Journal of Applied Biological Sciences
E-ISSN: 2146-0108 18(1): 33-41, 2024
DOI: https://doi.org/10.71336/jabs.1277
Research Article

CONTROL OF *SALMONELLA* ENTERITIDIS AND *SALMONELLA* KENTUCKY ON EGGSHELL WITH BACTERIOPHAGE APPLICATION

Başak Kızal[1], Naim Deniz Ayaz[2*], Gizem Çufaoğlu[2], Muammer Göncüoğlu[3],

[1]*Kırıkkale University, Graduate School of Health Sciences, Department of Food Hygiene and Technology Kırıkkale, Türkiye*
[2]*Kırıkkale University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, Kırıkkale, Türkiye*
[3]*Ankara University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, Ankara, Türkiye*

**Corresponding Author:*
E-mail: naimdenizayaz@kku.edu.tr

(Received 24th October 2023; accepted 27th January 2024; published: 31st January 2024)

ABSTRACT. *Salmonella* is one of the most common public health problems responsible for foodborne outbreaks, and contaminated table eggs are one of the primary sources of transmission. The aim of this study was to determine the effects of bacteriophage application for the control of *S.* Enteritidis and *S.* Kentucky on whole eggshells. Eggs were experimentally contaminated with low (~2 log cfu/g) and high (~4 log cfu/g) levels of *S.* Enteritidis and *S.* Kentucky. Eggs in the phage group were treated with phage cocktail suspension (SEnt1-2-1 and SKen1-1-1) (10^8 pfu/mL), and incubated at 20±1°C for 24 h. *Salmonella* counts were recorded at 1st, 3rd, 6th, and 24th hours. In the control groups of both *Salmonella* strains, the bacterial counts were changed by ±1.0 log cfu/g until the 6th hour. In the 24th hour, almost 2 log cfu/mL reductions were observed except for the *S.* Kentucky high contaminated group (KK4). *Salmonella* counts remained under the detection limit (1.0 log cfu/g) from the beginning of the first hour in all bacteriophage treated groups. In conclusion, the phage cocktail reduced *Salmonella* on eggshells for both two contamination levels (log 2 and log 4). Therefore, in laying, breeding farms and egg processing facilities, phage application on the eggshell surface can be suggested to reduce the *Salmonella* load.

Keywords: *Bacteriophage, biocontrol, eggshell, Salmonella, Enteritidis, Kentucky.*

INTRODUCTION

Salmonella is one of the most common public health problems responsible for foodborne outbreaks worldwide. According to the CDC (Centers for Disease Control and Prevention) reports, *Salmonella* is the organism that most frequently causes foodborne infections [1]. Besides, *Salmonella* contributes to the economic load of developed and developing countries due to the health expenditures of disease [2]. The bacterium often transmits to humans through animal originated foods such as meat, eggs, and milk [3].

Contaminated table eggs are reported as one of the main sources of foodborne salmonellosis [4]. *Salmonella* Enteritidis is one of the two major serotypes responsible for foodborne *Salmonella* outbreaks in the world [5]. On the other hand, *Salmonella* Kentucky was reported as the most frequent serotype in laying hens according to the data

of the National *Salmonella* Control Program of Türkiye [6]. Especially, *Salmonella enterica* subsp. *enterica* serovar Enteritidis is of concern for many egg producers worldwide [7]. It has been reported that *Salmonella* serotypes, especially *S.* Enteritidis, can also be transmitted from infected laying hens to egg yolk by transovarian transmission [8, 9]. Also, the eggshell contains many pores which provide a pathway for gas transfer for the embryo. However, these channels can also allow bacteria to pass through shell and get into the interior which the nutrient-rich part of the egg [10]. At the same time, contamination of the eggshell surface with *Salmonella* may also cause cross-contamination in the period from post-laying to consumption. Therefore, the control of zoonotic pathogens on the eggshell plays a significant role in maintaining the farm-to-fork food safety chain.

Salmonella transmission poses a threat to human health because overgrowth of *Salmonella* in eggs doesn't cause odor, consistency, and color changes in egg contents [11]. With vaccination programs for laying hens, some achievements were obtained and some reductions in the incidence of human *S.* Enteritidis infections were accomplished. However, no type of vaccine has consistently provided an insurmountable barrier to infection, especially at *Salmonella* contamination level [12]. On the other hand, antibiotic treatment may be one way to reduce the prevalence of *Salmonella* in laying flocks. As with many other pathogens, antibiotic resistance is a big problem for *Salmonella* as well [13]. The overuse and misuse of antibiotics led to an increase in drug resistance, including disinfectants. Moreover, biofilm formations and complex equipment especially in laying hen farm level make disinfectants more difficult to reach *Salmonella* cells [14, 15]. In this point of view, some other alternative solutions are needed to apply, and bacteriophages could be considered one of them. Bacteriophages have been used for various purposes such as treatments of diseases (therapy), decontamination in foods (biocontrol), sanitation of surfaces and equipment in contact with food (biosanitation) and extend the shelf life of ready-to-eat foods (biopreservation) [16]. In this concept, this study aimed to investigate the control of *S.* Enteritidis and *S.* Kentucky in whole eggshells with a bacteriophage cocktail.

MATERIALS AND METHODS

Materials

Eggs were purchased from markets in Ankara on the study day and were taken to the laboratory in their original packaging. *S.* Enteritidis ATCC 13076 and previously isolated *S.* Kentucky [6] strains were used as reference strains. Also, two lytic bacteriophages encoded SEnt1-2-1 and SKen1-1-1, which were isolated and characterized in a previous study (TÜBİTAK, Project number: 121Z447), were used (Figure 1). Bacteriophages and reference *Salmonella* strains were obtained from the culture collection of Kırıkkale University, Department of Food Hygiene and Technology.

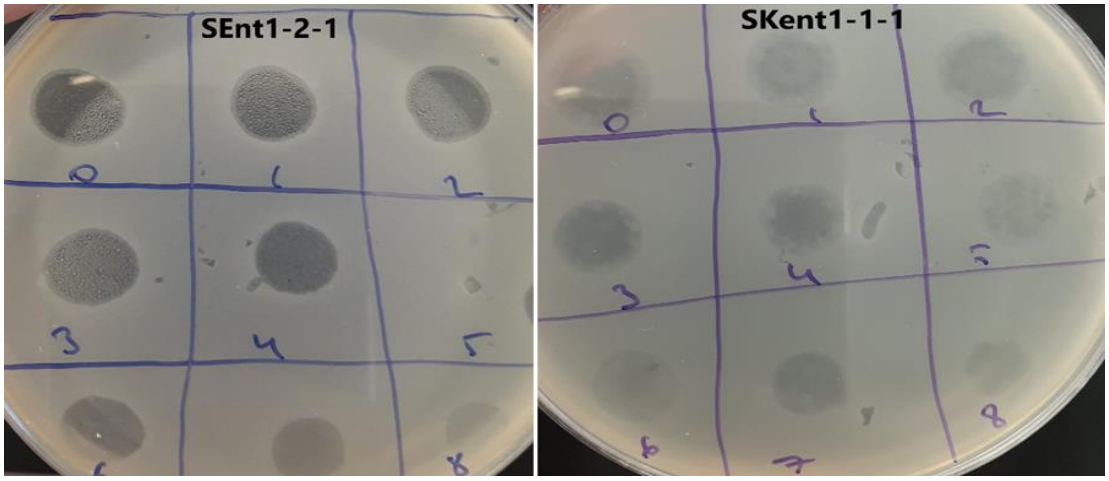

Figure 1. *Lytic effect of serially diluted bacteriophages SEnt1-2-1 and SKent1-1-1 on host Salmonella strains.*

Preparation of Salmonella cultures

Salmonella strains were enriched in Tryptic Soy Broth (TSB, Oxoid CM0129) overnight. Then, serial dilutions were prepared and in order to prepare the bacterial inoculums in the targeted cfu/mL strains were measured in a spectrophotometer (Thermo Scientific Multiskan GO, Finland) at 600 nm wavelength (OD_{600}). Colony counts of bacteria cultured were confirmed on Xylose Lysine Deoxycholate agar (XLD, Oxoid CM0469) by plating [17].

Preparation Salmonella phages

Phage susceptibility profile tests of bacteriophages were previously determined (TUBITAK Project number: 121Z447). Accordingly, the bacteriophages SEnt1-2-1 and SKen1-1-1 which showed the widest lytic spectrum on *S.* Enteritidis and *S.* Kentucky isolates were selected for this study. Each phage was propagated in log-phased host *Salmonella* cell cultures enriched in TSB and incubated at 37 °C for 24h. The next day, they were centrifuged (Hermle, Z326K, Germany) at 5000 rpm for 3 min, passed through a sterile 0,22 µm diameter millipore filter, and transferred into sterile tubes.

The titer of each phage was determined by the spot test method on double layer Luria Bertani agar (LB, Merck, 110283). In brief, serial dilutions of *Salmonella* phages were prepared in TSB, and inoculums were spotted on LB agar contaminated with bacterial cultures. Petri dishes were incubated at 37ºC for 24 hours and plaques (clear zones) on the agar were counted to determine phage titers (pfu/mL).

Preparation of eggs used in the experiment

The shells of whole eggs were disinfected with 70% ethyl alcohol followed by boiling distilled water to eliminate the possible bactericidal effect of alcohol. After drying, eggs were contaminated with *S.* Enteritidis or *S.* Kentucky separately for the control (C) and phage (P) groups as shown in Figure 2. Two contamination levels were preferred (2 and 4 log cfu/mL) for the experiment so, consequently, a total of 36 eggs were used.

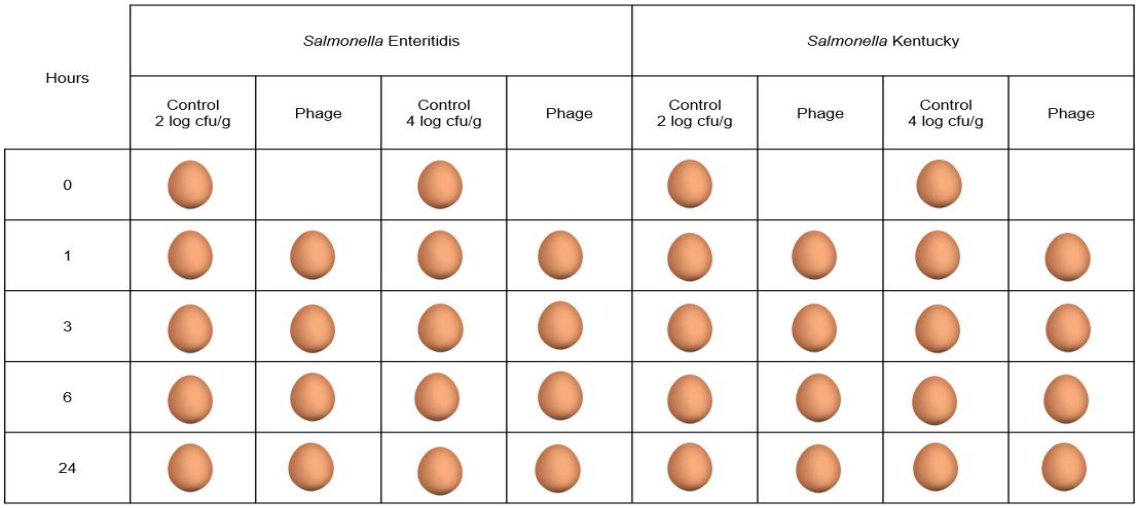

Figure 2. *Experimental design of Salmonella phage application in eggshells.*

Contamination and incubation of Salmonella on eggshells

The outer surface of each eggshell was individually contaminated with 0.05 mL of *Salmonella* fresh culture in sterile sample bags with a final titer of approximately 2 log cfu/g and 4 log cfu/g. Eggs of the phage groups were treated with one mL of 10^8 pfu/mL phage suspension. Control groups were treated with sterile distilled water instead of bacteriophage suspension (Table 1). In the Turkish Food Codex, it is not obligatory to refrigerate the eggs until the 18th day after the laying date. For this reason, it is common to keep eggs at room temperature for the first 18 days in warehouses and markets. Since it further encourages *Salmonella* growth, the possible worst scenario was preferred in our study and eggs were incubated at room temperature (20±1°C) for 24 h. During this period, at the first hour, third, sixth, and twenty fourth hour eggs were rinsed in peptone water in sterile plastic bags, serial dilutions were prepared, and plated on XLD agar. After incubation at 37°C overnight, colonies were counted in order to determine the reductions in the number of *S.* Enteritidis and *S.* Kentucky on the eggshells. The presence of *Salmonella* in the samples (bacteriophage-treated experimental groups) whose *Salmonella* counts could not be detected was confirmed method by subjecting them to the pre-enrichment process [18].

Table 1. *The experimental design of phage and control groups.*

	Phage group								**Control group**							
Amount of phage added to groups (pfu/g)	10^8								-							
Salmonella contamination level (cfu/g)	10^2				10^4				10^2				10^4			
Mol for 10^8 pfu/g phage	10^6				10^4				-				-			
Plating time (hours)	1	3	6	24	1	3	6	24	1	3	6	24	1	3	6	24

MOI: Multiplicity of Infeciton

Determination of Salmonella reductions and statistical analysis

The experimental trials of the study were performed as two separate experiments, and the cultivation on plates was carried out in three parallels. The reduction in *Salmonella* counts in the bacteriophage-treated experimental groups was compared with the phage-free control group. IBM SPSS Statistics 25 software was used to determine whether the reductions were significant using the One-way ANOVA Test. Results with a p-value < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

In this study, control of *S.* Enteritidis and *S.* Kentucky with two lytic *Salmonella* phages (SEnt1-2-1 and SKen1-1-1) in eggshells was investigated. The reductions of *Salmonella* in the phage groups were compared with the phage-free control groups, and the differences between the colony counts are given in Table 2. Two contamination levels were aimed in the experiment (log 2 and log 4) in order to achieve two MOIs. Accordingly, the initial contamination levels of egg samples were 2.60 log cfu/g and 4.23 log cfu/g for *S.* Enteritidis, 2.30 log cfu/g, and 4.04 log cfu/g for *S.* Kentucky. In the control groups of both *Salmonella* strains, the bacterial counts were changed by ±1.0 log cfu/g until the sixth hour. In the 24th hour, almost 2 log cfu/mL reductions were observed except for the *S.* Kentucky high contaminated group (KK4) (Table 2). This decrease may be due to various reasons. Such as in the study, eggshells were enriched with overnight incubation and were probably contaminated with *Salmonella* at the end of the logarithmic growth phase or in the stationary phase, so the decreases seen in the control group may be possible towards the end of the storage period. Moreover, it is known that *Salmonella* can switch to the viable but nonculturable (VBNC) form in conditions that are not suitable for their reproduction and that they can maintain their viability in this form, but cannot be cultured [19]. However, study findings have shown that eggshells are not an environment that supports the growth of *Salmonella* at room temperature (20±1°C). Importantly, in all of the phage treated groups *Salmonella* counts remained under the detection limit of 1.0 log cfu/g ($p<0.05$). Hence, the phage cocktail of SEnt1-2-1 and SKen1-1-1 was found effective against *Salmonella* in eggshells for two contamination levels (log 2 and log 4).

Table 2. *Salmonella counts on XLD agar for phage and control groups.*

	***S.* Enteritidis counts (log cfu/g)**				***S.* Kentucky counts (log cfu/g)**			
Hour	**EK2**	**EF2**	**EK4**	**EF4**	**KK2**	**KF2**	**KK4**	**KF4**
0	2.60		4.23		2.30		4.04	
1	2.30	<1.0*	4.17	<1,0*	3.60	<1.0*	3.47	<1.0*
3	2.20	<1.0*	3.94	<1,0*	3.83	<1.0*	3.59	<1.0*
6	3.30	<1.0*	4.20	<1,0*	1.20	<1.0*	3.50	<1.0*
24	1.00	<1.0*	2.51	<1,0*	1.11	<1.0*	3.62	<1.0*

: Not detected, under the detection limit of 1.0 log cfu/g., **EK2: Eggs contaminated with S. Enteritidis at approximately 10^2 cfu/g, **EF2:** Bacteriophage SEnt1-2-1 applied eggs contaminated with S. Enteritidis at approximately 10^2 cfu/g, **EK4:** Eggs contaminated with S. Enteritidis at approximately 10^4 cfu/g, **EF4:** Bacteriophage SEnt1-2-1 applied eggs contaminated with S. Enteritidis at approximately 10^4 cfu/g, **KK2:** Eggs contaminated with S. Kentucky at approximately 10^2 cfu/g, **KF2:** Bacteriophage SKen1-1-1 applied eggs contaminated with S. Kentucky at approximately 10^2 cfu/g, **KK4:** Eggs contaminated with S. Kentucky at approximately 10^4 cfu/g, **KF4:** Bacteriophage SKen1-1-1 applied eggs contaminated with S. Kentucky at approximately 10^4 cfu/g*

In our study, 36 eggs were washed in boiling water and disinfected with 70% ethanol, then eggshells were contaminated with *S.* Enteritidis and *S.* Kentucky at two different levels of 2 and 4 log cfu/g. Distilled water was applied to the control group's eggshells, while a phage cocktail was applied to the test groups at the level of 8 log pfu/mL. Our results showed that lytic phage cocktail application was achieved to reduce *Salmonella* contamination under the detection limit of 1.0 log cfu/g on the surface of whole eggshell ($p<0.05$). In a study conducted in India with a similar experimental design, 40 eggs were washed and disinfected with 70% ethanol and then divided into 4 groups of 10 eggs each. Eggs in the first two groups were infected with 4×10^5 cfu/mL *Salmonella* Enteritidis and treated with phages at 0.01 MOI. Eggs were incubated at 37°C for up to 2 hours. The authors reported that after 30 minutes phages were able to reduce the bacterial load by 99.9% (~3 log cfu/g) [20]. In another study, *Salmonella* solution of 10^7 cfu/mL and bacteriophage preparation of 10^8 pfu/mL were applied to the eggshells. For the control group, at the initial point, bacterial count was recorded 5.34±0.64 log cfu/mL, whereas after phage application the contamination level decreased to 3.41±0.79 log cfu/mL with a reduction level of 1.93 log. Then the eggs were incubated for 7 days at 4°C. At the end of this period, the concentrations of *Salmonella* Enteritidis for the control and phage groups were recorded as 5.33±0.55 log cfu/mL and 3.09±0.02 log cfu/mL, respectively. Consequently, the level of reduction was determined as 2.24 log. The result showed that a reduction of more than 2 logs was achieved in 7 days with phage treatment at 10 MOI. Additionally, when 10^5 log cfu/mL *Salmonella* solution and 10^8 pfu/mL bacteriophage solution were used, the reduction was raised up to 4.2 log at the same incubation conditions [21]. Although it has a different matrix from our study, As previously reported by Yi et al. [22], 10 mL of homogenized egg samples were contaminated with 10^3 cfu/mL *Salmonella* Typhimurium and Enteritidis. Then the liquid eggs were inoculated with two phages at 100 MOI. In the study, both phages (OSY-STA and OSY-SHC) exhibited lytic activity against *Salmonella* Typhimurium and *Salmonella* Enteritidis serovars. The treatment resulted in more than 1.8 and 2.5 log cfu/mL reduction in *Salmonella* Typhimurium and *Salmonella* Enteritidis, respectively [22]. In another study, liquid homogenized egg samples were inoculated with 100 µl of 10^5 cfu/mL *Salmonella* Typhimurium or Enteritidis and treated with 100 µl phage cocktail at a concentration of 10^7 pfu/mL. Phages were heat stable (55°C) and the efficacy of the phage cocktail was determined by counting the viable *Salmonella* population after 24 h incubation at 4°C. Phage cocktail application combined with heating treatment up to 55°C resulted in more than 2.5 log cfu/mL reduction in *Salmonella* Enteritidis [22]. Control of *Salmonella* was also investigated in some laying hen environments Evran et al. [23] investigated the effectiveness of a *Salmonella* cocktail in drinking water, shavings, and plastic surfaces in laying hen environment. According to the data, the phage cocktail could reduce the amount of *Salmonella* in drinking water by up to 2.80 log, shavings by up to 2.30 log, and plastic surfaces by up to 2.31 log. Phage mixtures have been found by the authors to be a promising substitute for minimizing *Salmonella* contamination in chicken environments. In another study, a lytic phage, LP31, was isolated from fecal samples of poultry. LP31 was found to reduce the number of *S.* Enteritidis in chick feces (2.14 log cfu/g) and on metal surfaces with a number of 0.951 log cfu/mL. In particular, it has been observed that LP31 can remove biofilms almost completely formed by *S.* Pullorum and *S.* Enteritidis within one hour. The authors claimed that LP31 has a well controlled effect against biofilms and antibiotic-resistant *Salmonella*. This makes the LP31 an effective biocontrol agent to control the spread of *Salmonella* in the food processing plants especially in the

poultry sector [24].

The human population and the need for animal food are increasing day by day. On the other hand, the limitation of animal production is discussed around the world due to problems related to crops, water, and land use, as well as greenhouse gas emissions and animal welfare. This situation brings with it the risk of food security [25, 26]. For this reason, how much we should compromise on food safety to ensure food security is among the questions that need to be answered in the coming period. About this question, the necessity of a zero tolerance limit for *Salmonella* in whole eggs is a matter of debate. In our study, we succeeded in reducing the presence of *S.* Enteritidis and Kentucky on the surface of eggshells below the detection limit with bacteriophage application. Thus, we think that the risk of cross-contamination from eggshells during the packaging, distribution, preservation, and food production (in food production plants and the kitchen) can be significantly reduced.

CONCLUSION

In the study, it was observed that the counts of *S.* Enteritidis and *S.* Kentucky decreased below the detection limit of 1.0 log cfu/g on the surface of eggshells from the first hour of application of phages SEnt1-2-1 and SKen1-1-1. In conclusion, bacteriophage treatment can be considered promising in the control of *S.* Enteritidis and *S.* Kentucky on eggshells contaminated with low and high levels. Phage application to the eggshell surface can be used to reduce *Salmonella* load in laying hen farms and egg processing plants. Nevertheless, it is also important to investigate various application models, conditions, and different serotypes in future studies. As a result of our study, it was determined that bacteriophage application to eggs contaminated with low and high levels of *S.* Enteritidis and *S.* Kentucky can effectively provide biocontrol.

Acknowledgement. This article contains the findings of Başak KIZAL's Master thesis of Kırıkkale University Graduate School of Health Sciences, Department of Food Hygiene and Technology.

Conflict of Interest. The authors declared that there is no conflict of interest.

Authorship Contributions. Concept: N.D.A., B.K., Design: N.D.A., B.K., M.G., G.C., Data Collection or Processing: B.K., N.D.A., Analysis or Interpretation: B.K., N.D.A., Literature Search: B.K., N.D.A., Writing: N.D.A., B.K., M.G., G.C.

Financial Disclosure. This research received no grant from any funding agency.

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