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Isolation and Characterization of Lytic Phage against Salmonella Typhimurium

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Abstract

Significant prevalence of antibiotic resistance in Salmonella has been observed, causing global concern that it may lead to more severe health effects. The use of bacteriophages as an alternative treatment tool for the management of bacterial infections has recently received new attention. This study aimed to identify a Salmonella typhimurium-specific phage from chicken farms. The ability of lytic phage SAL 10 to stop the growth of bacteria was verified. A series of phage analyses were conducted to verify their physical characteristics, such as temperature, pH, and host range. The phage was more stable at 37–50°C and at pH 4–7. During the first 4 h of infection, phage SAL 10 inhibited the host bacterial growth. Following 24 h of incubation at 37°C, phage titration was reported to be in the range of 10^3–10^8 PFU/mL in all experiments. Moreover, the morphological properties of the phage were determined using transmission electron microscopy, and the phage SAL 10 was considered to belong to the order Caudovirales and family Siphoviridae. Through the results presented in this research, SAL 10 phage can be used as a successful alternative to antibiotics.

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Introduction

Antibiotics are one of the most important scientific discoveries as they are used to treat the bacterial and fungal infections that affect animals, humans, and plants [1]. They have been used for approximately 70 years and, in turn, they have decreased the incidence of infectious disease-related illnesses and deaths. However, recently, there has been an increase in antibiotic-resistant bacteria, which has become a significant health issue [2]. This occurs when antibiotics fail to kill the target bacteria because it has evolved to become resistant to this treatment. These bacteria can multiply and lead to colonies of antibiotic-resistant organisms [3]. As a result, millions of individuals become infected with multiple-drug-resistant bacteria every year, which leads to numerous deaths [4]. The World Health Organization (WHO) has published an A-list of pathogens, which consists of the most dangerous types of resistant bacteria that affect human health and comprises 12 families of bacteria. Among these, Salmonella is considered among the most severe hazards to our health [1] *S. typhimurium*, considered part of the Enterobacteriaceae family, is a gram-negative bacterium, which can cause many diseases [5]. Salmonella is transmitted through contaminated water or undercooked food; in turn, it leads to infection in the gastrointestinal tract. Poultry is an important reservoir for Salmonella bacteria [6] as it transfers these bacteria through the food chain [7, 8]. In 2009, an outbreak of Salmonella occurred in the United States of America, affecting 714 people [3]. Recently, Salmonella has developed resistance to antibiotics [9]. In response to this potential issue, renewed emphasis has been placed on bacterial viruses called bacteriophages [1]. Bacteriophages can be defined as the viruses that specifically affect bacterial cells. The term “bacteriophage” signifies “eater of microorganisms” [10].As such, these viruses are essential in maintaining ecosystem balance [11, 12]. They are naturally occurring organisms that can be found in all ecological niches. Moreover, they are found throughout the body, over the oral cavity, the digestive tract, the skin, as well as the vagina [13]. Bradley's study in 1967 was a breakthrough, and it remains the basis for the modern-day bacteriophage classification system. As their genetic material, Phages contain either RNA or DNA [14]. Although the bacteriophage structure varies between phages, the majority of the ones that have been identified share certain basic features. The fundamental distinction between phages is the presence or absence of a ‘tail’ component [15]. Phages have either a lysogenic or a lytic life cycle [16]. Lytic cycle are distinguished by the adhering of phages to the bacterial cell,

using the genetic material of host bacterium for multiplying, and releasing an enzyme that lyses the cell. Consequently, new phages are released into the environment, which makes them ideal for use in phage treatment [17, 18, 19]. In terms of phage therapy, bacterial viruses are considered unique and effective for treating bacterial infections, especially the ones that have occurred as a result of drug-resistant bacteria [20]. There are several reasons for this uniqueness. First, phages act against antibiotic-resistant bacteria; notably, they can be used alone or with antibiotics and other medications [21]. Second, in most cases, only one dose of a certain phage is needed in treatment because it can multiply and increase in number during the treatment [22]. Moreover, phages are plentiful and found in many places [23]. In addition, they are not harmful or toxic to humans, animals, plants, or the atmosphere because they are incapable of infecting eukaryotic cells [24,25]. Bacteriophages can endure in severe conditions and their virulence does not end until they have sharply reduced the amount of host bacteria [26]. Nevertheless, there remains a gap in research conducted on phages around the world, and not much is known about their capacity and diversity in the natural environment [27] even though phages outnumber bacterial cells by tenfold [28].

Animal farms include a variety of components – such as soil, wastewater, animal feces, and animals – that may be an essential source of phages and their bacterial hosts [29]. In turn, chickens are a significant source of Salmonella, a bacterial disease that can contaminate human food and cause food-borne diseases [7,8]. In 2013, phages (Eϕ151, Tϕ10, and Tϕ11) were isolated from chicken feces. A study [30] found that the populations of *S. typhimurium* and S. enteritidis were reduced by over 70% after phagocytic therapy, as compared to those of controls. Thus, this study supports using phages as bio-sanitizers in the food industry [30,31]. Conducted a study involving isolation of 21 lytic Salmonella phages and their subsequent screening against *S. typhimurium* strain E4231. The phage cocktail that the researchers used in an experimentally-contaminated sample of meat substantially reduced the viable count of *S. typhimurium* in the experimental group with comparison to that in the control group. In this study, we isolated and characterized SAL 10 a lytic phage against *S. Typhimurium*, this work aims to use phage as alternative biocontrol tool against *S. Typhimurium* in chicken.

Materials and Methods

Sample Collection

In September of both 2020 to 2021, soil samples (soil mixed with water and animal waste) were randomly obtained from chicken farms in Jeddah, Saudi Arabia. For sample collection, 5 g of surface soil were placed into sterile. Bagged samples were labeled to reflect their source and location and then they were refrigerated at 4°C. Subsequently, these samples were brought to a laboratory The King Fahd Medical Research Center (KFMRC) for bacteriophage isolation.

Bacterial strains

We purchased a strain *Salmonella enterica* serovar *typhimurium* from American Type Culture Collection (ATCC) 14028 (in Gaithersburg, Maryland). In turn, this strain was isolated from the pools of heart and liver tissues of 28-day-old chickens. The isolates were then grown either in standard nutrient broth or a nutrient agar medium (Oxoid®, Hampshire, England). Furthermore, the culture was kept in 18% glycerol at a temperature of −80°C.

Antibiotic susceptibility assay

The bacteria were suspended in 4 mL of nutrient broth (Oxoid®, Hampshire, England) for 24 h before the experiment began. The turbidity of this culture was adjusted to 0.5 McFarland through augmenting the amount of bacteria if it was too low or by diluting the substance with a mixture of sterile saline if it was too high. The dry surface of a Mueller–Hinton agar plate was inoculated three times by rubbing the swab across the surface; the plate was rotated 60° each time to achieve the uniform dispersion of the inoculum. We then placed antimicrobial-impregnated discs upon the agar surface. Then, the discs were placed on an MH agar plate that, from center to center, was more than 24 mm away.

Subsequently, we inverted the plates and put them in an incubator for 24 h at 37°C. In turn, we used a ruler to measure the zone diameters to the closest millimeter.

The bacteria were tested against 12 antibiotics (Merseyside, U.K.), namely ciprofloxacin (CIP) 5 µg, amikacin (AK) 30 µg, cefoxitin (FOX) 30 µg, ceftazidime (CAZ) 30 µg, imipenem (IMI)10 µg, piperacillin (PRL) 100 mg, amoxicillin (AUG) 30 µg, cephalothin (KF) 30 µg, gentamicin (GM)10 µg, aztreonam (ATM) 30 µg, ampicillin (AP) 10 µg, and cotrimoxazole (TS) 25 µg. Afterwards, we measured the inhibition zones and we recorded them in millimeters.

Phage Isolation and Purification

Phage isolation

S. typhimurium ATCC 14028 was selected to be the host for the phage isolation. Soil samples from chicken farms served to isolate the lytic bacteriophages. Two grams of the soil sample were suspended in 20 mL Phosphate buffered Saline (PBS Gibco™ 70011044, UK) and incubated overnight to remove solid matter. We then filtered the suspension by utilizing a disposable syringe filter that had a pore size of 0.22 mm (Axiva, Faridabad, India). The filtrate was then combined with the incubated culture of *S. typhimurium* treated with 10 mM $CaCl_2$ and 0.5 mM $MgSO_4$. We incubated these enriched samples for 48 h at 37°C and then shook them at 120 rpm before centrifugation for 10 min at 10,000 × g. In order to eliminate any residual bacterial cells, we filtered the supernatant using a disposable syringe filter that had a 0.22 mm pore size (Adams, 1959). We placed the plaque-forming filtrates at 4°C in PBS and used this as the bacteriophage lysate solutions in the remainder of the research (Poxleitner et al., 2017). For phage purification: the double-layer agar was used described by Maszewska & Różalski (2019) to get pure phage SAL 10. In this method both phage serial dilution and bacterial host culture were prepared as will described shortly to utilized them in double layer agar.

Preparation of phage serial dilution for double agar experiment
The serial dilutions of phage were prepared as follows: 900 µL

of PBS and 100 µL of phage were mixed using a vortex (Labnet, U.S.A) to prepare a10^{-1} dilution. Then, 100 µL of this dilution was combined into 900 µL of PBS to prepare a 10^{-2} dilution. In turn, this dilution process was repeated until reaching a 10^{-6} dilution.

Preparation of bacterial culture for double agar experiment Four milliliters of nutrient broth was inoculated with bacterial *S. typhimurium* and it was then incubated at a temperature of 37 ˚C for 24 h (note: this was done one day before the actual experiment). Then, 60 µL of $CaCl_2$ was added to and mixed with this bacterial culture.

The double-layer agar experiment 100 µL was taken from the previously prepared culture and it was added to and mixed with 100 µL of the 10^{-1},10^{-2}, 10^{-3}...10^{-6} phage solutions. All the tubes were incubated for 20 min at a temperature of 37°C. Subsequently, each tube containing bacteria and phage was mixed with another tube that contained 5 mL of soft agar and then it was transferred to a nutrient agar plate. A rest period of 10–15 min for the plates was taken to ensure that the mixture solidified; after this was accomplished, all of them were placed in the incubator at 37 ºC for 24 h.

The double-layer agar was replicated thrice until it derived a single plaque of pure phage (meaning that it was the same size) [33]. Finally, PBS was withdrawn from the dish after 24 h, purified using a 0.22 mm filter, and then it was stowed as pure phage at a temperature of 4°C [32].

Spot tests assay

A spot test is used to determine whether a phage sample can infect a bacterium [34]. This test is performed by dropping a small drop or "spot" of bacteriophage onto a plate that has been inoculated with the bacteria.

Preparation of bacterial culture for the spot test Four milliliters of nutrient broth was inoculated with bacterial *S. typhimurium* and it was then incubated at a temperature of 37 ˚C for 24 h (note: this was done one day prior to the actual experiment). Then, 60 µL of $CaCl_2$ was added to and mixed with this bacterial culture.

The spot test experiment

100 µL of the prepared bacterial culture was inserted into a tube that contained 5 mL of soft agar and the blend was then transmitted to a nutrient agar plate. There was a 10-15 min rest period for the plates to ensure that the mixture solidified. After solidification, we combined 100 µL of the phage drop with the agar surface; subsequently, the plates were put into the incubator at 37°C for 24 h. The next day, the plates were examined for lytic phages. A positive spot test outcome was determined in cases when a clear plaque was observed. Thus, phages that resulted clear plaques were considered virulent and able to infect the bacteria. A negative spot test indicated that the bacteria grew normally, and that the phage was not able to infect the bacteria.

Bacteriophage titer determination

A tenfold dilution of the bacteriophage lysate solution was made in PBS, and to find the titer of the phage, a double-agar overlay assay was used. Plates that had concentrations between 30 and 300 PFU/mL were selected to ascertain the bacteriophage titer in the complete suspension.

Bacteria reduction assay

In a 96-well microtiter plate, we added a 200 µL of an overnight pure culture of *S. typhimurium*. Further, 200 µL of the *S. typhimurium* culture was mixed with 200 µL of the pure phage SAL 10 (1 x 10^7 CFU/mL) and placed in another well. Finally, 200 µL of nutrient broth was added to another well, and this served as the control. The plates were then placed in the incubator for 24 h at a temperature of 37°C, and it was shaken at a speed of 100 rpm. Subsequently, we measured the absorbance at 600 nm (OD_{600}) at 2-h intervals for 12 h to detect changes in the turbidity of the mixture. The same volume of nutrient broth was then combined with the log phase bacterial cultures, and this served as the negative.

Characterization of *S. typhimurium* phage

Host range of the bacteriophage

In order to determine the host range of the lytic phage SAL 10, a spot assay was performed. This was done using seven bacterial strains, namely Shigella sonni (ATCC 25931), Klebsiella oxytoca (ATCC 49131), Pseudomonas aeruginosa (ATCC 9027), Escherichia coli (ATCC 25922), Staphylococcus aureus (ATCC 12600), Proteus vulgaris (ATCC 49132), and Enterococcus faecalis (ATCC 29212). The emergence of the spot was seen after the plates had been placed in the incubator overnight at a temperature of 37°C [34]. The outcomes were distributed into 2 categories according to their degree of clarity: no plaques (−) and plaques that were clear (+).

S. typhimurium phage Thermal stability

The *in vitro* thermal inactivation point of SAL 10 phage was figured out using the technique reported by [35] with minor changes by subjecting the purified phage lysate to temperatures between 37°C and 90°C. Briefly, an Eppendorf tube containing 500 µL of pure phage lysate (1 × 10^8 PFU/mL) was placed in a water bath warmed at a range of levels of warmth (37°C through 90°C) for 2 h. We then measured the bacteriophage titer through the utilization of the double-agar overlay technique.

S. typhimurium phage pH stability

We tested the capacity of the SAL 10 phage to endure at various pH levels by subjecting each phage suspension to modified pH values ranging between 2 and 14 using 0.1 M HCl/NaOH at a temperature of 37°C for 1 h. Again, we utilized the double-layer agar technique to establish the phage titer in each solution.

Using transmission electron microscopy (TEM) for bacteriophage morphological analysis

Concentrated phage stocks are necessary for electron microscopy. Therefore, we generated fresh high-titer stocks by creating 10 plates of our derived phage stock utilizing the overlay technique to attain confluent lysis plates. We then placed them in the incubator overnight to replicate ideal host conditions.

Subsequently, we placed 5 mL PBS on each plate and stirred it for 24 h at 25°C. After scraping the liquid and soft agar into disposable centrifuge tubes, we centrifuged the samples at 3000 × g for 15 min. We then utilized a 0.22 mm filter in order to filter it. Further, we portioned 50 µL of glutaraldehyde to each tube containing the pure phage [36]. A high-titer phage lysate (5 µL) was placed onto copper grids for 90 s to coat the grids thoroughly. We then eliminated the extra liquid. Filter paper was used to further absorb liquid from the grid. In turn, uranyl acetate (2%) was added to the grids for 30 s to allow negative staining. The grids were allowed to dry before imaging (JEOL, Tokyo, Japan) [37].

Statistical analysis

To evaluate the difference in bacterial growth in the different groups (bacteria without phage, bacteria with phage, and control group) at 0, 1, 2, 3, and 6h time points, we used a two-way repeated measures ANOVA to analyze our data. Moreover, a one-way repeated measures ANOVA was subsequently implemented to check the change with time. Furthermore, we did a one-way ANOVA to check the variance in bacterial growth amid all three sets at each period of time. The statistical significance was prescribed at ($P<0.001$) In addition, We implemented a two-way ANOVA to evaluate the influences of different phage dilutions (10^{-1}, 10^{-2}, and 10^{-3}) and different pH values (4, 7, and 14) on the bacterial growth. A one-way ANOVA was also used to check the horizontal change in the bacterial counts at different pH values. Moreover, we utilized a one-way ANOVA to check the disparity in the bacterial counts at three different phage dilutions (10^{-1}, 10^{-2}, and 10^{-3}) at each pH value. A two-way ANOVA was also conducted to evaluate the consequences of the different phage dilutions (10^{-1}, 10^{-2}, and 10^{-3}) and different pH values (4, 7, and 14) on the bacterial growth. A one-way ANOVA was used to check the horizontal change in the bacterial counts at different pH values. Moreover, we also utilized a one-way ANOVA to check the variance in the bacterial counts at three different phage dilutions (10^{-1}, 10^{-2}, and 10^{-3}) at each pH value.

Results

Antibiotic sensitivity of *S. typhimurium*

The results showed that *S. typhimurium* was resistant to three antibiotics: AK (30 mg), CIP (5 µg), and FOX (30 µg). The strain was also sensitive to CAZ (30 mg), IMI (10 µg), PRL (100 mg), AUG (30 mg), cephalothin (KF), GM (10 mg), and ATM (30 mg). In turn, the effect was intermediate for AP and TS (**Figure 1 and Table 1**).

Bacteriophage isolation and morphology

A spot test was conducted using *S. typhimurium* bacteria as a host. The test yielded tiny, transparent plaques with <1 mm diameter; this phage was named as SAL 10 (**Figure 2**).

Phage titer

The bacteriophage titers were measured following 24 h of being in the incubator at a temperature of 37°C with the host bacteria. They were in the range of 10^3–10^8 PFU/mL. The bacteriophage titers in all the experiments conducted were estimated using the titration formula: $\frac{PFU}{mL} = (Number\ of\ plaques) / (D \times V)$

Type of phage	The Equation	Total PFU
SAL 10 titer	$\frac{300\ PFU\ /\ mL}{0.1\times10^{-5}}$	3×10^8 PFU/mL

Bacteria reduction assay

To evaluate the phage's ability to lyse the host strain *S. typhimurium*, the bacteria were cultured in LB broth and, in turn, they were infected with phage SAL 10. Further, the growth of the bacteria was tracked by calculating the optical density at OD_{600}. The optical density of the bacterial culture was reduced, indicating that the bacterial growth had been inhibited by phage infection (**Table 2, Figures 3 and 4**). In the *S. typhimurium* strain, the lysis kinetics of SAL 10 were determined approximately 60 min after the infection.

Statistical analysis

We implemented a two-way repeated measures ANOVA to evaluate the difference in bacterial growth in the different groups (bacteria without phage, bacteria with phage, and control group) and with consideration of multiple junctures of time (0, 1, 2, 3, and 6 h). The overall difference in bacterial growth between the groups was highly significant.

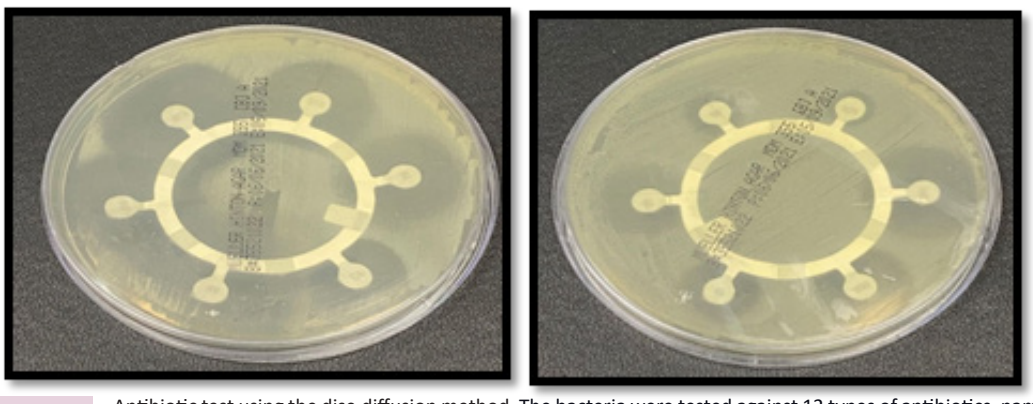

Figure 1 Antibiotic test using the disc-diffusion method. The bacteria were tested against 12 types of antibiotics, namely amikacin (AK), ciprofloxacin (CIP), cefoxitin (FOX), ceftazidime (CAZ), imipenem (IMI), piperacillin (PRL), amoxicillin (AUG), cephalothin (KF), gentamicin (GM), aztreonam (ATM), ampicillin (AP), and cotrimoxazole (TS). The results showed that *S. typhimurium* was resistant to three antibiotics: AK, CIP, and FOX.

Table 1. Complete list of antibiotic tests. The inhibition zones were measured and recorded in mm. The labels are as follows: resistant: R; sensitive: S; intermediate: I

Antibiotic	Inhibition zone diameter (mm)	R or S
Ceftazidime (CAZ) 30 mg	24	S<21
Imipenem (IMI) 10 µg	30	S ≥ 23
Piperacillin (PRL) 100 mg	30	S > 18
Ciprofloxacin (CIP) 5 µg	20	R<20
Aztreonam (ATM) 30 mg	30	S ≥ 21
Cotrimoxazole (TS) 25 mg	20	I
Amikacin (AK) 30 mg	25	R
Amoxicillin (AUG) 30 mg	20	S > 18
Cefoxitin (FOX) 30 µg	9	R ≤ 14
Cephalothin (KF)	10	S
Ampicillin (AP) 10 mg	12	I 12-13
Gentamicin (GM)10 mg	25	S ≥ 15

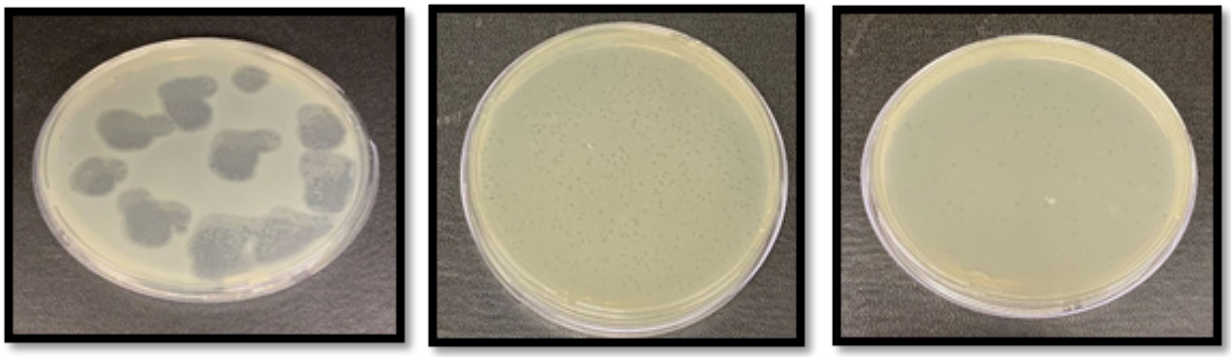

Figure 2 (A) Spot assay and (B, C) serial dilutions for phage SAL 10 at 10^5 and 10^6 PFU/mL. The characterization of the isolated bacteriophage plaques revealed clear, tiny (<1 mm diameter) plaque.

Table 2. The ability of phage SAL 10 to lyse the host strain *S. typhimurium*. Analysis of variance: ANOVA

Time (h)	Bacterial growth with time (h) (Mean ± standard deviation: SD)			
	Bacteria	Bacteria with phage	Control	ANOVA
0	0.803±0.001 b	0.546±0.002 e	0.096±0.000 j	< 0.001***
1	0.80±0.001 b	0.50±0.002 g	0.10±0.000 j	< 0.001***
2	0.80±0.001 c	0.49±0.001 h	0.10±0.000 j	< 0.001***
3	0.76±0.004 d	0.49±0.003 i	0.10±0.000 j	< 0.001***
6	0.86±0.002 a	0.51±0.001 f	0.10±0.000 j	< 0.001***
P-value	< 0.001***	< 0.001***	> 0.05 ns	
Repeated measures ANOVA				
Corrected model	< 0.001***			
Group	< 0.001***			
Time	< 0.001***			
Group x Time	< 0.001***			

*, significant at $P<0.05$; **, significant at $P<0.01$; ***, significant at $P<0.001$; NS, non-significant at $P > 0.05$.

In addition, the difference in bacterial growth between the time points listed above was highly significant, and a significant change was induced in the bacterial growth by the interactions amid the treatment groups and the span of time (P<0.001).

Next, a one-way repeated measures ANOVA was carried out to check the change over time, which was highly significant in the bacterial groups without phage (P<0.001) and in the bacterial groups with phage (P<0.001); however, there was no significant change in the control group with regard to time (P > 0.05).

A one-way ANOVA was implemented to check the disparity in bacterial growth among the 3 treatment groups along every interval of time. There was a highly significant difference among the three treatment groups at all time points.

Host range of phage

Host ranges *S. typhimurium* of isolated phages against various strains were analyzed. All tests were carried out at a temperature of 37°C. The phages showed lytic activity against only one species (*E. coli*) out of the seven species analyzed, which were comprised of S. sonni (ATCC 25931), *K. oxytoca* (ATCC 49131), S. aureus (ATCC 12600), *E. coli* (ATCC 25922), *E. faecalis* (ATCC

29212), P. aeruginosa (ATCC 9027), and P. vulgaris (ATCC 49132) (**Table 3**). Positive numbers (+) show that the phage has infected the bacteria; negative numbers (-) show that the bacteria grew normally, and the phage was not able to infect it.

Thermal stability of phage *S. typhimurium*

The results of the thermal stability test of phage *S. typhimurium* reflected a significant disparity in the growth of bacteria induced by different temperatures (***P<0.001), phage dilutions (***P<0.001), and the interaction among phage dilutions and

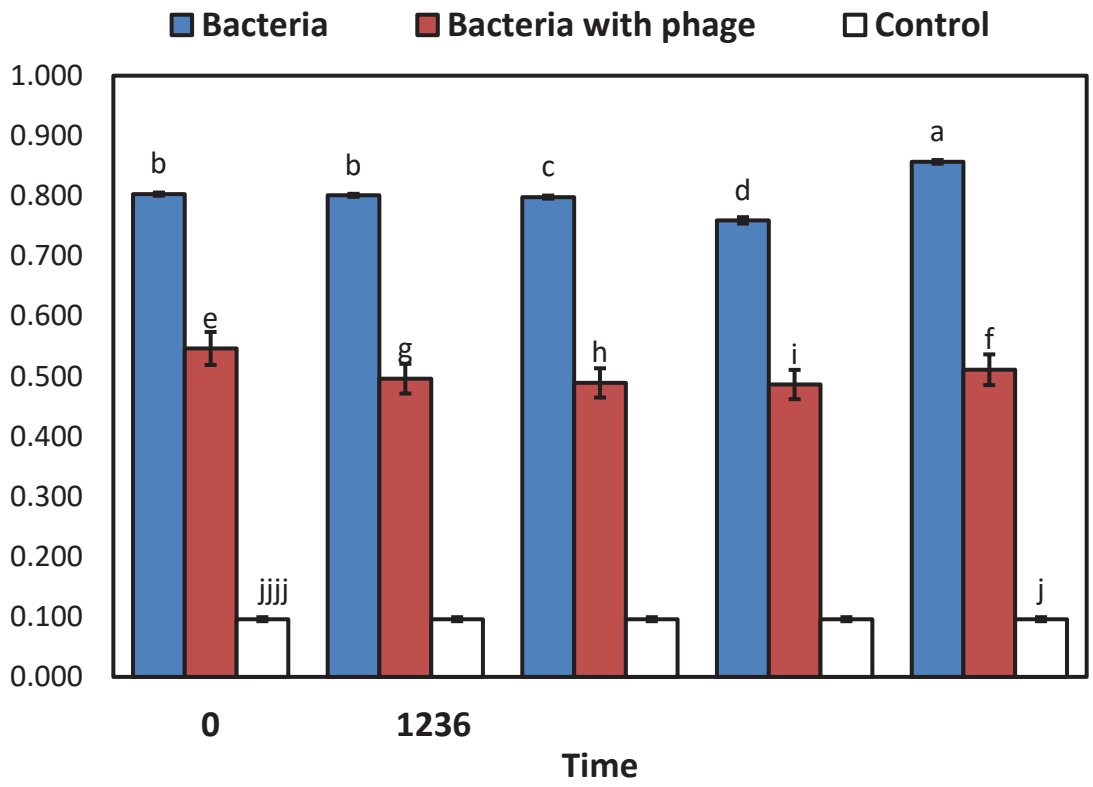

Figure 3 Bar chart showing the ability of SAL 10 phage to lyse the host strain *S. typhimurium*. When different letters occur subsequent to the means, this indicates that they are are significantly dissimilar, according to Duncan's Multiple Range Test (DMRT)

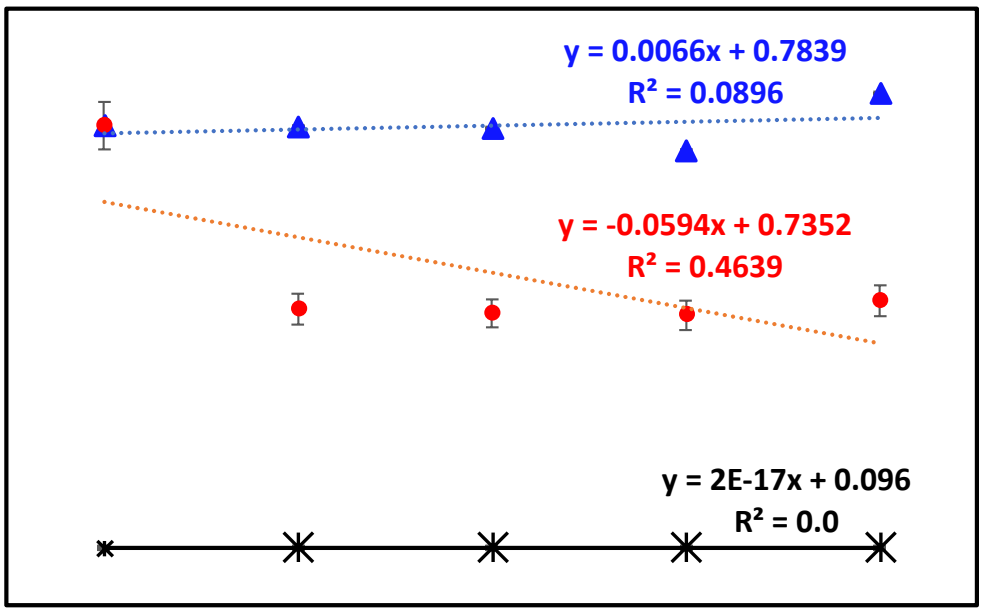

Figure 4 Regression trendline that shows the interrelationship between time (x-axis) and bacterial count (y-axis)

temperature (***P<0.001). The data in **Table 4** and **Figure 5** are presented as the mean ± SD. The results revealed that 37°C was the optimal temperature for phage SAL 10. Moreover, phage SAL 10 was highly stable between 40°C and 50°C and it was inactivated at 60°C.

A two-way ANOVA was utilized to evaluate the influence of different phage dilutions (10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}) and diverse temperatures (37, 40, 50, and 60°C) on the bacterial growth. A statistically significant difference was induced by different phage dilutions (P<0.001) and temperatures (P<0.001). Moreover, a significant change was induced in the bacterial growth by the interaction between phage dilutions and temperatures (P<0.001).

Subsequently, we performed a one-way ANOVA to check the vertical change in the bacterial growth at different temperatures, which was highly significant at phage dilutions 10^{-3}, 10^{4}, 10^{5}, and 10^{-6}, all of which were (P<0.001).

Table 3. Host ranges *S. typhimurium* of isolated phages against different strains. Phages showed lytic activity against one species out of the seven examined.

S. ssonni (ATCC 25931)	*K. oxytoca* (ATCC 49131)	*E. coli* (ATCC 25922)	*S. aureus* (ATCC 12600)	*P. aeruginosa* (ATCC 9027)	*E. faecalis* (ATCC 29212)	*P. vulgaris* (ATCC 49132)
–	–	+	–	–	–	–

Table 4: Phage's ability to lyse the host strain *S. typhimurium*. Analysis of variance: ANOVA

Temperature	Bacterial growth / temperatures at different phage dilutions (Mean ±SD)				
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	ANOVA
37°C	$2.96x10^{4}±2.13x10^{2}$	$9.8x10^{4}±2.65x10^{3}$	$1.32x10^{6}±1.01x10^{5}$	$1.03x10^{7}±5.5x10^{5}$	< 0.001***
40°C	$2.96x10^{4}±3.6x10^{2}$	$1.83x10^{5}±2.14x10^{4}$	$1.40x10^{6}±1.0x10^{5}$	$0.00x10^{0}±0.00x10^{0}$	< 0.001***
50°C	$1.90x10^{4}±1.70x10^{3}$	$1.37x10^{5}±1.53x10^{4}$	$9.90x10^{5}±1.00x10^{4}$	$0.00x10^{0}±0.00x10^{0}$	< 0.001***
60°C	$0.00x10^{0}±0.00x10^{0}$	$0.00x10^{0}±0.00x10^{0}$	$0.00x10^{0}±0.00x10^{0}$	$0.00x10^{0}±0.00x10^{0}$	< 0.001***
***P*-value**	< 0.001***	< 0.001***	< 0.001***	< 0.001***	< 0.001***
Two-way ANOVA					
Corrected model	< 0.001***				
Phage dilutions	< 0.001***				
Temperature	< 0.001***				
Phage dilutions x temperature	< 0.001***				

*, significant at $P < 0.05$; **, significant at $P < 0.01$; ***, significant at $P < 0.001$; NS, non-significant at $P > 0.05$.

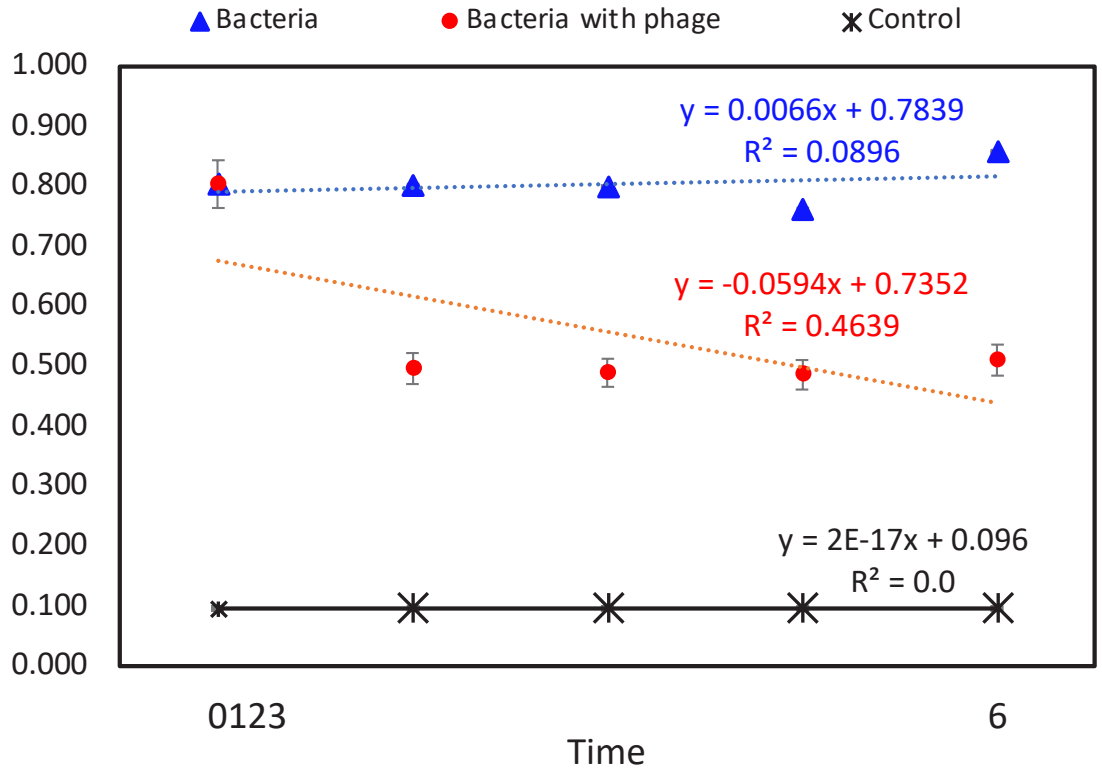

Figure 5 Bar chart showing the thermal stability of phage *S. typhimurium.* Bars in which different letters are subsequent are significantly dissimilar according to DMRT.

Moreover, to check the horizontal disparity in the bacterial growth at four phage dilution (10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}), a one-way ANOVA was used, at each temperature. The results reflected a highly significant disparity in the bacterial growth among the four phage dilutions at temperatures 37°C, 40°C, 50°C, and 60°C, which were again all (P<0.001).

A correlation matrix showing the relationship between the effect of the temperature and the different phage dilutions on the bacterial growth is presented in **Figure 6**. Blue reflects that there is a positive correlation; in turn, a negative correlation comes across as red; finally, when there are boxes, this reflects that there is a significant correlation. Temperature was strongly, negatively (inversely), and significantly correlated with increasing temperature, as shown by both the Pearson's correlation (**Figure 6**) and the linear regression (**Figure 7**) (a, b, c, d represent the results of DMRTs, which is a post hoc (post ANOVA) test. This test is able to perform further comparison between subgroups. It is used to compare any two bars with similar letters that indicate a non-significant difference; in turn, bars with different letters indicate significant difference. e.g., d, d = not significant. c, d = significant. a, b = significant y: y-axis, which indicates the bacterial growth; R^2: determination coefficient which corresponds to the correlation coefficient; 0.6–0.9: strong correlation; 0.3–0.5: moderate correlation; 0.1–0.25: weak correlation; 0: indicates that there is zero correlation.

pH stability of phage *S. typhimurium*

We performed a two-way ANOVA to evaluate the influences of different phage dilutions (10^{-1}, 10^{-2}, and 10^{-3}) and different pH values (4, 7, and 14) on the bacterial growth (**Table 5**). The differences in bacterial counts at different phage dilutions (10^{-1}, 10^{-2}, 10^{-3}) were shown to be highly significant (***P<0.001); moreover, different pH values (4, 7, 14) induced significant differences in bacterial counts (P<0.001). Furthermore, the interactions between different phage dilutions and pH induced a significant change in the bacterial growth (P<0.001).

To check the horizontal change in the bacterial counts at different pH values, a one-way ANOVA was used. This reflected that there was a highly significant disparities in the bacterial count at different tested pH (4, 7, 14) and at different phage dilutions 10^{-1} (P<0.001), 10^{-2} (P<0.001), and 10^{-3} (P<0.001).

A one-way ANOVA was also utilized to check the difference in the bacterial count at three different phage dilutions (10^{-1}, 10^{-2}, and 10^{-3}) at each pH. This reflected a highly significant difference in the bacterial count at 3 phage dilutions at pH 4 (P<0.001) and pH 7 (P<0.001). However, the bacterial count at different phage dilutions at pH 14 (P<0.001) showed no significant difference (**Figure 8**).

Bacteriophage morphology analysis using TEM

We established the morphology of the virion by utilizing TEM and negative staining. They were found to be tailed phages belonging to the order Caudovirales. In turn, we also determined that the isolated phage SAL 10 belonged to the Siphoviridae family. We came to this conclusion because of the existence of an isometric head as well as the fact that the tail was long and non-contractile (**Figure 9**).

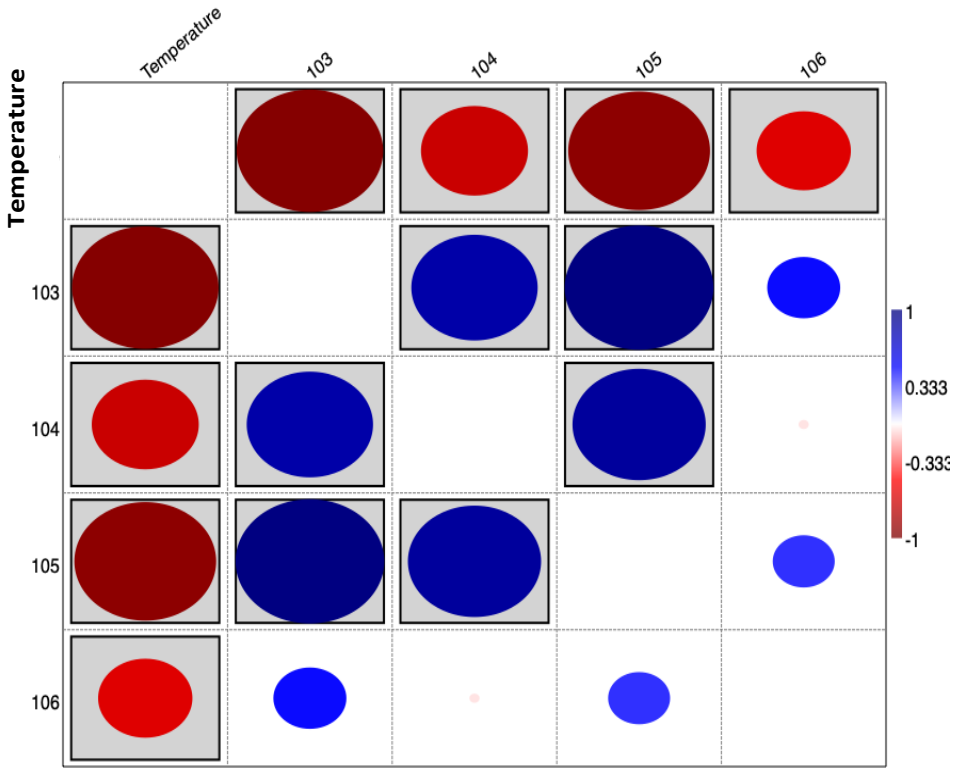

Figure 6 Correlation matrix showing the relationship between temperature and different phage dilutions on the bacterial growth. Blue reflects a positive correlation; negative correlation is reflected by red, while boxes denote a significant association.

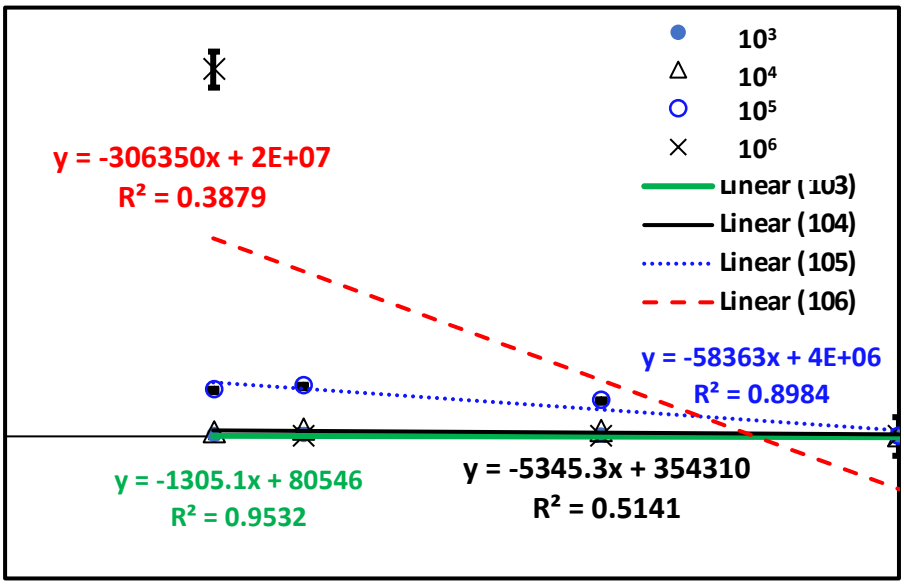

Figure 7 Regression trendline showing the relationship between increasing temperature and different phage dilutions on the bacterial growth.

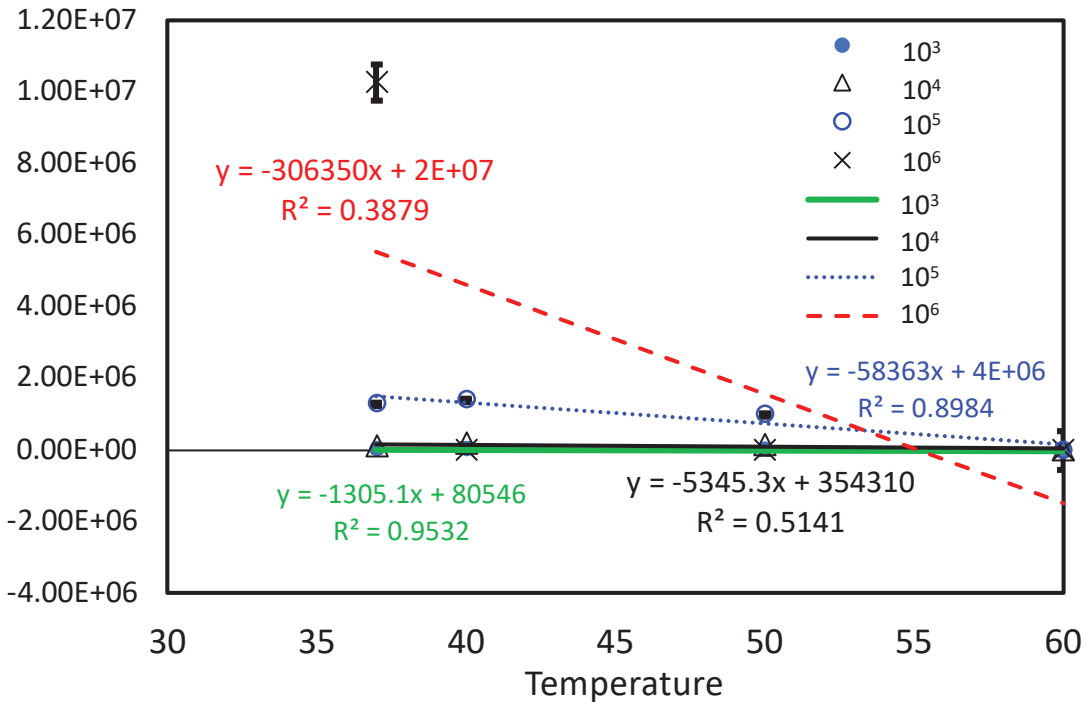

Figure 8 Bar chart presenting the effects of different levels of pH (4, 7, 14) on the bacteria isolates

Discussion and Conclusion

Bacteriophages have been regarded as promising antibacterial therapies for treating numerous infectious illnesses in humans since they were discovered [38].

Initially, bacteriophages were used in clinical settings to treat acute intestinal illnesses [10] as well as other ailments, including skin infections [39]. Subsequently, surgical therapy implemented bacteriophages to treat purulent wounds and postoperative infections [40,41]. At this time, several organizations, universities, and institutes are investigating phage therapy for mammals, including human beings [42]. The existence of bacteriophages is tightly connected with their natural hosts. In the current research, soil samples were collected from chicken farms to isolate lytic

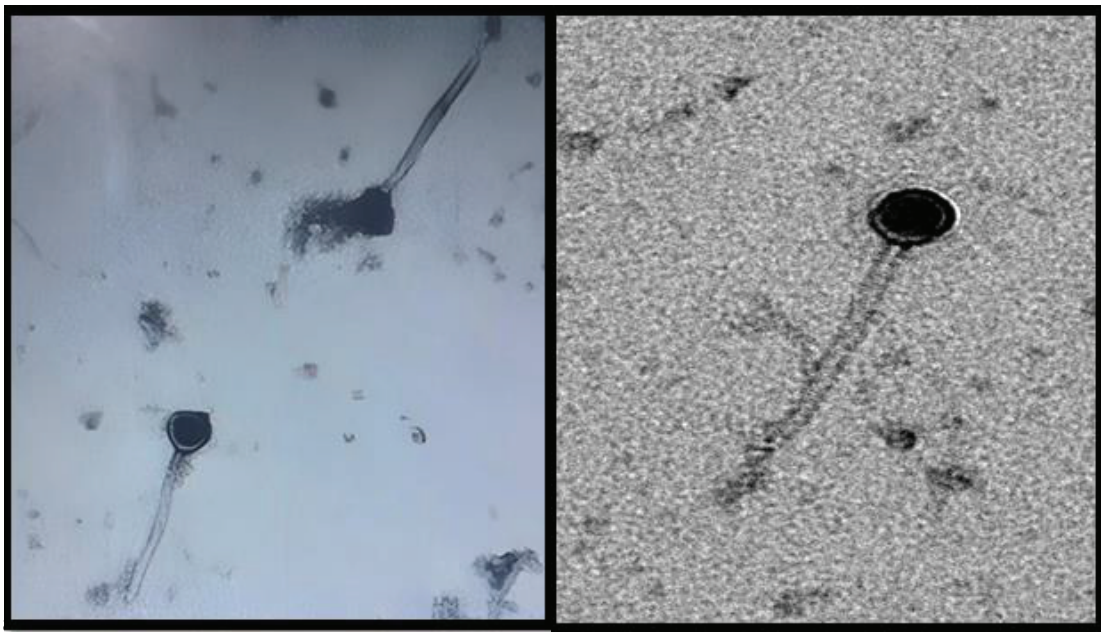

Figure 9 Transmission electron micrographs of negatively stained bacteriophages. TEM analysis of the purified phage SAL 10 reflected that SAL 10 was from the *Siphoviridae* family. Its tail is non-contractile and long; moreover, it has an isometric head. (Scale bar = 200 nm)

Table 5. The bacterial strain count presented as the mean ± SD at different phage dilutions and pH (4, 7, 14).

Phage dilutions	Bacterial strain count at different pH (Mean ± SD)			
	4	7	14	ANOVA
10^{-1}	2.96 x10^2±3.61 bc	3.22x10^2+2.08 bc	0.00+0.00 c	< 0.001***
10^{-2}	1.03 x10^2±4.00 bc	4.24 x10^2+498.54 b	0.00+0.00 c	< 0.001***
10^{-3}	0.00+0.00 c	9.80x10^3+264.58 a	0.00+0.00 c	< 0.001***
P-value	< 0.001***	< 0.001***	> 0.05 ns	
Repeated measures ANOVA				
Corrected model	< 0.001***			
Phage dilutions	< 0.001***			
pH	< 0.001***			
Phage dilutions x Time	<0.001***			

*, significant at $P < 0.05$; **, significant at $P < 0.01$; ***, significant at $P < 0.001$; NS, non-significant at $P > 0.05$.

bacteriophages against *S. typhimurium*. The *S. typhimurium* bacteria were isolated from liver and heart tissues taken from 28-day-old chickens. The morphological characterization of the isolated bacteriophage plaques revealed that all of them formed clear, tiny (<1 mm diameter) plaques. Host range is an essential factor to consider when choosing phages for phage therapy [43]. Our results indicated that the isolated bacteriophages had a narrow range of activity. Moreover, they showed that pH as well as temperature can influence the efficacy of bacteriophage treatments of pathogenic microorganisms [44]. Greater temperatures have the ability to cause irreparable harm or the denaturation of viral particles [45]. In this study, the stability of the isolated phages was tested by subjecting them to different temperatures. The temperatures for incubation were derived with consideration of the normal temperature of the living organisms, which ranges from 37 to 40°C. The temperature stability test results indicated that each of the bacteriophages was able to stay reasonably stable between 37 to 50°C; in turn, they were inactivated at temperatures above 50°C. The pH stability test results reflected that the studied materials remained reasonably stable at pH values between 6 to 8; in turn, they were inactivated at pH 14. These conclusions support those of the previous report from [46]. The capability of phages to persist at such pH and temperatures implies that it would be possible to use them as therapeutic agents in living organisms. Furthermore, this study determined that the bacterial growth was inhibited by phage infection. For *S. typhimurium*, the lysis kinetics of SAL 10 were determined approximately 60 min after the infection; moreover, the culture's optical density was reduced. The bacterial growth slightly increased after 6 h. Once again, our results are complimentary with those of previous studies, which were performed by [47 and 48]. Undoubtedly, phage treatment is a promising approach that is poised to tackle antibiotic resistance. Multiple researches have highlighted the potential use of therapeutic phages both *in vitro* and *in vivo*; however, more evidence is required to establish a solid regulatory case for its clinical use. Moreover, there are still significant obstacles to phage treatment, notably regulatory policy management [49].

Finally, this study can aid in providing information regarding utilizing these phages as a successful substitute for antibiotics against *S. typhimurium*. his study presents biological analyses of SAL 10. Furthermore, our study revealed that phage SAL 10 has antimicrobial activity against *S. typhimurium*. This implies that it could be used as a therapeutic agent.

In our study, we isolated only a few predators (phages) that we encountered over a short period of time. These constraints are related primarily to time and money. Therefore, more comprehensive studies that consider more phages and different timeframes are required to fill this gap. Moreover, if the sample size were to be increased, it would achieve better results.

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