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Collateral sensitivity-driven phage cocktail effectively enhances the antibacterial activity against Salmonella enteritidis in chickens

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Research Article

Keywords: Phage cocktails, collateral sensitivity, phage-host coevolution, Salmonella enteritidis, drug-resistant bacteria control

Posted Date: June 30th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9877629/v1

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Additional Declarations: No competing interests reported.

Abstract

Phages show promising application prospects as antibacterial agents for the treatment of drug-resistant bacterial infections. However, the rapid evolution of phage resistance in bacteria has compromised the therapeutic efficacy. Here, we first screened a broad-host-range phage PC13 based on the *Salmonella*-phage library. Then, phage-resistant mutants were isolated by serial co-culture passages of PC13 with *Salmonella enteritidis* C2. Finally, phages PC11 and PC16 capable of efficiently lysing PC13-resistant mutants were identified by cross-infection assays. Whole-genome sequencing analyses were performed on the phages and resistant strains to decipher the potential molecular mechanisms of phage-host interactions and collateral sensitivity. The bacteriostatic and therapeutic effects of the triple phage cocktail (PC13 + PC11+PC16) against *S. enteritidis* C2 were evaluated through in vitro bacteriostatic assays and *in vivo* chicken infection models. The results showed that PC13 lysed 90% (81/90) of chicken-derived *Salmonella* isolates. Both PC11 and PC16 exhibited an 80% lysis rate against 20 PC13-resistant mutants. The phage cocktail significantly prolonged the bacteriostatic time against *S. enteritidis in vitro* compared to individual phages ($p < 0.01$). Comparative genomic analysis revealed that 85% of the PC13-resistant mutants harbored missense, nonsense, or frameshift mutations in genes associated with lipopolysaccharide (LPS) core oligosaccharide synthesis. *Salmonella* evaded PC13 infection by altering LPS structure via these mutations, but these genetic changes simultaneously exposed the specific binding receptors of PC11 and PC16, resulting in collateral sensitivity of the resistant mutants to the latter two phages. In the chicken *S. enteritidis* infection model, compared with the *Salmonella* treatment group, the fecal *Salmonella* load of chicks in the cocktail treatment group decreased by about 4.0 log CFU/g, and there was no significant difference in body weight changes compared with control group. In conclusion, a phage cocktail against *S. enteritidis* with synergistic bacteriostatic effects was rationally formulated based on the collateral sensitivity of phage-host coevolution, which provides theoretical and practical strategies for developing highly efficient phage therapy.

Introduction

Salmonella is a critical zoonotic pathogen and the problem of its multidrug resistance has become increasingly severe due to the extensive use of antibiotics in poultry (Farhat et al. 2024). As antibiotic development lags behind the accelerating evolution of bacterial drug resistance, there is an urgent need to develop novel sustainable antibacterial strategies to combat *Salmonella* infection and prevent dissemination of drug resistance. Virulent phages, with their high specificity, inherent biosafety and potent bactericidal activity, offer a promising alternative to antibiotics for controlling multidrug-resistant bacterial infections in poultry and clinical medical fields (Gordillo et al. 2019).

While the high specificity of phages enables targeted antibacterial activity, this same property carries inherent trade-offs: a restricted host range and the rapid emergence of phage resistance in bacteria, both of which have severely restricted the application of phage therapy (Palma et al. 2024). For example, stable resistant mutants typically emerge within 6–8 hours of continuous co-culture of a phage with

Salmonella, resulting in complete abrogation of bacteriostatic effect. This rapid development of phage resistance is the primary cause of therapeutic failure in clinical phage therapy (Labrie et al. 2010). Phage cocktails, consisting of multiple phages, are currently the most widely adopted strategy to address these limitations. Compared with single phages, cocktails can effectively broaden the host range, improve bactericidal efficiency, and delay or even block the emergence of bacterial phage resistance (Nale et al. 2016). However, the formulation of most current phage cocktails relies on simple superposition of lysis spectra or empirical combination, lacking systematic theoretical guidance on the laws of phage-host coevolution, resistance evolution mechanisms, and phage receptor recognition characteristics. This leads to common defects of formulated cocktails, including overlapping receptor recognition targets, insufficient synergistic effects, failure of long-term inhibition of resistant strains, as well as high variability and instability of *in vivo* therapeutic effects (Lood et al. 2022). It has been reported that more than 60% of empirically formulated phage cocktails still show high frequency of resistant mutants after 10 serial co-culture passages with host bacteria (Kaneko et al. 2026).

The collateral sensitivity based on the theory of phage-host coevolution has provided a brand-new perspective for the rational design of phage cocktails (Acton et al. 2024). Collateral sensitivity refers to the phenomenon that when bacteria develop resistance mutations against one phage, the alteration of their surface receptor structure simultaneously enhances their sensitivity to other phages. The fitness cost of resistance evolution can reduce the probability of bacterial escape from phage infection (Mu et al. 2025). This mechanism lies in the selection of phage combinations that target different receptors and have mutually exclusive effects on resistance mutations: the receptor mutations that occur in bacteria to evade infection by the first phage will inevitably lead to high sensitivity to the second and third phages, thus forming an "evolutionary trap" and blocking the emergence of resistant strains (Strathdee et al. 2023). Studies have confirmed that phage cocktails designed based on collateral sensitivity can significantly prolong the bacteriostatic effect against pathogenic bacteria such as Klebsiella pneumoniae (Yoo et al. 2023), Pseudomonas aeruginosa (Yang et al. 2020), and Salmonella (Acton et al. 2024), and reduce the emergence frequency of resistant strains.

Lipopolysaccharide (LPS) and outer membrane proteins (OMPs) are common adsorption receptors of Salmonella phages. Among them, more than 70% of phages against avian Salmonella use LPS as the primary recognition target, and the O-antigen, core oligosaccharide can be used as specific adsorption receptors for phages (Martinez-Soto et al. 2024). When bacteria evade phage infection through mutations in LPS synthesis-related genes, it often leads to truncation of LPS structure or exposure of outer membrane proteins, thereby resulting in collateral sensitivity to phages targeting other receptor sites (Acton et al. 2024). However, there are few studies on the rational design of phage cocktails against chicken-derived Salmonella based on collateral sensitivity, and its molecular mechanism and in vivo therapeutic effect are still limited.

In this study, based on our resource library of chicken-derived *Salmonella* and phages, the phenomenon of collateral sensitivity during phage-host coevolution was explored. Phage combinations that target different receptors and can induce collateral sensitivity in *Salmonella* were screened, and an optimized

triple phage cocktail was rationally constructed. Its bacteriostatic activity and resistance inhibition effect against *S. enteritidis* were evaluated through *in vitro* assays, the molecular mechanism of its synergistic bacteriostatic effect was deciphered combined with whole-genome sequencing, and its therapeutic effect was verified by the model of chicken infected with *S. enteritidis*. This study aims to provide theoretical and experimental basis for phage therapy to prevent and control *Salmonella* infection in poultry.

Materials and Methods

Salmonella strains and culture conditions

A total of 90 *Salmonella* strains used in this study were isolated from clinical intestinal lesion samples of chickens from chicken farms in China. All strains were identified as *Salmonella enteritidis* by biochemical identification, 16S rDNA sequencing, and serotyping. Strains were inoculated into Luria-Bertani (LB) medium and cultured overnight at 37°C with shaking at 200 rpm. Then, the culture was transferred to fresh LB medium with 3% inoculation and cultured to OD_{600nm} of about 0.5 (~ 10^8 CFU/mL) for subsequent experiments.

Isolation and purification of phages

Salmonella phages were isolated from farms or domestic sewage using the double-layer agar plate method as previously described (Mugo et al. 2025). Briefly, Sewage samples were centrifuged at 1000 rpm to remove precipitates. The supernatant was mixed with 2×LB (v/v = 1:1), followed by the addition of 5% *Salmonella* culture. The mixture was incubated overnight at 37°C with shaking at 200 rpm. Then, the culture was centrifuged at 10,000 rpm for 5 min at 4 ^{o}C and the supernatant was filtered through a 0.22 μm filter. 100 μL of the filtrate was mixed with 100 μL *Salmonella* culture in 5 mL of 0.75% LB semi-solid agar, which was then spread on 1.5% LB agar plates. After the top agar had solidified, plates were incubated overnight at 37°C. Single plaques were picked and subjected to three consecutive rounds of purification by the double-layer agar method. Purified phages were propagated in liquid cultures of *Salmonella*. The phage suspension was obtained by centrifugation and filtration of the supernatant, and stored at 4°C.

Biological characterization of phage

The pH and temperature stability of phages were determined according to the method described by Ross A et al. (Ross et al. 2016). The pH of SM buffer was adjusted to 2.0–12.0 using 1 M HCl or NaOH, then, 100 μL of phage suspension was added to 900 μL of SM buffer at each pH, mixed and incubated at 37°C for 1 h. The phage titer was determined by the double-layer agar plate method to evaluate the pH stability of phages. For thermal stability assays, 1 mL of phage suspension were incubated at temperatures ranging from 25–95°C in a water bath for 1h. After incubation, the phage titer was also determined by the double-layer agar plate method to evaluate the temperature stability of phages. For

transmission electron microscopy (TEM) analysis, a copper grid was immersed in phage suspension for 5 min, then taken out and dried on filter paper for 2 min, and the grid was negatively stained with 3% (w/v) phosphotungstic acid for 2 min. After air-drying for 5 min, the phage morphology was observed using a TEM operating at an accelerating voltage of 80 kV.

Determination of phage host range

The host range of phages was determined using the spot lysis assay as previously described (Zhao et al. 2024). 200 μL of mid-exponential phase bacterial culture (~ 10^8 CFU/mL) was added to 5 mL of 0.75% semi-solid LB agar. The mixture was poured onto 1.5% LB agar plates. After the top agar had solidified, 3 μL of phage suspension (~ 10^8 PFU/mL) was spotted onto the plates. The plates were allowed to air-dry at room temperature, and then incubated overnight at 37°C. The lytic activity of phages against each strain was evaluated according to the formation of visible plaques, and the lysis rate was calculated as follows:

Lysis rate (%) = (number of lysed strains / total number of tested strains) ×100%

In vitro bacterial growth inhibition assay

The lytic activity of phages against *Salmonella* was determined using a microplate-based growth inhibition assay as previously described (Mu et al. 2025). Briefly, 100 μL of mid-exponential phase *Salmonella* culture (adjusted to ~ 1×10^7 CFU/mL) was added to each well of a 96-well microplate. For the experimental group, 100 μL of phage suspension was added to MOI = 1.0; the negative control group was added with 100 μL of sterile LB; and the blank control group was only added with 200 μL of LB. The microplate was placed in an automatic microbial growth curve analyzer, and incubated statically at 37°C for 24 h. The optical density at 600 nm (OD_{600nm}) value was measured every 1 h. All experiments were performed in triplicate, and the mean values ± standard deviations were calculated.

Isolation of phage-resistant mutants

S. enteritidis C2 was used as the wild-type strain for the isolation of PC13-resistant mutants. Mid-exponential phase C2 culture was mixed with phage PC13 suspension at MOI = 10. The mixture was co-cultured with shaking at 200 rpm at 37°C for 48 h, followed by five consecutive serial passages. For each passage, 1% of the previous culture was transferred to fresh LB and incubated under the same conditions. After the fifth passage, the bacterial culture was serially diluted and spread onto LB agar plates. The plates were incubated upside down at 37°C overnight. Single colonies were picked and purified by three successive rounds of streaking on LB agar plates. Resistance to PC13 was verified using the spot lysis assay: strains without plaque formation on the plates were identified as PC13-resistant mutants.

Phage DNA extraction, sequencing and annotation

Phage genomic DNA was extracted from high-titer phage suspension (10^9 PFU/mL) using Vazyme Phage Genomic DNA Extraction Kit (Vazyme Biotech Co., Ltd, China) according to the manufacturer's instructions. Whole-genome sequencing was performed on the Illumina NovSeq 6000-PE150 platform (Illumina Inc., USA). The genome sequence was assembled using SPAdes (Bankevich et al. 2012); VICTOR (Meier-Kolthoff et al. 2017) was used for biological classification and phylogenetic analysis based on the whole-genome sequence of phages; Pharokka (Bouras et al. 2023) was used to predict open reading frames (ORFs) and annotate gene functions; the PhageRBPDetect algorithm model (Boeckaerts et al. 2022) was used to predict the receptor-binding proteins (RBPs) of phages; the algorithm model described by Unnikrishnan, V.K. et al. (Unnikrishnan et al. 2023) was used to predict the potential receptors of RBPs.

Salmonella genomic DNA extraction and whole-genome sequencing analysis

Genomic DNA was extracted from wild-type *S. enteritidis* C2 and 20 independent PC13-resistant mutants using a bacterial genomic DNA extraction kit (TIANGEN Biotech Co., Ltd., China) according to the manufacturer's instructions. Whole-genome sequencing was performed on the Illumina NovSeq 6000-PE150 platform. Genome assembly was completed using SPAdes (Bankevich et al. 2012), and genome functional annotation was performed using Bakta (Schwengers et al. 2021). Comparative genomic analysis was performed using Breseq (Deatherage et al. 2014) with the complete genome of wild-type *S. enteritidis* C2 as the reference. This analysis identified single nucleotide polymorphisms (SNPs) and insertion/deletion (InDel) mutations present in the resistant mutants, and the affected genes were subsequently annotated.

Chicken infection model and in vivo phage cocktail therapeutic efficacy assay

One-day-old Roman gray chicks were used to establish the *S. enteritidis* infection model. Chicks were randomly divided into three groups (n = 6 per group): uninfected control group, infected control group, and phage cocktail treatment group. Chicks in the infected group and treatment group were orally inoculated with 100 μL of *S. enteritidis* C2 suspension containing 1×10^2 CFU via gavage. The uninfected control group received 100 μL of sterile phosphate-buffered saline (PBS) by gavage. At 1 h post-infection (hpi), chicks in the treatment group were orally administered 100 μL of phage cocktail suspension at a titer of 1×10^8 PFU. Survival rate, body weight changes and fecal *Salmonella* loads were monitored daily for 7 consecutive days. To quantify fecal *Salmonella* loads, 0.1 g of fecal samples were collected from each chick, and homogenized in 1 mL of sterile PBS. Serial ten-fold dilutions were prepared, and 100 μL aliquots of appropriate dilutions were spread onto *Salmonella*-selective SSR agar plates. Plates were incubated at 37°C overnight, and the number of *Salmonella* colonies on the plates was counted. *Salmonella* loads were calculated as CFU/g of feces.

Statistical analysis

Statistical analysis in this study was performed using R software (v4.5.2; R Foundation for Statistical Computing, Vienna, Austria). *p*-values were calculated by one-way analysis of variance (ANOVA) and two-tailed unpaired Student's t-test. A *p*-value less than 0.05 indicates that there is statistical significance.

Results

Screening of broad-host-range Salmonella phages

A total of 30 *Salmonella* phages were isolated and purified from environmental samples in this study. Their host range and lytic activity against a panel of 90 chicken-derived clinical *Salmonella* isolates were evaluated by the spot lysis assay. The phage-host interaction heatmap (Fig. 1A) showed that 1877 positive lytic phenotypes were detected among 2,700 groups of potential phage-host interactions. Each *Salmonella* strain could be lysed by at least 6 different phages, while the lysis range of a single phage was 43 to 81 strains. Phage PC13 displayed the broadest host range, lysing 81 of the 90 strains (90%). Five phages with the most robust lytic activity were selected for further evaluation (Fig. 1B). *In vitro* growth inhibition assays demonstrated that PC13 at MOI = 1.0 completely suppressed the growth of *S. enteritidis* C2 over a 6 h period (Fig. 1C). Based on the comprehensive host range and lytic performance, PC13 was selected as the core phage for subsequent construction of phage cocktails.

[Fig. 1]

Screening of phages against PC13-resistant mutants

Following five consecutive serial passages of *S. enteritidis* C2 in the presence of phage PC13, 20 independent PC13-resistant mutants were isolated and confirmed by double-layer agar assay. The lytic activity of the 30 phages against these 20 resistant mutants was evaluated. Cross-infection matrix (Fig. 2A) showed that 182 positive lytic phenotypes were detected among 600 potential interactions between the phage library and resistant strains. On average, each resistant mutant was susceptible to 9 distinct phages. Phage PC11 displayed the broadest lysis spectrum, with a lysis rate of 95% against the 20 PC13-resistant mutants (Fig. 2A), representing the most potent lytic activity against this mutant collection. The *in vitro* growth curve results showed that, the PC13 + PC11 combination significantly prolonged the bacteriostatic activity against *S. enteritidis* C2 compared to PC13 alone, and was superior to other phage combinations tested (Fig. 2B). These results indicate that PC11 effectively lyses PC13-resistant mutants, and its combination with PC13 significantly improves the bacteriostatic efficacy.

[Fig. 2]

Optimization and screening of triple phage cocktail

To further enhance the bacteriostatic efficacy of the phage cocktail, additional phages were screened from the phage library to construct triple combinations with the PC13 + PC11 binary cocktail. Growth curve analysis revealed that supplementation of the PC13 + PC11 combination with phage PC16

significantly prolonged the bacteriostatic period against *S. enteritidis* C2 ($p < 0.01$), resulting in complete growth inhibition throughout the 24 h observation period (Fig. 3A). Quantitative area under the curve (AUC) analysis further confirmed that although the triple combination of all tests showed different degrees of enhanced antibacterial activity relative to the PC13 + PC11 (as shown by its reduced AUC value), the PC13 + PC11+PC16 combination showed the lowest AUC value (Fig. 3B). Collectively, these results demonstrated that the triple combination of phages PC13, PC11 and PC16 was identified as the optimal phage cocktail formulation against *S. enteritidis* C2.

[Fig. 3]

Biological properties and whole-genome sequencing analysis of phages

TEM observation results showed that PC13, PC11 and PC16 all had typical tailed phage morphology. PC13 and PC16 belong to the *Siphoviridae*, and PC11 belongs to the *Podoviridae* (Fig. 4A, D). All three phages remained stable within the pH range of 4–11, with no significant decrease in titer. After incubation at 37–65°C for 1 h, the titer still maintained above 90% of the initial level, showing excellent pH and temperature stability.

Whole-genome sequencing results showed that PC13 genome had a full length of 43,286 bp with a GC content of 45.82%; PC11 genome had a full length of 39,572 bp with a GC content of 44.36%; PC16 genome had a full length of 41,693 bp with a GC content of 43.95% (Fig. 4B, C). The genomes of all three phages only encoded lysis-related functional proteins, and did not carry virulence genes, antibiotic resistance genes, or lysogeny-related genes, which conformed to the characteristics of virulent phages and biosafety requirements. Prediction of phage receptor-binding proteins (RBPs) and their cognate host receptors is summarized in Table 1. The RBPs of PC13 target LPS O-antigen, the RBPs of PC11 target LPS core oligosaccharide, and the RBPs of PC16 target outer membrane porin RopA1 and vitamin B_{12} transporter BtuB, respectively. The three phages utilize different host receptor molecules for infection, which may be the molecular basis for their observed synergistic antibacterial activity and collateral sensitivity.

Table 1
Predicted receptors of the receptor-binding proteins (RBPs) of the three phages

Phage	RBP	Predicted receptors
PC13	Tail protein (828 aa)	LPS O antigen
	Tail spike protein (554 aa)	LPS O antigen
PC11	Tail fiber protein (812 aa)	LPS core
	Tail fiber protein (378 aa)	LPS core
PC16	Central tail fiber J (949 aa)	Outer membrane porin RopA1
	Tail fiber protein (1071 aa)	BtuB

[Fig. 4**]** [Table 1]

Molecular mechanism of synergistic bacteriostatic effect of the triple phage cocktail

To elucidate the molecular mechanism of the significantly improved bacteriostatic efficacy of the triple phage cocktail, whole-genome resequencing and comparative genomic analysis were performed on wild-type *S. enteritidis* C2 and 20 PC13-resistant mutants. The results showed that 17 out of the 20 resistant mutants (85%) harbored mutations in genes involved in LPS core oligosaccharide synthesis, including missense, nonsense, and frameshift mutations in *rfaJ*, *rfbN*, and *rfe* (Fig. 5A). These genes encode enzymes essential for the assembly and modification of *Salmonella* LPS and O-antigen, indicating that the LPS O-antigen serves as the primary adsorption receptor for phage PC13.

Spot assay validation further confirmed these findings (Fig. 5B). Phage PC13 completely lost infectivity against *rfc, rfbN, and rfaJ* mutant strains, but retained full infectivity against the purD mutant. In contrast, phages PC11 and PC16 maintained robust lytic activity against all tested LPS-deficient mutants, indicating they utilize receptors distinct from the LPS O-antigen. Sequence analysis of the receptor-binding proteins (RBPs) from the three phages showed that pairwise sequence identities were all below 35% (32% between PC13 and PC11, 19% between PC13 and PC16, and 21% between PC11 and PC16), indicating negligible sequence homology (Fig. 5C). Receptor-binding domain (RBD) alignment further demonstrated that PC13 and PC11 possess conserved G-X-S motifs characteristic of LPS-binding RBPs, while PC16 contains unique R-X-Y motifs specific for outer membrane protein binding (Fig. 5D). Based on the above results, a possible mechanism for the synergistic antibacterial effect of the three phages is proposed: Loss-of-function mutations in LPS core oligosaccharide synthesis genes cause truncation and deletion of *Salmonella* LPS O-antigen, which exposes the LPS core oligosaccharide and outer membrane proteins originally shielded by O-antigen. These exposed structures are the specific adsorption receptors of PC11 and PC16 (Table 1). Therefore, the LPS mutations that *Salmonella* acquires to evade PC13

infection simultaneously resulted in significant collateral sensitivity of the resistant mutants to PC11 and PC16.

[Fig. 5]

In vivo therapeutic efficacy evaluation of the phage cocktail in chicks

To validate the *in vivo* therapeutic effect of the phage cocktail, a *S. enteritidis* C2 infection model was established in chicks, and the results are shown in Fig. 6. Chicks in the infected control group showed typical clinical symptoms of enteritis such as depression, ruffled feathers, and diarrhea; while chicks in the phage cocktail treatment group exhibited no obvious clinical symptoms, and were no different from the healthy control group (Data not shown).

Fecal *Salmonella* loads of chicks in the phage treatment group decreased significantly on 1 day post-treatment (dpt), dropping to approximately 10 CFU/g by 3 dpt and remaining at this level for the duration of the experiment. In contrast, fecal *Salmonella* loads in the infected control group remained stable at 10^5-10^6 CFU/g throughout the entire observation period (Fig. 6A).

Body weight analysis revealed that chicks in the phage treatment group exhibited normal growth, with body weight gain not significantly different from the healthy control group ($p > 0.05$). In contrast, the infected control group showed significant growth retardation, with their 8-day post-infection body weight markedly lower than the healthy control group ($p < 0.01$) (Fig. 6B). These results indicated that the phage cocktail significantly reduces intestinal *Salmonella* burden in chicks, reverses infection-induced growth inhibition, and exhibits excellent *in vivo* therapeutic efficacy and biosafety.

[Fig. 6]

Discussion

Phage therapy is a effective alternative strategy for the prevention and control of multidrug-resistant *Salmonella* infection, yet the rapid development of bacterial resistance to phages has limited its therapeutic efficacy. In this study, based on the collateral sensitivity mechanism of phage-host coevolution, a triple *Salmonella* phage cocktail targeting different receptors was rationally designed. The cocktail effectively inhibited *Salmonella* growth for at least 24 h *in vitro*; in the *in vivo* chicken infection model, it significantly reduced *Salmonella* in the intestinal tract, reverse the growth inhibition caused by infection, and effectively alleviated clinical symptoms and restored normal growth performance. This study provides a rational design strategy and experimental basis for phage-based prevention and control of *Salmonella* in poultry.

Compared with single phages, phage cocktails offer advantages for bacterial infection control, such as broadening the host range and improving the coverage rate of pathogenic strains (Martinez-Soto et al. 2024; Palma et al. 2024); prolonging the inhibition time of bacterial growth (Kaneko et al. 2026); reducing the mutation frequency of phage-resistant mutants, thereby improving the bactericidal efficacy (Yoo et

al. 2023). Although the single broad-host-range phage PC13 had a lysis rate of 90% against 90 chicken-derived clinical *S. enteritidis* isolates, it could only effectively inhibit the growth of the host within 6 h *in vitro*, and the bacteriostatic effect was completely lost after 6 h due to the emergence of resistant strains (Fig. 1). In contrast, the PC13 + PC11+PC16 triple cocktail designed based on collateral sensitivity could completely inhibit bacterial growth within 24 h, and no resistant strains were detected, which demonstrated the advantages of the rational formulation strategy over single phages (Fig. 3).

The development of most phage cocktails relies on the simple superposition of lysis spectra and empirical combination, and generally neglecting the principles of resistance evolution and fitness trade-offs during phage-bacteria coevolution, resulting in common limitations, including overlapping receptor targets, insufficient synergistic effect, and suboptimal long-term bacteriostatic activity (Mu et al. 2025; Zhao et al. 2024). It has been reported that several phages were screened to formulate cocktails according to the phage host spectrum and principles of coevolution, and the bacteriostatic effect was determined by bacterial growth curve, but the phage-bacteria interaction mechanism was not explored, which could not rationally guide the design of specific cocktail formulations (Kaneko et al. 2026). Among the published phage cocktail studies, more than 70% of empirically formulated preparations can screen out stable resistant strains after 5 serial co-culture passages with host bacteria. The reason is that the targeted design of phage combinations is not carried out for the resistance escape path of bacteria (Kaneko et al. 2026).

With the growing interest in phage therapy, investigation into the mechanisms underlying phage-bacteria interactions have advanced significantly. Since the evolutionary potential of bacteria is much higher than that of phages (Acton et al. 2024), bacteria can easily evolve phage-resistant mutants under the selection pressure of phages (Mugo et al. 2025; Palma et al. 2024). Among them, the structural mutation of host surface receptors is the main mechanism for bacteria to evade phage infection, accounting for more than 70% of all resistance mutations (Hampton et al. 2020; Labrie et al. 2010). The emergence of bacterial phage resistance usually comes with a fitness cost (Zhao et al. 2024), and collateral sensitivity, as a form of fitness cost, has important value in phage application. This study showed that among the 20 phage-resistant mutants, 85% of the PC13-resistant strains had missense, nonsense, or frameshift mutations in genes related to LPS O-antigen synthesis, indicating that LPS O-antigen is the main potential receptor of PC13. *Salmonella* blocks the adsorption of PC13 by mutating LPS synthesis genes to truncate or delete LPS O-antigen, which is consistent with the reported phage resistance mechanism of *Salmonella* (Acton et al. 2024; Martinez-Soto et al. 2024).

Notably, the emergence of bacterial phage resistance is usually accompanied by a significant fitness cost, and collateral sensitivity is the manifestation of this evolutionary trade-off (Mu et al. 2025). In this study, through receptor prediction and comparative genomic analysis, the molecular mechanism of the synergistic bacteriostatic effect of the triple cocktail was clarified: the RBPs of PC13 specifically target LPS O-antigen, the RBPs of PC11 target LPS core oligosaccharide, and the RBPs of PC16 specifically target outer membrane porin RopA1 and vitamin B12 transporter BtuB (Table 1). The receptor recognition motifs of the three phages have no sequence homology, and the targets are completely

independent (Fig. 5C, 5D). The possible mechanism for the significantly enhanced bacteriostatic effect of the triple cocktail against *Salmonella* is as follows: to evade the infection of PC13, *Salmonella* leads to the deletion of O-antigen structure through mutations in LPS synthesis-related genes. Although it successfully blocks the adsorption of PC13, it also fully exposes the LPS core oligosaccharide and outer membrane protein receptors originally shielded by O-antigen, which are the specific adsorption targets of PC11 and PC16, and finally leads to significant collateral sensitivity of the resistant strains to the latter two phages.

This mutually exclusive effect of "resistance mutation equals sensitization" makes it impossible for *Salmonella* to evade the killing of the three phages simultaneously through a single mutation, forming an irreversible "evolutionary trap", thus achieving a long-term bacteriostatic effect (Fig. 3). It has been found that after *Salmonella* develops resistance to phages targeting LPS O-antigen, it will show significant collateral sensitivity to phages targeting LPS core oligosaccharide and outer membrane proteins (Acton et al. 2024). Therefore, the use of collateral sensitivity can guide the design of phage cocktails or synergize with antibiotics to improve therapeutic effects.

In this study, based on the collateral sensitivity mechanism, three phages were screened to formulate a cocktail, and the *in vitro* bacteriostatic results showed that it significantly extend the duration of bacteriostasis (Fig. 3). The triple phage cocktail showed excellent therapeutic effect in the chicken *Salmonella* infection model. The fecal *Salmonella* loads of chicks in the treatment group dropped to 10 CFU/g on day 3 post-infection, and there was no significant difference in body weight growth between the treatment group and the healthy control group, which completely reversed the growth inhibition and clinical symptoms caused by *Salmonella* infection (Fig. 6). Torkashvand et al. (Torkashvand et al. 2024) similarly reported that phage therapy not only reduces *Salmonella* from broilers but also significantly promotes body weight gain. This effect may be attributed to the fact that phage therapy avoids the disruption of the normal intestinal microbiota caused by antibiotics, while reducing inflammatory responses and nutrient consumption associated with bacterial infection.

As one of the core virulence factors of *Salmonella*, the deletion of LPS O-antigen will not only lead to collateral sensitivity, but also significantly reduce the intestinal colonization ability, anti-host immune phagocytosis ability, and environmental adaptability of *Salmonella* (Krzyżewska-Dudek et al. 2024). LPS-deficient resistant strains exhibit increased sensitivity to pH and temperature stress, surviving only within a narrow range of environmental conditions, and show dramatically enhanced susceptibility to antibiotics (Zeng et al. 2024). In addition, the fitness costs also associated with OMP receptor mutations, such as when bacteria acquire *btuB* mutations to evade infection by BtuB-targeting phages, they simultaneously lose the ability to actively uptake vitamin B_{12}, resulting in severe growth restriction under low-nutrient conditions and a 121-fold reduction *in vivo* virulence (Lv et al. 2026). This dual fitness cost further improves the *in vivo* therapeutic effect of the cocktail in this study, which may also be one of the important reasons why chicks in the treatment group can completely eliminate intestinal *Salmonella* within 3 days. It has been shown that phage cocktails against Klebsiella pneumoniae designed through collateral sensitivity can significantly improve the therapeutic effect on mice infected with *Klebsiella*

pneumoniae (Yoo et al. 2023). It has also been reported that compared with single phage co-culture, the co-culture of SPLA1a and SPLA5b with bacteria can more effectively inhibit the emergence of phage resistance (Acton et al. 2024). Therefore, the identification of host susceptibility and resistance genes and the understanding of infection dynamics are key steps in the design of phage cocktails against specific bacterial pathogens. The results of this study demonstrate the application potential of using collateral sensitivity to improve the efficacy of phage therapy against *Salmonella* infection, and provide a sophisticated and effective strategy for addressing phage resistance evolved in *Salmonella*.

Declarations

Funding information

This work was funded by the National Key Research and Development Program of China (2025YFC3408500), and the postdoctoral project from Qingdao, Shandong Province, China (QDBSH20250102265), and Qingdao special project of science and technology for benefiting the people (24-1-8-xdny-6-nsh).

Author contributions

Huzhi Sun: Investigation, Data curation, Formal analysis, Writing – original draft. Yuanyang Zhao: Data curation, Formal analysis. Yanxin Yan: Data curation, Formal analysis. Guangqin Liu: Data curation. Qiang Pan: Data curation, Formal analysis. Huiying Ren: Conceptualization, Investigation, Project administration, Data curation, Writing – review & editing. Shaowen Li: Data curation, Writing – review & editing.

Conflicts of interest

No competing interests have been declared.

Ethical statement

All animal experiments in this study were approved by the Laboratory Animal Ethics Committee of Qingdao Phagepharm Bio-tech Co., Ltd (Ethics Approval No.: NABT-AE-2025-016) and were strictly implemented in accordance with the relevant national standards for laboratory animal welfare and ethics, as well as the 3R principles. The clinical Salmonella isolates used in this study were obtained from routine isolation and identification of clinical disease samples, did not involve human subjects, and required no informed consent.

Data Summary

The genome sequences of the three phages are available in NCBI under the accession numbers (PC13: ON381768; PC11: PZ302086; PC16: NC_103563). All data generated or analyzed during this study are included in this article.

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Figures

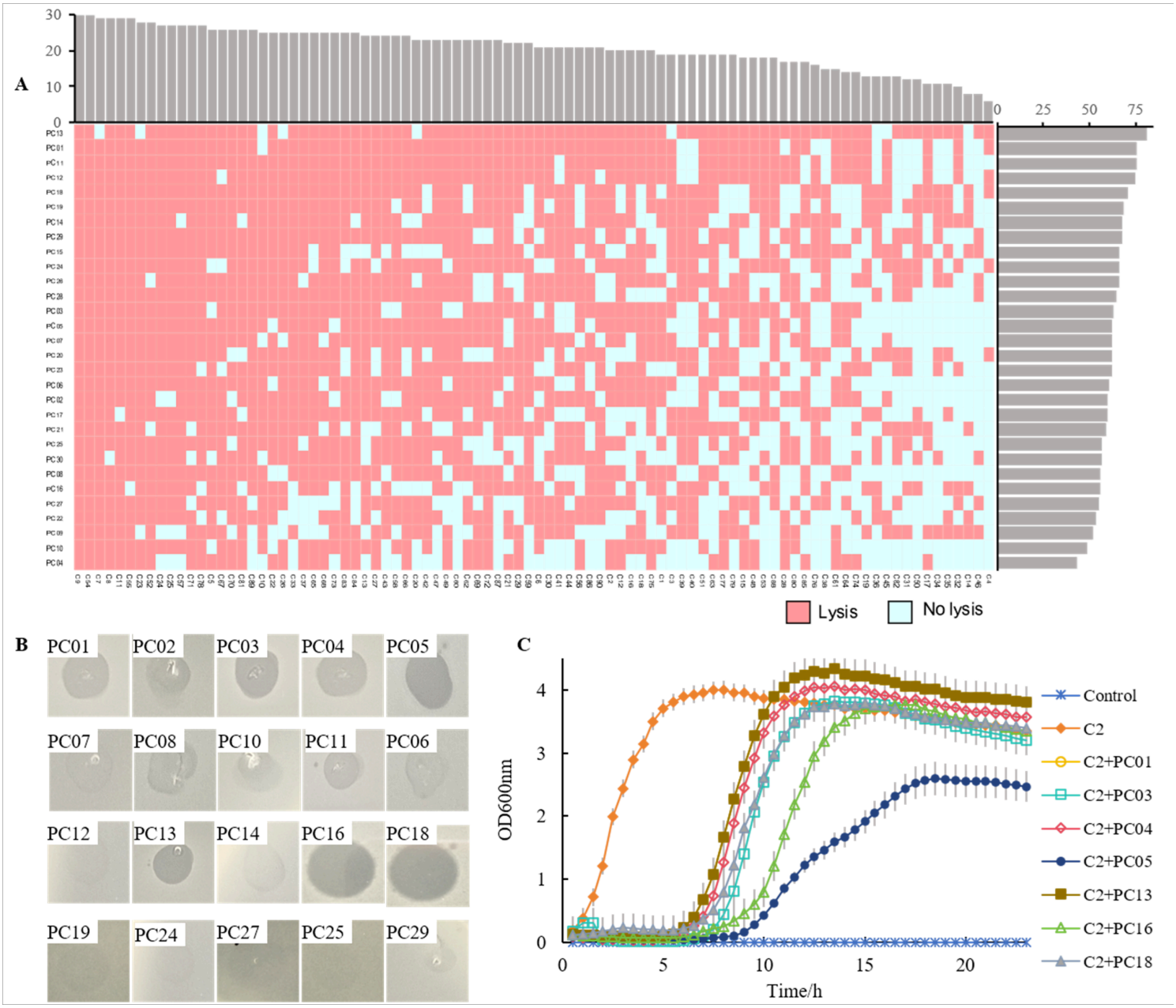


Figure 1

Screening of broad-spectrum Salmonella phages. (A) Host range and lytic activity heatmap; (B) Representative spot assay results; (C) *In vitro* lytic kinetic curves against *S. enteritidis* C2.

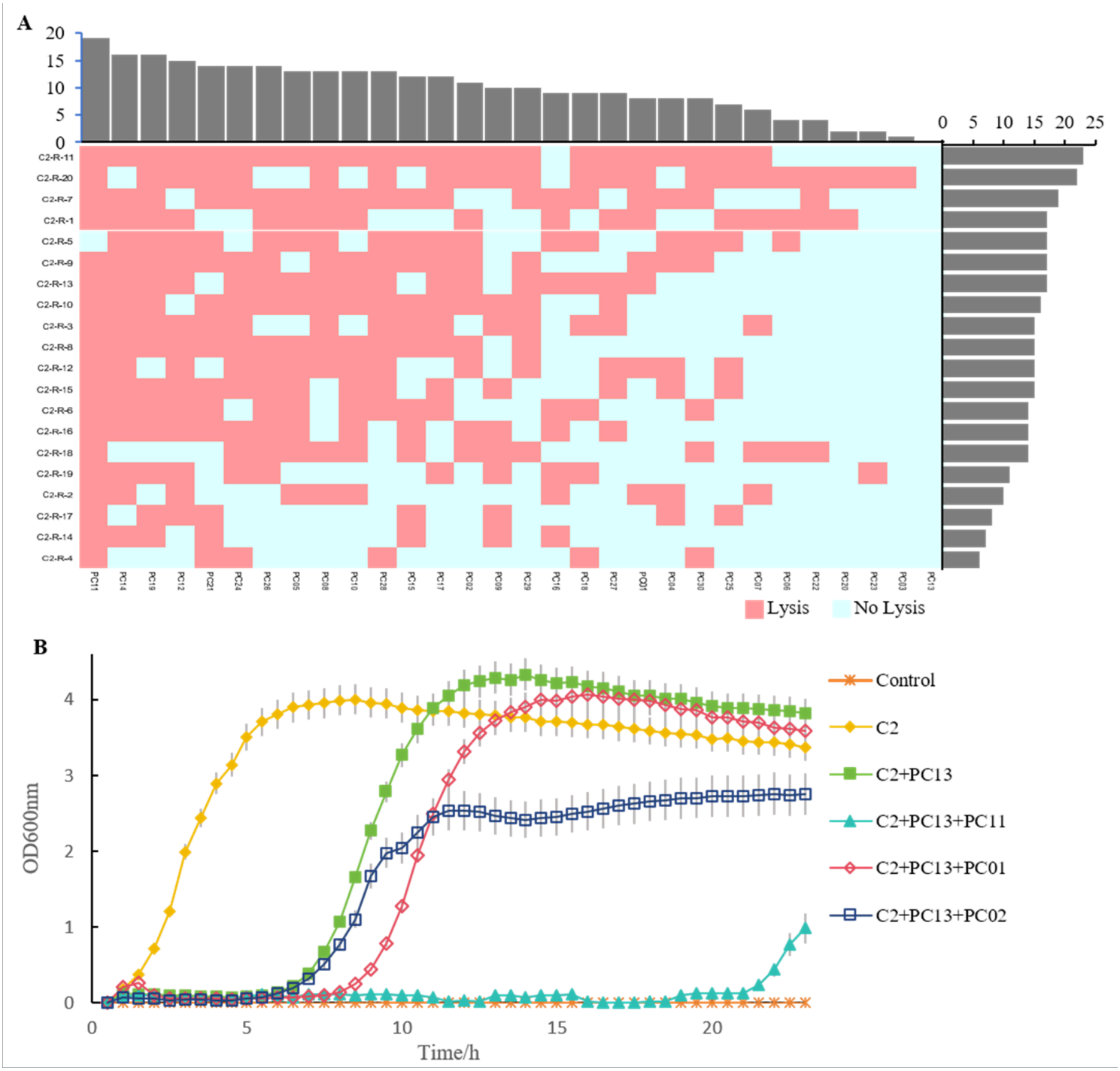


Figure 2

Selection and functional validation of phages with lytic activity against PC13-resistant *S. enteritidis* C2. (A) Cross-infection lytic profile heatmap of 30 phages against 20 PC13-resistant *S. enteritidis* C2; (B) *In vitro*bacteriostatic kinetic curves of different phage combinations against *S. enteritidis* C2.

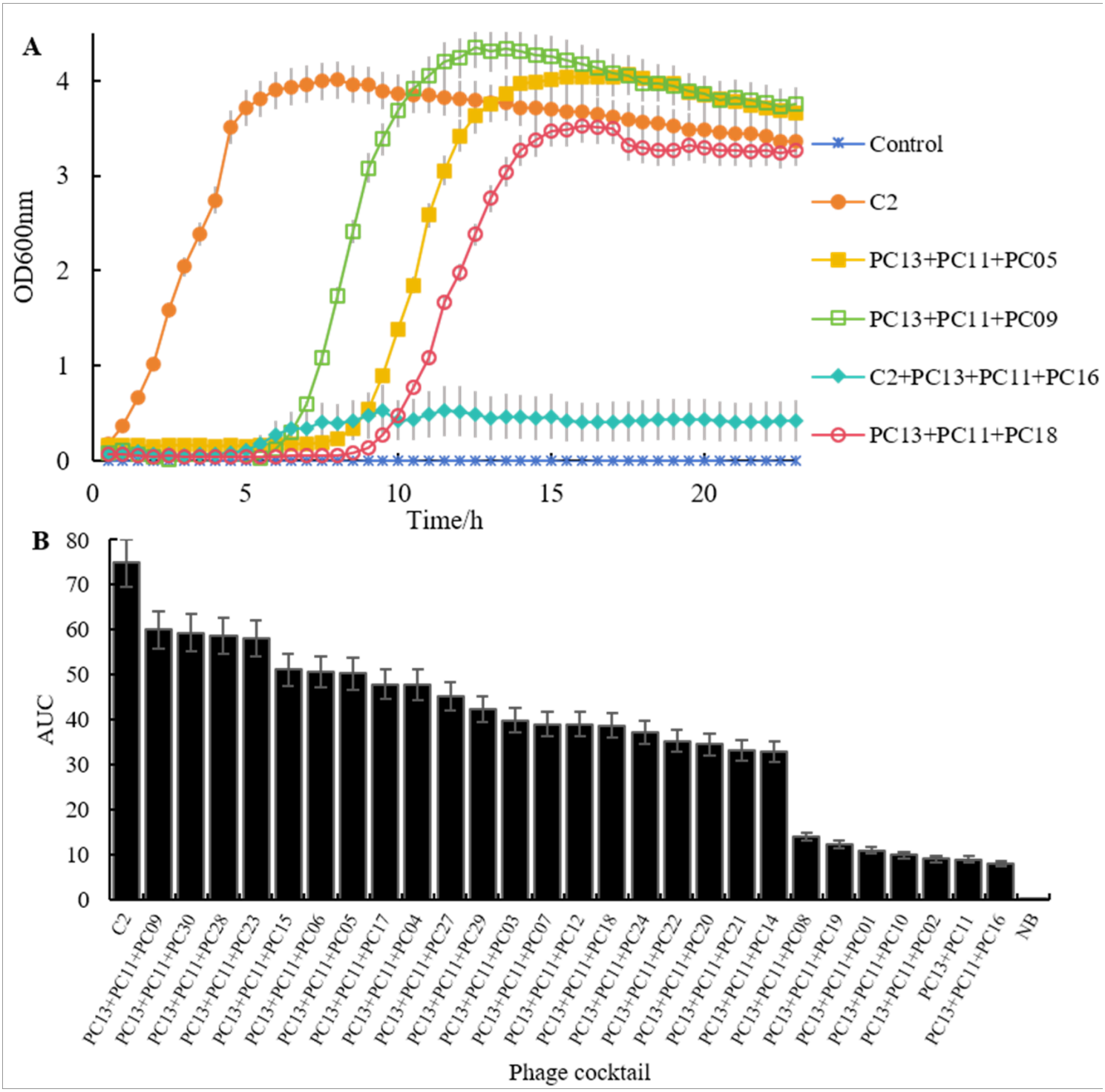


Figure 3

Optimized screening of triple phage cocktails. (A) Growth inhibition curves of different triple phage combinations against *S. enteritidis* C2; (B) Area under the curve (AUC) analysis of 24 h bacteriostatic curves for various phage combinations.

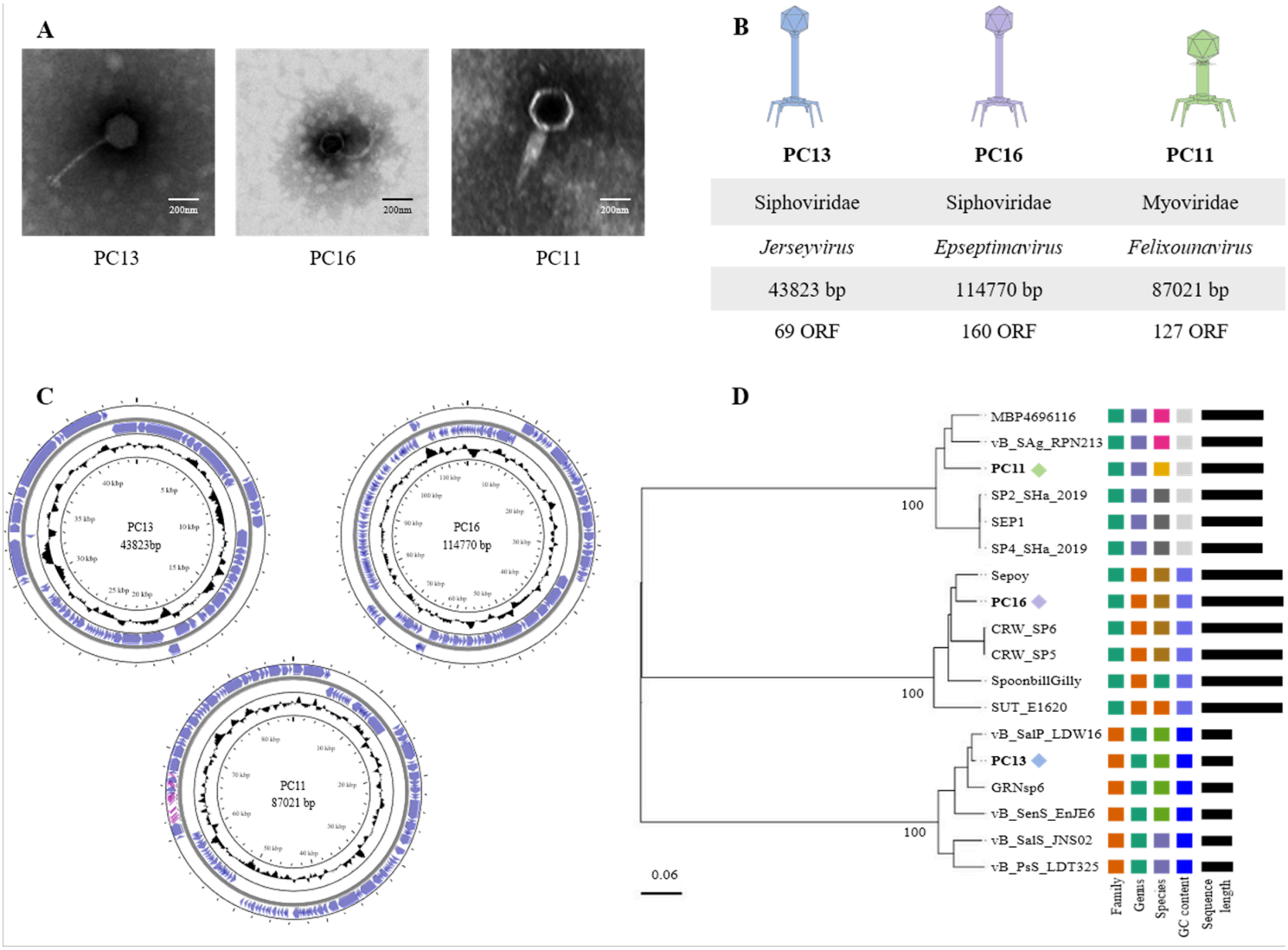


Figure 4

Morphological, physiological and genomic characterization of three representative Salmonella phages (A) Transmission electron microscopy (TEM) images of phages PC13, PC16, and PC11; (B) Taxonomic classification, genome size and open reading frame (ORF) number of the three phages; (C) Circular visualization of the complete genome maps of phages PC13, PC16, and PC11; (D) Phylogenetic tree analysis of the three phages and their closely related homologs

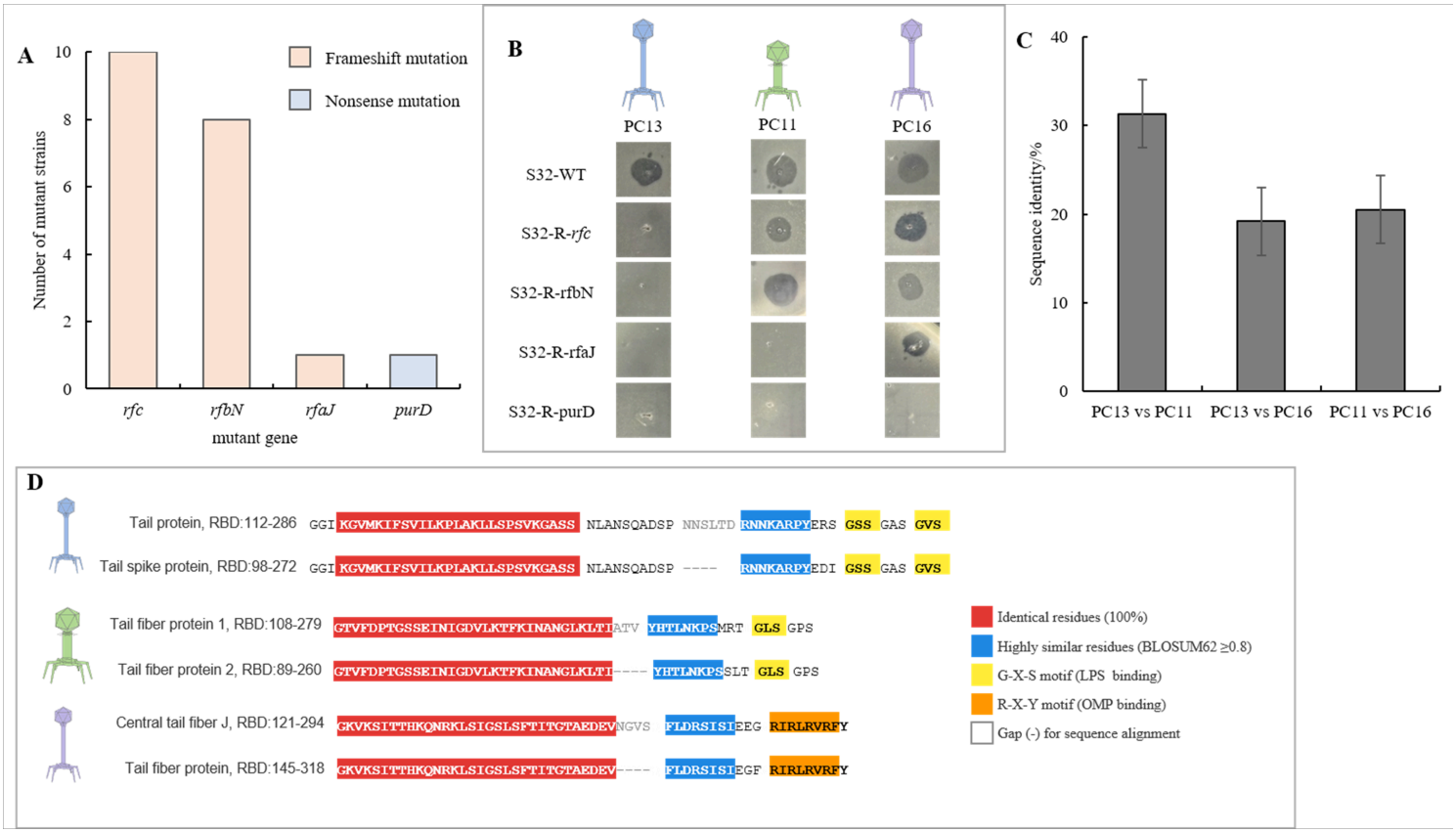


Figure 5

Molecular mechanism underlying the synergistic bacteriostatic effect of the triple Salmonella phage cocktail. (A) Statistical analysis of mutation types and counts in PC13-resistant strains, focusing on LPS core oligosaccharide synthesis-related genes; (B) Spot assay validation of lytic activity of the three phages against wild-type and PC13-resistant mutant strains; (C) Sequence identity analysis of RBPs among the three phages, showing minimal inter-phage sequence homology; (D) Multiple sequence alignment of receptor-binding domains (RBDs) of the phage RBPs, with annotated conserved functional motifs associated with host receptor recognition.

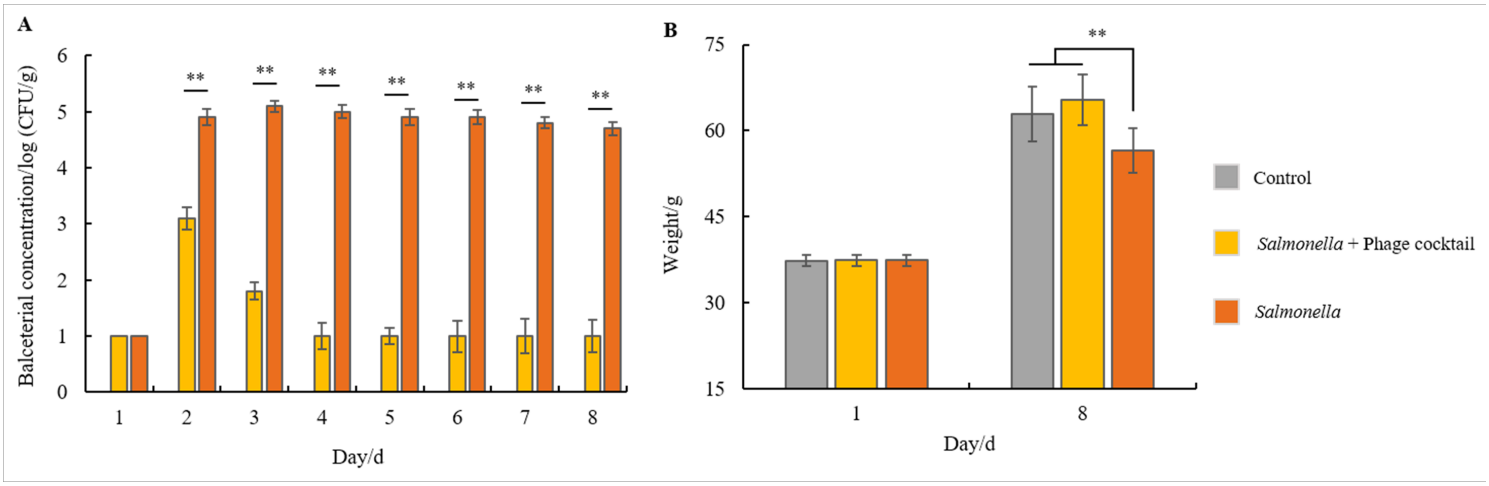


Figure 6

In vivo therapeutic efficacy of the phage cocktail against *S. enteritidis* C2 infection in chicks. (A) Dynamic changes in fecal *Salmonella* load (log CFU/g) in chicks of the control, *Salmonella*-infected, and phage cocktail-treated groups over the 8-day experimental period; (B) Body weight changes of chicks in different treatment groups throughout the trial.