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Physiological Profiling of Lytic Bacteriophages and their Efficiency against *Salmonella* Enteritidis on Chicken Breast Cuts

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ABSTRACT

Worldwide, antibiotic-resistant *Salmonella* Enteritidis being accountable for major foodborne *Salmonella* outbreaks, is a significant concern for public health and the agro-food sector. The use of bacteriophages, as a potential replacement, for antibiotics and as food antimicrobial agents to potentially target foodborne pathogens is gaining augmented attention. This study's goal was to isolate and physiologically characterize lytic bacteriophages against Enteritidis, and to ascertain their efficacy in modulating bacterial population on chicken breast cuts in the form of a phage cocktail. Out of 17 isolated bacteriophages, three bacteriophages (SEPL01, SEPL13, SEPL20) exhibited maximum lytic potential and broad lytic spectrum. Transmission electron microscopy analysis classified SEPL01 as a Siphovirus and SEPL13 and SEPL20 as Myoviruses. In liquid culture, phage virulence activity was rapid and high at a high (100) multiplicity of infection with phage SEPL01 depicting the best activity among all. Compared to the solophage suspensions at high MOI, the phage cocktail comprising all three phages did not let the bacteria regrow until 24 h overcoming bacterial resistance. All the three bacteriophages demonstrated a broad host range within the genus *Salmonella* (Enteritidis, Typhimurium, Gallinarum, and Pullorum) embracing 100 percent activity with no heterologous activity against non-*Salmonella* serotypes tested. Latent time period of 15, 15, and 20 min was documented for SEPL01, SEPL13, and SEPL20, respectively, with an average burst size of 110, 32, and 63 PFU CFU^{-1}, respectively. All the three bacteriophages were tolerant of temperature range 4°C-70°C and pH between 3-12, thus providing a broader window of their application throughout the food chain. On chicken breast cuts, phage mix efficiently supported sustained (3 days) bacterial reduction of 1.47 log units and 2.72 log units at MOI 100 and 10,000, respectively with greater MOI providing greater reduction. For standardizing the use of bacteriophages, physiological characterization is a crucial constraint. Owing to the strong lytic regime and spectrum, bacteriophages proved to be ideal for their use as an alternative or addition to conventional antimicrobials used in the food industry.

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AAS and MR conceptualized the idea. AK, AAS and RHD designed methodology. AK and MIR did formal analysis. AK and SR curated data. AK and SR did statistical analysis. AK, RHD, AAS, and MIR did original draft preparation. AAS, MR, WS and SR reviewed the manuscript.

Key words
Bacteriophages, *Salmonella* Enteritidis, Phage cocktail, Virulence, Chicken breast cuts

INTRODUCTION

Salmonella is a globally important zoonotic pathogen that has been associated with foodborne illnesses by the consumption of contaminated products of both animal origin (poultry meat, eggs, dairy, etc.) and of non-animal origin (fruits, fresh vegetables, etc.) (Paudyal *et al*., 2017). According to the centre for disease Control, *Salmonella* is responsible for more foodborne illnesses than any other pathogen (CDC, 2021). The non-typhoidal *Salmonella* species including Enteritidis are a leading bacterial cause of acute gastroenteritis both in children under 5 years and in the general population (Majowicz *et al*., 2010). The non-typhoidal *Salmonella* has been reported to cause 93 million cases of acute gastroenteritis annually across the globe (Islam *et al*., 2020). The scenario gets further complicated with the rise in antimicrobial resistance (AMR) a global phenomenon driven by antimicrobial use (AMU). The global stats suggested that AMU in food-producing animals far outweighs its consumption in human medicine (Eng *et al*., 2015; Mohsin *et al*., 2019). Mounting evidence suggested that the imprudent prophylactic use of antibiotics in poultry is common veterinary practice in countries with limited resources, to overcome managemental deficiencies

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or simply to improve growth efficiency (Mohsin *et al*., 2019).

Not surprisingly, growing concern about the consumer's perspective on the quality and safety of the poultry products is a continuous issue for the strategic future of poultry industry (Hafez and Attia, 2020). Regardless of better preventive and control procedures placed at various levels of food production from farm to fork, foodborne Salmonellosis is still a major threat. Different physical, chemical, and biological interventions implanted to enhance food safety despite having effective outcomes pose major limitations. Moreover, unrestrained use of antimicrobials has also lead to bacterial resistance against many antimicrobials (Gorissen *et al*., 2015; Milbradt *et al*., 2014; Moye *et al*., 2018). So, it is very imperative to delve into new effective, environmentally friendly ways to manage *Salmonella* spp. throughout the food chain. There is a renewed interest in the application of lytic bacteriophages and phage-derived proteins in modern veterinary medicine to combat bacterial pathogens (Atterbury and Barrow, 2021; Fernández *et al*., 2018; Żbikowska *et al*., 2020).

Our study was intended to isolate lytic bacteriophages from sewage water and to study their physiological properties including growth kinetics, virulence ability, lytic spectra, and thermal and pH tolerance to characterize them for optimal use. Additionally evaluated the efficiency of phage mix in reducing Enteritidis number on chicken breast cuts.

MATERIALS AND METHODS

Bacterial strain

For this study, the reference poultry field isolate of *Salmonella enterica* subsp. *enterica* serovar Enteritidis used to isolate bacteriophages (procured from *Salmonella* culture collection under the Punjab Agriculture Research Board project at the Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan) was reconfirmed by traditional biochemical practices (Nair *et al*., 2015) followed by serotyping using specific antisera in accordance with Kauffman n-White Scheme (Grimont and Well, 2007; ISO 6579-3, 2014) and finally reassessed by a PCR based test targeting a *Salmonella* difference region (Sdf I), highly specific for serotype Enteritidis (Mir *et al*., 2015; Sadiq *et al*., 2020).

Bacteriophage isolation and screening

For isolation of bacteriophages a total 30 sewage water samples collected from the poultry farms situated in District Lahore were centrifuged at 9000 x g for 15 min to settle down major debris in them, and then filtered using a 0.2 µm PVDF syringe filter the overnight grown culture of *Salmonella* Enteritidis was mixed in equal 1:1 volume in 1X tryptone soya broth (CM0129 Oxoid, UK) and incubated at 37°C overnight with shaking at 120 RPM followed by centrifugation at 9000 x g for 15 min and filtration through 0.2 µm PVDF syringe filters. Samples were screened for the presence of bacteriophages using spot assay by spotting 10 µL volume over bacterial lawn plates. After overnight incubation at 37°C plates were screened for clear zones of lysis (Bao *et al*., 2011; Hungaro *et al*., 2013; Parra and Robeson, 2016).

Purification and titration of viral particles

For purification, 100 µL phage lysates were 10-fold serially diluted with 100 µL of bacterial log phase culture added to every dilution afterward followed by pouring mixtures separately along with 0.75% semi-solid soft agar onto Tryptone Soya Agar (CM0131 Oxoid, UK) plates. After overnight incubation at 37 °C, plates were analyzed for plaque morphology. Plaque with distinct morphology was purified 3 to 5 times using agar overlay method after picking plaque with the help of sterile tip cut to plaque size. Additionally supplemented with 30µl volume of 1*m*M $MgCl_2$ and $CaCl_2$ each. Titration of bacteriophages was also performed using Double Agar Overlay method (Bao *et al*., 2011; Hungaro *et al*., 2013; Parra and Robeson, 2016).

Electron microscopy of bacteriophages

Purified bacteriophages were propagated in tryptone soya broth (CM0129 Oxoid, UK) to high titer (10^{12} PFU/mL) and samples were sent to the National Institute of Biotechnology and Genetic Engineering (NIBGE), Faisalabad for analysis. At NIBGE, samples were fixed onto copper grids, washed with 0.1 M ammonium acetate solution (pH 7.0) three times, and then negatively stained with 5% uranyl acetate. The observation was accomplished under JEOL JEM 1010 Transmission Electron Microscope (100 kV).

In vitro *experiment to check phage virulence at different multiplicity of infection in liquid medium*

For this assay, an overnight grown *S.* Enteritidis culture was diluted to get an OD of 0.2 using spectrophotometer (Specord 200 Plus, Analytikjena, Germany) at 600nm. At this stage, equal volumes 1:1 of bacteria and phage (at different Multiplicity of infection 1, 10, 100) were added to a flask containing 50ml of tryptone soya broth (CM0129 Oxoid, UK) and incubated at 120 RPM at 37°C. Positive control served as bacterial control with only bacteria inoculated (phage free). After every 2 h, up to 24 h, 2 ml of the mixture was drawn to see the variation in OD_{600}

(Hamza *et al*., 2016).

Profiling of host lytic spectrum

Isolated bacteriophages were tested for their ability to infect other related serotypes and genera (already confirmed) including *Salmonella* Pullorum (n=3), *Salmonella* Gallinarum (n=4), *Salmonella* Typhimurium (n= 4), *Salmonella* Enteritidis (n=8) procured from culture collection under PARB 680 project, *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 23235 using spot assay technique as described earlier (Atterbury *et al*., 2007; Clokie *et al*., 2009). After incubation at 37°C for 24 h, the extent of lysis was recorded.

Phage adsorption rate and single step-growth kinetics

The adsorption rate was determined following the method adopted by (Czajkowski *et al*., 2014; Sadekuzzaman *et al*., 2018) with few modifications. For that phages were added to Enteritidis culture at 100 multiplicity of infection and after shaking incubation of 10 min at 37°C the suspension was centrifuged for 5 min at 7000 x g and the filtered supernatant was evaluated for unadsorbed phages by plaque assay. Percentage of adsorbed phages was calculated as follows: Total applied phages -free phages/ total applied phages) × 100

To determine the latent time period and burst size for phages a protocol of Bao *et al*. (2011), and Hamza *et al*. (2016) was adopted with little modifications. Phage suspensions were added to Enteritidis host culture at 100 MOI for each phage separately up to a total volume of 30 mL. After shaking incubation of 10 min at 37°C the mixture was centrifuged at 7000 x g for 5 min at 4°C to separate free phages that have not been adsorbed. The pellet was then resuspended in 30 mL of tryptone soya broth (CM0129 Oxoid, UK). After every 5 min, up to 60 min samples were withdrawn and plaque assay was performed to enumerate phages and a graph was plotted between PFU against time and the latent time period and burst size were calculated from it excluding 10 min of pre-incubation.

Assessment of phage tolerance at different temperatures and pH

For assessment of phage stability at different temperatures, a method by Litt and Jaroni (2017) was adopted with minor modifications. Tolerance of isolated lytic bacteriophages was tested against different temperatures ranging from 4°C to 80°C. For that, phage suspension of known titer (8 logs PFU/mL) was placed in a water bath set at a specified temperature and at 4°C for an h and a decline in bacteriophage titer was counted using the double agar overlay method as described above. Likewise, tolerance of bacteriophages was tested against different pH ranging from 3 to 12 according to the method adopted by Dallal *et al*. (2019) with few modifications. For that, isolated bacteriophage, suspensions were added to Tryptone soya broth (CM0129 Oxoid, UK) at desired pH adjusted using HCl and NaOH for an h and change in titer of bacteriophages was tested similarly using the Agar overlay method.

Efficacy of phage cocktail against Salmonella *Enteritidis on chicken breast cuts*

Chicken breast samples bought from a local supermarket, were aseptically cut into pieces weighing approximately 30 g each. To eliminate suspected *Salmonella* and to lesson microbial load, chicken breast pieces were subjected to chlorine treatment (50 ppm solution) for 5 min (Petsong *et al*., 2019). Three pieces were randomly picked to test for presence of *Salmonella*, confirmed by standard microbiological procedures as discussed above. To check efficacy of phage cocktail, chicken breast pieces were first artificially contaminated with *S.* Enteritidis. For this, cell pellets of overnight grown *S.* Enteritidis culture were resuspended in phosphate buffer saline and *S.* Enteritidis concentration was adjusted to 10^7 CFU/mL. All the breast pieces were dipped in for 15 min and then dried in safety cabinet for 30 min for bacteria to adhere to the breast pieces. Phage cocktail was prepared by mixing all the three bacteriophages at high titer 10^{11} PFU/mL each in equal ratio (1:1:1). This cocktail stock was then used to dilute the cocktail to required concentration 10^9 PFU/mL (for 100MOI) and 10^{11} PFU/mL (for 10,000MOI).

Grouping

Chicken breast cuts were divided in to four groups, group 1 consisted of non-treated chicken breast cuts, only spiked with *S.* Enteritidis (positive control), group 2 includes chicken breast cuts with neither phage treatment nor bacterial contamination (negative control). Group 3 comprised of cuts which were first artificially contaminated with *S.* Enteritidis and then dipped in 10^9 PFU/mL (100MOI) phage cocktail solution. However, in group 4, chicken breast cuts were immersed in 10^{11} PFU/mL (10,000MOI) cocktail solution. All the chicken breast cuts were dipped in phage cocktail solution for 10 min and then placed at 4°C for 72 h.

Salmonella *titration*

To analyze effects of phage cocktail on *S.* Enteritidis reduction, after every 24 h up to 72 h, chicken breast cuts were picked in triplicates and washed in 30mL 0.9% normal saline. Washed solution was centrifuged at 9000 xg for 15 min for bacteria to settle down and to separate from phages and cell pellets were resuspended again in 0.9% normal

saline, serially diluted (10-fold) and plated on *Salmonella-Shigella* Agar (CM0099 Oxoid, UK). After incubation at 37°C for 24 h count was performed (Augustine and Bhat, 2015; Bao *et al*., 2015).

Statistical analysis

All the experiments were performed thrice, and results were described as mean values along with error bars representing standard deviation. Data achieved from food trail was arranged in Microsoft Excel spread sheets (Microsoft 365). To observe change in bacterial concentrations as a result of application of different phage concentrations on chicken breast cuts, recorded data was examined using Two-Way ANOVA along with post-hoc test (Tukey's test). Statistical significance was considered at ($P \leq 0.05$).

RESULTS

The reference strain of *Salmonella* used in this study to isolate bacteriophages was isolated from poultry and characterized as *Salmonella enterica* subsp. *enterica* serovar Enteritidis by biochemical techniques, serotyping (1,9,12:g,m:-) and PCR based test (yielded an amplicon of 304bp indicating the presence of Enteritidis specific (Sdf I region) (Data not shown).

Out of 30 sewage water samples, a total of seventeen Enteritidis phages were isolated, three phages out of them (designated as SEPL01, SEPL13, SEPL20) were selected for further use in the study. Phage SEPL20 showed medium to large-sized (ranging in diameter between 4 to 4.5 mm) clear plaques, Phages SEPL01 and EPL13 showed small size (ranging in diameter between 1 to 1.5 mm) plaques. whereas plaques showed by SEPL13 were clear and by SEPL01 had a halo around the central clear point (Fig. 1A, 1B, and 1C).

Transmission electron microscopy analysis revealed that all the three phages are tailed and belong to order caudovirales of dsDNA. Among them, phage SEPL01 belongs to the family Siphoviridae as it possesses an icosahedral head and a long noncontractile tail with a head of 143.7 nm and a tail of 293.7 nm in length (Fig. 1A). Whereas phage SEPL13 and SEPL20 had standard morphological features like the Myoviridae family having an icosahedral head and a contractile tail (Fig. 1B, 1C). Phage SEPL13 and SEPL20 heads were measured to be 192.8 and 166.6 nm and tails length was recorded to be 172.8 and 183.3 nm in length, respectively.

To analyze which dose of bacteriophage is appropriate for the maximum killing of Enteritidis, SEPL01, SEPL13 and SEPL20 were tested at MOI 1, 10, and 100. Absorbance (OD_{600}) values of control having only bacteria no phage increased rapidly throughout 24 h manifolds. Figure 2A shows the killing pattern of solophage SEPL20 which inhibited the growth of Enteritidis for 14 h after which a boost in absorbance was sighted (ranging from 0.4 to 0.7). Figure 2B shows the lytic ability of solophage SEPL13 in which bacterial suppression was observed up to 18 h with an enhance in absorbance onwards (from 0.5 to 0.6). Figure 2C shows the absorbance readings of solophage SEPL01, it shows a less steep increase in absorbance (up to 0.4) observed at the 20th h. All the three phages were further tested in form of a cocktail at MOI 100. In Figure 2D it can be seen that in form of a cocktail bacteriophages did not let bacteria regrow up till 24 h as compared to individual phages at the same MOI.

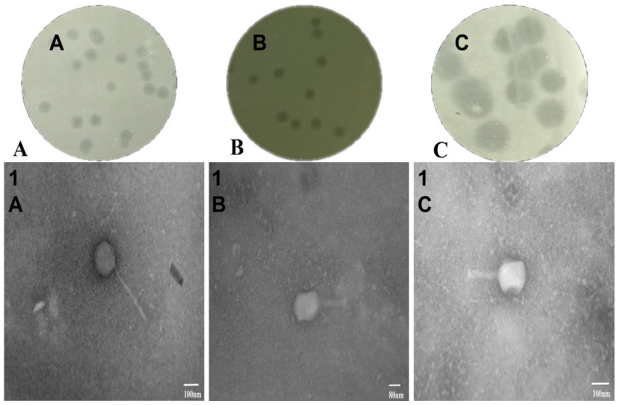

Fig. 1. Plaque morphologies of phage SEPL01 with a halo (A), SEPL13 (B) and SEPL20 (C). Electron Micrographs of phage SEPL01 (1A), SEPL13 (1B), and SEPL20 (1C).

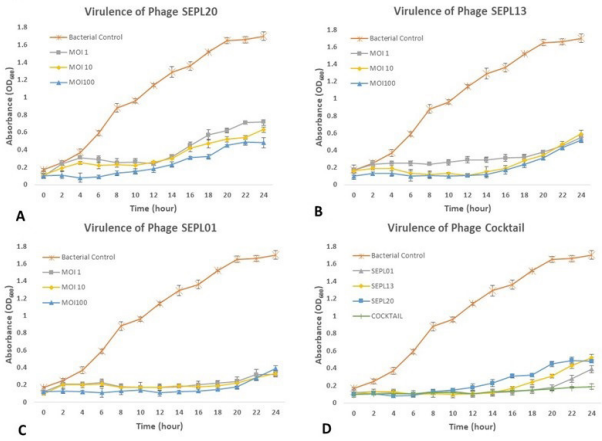

Fig. 2. Comparative virulence of bacteriophages at MOI 1, 10, and 100 (A), Virulence of Phage SEPL20 (B), Virulence of phage SEPL13 (C), Virulence of phage SEPL01 (D) Virulence of phage cocktail, All the data were mean of three independent experiments ± standard deviation.

SEPL01, SEPL13, and SEPL20 were found lytic to all four serotypes of *Salmonella* tested *viz*. Enteritidis (n=8), Typhimurium (n=4), Gallinarum (n=4), and Pullorum (n=3) with the varied extent of clearance on spot (Table I). Bacteria related to another genus including *Escherichia coli* 25922, *Staphylococcus aureus* ATCC® 23235 showed no susceptibility to any of the three phages.

Table I. The lytic spectrum of phages (SEPL01, SEPL13, SEPL20).

Bacterial strain	SEPL01	SEPL13	SEPL20
Salmonella Typhimurium PARBT02	+++	+++	+++
Salmonella Typhimurium PARBT05	+++	+++	++
Salmonella Typhimurium PARBT26	++	++	+++
Salmonella Typhimurium PARBT34	+++	++	++
Salmonella Enteritidis ATCC 13076	+++	+++	+
Salmonella Enteritidis PARBE05	Host	Host	Host
Salmonella Enteritidis PARBE09	++	+++	++
Salmonella Enteritidis PARBE13	+++	+++	+++
Salmonella Enteritidis PARBE14	+++	++	+++
Salmonella Enteritidis PARBE25	++	+++	+++
Salmonella Enteritidis PARBE27	+++	+++	++
Salmonella Enteritidis PARBE31	+++	+	+
Salmonella Pullorum SP1628	+++	++	+
Salmonella Pullorum PARBP22	++	+	+
Salmonella Pullorum PARB07	++	+	+
Salmonella Gallinarum PARBG07	+++	++	+
Salmonella Gallinarum SG1788	+++	++	++
Salmonella Gallinarum SG2812	+	++	++
Salmonella Gallinarum SG1610	+++	+++	+
Escherichia coli, ATCC 25922	-	-	-
Staphylococcus aureus ATCC 23235	-	-	-

Full clear lysis (+++), Full lysis with few resistant colonies (++), Appearance of few plaques (+), No lysis (-).

In this study, almost half of the phage particles got adsorbed to the bacterial cells within 10 min. The individual adsorption rate observed was 54%, 48%, and 56% for phage SEPL01, SEPL13, and SEPL20, respectively after 10 min. Further, single step growth curve revealed that the phage SEPL20 had an average burst size of 63 PFU CFU^{-1} with a latent time period of around 20 min shown in Figure 3A. Phage SEPL13 showed an average burst size of 32 PFU CFU^{-1} and maintained a latent state for almost 15 min shown in Figure 3B. However, phage SEPL01 had an average burst size of 110 PFU CFU^{-1} with a latent time period of around 15 min can be seen in Figure 3C. With an average latent period of 15 to 20 min, all three phages showed an average burst size of between 32 to 110 PFU CFU.

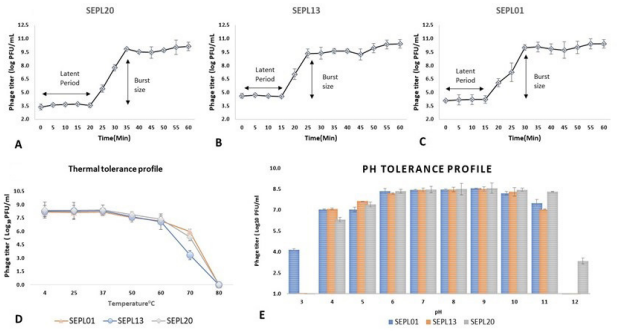

Fig. 3. Growth curves of phage SEPL20 (A), phage SEPL13 (B), and phage SEPL01 (C). D shows tolerance profile of all three phages against different temperatures. E tolerance profile of all three phages against different pH. Data showed were means ± standard deviation of three independent experiments.

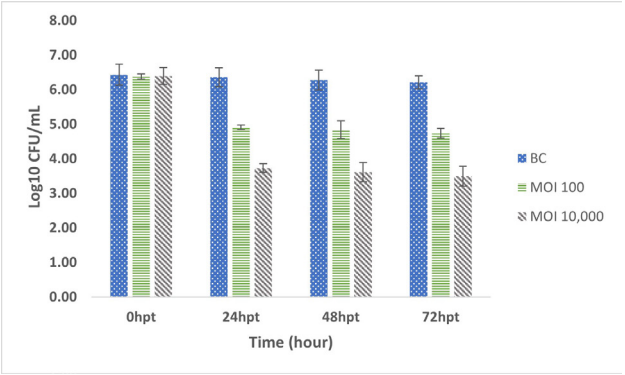

Fig. 4. Efficacy of phage cocktail against *Salmonella* Enteritidis on chicken breast cuts, data presented are mean of three separate experiments ± standard deviation

In comparison to the phage titer at the start of the experiment which was 10^8 PFU/mL, all the three phages (SEPL01, SEPL13, and SEPL20) showed no significant change in titers at 4°C, 25 °C, and 37 °C after 1 h of incubation. At 50 °C and 60 °C temperatures, phages showed around 1 log reduction. However, at 70 °C, three phages showed a varying degree of tolerance with 2.2, 5.0 and 3.1 log reduction observed in the case of SEPL01, SEPL13, and SEPL20, respectively. No phage recovery was observed at the highest temperature of 80°C tested in this study. The tolerance profile of all three phages at different temperatures can be seen in Figure 3D.

All the three bacteriophages showed relatively stable titer as compared to the starting phage titer (10^8 PFU/mL)

when tested at pH from 4 to 11. However, at low pH of 3 and high pH of 12 the tolerance was highly varied. At pH 3 phage SEPL01 showed a 4.3 log reduction and the phage titer of SEPL13 and SEPL20 was below the detection limit (10^1 PFU/mL). Upon exposure to pH 12 only phage, SEPL20 showed survival with 5.1 log reduction while the other two phages showed no viability. The pH tolerance profile of all three phages at different pH is shown in Figure 3E.

Change in initial concentrations of *S.* Enteritidis on chicken breast cuts, due to application of phage cocktail at different concentrations over a period of 3 days at 4°C, is shown in Figure 4. Chicken breast cuts in the negative control group showed no recovery of Enteritidis. On cuts that were only contaminated with Enteritidis without any phage treatment, the Enteritidis count recorded was 6.43 Log_{10} CFU/ mL and 6.21 Log_{10} CFU/ mL at 0 and 72 h, respectively. At MOI of 100, Enteritidis concentration decreased significantly ($P \leq 0.05$) from 6.36 Log_{10} CFU/ mL to 4.74 Log_{10} CFU/ mL with an overall reduction of 1.47 log units over a period of 3 days/72 h. Comparably, when the phage cocktail was applied at an MOI of 10,000, a greater reduction of 2.72 log units ($P \leq 0.05$) was observed as the Enteritidis count drops from 6.40 Log_{10} CFU/ mL to 3.49 Log_{10} CFU/ mL over a period of 72 h at 4°C.

DISCUSSION

Food-borne Salmonellosis triggered by non-typhoidal *Salmonella* is emerging public health issue that has troubled the food industry as well as occupied health sector (Abdel-Maksoud *et al*., 2015). Absurd and excessive use of antibiotics in poultry sector, as a substitute to environmental hygiene and subjecting birds to sub-therapeutic antibiotic dozes, is leading to antibiotic resistance in return failure to clinical antimicrobial therapy (Ma *et al*., 2021). Irrespective of many stratagems used in war to control *Salmonella* throughout the whole food production chain, Salmonellosis spreading from food is still among the most common zoonotic diseases (Hafez and Attia, 2020). To have emphatic outcomes, people are moving towards substitution of present therapies which highlights the use of bacteriophages which is marking as low cost high effective approach (Bardina *et al*., 2012).

Formerly, many studies have reported isolation of *Salmonella* specific bacteriophages from sewage water demonstrating their high presence in host rich environment as sewage water serves as a rich source of enteric pathogens including *Salmonella* Enteritidis (Hungaro *et al*., 2013; Nabil *et al*., 2018; Parra and Robeson, 2016; Yildirim *et al*., 2018). Based on our information this is the first study in Pakistan that involves physiological assessment/ characterization including biological parameters of Enteritidis specific lytic phages and their use to check Enteritidis modulation on chicken breast meat cuts.

Based upon phage virulence and broader lytic spectrum (discussed below), out of seventeen isolated phages, three phages (SEPL01, SEPL13, SEPL20) were selected for further use. All the three phages produced round and clear plaques ranging in size from 1 to 4.5 mm. There was a halo observed around a central clear point in the case of phage SEPL01. This halo zone refers to the possession of an enzyme EPS depolymerase by the phage which aids them to depolymerize a biofilm or exopolysaccharide (Islam *et al*., 2020). As per TEM analysis, all the three bacteriophages were found morphologically similar to members of order Caudovirales (Myoviridae and Siphoviridae family). Many studies have found *Salmonella* phages as members of the Siphoviridae and Myoviridae family (Caudovirales order). All acquiring potential to be used as biocontrol agents against *Salmonella* (Jung *et al*., 2017; Kim *et al*., 2020; Merwad and Abdel-Haliem, 2018; Nováček *et al*., 2016).

Bacteriophages to be used as biocontrol agents should have a broad host range and virulent lytic potential. Phage virulence assay and host spectrum studies were performed side by side to select the best phages. Three phages (SEPL01, SEPL13, SEPL20) were found to be more virulent and suppressed the bacterial growth for up to 20, 18, and 14 h respectively. MOI 100 gives rapid results by totally inhibiting initial bacterial growth (required for a phage which is to be used as a biocontrol agent) whereas MOI 1 and 10 also provided good results but delayed in time with initial rise in bacterial growth. Issue of regrowth was overcome by the use of cocktail which, by far, gave the best results by not letting bacteria regrow till 24 h with bacterial mass staying up to 0.2 (OD_{600}). Study by (Islam *et al*., 2019) also narrated the similar results that phage LPST153, LPSTLL, and LPST94 inhibited *Salmonella* growth for 5 to12 h, however, the cocktail showed extended *Salmonella* inhibition for continuous 20 h. Nale *et al*. (2020) also stated that when individual phages were combined in the form of a cocktail against *Salmonella*, the OD_{600} value decreased to 0.1 and remained consistent until the end of the experiment (24 h). Hungaro *et al*. (2013) also supported the concept that for significant bacterial reduction greater MOI is required.

SEPL01, SEPL13, and SEPL20 showed impressive host range by infecting 100 percent (19/19) of tested *Salmonella* isolates with 0 percent heterologous infectivity. Findings indicate their use as potential broad-spectrum biocontrol agents targeting *Salmonella spp*. Phage LPST153 showed broad-spectrum lytic potential by lysing all thirty widespread *Salmonella* serotypes along with potential to decontaminate chicken meat (Islam *et al*.,

2020). Another study revealed the same pattern of the lytic spectrum of phage wksl3 which showed 100 percent lysis against 32 and 36 strains of Enteritidis and Typhimurium, respectively (Kang *et al*., 2013).

One of the critical steps of the bacteriophage infection cycle is adsorption as it initiates the infection cycle (Bertozzi-Silva *et al*., 2016). A shorter lysis time is related to those phages which show a high adsorption rate (Shao and Wang, 2008). Three bacteriophages (SEPL01, SEPL13, and SEPL20) in this study showed an average of 50% adsorption to host bacteria within 10 min showing a high adsorption rate/specificity and indicating their potential for use as an effective antibacterial agent. (Sadekuzzaman *et al*., 2018) mentioned the adsorption rate of two *Salmonella* phages BP1369 and BP1370 to be more than 50% in 10 min. To select bacteriophages for their use as effective biocontrol agents, replication dynamics including bursts size and latent time period both correlate positively in bacterial killing (Sinha *et al*., 2018). It is said that phages with short latent time and high burst size make them a potential candidate for biocontrol and therapeutic activities (Mateus *et al*., 2014). In this study, with an average latent period of 15 to 20 min all three phages showed an average burst size of between 32 to 110 PFU CFU^{-}. Results of a study by (Huang *et al*., 2018) were in concordant to our study stating the latent time period of 20 min with a burst size of approximately 94 PFU CFU^{-}. In another study, four *Salmonella* specific lytic bacteriophages belonged to Siphoviridae and Myoviridae family showed burst sizes of 30 to 294 PFU CFU^{-} with an average latent period of 5 to 30 min (Kim *et al*., 2020).

In this study, all three bacteriophages (SEPL01, SEPL13, and SEPL20) withstand the temperature from 4°C to 60°C, with varying degree of tolerance to 70°C and complete sensitivity to 80°C. However, the said phages showed relatively stable titter between 4 to 11 pH. Bacteriophages showing tolerance over a wide range of temperature and pH provides a broad window of their application Huang *et al*. (2018). Phage LSE7621 showed 90 to 95% stability at pH 4 to 11 and 30°C to 50°C with no viability below and above this pH and at70°C and 80°C. However, in this study, phages SEPL01 and SEPL20 showed varied stability at pH 3 and 12, respectively, whereas all three were found somewhat stable at 70 °C (Liu *et al*., 2020). A similar study by Dallal *et al*. (2019) reported stability of phage from pH 3 to 11 with optimal pH from 6 to 8. Whereas at a temperature above 60°C lead to no viability of phage with good survival between 4°C to 60°C. A study by Abdelsattar *et al*. (2021) also agrees with the present study in which they demonstrated the stability of phage till 70°C (Abdelsattar *et al*., 2021).

In this study potential ability of phage cocktail to modulate Enteritidis viability on chicken breast cuts was assessed at 4°C (refrigerating temperature) for a period of 3 days simulating conditions in food processing facilities mainly slaughtering plants. Findings of the experiment revealed an overall Enteritidis reduction of 1.47 log units and 2.72 log units upon phage cocktail application at MOI 100 and 10,000, respectively, after 3 days of incubation at 4°C. Use of phage cocktail at high multiplicity of infection provided greater reduction. Comparing 24-h activity of phage cocktail, significant reduction of 1.45 and 2.63 log units at MOI 100, and 10,000, respectively was observed after first 24 h followed by slow reduction. Phage cocktail in this study showed greater efficiency than previously reported phage cocktail that reduced Enteritidis by 0.9 log10 cfu/g and Typhimurium by 2.2 log10 cfu/g at MOI 1000 after 7days at 4°C (Spricigo *et al*., 2013). Our study was also in coherent with the study by Zinno *et al*. (2014) in which they also declared greater *Salmonella* reduction of 1.73 log in first 24 h using MOI 10^7 on sliced chicken breast by phage P22. Literature revealed that to obtain effective/high bacterial reduction on food items stored at low temperature (4°C) high doses/high MOI of phages would be required owing to slow bacterial growth and considering immobility of phages on solid food items (Bao *et al*., 2015; Huang *et al*., 2018; Islam *et al*., 2019).

CONCLUSION

Present study findings advocated that phage SEPL01, SEPL13, and SEPL20 chosen from a group of phages owing to their extensive host range against four *Salmonella* serotypes and highest lytic ability revealed tremendous potential as phage cocktail to be used as a biocontrol agent to battle foodborne pathogen Enteritidis to death in vitro and on chicken breast cuts.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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