformaldehyde for 24 h at room temperature. Then the liver samples were embedded by paraffin, and slides were stained with hematoxylin-eosin (HE) stain.

Serological and Cytokine Testing in Blood Serum

Serum samples were collected by centrifugation and subjected to serological testing and enzyme-linked immunosorbent assay (ELISA) for interleukin-6 (IL-6) and interferon gamma (IFN- γ) (Cloud-Clone Corp., China). The *S. Pullorum* and *S. Gallinarum* multivalent antigen used for the agglutination test was purchased

from Beijing Zhonghai Biotech Co. Ltd in China. The assays were performed as recommended by the manufacturers.

16S rRNA Amplicon Sequencing and Bioinformatic Analyses

Extraction of DNA from preweighed cecal content samples was performed using SDS method. V3-V4 hypervariable region of 16S *rRNA* gene was amplified using forward primer 341F and reverse primer 806R with dual-index barcodes by TruSeq DNA PCR-free sample preparation kit (Takahashi et al., 2014). The purified amplicons were quantified by using Qubit Fluorometer (ThermoFisher Scientific, Carlsbad, CA) and subjected to Illumina NovaSeq6000 desktop sequencer. Reads were filtered using Qiime and removed chimeric reads to obtain effective tags (Hall and Beiko, 2018). The above quality-controlled sequences were then clustered OTUs and annotated against SSUrRNA database using a 97% similarity cutoff (Quast et al., 2013). Alpha diversity (Shannon's diversity index and Faith's phylogenetic diversity) and beta diversity (weighted and unweighted UniFrac) were calculated using Qiime (Lozupone and Knight, 2005). The functional contribution of the bacteria was predicted based on OTUs using the Tax4Fun package in R software (Asshauer et al., 2015).

Statistical Analysis

Statistical significance was determined based on one-way analysis of variance (ANOVA) in appropriate condition. Significance was determined at $P < 0.05$.

RESULTS

Phage Therapy Improved Body Weight Loss of Chicks Infected With *S. Pullorum*

As shown in Figure 1, infection with 2×10^6 CFU/chick of the hypervirulent *S. Pullorum* strain 20JS04 had a significant negative effect on body weight of young SPF chicks ($55.62 \text{ g} \pm 2.75$, $P < 0.01$). Phage alone group did not show any abnormal behavior or obvious symptoms within 7 d, as comparing to chicks in control group ($62.82 \text{ g} \pm 4.82$), and *Salmonella* phage CKT1 had no significant negative effects on body weight of chicks ($61.12 \text{ g} \pm 4.13$). When the infected chicks were treated with a single dose of phage (10^7 PFU/chick) after *S. Pullorum* challenge, the body weight of chicks was markedly increased ($58.49 \text{ g} \pm 4.62$), close to the weight level of chicks in control group.

Phage Therapeutic Effect on the Liver and Spleen of Chicks Infected With *S. Pullorum*

As shown in Figure 2, *Salmonella* infection did not affect liver/body weight ratio of chicks, but resulted in

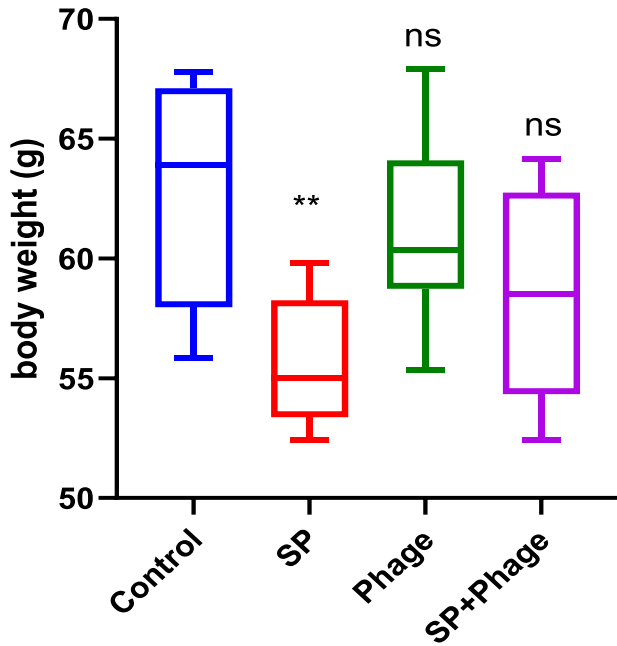


Figure 1. Phage therapeutic effect on the body weight of SPF chicks infected with *S. Pullorum*. $n = 6$ chicks. ** $P < 0.01$ (One-way ANOVA); ns, no significance.

significantly increased spleen/body weight ratio ($P < 0.05$) on d 3 postinfection. However, chicks challenged with *Salmonella* showed significantly elevated both liver/body ($P < 0.0001$) and spleen/body ($P < 0.05$) weight ratios on d 6 postinfection compared with that of the control group, indicating that the liver and spleen of chicks might be enlarged (Figures 2D and 2E). For phage alone group, no difference was observed on both liver/body and spleen/body weight ratios on d 3 postinfection, but a significant reduction ($P < 0.01$) in liver/body weight ratio occurred on d 6 postinfection. For the infected chicks treated with phage, the liver/body weight ratio was significantly lower than those infected chicks without phage treatment ($P < 0.01$), but still higher than that of the control group on d 6 postinfection; the spleen/body weight ratio was also increased but showed no difference between *Salmonella* challenged chicks treated with or without phage CKT1 on d 6 postinfection. The average loads of *S. Pullorum* in liver and spleen reached 2×10^5 CFU/g and 5.4×10^5 CFU/g on d 3 postinfection, respectively, which were higher than that on d 6 postinfection (2.7×10^4 CFU/g and 8.9×10^4 CFU/g, respectively; Figures 2C and 2F). However, phage treatment just resulted in a reduction of 39.3% in bacterial loads of

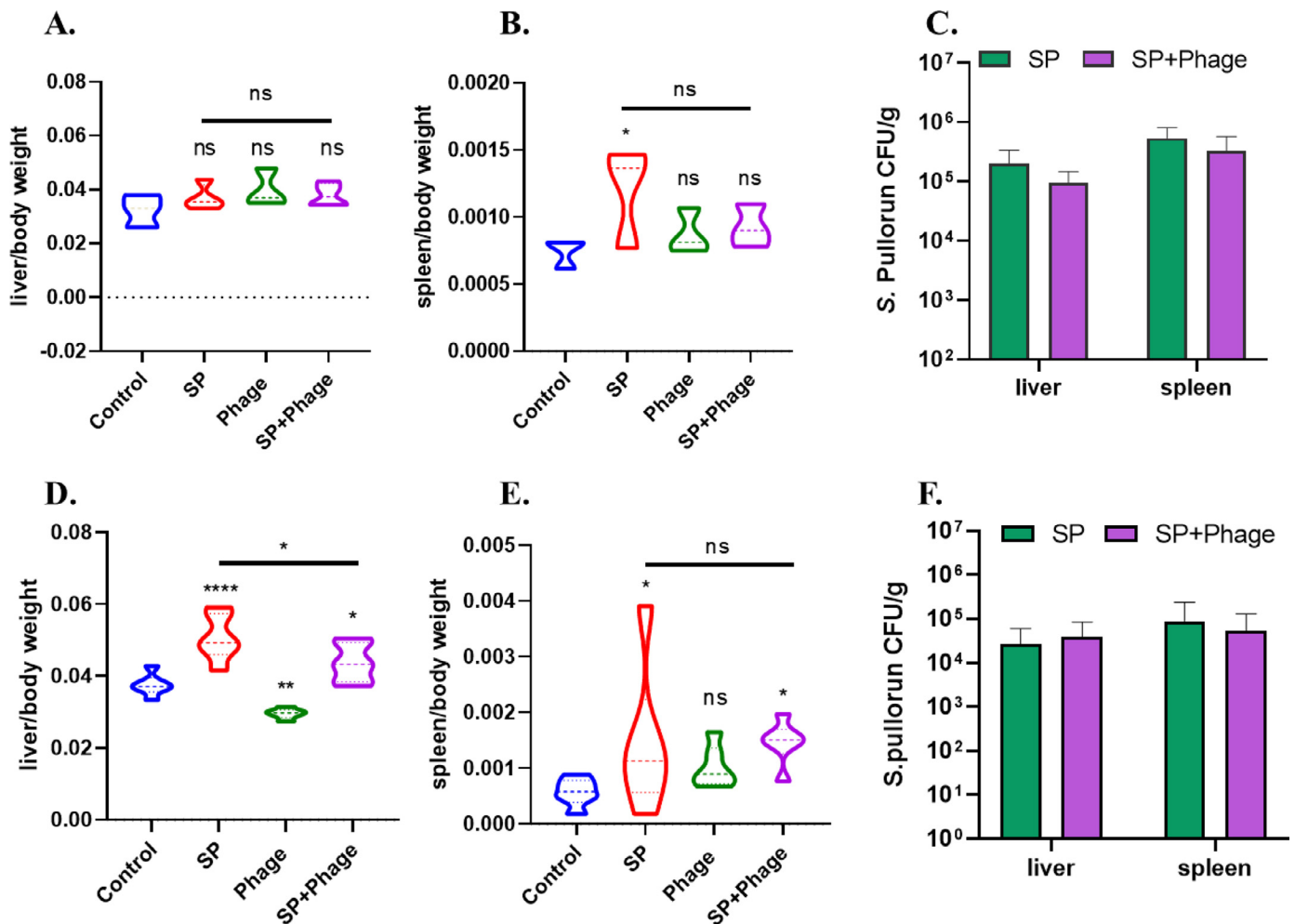


Figure 2. Phage therapeutic effect on liver and spleen of chicks infected with *S. Pullorum*. Phage effect on the liver/body weight ratio (A) and spleen/body weight ratio (B) on d 3 postinfection. $n = 6$ chicks. (C) Bacterial loads in liver and spleen on d 3 postinfection. Phage effect on the spleen/body weight ratio (D) and spleen/body weight ratio (E) on d 6 postinfection. $n = 6$ chicks. (F) Bacterial loads in liver and spleen on day 6 post infection. *, $P < 0.05$ (One-way ANOVA); **, $P < 0.01$; ****, $P < 0.0001$; ns, no significance.

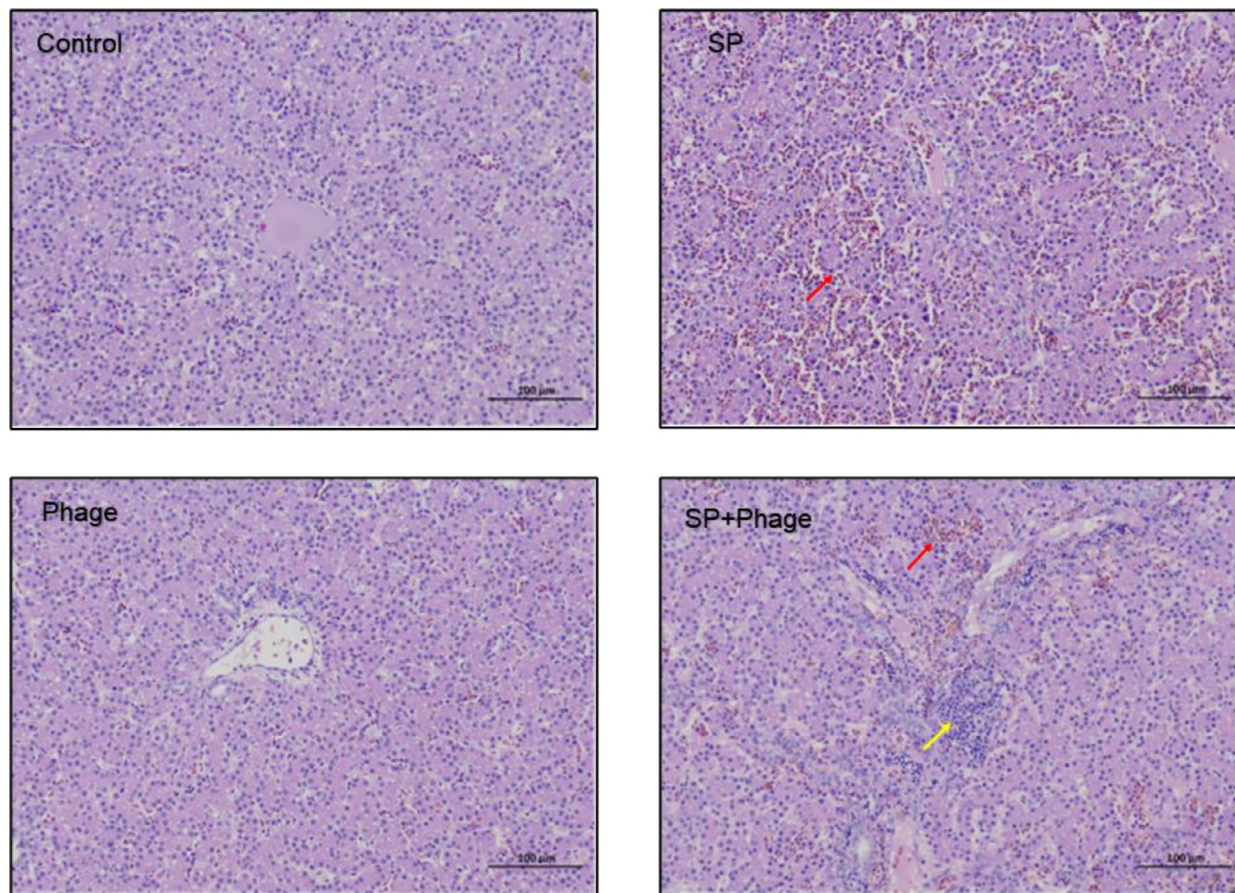


Figure 3. Phage therapeutic effect on pathological changes of liver. Massive hepatic sinusoidal dilatation and congestion (red arrow) in only *Salmonella* challenged chicks, whereas only a moderate amount of hepatic sinusoidal dilatation and congestion (red arrow) and small focal infiltration of inflammatory cells around the portal area in liver (yellow arrow) were observed in infected chicks after phage treatment.

spleen (5.4×10^4 CFU/g) on d 6 postinfection, no significant difference in *Salmonella* numbers in both liver and spleen were found between infected chicks treated with or without phage CKT1 on d 6 postinfection.

Compared with the normal liver of chicks in control group, enlarged liver in *Salmonella* challenged group displayed many white necrotic spots, while infected chicks treated with phage showed less necrosis. There were massive hepatic sinusoidal dilatation and congestion in only *Salmonella* challenged chicks. In contrast, besides small focal infiltration of inflammatory cells around the portal area in liver, only a moderate amount of hepatic sinusoidal dilatation and congestion were observed in infected chicks in phage treatment group (Figure 3). It was noteworthy that, the liver in phage control group had no obvious pathological changes but the liver size became smaller, which may be related to chicken wings injury due to unexpected air-filtered isolation cabinet failure.

Phage Persistence in Chicks

As shown in Figure 4A, phage particles can be detected in the liver and spleen of chicks in phage alone group, suggesting that *Salmonella* phage CKT1 was able to enter organs, although the phage titers in both liver and spleen were less than 10^3 PFU/g after 3 d

administration. In addition to approximately 10^6 PFU/g of phage particles were remained in cecum of infected chicks after 3 d of phage administration, *Salmonella* phage CKT1 was also detected in both liver and spleen at concentrations of around 10^4 PFU/g for at least 3 d. However, phages could not be detected in cecum, liver, and spleen after 6 d of phage treatment. It is noteworthy that phage titers in organs of chicks in phage treated group were generally higher than that in phage alone group, indicating that phage CKT1 was propagated on colonized *S. Pullorum* in organs.

Phage Therapy Affected Serum Cytokine Levels

Serological analysis showed that no antibodies to *S. Pullorum* was detected in any *Salmonella* challenged group on d 6 postinfection. Among three tested cytokines, the serum level of IFN- γ was very low, beyond the detection limit of ELISA kit used in this study. As shown in Figure 4B, infection with *S. Pullorum* caused a 11.9-fold ($P < 0.0001$) and 6.4-fold ($P = 0.33$) increase in the serum levels of IL-6 at d 3 and 6 postinfection. There was also a significant upregulation (4.8-fold, $P < 0.05$) of serum IL-6 following only phage administration on d 3 postinfection, but the IL-6 level was just 1.7-fold higher ($P = 0.90$) on d 6 postinfection. In terms of the

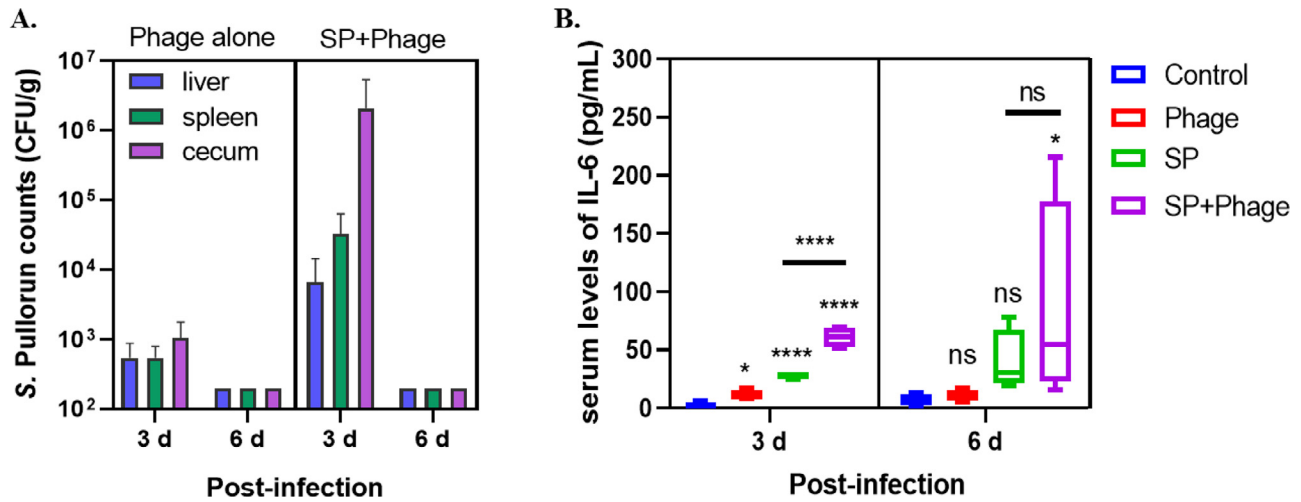


Figure 4. (A) *Salmonella* phage CKT1 persistence in liver, spleen and cecum of chicks in both phage alone group and phage therapy group on d 3 and 6 postinfection. (B) *Salmonella* phage CKT1 effect on serum levels of IL-6 on d 3 and 6 postinfection. *, $P < 0.05$ (One-way ANOVA); ****, $P < 0.0001$; ns, no significance.

serum levels of IL-6 in infected chicks after phage treatment, there was a 25.8-fold ($P < 0.0001$) and 13.8-fold ($P < 0.05$) increase on d 3 and 6 postinfection, respectively. Compared with the level of serum IL-6 of infected chicks without phage treatment, phage therapy significantly promoted higher levels of IL-6 production (2.2-fold, $P < 0.0001$) on d 3 post infection, and resulted in a 2.1-fold increase although no statistical difference on d 6 postinfection were found.

Phage Therapy Normalized Altered Intestinal Microbiome Caused by Hypervirulent *S. Pullorum*

To determine whether phage treatment altered the gut metagenome of chicks, 16S *rRNA* gene profiling was carried out on cecal content samples. At the phylum level on d 6 postinfection, *Firmicutes* and *Proteobacteria* were predominant in gut microbiota of chicks from all groups. However, a higher relative abundance of *Proteobacteria* (0.56, $P < 0.0001$) and less abundant *Firmicutes* (0.41, $P < 0.0001$) was observed in only *Salmonella* challenged group than in the control group (0.19 and 0.79 respectively). In contrast, there was no significant difference in bacterial phyla between phage alone and phage therapy group (Figure 5A). The phylogenetic tree showed that the bacterial communities were divided into 2 major groups, *Salmonella* challenged group form a single branch, whereas phage alone, phage therapy and control groups were distributed on the same large branch (Figure 5A). Chicks between phage alone and control group showed a smaller weighted UniFrac distance, indicating single phage administration did not affect the normal gut microbiota structure of chicks. The beta diversity distance between only *Salmonella* challenged group and control group was statistically significantly larger compared to that between control group and other phage groups, implying *Salmonella* challenge significantly altered the gut microbiota

structure of chicks (Figure 5A). Of the top 10 bacterial taxa at the genus level in control group, *Lachnoclostridium* (0.285) and *Blautia* (0.156) were predominant, followed by *Escherichia-Shigella* (0.089) and *Ruminococcus* (0.082), which was similar to that of phage alone group. In contrast, the relative abundances of *Lachnoclostridium* (0.048) and *Blautia* (0.059) in only *Salmonella* infected chicks were reduced substantially, resulting in significantly increased *Escherichia-Shigella* (0.225, $P < 0.0001$) and *Klebsiella* (0.216, $P < 0.0001$) becoming the predominant bacterial taxa (Figure 5B). Interestingly, the relative abundances of *Escherichia-Shigella* (0.034, $P < 0.0001$) and *Klebsiella* (0.064, $P < 0.0001$) decreased significantly after *Salmonella* phage treatment against infected chicks, accompanied by *Lachnoclostridium*, *Ruminococcus*, *Lactobacillus* and *Pseudoflavonifractor* increased remarkably compared to that of only *Salmonella* challenged group. However, the relative abundances of *Blautia* and *Enterococcus* in phage treatment group were still lower than that of control group (Figure 5B, Figure S1).

Alpha diversity comparison between *Salmonella* challenge and phage therapy groups showed significant differences in Shannon indices, indicating phage administration increased species richness in the cecum of chicks and made the distribution of species more even (Figure 6A). Cluster analysis of the metabolism functions in level 2 revealed that abundance of glycan biosynthesis and metabolism genes and infectious diseases genes in only *Salmonella* challenged group were increased greatly compared to other groups, followed by the increase of cellular community prokaryotes genes, amino acid metabolism genes, xenobiotics biodegradation and metabolism genes and terpenoids and polyketides metabolism genes. In contrast, above genes were reduced to the similar level as the control group after introduction of phage on infected chicks, which was also accompanied by an increase in genes mainly related to transport and catabolism, transcription, and replication and repair. The profile of expressed genes was general

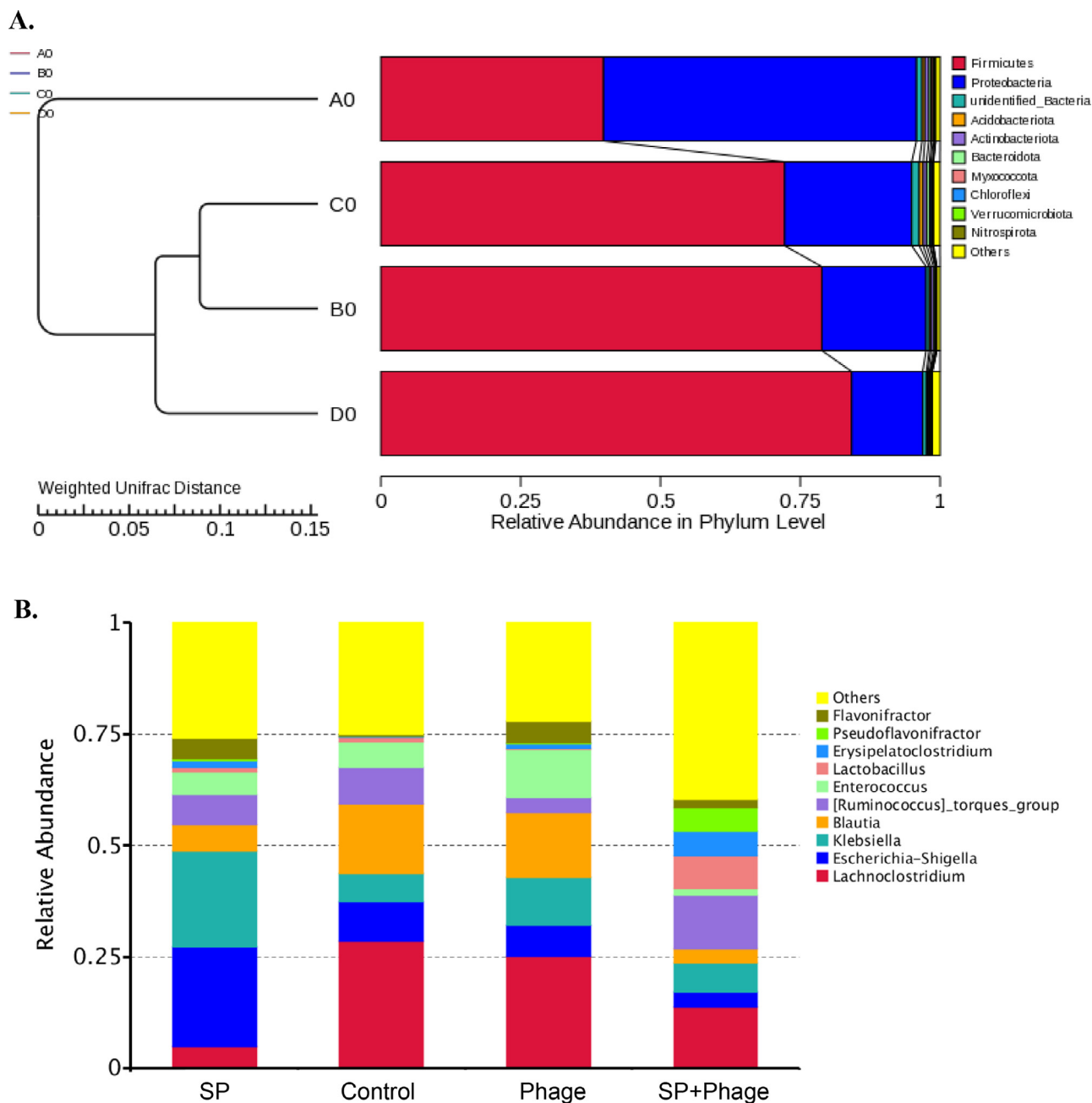


Figure 5. (A) UPGMA tree based on weighted UniFrac distance at the phylum level. On the left is the UPGMA cluster tree structure, on the right is the species relative abundance distribution at the phylum level for each sample. A0: SP, B0: Control, C0: Phage, D0: SP + Phage; (B) *Salmonella* phage CKT1 therapeutic effect on the gut metagenome of chicks at the genus level. The relative abundances of the top 10 genera were displayed.

similar between phage alone group and the control group, and cell growth and death genes and cell motility genes were both highly expressed in both 2 groups (Figure 6B).

DISCUSSION

Hypervirulent arthritis-causing *S. Pullorum* is widespread in Chinese native chicken flocks with swollen joint and lameness, leading to severe economic losses (Guo et al., 2019). The problems associated with multi-drug-resistant *Salmonella* and the regulations concerning the use of antimicrobials in animal production have

led to a resurgence of interest in phage therapy. In this study, we assessed the therapeutic efficacy of *Salmonella* phage CKT1 against hypervirulent *S. Pullorum* strain 20JS04 which were isolated from Chinese native chicken breeders with arthritis symptoms. We used the LD₅₀ dose of *Salmonella* in Chinese native Qingjiaoma chicks to infect SPF White Leghorn chicks, although this dose did not cause arthritis symptoms, significant body weight loss was still observed in SPF chicks, similar to the symptom appeared in Qingjiaoma chicks (Li et al., 2021). Besides, this hypervirulent *S. Pullorum* strain also induced enlarged liver and spleen and massive hepatic sinusoidal dilatation and congestion in young chicks on d 6 postinfection. When

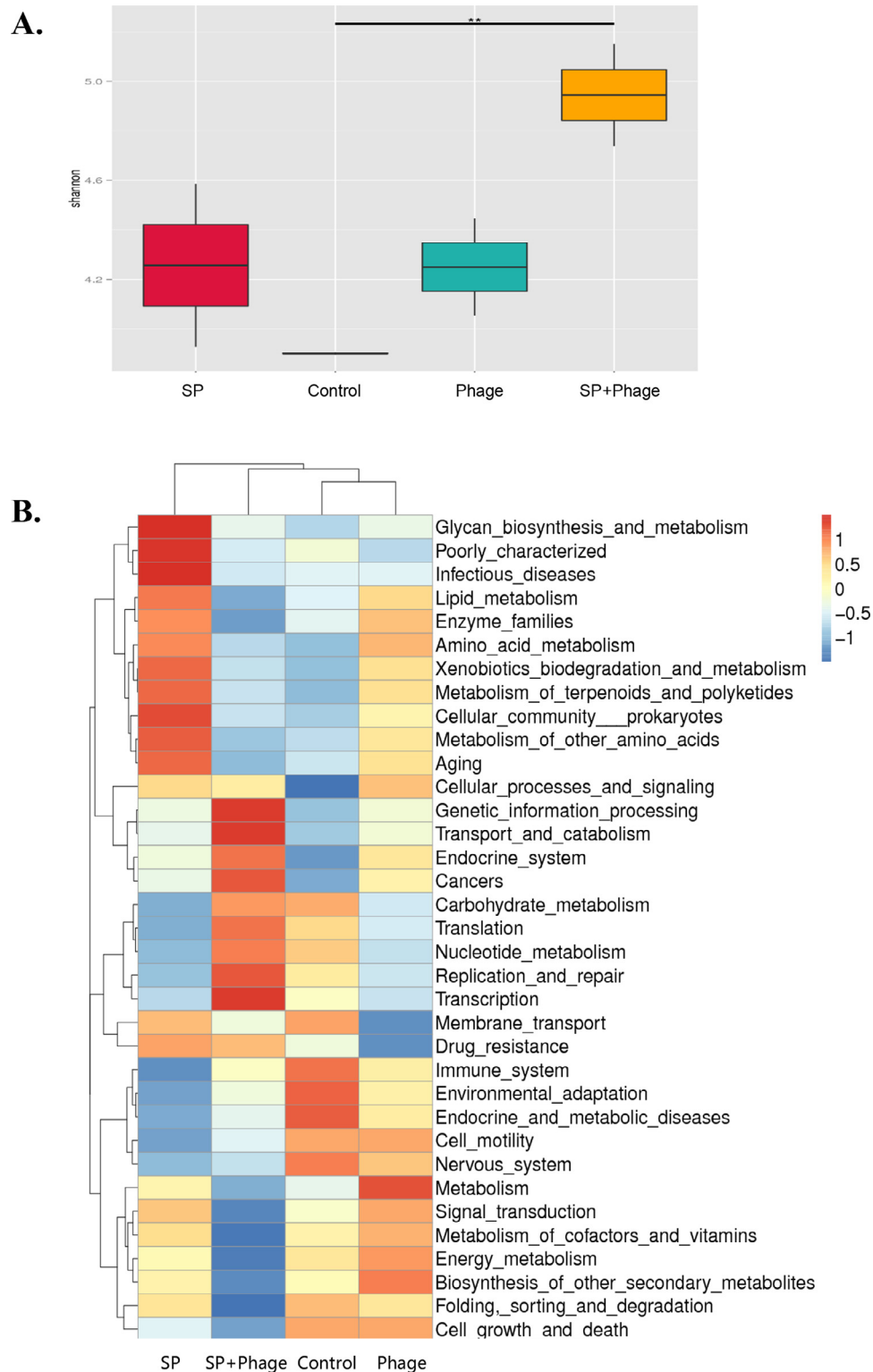


Figure 6. (A) Alpha diversity comparison between *Salmonella* challenge, phage alone, phage therapy and control groups with a box plot of the Shannon index. (B) Cluster heatmap of annotated metabolism functions in level 2 by Tax4Fun. **, $P < 0.01$ (Wilcoxon rank-sum test).

bacteriophages were administrated to chicks after oral *Salmonella* infections, organ damage was effectively reduced. However, bacterial loads in liver and spleen were not significantly decreased after phage treatment. Many studies showed that frequent phage treatments or a single administration of high titer of phage, and especially prior to colonization of the intestinal tract by

Salmonella, could result in effective bacterial reduction (Borie et al., 2008; Bardina et al., 2012). Tie et al. (2018) demonstrated that a single oral administration of *Salmonella* phage YSP2 2 h after bacteria challenge with approximately 10^9 PFU/chick could protect chickens against diarrhea and relieve hemorrhage in intestine and liver tissue significantly.

Nabil et al. (2018) showed that successive phage treatments applied within a short time period infection were able to clear *Salmonella* from infected chicks. Our results suggested that a single phage administration after *Salmonella* infection was not sufficient to reduce bacterial loads in organs significantly. One reason may be that phage particles tend to be flushed from the organs of chicks over time by the immune system. Successive phage treatments may be efficacious in reducing *S. Pullorum* colonization in poultry.

Cytokines are key communication molecules between host cells in the defense against *Salmonella*. Infection with *Salmonella* leads to the activation and recruitment of neutrophils and macrophages and the production of proinflammatory cytokines. The Th1 cytokine IFN- γ has important roles in innate and acquired immunity, such as inducing antimicrobial responses in macrophages (Schroder et al., 2004). ELISA results showed little or no induction of serum IFN- γ in both *Salmonella* challenged chicks and phage treated chicks. This observation illustrated that the low-level of IFN- γ may be permissive for systemic spread of this hypervirulent *S. Pullorum* strain. IL-6 is a pleiotropic cytokine that has proinflammatory activity via the induction of acute phase protein synthesis, and it is important in the development of adaptive immune responses (Tanaka et al., 2014). The serum level of IL-6 was remarkably increased in chicks by 3 d postinfection, suggesting that IL-6 was involved in triggering an inflammatory response to *S. Pullorum* infection. Further administration of phages on infected chicks promoted the production of serum IL-6, which may be related to phage-induced bacterial lysis. Many previous studies showed that antibacterial activity of phages combined with the host's own immune defenses led to asymptomatic eradication of bacterial infections (Roach et al., 2017; Krut and Bekeredjian-Ding, 2018). Recent literature also provides evidence that even phages that target the same bacterial pathogen may yield different effects (Shiley et al., 2017). However, there are few studies focused on phage effects on the immunity of body, especially in food animals. To screen the best candidate phages for systemic infections of *Salmonella* in poultry, it will be essential in future studies to investigate the overall animal host immune response against the bacteriophage activities as well as responses at the cellular levels of the host.

The microbiome of the broiler chicken gastrointestinal tract has been amply demonstrated to be important for the health of host, as it has a positive impact on the immune system, the physiology of the gastrointestinal tract, and productivity (Clavijo and Florez, 2018). Although the use of phages could reduce the *Salmonella* load in the cecal content, how phages modulate the intestinal microbiome of chicken intestines is still unclear. Here we showed that *Salmonella* challenge disrupted the normal gut microbiota structure of chicks, and the use of phage CKT1 significantly reduced the *Escherichia-Shigella* and *Klebsiella* populations, permitting the proliferation of beneficial microbiota including *Lachnoclostridium*, *Ruminococcus*, *Lactobacillus*, and

Pseudoflavonifractor. It should be noted that *Salmonella* phage CKT1 specifically infected *Salmonella* and body weight loss was not fully recovered after phage therapy. The still lower abundances of *Blautia*, a genus of anaerobic bacteria with probiotic characteristics, and opportunistic pathogen *Enterococcus* in phage treatment group suggested that phage therapy shaped the abnormal gut microbiome of chicks to a certain extent. Interestingly, *Akkermansia*, a mucin-degrading bacterium that inversely correlates with body weight in rodents and humans (Everard et al., 2013), is increased after *Salmonella* challenge, indicating that increased *Akkermansia* may also contribute to the body weight loss caused by *Salmonella* infection in young chicks. To the best of our knowledge, the inverse correlation of *Akkermansia* abundance with body weight in chicks has not been reported to date. Moreover, the increased levels of glycan biosynthesis and lipid metabolism genes and infectious diseases genes in only *Salmonella* challenged group reflected *Salmonella* infection and adaptation strategy to gain a growth advantage over the intestinal microbiota. Phage therapy led to a reduction of infectious diseases genes and an increase in genes mainly related to transport and catabolism, transcription, and replication and repair, suggesting reduced metabolic activity of pathogenic bacteria and alterations in bacterial diversity and catabolism of the other microbiota. Interestingly, Upadhaya et al. (2021) assessed the dietary usage of a bacteriophage cocktail in broiler chickens without bacterial challenge. They found that phage cocktail improved broiler weight and 0.05% phage addition was sufficient for supporting immune organs, bursa and spleen as well as enhancing gut microbiome. These results indicated the potential of phages as a substitute antibiotic growth promoter in broiler chickens.

In summary, we investigated the therapeutic efficacy of *Salmonella* phage CKT1 against hypervirulent arthritis-causing *S. Pullorum* in a chick model. The results showed that single phage treatment after *S. Pullorum* infection significantly improved the body weight loss of chicks, possibly by modulating the abnormal intestinal microbiome, indicating *Salmonella* phage CKT1 could be considered as a potential alternative to antimicrobial growth promoters on poultry farms.

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DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2021.101668.

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