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Application of the lytic bacteriophage Rostam to control *Salmonella* Enteritidis in eggs

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Abstract:

Foodborne *Salmonella* Enteritidis infections place human health at risk, driven by regular outbreaks and individual cases by different contaminated food materials. This study was conducted to characterize and employ a single bacteriophage as a potential biocontrol agent. Phage Rostam was isolated, characterized and then applied as biocontrol agent against *S.* Enteritidis in liquid whole eggs and eggshell. Rostam is a novel myovirus belonging to the *Rosemountvirus* genus and active against *Escherichia coli* and *Salmonella* spp. Rostam is stable in a pH range from 4-10, a salt concentration of 1-9%, whereas UV radiation gradually reduces phage stability, and its 53kb genome sequence indicates this phage does not contain known toxins or lysogeny-associated genes. Its latent period is short with a burst size of 151 PFU/cell, under standard growth conditions. Killing curves indicate that at higher multiplicities of infection (MOI), the reduction in *S.* Enteritidis count is more pronounced. Phage Rostam (MOI 10,000) reduces *S.* Enteritidis growth to below the detection limit at 4° C in both liquid whole eggs and on the eggshell within 24 h. Due to its high lytic activity and stability in relevant conditions, Rostam has the potential to be an efficient biopreservative for egg and egg products.

Keywords:

Bacteriophage, Foodborne pathogen, isolation, Characterization, liquid whole egg, eggshell

1. Introduction

Salmonella spp. includes some of the most important foodborne pathogens that cause disease through consuming contaminated foods. Salmonellosis is characterized by nausea, vomiting, abdominal pain, fever, and headache and it causes 93 million cases of gastroenteritis and 155,000 deaths annually (El-Dougdoug et al., 2019; Z. Li et al., 2020). Although 2,500 serovars of *Salmonella* have been identified, Enteritidis and Typhimurium are the most prevalent food-related serovars, responsible for 50% of all salmonellosis cases (Fatica and Schneider, 2011). *Salmonella* spp., which causes human infection, has been isolated from a wide range of foods, including eggs and egg products, meat, fish, dairy, fruits and vegetables (EFSA, 2022). Eggs and their products have been considered as the major source of *Salmonellosis* outbreaks in 2021 (EFSA, 2022; Pinedo et al., 2022). In addition, in 2018, 45.6% of salmonellosis was accrued due to the consumption of eggs (EFSA/ECDC, 2019). The U.S. Food and Drug Administration (FDA) has placed the egg in second place of most risky foods for foodborne diseases (Klein et al., 2009). In particular, *S.* Enteritidis is the most prevalent isolated serovar from egg contents (Whiley and Ross, 2015). In addition, a systematic review and meta-analysis of the incidence of *Salmonella* spp. contamination in eggs in Iran revealed a frequency of 6.87 % for *Salmonella* spp., and 6.20 % for *S.* Enteritidis infection. This indicates that *S.* Enteritidis has the highest incidence in eggs, in Iran (Hosseininezhad et al., 2020).

Over the past decades, the excessive use of antibiotics by humans has led to an increase of antibiotic-resistant bacteria, such as antibiotic-resistant *Salmonella* spp. (Bajpai et al., 2012). According to some reports, more than 100,000 *Salmonella* infection cases occur annually due to resistant strains (CDC, 2013). In 2017 the World Health Organization (WHO) placed Salmonella resistance to fluoroquinolone on the worldwide priority list of antibiotic-resistant bacteria

requiring research and novelty (Tacconelli, 2017). Therefore, finding and using a new alternative to antimicrobials instead of antibiotics to control this pathogen seems critical.

Physical, chemical, and biological methods have been employed to prevent and control *S.* Enteritidis contamination of food. For instance, irradiation is one of the most reliable physical ways of controlling food infections, but its side effects include lipid oxidation which results in unpleasant odor, color & texture alterations and nutritional loss (Ahn et al., 2006; Chen et al., 2012; Jo, et al., 1999). The most widely used chemical sanitizer is chlorine, which kills bacteria and sanitizes food processing environments by reducing pathogenic microorganisms. However, chlorine is classified as a health hazard chemical due to the formation of carcinogenic compounds known as trihalomethanes in the presence of organic materials, so its application in the food industry is strictly restricted (Richardson, 2003; Sofos and Smith, 1998). In addition to being a threat to human health, this chemical contains environmentally hazardous chemicals (Byelashov and Sofos, 2009; Ölmez and Kretzschmar, 2009). Conventionally, thermal processing is the most effective method to control *Salmonella* spp. in egg products, which has significant adverse effects on egg quality (Schuman et al., 1997).

A limited number of studies have assessed the potential of using lytic phages as a biocontrol agent to overcome *S.* Enteritidis contamination in eggs. Bacteriophages (or phages) specifically target and propagate inside their host bacteria and eventually release many progeny phages after host lysis, and they do not cause any damage to co-existing microbiota, plants, or animal cells (Montso et al., 2021; Richards et al., 2019). In 2006, the FDA designated phages as Generally Recognized As Safe (GRAS) for use in food, and they are being used in commercial products such as PhageGuard^{TM}, Salmonelex^{TM}, and SalmoFresh ^{TM}. SalmoFresh ^{TM}, for example, is a cocktail of six lytic phages against *Salmonella* Enteritidis (*S.* Enteritidis) and studies have shown

that it efficiently decreases *Salmonella* on cucumber, fresh fruit and chicken (Guo et al., 2021; J. Li et al., 2021). It is important to note that compared to antibiotics, phages can be isolated from various sources, have high specificity, safety, and high antibacterial effects. Therefore, this study aimed to isolate and evaluate the characteristics and stability of a lytic phage against *S.* Enteritidis by applying it as a phage-based antibacterial agent on the eggshell and in a liquid whole egg contaminated with *S.* Enteritidis. This study can be framed in the development of antibiotic-free decontamination methods while maintaining the value and quality of food.

2. Materials and Methods

2.1. Bacteriophage isolation, purification, and propagation

Phage isolation was performed based on a Double Layer Agar assay (DLA) as previously described by Wang et al. (2017) with some modifications. Five sewage water samples were initially collected from one of the chicken slaughterhouses in Shiraz, Iran. Samples were collected aseptically, transported to the food hygiene laboratory, and held at 4°C for 24 hours to allow debris and big particles to settle. Subsequently, 10 mL of each sample were centrifuged at 6,000 × g for 10 min at 4°C, and the supernatant was filtered through 0.22 µm syringe filters. 100 µl of each filtered sample and 300 µl of an overnight culture of *S.* Enteritidis PTCC 1787 was added to 50 ml of tryptic soy broth (TSB) medium and incubated at 37°C with 160 rpm for 24 hours. After incubation, the suspension was centrifuged at 4°C for 10 minutes at 6,000 × g and filtered again. To isolate potential phages from this filtered sewage, it was first serially diluted in SM buffer (0.05 M Tris-HCl, pH 7.5, 0.1 M NaCl, 0.008 M $MgSO_4$, and 0.01% gelatin). Next, 100 µl of each dilution was mixed with 300 µl of overnight bacterial suspension in 4 ml of soft

agar (TSB broth, agar powder 0.5% [w/v]) and then poured onto TSA (TSB broth, agar powder 0.9% [w/v]) plates (double agar overlay method). After 15 minutes, when the top layer was completely set, plates were incubated overnight (18 hours) at 37°C. When clear plaques (lack of bacterial growth) were observed, a single plaque was picked and dissolved in 3 ml SM buffer and incubated at 37°C and 160 rpm for 30 min. The suspension was filtered using 0.22 μm syringe filters and diluted in SM buffer, and it was re-cultured using the DLA method. This process was repeated at least three times to ensure the purity of the phage. To propagate the isolated phage for subsequent use and storage, the method presented by Bonilla et al. (2016) was employed with slight modifications. First, 10 ml of a fresh bacterial culture with an OD_{600nm} of 0.1 (approximately 10^8 CFU/ml) was added to 100 ml of TSB supplemented with 10 mM $MgSO_4$, and the mixture was incubated at 37°C for 1 hour. After incubation and bacterial adaptation, 100 μl of phage suspension (> 10^7 PFU/ml) was added to the culture medium, which was subsequently incubated for about 5 hours at 37°C and 160 rpm. The mixture was centrifuged at 6,000 × g for 10 minutes, the supernatant was filtered, and the phage titer was subsequently measured using the DLA method.

2.2. Transmission electron microscopy (TEM)

Transmission electron micrographs were made as described by Martino and colleagues (Martino et al., 2021). Briefly, 10 μL of the pure phage stock was deposited on carbon and formvar-coated 400 mesh grids (Gilder, Grantham Lincolnshire, England) and negatively stained with aqueous 0.5% w/v uranyl acetate. Observations and photographs were made using a Philips CM 10 transmission electron microscope (Eindhoven, The Netherlands), operating at 60 kV.

2.3. Genome sequencing and annotation

To isolate the phage genome, the method of Kot (2018) was followed, with minor modifications. Briefly, 900 µl of a high titer phage stock was mixed with 100 µl of 1X DNase I buffer (ThermoFisher Scientific, Waltham, MA, USA). The solution was transferred into a 0.45 µm ultrafiltration spin column (Merck Millipore, Burlington, MA, USA). From that point on, the published protocol was followed. Sequencing was performed on an Illumina (San Diego, CA, USA) MiniSeq device, using a library prepared with the Nextera Flex DNA library kit (Illumina). After assembly of the raw sequencing data using SPAdes (Bankevich, et al., 2012), BLASTn (Altschul et al., 1990) and Viptree v1.9 (Nishimura, et al., 2017) were used to identify the most related phages. The phage's taxonomy was further analyzed by calculating the intergenomic similarities using VIRIDIC (Moraru et al , 2020). Next, annotation was performed with RASTtk (Brettin, et al., 2015) followed by manual curation using BLASTp and HHPred (Söding et al., 2005). The genome was visualized with Easyfig (Sullivan et al.,2011). The data were submitted to NCBI GenBank and are available under accession number OP132241.

2.4. Host range and Efficiency of Plating (EOP)

To assess the host range of the phage, 40 bacterial strains, including 13 reference and 27 wild strains, were utilized. All wild strains were present at the food laboratory of the School of Nutrition and Food Science, Shiraz University of Medical Sciences, and had been previously isolated from retail and confirmed by biochemical and molecular (PCR) tests. Each bacterial strain was plated on a TSA medium, and 10 µl (108 PFU/ml) of bacteriophage suspension was spotted in triplicate on plates. *S.* Enteritidis PTCC 1787 was also spotted as a control positive strain to compare with the results of other strains. In addition, a sterile SM buffer was spotted on each tested strain to differentiate the real clean zone from the sterile SM buffer drop (negative

control). The plates were incubated at 37 ° C overnight and observed for a clear zone the next day.

For each bacterial strain that showed a clear zone in the host range test, an efficiency of plating (EOP) test was performed. All selected strains and the primary host were treated with phage dilution (1:9) and plated using the DLA method to determine the phage titer of each bacterial strain. The EOP was determined by dividing the phage titer of each bacterial strain by that of the primary host and was done thrice for each strain.

2.5. Phage stability over a range of temperatures, pH, NaCl, and UV radiation

The stability of phage was assessed under various relevant environmental conditions, including temperature, pH, NaCl, and UV radiation, using a modified version of the method described by Wang et al. (2017), carried out in triplicate. First, the impact of temperature on phage stability was determined by adding 10 µl of phage suspension (10^8 PFU) to 990 µl of pre-warmed SM buffer at 20, 40, 60, and 80°C. The tubes were then maintained at the specified temperatures for 60 minutes. After exposure for one hour to the determined temperatures, each tube was cultivated using the DLA, and their titers were determined. In addition, titer of phage stock that stored at 4°C was measured as the control group. The phage stability at various pH was evaluated by adding 10 µl (10^8 PFU) of phage suspension to 990 µl SM buffer that adjusted its pH between 2 and 12 by 1 M of NaOH or HCl. Then they were kept at 37°C for 60 minutes before the phage titer was determined. The impact of different NaCl concentrations on phage stability was assessed as mentioned in the previous steps using 990 µl of SM buffer supplemented with 1%, 3%, 5%, 7%, 9%, 11% and 13% NaCl and incubated for 60 minutes. Phage stability was measured for 0, 10, 20, 30, 40, 50, 80 and 120 minutes of exposure to UV radiation (UV light 20 w). For this purpose, phage suspension (10^8 PFU) at an equal amount of

1ml was poured into 12 well plates and it was placed under UV light. At each time point, phage suspension was taken from a well, and after dilution in SM buffer, the phage titer was measured as described previously.

2.6. Phage adsorption assay

One ml of fresh host bacteria (10^8 CFU/ml) was poured into a micro-tube and mixed with 10 µl of phage (10^8 PFU/ml) to achieve an MOI of 0.01; then, the mixture was added to 9 ml TSB. To determine the phage titer at t=0, 1 ml of sterile medium (without bacteria) was added to the same volume of phage suspension. The phage-host mixture was placed at 37°C and 100 µl aliquots were taken after 5 and 10 min, then mixed rapidly in 900 µl SM buffer, centrifuged and passed through a 0.22 µm filter. The filtered liquid was cultured using the DLA method, and the titer of the free phage was calculated. The percentage of free phage at every given time was determined by dividing the phage titer at 5 and 10 minutes by the phage titer at time zero (Khalatbari-Limaki et al., 2020).

2.7. One-step growth curve

At the beginning, 1 ml of fresh bacterial culture (1.7×10^8 CFU/ml) was mixed with 1 ml of phage suspension (4.2×10^6 PFU/ml) to obtain an MOI of approximately 0.01. The mixture was allowed to absorb the phage onto the bacteria for 10 minutes at room temperature before being centrifuged for 1 minute at 10,000 × g. The supernatant containing unabsorbed phages was discarded, and the pellet was resuspended in 10 ml of TSB incubated at 180 rpm at 37°C for 90 minutes in a shaker incubator. At 10-minute intervals, 100 µl aliquots of the mixture were taken and mixed with 900 µl SM buffer and then centrifuged at 10,000 × g for one minute and the supernatant was filtered by a 0.22 µm filter. The phage titer was measured using the DLA by

serially diluting the filtered sample in SM buffer (Wang et al., 2017). The burst size was determined by one-step growth curve using the following formula:

$$\textbf{Burst size} = \frac{\textbf{phage titer at the end of burst cycle} - \textbf{initial phage titer}}{\textbf{phage added} - - \textbf{initial phage titer}}$$

2.8. Killing curve at different MOIs

Killing curves were designed to determine the impact of phage at different MOIs on the growth of *S*. Enteritidis as the host bacterium. After preparing 10^4 CFU/ml on log-phase host bacteria and dividing it into 1 ml aliquots, 100 µl of phage suspension with varying titers was added to the host bacteria suspension to get MOIs of 0.001, 0.1, 1, 10, 100, and 10,000. In addition, 100 µl of phage-free buffer was added to the bacteria as a positive control. Microtubes were gently shaken and dissolved in 8.9 ml of TSB culture media before being incubated at 37˚c with 160 rpm. 100 µl from each flask was taken at times 0, 1, 3, and 6 h, and after dilution in normal saline, they were plated on TSA and then placed in an incubator at 37°C for 24 hours (Wang et al., 2017).

2.9. *In vitro* phage-bacterium challenge test (low temperatures)

Fresh host bacteria were diluted to 10^5 CFU/ml, and 100 µl of this suspension was mixed with 100 µl of phage with different titer to obtain MOIs of 10, and 10000, which were then added to flasks containing 9.8 ml TSB and incubated at 4 and 25°C. The control group had the same quantity of bacteria, and 100 µl of phage-free SM buffer was added to 9.8 ml of culture medium. The control negative was conducted similarly, but without bacteria, and all samples were incubated at 4 and 25°C. to determine the count of host bacteria, 1 ml of each flask at 0, 3, 6, and 24 hours after incubation was added to 9 ml PBS and then diluted (1:9), plated on TSA, and

incubated at 37°C for 24 hours. The bacterial count for each time point was determined in triplicate.

2.10. Biocontrol of *S.* Enteritidis in liquid whole eggs

Fifty intact fresh eggs were purchased from the store and soaked in 70% alcohol for 25 minutes for sanitization. After breaking the eggs with sterile tweezers, their contents were transferred to sterile bags and homogenized with a stomacher for one minute. Next, aliquot volumes of 9.8 ml of the homogenized whole egg liquid were placed in 15 ml sterile falcon tubes (Yi et al., 2021). The falcon tubes were divided into 4°C and 25°C temperature groups and inoculated with 100 µl of host bacteria (10^6 CFU/ml) and 100 µl phage with different titer to obtain two different MOIs (10 and 10,000). For the control group (phage-free), an equivalent volume of bacterial suspension was mixed with 100 µl of sterile SM buffer and added to the egg liquid. Also, the control negative was evaluated using the same procedure as before and was followed only with the difference that 100 µl of phage suspension with 100 µl of sterile PBS (without bacteria) was added to the same volume of homogenized whole egg liquid. All previous procedures were conducted without bacteria or phage to ensure the sterility of eggs and SM buffer (Li et al., 2020). For an even distribution of phage and bacteria throughout the liquid, the content of each falcon tube was vortexed and stored in two different groups of 4°C and 25°C. To determine the bacterial count at time 0, 3, 6, 24, 48 and 120 hours after incubation, 1 ml of each falcon tube was sampled and dissolved in 9 ml PBS, diluted, and then plated on TSA and incubated for 24 hours at 37°C. The experiments were performed in triplicate and the bacterial count was counted similar to the previous steps.

2.11. Biocontrol of *S.* Enteritidis on eggshell

Intact eggs were placed in boiling water for 30 minutes to eliminate any bacterial and fecal contamination. Simultaneously, fresh *S.* Enteritidis culture with OD_{600nm}=0.1 (approx. 10^8 CFU/ml) was centrifuged at 4,000 × g for 10 minutes, and after discarding the supernatant, the pellet was dissolved in an equal amount of PBS buffer. In two containers, one containing bacteria in PBS (10^6 CFU/ml) and another containing phage (10^9 PFU/ml) in SM buffer was prepared. After chilling at ambient temperature, whole, unbroken eggs were soaked in a container containing bacteria for 30 minutes before being placed on sterile trays to dry. The eggs were then immersed in a bacteriophage container for 5 seconds and allowed to dry for 30 minutes on a sterile tray. At the end, all eggs were separated into two groups for incubation at temperatures of 4°C and 25°C. The control group eggs (control positive) were prepared as described above, but with the difference that eggs after being immersed in *S.* Enteritidis containers, were immersed in sterile SM buffer (phage-free). Also, the control negative eggs group (bacteria-free) was prepared as described for other groups with the difference that they were firstly immersed in sterile PBS, then after drying, eggs were immersed in the phage container. In addition, the phage-bacteria-free eggs group was also prepared to ensure the sterility of the eggs. All groups were sampled at 0, 3, 24, 48, and 120 hours following the start of incubation at the specified temperature. To count the bacteria on the surface of the egg, each egg was thoroughly washed with 50 ml of PBS for 10 minutes, then 1 ml of solution was diluted (1:9) in PBS, plated in TSA, and incubated at 37°C for 24 hours. All groups had their bacterial counts done in triplicate (Cao et al., 2022; Makalatia et al., 2018).

2.12. Phage persistence in whole liquid eggs and eggshell

To determine phage persistence in whole liquid egg and eggshell, as the previous steps were conducted, from the control negative group (only phage) at each time point, 100 µl of the sample

were mixed in 900 µl, SM buffer serially diluted and then DLA method performed to measure the phage titer.

2.13. nalysia Statisticals

All experiments were done in triplicate. After converting bacterial and phage numbers to the logarithm of CFU/ml and PFU/egg, results are shown as Mean ± SD. In order to compare between groups, the One-Way ANOVA test was performed. Signi icant differences were discriminated using Duncan's test with significance set at $P < 0.05$. All data were analyzed using SPSS 16.0 (SPSS Inc., Chicago IL, USA).

3. Results

3.1. Phage Rostam morphology, host range and EOP

One phage was isolated against *S.* Enteritidis from a poultry slaughterhouse sewage water sample, displaying large, clear plaques. We selected the name Rostam for the phage, based on a mythical Iranian hero. Rostam showed a myovirus morphology with an elongated head (Figure 1). As shown in Table 1, for 20 out of 40 strains (50%), a clear zone was seen in a spot assay test, including several *Salmonella* and *E. coli* strains. The EOP assay was performed for a host positive strain to eliminate lysis from without and evaluate the efficiency of phage to each strain compared with the primary strain (Table 1). Phage Rostam displayed a high (EOP > 0.5), medium (0.5 > EOP > 0.1) and low (EOP < 0.1) efficiency on 5, 3 and 12 strains, respectively.

3.2. Whole-genome sequencing of Rostam

The whole genome of Rostam was analyzed, showing a dsDNA genome of 53,028 bp. BLASTn analysis revealed many similar *Salmonella* phages, while a Viptree analysis pointed towards *Salmonella* phages SE13 (NC_048763), birk (NC_048864), yarpen (NC_048863) and UPF_BP2 (NC_048649) as closest relatives. The intergenomic distance between Rostam and these related *Salmonella* phages was calculated using VIRIDIC (Supplementary Figure 1), proving phage Rostam belongs to the same species as yarpen, birk and UPF_BP2 of the *Rosemountvirus* genus, which is not yet classified into a new taxonomic family of the *Caudoviricetes* class.

Annotation of the genome (Figure 2) did not reveal any lysogenic lifecycle-related genes, nor antibiotic resistance genes or virulence-related genes. Therefore, we can conclude Rostam is a strictly lytic phage, fit for biocontrol purposes.

3.3. Stability of phage Rostam under different conditions

Figure 3A demonstrates that there was no statistically significant difference in phage titer between 4°C and 40°C ($P > 0.05$). After 60 minutes of incubation at 60°C, the phage titer was reduced by 0.68 log PFU/ml compared to the control sample ($P < 0.05$), while at 80°C, it decreased by 5.81 log PFU/ml ($P < 0.05$). The pH stability of the phage was then evaluated (Figure 3B). At pH 2, no phage was visible, but the phage remained stable between pH 4 and 10 ($P > 0.05$). At pH=3 and 12, a decrease in phage titer of 1.63 and 1.87 log PFU/mL ($P < 0.05$) were observed compared to the standard SM buffer (pH=7.5), respectively. Regarding phage tolerance to NaCl (Figure 3C), no significant differences were observed in phage titer in the 1-9% NaCl range ($P > 0.05$). However, increasing the NaCl concentration to 11% and 13% decreased the phage titer by 0.7 and 0.87 log PFU/mL, respectively, compared to the standard SM buffer ($P < 0.05$). UV radiation gradually reduced phage titer over time (Figure 3D). In the first 40 minutes, the phage titer decreased by 0.47 log PFU/mL ($P < 0.05$), and at 120 minutes of

UV exposure, a decrease of 1.61 log PFU/mL ($P < 0.05$) was observed. These microbiological properties appear well adapted for its potential purpose in biocontrol.

3.4. Phage Rostam adsorption, one-step growth curve and kill curve

In the adsorption assay, the phage was almost completely adsorbed to its host bacterium within the first five minutes (99.13% adsorption), with only 0.87% of the initial free phage remaining unabsorbed. The one-step growth curve was plotted for the phage (Figure 1B), and it revealed a latent period of approximately 10 min and the burst size was determined at 60 minutes was 151 PFU/cell under standard growth conditions. For the kill-curve test, as shown in Figure 1C, the inhibitory effect of the phage on the host bacterium increased with increasing the MOI. Therefore, at hour 6, bacterial counts in all MOI were significantly different from the control group ($P < 0.05$). According to the results, for MOIs 1 and lower, the antibacterial effect was similar, with no significant differences between MOIs 0.1 and 0.001 at 1 and 6 hours ($P > 0.05$). Also, at hour 3, the difference between MOI 1 and 0.1 was not significant ($P > 0.05$). The reduction of the bacterial count at MOI 1000 and 0.01 and 0.001 at hour 6 compared to the control sample were 4.62, 1.05, and 0.76 log CFU/ml ($P < 0.05$), respectively.

3.5. Lytic activity of Rostam at lower temperature *in vitro*

Significant bacterial reductions ($P < 0.05$) were observed for all evaluated MOIs at both 25°C and 4°C (Figure 4). At 4°C bacterial counts were below the limit of detection ($P < 0.05$) after 3 and 6 hours at MOIs of 10,000 and 10, respectively. The maximum reduction of the bacterial count was reported at 25°C for MOI 10,000 with a decrease of log 4.83 CFU/ml after 24 hours ($P < 0.05$), and for MOI=10 the reduction was 2.87 log CFU/ml at 25°C ($P < 0.05$). At 4°C, the

control group maintained a stable count for 24 hours ($P > 0.05$), and at 25°C, the count increased by 4.27 log CFU/ml, compared to initial bacterial count (time=0) ($P < 0.05$).

3.6. Biocontrol of *S.* Enteritidis in liquid whole eggs by Rostam

The ability of the phage application was assayed in the liquid whole egg. After five days, the application of the phage against *S.* Enteritidis significantly decreased the bacterial count at both 4°C and 25°C ($P < 0.05$) (Figure 5A-B). For phage-treated groups kept at 4°C, there was a significant reduction in bacterial count ($P < 0.05$), at MOI 10,000 and 10; after 24 and 48 hours, the bacterial counts were for both MOIs under detection limit. The highest reduction at 25°C was 4.48 log CFU/ml ($P < 0.05$) and was observed at MOI 10,000. In addition, the reduction at MOI 10 for 25°C was 1.82 log PFU/ml ($P < 0.05$), compared to the control sample. At 25°C in the control group, the bacterial count increased by 4.54 log CFU/ml during the first 24 hours, while at 4°C it was stable for five days at around 4 log CFU/ml. The phage titer of control negative along with searching for any cross-contamination showed that the liquid whole egg had no contamination during five days of storage and the phage titer had no significant difference during five days of storage in the liquid whole egg at 4°C, whereas at 25°C it decreased by 1.4 log PFU/ml at 120 hours after storage (Figure 5C).

3.7. Biocontrol of S. Enteritidis on eggshells by Rostam

On eggshells, the biocontrol impact of the phage against *S.* Enteritidis infection was evaluated. In the first six hours, the reduction in phage-treated groups was 1.06 and 1.01 log CFU/egg for 4 ° C and 25 ° C, respectively. Figure 6A-B demonstrates that the bacteria were no longer detectable in the phage-treated group after 24h at 4°C and 48h at 25°C. After 120h, the bacterial count of control positive samples (phage-free) at 4°C and 25°C decreased by 1.7 and 1.1 log CFU/egg,

whereas the phage titer in the control negative group (only phage) decreased by 4.01 and 5.84 log PFU/egg (Figure 6C).

4. Discussion

In 2020, Eggs and its products were responsible for 44.0% of outbreaks that caused by *Salmonella* in the EU (EFSA, 2021). In Iran, there is a high prevalence of *Salmonella* in eggs, notably the Enteritidis strain (Hosseininezhad et al., 2020). In this study, we isolated a novel phage intending to control *S.* Enteritidis contamination on eggshell and whole liquid eggs. After isolating the phage from one of its best sources (Carey-Smith et al., 2006;Z. Li et al., 2020), sewage, the isolated phage was characterized, and then its efficiency in controlling *S.* Enteritidis in food was measured.

The ability of the phage to affect several *Salmonella* and *E. coli* strains, which are among the most virulent foodborne pathogens, offers the phage the potential to inhibit contamination caused by multiple bacterial strains. Since the serotypes of wild bacteria used in the host range test were unavailable, some of these strains may be the same. Therefore, the isolated phage can have a much narrower host range, which is a downside of the present study. In addition to the host range, the latent period and burst size tests are essential for assessing the lytic ability of the phage against its host bacteria. However, the optimal characteristics may change depending on the purpose of the phage application (Abedon, 1989; J. Li et al., 2021). Compared to previous studies, the phage has a short latent period (10 minutes), indicating that it reaches the stage of killing its host quickly (Kim et al., 2018; Mahmoud et al., 2018; Pham-Khanh et al., 2019; Yi et al., 2021). Since phages with a large burst size, such as the isolated phage, often need more time to achieve the burst size, it is suggested to use them when enough time is available for treatment (Abedon, 2009).

Several intrinsic and extrinsic factors, including pH, temperature, NaCl, and UV radiation, were simulated to evaluate phage stability. The evaluation of phage stability revealed its stability in a pH range of 4-10, which is consistent with most prior research, particularly given that a large variety of foods, including liquid whole eggs, fall into this pH range (Abhisingha et al., 2020; Whiley et al., 2015). The identification of the phage after 60 minutes of exposure to 80˚C demonstrated that this phage is among the heat-resistant phages (Bao et al., 2015; Islam et al., 2020). Stability to UV radiation and NaCl indicated the phage could persist in different food materials and during various processes employed in the food industry, particularly in liquid eggs and eggshells.

Bacteriophages kill their host bacterium by multiplying within the host cell or by adsorbing a large number of phages to the host. The key to combating infections is likely phage absorption, not their replication since the absorption is done quickly and in one impulse (Abedon, 2009). A large part of the sharp reduction of phage in MOIs of 100 and 10,000 during the first 60 minutes of the kill-curve assay can be due to the lethality of phage absorption. In addition, after 60 minutes, the trend of phage antimicrobial effects in various MOIs is relatively comparable since the antibacterial activity of phage is the result of multiplication rather than absorption during this period. These results are consistent with previous studies, and phage behavior at high MOI has been attributed to "lysis from without" in several studies (Z. Li et al., 2020; Wang et al., 2017). Lysis from without is caused by the adsorption of large numbers of phage to the surface of the host bacterial cells, causing envelope damage and, consequently, cell death without the need for phage multiplication (Abedon, 2011; Duc et al., 2020). Since no tests were performed in this study to confirm that the phage may initiate lysis from without, the more general term “abortive

infection" is used, which covers all situations in which the phage causes the death of its host bacterium without replication (Abedon, 2011).

The phage application at low temperatures (4°C) in TSB revealed its bactericidal ability at these temperatures, which was consistent with earlier research, and is a valuable characteristic in terms of biocontrol applications. In addition to the stability and antibacterial action of the phage at 4°C, this temperature inhibited the growth of bacteria, resulting in total control of *S.* Enteritidis.

It should be considered, according to the USDA risk assessment, one in every 3,600 eggs is naturally infected annually, with an infection rate between 1-100 bacterial cells per egg (USDA-FSIS, 2005). Also, USDA estimates that only 15% of contaminated eggs are exposed to infection > 1 log growth (USDA-FSIS, 2005). Considering growth factors during storage, the high contamination of eggs in this study with *S.* Enteritidis (10^4 CFU/egg and 10^4 CFU/ml) was much higher than the worst-case scenario for an egg under natural conditions. To treat a high level of bacterial contamination, the lethality of the phage by absorption alone is not enough, and it is necessary to rely on the multiplication of the phage inside the cell to kill the host (Abedon, 2009). The application of the phage for the treatment of whole liquid and eggshell demonstrated, similar to previous studies, that the isolated bacteriophage can be very efficient in treating *S.* Enteritidis, particularly in high MOIs and temperatures of 4 °C (Duc et al., 2020b; Z. Li et al., 2020; Yi et al., 2021).

In accordance with an *in vitro* test, biocontrol of *S.* Enteritidis in the liquid whole egg at 25°C demonstrated the regrowth of bacterial cells at this temperature. Although the bactericidal impact of the phage at 25°C in liquid whole eggs in the treated sample at MOI 1,000 was quite significant (4.48 log CFU/ml), on day five, the bacterial count returned to its initial level of 10^4 CFU/ml.

Based on the types of food and the abortive infection of phages at high titers, the use of high MOIs is beneficial for biocontrol, especially because phage with high titer is much more effective in treating a minimal amount of bacterial contamination. Furthermore, in addition to being solid and liquid, various food items may lower the probability of phage and bacteria interacting, and the food itself may include factors that inhibit or boost phage lytic activity.

As in some earlier studies, the use of phage for the biocontrol of *S.* Enteritidis on eggshell yielded acceptable results, particularly at 4 °C, for example, based on results presented by Spricigo et al. (Spricigo et al., 2013), the decrease in *Salmonella* count (combination of *S.* Enteritidis and *S.* Typhimurium) on the eggshell in two hours was 0.9 log CFU/cm^2 at room temperature (Z. Li et al., 2020; Yi et al., 2021). Conditions of phage biocontrol on eggshells were slightly different because bacterial cells were unable to regrow at both 4 and 25°C, potentially because of the lack of essential nutrients on the cleaned and heated eggshells.

Based on the findings of the present study and the recommendation to store the egg or its liquid at a temperature of 4 °C, it appears that phage is a realistic choice for the safety of eggs and can be applied via spraying, dipping, or mixing throughout the entire food chain, from farm to fork. Phage genome analysis showed that phage Rostam, in addition to being strictly lytic, could be used in the food industry as a safe biocontrol agent against *S.* Enteritidis. There have been studies performed in the subject of phage safety, but the most significant problem of employing phage in food may be convincing customers to take a food that is containing the virus, even though this virus is naturally prevalent in the food materials (Kazi & Annapure, 2016).

5. Conclusions

In summary, the anti *S.* Enteritidis phage was isolated and characterized in this study. *Rosemountvirus* Rostam indicated good characteristics, including rapid adsorption, short latent period, large burst size, stability at various pH, thermal, NaCl, UV radiation, and high lytic ability at low temperatures, especially at 4°C. The results of application of Rostam to treat *S.* Enteritidis contaminated whole egg liquid and shell indicate that the isolated phage can potentially be used for biocontrol in different food matrixes.

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Table 1. Host range and efficiency of plating (EOP) of the phage.

Bacterial strain	Source	Identification Method	Spot test[a]	EOP[b]
Escherichia coli EPC(M) o55:K 59	PTCC 1269		+	Low
Escherichia coli	PTCC 1533		+	Medium
Escherichia coli	PTCC 1551		+	Medium
Escherichia coli	ATCC 35218		-	
Escherichia coli	PTCC 1330		+	High
Escherichia coli	PTCC 1399		+	Low
Escherichia coli	PTCC 1270		+	Low
Escherichia coli	PTCC 1276		-	
Salmonella enterica subsp. *enterica* Typhimurium	ATCC 700720		+	High
Mycobacterium kansasii Hauduro	ATCC 25222		-	
Staphylococcus aureus subsp. *aureus* Rosenbach	ATCC 29213		-	
Staphylococcus aureus subsp. *aureus* Rosenbach	ATCC 6538		-	
Pseudomonas aeruginosa	PTCC 1555		-	
Salmonella 2	Retail	PCR	+	High
Salmonella 248.1	Retail	PCR	+	Medium
Salmonella 31	Retail	PCR	+	Low
Salmonella 44	Retail	PCR	+	Low
Salmonella 44	Retail	PCR	+	Low
Salmonella 42	Retail	PCR	+	High
Salmonella 43.1	Retail	PCR	+	Low
Salmonella 79	Retail	PCR	-	
Salmonella 13	Retail	PCR	-	
Salmonella 259.1	Retail	PCR	+	High
Salmonella 53	Retail	PCR	-	
Salmonella 280.1	Retail	PCR	+	Low
Salmonella 251.1	milk	PCR	-	
Escherichia coli 94	milk	PCR	+	Low
Escherichia coli 95	milk	PCR	+	Low
Escherichia coli 96	milk	PCR	+	Low
Escherichia coli 105	milk	PCR	+	Low
Listeria 88	milk	PCR	-	
Enterococcus		PCR	-	
klebsiella		PCR	-	
Micrococcus 6		PCR	-	
Pseudomonas		PCR	-	
Proteus	Retail	PCR	-	
Staphylococcus 8	Retail	PCR	-	
Staphylococcus 187	Retail	PCR	-	
Staphylococcus 272	Retail	PCR	-	
Staphylococcus 257.1	Retail	PCR	-	

[a] Host range result: (+) clear lysis, (-) without lysis.

[b] Efficiency of Plating (EOP) result: EOP >0.5, medium impact: 0.1 <EOP <0.5 and low impact: EOP < 0.

Figure 1. The bacteriophage morphology and *in vitro* phage-bacterium challenge test: (**A**) Morphology of the *Salmonella* bacteriophage, examined by transmission electron microscope; (**B**) One-step growth curve; (**C**) The kill curve assay of the phage at different MOIs against *S.* Enteritidis (PTCC 1787) based on bacterial count. Values represents mean with standard deviation of three experiments.

Figure 2. Phage Rostam genome map and comparison to the related phage UPF_BP2 using BLASTn (greyscale). Each arrow represents a coding sequence. Linked, genes encoding packaging and lysis-associated proteins are displayed, in green structural proteins and in blue DNA- and metabolism-associated proteins (adapted from EasyFig).

Figure 3. stability of the phage in temperature, pH, NaCl, and UV radiation: (**A**) temperature; (**B**) pH; (**C**) NaCl; (**D**) UV radiation. Values represents mean with standard deviation of three trials.

Figure 4. *In vitro* phage-bacterium challenge test: (**A**) bacterial count at 4°C; (**B**) bacterial count 25°C. Values represents mean with standard deviation of three trials. * Significant at $P < 0.05$.

Figure 5. Biocontrol of *S.* Enteritidis in liquid whole eggs: (**A**) bacterial count at 4°C; (**B**) bacterial count 25°C; (**C**) phage persistence at 4 and 25°C. Values represents mean with standard deviation of three trials. * Significant at $P < 0.05$.

Figure 6. Biocontrol of *S.* Enteritidis on eggshell: (**A**) bacterial count at 4°C; (**B**) bacterial count 25°C; (**C**) phage persistence at 4 °C and 25°C. Values represents mean with standard deviation of three trials. * Significant at $P < 0.05$.

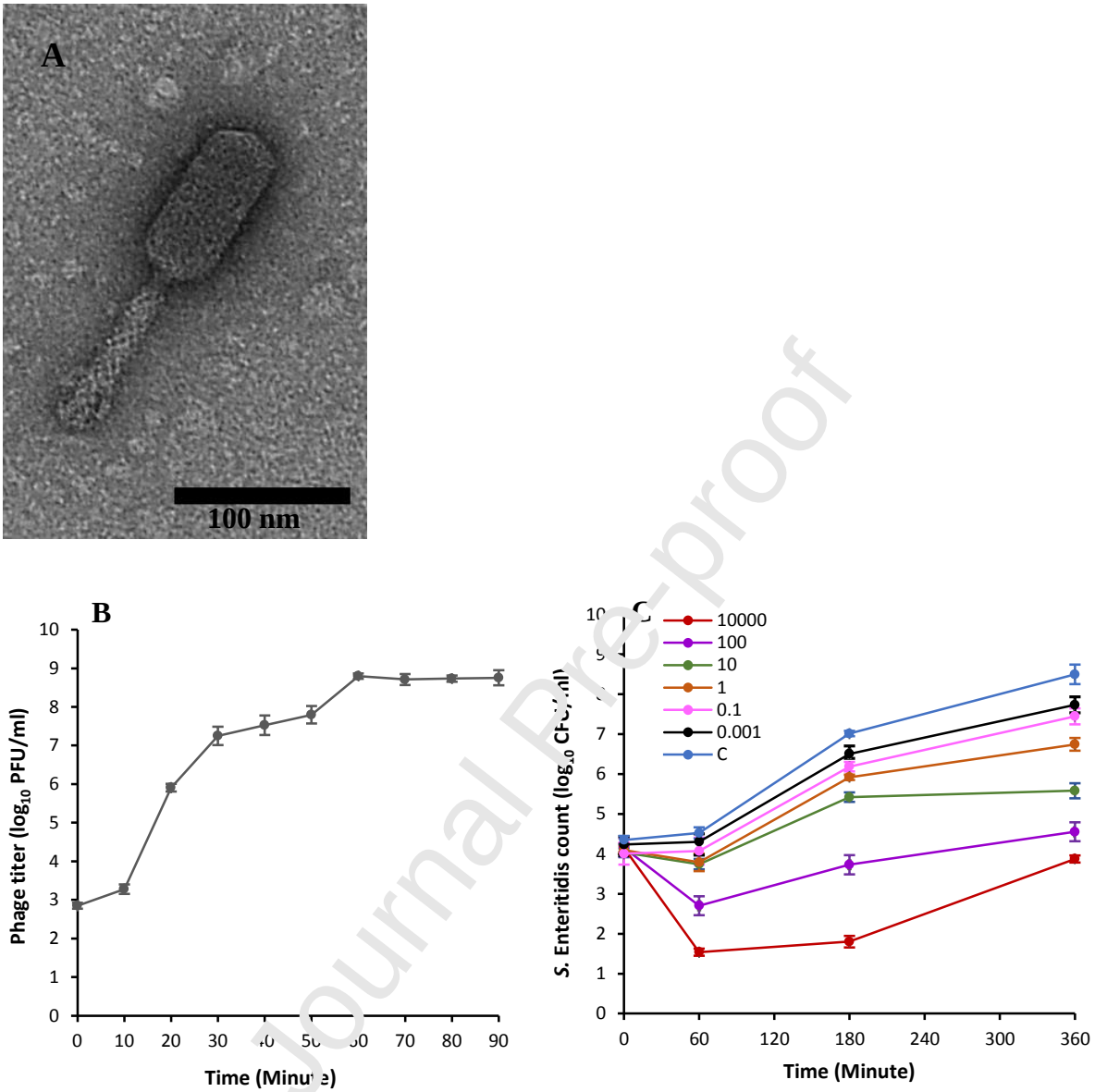

Fig. 1

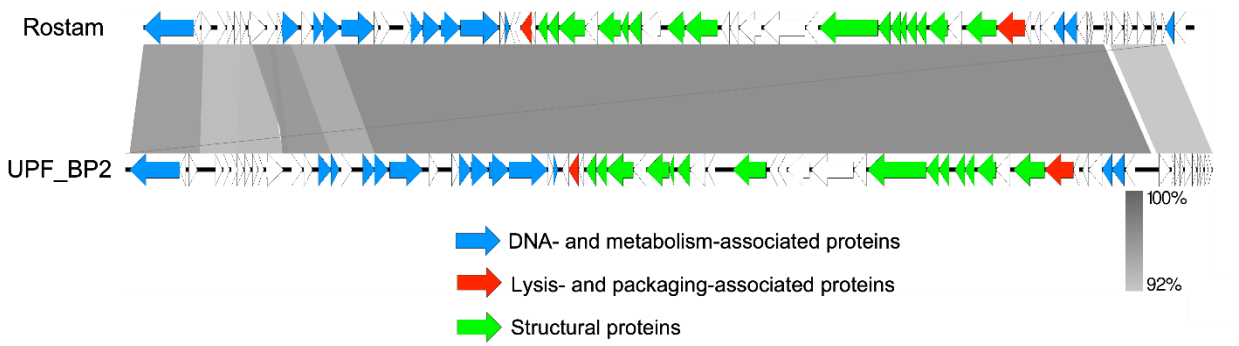

Fig.2

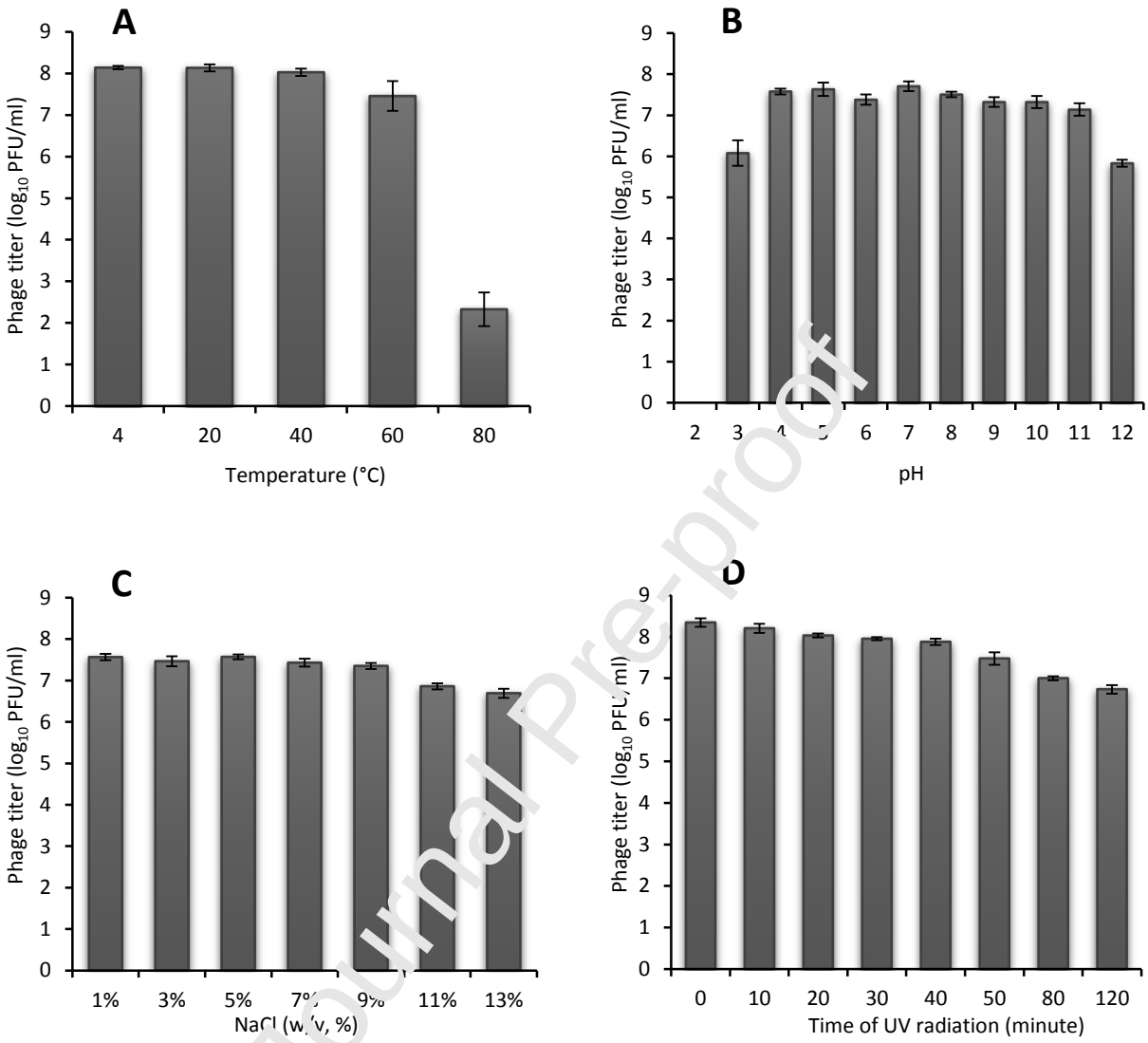

Fig.3

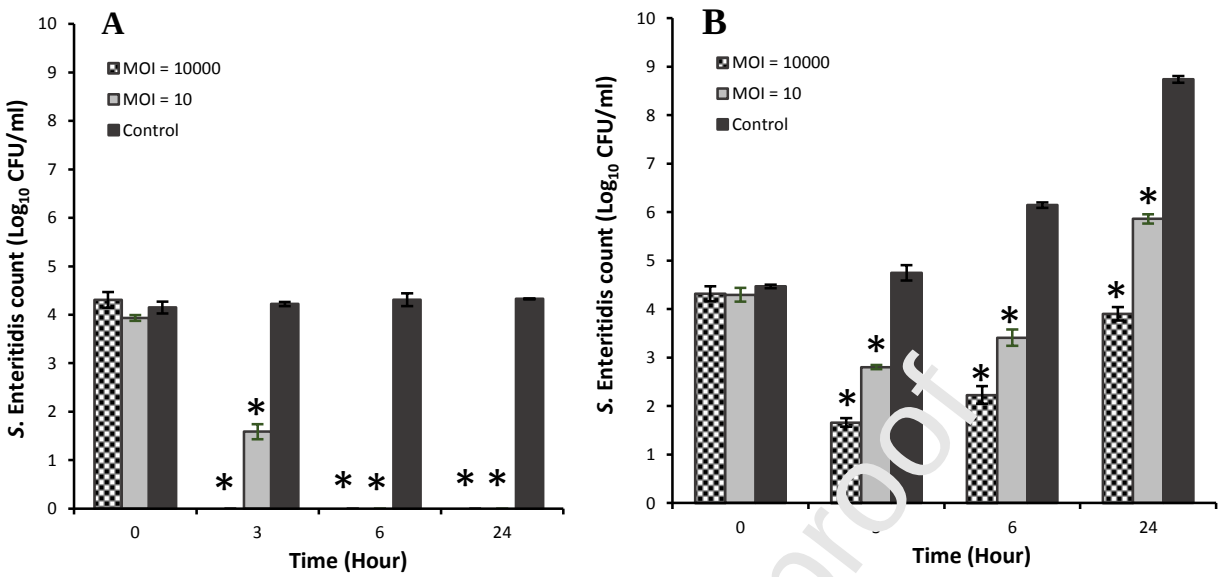

Fig.4

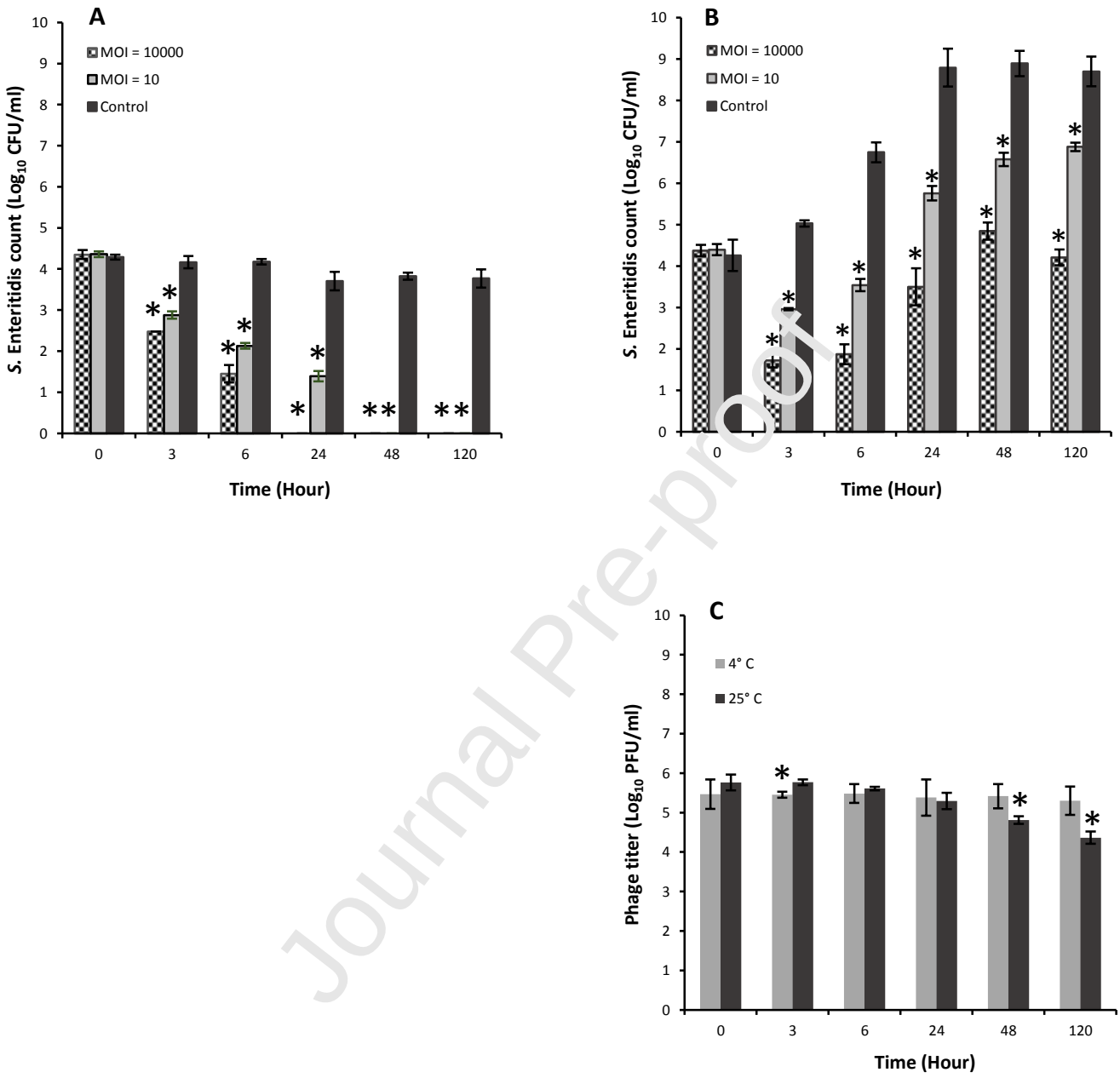

Fig.5

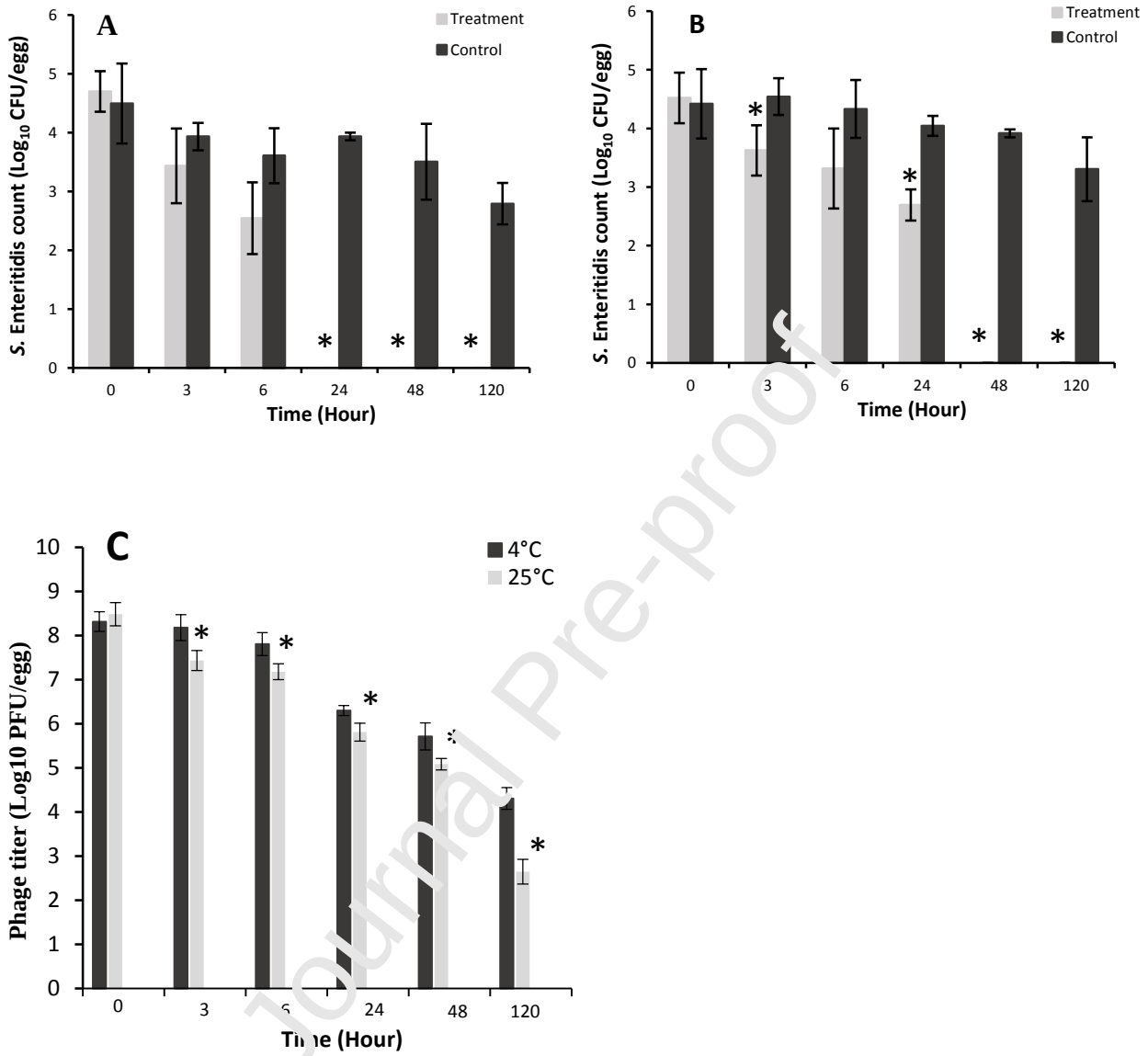

Fig.6

Declarations of interest

None.

Highlights

- Bacteriophage Rostam is highly lytic, adsorb fast and stable under different environmental circumstances.
- Bacteriophage Rostam has 53kb genome sequence with no toxins or lysogeny-associated genes and it is suitable for biocontrol purposes.
- In liquid whole egg phage Rostam reduced the number of *S.* Enteritidis at 25°C by 4.48 log units and at 4°C to below the detection limit.
- In eggshell bacteriophage reduced the number of *S.* Enteritidis at 25 ° and 4 ° C to below the detection limit.