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Biological and genomic characterization of a polyvalent phage PSH-1 against multidrug-resistant *Salmonella Enteritidis*

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Abstract

Background Bacteriophage has been renewed attention as a new antibacterial agent due to the limitations of antibiotic treatment. Bacteriophages are generally thought to be highly host specific and even strain specific, but a small number of polyvalent bacteriophages have been found to infect bacteria of different genera.

Results In this study, a virulent lytic bacteriophage (named *Salmonella* phage PSH-1) of *Salmonella Enteritidis* was isolated from the sewage samples of a large-scale pig farm, PSH-1 demonstrated lytic activity against four multidrug-resistant *Salmonella Enteritidis* isolates and *Escherichia coli*, and then its biological characteristics, genome and bacteriostatic ability were investigated. The results showed that the initial titer of PSH-1 was 1.15×10^{10} PFU/mL and the optimal multiplicity of infection (MOI) was 0.01, PSH-1 has stable activity in the range of pH 3.0–11.0. One-step growth curve showed that its latent period was 20 min, burst time was 80 min, and the burst was 495 particles. The whole-genome sequencing results revealed phage PSH-1 had a linear dsDNA with 48,466 bp length. The G/C content was 45.33%. Non-coding RNA genes and virulence factors were not found. Eighty- five open reading frames (ORFs) were identified after online annotation. By tests, the use of phage could succeed in controlling the artificial *Salmonella* contamination in milk at a range of temperatures.

Conclusions This study reports a novel *Salmonella Enteritidis* phage PSH-1, which has a robust lytic ability, no virulence factors, and good stability. The characterization and genomic analysis of PSH-1 will develop our understanding of phage biology and diversity and provide a potential arsenal for controlling of salmonellosis.

Keywords Multidrug-resistant *Salmonella Enteritidis*, Polyvalent phage, Biological characteristics, Genomic analysis, Food bacteriostatic test

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Introduction

The gram-negative bacterium *Salmonella* is the causative agent of salmonellosis in humans and animals characterized by enteritis and septicemia, and it is one of the most important zoonotic pathogens worldwide [1]. In the United States, non-typhoidal *Salmonella* (NTS) is the leading cause of bacterial foodborne illness with $4.1 billion lost to NTS illness yearly [2, 3]. A survey in the UK showed that *Salmonella typhimurium* and *Salmonella enteritidis* infections accounted for more than 75% of human *Salmonella* infections [4]. Annually, around 200 million to 1 billion cases of *Salmonella* infections are recorded worldwide, with 93 million cases of gastroenteritis and 155,000 deaths; among them, approximately 85% of the cases are associated with the consumption of contaminated food [5]. Therefore, it is urgent to find a new effective antibacterial method to prevent and treat *Salmonella* infection.

The emergence of antibiotic resistance has shown an increasing trend of 20–30% per decade [6]. Multidrug-resistant *Salmonella* has also been found in several previous studies. It has been reported that 30.9% of *Salmonella* isolated from broiler farms were resistant to streptomycin, of which 13.9% were resistant to tetracycline, 12.6% to gentamicin, and 8.6% to sulfamethoxazole trimethoprim [7]. For example, multidrug resistance was detected in 17% of broilers in Egypt, with the highest resistance to neomycin (100%), nalidixic acid and cefoxitin (95%), norfloxacin (86.3%), cefotaxime (77.2%), amikacin (72.7%), erythromycin (68.1%) and chloramphenicol (40.9%) [8]. Similarly, in the United States, 19.6% of infant streptococcus isolates from animals were multidrug resistant, with the highest resistance to aminoglycosides, chlorophenols, beta-lacamides, and tetracyclines [9].

In recent years, bacteriophage as an alternative to antibiotics, has come into people's attention because of phage's ability to specifically lyse bacteria and easily obtained and safety [10]. In 2010, a phage product named BioTector appeared abroad as a word additive to control *Salmonella* contamination in feed [11]. In 2013, Micreos launched the world's first human health product based on phage lyase, Staphefekt™, which has been shown to inhibit the growth of *Staphylococcus* aureus on the skin, relieve associated symptoms, and do not harm beneficial microorganisms on the skin [12]. In 2017, a phage product "Happy shrimp", which is mainly used for the prevention and treatment of shrimp premature death syndrome, was launched by China's Nanjing Feijilke Biotechnology Co., LTD [13]. Thanchanok reported that the isolated bacteriophages vB_AdhM_DL, vB_AdhS_TS3, vB_AdhM_TS9, and vB_AdhS_M4 had obvious inhibitory effect on the growth of *Aeromonas Dhakensis* [14]. A phage cocktail demonstrated high efficiency in controlling various serovars of *Salmonella* historically linked to contamination on these broiler farms [15]. Nowadays, the advantages of bacteriophage-based biological control methods have been generally accepted, making bacteriophages expected to be developed as a new antibacterial agent for the prevention and control of *Salmonella* [25].

In this study, four multi-drug resistant *Salmonella Enteritidis* isolates were used as the hosts, and a strain of *Salmonella Enteritidis* phage PSH-1 was isolated from sewage samples, and its biological characteristics and whole-genome were studied to provide a theoretical basis for the development of phage antibacterial preparations and to provide new method for the prevention and control of *salmonella* infection.

Materials and methods

Sample collection and tested strains

A total of 40 samples (20 sewage samples and 20 fecal samples) were collected in 4 feedlots from Henan, China, in 2019 (The sample collection was approved by the pig farm and met the requirements of the Animal Ethics Committee of Henan Agricultural University, and the collection of sewage and fecal samples were based on published studies [16]). Samples were processed within 48 h. One hundred and thirty-four tested strains (54 *Salmonella Enteritidis* strains and 10 strains each of *Escherichia coli*, *Streptococcus pneumoniae*, *Actinobacillus pleuropneumoniae*, and *Haemophilus parasuis*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*) were from the strain collection of our laboratory. Four multi-drug resistant *Salmonella Enteritidis* strains (Table 1) of which were used as host strains to isolate phages, and the rest were used to determine host range.

Susceptibility testing and detection of resistance genes

The minimum inhibitory concentrations (MICs) of 13 antimicrobial agents [Amoxicillin, Cephalosporin, Cefquinome, Gentamicin, Amikacin, Oxytetracycline, Doxycycline, Florfenicol, Colistin, Sulfamethoxazole/trimethoprim (5/1), Enrofloxacin, Olaquindox, Mequindox] for 4 host strains were determined using the broth microdilution method, and the results were interpreted following the guidelines of the Clinical and Laboratory Standards Institute [17]. *Escherichia coli* ATCC 25,922 was used as control strains. Multiple drug resistance genes (*mcr-1*, *floR*, *rmtB*, bla_{CTX-M}) were screened by polymerase chain reaction (PCR) for the 50 *Salmonella* strains involved, and primers were listed in Table S1.

Isolation, identification and purification of phage

The phage isolation method is as described above [18]. First, the phage was purified several times by a double agar plate until multiple individual transparent plaque

Table 1 MIC of 13 antimicrobial drugs against 4 multi-drug resistant *Salmonella Enteritidis*

Antibiotics	4 strains of multi-drug resistant Salmonella MIC(μg/mL)			
	SA209	SH01	SH20	SH171
Amoxicillin	> 512	8	512	512
Cephalosporin	512	128	256	2
Cefquinome	256	256	32	1
Gentamicin	8	< 1/2	64	128
Amikacin	8	< 1/2	< 1/2	8
Oxytetracycline	512	512	512	256
Doxycycline	32	128	32	32
Florfenicol	> 512	16	128	256
Colistin	< 1/2	< 1/2	1	8
Sulfamethoxazole /trimethoprim (5/1)	512	512	512	> 512
Enrofloxacin	1	< 1/2	< 1/2	< 1/2
Olaquindox	64	32	4	128
Mequindox	16	64	4	16

The table shows the MIC values of 13 antibacterial drugs against 4 host *Salmonella Enteritidis*: SA209, SH01, SH20, and SH171 are all multi-drug resistant bacterias

Amoxicillin (content: 80%), Cephalosporin (content: 80%), Cefquinome (content: 80%), Gentamicin (content: 62%), Amikacin (content: 85%), Oxytetracycline (content: 80%), Doxycycline (content: 80%), Florfenicol (content: 98%), Colistin (content: 95%), Sulfamethoxazole-trimethoprim (content: 98%), Enrofloxacin (content: 90%), Olaquindox (content:80%), Mequindox (content: 90%)

appeared on the separation plate. A single transparent and regular shaped plaque was selected, the isolated phage was verified by "drop method", and then the isolated phage was cultured and multiplied for several times, and finally stored with glycerol for use [19].

Identification of phage nucleic acid type

The phage concentrate obtained by the PEG precipitation method as described in the literature [19]. The phage genome was extracted from the concentrated solution by Virus DNA/RNA Kit (TIANamp Virus DNA/RNA Kit: DP315, TIANGEN Biotech (Beijing)Co., Ltd.), and was identified by DNase I and RNase A: experimental group: 12 μL genome + 2 μL DNase I/RNase A + 6 μL ddH20; control group: 12 μL genome + 8 μL ddH20, metal bath at 37℃ for 30 min, verified by 1% agar-gel electrophoresis.

Transmission electron microscopy

The phage culture was concentrated and collected, and the phage was negatively stained with 2% phosphotungstic acid, and then the phage morphology was observed by a projection electron microscope. (JEM-1400, Japan).

Biological characteristics of the phage

The biological characteristics of PSH-1 mainly include the killing curves of the phage at different multiplicities of infection (MOI), its thermal stability, and pH stability. The double-layer agar plate method was used to determine phage titer and optimal MOI [16]. The MOI of phage PSH-1 has undergone some modifications as described earlier [21]. The concentration of *Salmonella Enteritidis* SH01 was set at 10^8 CFU/mL. A total of 6 different MOIs, including 0.001, 0.01, 0.1, 1, 10 and 100, were set. Then the optimal MOI was determined by measuring phage titer.

The pH stability of phage PSH-1 was determined by incubation in LB broth adjusted to pH 2–12 for 2 h as described previously [22], and the experiment was repeated three times. The thermal stability of PSH-1 was determined by incubating LB broth adjusted to 40–80 °C for 1 h, as previously described [23]. A double layer agar plate was used in the thermal stability and pH stability assay.

Table 2 The correlation between phage lysis profile and bacterial resistance genes

Bacterial strains	Resistance genes	Number of strains	Number of lytic strains	Lytic Rate (%)
Salmonella Enteritidis	*mcr-1*	21	14	66.7
Salmonella Enteritidis	*floR*	50	32	64.0
Salmonella Enteritidis	*rmtB*	37	27	72.9
Salmonella Enteritidis	bla_{CTX-M}	23	14	60.9

a: *mcr-1*: the polymyxin resistance gene; *floR*: the chloramphenicol resistant gene; *rmtB*: the aminoglycoside resistance gene; bla_{CTX-M}: the β-lactamase-encoding gene

One step growth curve of the bacteriophage

The one-step growth curve of PSH-1 is described previously [24]. PSH-1 and *Salmonella Enteritidis* SH01 culture were mixed at the ratio of 0.01, incubated at 37℃ for 15 min, centrifuged at 5000 g for 10 min, washed and precipitated with LB broth twice. Then the LB broth preheated at 37℃ was precipitated and suspended and cultured in a constant temperature shaking table at 37℃. Samples were taken at 0, 5, and 10 min, and then samples were taken every 10 min to detect the PSH-1 titer, and the experiment was conducted three times.

Phage host range

The host range of phage PSH-1 was determined according to the previous description, with some modifications [25]. A total of 90 strains were selected (Table 2) to determine the host range, and the spot method was used to observe whether there was clear plaque production.

DNA sequencing, assembly, annotation and bioinformatics analysis of the phage genome

In the process of phage genome sequencing and subsequent analysis, the third-generation sequencing platform PromethION from Oxford Nanopore Technologies (ONT) was utilized in conjunction with its proprietary kits (SQK-LSK109, EXP-NBD104/114), specifically

designed for constructing high-quality phage libraries. The constructed libraries were loaded onto the R9.4 sequencing flow cell, followed by a prolonged sequencing run of 48 to 72 h on the PromethION sequencer to capture the integrity and complexity of the phage genome. Prokka (version 1.13) software was employed to predict the coding genes of the assembled phage genome, identify open reading frames (ORFs) within the genome, and predict their functions. The Island Viewer 4 website (http://www.pathogenomics.sfu.ca/islandviewer/) was used to predict genomic islands present in the genome. RepeatMasker (version 4.1.0) software was leveraged to identify repetitive sequences in the phage genome. Detailed annotation of phage gene functions was performed using hmmer (version 3.3.1) software in conjunction with multiple databases such as GO, Pfam, TIGRFAM, and Refseq. The Antibiotic Resistance Genes Database (ARDB) and Diamond blastp (version 2.9.0) tool were utilized to identify resistance genes in the phage genome and their associated drug targets. Based on the Virulence Factors Database (VFDB), Diamond blastp was applied to annotate virulence genes in the phage protein sequences. SignalP (version 5.0) was used to analyze the protein sequences of all predicted genes, identifying proteins containing signal peptides, while tmhmm (version 2.0) was employed to predict transmembrane proteins. SnapGene Viewer (version 4.2.3.0) was utilized to generate circular genome maps of the phage, providing a visual representation of the genome structure and gene locations. Software and database information is shown in Table S3.

Comparative genomic analysis of phage PSH-1

The whole genome of PSH-1 was compared by BLAST (BLAST: Basic Local Alignment Search Tool (nih.gov)), and the genomes of 20 strains phage with more than 90% homology or more than 60% coverage were selected and downloaded to construct the genome-wide evolutionary tree. Then use the CD - HIT (https://sites.google.com/view/cd-hit) for 20 phages comparing genome-wide pin - two nucleotide sequences, Cluster analysis using Chiplot (https://www.chiplot.online/). At the same time, comparative genomic analysis of 5 *Salmonella* phages and 2 *Escherichia coli* phages with high homology to PSH-1 was performed.

Table 3 The host range of phage PSH-1

Bacterial strains	Number of strains	Number of lytic strains	Lytic Rate (%)
Salmonella	50	33	66
Escherichia coli	10	3	30
Streptococcus	10	0	0
Actinobacillus pleuropneumoniae	10	0	0
Haemophilus parasuis	10	0	0
Klebsiella pneumoniae	10	0	0
Acinetobacter baumannii	10	0	0
Staphylococcus aureus	10	0	0
Pseudomonas aeruginosa	10	0	0

In vitro antibacterial test of phage

The antibacterial activity in milk (Inner Mongolia Yili Industrial Group Limited by Share Ltd, protein percentage 5%, fat percentage 6%, carbohydrate 2%, Na 3%, Ca13%) was tested in vitro. The milk was sterilized at 121 ° C for 15 min, then 5 ml of milk was added to each tube. The phage was added to the milk test tube to make the final concentration of phage about 10^9 PFU/mL. Then, in the 4℃ environment, the bacteria were inoculated 24 h later, so that the final concentration of bacteria was about 10^5 CFU/mL. The test tubes were placed at 4℃, 25℃, 37℃, respectively, after adding bacteria and after 1, 2, 3, 4, 5, 6, 7 days after inoculation to determine the viable bacteria count (CFU/mL).

Results

Susceptibility testing

The minimum inhibitory concentrations (MICs) of 13 antimicrobial agents for 4 host strains showed the 4 host *Salmonella Enteritidis* strains (SA209, SH01, SH20, and SH171) were all multi-drug resistant bacteria, and their resistance to multiple antibacterial drugs varies (Table 1).

Detection of resistance genes

Among the 50 *Salmonella Enteritidis* strains tested, the number of strains carrying four drug resistance genes (*mcr-1*, *floR*, *rmtB*, bla_{CTX-M}) was 21, 50, 37, 23, respectively (Table 3).

Isolation and purification of phage PSH-1

As many as 40 samples were tested for the presence of bacteriophages using four multidrug-resistant *Salmonella Enteritidis* as hosts. Finally, a *Salmonella Enteritidis* phage (PSH-1) was obtained by double-layer plate method and spot method. 1 out of 40 samples tested, resulting in an isolation rate of 2.5%.

Phage nucleic acid type identification

Agarose gel electrophoresis showed that the nucleic acid of PSH-1 was sensitive to DNase I, but not to RNase A (Figure. S1), which suggested that the nucleic acid of PSH-1 is DNA.

Transmission electron microscopy

The results of electron microscopy showed that phage PSH-1 had a polyhedral head, with a head of approximately 69.3 nm and a tail of approximately 203.2 nm (Fig. 1). According to the latest classification of viruses

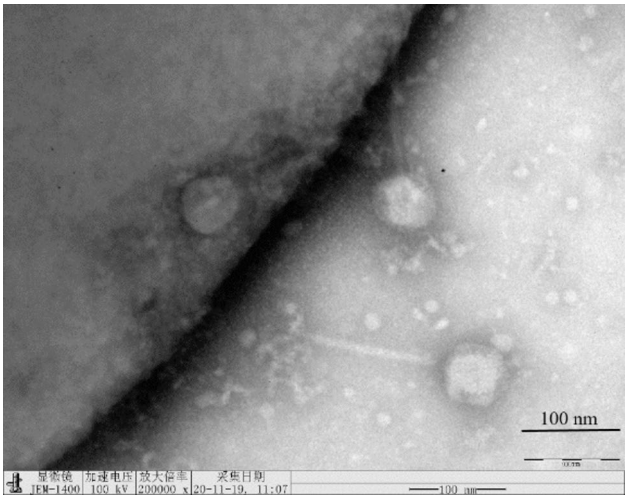

Fig. 1 The transmission electron microscopy of phage PSH-1. Phage PSH-1 had a polyhedral head, with a head of approximately 69.3 nm and a tail of approximately 203.2 nm

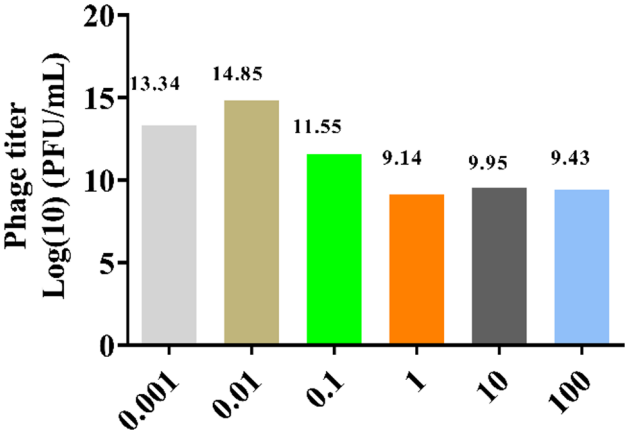

Fig. 2 Determination of MOI of phage PSH-1. Among the 6 different MOI (multiplicity of infection) set, when MOI = 0.01, the titer of bacteriophage PSH-1 was significantly higher than others

in ICTV2023, PSH-1 belongs to *Duplodnaviria Realm*, *Heunggongvirae Kingdom*, *Uroviricota Phylum*, *Caudoviricetes Class*, *Jameshumphriesvirinae* Subfamily *Sircambvirus Genus*, *Sircambvirus justaphage Species*.

Biological characteristics of the phage

After 6 h of mixed culture of phage PSH-1 and *Salmonella Enteritidis* SH01 according to different MOI, the titer of phage was shown on Fig.2 . When MOI was 0.01, the titer of phage was the highest, which was 8.5×10^{14} PFU/mL. Therefore, the optimal MOI of phage PSH-1 infected with *Salmonella Enteritidis* SH01 was considered to 0.01. This result indicate that PSH-1 can exert bactericidal effect when infecting the host *Salmonella Enteritidis* through very small amounts.

The growth of the phage PSH-1 at different temperatures (40 ℃, 50 ℃, 60 ℃, 70 ℃ and 80 ℃) was

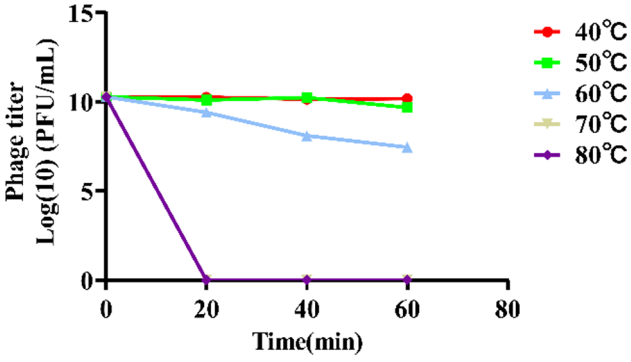

Fig. 3 Thermal stability of PSH-1. Thermal stability: phage particles were incubated at various temperatures as indicated for 2 h. Then the samples were collected after 20, 40 and 60 min

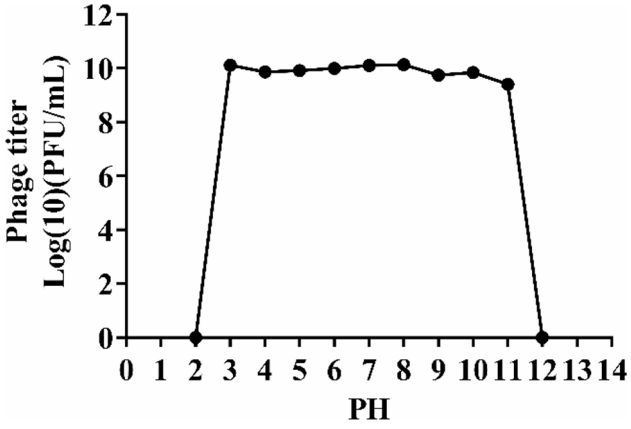

Fig. 4 The PH stability of PSH-1. PH stability: phage particles were incubated under diferent pH conditions for 2 h

determined (Fig. 3). The titer of the phage remained more or less unchanged at 40 and 50 °C, while the phage titer gradually decreased at 60 °C. In addition, the phage titer decreased rapidly at 70 °C and 80 °C, and the activity could not be detected after 20 min. Meanwhile, the growth of phage PSH-1 was measured at the pH range of 2 to 12 (Fig. 4). The results showed that PSH-1 exhibited tolerance at pH values of between 3 and 11, however, the phage titers decreased sharply at the acidic (pH 1–2) or alkaline (pH 12–14) conditions, which indicated that the phage was active at a wide pH range.

These results indicated that PSH-1 had excellent environmental tolerance and could exist stability under high temperature and strong acid and alkali conditions. At the same time, this is a very important property, the stable existence of PSH-1 can ensure that it can still play a strong bactericidal ability in the face of complex environments in future clinical applications.

One step growth curve of the bacteriophage PSH-1

In the first 20 min, the titer of phage PSH-1 showed a downward trend, then increased rapidly within 80 min,

before reaching stationary phase after 100 min, with a titer of 10^{12} PFU/mL. This indicated that the latent period of PSH-1 was 20 min, the burst time was 80 min, and the burst was 495 virions (Fig. 5).

Phage host range

Among the 90 strains tested, 50 strains of multidrug-resistant *Salmonella Enteritidis* and 10 strains each of *Escherichia coli*, *Streptococcus*, *Actinobacillus pleuropneumoniae*, and *Haemophilus parasuis* were included, the phage PSH-1 was able to lyse 33 strains of *Salmonella* (66%) and 3 strains of *Escherichia coli* (30%), and can't lyse the remaining strains (Table 3) (Figure.S3). On the other hand, among the 50 *Salmonella Enteritidis* strains, there have 21 strains carrying the *mcr-1* gene, 37 strains carrying the *rmtB* gene, 23 strains carrying the bla_{CTX-M} gene and 50 strains carrying the *floR* gene. Phage lysis rates for *Salmonella* carrying different resistance genes (*mcr-1*, *rmtB*, bla_{CTX-M}, *floR*) were 66.7%, 72.9%, 60.9% and 64%, respectively (Table 2).

Phage whole genome analysis and functional annotation

The results of whole genome sequencing show that: sequencing through the Nanopore third-generation sequencing technology platform of Wuhan Bena Technology Co., LTD., the total amount of data obtained is as follows: There were 20,081 reads with a total length of 1,000,054,226 bp, among which the longest reads was 103,914 bp, the shortest reads was 48,109 bp, and the average reads length was 49,801 bp. The final assembly results showed that the full length of PSH-1 genome was 48,466 bp, the G+C content was 45.33%, and the GenBank entry number was OR260414.

The results of online annotation by RAST (RAST Server - RAST Annotation Server (nmpdr.org)) indicate that there are 85 open reading frames (ORFs) in the whole genome sequence of phage, of which 57 ORFs are hypothetical proteins. Five were structure-related proteins (including Phage coat protein, Phage tailspike, Phage fibritin (wac) protein), nine were related to phage replication and metabolism, two were lysed proteins, and two were packaged proteins. It also contains 16 enzyme restriction sites, such as *HindIII*, *ScaI*, *SwaI*, *BamHI*, etc. (Fig. 6).

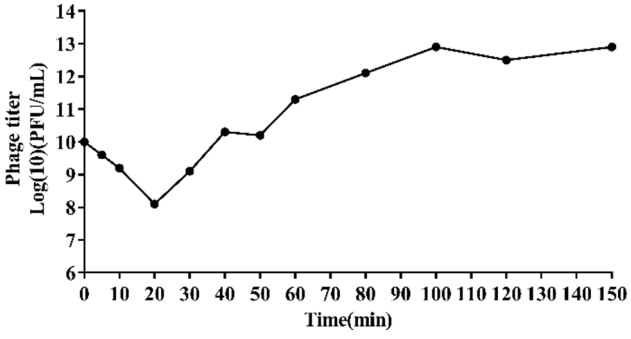

Fig. 5 The phage PSH-1 one-step growth curve. The PSH-1 and the *Salmonella Enteritidis* SH01 culture were mixed at a MOI of 0.01. The titer of the phage PSH-1 was determined every 10 min. The latent period of PSH-1 was 20 min, the burst time was 80 min, and the burst was 495 particles

VFDB annotation (virulence Factors Database, VFDB) : Diamond blastp was used to annotate the target protein sequence based on the VFDB database. Phage PSH-1 genome was not found to contain major virulence factors of characteristic bacterial pathogens.

Comparative genomic analysis of phage PSH-1

The similarity of the nucleotide sequence of the PSH-1 genome to that of other phages was calculated by sequence alignment (BLASTN) using default Settings. It can be seen from the obtained results that PSH-1 has high similarity with *Salmonella* phage. Twenty phages with high similarity to PSH-1 (Table S2) were selected and MEGA11(Contents --MEGA manual (megasoftware.net)) was used to perform genome comparison and construct genome phylogenetic tree (Fig. 7). Through BLAST comparison, it was found that the genome similarity of PSH-1 to PST472 (GenBank accession: NC_054644.1) was the highest, which reached 99.22%. At the same time, the genome comparison coverage rate of *Salmonella* PSH-1 phage LPST10 (GenBank accession: NC_054645.1) was 77%, which was the highest compared with PSH-1. However, MEGA11 genome comparison and phylogenetic tree construction showed that KFS_SE2 (GenBank accession: NC_054641.1) is closely related to PSH-1, while PSH-1 is closely related to LPST10, but VSt472 is relatively low related. At the same time, phylogenetic tree showed that PSH-1 was also related to *E. coli* phage C1 and vB_EcoS_swi2 and *Shigella* phage DS8.

In phylogenetic tree, we can see that phage KFS-SE2 and PSH-1 are closely related. After ppair comparison of the genomes of 20 strains of phages, a heat map was formed using genomic similarity. As shown in Fig. 8 (a), the color coding allows a rapid visualization of the clustering of the phage genomes based on intergenomic similarity: the more closely-related the genomes, the darker the color. it was found that phage Pu29, D10, VSt472 had the highest similarity rate with PSH-1, reaching 99%. At the same time, PSH-1 had a high similarity rate with *E. coli* phage C1. The results of genomic collinear comparison analysis between PSH-1 and seven highly similar phages are shown in Fig. 8 (b). Although the genomes are highly similar, there are still differences in gene modules of specific functions, such as tail fiber genes.

In vitro antibacterial testing of phage

As shown in Fig. 9(A, B and C), with ca. 1×10^5 CFU/mL contamination level, *Salmonella Enteritidis* was capable to grow rapidly in milk untreated by phages at relatively

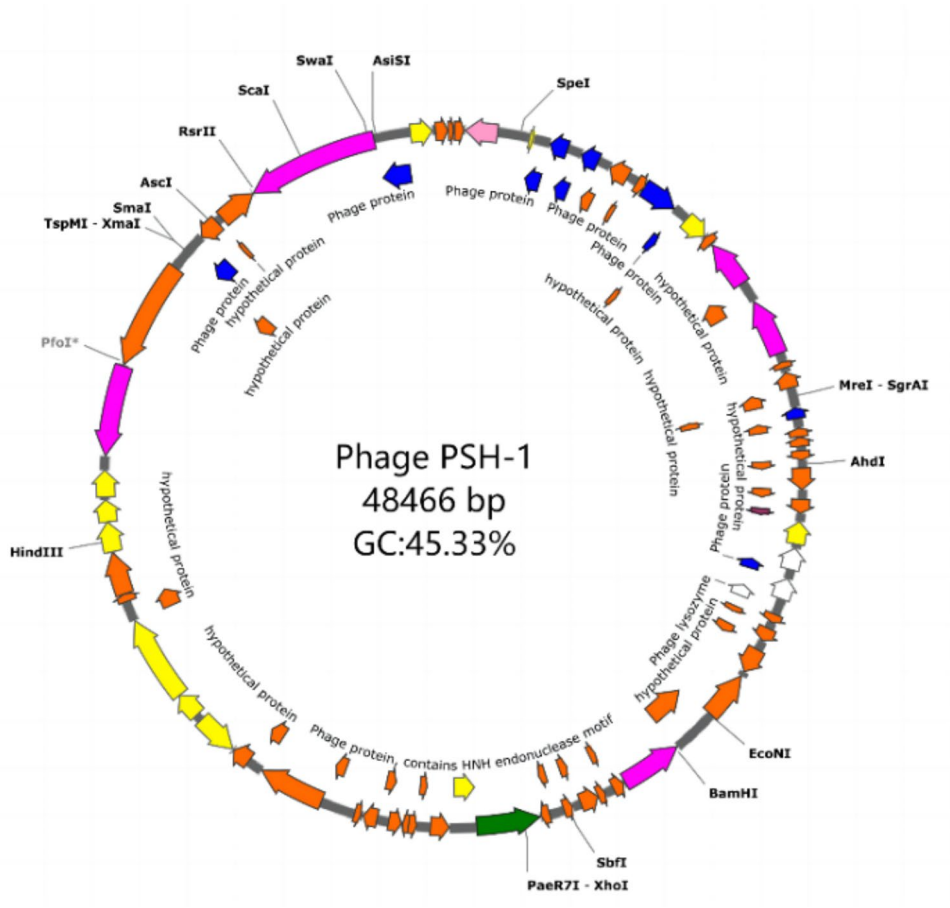

Fig. 6 Annotation of PSH-1 gene structure of phage. Arrows represent gene directionality, and colors represent different domains or functional genes: orange, assuming protein-related genes; Pink, structural protein related genes; Yellow, replication and metabolism related genes; Green, packaging protein gene; White, lyase related genes; Blue, unknown gene

warm temperatures (25–37 ℃), and could multiply to ca. 10^9 CFU/mL or g after only one day of bacterial inoculation. Oppositely, the *Salmonella Enteritidis* was completely controlled and barely detected in milks protected by phages at 37℃ temperatures from the first day to the end of experiments .The same effect was achieved at 25 ℃ at the end of the second day.

Discussion

Salmonella is a natural inhabitant of the gastrointestinal tract of animals and abundant in animal feces, various waste effluents typically provide the best source for phage isolation [26]. It is reported that the separation rate of sewage samples (47.2%) was much higher than stool samples (26.1%) among 1,100 samples in Jiangsu [27]. In this study, swine manure and sewage samples were collected to isolate the lytic phage against a selected group of *Salmonella Enteritidis*. However, only one lytic phage of *Salmonella Enteritidis* was isolated from the sewage sample. The overall isolation rate (2.5%) of among swine manure and sewage samples in this study was considerably lower than that reported in other study (47.2%; 26.1%) [27], and it was slightly lower than that reported in other study(6.6%) [28] and (7.1%) [29]. The difference can be explained that samples collected from different isolation sources and different regions. It is worth noting that the difference of host strains is the significant reason for the isolation rate.

At the same time, from our study and other studies that have been reported, we can find that there is indeed a higher phage separation rate in sewage samples than in fecal samples. This phenomenon can be attributed to several factors. Firstly, the nature of the samples themselves plays a crucial role. The sewage environment offers more conducive conditions for bacterial survival than the fecal environment. Since wild phages rely on the bacteria present in their surroundings for survival and propagation, this could explain why the phage fraction rate is lower in fecal samples compared to sewage samples. Moreover, the treatment of the samples differs significantly. Sewage samples, after filtration, can be directly mixed with the host bacteria culture medium, allowing the wild phages within to come into direct contact with the host bacteria. Conversely, fecal samples require a more intricate

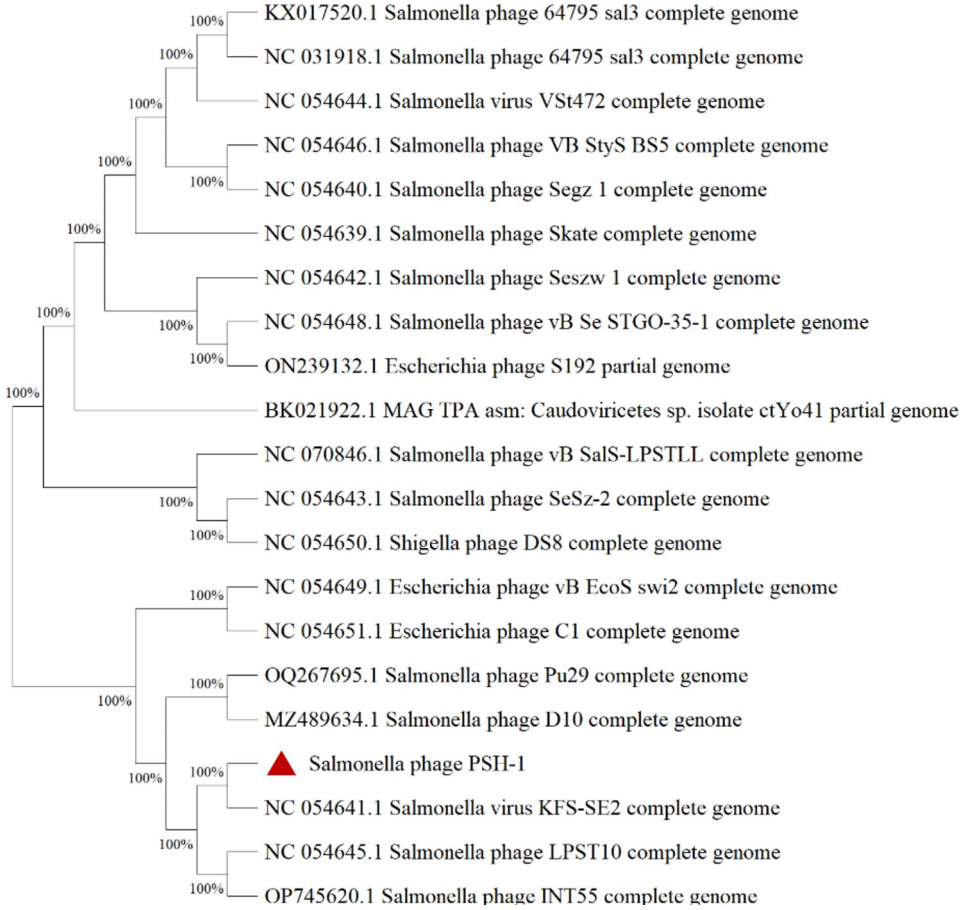

Fig. 7 Phylogenetic analysis of PSH-1 of *Salmonella Enteritidis* phage. The genome sequence of phage with high homology was downloaded by whole genome BLAST comparison, and phylogenetic analysis was performed by MEGA11

process, involving crushing, soaking in SM buffer for an extended period of 8–12 h, followed by centrifugation of the supernatant and subsequent mixing with the host bacteria culture medium for culturing. This intricate treatment process may lead to partial loss of phages, thereby contributing to the lower isolation rate observed in fecal samples. In summary, it can be inferred that the content of wild phages in fecal samples is lower than in sewage samples, and the complex treatment process of fecal samples may result in the loss of some phages, ultimately leading to a reduced separation rate.

The phage PSH-1 has a wide host range. It can be seen that the phage PSH-1 has a strong ability to lyse clinically isolated multi-drug resistant *Salmonella Enteritidis* stains, and not affected by the resistance genes carried by the host strain. In general, the strict compatibility between a phage and its host requires the phage to complete its infection cycle, which limits the host range of the vast majority of phages to potentially very narrow [30]. Therefore, phages with a wider host range could be very attractive from an application point of view, as they can target more pathogenic strains at the same time [31]. From an application and molecular point of view, our understanding of the molecular and evolutionary complexity of polyvalent phages is still limited to a few examples [32], and phage research still needs more polyvalent phages to be discovered and explored. PSH-1 does not encode any antibiotic resistance genes, virulence factors, or integrases, indicating a strict lytic lifestyle that makes PSH-1 a suitable candidate for phage therapy and biological control. Therefore, we still need to conduct more in-depth exploration of PSH-1 in order to provide more ideas for the development of polyvalent phages.

In this study, the titer of purified phage PSH-1 was 1.15×10^{10} PFU/mL, which indicated that PSH-1 was a high-titer phage [20]. The optimal multiplicity of infection of phage PSH-1 was 0.01, and it meant that when the bacteriophage PSH-1 infected the host bacteria, only needed a small dose can cause great damage, this is also the advantage of bacteriophages over antibiotics [25]. According to the one-step growth curve, its latency time was 20 min, the burst time was 80 min, and the burst was 495 particles. Compared with ϕst1 (its latency time was 50 min, the burst time was 70 min, and the burst

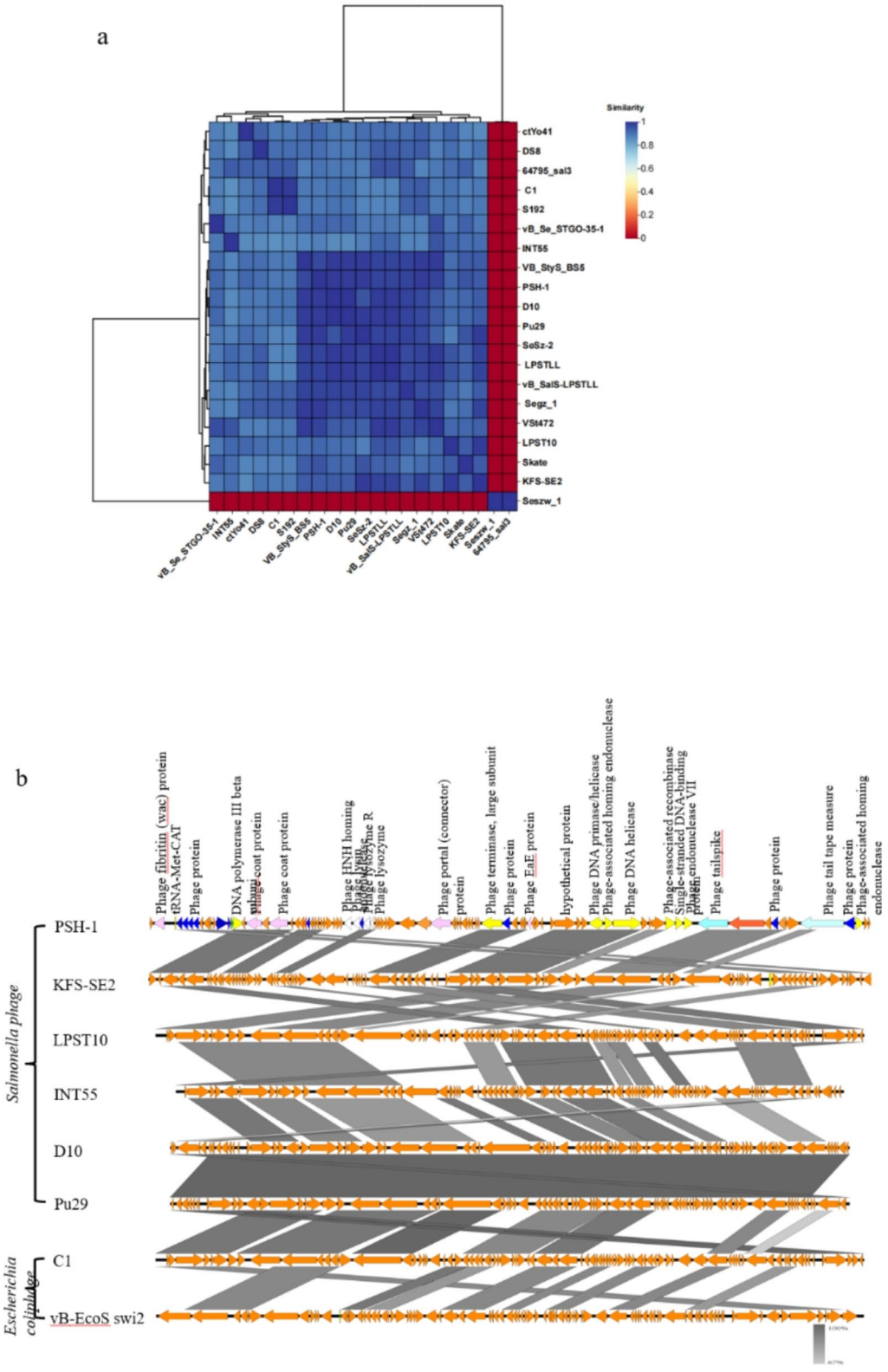

Fig. 8 Genomic comparison at the nucleotide level of PSH-1 to the other similar phages. (**a**) A genome-wide pin-two comparison of 20 phage nucleotide sequences was then used to generate a clustering heat map using Chiplot. (**b**) Organization and functional modules of the eight phage genomes. Arrows represent gene directionality, and colors represent different domains or functional genes: orange, assuming protein-related genes; Pink, structural protein related genes; Yellow, replication and metabolism related genes; Green, packaging protein gene; White, lyase related genes; Blue, unknown gene

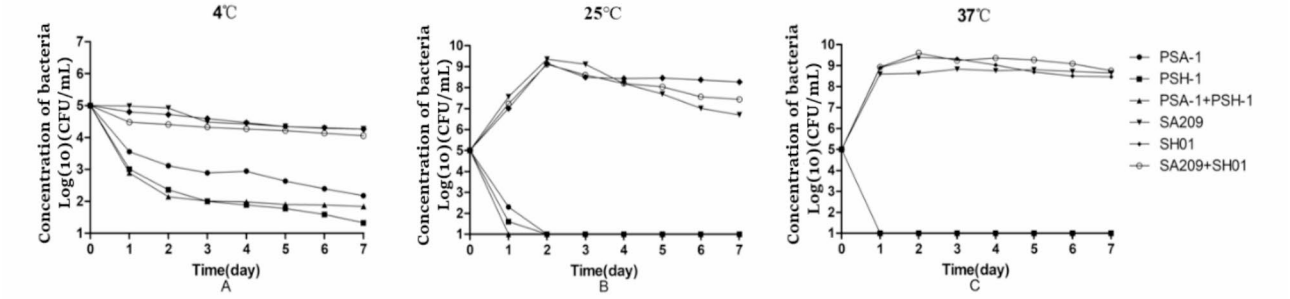

Fig. 9 In vitro antibacterial test of phage. The abscissa is time, and the ordinate is the logarithm of the number of bacteria. The curve connecting the solid squares is the growth curve of *Salmonella Enteritidis* in milk containing the phage PSH-1; the curve connecting the solid diamonds is the growth curve of *Salmonella Enteritidis* in the milk without the phage PSH-1

was 22 particles), it could be seen that the incubation period of bacteriophage PSH-1 was short, but the burst time was long, and the burst was much larger than that of bacteriophage ϕst1, it indicated that bacteriophage PSH-1 may exerted a greater bactericidal effect in clinical applications [33] .The optimum pH and temperature of PSH-1 were in the range of pH (3–11) and temperature (<60 ℃). It had reported that the lytic phage of *Salmonella* enterica, SaFB14 grew between pH 3–10 [34], and a strain of *Salmonella typhimurium* ΦBLCC8-0050-3 [29] could maintain activity under the conditions of temperature (30–50 ℃) and pH (5–10), which suggested that PSH-1 could survive in more hostile environments than SaFB14 and ΦBLCC8-0050-3 [35]. In short, physical and chemical stability are the basis for phages to be used as clinical antimicrobial agents [36]. These indicate that phage PSH-1 has potential application value in rapid diagnosis and treatment of clinical infections.

Genomic studies are beneficial to a deeper understanding of *Salmonella Enteritidis* phages at the genetic level. The genome of PSH-1 consists of double-stranded DNA with a total length of 48,466 bp and a G+C content of 45.33%. It has strong cracking property to Campylobacter jejuni. The phage has a total of 85 ORFs and 3 trnas, of which 28 ORFs are functional. In addition, phylogenetic trees constructed with whole genome sequences showed that PSH-1 was closely related to *Salmonella* phage KFS_SE2. In addition, after the whole genome BLAST comparison of PSH-1, we found that the genome of PSH-1 was very similar to that of *Escherichia coli* phage C1 and vB_EcoS_swi2, which were 88.65 and 94.82%, respectively, and the comparison coverage was about 60%. Moreover, certain affinity was also reflected in the phylogenetic tree, which was an interesting phenomenon. At the same time, this phenomenon also confirmed that PSH-1 of *Salmonella Enteritidis* phage had certain lytic ability to *E. coli* during the determination of the host range of phages, and it was also worthy of our further exploration.

The receptor is the main determinant of phage host range [30]. The specificity of the interaction depends on the RBP structure, which is located at the tip of the tail spike or fiber and primarily determines which hosts the phage can bind to [32]. In the genome of phage PSH-1, ORF77 encodes PSH-1 tail spines. After Gene function annotation through Gene Ontology, it was found that it may bind PSH-1 to host bacteria cells by recognizing host bacteria receptors, and bind enzymes to degrade host cell structures to enable PSH-1 to enter the host. We hypothesized that ORF77 is mainly involved in the recognition and binding of PSH-1 to host bacteria. This is similar to the discovery of ORF31 in the phage S144 genome, which encodes the tail fibers of phage S144, which, interestingly, is also a polyvalent phage. ORF38 and ORF39 encode Phage lysozyme R and Phage lysozyme of PSH-1, respectively, and gene function annotation indicates that their main functions can regulate phage lysozyme activity, lytic transglycosylase activity and lyase activity. Involved in cell wall macromolecular catabolism and cell lysis processes as well as bacterial defense responses. We know that one of the characteristics of phages is that they can vary with the variation of the host bacteria, and we can also speculate that there is an interaction between ORF38, ORF39 and ORF77, which can enable PSH-1 to infect *Salmonella Enteritidis* and *Escherichia coli*, but this speculation needs to be further explored and verified.

Although the genomes of different bacteriophages are highly similar, there are also differences in certain genes, such as those that encode structural proteins in the tail of the bacteriophage. It has been shown that the tail fiber genes are the most varied part of the phage genome, and they usually have a Mosaic structure, especially in the C-terminal domain that determines receptor recognition and phage specificity [37, 38]. In a group of 61 strains of Salmonella belonging to 34 different serotypes, SE4 had a narrower host range than phage SE13 (10 fewer strains infected) [39]. Similarly, this may be one of the reasons

why PSH-1 is able to infect both *Salmonella Enteritidis* and *Escherichia coli*.

Foods involved in outbreaks of salmonellosis are mainly eggs, poultry and other meats, raw milk, etc. By tests, the use of phage PSH-1 could succeed in controlling the artificial *salmonella* contamination in milk at a range of temperatures. Similarly, Yajie Cao et al. studied the effects of *salmonella* phages PSE-D1 and PST-H1 on controlling contamination of three food products (eggshell, sausage, and milk) with *Strep enteritidis* CVCC1806 and *Salmonella typhimurium* CVCC3384, and their results showed that, compared to phage-free groups, PSE-D1 and PST-H1 inhibited the growth of their host strain significantly. A significant reduction of host bacteria titers (1.5 and 1.9 log10 CFU/sample, $p<0.001$) on eggshells was observed under PSE-D1 and PST-H1 treatments, respectively. Furthermore, administration of PSE-D1 and PST-H1 decreased the counts of bacteria by 1.1 and 1.2 log10 CFU/cm2 ($p<0.001$) in sausages as well as 1.5 and 1.8 log10 CFU/mL ($p<0.001$) in milk, respectively [40]. Ya-Ke Li et al. showed that the use of phage JN01 significantly reduced the amount of *Escherichia coli* O157: H7 in milk and raw beef samples, suggesting that phage JN01 may have a natural potential as a biocontrol agent for *Escherichia coli* O157: H7 in food [41]. These showed a well effectiveness of the isolated phages for controlling salmonella contamination in some salmonellosis outbreak-associated food vehicles regardless at cool or warm temperatures. Additionally, in these tests, phages were used with pre-addition instead of post-treatment, this might differ from previous studies; however, our results have demonstrated the feasibility of phages as "food additives" for controlling contamination in some foods.

The biological characteristics (temperature, PH, growth characteristic and MOI) of the phage are necessary conditions for producing a new bacteriophage preparation. At present, there are certain basics in producing a new bacteriophage preparation, the safety of phages was also determined through genomic analysis, however, it still faces many difficulties and challenges. Can the number of infective phage particles in the preparation exist stably? Can the titer of bacteriophage remain stable in the phage PSH-1 solution treatment or storage? Resolving these issues was essential in producing a new bacteriophage preparation. In addition, further research will be required to determine the dosage form, administration dosage, interval and method of administration.

Conclusions

The phage PSH-1 isolated in this study has a strong lytic ability to multidrug-resistant *salmonella*. After drug resistance gene detection of tested *Salmonella Enteritidis*, we found that the lytic ability of phage PSH-1 to host *Salmonella Enteritidis* was not affected by the type and number of drug resistance genes carried by the strain. Similarly, PSH-1 has good biological characteristics, and the whole genome analysis found that PSH-1 does not carry any drug resistance genes, virulence genes and allergen genes related to pathogenic bacteria, which can be used as an important means to treat multi-drug-resistant *Salmonella Enteritidis* infection. And we also found that phages have the potential to be used as "food additives".

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-024-03489-w.

Supplementary Material 1

Author contributions

In this study, Gong-zheng Hu planned the research design and reviewed the articles, the test operation, data collection, analysis and article writing were completed by Shuai-hua Li, software provision and data analysis guidance were completed by Jun-kai Zhang and Kai-fang Yi, and Jian-hua Liu, Hua Wu and Ya-jun Zhai jointly completed the work of trial guidance and review.

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Data availability

Data is provided within the manuscript and supplementary information files.

Declarations

Ethics approval and consent to participate

This article does not include any studies involving humans or animals.

Consent for publication

Not Applicable.

Competing interests

The authors declare no competing interests.

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