

# Exploring the Potential of Lytic Phage Dsi15 with Broad Host Range for Controlling *Salmonella* in a Food Matrix

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*Salmonella* is a major food-borne pathogen associated with outbreaks of enteric diseases. Antibiotic-resistant ability of *Salmonella* is a major concern because of abuse of antibiotics may pose bring risk to humans and animals. This study aimed to isolate and characterize a lytic bacteriophage from a sewage sample with the potential to biologically control instead of chemically control *Salmonella* in food contamination. Phages are intracellular bacterial viruses; they are obligate and host-specific, and can infect only prokaryotes. Spot test and efficiency of plaque formation (EOP) data indicated that phage Dsi15 had a wide host range and broad lytic ability within the *Salmonella* spp. Phage Dsi15 confirmed extensive pH (4-11) tolerance, and lengthened thermal (30-50°C) stability. We experienced the effectiveness of phage Dsi15 as a biological control in a food metric when lettuce contaminated with *S. Enteritidis* ATCC 13076, treated with phage Dsi15 at a multiplicity of infection (MOI) of 1000 at 25°C. The ability of phage Dsi15 to control *Salmonella* in lettuce was recognized. Treated with phage Dsi15 *Salmonella* count decreased by 1 log CFU/cm<sup>2</sup> at 25°C in lettuce by MOI of 1000. Our findings showed that phage Dsi15 has potential efficiency as a biological control against pathogenic *Salmonella* in a food matrix.

**Keywords:** Phage, Dsi15, *Salmonella*, Biological control, Food matrix

## INTRODUCTION

The diarrhoeal disease salmonellosis caused by *Salmonella* is one of the most prevalent food-borne illnesses worldwide, and outbreaks are characteristically connected with the consumption of foods contaminated by *Salmonella* spp. The main symptoms of the disease, primarily caused by *Salmonella* infections, include abdominal pain, vomiting, inflammatory diarrhea, fever, and headache (1, 2). According to the most recent reports, major food-borne illnesses are caused by nontyphoidal *Salmonella* in the United States. *Salmonella*, responsible for 11% of illnesses, 35% of total hospitalizations, and 28% of deaths associated with food-borne illnesses, has been reported annually in the United States (3, 4). *Salmonella* contaminates a broad variety of food products like, meat, egg, milk, vegetables, and different types of fruit juices, and its source of contamination ranges from humans through food processing facilities (5, 6).

The heat treatment and chemical preservatives can control pathogens, including different *Salmonella* serovars, in food (7, 8). However, the risks of adverse side effects bestowed by chemical preservatives and heat treatment are deterring (9) resembling heat treatment can destroy vitamins and other nutritional values of foods (10) and produced glycation end products that have health-threatening advanced (11) also altered the food's taste and flavors. The control of *Salmonella* by using antibiotic treatment of patients with acute *Salmonella* gastroenteritis is a core way (12) but food is a major

source of antimicrobial-resistant *Salmonella* infections in humans, and these infections are linked with antibiotics used on farms (12). The use of antibiotics is an ordinary common practice for the treatment of diseases, but the extreme use of antibiotics may lead to the emergence of antibiotic-resistant bacteria (13, 14).

Phages are obligate, because of targeted pathogen host specificity, rapid killing, and self-replicating ability, and also present within the boundaries of bacterial specificity (15). Bacteriophages do not cause harm to eukaryotic cells and provide a high level of safety (16). In the present study, we isolated and characterized a new phage, Dsi15, for controlling *Salmonella* in lettuce. Our promising results suggested a prospective approach to preventing and controlling the contamination of *Salmonella* in diverse food matrices.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions:** *Salmonella enterica* serovar Enteritidis ATCC 13076 was used for isolating the phage. A total of 10 different strains of *Salmonella* and 2 *E. coli* strains were used to provide as host strains (Table 1). All bacterial strains were cultured by the streak plate method onto tryptic soy agar (TSA, Difco™, BD, USA). Then they were transferred to 1.5 ml eppendorf tube containing tryptic soy broth (TSB) +18% glycerol and stored at -80°C. Before the experiment, each strain was grown in tryptic soy broth (TSB) and incubated at 37°C to acquire a fresh overnight culture.

**Enrichment, isolation, purification, and preparation of phages:** According to the method described by (17), phage Dsi15 was isolated from a sewage sample collected at Shenzhen, China. For enrichment, *S. Enteritidis* ATCC 13076 was used as the host strain. Two hundred microliters of overnight cultures were inoculated into 10 mL TSB and incubated at 37°C shaker at a speed of 160 rpm for 6-8 hours to reach the exponential growth phase. 10 mL *Salmonella* cultures were mixed with 40 mL 2xYT broth medium, and the filtered sample was used to

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amplify the collected phages. Amplified phages were isolated by centrifugation at 5000xg for 5 min and filtration using 0.22 µm pore size disposable sterile syringe filters. Both large and small phage plaques were picked. To do so, a dilution series of isolated phage samples was assessed on plates covered in a lawn of target bacteria. Individual plaques were picked and re-purified for 3 consecutive passages. The double-layer agar method was used to determine the titer of the phage stock. Dilutions of the phage stock (100 µL each) were made in sterile SM buffer, mixed with a suspension of exponential phase *Salmonella*, and added to 4 mL of molten (45°C ≤ temperature ≤ 50°C) TSB agar (0.7%). The mixture was then poured onto surface-dried TSA agar plates. Then, the plates were incubated at 37°C for 24 hours, and the number of plaques was counted on plates with 10–300 plaques. The purified phages, named Dsi15, were then stored at 4°C for further experimental purposes.

**Determination of host range:** The host range of phage Dsi15 was determined by spotting 5 µL of phage lysates onto lawns of test strains. The TSA plates containing lawns of test strains were prepared with a mixture of 100 µL and 4 mL of 0.7% agar for the overlay. The plates were incubated at 37°C for 24h, and bacterial lysis was recorded as follows: +; presence of phage growth clearing, -; absence of phage. Further determination of the efficiency of plaque formation (EOP) was conducted using a previously described method, which measured the phage titer of the test strain relative to the phage titer of the host strain (18). EOP of phage Dsi15 was identified on the tested strains from a total of 12 strains (Table 1).

**pH and thermal tolerance of the phage Dsi15:** To determine the effect of pH on the stability of phage Dsi15, phage lysates ( $1 \times 10^7$  PFU/mL) were mixed in different tubes containing sterile buffer peptone water (BPW) of different pH values, 2 to 13, adjusted by using NaOH or HCl, and incubated at 37°C for 60 min. Then, phage samples were taken out, serially diluted, and titers were determined using *S. Enteritidis* ATCC 13076 as a host by using the double-layer agar method (19). For thermal stability, samples of the phage Dsi15 lysates ( $1 \times 10^7$  PFU/mL) were incubated at 30°C, 40°C, 50°C, 60°C, 70°C, and 80°C, for either 30 or 60 minutes. Then, aliquots were taken out to determine the titers of phage by using the double-layer agar method (20).

**Biological control of *Salmonella* in lettuce by using phage Dsi15:** Lettuce was obtained from a local supermarket. Inner leaves were cut into pieces using a sterile, sharp knife. Lettuce was cleaned with distilled water and 70% alcohol swab for the initial surface decontamination. Lettuce leaves were cut into pieces (1 cm<sup>2</sup> in diameter). These leaves were sterilized under UV exposure for 20 min. Sterility was ensured by exposing a leaf sample to a fresh TSA plate. Place the sample in the center of a sterile Petri dish, flat-laid, flat test surface facing up, with a micro tips  $10^3$  cfu/mL test bacteria (*S. Enteritidis* ATCC 13076) randomly drop on the surface of lettuce samples, to obtain samples of the artificial pollution of the *Salmonella*, to make the bacteria fully adsorbed to the sample surfaces, wait 60 min for dry. Using phage Dsi15 to treat the lettuce samples, take the proportion of MOI=1000 of phage drip on the sample, as far as to cover the location of the drop and bacteria. The control group received the same volume of PBS buffer without phage solution. Cover the petri dishes with samples and place the samples in 25°C incubators for 48h. The aliquots were taken out after the 0,1,2,3, and 4 hours of incubation and suspended in 1mL sterile PBS buffer solution. Suspended samples were homogenized with sterile bars and vortexed for 30 sec. Viable bacteria counts (CFU/mL) were determined by serial dilution and enumeration by direct spread plate techniques (21).

**Statistical analysis:** Bacteria and phage counts were determined by duplicate plating, and all experiments were performed in at least triplicate. Results are presented as mean value, and the standard deviation of the mean is indicated by error bars. Statistical analyses were performed with PRISM software. Comparisons were analyzed using nonparametric one-way analysis of variance (ANOVA) with Bonferroni's multiple-comparison post-test.

## RESULTS AND DISCUSSION

**Isolation of phage Dsi15:** A new *Salmonella* phage Dsi15 was successfully isolated from sewage samples by using *S. Enteritidis* ATCC 13076 as a host bacterium. Phage Dsi15 could form clear plaques on the host lawn, with a diameter of about 0.5 mm (Figure 1). This study isolated broad-spectrum and lytic *Salmonella*-specific bacteriophages from natural sources, with the clear expression of its collection of phages to biocontrol *Salmonella*. It has been professed that the presence of bacteriophages is tightly amalgamated with their natural hosts. Because *Salmonella* is a natural inhabitant of the gastrointestinal tract of animals, it can easily contaminate

food products of animal origin and plenty in animal feces. Manifold waste effluents typically provide cattle manure, and sewage samples were collected at various time points to isolate the lytic phages against *S. Typhimurium*. Sewage samples were used for the isolation of bacteriophages against *Salmonella* (22) and they also isolated phage against *S. Enteritidis* ATCC 13076. Broad host spectrum phages from sewage are suitable for finding lytic phages (23).

**Spot testing:** Phage Dsi15 was able to lyse its host strains throughout the purification process. The host range by spot test of phages in this study was determined by using 12 strains of 10 *Salmonella* and 2 *E. coli* (Table 1). Phage Dsi15 had a broad host range and lysed 100% strains tested of *Salmonella*. Dsi15 phage can form apparently translucent spots on the bacterial lawns of all eleven tested *Salmonella*. Dsi15 did not infect any *E. coli* tested. However, spot test data (Table 1) indicated a strong infection ability of Dsi15. Sized about 0.5-1 mm in diameter, phage Dsi15 infected many strains of *Salmonella*, like the previously reported phage LPSTLL (24, 25). Previously isolated *Salmonella* phage LPST94 (26) has also exhibited a broad host-spectrum virulence capacity, matching Dsi15.

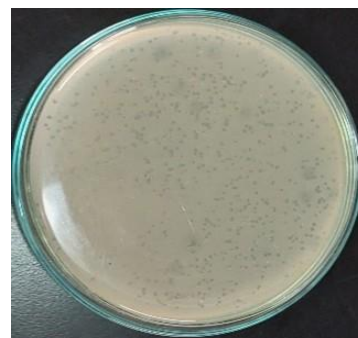


Figure 1: Phage Dsi15 plaques for morphology.

**EOP:** Phage Dsi15 was further analyzed to determine the host range by EOP. The host range by EOP assay was conducted against a total of 10 *Salmonella* and 2 *E. coli* (Table 1). Dsi15 was efficient in generating plaques against 10 *Salmonella* strains. Dsi15 was found to have the broadest spectrum of host range activity. The highly infectious ability of EOP values from 0.5 to 1 against all *Salmonella* strains. Wide host range phages against *Enteritidis*, with clear plaques, were isolated from sewage effluents (27, 28). The authors also indicated that phages isolated from fecal samples were unable to find a wide host range and had the ability to infect only 20% of the tested strains of carrier culture phages (29). Swine feces from commercial farms were observed by and reported that phages were omnipresent in pig manure, but the population of lytic phages against *Salmonella* was very low (30). Phages from sewage were capable of infecting a broad host range and not highly serovar specific (21) or genus-restricted (31).

Table 1: Host range of phage Dsi15.

Bacteria	Strains	Dsi15/Spot Test	Dsi15/EOP	Sources
<i>S. Enteritidis</i>	ATCC 13076	+	1	ATCC
<i>S. Typhimurium</i>	ATCC 14028	+	1	ATCC
<i>Salmonella</i>	SE01	+	1	Lab stock
<i>Salmonella</i>	SE02	+	0.7	Lab stock
<i>Salmonella</i>	SE03	+	1	Lab stock
<i>Salmonella</i>	SE04	+	0.9	Lab stock
<i>Salmonella</i>	SE05	+	1	Lab stock
<i>Salmonella</i>	SE06	+	1	Lab stock
<i>Salmonella</i>	SE07	+	0.6	Lab stock
<i>Salmonella</i>	SE08	+	1	Lab stock
<i>E. coli</i>	ATCC 25922	-	0	ATCC
<i>E. coli</i>	ATCC 43890	-	0	ATCC

**Thermal and pH tolerance of phage Dsi15 remains infective over the tested period:** Phage Dsi15 was a very stable phage, which showed a pH stability range of 4 to 11 (Figure 2). Phage Dsi15 titers stayed active at pH 4 to 11 (Figure 2). Phage Dsi15 titers were reduced when the pH was extreme as greater than 11 or very low as less than 4.

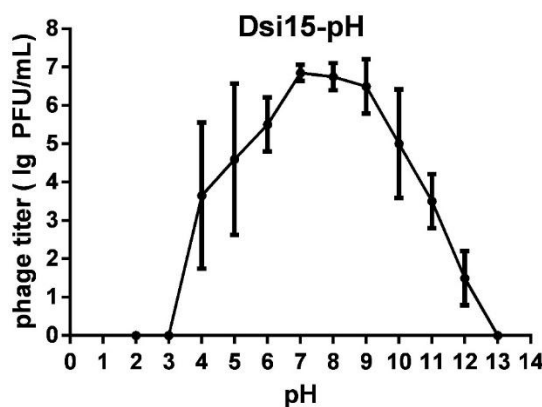


Figure 2: pH stability of Phage Dsi15.

Phage Dsi15 can also survive a high degree of thermal tolerance with an active titer as high as 60°C (Figure 3). The phage PFU number did not change at 50°C when heated at 60°C to 80°C for 30 minutes. Dsi15 phage titers were going to be reduced. The pH and thermal stability of Dsi15 were better than previously isolated phages pH range was 4 to 10, and the temperature at 40°C (32) and almost the same as the pH range 4 to 11, and thermal stability was 50°C (33). Heat and pH-resistant phage application affix benefits in efficiency against pathogens, as only heat or pH was unable to fully lyse the pathogens. This argument is supported by the information specified (34, 35), viewing the survival of pathogens even after heat treatment on meat products. Some other research demonstrated the prevalence of pathogens less than 100 CFU/g (36), but some are in disagreement with this and show even more than 100 CFU/g (37).

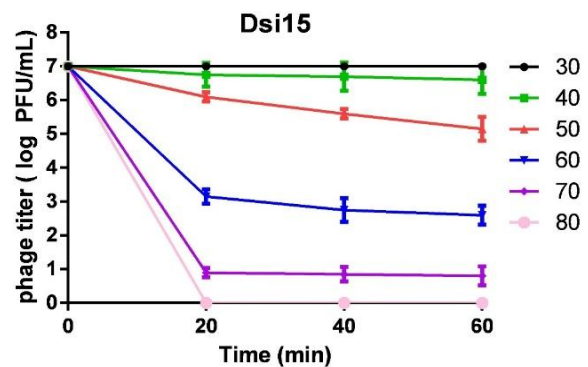


Figure 3: Thermal stability of phage Dsi15.

**Application of phage Dsi15 in controlling foodborne *S. enterica* in lettuce:** Vegetables, including lettuce, are often contaminated with foodborne pathogens such as *Salmonella*. No host organisms were isolated from uninoculated control samples. Dsi15 applied on lettuce leaves, we observed significant results as shown in Figure 4) employing *Salmonella* Enteritidis ATCC 13076.

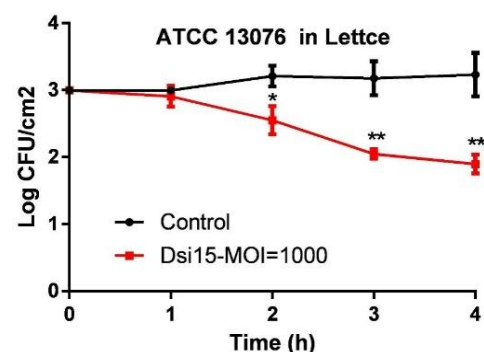


Figure 4: Effectiveness of phage Dsi15 in reducing the *S. Enteritidis* ATCC13076 in lettuce at 25°C. Values represent mean  $\pm$  standard deviation of three determinations. \*\*, Significant at  $P < 0.01$ ; \*, Significant at  $P < 0.05$ .

Relative to the non-treated controls, the viable *Salmonella* counts were reduced upon administration of Dsi15 at an MOI of 1000 at 25°C (Figure 4). These same studies showed that phage ECP-100 application significantly reduced the concentration of viable *E. coli* organisms on tomato slices by ca. 99% during storage at 10°C for 24 hours (38). These results are better than before, explained with independent observations showing a 0.5 log reduction when a distinct phage was applied against *Salmonella* on Chinese cabbage, 1 log reduction on lettuce (39), 1.37 log on mustard, and a 0.55 log<sub>10</sub> reduction on broccoli (40).

## CONCLUSIONS

*Salmonella* is one of the most important pathogens that is mainly associated with food-borne diseases and represents a global food safety issue. We isolated a newly characterized broad-host lytic phage Dsi15 against diverse *Salmonella* serovars. Phage Dsi15 demonstrated extensive pH tolerance and extended thermal stability, as well as an alternative prevention and biological control of *Salmonella* in the food matrix, such as lettuce. Our results indicate that treatment of artificially contaminated foods with phage Dsi15 significantly reduced the *Salmonella* in foods, signifying that phage Dsi15 could be potentially used for biological control of *Salmonella* in foods and the emergence of *Salmonella* epidemics.

## CONFLICTS OF INTEREST

The author has declared that no competing interests exist.

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