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*Microbiology*

# The Use of Bacteriophages for Decontamination of Experimentally Contaminated Chicken Breast Meat

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***Salmonella* spp. is one of the major cause of food-borne diseases and chicken meat is considered as the main source of *Salmonella* infection in humans. The present study evaluated the ability of bacteriophage preparation, composed of three lytic phages, in reducing *Salmonella* strain on chicken breast fillets at 25°C and 4°C. Multiplicities of infection were $10^4$ CFU/ml S. *typhimurium* and the efficacy of bacteriophage preparation was evaluated by application of two different concentrations $10^5$ PFU/ml and $10^8$ PFU/ml. Surface treatment of *Salmonella* inoculated chicken breast fillet samples with bacteriophages $10^8$ PFU/ml reduced *Salmonella* from 3.8 to 4 log CFU/ml ($P < 0{,}05$) after 1 and 24 h, respectively. $10^5$ PFU/ml Bacteriophage surface application on chicken breast fillets stored at room temperature (25°C) reduced the *Salmonella* counts from 0.6 to 2.2 log CFU/ml, compared to the untreated positive control. *Salmonella* counts were reduced by 0.2 and 0.6 log CFU/ml holding 1 and 24 h, stored at 4°C, that demonstrates the phage activity at refrigeration temperature. These results indicate the potential efficacy of the bacteriophage cocktail as a biological agent against *Salmonella* in raw chicken breast meat.** *© 2021 Bull. Georg. Natl. Acad. Sci.*

*Salmonella*, bacteriophage, decontamination, chicken, food safety

Most infections are associated with food consumption of animal origin, and poultry meat and meat products are considered to be the major carriers of *Salmonella* spp. to humans. *Salmonella* is often detected in meat and meat products, which are usually linked to outbreaks [1]. Several risk factors for horizontal transmission of *Salmonella* are identified, including a poor level of hygiene, the presence of rodents and insects on the farm, inadequate cleaning between the rotation of flocks, and contamination of the feed and drinking water. The contamination may occur throughout the poultry production chain, and processing steps such as head pulling and evisceration are considered as potential risk factors that contribute to the high incidence of *Salmonella* spp. in chicken carcasses [2]. At slaughter, the gastrointestinal tract may harbor *Salmonella* and may be damaged during the slaughter process, resulting in contaminated carcasses. Furthermore, cross-contamination can occur from a *Salmonella*-positive flock or the slaughter equipment to the carcasses of a

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*Salmonella*-free flock [3]. Although this bacterium is inactivated when these foods are properly cooked, *Salmonella* may survive for several days on surfaces on which the foods are processed and handled before cooking. If other foods, especially ready-to-eat foods (e.g., fruits and vegetables), come into contact with those contaminated surfaces, *Salmonella* may contaminate them and cause food-borne salmonellosis [4]. The presence of pathogens in chicken meat is an important issue due to a risk to public health. Therefore, several decontamination approaches including pre- and post-harvest applications have been used to eliminate or reduce pathogens [5]. Even if the decontamination of pathogens is ensured at the pre-harvest stage, cross-contamination at the post-harvest stage may still occur during further cutting, processing, packaging, storage, and transport [6].

Many decontamination technologies based on physical, chemical, and biological approaches have been subjected to scientific trials over the years in order to reduce the microbial contamination of meat [7]. But these strategies, while effective, have significant drawbacks, such as corrosion of the equipment, toxic chemical residues, and damage to the quality of foods [8].

The search for new decontamination technologies has recently gained the interest of the poultry industry. Bacteriophages are bacterial viruses that have great potential for the use as biocontrol agents in foods [9]. They offer a number of desired properties such as specificity for the target bacteria, inability to infect human cells, capacity for self-replication and self-limiting, and ubiquitous presence in nature, that makes them excellent tools for food safety purposes. The concept of reducing pathogens in food by using phages has mainly addressed raw and raw meat and ready-to eat (RTE) products, and also decontamination of carcasses [10]. Nowadays, there are some commercialized phage preparation against *Listeria monocytogenes*, *Salmonella*, *Shigella*, and *E. coli* that use phages as biopreservation agents of food [11]. The efficacy of different phage preparations to inactivate various foodborne pathogens, including *Salmonella*, has been studied in pre-harvest and post-harvest applications [12]. Salmofresh$^{TM}$, a commercial phage preparation (Intralytix, Inc., containing 6 lytic monophages) specifically targeted against *Salmonella*, has been approved as Generally Recognized as Safe product by the USFDA [13]. Pathogenic microorganisms in biofilm forms are the main sources of food contamination. Bacteria within biofilms have much higher resistance to antibiotics and have a successful strategy protecting them from unfavorable environmental conditions. Studies that appeared in the scientific literature in recent years demonstrate the prospects of using bacteriophages for the preventon of biofilm formation [14; 15]. The most recent studies by Kim et al. [16] revealed the efficacy of the bacteriophage cocktail in reducing *Salmonella enterica* subspecies enterica serovar *enteritidis* (*S. enteritidis*) in raw chicken breast meat at refrigeration temperatures (4$^o$C). Other studies showed the efficacy of a phage-based product called SalmoFreshTM (Intralytix Inc., USA) in reducing *Salmonella* on chicken breast fillets stored under aerobic and also modified atmosphere conditions (95% $CO_2$/5% $O_2$). All these studies suggest that lytic bacteriophages can work as effective agents for the biocontrol of foodborne pathogens on different food products. [17].

In this study, three previously characterized bacteriophages were investigated as a cocktail, to determine their biocontrol activity against pathogenic *Salmonella* strains on experimentally contaminated chicken breast meat.

## Materials and Methods

**Sample preparation.** Skinless chicken breast fillets were obtained from a grocery store. Before performing the experiment, chicken breast meat was checked for the absence of *Salmonella*. Meat samples were deposited in Brain-Heart Infusion (BHI) broth and incubated at 37°C for 24 h and then plated on Xylose Lysine Deoxycholate agar (XLD

agar) and kept overnight at 37°C to confirm the absence of the microorganism. Chicken breasts were aseptically cut into 3x2 cm (10 ± 0.5 g) sample size. The sliced samples were transferred into sterile containers.

**Bacteria and bacteriophage conditions.** *S. typhimurium* strain bacteriophages used in this experiments were isolated and characterized in the frame of the project "Efficacy of bacteriophages for prevention and treatment of Salmonella infection in poultry" [18]. Bacterial strain was subcultured on BHI broth and incubated at 37°C for 18-24 h until cultures reached an $OD_{600}$ of 0.5, approximately $10^8$ CFU/mL and stored at 4°C temperature until the further use. *S. typhimurium*-targeting phages, Sal-Phi13, Sal-Phi 18 and vB_Stm 21 were added the final concentration of $10^5$ or $10^8$ PFU/ml. Titre of the phages was determined by using a double layer agar method described by Adams [19]. Briefly, the solution titre was determined by plating 100 µl from 10-fold dilutions in SM buffer (100 mM NaCl, 8 mM MgSO4, 50 mM Tris-Cl, pH 7.2) with a 100 µl of a fresh log-phase *Salmonella* strain in the soft LB agar (0.6% agar) tempered to 45°C. The mixtures were vortexed and distributed over the surface of the hard LB agar (1.6% agar). Soft agar was allowed to solidify at room temperature and plates were inverted and incubated at 37°C for 24 h. Bacteriophage concentration in the stock solution was determined to be $10^{10}$ PFU/ml.

**Bacteriophage propagation and concentration.** The concentrated phage solutions were produced using the plate lysis and elution method as described by Sambrook and Russell [20] with some modifications. A top agar was prepared containing 1 mL of phage solution and 0.1 mL of a bacterial overnight culture in 3 mL of soft-agar and applied to the surface of BHI agar plates. After solidification of the top agar layer the plates were incubated at 37°C overnight. Afterwards, the propagation plates were examined and the degree of lysis recorded. Three mL of sterile SM buffer was added to the surface of each plate and the phages were harvested and centrifuged (45 min, 6000×g, 4°C) and the supernatant was then filtered through the 0.22-µm-pore-size membrane filter. Phage stocks were stored at 4°C until further use.

**Infective effect of S. *typhimurium*-phages against *Salmonella* in chicken breast fillets at 4°C and 25°C.** The phage cocktail and *S. typhimurium* suspensions were prepared as described previously. The meat pieces were divided into 3 groups (A, B and C) 9 pieces in each group. The samples from the groups A and B were inoculated with 100 µL ($2.2 \times 10^4$ CFU/mL) of the bacterial suspension and incubated at a room temperature for 10 min to allow bacterial attachment to the meat surface. Then, 1 ml phage cocktail (combination of three phages in an equal ratio) at $10^8$ PFU/ml and $10^5$ PFU/ml were separately pipetted onto the samples from the groups A and C. The group B - chicken breast samples only treated with S. *typhimurium* at levels of $10^4$ CFU/ml without phage treatment were used as positive controls and the group C - samples not inoculated with S. *typhimurium* and only inoculated with phage cocktail were used as negative controls, respectively. The prepared samples were stored at 4°C and 25°C for 1 h and 24 h.

**Determination of phage titres and Salmonella counts.** The samples were analyzed at 1 and 24 h of storage at 4°C and 25°C. At each sampling time, the samples were taken and stomached for 2 min after the addition of 20 mL of peptone water buffer. After the dilutions were prepared, the samples were analyzed to determine viable bacterial counts (CFU/ml). The number of *S. typhimurium* was determined by the pour plate method on XLD agar at 37°C for 24 h.

**Statistical analysis.** Bacterial counts were determined by triplicate plating, and all food experiments were independently performed at least

three times. Results are presented as mean values, and standard deviation of the mean is indicated by error bars. Student's t-test was used to determine the significance of cell count differences between controls and phage treated samples. For all analyses, significance was considered if $P \leq 0.05$.

## Results and Discussion

**Effect of S. *typhimurium*-phages against *Salmonella* in chicken breast fillets at 4°C and 25°C.** The chicken breast fillet sections used in this study were contaminated with $10^4$ CFU/ml of S. *typhimurium* and used to determine the decontamination effect of bacteriophages by means of comparison of this with untreated controls. Fig. 1 shows the reductions of *S. typhimurium* on experimentally-contaminated chicken breast fillet sections as a result of decontamination treatments. The bacteriophage cocktail applied to the experimentally-contaminated chicken meat at concentrations of $10^5$ and $10^8$ PFU/ml at 25°C led to significant reductions of Salmonella population ($P<0.05$). The treatment with $10^8$ PFU/ml caused the higher bactericidal activity by decreasing of Salmonella counts from 3.8 (control=6.8 log CFU/ml, $10^8$ PFU/ml=3 log CFU/ml) after 1 h to 4 log CFU/ml (control=8 log CFU/ml, $10^8$ PFU/ml=4 log CFU/ml) after 24 h storage, respectively. Whereas, applications at $10^5$ PFU/ml phage cocktail reduced cell counts by 0.6 log CFU/ml (control=5.2 log CFU/ml, $10^5$ PFU/ml=4.6 log CFU/ml) after 1 h and 2.2 log CFU/ml (control=6 log CFU/ml, $10^5$ PFU/ml=3.8 log CFU/m) after 24 h, respectively. One of the goals of this study was to evaluate phage activity during the short contact time in order to apply it before the freezing of carcasses.

Exposing of the chicken breast fillets to the phage for 24 h led to higher reductions when they were stored at room temperature (25°C) as compared to the storage at refrigerator temperature (4°C), but the values of Salmonella counts obtained after 1h of storage at both temperatures were similar and equalled 0.6 log CFU/ml. The *Salmonella* counts in $10^5$ PFU/ml phage-treated samples stored for 1h and 24 at 4°C were reduced by 0.2 and 0.6 log CFU/ml respectively, that demonstrates the phage activity at a refrigeration temperature (Fig. 2).

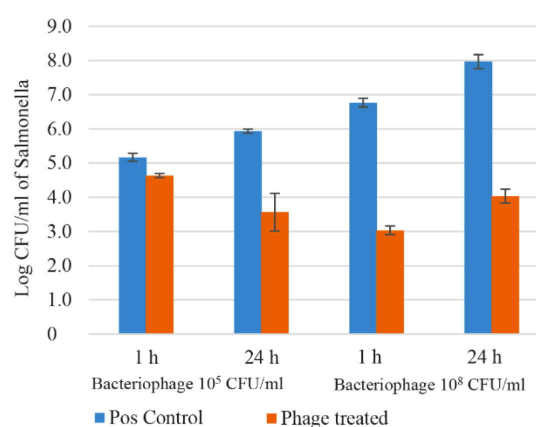


**Fig. 1.** Efficacy of phage in reducing *Salmonella* on chicken breast fillets stored at 25°C.

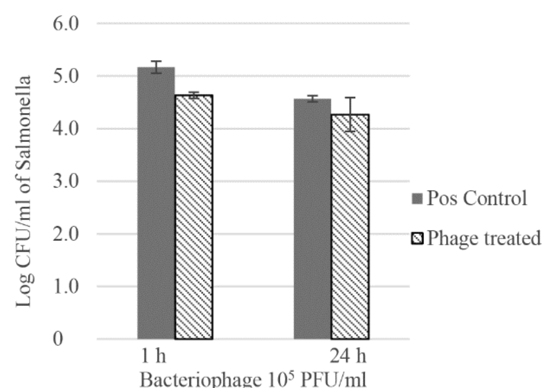


**Fig 2.** Efficacy of phage ($10^5$ PFU/ml) in reducing *Salmonella* on chicken breast fillets stored at 4°C stored 1 and 24 h.

*Salmonella* cells were not detected from the negative controls, indicating the absence of background *Salmonella*.

Bacteriophage applications for the decontamination of food have become an alternative method for the food industry due to the unique ability of infecting and lysing specific bacterial cells [10]. The effects of bacteriophages on *Salmonella* in different food matrixes were

demonstrated in various studies. Bacteriophage applications were also efficient in decreasing several *Salmonella* strains in poultry carcasses and parts.

In this study, we have demonstrated that bacteriophages were able to reduce *S. typhimurium* counts on chicken meat at refrigeration temperature and short contact time. The results of this study demonstrate that bacteriophages can remain viable for particular time periods when stored at low temperature and they might be applied for the purposes of decontamination. Our data are consistent with some published studies in which bacteriophages were used for the control of pathogens in foods [17]. In other studies, dealing with phage applications, the longer was time of incubation of the contaminated samples with phages, the greater were reductions in the host cell [21].

In this study, we also investigated the effect of temperature on growth inhibition of S. *typhimurium*. We observed that the phage activity varied at different temperatures, and for the most phages evaluated, at 25°C was revealed a better phage activity, than at 4°C. This is possibly related to the bacterial growth condition which is close to its optimum temperature. However, this does not hinder the phage application at low temperature, as demonstrated by other authors [6, 21].

Our results suggest that application of $10^8$ PFU/ml concentration of bacteriophage led to the reduction of *Salmonella* counts by up to 4 log CFU/ml as compared with application of $10^5$ PFU/ml bacteriophage which reduced *Salmonella* by 2.2 CFU/ml. It is supposed that the use of bacteriophages at higher concentrations may lead to the more efficient reductions of *Salmonella* contamination of poultry skin due to prevention of multiplication of bacterial cells.

In this study, the phage cocktail composed of three lytic bacteriophages significantly reduced *Salmonella* in chicken breast fillets. Our results demonstrate that bacteriophages have great potential of use as an effective tool for the biological control of the foodborne pathogens. Control of pathogenic microorganisms will help to reduce their frequency in the environment and food products, which in its turn will reduce the risk of spreading of foodborne diseases caused by this pathogen in humans.

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*მიკრობიოლოგია*

# ბაქტერიოფაგების გამოყენება ხელოვნურად დასნებოვნებული ფრინველის ხორცის დეკონტამინაციისთვის

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(წარმოდგენილია აკადემიის წევრის თ. სადუნიშვილის მიერ)

*Salmonella* spp. წარმოადგენს საკვებისმიერი დაავადებების ერთ-ერთ მთავარ გამომწვევს. საკვები პროდუქტებიდან ქათმის ხორცი ითვლება ადამიანებში სალმონელას ინფექციის ძირითად წყაროდ. სტატიის მიზანია ბაქტერიოფაგების გამოყენების ექსპერიმენტული შესწავლა ხელოვნურად დასნებოვნებული ფრინველის ხორცის დეკონტამინაციისთვის. ჩვენ შევისწავლეთ ბაქტერიოფაგური პრეპარატის, რომელიც შედგებოდა სამი ლიტიური ფაგისგან, უნარი ექსპერიმენტულ პირობებში შეამციროს სალმონელას შტამის რაოდენობა ქათმის მკერდის ფილეზე 25°C და 4°C ტემპერატურაზე. ექსპერიმენტში გამოყენებულია $10^4$ CFU/მლ S. *typhimurium* შტამი და ბაქტერიოფაგის ორი სხვადასხვა კონცენტრაცია $10^5$ PFU/მლ და $10^8$ PFU/მლ. სალმონელას შტამის აღნიშნული კონცენტრაციით ხელოვნურად დასნებოვნებული ქათმის მკერდის ფილეს ნიმუშების ზედაპირულმა დამუშავებამ ბაქტერიოფაგებით $10^8$ PFU/მლ შეამცირა სალმონელას რაოდენობა 3,8-დან 4 log CFU/მლ-ით, ($P < 0{,}05$) 1 და 24 საათის შემდეგ. $10^5$ PFU/მლ კონცენტრაციის ბაქტერიოფაგის გამოყენების შემდეგ შემცირდა ოთახის ტემპერატურაზე (25°C) სალმონელას რაოდენობა 0,6-დან 2,2 log CFU/მლ-მდე, ფაგით დაუმუშავებელ კონტროლთან შედარებით, შემცირდა სალმონელას რაოდენობა 0,2 და 0,6 log CFU/მლ-ით, 1 და 24 საათის განმავლობაში 4°C-ზე, რაც მიუთითებს ფაგის აქტივობას მაცივრის ტემპერატურაზე. მიღებული შედეგები მიუთითებს, რომ ბაქტერიოფაგებს აქვთ დიდი პოტენციალი გამოიყენებულ იქნეს, როგორც ეფექტური საშუალება საკვებისმიერი პათოგენების ბიოლოგიური კონტროლისთვის.

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