Post-harvest biocontrol of Salmonella *Enteritidis on Chicken breast meat and Shelleggs using multiphage cocktail*

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Summary
The study aimed to evaluate the efficacy of a phage cocktail to reduce *Salmonella* Enteritidis contamination on perishable food items viz. chicken breast meat and shell eggs using different concentrations. Initially, four bacteriophages €P54, €P59, €P66, and €P72 were isolated from sewage water using *Salmonella* Enteritidis as a target strain. €P54 and €P66 were found to be Myoviruses while €P59 and €P72 belonged to the Siphoviridae family. A phage cocktail was applied at a concentration of 100 and 10,000 multiplicity of infection (MOI) after artificially contaminating both food items with *Salmonella* Enteritidis. Results showed that, phage cocktail significantly ($p \leq 0.05$) reduced *Salmonella* Enteritidis count at both concentrations. However, the increased reduction was witnessed at 10,000 MOI. In comparison to untreated control, on chicken breast meat bacterial count was reduced to 1.94 and 3.17 Log_{10} cfu/g at 100 and 10,000 MOI respectively at 4°C. Similarly, on shell eggs, the bacterial count was reduced to 3.09 and 2.81 Log_{10} cfu/mL at 10,000 MOI at 4°C and 25°C respectively, while at 100 MOI there was less drop in bacterial count at both 4°C and 25°C. The results showed a better reduction at 4°C as compared to 25°C. Our data showed that the phage cocktail is an effective alternative and additional measure compared to conventional bacterial control methods for meat and eggs.

Introduction

Globally, to meet consumers' growing demand for poultry meat, the poultry industry is expanding with advancements. However, the factors that are posing a major challenge to the future of the food industry involve the emergence and re-emergence of foodborne zoonotic pathogens. Despite all strict measures placed to control *Salmonella* throughout the food production chain from farm to fork, salmonellosis is among the most common zoonotic diseases transferring from food to humans (Hafez and Attia 2020). Most cases of salmonellosis are not reported majorly in developing countries. According to a study, among cases of gastroenteritis, 93.8 million cases globally are due to *Salmonella* species with 155,000 deaths each year. Among estimated cases of gastroenteritis, 80.3 million were of foodborne origin (Mouttotou *et al.* 2017). One of the leading causes of salmonellosis in humans is generally due to consumption of poultry products i.e., meat and eggs contaminated with *Salmonella*.

In humans, *S.* Enteritidis and other diarrheal pathogens (Enteroinvasive *Escherichia coli*, *Campylobacter jejuni*, etc.) cause inflammatory

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diarrhea and abdominal cramps, nausea, vomiting, fever, chills, and mainly neutrophils are observed in the patient's stool (Antunes *et al.* 2016).

Along with adverse health effects, people also suffer the cost of illness, discomfort, inability to engage in routine activities, etc.

Poultry products get contaminated with *Salmonella* throughout the poultry production chain including production, processing, distribution, retail marketing, and even while cooking. (Aziz-ul-Rahman *et al.* 2017). Many strategies have been used to reduce microbial load on chicken meat and eggs including physical (use of steam, and water treatments), chemical (use of organic acids, chlorine-based, phosphate-based treatments), irradiation, and biological methods. Despite the effective reduction in the bacterial count, all these procedures have limitations including equipment corrosion, chemical toxicity, difficult chemical disposal, rejection of irradiated food, and adverse effects on sensorial and organoleptic properties (Nollet *et al.* 2006;Harrell *et al.* 2018; Moye *et al.* 2020). To have emphatic outcomes, people are moving towards finding another effective, environment-friendly, cost-effective, non-chemical control and preventive measure.

For the prevention of foodborne illness, the biocontrol agents of particular interest currently are lytic bacteriophages, being naturally available, self-replicating, self-limiting, and target specific renders them as a potential secure replacement (Vikram *et al.* 2021). Bacteriophages pose several advantages over antibiotics and other strategies to reduce *Salmonella* colonization in chickens and are proving to be the finest organisms in controlling pathogenic bacteria in farm-to-fork food supply chain system. Throughout the food production chain, lytic bacteriophages could be employed as pre-harvest (to decrease colonization of *Salmonella* at early stages of poultry production as a therapeutic and preventive measure) and post-harvest (as a biocontrol agent to minimize *Salmonella* contamination on the chicken carcass, eggs, etc.) pathogen control measure along with biosanitation (to disinfect equipment's, contact surfaces, etc.) and biopreservation (for pathogen-free storage and preservation of food) strategy to limit the transfer of bacteria to humans via the food chain (Endersen and Coffey 2020).

Many research studies have recommended the use of bacteriophages as a substitute in reducing *Salmonella* on the chicken carcass, eggs, and other poultry-derived products (Duc *et al.* 2018; Wei *et al.* 2019; Żbikowska *et al.* 2020). Phages being safe for humans is an excellent step towards new mitigation and biocontrol strategies for establishing safer food supply chain system.

Materials and methods

Bacterial Host and Bacteriophages

The host bacteria used in this study for the isolation and propagation of bacteriophages was *Salmonella enterica* subsp. *enterica* serovar Enteritidis, originally isolated from poultry intestines and confirmed through culturing, biochemical tests, serotyping, and PCR targeting the difference *Sdf1* region of the bacterial genome (Sadiq *et al.* 2020). Bacteriophages were isolated from sewage water according to protocol of Imklin and Nasanit (2020) with few modifications. For the initial screening of phages, spot assay techniques was used (Parra and Robeson 2016) and for phage purification and titration, plaque assay was performed (Kumar *et al.* 2020). Four purified bacteriophages €P54, €P59, €P66, and €P72 were selected for their use in phage cocktail preparation.

Morphological assay

High titer phage stock (10^{11} PFU/mL) was fixed onto copper grids. After negative staining with uranyl acetate (5%) phages were observed under Transmission Electron Microscope (JEOL JEM 1010) under 100 kV at National Institute of Biotechnology and Genetic Engineering (NIBGE), Faisalabad.

Preparation of food samples

Chicken breast meat and raw eggs purchased from the supermarket were first screened for the presence of natural *Salmonella* by standard microbiological techniques. Further to ensure the killing of any possible bacteria or to reduce background bacterial load, both food items were exposed to UV light for almost 20 minutes. Under sterile conditions, the chicken breast was cut into 2 cm × 2 cm squares almost 1 g.

Preparation of phage cocktail

For the preparation of the phage cocktail, all the four phages €P54, €P59, €P66, and €P72 were mixed in 1:1:1:1 concentration using titers 10^{11} PFU/mL and later was diluted according to the required concentration.

Phage cocktail treatment on chicken breast meat

This assay involved four groups shown in Table I. To begin, chicken breast pieces were first exposed with *S.* Enteritidis by pipetting 500 µL of 10^6 cfu/mL culture followed by drying in a safety cabinet for

almost 30 minutes for bacteria to adhere to the meat matrix. Then 500 µL of 10^8 PFU/mL and 500 µL of 10^{10} PFU/mL phage cocktail was pipetted on the surface to achieve 100 MOI and 10,000 MOI respectively. For the chicken pieces in positive bacterial control, a similar volume of 0.9% normal saline was pipetted and placed at 4°C (Bao *et al.* 2015; Islam *et al.* 2019a).

Phage cocktail treatment on shell eggs

Similarly, eggs were divided into 4 groups as mentioned in Table I. The experiment started with spiking the eggs with *S.* Enteritidis and for that eggs were dipped in *S.* Enteritidis culture (10^7 cfu/mL) for 15 minutes. Followed by drying in a safety cabinet a phage cocktail at 10^9 PFU/mL and 10^{11} PFU/mL respectively was sprayed onto the eggs for 100 MOI and 10,000 MOI phage treatment respectively along with 0.9% normal saline on eggs serving as positive bacterial control. Half the eggs were incubated at 4°C and half at 25°C (El-Shibiny *et al.* 2017).

Table I. *Food Assay Plan*

Treatment of phage cocktail to control Salmonella Enteritidis on shell eggs and on chicken breast meat				
Groups	Eggs (4°C)	Eggs (25 °C)	Chicken Breast pieces (4°C)	*Sampling*
Group 1 (Positive control, contamination with S. Enteritidis only)	15	15	15	Bacterial viable count at 0, 3, 6, 12 and after 24 hour
Group 2 (*Salmonella* Enteritidis + cocktail of phages at 100 MOI)	15	15	15	
Group 3 (*Salmonella* Enteritidis + cocktail of phages at 10,000 MOI)	15	15	15	
Group 4 (Negative control, no Enteritidis contamination nor cocktail treatment)	15	15	15	

Bacterial Enumeration

To titrate *S.* Enteritidis viable count, 3 chicken pieces/shell eggs were withdrawn at each time interval 0, 3, 6, 12, and 24 hours. Meat pieces were homogenized in 9 mL normal saline with the help of sterile glass bars followed by vortexing. While shell eggs were placed in a sterile zip lock bags having 10 mL normal saline and massaged for 1-2 minutes. All the liquid was then centrifuged at 9000 x g for 15 minutes for bacteria to settle down. The supernatant was removed to avoid phages and the pellet was resuspended again in normal saline. After serial dilution, specified volume was plated on Salmonella Shigella Agar (CM0099 Oxoid, UK) followed by incubation at 37°C for 24 hours for enumeration. The detection limit was set to 1 log cfu/g or 10 cfu/g (Islam *et al.* 2019b; Keerthirathne *et al.* 2020).

Statistical Analysis

Data attained from both experiments was organized in Microsoft Excel spread sheets (Microsoft 365). To analyze significant differences in bacterial numbers as a result of application of different phage cocktail concentrations (MOI100, MOI10,000) on different times (0h, 3h, 6h, 12h, 24h), data was analyzed with Two-Way ANOVA using Minitab 17.0.1. Differences between parameters were studied statistically significant at ($P \leq 0.05$). For pairwise comparison within groups post-hoc test (Tukey's test) was applied. To compare efficacy of bacteriophages on eggshells at different temperatures (4°C and 25°C), 2-sample t test was used. Descriptive statistics including standard deviation (SD), standard error (SE) of means, and upper and lower bounds of confidence level (95%) were also calculated.

Results

Salmonella Enteritidis Identification

Red color colonies with a black center were seen on Xylose-Lysine-Desoxycholate Agar. The antigenic formula of *S.* Enteritidis after treating with a series of polyvalent and monovalent antisera was interpreted according to WHO antigenic formula of *Salmonella* serovars, 9th edition and came out to be 1,9,12:g,m:-. Host bacterial strain along with three other isolates of *S.* Enteritidis SEPRI1, SEPRI2 (provided by Poultry research Institute, Islamabad), and one ATCC 13067 used as positive control yielded an amplicon of 304 base pairs in length against 100 bp ladder indicating presence of *S.* Enteritidis specific *Sdf I* region – can be observed in Figure 1.

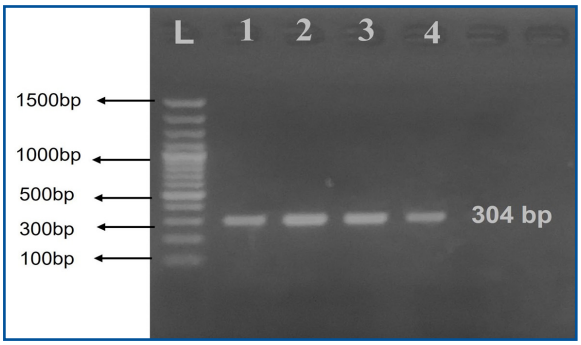

Figure 1. *PCR Confirmation of* Salmonella *Enteritidis (Left to right) (L) Ladder, (1) Host strain of* Salmonella *Enteritidis, (2), (3) SE PRI1 and SEPRI2 Strains of* Salmonella *Enteritidis provided by Poultry Research Institute, (4) ATCC® 13076* Salmonella *Enteritidis*

Bacteriophage Morphology Analysis

Electron micrographs of all four bacteriophages are shown in Figure 2. Two phages €P54 and €P66 had prolate icosahedron heads and short thick contractile tails indicating their resemblance to the members of Myoviridae family with heads of 188.4 and 174.6 nm in length whether the tails were of 180 and 150 nm in length respectively. The features of other two phages €P59 and €P72 were found similar to members of Siphoviridae family having isometric hexagonal heads (138 and 144.6 nm respectively) with icosahedral symmetry and long noncontractile tails (338 and 225 nm respectively).

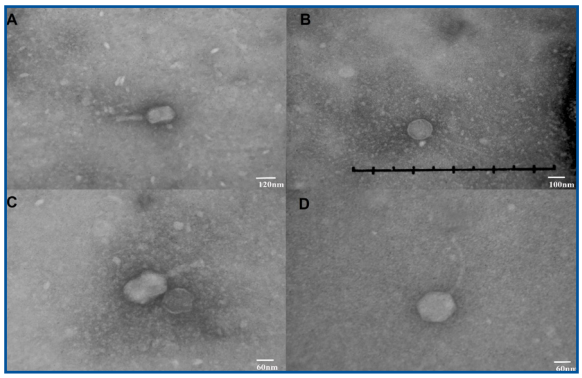

Figure 2. *Morphology Analysis of bacteriophages, (A) Electron Micrograph of phage €P54, (B) Electron Micrograph of phage €P59, (C) Electron Micrograph of phage €P66, (D) Electron Micrograph of phage €P72*

Phage cocktail potency to reduce S. Enteritidis load on contaminated chicken breast meat

Change in the *S.* Enteritidis count after treating chicken breast meat with phage cocktail at 100 and 10,000 MOI is shown Figure 3 (A). The mean count of the treated samples expressed in log values was compared with the mean bacterial control values to know bacterial reduction.No *S.* Enteritidis was recovered from samples in the negative control group. In the positive control group, in which meat pieces were only contaminated with *S.* Enteritidis, there was no distinct change in *S.* Enteritidis count and was observed to be 5.51 Log_{10} cfu /g at 0 hours and 5.40 Log_{10} cfu /g after 24 hours. At MOI 100, with phage cocktail application, the *S. S.* Enteritidis count decreased significantly ($P \leq 0.05$) to 4.30, 3.66, 3.56 and 3.46 after 3, 6, 12, and 24 hours respectively from 5.43 to 3.46 Log_{10} cfu /g with a total reduction of 1.94 log units after 24 hours as compared to the positive control. Similarly, phage cocktail at 10,000 MOI resulted in a decrease of *S.* Enteritidis count significantly ($P \leq 0.05$) from 5.48 to 3.48, 2.48, 2.32, and 2.23 Log_{10} cfu /g after 3, 6, 12, and 24 hours respectively with overall 3.17 log units of reduction as compared to the untreated positive control.

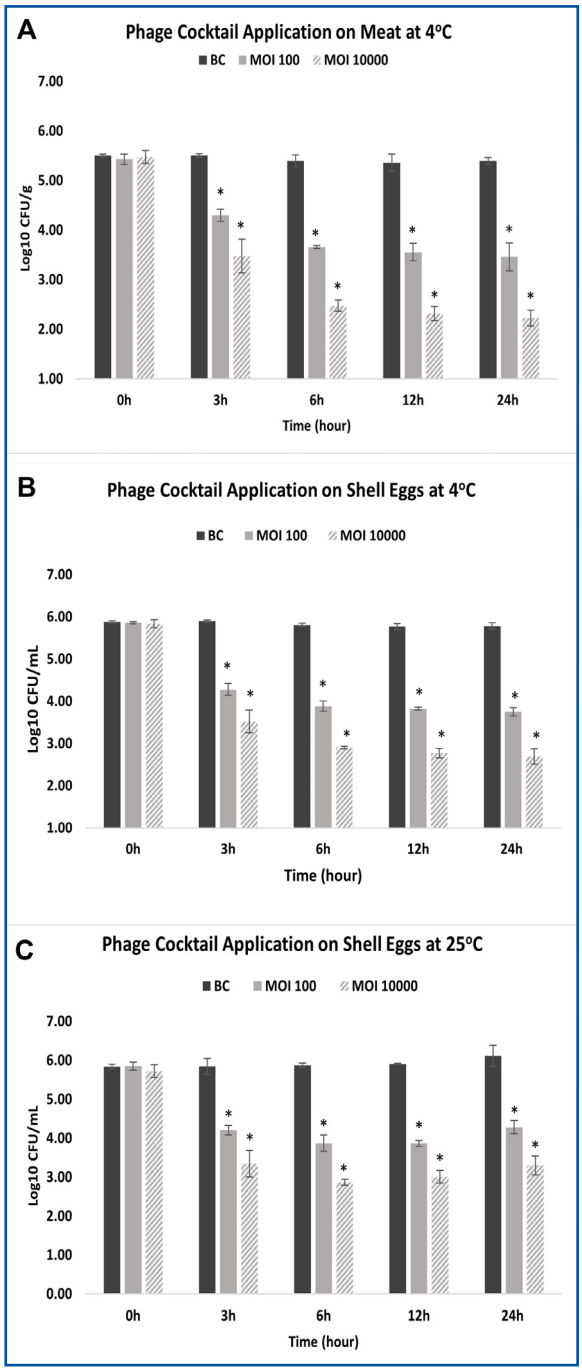

Figure 3. *Ability of Phage cocktail to reduce S. Enteritidis contamination (A) On chicken breast meat at 4°C, (B) On Shell Eggs at 4°C, (C) On Shell Eggs at 25°C. Data shown are the mean of three values ± Standard deviation. * Significant at P ≤ 0.05*

Phage cocktail potency to reduce S. Enteritidis load on Shell Eggs

Modulation in *S.* Enteritidis count on shell eggs at 4°C and 25°C after treatment with phage cocktail at 100 and 10,000 MOI is shown in Figure 3 (B), (C) respectively. No *S.* Enteritidis recovery from shell eggs in the negative control group. *S.* Enteritidis viable counts recovered from eggshells in positive control group retained at 4°C remained consistent over 24 hours with count recorded 5.88 Log_{10} cfu/

mL at 0 hours and 5.79 Log_{10} cfu/mL at 24th hour whereas a very slight increase (0.28 log cfu/mL) in *S.* Enteritidis count was observed from the eggshells during incubation at 25°C with count 5.84 Log_{10} cfu/mL recorded at 0 hours and 6.11 Log_{10} cfu/mL at 24th hour.

At 4°C with 100 MOI treatment, the count was reduced significantly ($P \leq 0.05$) to 4.28 and 3.75 Log_{10} cfu/mL after 3 and 24 hours respectively as compared to the positive control and resulted in an overall 2.04 log reduction after 24 hours. 10,000 MOI treatment also brought a significant ($P \leq 0.05$) reduction of 3.09 log compared to the positive control with count recorded to be 3.52 and 2.69 Log_{10} cfu/mL after 3 and 24 hours respectively.

Likewise, at 25°C, the change in count observed was 4.21, 3.88, 3.87, and 4.29 Log_{10} cfu/mL after 3, 6, 12, and 24 hours with an overall significant ($P \leq 0.05$) reduction of 1.83 logs compared to the positive control when phage cocktail was applied at 100 MOI. However, with 10,000 MOI treatment, *S.* Enteritidis contamination was significantly ($P \leq 0.05$) reduced by 2.81 logs compared to positive control with count noted to be 3.35, 2.87, 3.01, and 3.30 Log_{10} cfu/mL after 3, 6, 12, and 24 hours of incubation.

Discussion

The current study revolves around the development of bacteriophage-based addition to perishable food items to tackle the problem of *S.* Enteritidis contamination. In this study, all four lytic bacteriophages used in preparation of phage cocktail were found to be tailed bacteriophages confirmed through electron microscopy. Phage €P54 and €P66 showed similar morphology and were found to be Myoviruses whether phage €P59 and €P72 were found to be Siphoviruses. Many studies have demonstrated the biocontrol potential of Myoviruses and Siphoviruses against *Salmonella* (Kim *et al.* 2020; Merwad and Abdel-Haliem 2018).

Poultry meat and eggs comprises a considerable segment of present-day diets thus in this race to fulfill consumer demand there is tough competition for safer food production from farm to fork. With the emergence of multi drug resistant foodborne pathogenic bacteria the situation is turning out to be adverse (Esmael *et al.* 2021). In food industry, despite considerable traditional measures taken into consideration to eliminate *Salmonella* spp., the issue of food safety is still prevailing. For poultry processors and food safety researchers, minimization of bacterial load from poultry products is of major concern. So, scientists and researchers are looking for a more efficacious, cost-effective, and reliable approach (Guo *et al.* 2021; Kim *et al.* 2020). Among them bacteriophages are evolving as potential alternative to existing food decontaminating interventions being implanted in the food industry.

Our experimental strategy on chicken breast meat would mimic and is applicable in industrial and slaughtering plant settings with possible application of phages in chiller water, while pre-packaging and during storage of meat at 4°C. Phage cocktail was found highly efficacious when applied at a high MOI of 10,000 as compared to MOI 100 resulting in 3.17 and 1.94 log units' reduction respectively after 24 hours at 4°C. This study's results are coherent with many other studies (Bao *et al.* 2015) which showed that a phage cocktail at MOI 10,000 reduced the *S.* Enteritidis concentration on the chicken breast by 2.5 log cfu /sample after 5 hours at 4°C. In another study (Islam *et al.* 2019a), similar results were stated. *Salmonella* counts were dropped by around 3 log_{10} cfu/cm^2 with the application of phage cocktail at 10,000 MOI on chicken breast. Likewise, a phage cocktail of SP-1 and SP-3 at an MOI of 1000 was able to reduce *Salmonella* by 2.5 logs after 24 hours on chicken cuts. Other studies in which lower MOIs were used showed a lower reduction of *Salmonella* to be less than 3 logs (Augustine and Bhat 2015).

Assay on shell eggs were conducted to evaluate the ability of phage cocktail stored at 4°C (to depict refrigerating temperature) and at 25°C to mimic room temperature. Together at both temperatures the phage cocktail demonstrated a significant ($P \leq 0.05$) reduction in *S.* Enteritidis viable count on shell eggs as compared to the positive controls. When high titers of phage cocktail MOI 10,000 were applied on shell eggs an incredible reduction of 3.10 and 2.81 log units was achieved at 4°C and 25°C, respectively. However, a lower reduction of 2.04 and 1.82 log units was attained at lower titers of MOI 100 at 4°C and 25°C, respectively. At a temperature of 25°C *S.* Enteritidis count on treated shell eggs increased by 0.2 to 0.3 log units over 24 hours. This can likely be attributed to the restricted movement of phage particles owing to a solid matrix and finding no phage in contact with them. Additionally, favorable temperature enabled *S.* Enteritidis cells increase their number. A very few studies have demonstrated the application of phages on shell eggs. Among them (El-Shibiny *et al.* 2017) exhibited a reduction of *Salmonella* to an undetectable level after 2 days on eggs. Many other publications exhibited successful application of phages to several food items including ready-to-eat foods (Huang *et al.* 2018; Petsong *et al.* 2019; Spricigo *et al.* 2013).

It was observed that phages exhibit different behavior on different foodstuffs like in liquid food items, phages have been seen to reduce bacteria more effectively than solid food items, and among them again the composition of the material, protein and fat content, texture: smooth or rough may offer

refuge to bacteria. In this case, it becomes difficult for the phage to gain access to the bacteria in order to kill the bacteria, presenting a challenge for these particular foodstuffs.

In both experiments, a greater reduction was observed in beginning 6 hours and then the pattern of reduction was seen to be slowed down possibly because of restricted movement of phage particles on the solid food matrix. Considering less number of bacterial contamination on food items it was observed in this study that applying high concentrations from the very beginning will provide better chance for maximum bacterial contact leading to maximum bacterial reduction on solid food items. In this setup, phages opt for a phenomenon called lysis from without or passive therapy to cause bacterial cell lysis indicating no dependency on replicating host. This explains why at 4°C when the growth of host cells is negligible, phages are able to control pathogen (Bao *et al.* 2015; El-Shibiny *et al.* 2017; Guenther *et al.* 2009). Our experiment strongly supported this theory, as the phage cocktail, when applied at a greater MOI of 10,000, resulted in a greater overall reduction than when applied at a lower MOI of 100 on solid food items. Hence for effective reduction of bacteria on solid food elements, phage dosage, time of application, better application methods, and frequency should be optimized first.

Conclusion

In our study the phage cocktail successfully reduced *S.* Enteritidis contamination on chicken breast meat and shell eggs by around 3.2 logs. Thus, the results of this study emphasize on reconsideration of bacteriophages to be used as an alternative and additional method to reduce food contamination for a safer food production supply. Further studies are encouraged to further detail and refine phage-mediated foodborne pathogen control applications considering food matrix limitations.

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