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Discovery of Polyvalent Myovirus (vB_STM-2) Phage as a Natural Antimicrobial System to Lysis and Biofilm Removal of *Salmonella* Typhimurium Isolates from Various Food Sources

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Abstract: New and natural antimicrobials as an alternative control system are now an urgent need to overcome stubborn bacterial infections. *Salmonella* Typhimurium has become the most frequent serovar responsible for salmonellosis in humans around the world. The high antimicrobial resistance and biofilm production make this pathogen more dangerous. We aimed to isolate a broad lytic phage to prevent *Salmonella* infection and reduce its biofilms. Using *Salmonella* Typhimurium (ST-4) as a host, seven phages were isolated, and only three phages showed clear lytic plaques, two members of the *Siphoviridae* family (vB_STS-1 and vB_STS-3) and one of the *Myoviridae* family (vB_STM-2). The vB_STM-2 phage was the most potent broad lytic phage, infecting 100% of tested *Salmonella* Typhimurium serovars and non-*Salmonella* strains. Additionally, the vB_STM-2 phage was thermostable at −20 to 55 °C up to 24 h, while at 65 and 75 °C, a significant ($p < 0.05$) titer reduction was observed after 7 days. Moreover, the phage seemed to be stable at different pHs (4–11) after one to twelve hours (hrs), while increasing the time made the phage more sensitive to the alkaline medium rather than the acidic medium. Interestingly, the vB_STM-2 phage had the capacity to diminish or eradicate the biofilms of tested *Salmonella* Typhimurium, e.g., ST-4, ST-19, ST-30, ST-37, ST-45 and ST-49 by 81.2%, 76.4%, 43.6%, 41%, 39.8% and 93.4%, respectively, at a titer concentration of 10^6 PFU/mL. Eventually, the vB_STM-2 phage showed significant ($p < 0.05$) efficacy in the elimination of *Salmonella* Typhimurium (ST-4) from contaminated chicken breasts at both storage periods with high titer stability. The *Salmonella* count showed a severe decline from 7.00 ± 0.63 $\log_{10}$ CFU/cm^2 to 0.88 ± 0.17 $\log^{10}$ CFU/cm^2 on the seventh day of the short-term storage, and from 5.13 ± 0.44 $\log_{10}$ CFU/cm^2 to 1.10 ± 0.12 $\log_{10}$ CFU/cm^2 on day 27 of the long-term assay. In both periods, the phage titers remained stable, with insignificant ($p < 0.05$) loss. Therefore, this phage is considered a prime candidate to combat multi-drug-resistant *Salmonella* Typhimurium and its biofilms.

Keywords: *Salmonella* Typhimurium; multidrug-resistant; bacteriophages; phages; vB_STM-2

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1. Introduction

Salmonella is a Gram-negative, rod-shaped microorganism that is a member of the well-known bacterial *Enterobacteriaceae* family [1]. *Salmonella* can contaminate many types of foods (chicken, livestock, dairy, fruits, and vegetables), increasing the risk of eating contaminated food [2–5]. Many different serovars of *Salmonella* cause food poisoning, but the most dominant serovar is *Salmonella* Typhimurium [6].

Nowadays, *Salmonella* Typhimurium has become the most frequent serovar to be primarily responsible for salmonellosis in humans worldwide [7,8]. However, this serovar was not known until the mid-1990s, and its isolation from humans and animals has only

increased recently [9]. Salmonellosis is a considerable source of enteric disease for humans in many countries worldwide [10,11]. The majority of cases arise from eating contaminated food [12]. *Salmonella* spp. outlets are responsible for approximately 85% of foodborne diseases annually, leading to more than 100,000 deaths [10]. In addition, it was the most common pathogen associated with hospitalized patients diagnosed with bloodstream infections in Africa and Asia from 2008 to 2018 [13]. Salmonellosis is characterized by many symptoms, including fever, nausea, vomiting, abdominal cramps, and inflammatory diarrhea. Symptoms occur within 12 to 72 h and last from 2 to 7 days, with severe infections leading to hospitalization and death [1].

In addition, this bacterium was found to have a great ability to form surface-attached aggregates of communities embedded in extracellular polymeric substances (EPSs), known as biofilm [2]. Biofilm formation by bacteria is undesirable in many fields, including industry, agriculture, and medicine [14]. In short, biofilm increases the survival rate of bacteria from 1 to 1000 times and may weakens the effect of antibiotics or prevent their effects completely [15,16]. Antibiotics are the conventional control strategies to eliminate *Salmonella* serovars [1], but later resistance to these traditional antibiotics has emerged, which has led to the emergence of multidrug-resistant *Salmonella* spp. [17]. Therefore, it was necessary to find a promising alternative to control such resistant infections. Bacteriophages are natural bacteria viruses used as alternative antimicrobial agents (lytic type) to counter such multidrug-resistant bacteria [18].

Phages are highly capable against bacteria contaminating food products [19,20]. Due to their defense mechanisms and high diversity, many studies have reported the isolation and successful use of phages in the control of *Salmonella* [21–23]. Many previous studies have determined that *Salmonella* phages of a wide host range could control *Salmonella*, including *S. enteritidis* or *Salmonella* Typhimurium [24]. Although phages are usually species-specific [25], some polyvalent phages have been observed, especially among the phages of the *Enterobacteriaceae* family [26,27]. Phages with polyvalent behavior can infect strains from different genera or species, which is preferable to others [28].

Bacteriophages have been employed to control *Salmonella* in many foods, such as chicken [29,30], beef [31], pork [32], fruits [33], and dairy products [34].

This study aimed to isolate a lytic polyvalent bacteriophage to control multiple drug-resistant *Salmonella* Typhimurium (the most widely spread serovar) and combat its biofilm.

2. Materials and Methods

2.1. Salmonella Typhimurium Isolates Sources in the Study

A total of 23 *Salmonella* strains presenting a Typhimurium serovar previously isolated from food sources according to Silliker and Taylor [35] and ISO [36] were used in this study (Table 1). In addition, *Salmonella* Typhimurium (ST-4) was used as a lawn strain for phage isolation, propagation, and purification. All *Salmonella* used were previously isolated in the plant viruses and bacteriophage Lab of Botany and Microbiology Department, Faculty of Science at Al-Azhar University (Cairo, Egypt). *Salmonella* isolates were stored in 15% glycerol at −80 °C as testing stocks. For fresh culture, a colony formed by streaking on Tryptone Soya Agar (TSA) obtained from Oxoid (Hampshire, UK) was developed in 5 mL of Tryptone Soya Broth (TSB) also obtained from Oxoid, and left in the incubator for 16–18 h at 37 °C.

Table 1. Source, antibiotic-resistance profile, and biofilm characteristics of *Salmonella* serovars used in this study. *Salmonella* Typhimurium (ST-4) was used for phage isolation and propagation. All serovars were involved in the host range determination.

Serovars	Isolate ID	Source	Antibiotic-Resistance Profile	Optical Density of Cell Growth (OD_{620} nm)	Optical Density of Biofilm (OD_{570} nm)	Interpretation
Salmonella Typhimurium	ST-4	Chicken (Egypt)	Resistant	1.847	0.947	Strong
Salmonella Typhimurium	ST-7	Chicken (Egypt)	Resistant	0.998	0.411	Moderate

Table 1. *Cont.*

Serovars	Isolate ID	Source	Antibiotic-Resistance Profile	Optical Density of Cell Growth (OD_{620} nm)	Optical Density of Biofilm (OD_{570} nm)	Interpretation
Salmonella Typhimurium	ST-9	Chicken (Egypt)	Resistant	0.876	0.563	Moderate
Salmonella Typhimurium	ST-14	Bovine (Egypt)	Resistant	1.022	0.744	Strong
Salmonella Typhimurium	ST-16	Bovine (Egypt)	Resistant	0.773	0.117	Weak
Salmonella Typhimurium	ST-19	Bovine (Egypt)	Resistant	1.977	1.081	Strong
Salmonella Typhimurium	ST-21	Dairy products (Egypt)	Resistant	0.712	0.108	Weak
Salmonella Typhimurium	ST-22	Dairy products (Egypt)	Resistant	1.446	0.742	Strong
Salmonella Typhimurium	ST-27	Dairy products (Egypt)	Resistant	1.784	0.748	Strong
Salmonella Typhimurium	ST-30	Chicken (Egypt)	Resistant	1.709	0.904	Strong
Salmonella Typhimurium	ST-33	Chicken (Egypt)	Resistant	0.993	0.321	Moderate
Salmonella Typhimurium	ST-35	Chicken (Egypt	Resistant	1.003	0.411	Moderate
Salmonella Typhimurium	ST-36	Chicken (Egypt)	Resistant	0.878	0.103	Weak
Salmonella Typhimurium	ST-37	Chicken (Egypt)	Resistant	2.047	1.105	Strong
Salmonella Typhimurium	ST-38	Chicken (Egypt)	Resistant	0.788	0.119	Weak
Salmonella Typhimurium	ST-41	Bovine (Egypt)	Resistant	0.936	0.366	Moderate
Salmonella Typhimurium	ST-43	Bovine (Egypt)	Resistant	1.171	0.773	Strong
Salmonella Typhimurium	ST-45	Bovine (Egypt)	Resistant	1.664	0.817	Strong
Salmonella Typhimurium	ST-47	Bovine (Egypt)	Resistant	0.899	0.541	Moderate
Salmonella Typhimurium	ST-49	Bovine (Egypt)	Resistant	1.811	0.927	Strong
Salmonella Typhimurium	ST-53	Dairy products (Egypt)	Resistant	0.659	0.173	Weak
Salmonella Typhimurium	ST-55	Dairy products (Egypt)	Resistant	0.744	0.196	Weak
Salmonella Typhimurium	ST-56	Dairy products (Egypt)	Resistant	1.022	0.511	Moderate

2.2. Inoculums Preparation of Isolates

Salmonella strains were cultured into a TSB medium and incubated at 37 °C until a mid-logarithmic phase. The cells concentration was adjusted in CFU/mL against 0.5 McFarland turbidity standard (1.5×10^8 CFU/mL) [37].

2.3. Confirm the Identification of the Salmonella Typhimurium Used

Confirmation of *Salmonella* Typhimurium was performed in two major steps. The first step involved the morphological [38], biochemical [39,40], and serological (slide agglutination test) [41,42] characteristics. The second step, Biomerieux VITEK 2 identification system [27,28], automatically confirmed *Salmonella* Typhimurium.

2.4. Statuses of Antibiotic-Resistant Profiles of Salmonella Typhimurium Used

A total of 23 *Salmonella* strains presenting Typhimurium serovar were tested to determine the antibiotic-resistant profiles using disc diffusion assay as described by Bauer [43]. This assay was performed with the following tested antibiotics (the most widely used antibiotics in human medicine in Egypt): streptomycin (10 µg), kanamycin (30 mcg), flucloxacillin (5 mcg), tetracycline (30 mcg), levofloxacin (5 mcg), tobramycin (10 mcg), aztreonam (1 mcg), oxacillin (1 mcg), rifamycin (30 mcg), erythromycin (15 mcg), amoxicillin/clavulanic acid amc (20 µg), clindamycin (2 mcg), gentamicin (10 mcg), cephradine (30 mcg), ciprofloxacin (5 mcg), and ampicillin (10 mcg) which were obtained from El-Gomhouria co. for trading chemicals and medical appliances (Cairo, Egypt). The resistance or sensitivity of the tested bacteria to the antibiotics used was determined by measuring the growth inhibitory zone around each antibiotic disc after 24 h of incubation at 37 °C. The obtained results were designated as S (sensitive), I (intermediate sensitive), and R (resistant) based on the standardized protocols by the National Committee for Clinical Laboratory Standards (NCCLS) [44].

2.5. Biofilm Forming Capacity of Salmonella Typhimurium Isolates

The tissue culture plate method described by Stepanović et al. [45] was used for the qualitative assay of biofilm formation activity of all 23 *Salmonella* Typhimurium isolates. In this assay, biofilm formation was evaluated in 96-well tissue culture polystyrene plates, with flat bottoms and lids (Sigma-Aldrich, St. Louis, MO, USA). In total, 200 µL of TSB medium supplemented with 0.25% glucose was added into each well, plus 20 µL of 10^5 CFU/mL bacterial suspension. After overnight incubation at 37 °C, the plates were aspirated and washed using phosphate-buffered saline (PBS). The adsorbed bacteria were fixed by 95% ethanol to the polystyrene wells and then stained using crystal violet (0.1%) after removing the ethanol by washing. The dye was solubilized in 1% *w*/*v* SDS, and the optical densities were measured at $O.D._{570}$ nm photometrically by an ELISA reader (Sunrise™-TECAN, Männedorf, Switzerland) at the Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Cairo, Egypt. The assay was performed in triplicate. The formed biofilm was described as low, moderate, or strong, depending on the interpretation of Stepanović et al. [45].

2.6. Isolation and Purification of Salmonella Typhimurium Phages

2.6.1. Isolation Source

Twenty milliliters (20 mL) of sewage water (9 different samples) was obtained from Kafr El-Sheikh (N 30°56′45″ E 31°06′42″) governorate, Egypt.

2.6.2. Isolation and Enrichment

For the isolation of *Salmonella* Typhimurium phages, the method described by Bibi et al. [46] was used; 10-mL of centrifuged sewage water (supernatant) was mixed with an equal volume of TSB media (Difco™, BD, USA). At the mid-logarithmic phase of the host bacteria (*Salmonella* Typhimurium; ST-4), 100 µL was added to the mixture of sewage water and media, followed by overnight incubation (shaking at 270 rpm) at 37 °C. At 7000 (× *g*),

the previous mixture after incubation was centrifuged for 10 min.; the pellet was discarded, and through a sterile filter (0.45-µm), the supernatant was filtered and stored at 4 °C in a clean, sterile flask.

2.7. Phage Detection

Spot-Assay Technique

According to Capra et al. [47], a spot-test assay was performed. The overlay (4 mL of TSB supplemented with 0.7% agar) layer was prepared. In total, 100 µL of 24 h incubated bacterial culture (host bacterium) was inoculated into the previous layer (soft layer). The soft layer was poured over previously prepared solid trypticase soy agar (TSA) plates (overlay layer). Then, 10 µL from each enriched sample was spotted over the plates and incubated at 37 °C for 24 h. After the incubation period, phage activity appeared in the form of zones of complete or partial lysis on the soft layer. In CM phage buffer (0.73 g/L CaC_{l2}. 2 H_2O: 2.5 g/L $MgSO_4$. 7 H_2O; 0.05 g/L gelatin; 6 mL/L 1 M Tris buffer; pH 7.2), lysis zones were relocated separately under aseptic conditions.

Plaque Assay Technique

The double-layer agar method was performed by Sangha et al. [48] to assay phages' plaque formation. In TSB, ten-fold serial dilutions for phage lysate were performed. Then, 100 µL from each dilution of phage lysate was inoculated to 100 µL from the overnight host's culture (10^9 CFU/mL) and left in an incubator for 10 to 15 min. Next, the previous mixture was mixed gently, placed in a 4-mL soft agar medium, and then poured over the TSA plates. Afterward, the plates were incubated overnight at 37 °C, resulting in different plaques, whose sizes and shapes were quantified.

Purification, Propagation, and Titration

All these assays were performed by the double overlay method following Sangha et al. [48]. Using a sterile wire loop, an individual plaque was picked and then transferred to TSB inoculated with the bacterial host used in phage isolation (*Salmonella* Typhimurium; ST-4). To confirm the purity of the phage, the double overlay method was conducted at least three times. Regarding phage propagation, 100 µL of phage was added to 100 µL of bacterial lawn in TSB medium, followed by overnight incubation at 37 °C. Ten overlay agar plates were prepared for each isolate phage, and 3 mL of CM buffer was spread over each plate. In 50 mL clean, sterile tubes, the upper area of the soft layer in the plates was scribbled and transferred. The collected tubes were vortexed (Vortex-Genie-2; Inc., Bohemia, NY, USA) for 5 min after being left for 15 min. Tubes were centrifugated (7000× *g*/15 min) at 4 °C, the supernatant was filtered and then transferred into a clean, sterile tube to keep at 4 °C. A ten-fold serial dilution of the phage suspension was performed to calculate phage titers, and the resulting plaques were counted by the double overlay method, as previously explained.

Host Range Study

Using spot-testing, the host spectra of all isolated phages were studied per Capra et al. [47]. This assay was conducted on the bacterial isolates listed in Table 1, in addition to 13 other strains. The strains other than *Salmonella* included 5 of *Pseudomonas aeruginosa* (clinical and food sources), 3 of *Staphylococcus aureus* (food sources), 4 of *Escherichia coli* (clinical isolates), and 1 of *Klebsiella pneumoniae* (clinical source). The soft agar layer was inoculated with 100 µL fresh cultured strain and (1.5×10^9 CFU/mL) poured onto the overlay layer (TSA). After solidification, the phage suspension (1×10^7 PFU/mL) was dropped on the soft layer and then incubated for 24 h at 37 °C. Areas of spot-lysis on the tested strain were considered phage sensitive. The potent phage that showed the broadest host range in the spot test was confirmed by the efficiency of the plating (EOP) method, according to Mirzaei and Nilsson [49]. The test results of the EOP are interpreted in different patterns, namely, inefficient EOP <0.001; low efficiency EOP from 0.001 to <0.2;

moderate efficiency EOP from 0.2 to <0.5; and high efficiency EOP from 0.5 to 1.0. The experiments were performed in triplicates with standard deviation (±SD).

Transmission Electron Microscopy (TEM)

In total, 1 mL of purified high titer stock phages was centrifuged for 1 h /16,000× *g* at 4 °C, the supernatant was discarded, and the pellet was resuspended in CM phage buffer (20 µL). On the carbon grids (200 mesh) coated with formvar, 5 µL of phage suspension was added and fixed for 2 min. The examined phages were negatively stained (30 s) by 2% uranyl acetate, and the excess dye was removed by paper [50]. At the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt, the samples were examined by electron microscopy (Model Beckman 1010) operated at 60 KV [51].

Thermo and pH Stability of vB_STM-2 Phage

At different times (1, 2, 4, 12, 24, and 7 days), the stability of the vB_STM-2 phage was checked at different temperatures (e.g., −20, 4, 25, 37, 55, 65, and 75 °C) as well as pH levels (4, 7, 9, and 11). Both experiments were carried out per Philipson et al. [52] and Jamalludeen et al. [53] for temperature and pH stability. The initial titer of the phage was 7 $\log_{10}$, and any titer changes were detected during the experiments using the double-layer agar method of Sangha et al. [48]. Assays were performed in triplicate with a long standard deviation (± SD).

Biofilm Removal Activity of the vB_STM-2 Phage

The antibiofilm activity of the vB_STM-2 phage against the strongest biofilm-producing *Salmonella* Typhimurium listed in Table 1 was measured. Coincidentally, 6 isolates with codes ST-4, ST-19, ST-30, ST-37, ST-45, and ST-49 were the strongest and were derived from a meat source, i.e., chicken or bovine. According to Bekir et al. [54], the test was performed using 96-well tissue culture polystyrene plates with a flat bottom and a lid (Sigma-Aldrich, St. Louis, MO, USA). Then, 20 µL of tested bacterial culture (at mid-logarithmic phase) (2×10^6 CFU/mL) was inoculated into 200 µL TSB medium supplemented with 0.25% glucose in the plate wells. In some specific wells of the plate, 100 µL phage suspension (1×10^6 PFU/mL) was added and mixed well (wells of anti-biofilm). Thus, there were wells without phage addition in the plate, which contained bacteria and media (wells of biofilm), and wells to which we did not add the phage or bacteria (wells of negative control). Biofilm reduction by the phage was calculated according to Else et al. [55] and Kostaki et al. [56] by the following equation:

$$[(C - B) - (T - B)]/[(C - B)] \times 100$$

where C = refers to the optical density (OD_{620} nm) of the control result, B = refers to the optical density (OD_{620} nm) of the blank (TSB), and T = refers to the optical density (OD_{570} nm) of phage-treated wells.

2.8. Bacterial Challenge Measurements

In Broth Medium (Culture Clearing)

Tests of the bacterial challenge were carried out to detect the activity of the vB_STM-2 phage in confronting *Salmonella* in a liquid medium. In brief, 3 clean flasks, each with 100 mL sterile TSB, were prepared. First, 1 mL (6 $\log_{10}$ CFU/mL) fresh *Salmonella* Typhimurium (ST-4) culture was inoculated into only 2 flasks of the three, while the latter remained unchanged. Next, 1 mL phage (8 $\log_{10}$ PFU/mL) was injected into one of the flasks implanted with bacteria (treated flask), while the other implanted one was injected with sterile distilled water (positive control flask). The flasks containing the TSB medium remained a negative control. Finally, the three flasks were placed overnight in an incubator shaker (220 rpm) at 37 °C, with 2 mL of each flask taken at 0, 1, 3, 6, 12, and 24 h to determine the count of *Salmonella*. For accuracy comparison, 1 mL was examined by spectrophotometry (OD_{620} nm), while the other was examined by counting ($\log_{10}$) *Salmonella* on TSA plates. The tests were performed in triplicate with standard deviation (±SD).

2.9. Preparation of the Food Sample Used in the Study

The first step: in a local supermarket, chicken breasts (used as a food model) with a recent production date were purchased, placed in sterile bags, and then transferred in an icebox to the laboratory to carry out the experiment. Using a sterile meat cutting board in the laminar flow, chicken breasts were sliced into small squares (2 × 2 cm) with a clean, sterile knife [57]. These squares were sterilized for 3 h with 70% ethanol, washed three times with sterile water, and then left to dry for half an hour. After drying, all squares were examined on TSA plates according to FDA [58]; any squares that showed microbial growth were excluded, and the remaining squares were used.

The second step: the used squares were divided into 2 sections; each section had 3 groups of squares. The first section was prepared for the short-term experiment (7 days) as follows: in two of the three groups, *Salmonella* Typhimurium culture (ST-4) was inoculated (7 $\log_{10}$ CFU/cm^2), while the third remained the same. The second section was prepared for the long-term experiment (27 days) as follows: similar to the above, *Salmonella* Typhimurium culture (ST-4) was inoculated (6 $\log_{10}$ CFU/cm^2) in 2 groups, while the other remained the same. The two experiments were performed in triplicate with standard deviation (±SD).

2.10. Effectiveness and Stability of the vB_STM-2 Phage in Food Preservation

Short-term assay: one of the two groups inoculated with bacteria from the first section of groups was treated with a phage suspension (8 $\log_{10}$ PFU/cm^2) (inoculated, treated group), while the second inoculated group remained without treatment (inoculated, un-treated group). The remaining uninfected third group was not treated (non-inoculated, un-treated group). Squares of the three groups were placed independently in sterile Petri dishes, covered with sterile plastic rubber, and kept at 4 °C. Colonies of *Salmonella* ($\log_{10}$ CFU/cm^2) and titers of phage (PFU/cm^2) were counted at days 0, 1, 2, 4, and 7 of the storage period to judge the ability of the phage to inhibit *Salmonella*, as well as its stability.

Long-term assay: as in the previous experiment, groups of chicken breasts were treated in the same way here but were kept for 27 days at −20 °C. In addition, both the counting of the *Salmonella* colonies and titers of the phage were carried out at days 0, 2, 7, 14, 21, and 27 of the storage period.

2.11. Recovery of Inoculated Salmonella from Food

On the days specified in both experiments to check the chicken breast squares, they were taken out and left for appropriately an hour at 37 °C. Each sample was placed into a sterile bag containing 2 mL sterile phosphate-buffered saline (PBS). The sample was homogenized by a sterile flattened rod and vortexed [59]. The homogenized sample was centrifuged for 10 min at 3000× *g* [60], the supernatant was discarded to avoid plating the bacteriophage. A pellet containing precipitated bacteria was mixed with sterile peptone water (PW) and then serially diluted (10-fold). Then, 100 µL was spread over TSA and xylose lysine desoxycholate (XLD; Oxoid) agar plates from each dilution. For a day or two, the plates were incubated at 37 °C. After incubation, the suspected *Salmonella* colonies were counted, and the biochemical/serological tests were performed [35,41].

2.12. Statistical Analysis

All experiments were performed in triplicate with standard deviation (±SD), where three independent samples/assays were taken in each replicate. For bacteria and the phage, the data were converted into $\log_{10}$ form. Statistical Package for the Social Sciences version 26.00 (SPSS) was the statistical program used at a probability level of 0.05. One-way ANOVA was used to achieve quantitative analyses with the least significant difference (LSD) test variance analysis. Graphs were produced with GraphPad Prism 8.

3. Results

3.1. Bacterial Isolates, Identification, Antibiotic-Resistant and Biofilm Behavior

Suspect *Salmonella* Typhimurium colonies were 1–2 mm in diameter with an off-white color and circular shape in the TSA plates. The colonies appeared to be red with black centers in some, with 2–3 in some diameter on the XLD plates. The biochemically identified cells with the oxidase, Indole, and Vogues–Proskauer (VP) tests were Gram-negative type rods, with negative interactions. The biochemical tests that showed positive results were urease, methyl red (MR), and a reduction of nitrate to nitrite. The slide agglutination test used to determine the serotype level of *Salmonella* showed that all isolates were given a positive reaction with both antisera of O (poly O) and polyvalent H (poly H). In the affirmative identification by the Biomerieux VITEK 2 system, the results of the 23 isolates were all *Salmonella* Typhimurium. The antibiotic sensitivity profile test of *Salmonella* Typhimurium showed at least six antibiotics (ampicillin, ciprofloxacin, flucloxacillin, gentamicin, erythromycin, and aztreonam) of different groups, to which all isolates were resistant. Additionally, the 23 *Salmonella* Typhimurium isolates showed different abilities to form the biofilm, as 10 (43.47%) isolates were strong in their production, seven (30.4%) were of moderate capacity, and the remaining six (26%) isolates showed a weak ability to form it.

3.2. Most Potent Lytic Phages Isolation and Characterization

Using *Salmonella* Typhimurium (code; ST-4) as a host, seven phages were isolated from sewage water samples. Although the seven isolated phages can lyse their host with different sizes and shapes of the plaques, only three (42.8%) phages showed clear lytic plaques. The three phages with clear plaques were named vB_STS-1, vB_STM-2, and vB_STS-3, and they were the only ones that were purified and propagated (titers ranged from 10^7 to 10^{11} PFU/mL). Isolate vB_STM-2 was chosen as the most potent lytic phage, based on the clarity of the plaques. The plaques of the vB_STM-2 phage appeared clear, large in size (approximately $\simeq$ 4.5 mm), and semi-regular circular in shape (Figure 1A). Morphotype characters of the three phages were examined, and the TEM images showed that both vB_STS-1 (Figure 1B) and vB_STS-3 (Figure 1D) were members of the *Siphoviridae* family. Siphoviruses had an icosahedral head that measures approximately 61.32 nm for vB_STS-1 and 47.62 nm in diameter. Both phages contained a long, non-contractile tail with a length of 167.21 nm for vB_STS-1 and 279.36 nm for vB_STS-3. On the other hand, the vB_STM-2 phage was classified as a myovirus, where it belongs to the *Myoviridae* family, per the criteria established by the International Committee on Taxonomy of Viruses. The TEM image of the vB_STM-2 phage (Figure 1C) showed that the phage contained a round head in shape with isometric symmetry (51.27 nm in diameter) and a contractile sheathed tail (94.42 nm in length).

3.3. Selection of a Broad Host Spectrum and Polyvalent Phages

The host range of three lytic phages (vB_STS-1, vB_STM-2, and vB_STS-3) was determined by spot test and EOP to confirm their host range (Table 2). However, the vB_STS-1 and vB_STS-3 phages could lyse most of the *Salmonella* Typhimurium isolates, and they could not infect any other bacterial strains. The results indicated that vB_STS-1 lysed 65% (15/23) of *Salmonella* isolates. However, it did not show high EOP except in 5 out of 15 positive spot-tests. The vB_STS-3 phage showed a positive spot test with only 52% (12/23) of *Salmonella*, with high EOP in two isolates. Phage vB_STM-2 showed important results, in which all *Salmonella* Typhimurium isolates were lysed, with high EOP in 17 out of 23 *Salmonella* isolates, plus three non-*Salmonella* strains. Interestingly, the vB_STM-2 phage showed a polyvalent behavior, as it could infect non-*Salmonella* strains, including *P. aeruginosa*, *S. aureus*, and *E. coli*, while *K. pneumoniae* was unable to be infected. Due to the strong lytic ability of the vB_STM-2 phage, polyvalent behavior was chosen to complete the study and the *Salmonella* challenge experiments.

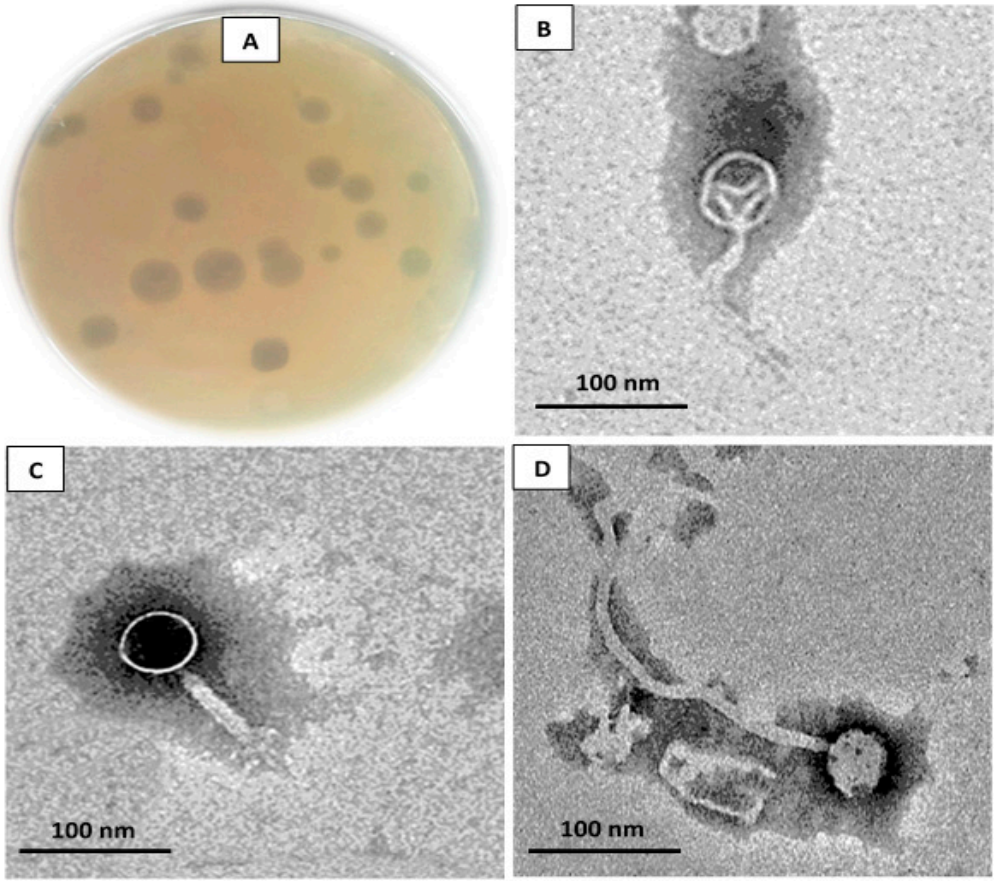

Figure 1. *Salmonella* Typhimurium phage characterization. (**A**) Clear, large circular plaques (≃4.5 mm in diameter) were produced on the double-layered agar plate. Electron micrographs at 80,000× of (**B**) vB_STS-1 phage (Siphovirus), (**C**) vB_STM-2 phage (Myovirus) and (**D**) vB_STS-3 phage (Siphovirus).

3.4. Thermo and pH Stability of the vB_STM-2 Phage

The thermal and pH stability of the vB_STM-2 phage was determined to identify the extent of the phage's resistance to different temperatures (Figure 2A) and pH (Figure 2B).

Thermal stability: the vB_STM-2 phage showed resistance at measured temperatures from −20 to 55 °C up to 24 h. The original activity of the phage was also not adversely affected at 65 °C, where insignificant ($p < 0.05$) titer reduction was observed at 1.89 ± 0.21 and 2.78 ± 0.33 $\log_{10}$ PFU/mL after 12 and 24 h, respectively. In contrast, a temperature of 65 °C negatively affected the phage significantly ($p < 0.05$) when incubated for 7 days, and the titer reduction was 4.59 ± 0.36 $\log_{10}$ PFU/mL. Furthermore, a negative effect on the phage titers was observed at 75 °C after 7 days of incubation, whereas 6.01 ± 0.43 $\log_{10}$ PFU/mL was reduced from the initial phage titers (7 $\log_{10}$ PFU/mL).

pH stability: Similarly, the vB_STM-2 phage seemed extremely stable at different pH levels from 4 to 11 after incubation times from 1 to 12 hrs. At other time points, the experiment showed that the vB_STM-2 phage was more sensitive to the alkaline than the acidic medium. At an acidic pH of 4 the phage titers were insignificantly ($p < 0.05$) reduced by 0.26 ± 0.1 and 0.34 ± 0.03 $\log_{10}$ PFU/mL after 24 h and 7 days. At a pH of 11, the phage lost 3.13 ± 0.41 $\log_{10}$ PFU/mL of infective ability after 24 h, while the titers were collapsed by 6.18 ± 0.47 $\log_{10}$ PFU/mL after 7 days.

3.5. Biofilm Removal Effect of the vB_STM-2 Phage

The effectiveness of the vB_STM-2 phage against the strongest biofilm-producing *Salmonella* Typhimurium (ST-4, ST-19, ST-30, ST-37, ST-45, and ST-49), as listed in Table 1,

was quantitively determined (Figure 3G). *Salmonella* Typhimurium ST-4 (host strain) biofilm (0.979 OD_{570} nm) was removed by 81.2%, 70.5%, 61% and 34.7%, respectively, at 10^6, 10^5, 10^4, 10^3 and 10^2 PFU/mL of phage titers, with no effect on cell growth (1.847 OD_{620} nm), (Figure 3A). Treatment of *Salmonella* Typhimurium ST-19 resulted in a reduction in biofilm (1.081 OD_{570} nm) by 76.4%, 60.3%, and 10% with respective titers of 10^6, 10^5, and 10^4 PFU/mL, while the cell growth remained stable (1.977 OD_{620} nm) (Figure 3B). The antibiofilm effect of the vB_STM-2 phage on both *Salmonella* Typhimurium ST-30 and *Salmonella* Typhimurium ST-37 was closely related, whereas at titers of 10^6, 10^5, and 10^4 PFU/mL, the biofilm was disrupted by 43.6%, 21.2%, and 10.1%, respectively, for *Salmonella* Typhimurium ST-30 (0.904 OD_{570} nm) and by 41%, 15.7%, and 9.6%, respectively, for *Salmonella* Typhimurium ST-37 (1.105 OD_{570} nm), (Figure 3C,D). For each previous bacterium, no effect on cell growth was observed at the previously used phage titer concentration. Phage vB_STM-2 exhibited moderate to weak strength in the biofilm (0.817 OD_{570} nm) removal of *Salmonella* Typhimurium ST-45. The maximum effect reached only 39.8% at a 10^6 PFU/mL concentration, while it decreased to 28.2% and 15.4% at 10^5 and 10^4 PFU/mL, respectively (Figure 3E). Phage vB_STM-2 showed its strongest biofilm removal activity when applied with *Salmonella* Typhimurium ST-49, and the cell growth (1.811 OD_{620} nm) remained the same. The biofilm (0.927 OD_{570} nm) of this bacterium was disrupted by 93.4%, 81%, 73.6% and 59.4% at 10^6, 10^5 and 10^4 PFU/mL of phage titers, respectively (Figure 3F).

Table 2. Spot test and EOP by the vB_STS-1, vB_STM-2, and vB_STS-3 phages against 23 *Salmonella* Typhimurium serovars and other bacterial strains.

Bacteria	Phages						Bacteria	Phages					
	vB_STS-1		vB_STM-2		vB_STS-3			vB_STS-1		vB_STM-2		vB_STS-3	
	ST	EOP	ST	EOP	ST	EOP		ST	EOP	ST	EOP	ST	EOP
Salmonella Typhimurium (ST-4) Host	+	**M**	+	**H**	+	**H**	*Salmonella* Typhimurium ST-47	+	**L**	+	**H**	+	**L**
Salmonella Typhimurium (ST-7)	+	**H**	+	**H**	+	**M**	*Salmonella* Typhimurium ST-49	—	**N**	+	**M**	—	**N**
Salmonella Typhimurium (ST-9)	—	**N**	+	**M**	—	**N**	*Salmonella* Typhimurium (ST-53)	+	**H**	+	**H**	+	**M**
Salmonella Typhimurium (ST-14)	—	**N**	+	**H**	—	**N**	*Salmonella* Typhimurium (ST-55)	—	**N**	+	**M**	—	**N**
Salmonella Typhimurium (ST-16)	+	**H**	+	**H**	+	**M**	*Salmonella* Typhimurium (ST-56)	+	**H**	+	**H**	+	**M**
Salmonella Typhimurium (ST-19)	+	**M**	+	**H**	+	**M**	*P. aeruginosa* (PsaCI-1)	—	**N**	+	**M**	—	**N**
Salmonella Typhimurium (ST-21)	—	**N**	+	**M**	+	**M**	*P. aeruginosa* (PsaCI-2)	—	**N**	+	**M**	—	**N**
Salmonella Typhimurium (ST-22)	—	**N**	+	**H**	—	**N**	*P. aeruginosa* (PsaFI-1)	—	**N**	+	**H**	—	**N**
Salmonella Typhimurium (ST-27)	+	**M**	+	**H**	+	**M**	*P. aeruginosa* (PsaFI-2)	—	**N**	+	**H**	—	**N**

Table 2. *Cont.*

Bacteria	Phages						Bacteria	Phages					
	vB_STS-1		vB_STM-2		vB_STS-3			vB_STS-1		vB_STM-2		vB_STS-3	
	ST	EOP	ST	EOP	ST	EOP		ST	EOP	ST	EOP	ST	EOP
Salmonella Typhimurium (ST-30)	+	**H**	+	**H**	—	**N**	*P. aeruginosa* (PsaFI-1)	—	**N**	+	**H**	—	**N**
Salmonella Typhimurium (ST-33)	—	**N**	+	**M**	—	**N**	*S. aureus* (SaFI-1)	—	**N**	+	**L**	—	**N**
Salmonella Typhimurium (ST-35)	+	**H**	+	**H**	+	**H**	*S. aureus* (SaFI-2)	—	**N**	+	**M**	—	**N**
Salmonella Typhimurium (ST-36)	—	**N**	+	**M**	—	**N**	*S. aureus* (SaFI-3)	—	**N**	+	**L**	—	**N**
Salmonella Typhimurium (ST-37)	+	**M**	+	**H**	—	**N**	*E. coli* (EcCI-1)	—	**N**	+	**M**	—	**N**
Salmonella Typhimurium (ST-38)	+	**M**	+	**H**	+	**M**	*E. coli* (EcCI-2)	—	**N**	+	**M**	—	**N**
Salmonella Typhimurium (ST-41)	+	**L**	+	**H**	—	**N**	*E. coli* (EcCI-3)	—	**N**	+	**M**	—	**N**
Salmonella Typhimurium (ST-43)	+	**L**	+	**H**	—	**N**	*E. coli* (EcCI-4)	—	**N**	+	**M**	—	**N**
Salmonella Typhimurium (ST-45)	+	**M**	+	**H**	+	**L**	*K. pneumoniae* (KpCI-1)	—	**N**	—	**N**	—	**N**

The brackets inside the bacterial column in the table indicate the isolate ID, ST: spot-assay technique, +: positive reaction (the isolate caused lysis by the phage), −: negative reaction (the isolate does not cause lysis by the phage), EOP; efficiency of plating, H: high efficiency of plating (0.5–1.0), M: moderate efficiency of plating (0.2–0.4), L: low efficiency of plating (0.001–0.1), N: no efficiency of plating (inefficient) ($p < 0.05$).

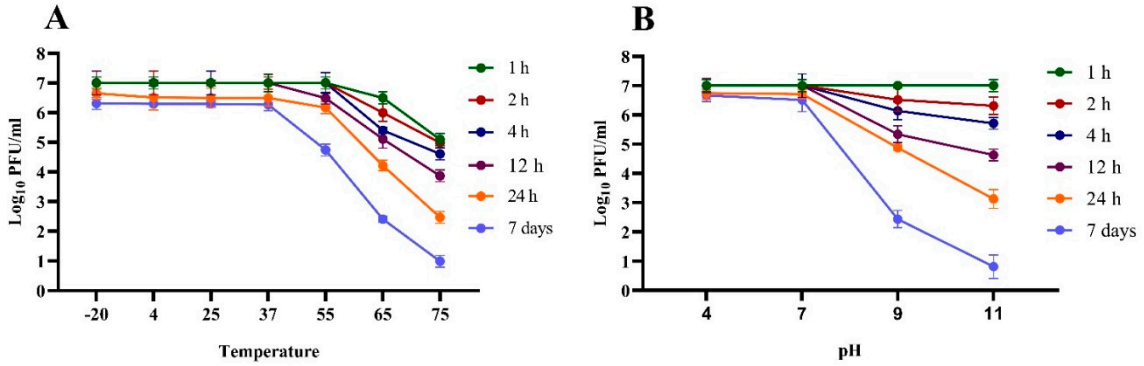

Figure 2. Virion stability of the vB_STM-2 phage at different times (1, 2, 4, 12, 24 h and 7 days) (**A**) Thermal tolerance at (−20, 4, 25, 37, 55, 65 and 75 °C, and (**B**) pH values of 4, 7, 9 and 11. The two experiments were performed in triplicate with standard deviation (±SD). One-way ANOVA was used to achieve quantitative analyses with the least significant difference (LSD) test variance analysis at $p < 0.05$.

3.6. The Ability of the vB_STM-2 Phage as a Natural Antibacterial
Lytic Potency in TSB Medium (Culture Clearance)

The results illustrated in Figure 4A clearly show the efficiency of the vB_STM-2 phage in controlling *Salmonella* in a liquid medium. The optical density (OD_{620} nm) of *Salmonella* Typhimurium culture ST-4 showed 1.65 ± 0.31 OD_{570} nm at the end of the experiment. On the other hand, the phage-treated culture flask showed no absorbance at any measurement times, except at the first hour (0.10 ± 0.01 O_{D570} nm) of incubation. In addition, the results obtained from counting the plates confirm the lytic ability of the vB_STM-2 phage. At 3 h of incubation, the colony count was 0.19 ± 0.03 $\log_{10}$ CFU/mL, while no colonies were detected at other times of the experiment (B).

3.7. Control of Salmonella in Food and Phage Stability

In short (7 days) and long (27 days) term assays, both the efficiency and stability of the vB_STM-2 phage were measured in food (chicken breast squares 2 × 2 cm).

Short-term assay: the recoverable $\log_{10}$ of *Salmonella* Typhimurium (ST-4) in non-treated phage sections was significantly ($p < 0.05$) increased by increasing the time. Therefore, the titer was 0.81 ± 0.11 $\log_{10}$ CFU/cm^2 on day one, 3.27 ± 0.24 $\log_{10}$ CFU/cm^2 on day two, 4.55 ± 0.37 $\log_{10}$ CFU/cm^2 on day four, and 7.00 ± 0.63 on the last day. Otherwise, the recoverable $\log_{10}$ of *Salmonella* Typhimurium (ST-4) from phage-treated chicken breast cuts was significantly ($p < 0.05$) decreased, and it was only 0.88 ± 0.17 $\log_{10}$ CFU/cm^2 on day 7. In other words, the vB_STM-2 phage reduced *Salmonella* by 6.12 ± 43 $\log_{10}$ CFU/cm^2 on day 7. Nevertheless, the phage showed high stability throughout the experiment times with a recovery titer of 6.87 ± 43 $\log_{10}$ PFU/cm^2 on day 7, losing only 1.13 ± 0.16 $\log_{10}$ PFU/cm^2 from the initial titer (Figure 5A).

Long-term assay: In this experiment, the duration was increased to 27 days in order to ensure the effectiveness and stability of the vB_STM-2 phage. On days 2 and 27, the $\log_{10}$ recovery of *Salmonella* from the non-treated chicken breast section was 0.92 ± 0.08 $\log_{10}$ CFU/cm^2 and 5.13 ± 0.44 $\log_{10}$ CFU/cm^2, respectively. In contrast, there was a highly significant decrease ($p < 0.05$) in the count of *Salmonella* recovered from chicken breast cuts treated with phage. At the same two previous times, only 0.11 ± 0.02 $\log_{10}$ CFU/cm^2 was recovered on day 2, while 1.10 ± 0.12 $\log_{10}$ CFU/cm^2 was recovered on day 27. It is worth noting that the vB_STM-2 phage showed a high stability strength, whereas there was a slight shortage of titers at all times, even on the last day. Accordingly, on day 27, the recovered phage titers were 5.88 ± 0.41 $\log_{10}$ PFU/cm^2, and only 2.12 ± 0.22 $\log_{10}$ PFU/cm^2 (insignificant) of the original titers were lost (Figure 5B).

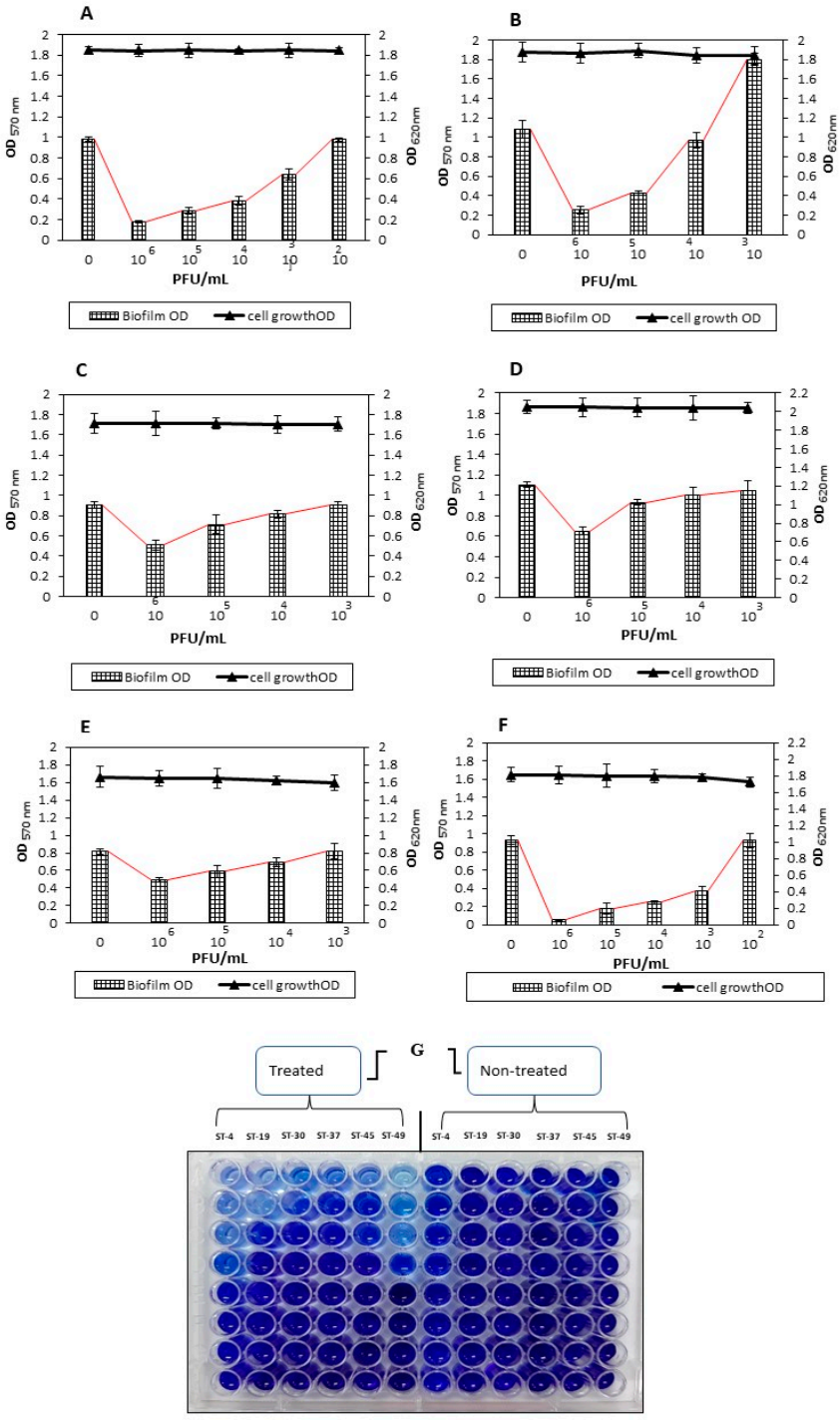

Figure 3. The qualitative anti-biofilm capacity of the vB_STM-2 phage in 96-well microplate against biofilms of *Salmonella* Typhimurium isolates (**A**) ST-4, (**B**) ST-19, (**C**) ST-30, (**D**) ST-37, (**E**) ST-45, (**F**) ST-49 and (**G**) 96-wells of tissue culture plates. The vertical wells of the plate on the left are the antibiofilm (phage-treated) wells, while those on the right re the biofilm controls (without adding phage) for these bacteria. One-way ANOVA was used to achieve quantitative analyses with the least significant difference (LSD) test variance analysis at $p < 0.05$.

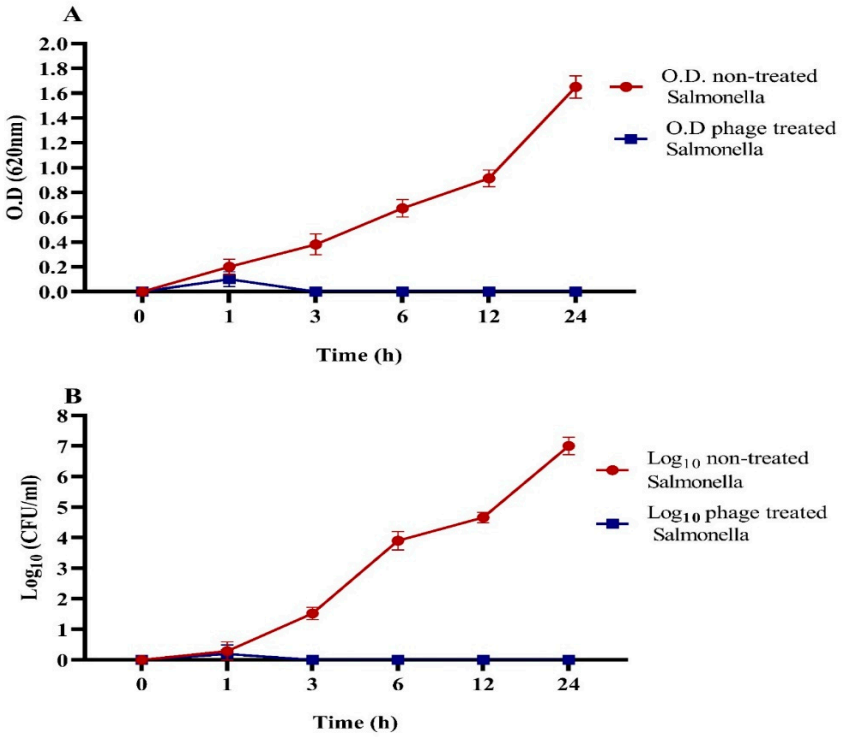

Figure 4. The lytic ability of the vB_STM-2 phage in the challenge of *Salmonella* Typhimurium ST-4 serovar in (**A**) TSB medium (absorbance of OD_{620} nm) and (**B**) TSA medium (colonies count CFU/mL). Experiments were performed in triplicate at each time point with a standard deviation (±SD). One-way ANOVA was used to achieve quantitative analyses with the least significant difference (LSD) test variance analysis at $p < 0.05$.

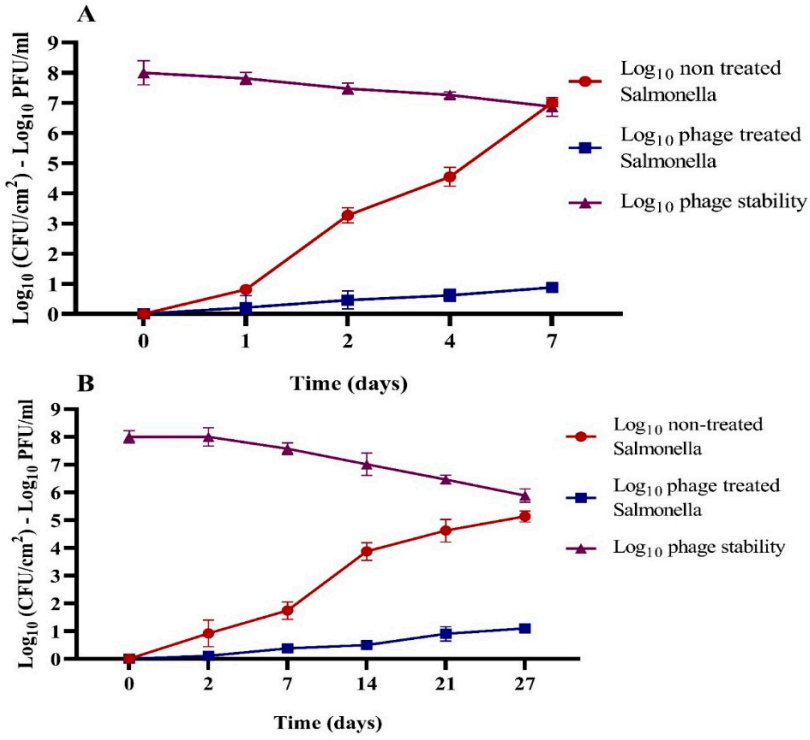

Figure 5. Efficacy and virion stability of the vB_STM-2 phage in eliminating *Salmonella* Typhimurium ST-4 serovar after (**A**) 7 days at 4 °C, and (**B**) long-term assay (27 days) at −20 °C. One-way ANOVA was used to achieve quantitative analyses with the least significant difference (LSD) test variance analysis at $p < 0.05$.

4. Discussion

The emergency of multiple-drug resistant (MDR) bacteria in food has become a major and complex problem for both producers and consumers, posing a serious threat to human public health [17,61,62]. For example, *Salmonella* Typhimurium has become the most frequent serovar primarily responsible for salmonellosis in humans worldwide [7,8]. Antibiotics are a well-known strategy to control *Salmonella* [1], but it has acquired resistance to these antimicrobials [17], making it more difficult to resist disease outbreaks [63].

Therefore, this study aimed to search for a safe and effective alternative to control multidrug-resistant *Salmonella* Typhimurium and its biofilm by broad-spectrum phage isolation with the string lytic effect.

In this study, *Salmonella* Typhimurium serovars previously isolated from food sources were confirmed biochemically, serologically, and using the Biomerieux VITEK 2 system. Additionally, all *Salmonella* Typhimurium serovars used were multi-drug resistant, and nearly half showed strong biofilm production behavior. Many previous studies are in line with our results, where it was found that *Salmonella* Typhimurium, *S. enterica*, and *S. enteritidis* serovars are the most prevalent for the occurrence of salmonellosis outbreaks worldwide [8,64]. Many other studies have also confirmed that the effect of the front-line antibiotics used to arrest *Salmonella* have become weak or negligible due to bacteria gaining resistance [65–67]. In accordance with previous results, antibiotic-resistant genes were common in *Salmonella* Typhimurium [68]. The European Food Safety Authority (EFSA) announcement that 64.2% of 123 *Salmonella* Typhimurium strains obtained from pig carcasses were resistant to many antibiotics is concerning [69]. It is worth noting that bacteria may acquire this resistance due to incorrect use, including a lack of need for antibiotics in many cases [70–72]. The mechanism by which bacteria can resist the action of antibiotics may be the over-expression of efflux pumps that lead to the active efflux of antibiotics from the bacterial cell and restrict the antibiotics to their target sites by decreasing the selective permeability of the bacterial cell wall. In addition, bacteria may produce target enzymes to circumvent the drug's antibacterial activity [73–75]. *Salmonella* Typhimurium (ST-4) was used to isolate phage, as it is the strongest isolate for biofilm production. More than one study states that biofilm is a rising challenge as it gives the pathogen more resistance to antibiotics. Hence, it is difficult to eradicate [76–78]. Out of the seven isolated phages, only three (42.8%) phages showed clear lytic plaques, which were named vB_STS-1, vB_STM-2, and vB_STS-3. Among the three isolates, vB_STM-2 belonging to the *Myoviridae* family was chosen to achieve the desired goal of this study (most potent lytic phage). The results of the lytic activity indicated that 100% of *Salmonella* Typhimurium isolates and non-*Salmonella* strains were lysed, including *P. aeruginosa, S. aureus*, and *E. coli*. These results align with previous findings in which phages have been developed to deal with MDR *Salmonella* Typhimurium [32,79]. These findings are in accordance with previous works by Park et al. [26]. Parra and Robeson [27] reported that some polyvalent phages were observed, especially among the phages of the *Enterobacteriaceae* family. In addition, the study by Malki et al. [28] showed that phages with polyvalent behavior could infect strains from either different genera or species, which is preferable to others. In addition to controlling bacterial resistance by phages, there is recent literature to overcome the problem of bacterial resistance using metal-based antibacterial compounds instead of conventional antibiotics [80].

The previous literature indicated that the *Myoviridae* family's phages could potentially control *Salmonella* [81,82].

Here, vB_STM-2 showed a broad host range of other phages, which is an important charitarian of the use of phages in the treatment of infection, as reported by previous studies [83,84]. In addition, the phage's polyvalent behavior makes it a preferred choice for use in biocontrol applications [85–87].

The differences between phages in their host range may be due to restriction endonucleases changes, non-specific binding receptors [88,89], and the formation of resis-

tance to phage through a variety of mechanisms, known as insensitive bacterial mutants (BIM) [90,91].

In the phage thermal and pH stability study, the vB_STM-2 phage showed durability over a wide range of temperatures and at different pH levels. Consistent with previous studies, phages are stable at various temperatures, pHs, and biotic conditions [92,93]. Additionally, our findings are in agreement with those of Jamalludeen et al. [53]. They decided that most of the phages could resist different pH levels (5–9) without affecting their stability or structure. However, extreme degrees may lead to thickening, deposition, or aggregation of the phages [94].

Our data imply that the vB_STM-2 phage can diminish or eradicate the tested *Salmonella* Typhimurium (ST-4, ST-19, ST-30, ST-37, ST-45, and ST-49) biofilms. In a previous work [82] on *Salmonella* Typhimurium biofilm, the phage used was able to reduce the biofilm by 44–63%. Here, the removal rate reached 93.4% (e.g., *Salmonella* Typhimurium ST-49). Concurrent studies confirm the ability of phages to eradicate biofilms in vitro assays [82,95,96]. In a previous study, the biofilms of multi-drug resistant bacteria were overcome by natural antimicrobial agents [86,97]. In an attempt to find an explanation for how the phage attacks the biofilm, it may be due to the phage's ability to infect cells embedded in biofilm throughout their environment [89]. In addition, the high number of cells within the biofilm protect the phage virions from infection [89]. The mechanism of this action is the penetration of the EPS layers of biofilms, which become less hard [98], less consistent [99,100], or no longer compact [101], and the production of EPS depolymerizes, which can break down the biofilm matrix [102,103].

Our results indicated that the vB_STM-2 phage showed efficiency in inhibiting *Salmonella* in a phage-treated culture flask (culture clearance). In a liquid medium, phage-treated *Salmonella* culture showed no absorbance after 3 h of incubation. Following our results, other researchers isolated phages named FGCSSa1, LPST10, LPST18, and LPST23 against *Salmonella* Typhimurium PT160, which could inhibit growth from growth 2 to 6 hrs [104].

The phage application results showed the efficacy and stability of the vB_STM-2 phage in the elimination of *Salmonella* Typhimurium (ST-4) from the chicken breast section with significant ($p < 0.05$) virion stability. In addition, the severe *Salmonella* count declined in both the short-term (7 days) and long-term (27 days) assays. Hence, the decrease was from 7 ± 0.63 $\log_{10}$ CFU/cm^2 to 0.88 ± 0.17 $\log_{10}$ CFU/cm^2 on the seventh day of the short assay, while the count was diminished significantly ($p < 0.05$) from 5.13 ± 0.44 $\log_{10}$ CFU/cm^2 to 1.1 ± 0.12 $\log_{10}$ CFU/cm^2 on day 27 of the long-term assay. Interestingly, phage titers remained stable in both periods, with slight insignificant ($p < 0.05$) losses. Many previous studies on *Salmonella* have observed that phages could reduce counts in different food matrices [59,105–107]. Consistent with our data, a recent study exhibited the stability of phages against *Salmonella* in chicken breast [82].

5. Conclusions

We can conclude from the obtained results that three phages that showed clear lytic plaques, namely, vB_STS-1, vB_STM-2, and vB_STS-3, were isolated, the most potent of which was vB_STM-2, which belongs to the *Myoviridae* family. This phage exhibited strong stability at different temperatures and a wide range of pH values. The isolated phage showed high efficacy in reducing *Salmonella* Typhimurium below the detectable limit throughout liquid media and chicken breast challenges. Additionally, it showed a potential effect in the elimination/reduction of its biofilm. Therefore, this phage is considered a prime candidate for combating multi-drug-resistant *Salmonella* Typhimurium and its biofilms.

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H.E.S. and M.A.A.; validation, I.M.A.A., A.R.S., A.A.H., E.E.R., H.E.S. and M.A.A.; visualization, I.M.A.A., A.R.S., A.A.H., E.E.R., H.E.S. and M.A.A.; writing—original draft, I.M.A.A., A.R.S. and A.A.H.; writing—review and editing, I.M.A.A., A.R.S., A.A.H., E.E.R., H.E.S. and M.A.A. All authors have read and agreed to the published version of the manuscript.

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