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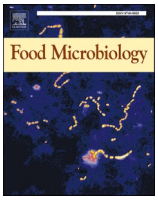

Salmonella Typhimurium DT 104 response to Lytic bacteriophage and Lactobionic acid on raw chicken breast

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ABSTRACT

Bacterial food poisoning cases due to *Salmonella* have been linked with a variety of poultry products. This study evaluated the effects of a *Salmonella*-specific Lytic bacteriophage and Lactobionic acid (LBA) on *Salmonella* Typhimurium DT 104 growth on raw chicken breast meat. Each chicken breast was randomly assigned to a treatment group (Control, DI water, phage 1%, phage 5%, LBA 10 mg/mL, LBA 20 mg/mL, and phage 5% + LBA 20 mg/mL) with four chicken breasts per group. Samples were inoculated with 10^6 CFU/mL of *Salmonella* and stored at 4 °C for 30 min. The inoculated chicken breasts were randomly assigned to different storage time (0 h, 1 h, 24 h, or 48 h). Both time and treatment showed significance reduction ($P < 0.0001$) of microbial growth. The weight loss was significantly different ($P < 0.0001$) between treatments. The LBA treatments were not effective when compared to the control group, but Lytic bacteriophage significantly reduced the amount of microbial growth.

1. Introduction

Consumption of poultry meat has been steadily increasing worldwide, and the United States is the second-largest consumer of poultry meat – 48.8 kg per person per year (Anonymous, 2018). The consumption has been increased by 6 kg per person per year when compared to the same trend for the year 2000 (National Chicken Council, 2018). Because of these increasing trends, ensuring microbial safety of poultry meat and its products is of utmost importance. Some of the microbiota present in poultry surface or its gut is pathogenic to humans, which can be present at the time of poultry slaughter and may cross-contaminate slaughterhouse environment, cutting equipment, poultry parts, and products.

Bacterial food poisoning is a major health problem that annually affects millions of people worldwide. There is a diverse group of pathogenic bacteria that can cause bacterial food poisoning, but *Salmonella* spp., *Listeria monocytogenes*, enterotoxigenic *Staphylococcus aureus,* and *Campylobacter* spp. are the most common bacterial food contaminants (Goncalves-Tenorio et al., 2018). Among the diverse group, *Salmonella* bacteria are the most frequent causes of foodborne illness and death in the United States. Out of 2300 serotypes, *Salmonella* Enteritidis and *Salmonella* Typhimurium account for almost half of all human infections in the U.S. (United States Department of Agriculture, 2014). Despite industrial efforts to control the pathogen, the numbers of illnesses in the U.S. are not declining as one would hope. Poultry, specifically chickens, has been known to be the primary source of foodborne pathogens (Goncalves-Tenorio et al., 2018). In 2018 alone, there were 12 multistate *Salmonella* outbreaks, which indicates that current intervention plans are inadequate (Eskin, 2018). In 2019, the Centers for Disease Control and Prevention investigated 13 multistate *Salmonella* infection outbreaks. They found that these outbreaks were closely linked with backyard poultry handling. Over 1100 people contracted *Salmonella* from 49 states, and two people died (Center for Disease Control and Prevention, 2019a).

Risk groups include all ages, however immune-compromised, elderly, and infants are at a higher risk of contracting *Salmonella* infection. These pathogens are common colonizers of poultry, and though the host is often asymptomatic, they can cause Salmonellosis if they are ingested by humans. Salmonellosis is the disease process that results when a host is infected with pathogenic *Salmonella* spp. and it can result in gastroenteritis, septicemia, or enteric fever. Gastroenteritis symptoms often start 6–48 h after the contaminated food is ingested, and the fever, diarrhea, and abdominal cramps can last for 2–7 days (Roth, 2013). Gastroenteritis is the most common presentation of Salmonellosis and

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was commonly treated with antibiotics (Center for Disease Control and Prevention, 2019b). However, many of the pathogenic strains of *Salmonella* have developed antibiotic resistance and thus present a problematic case for treatment (Nair et al., 2018). The easiest way to reduce the number of people infected by *Salmonella* spp. is to focus on ways to reduce the presence of these bacteria on poultry.

As a result, research has been targeted on treatments to reduce surface contamination by pathogenic bacteria. Besides, the emergence of antibiotics resistant *Salmonella* is on the rise in the last few decades. One method of controlling *Salmonella* outbreaks is to reduce the concentration of *Salmonella* in the poultry before it is consumed. This can be partially accomplished by thoroughly cooking poultry before consumption, but more steps can be taken to reduce overall *Salmonella* presence in raw poultry. Antibiotics can be used to treat septicemia and enteric fever caused by non-typhoidal *Salmonella* as well as Typhoid fever that results from infection by *Salmonella* Typhi. However, antibiotics should not be used for self-limiting gastroenteritis caused by *Salmonella*, as it does not decrease the length of the illness and can contribute to antibiotic resistance (Roth, 2013).

Biocontrol of *Salmonella* using specific *Salmonella*-targeting lytic bacteriophages was suggested as a way to reduce the *Salmonella* concentration on food products, which will slow the decay of the food as well as prevent the spread of pathogenic bacteria that could cause disease (Oh and Park, 2017). This is particularly useful because the Lytic bacteriophage that targets the bacterial pathogens has no effect on humans and does not contribute to antibiotic resistance of the bacteria.

Another suggested tool for food-safe microbial reduction is Lactobionic acid (LBA), which is a natural polyhydroxy acid (Kang et al., 2019, 2021). Its effects were tested against *Staphylococcus aureus* and found to be significant in its reduction of microbial growth at concentrations between 15 mg/mL and 50 mg/mL (Cao et al., 2019; Kang et al., 2020). Since *Staphylococcus aureus* is a gram-positive bacteria and *Salmonella* Typhimurium is a gram-negative bacterium, the effects of LBA on *Salmonella* growth must be tested and not assumed to be the same as on *Staphylococcus aureus*. Hopefully, using antimicrobials such as bacteriophage and LBA can reduce the incidence of bacterial resistance. Therefore, this study's primary objective is to use Lytic bacteriophages and LBA individually or in combination to reduce the colony-forming units (CFU) of *Salmonella* Typhimurium from raw chicken breast. Thus, to validate the intervention strategies and improve the microbial safety of poultry meat products.

2. Materials and methods

2.1. Experimental design

Salmonella Typhimurium (*S*. Typhimurium) DT 104 was obtained from the Department of Food Science and Nutrition, California Polytechnic State University, San Luis Obispo, California. The *S*. Typhimurium isolate was maintained on Luria-Bertani (L.B.) broth throughout the experiment. Raw chicken breasts were obtained from a nearby grocery outlet and were carried under ice (<4 °C) to the food safety lab at the University. The raw breasts (5 cm × 5 cm, n = 28) were experimentally inoculated with *S*. Typhimurium DT 104 (10^6 CFU/mL for 30 min attachment at 4 °C) cultured in 10-mL volumes of L.B. broth overnight. The concentration of inoculum was quantified prior to inoculating the chicken breast. Microbial sponge sticks for environmental sampling (EZ Reach™ Sponge Sampler, World Bioproducts) were used to swab the chicken breast surface. Raw chicken breasts were randomly allotted to one of the seven treatments (Control, DI water, 1% phage, 5% phage, 10 mg/mL LBA, 20 mg/mL LBA, 5% phage + 20 mg/mL LBA, n = 4) per replication. The solutions of 1% (2×10^7 CFU/mL – on meat surface – data not shown) or 5% (1×10^8 CFU/mL – on meat surface – data not shown) Lytic bacteriophages (PhageGuard® S, Micreos Food Safety, the Netherlands) and 10 mg/mL or 20 mg/mL of LBA (Millipore Sigma, St. Louis, MO) were made based upon the manufacturer's recommendation. Treated pieces of chicken breast were kept under refrigeration temperature for 48 h. Serial dilution was followed to construct an approximate density of 10^2 and 10^3 CFU/cm^2 on the media plate. Dilutions were spread plated on Xylose Lysine Deoxycholate agar (XLD) for the isolation of *Salmonella* species, and the plates were incubated at 35 ± two °C for 20 h.

2.2. Bacterial strain and culture preparation

Salmonella Typhimurium DT 104 was cultured and maintained in Luria-Bertani (L.B.) broth for the entirety of the experiment. Strain DT 104 was plated on Xylose Lysine Deoxycholate agar (XLD), where it formed a black, round colony that signifies the production of hydrogen sulfide by the bacteria.

2.3. Treatment, product preparation, and storage

5 cm × 5 cm plastic templates were used to cut the raw chicken breast into equal-sized portions (n = 28). The experiment was ran three consecutive times (N = 84). The description below is for a single run of the experiment.

The entire surface of each portion of raw chicken was inoculated with 10^6 CFU/mL of *Salmonella* Typhimurium DT 104 (in L.B.) and allowed to rest at 4 °C for 30 min to facilitate attachment of the bacteria to the surface of the chicken breast samples. Chicken portions were randomly assigned to one of seven treatment groups (A-G). The treatment groups are as follows: Group A – Control, Group B – DI water, Group C – phage 1%, Group D – phage 5%, Group E – Lactobionic Acid (LBA) 10 mg/mL, Group F – LBA 20 mg/mL, and Group G – phage 5% + LBA 20 mg/mL.

Each chicken breast sample (n = 28) was weighed, inoculated with its treatment, placed in a plastic weigh boat and placed on a plastic serving tray for easy transfer to and from the refrigerator. All seven treatment groups (n = 4 per group) were stored in the refrigerator at 4 °C for the entirety of the experiment.

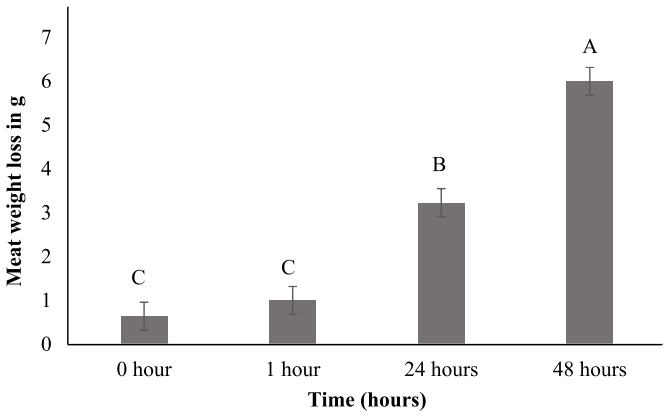
2.4. Sample collection

3M sponge sticks were used for a sample collection from each raw chicken portion. Within each treatment group, one chicken portion was randomly selected to be removed and sampled at 0 h, 1 h, 24 h, and 48 h. For each sample, the 3M collection bag was filled with 10 mL of Buffered Peptone Water (BPW), and the sponge stick was soaked with BPW. The sponge was squeezed inside the bag to remove the excess liquid, and the sponge was run over the entire surface (25 cm^2) of the raw chicken portion to collect any *Salmonella* on the surface. The sponge stick was returned to the collection bag and repeatedly squeezed in the liquid for 2 min to ensure that any *Salmonella* on the sponge was transferred into the BPW broth.

Serial dilutions of each sample were made by removing 1 mL of the sample and mixing it into 9 mL of BPW in a test tube to obtain ten-fold dilutions until the approximate density of 10^2–10^3 CFU/mL was reached. Two dilutions were plated in duplicate on XLD for each of the treatments at each sampling time and incubated at 35 ± 2 °C for 20 h. Plate counts were performed on each plate, and CFU/mL was calculated for each plate using the dilution factor. For duplicate plates where the plate count was between 30 and 300 CFU, the CFU/mL were averaged. The CFU data were $\log_{10}$ transformed before the statistical analysis.

2.5. Statistical analysis

A randomized complete block design was used to design this experiment where the number of repeated replications were considered blocks. Data were analyzed using the SPSS statistical software (IBM Corporation). Univariate Analysis of Variance was used for treatment time and treatment*time interactions coupled with Tukey's least



[A, B, C] Groups lacking common superscript letters are different ($P<0.05$).

Fig. 1. Meat weight loss (g) over a 48-h time period after treatment application. .

Table 1
ANOVA results from the univariate analysis of raw chicken breast weight loss. There was no interaction between treatment and time for meat weight loss. However, the weight loss was significantly different ($P < 0.0001$) between treatments compared to the length of time the treatments were applied.

Tests of between-subjects effects						
Source	Type III sum of squares	df	Mean square	F	Sig.	Partial Eta squared
Treatment	5.357	6	.893	.633	.703	.119
Time	256.911	3	85.637	60.705	.000	.867
Treatment * Time	35.214	18	1.956	1.387	.213	.471
Error	39.500	28	1.411			

R Squared = .883 (Adjusted R Squared = .770)

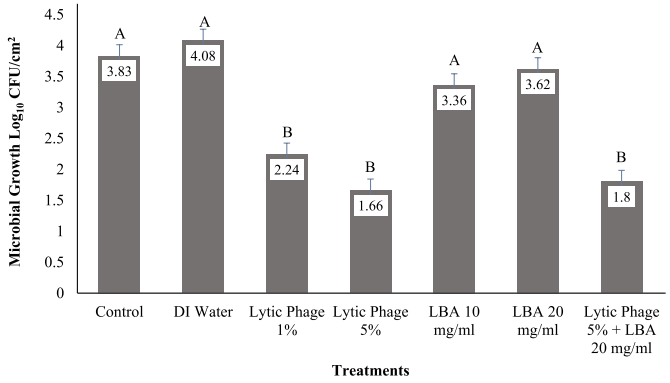
Table 2
ANOVA result from SPSS univariate analysis of the decrease in bacterial count. There was no interaction between time and treatment ($P = 0.86$). However, both time and treatment showed significance in their main effects ($P < 0.0001$).

Tests of between-subjects effects					
Dependent variable: *Salmonella* Typhimurium counts in Log_{10} CFU/cm^2					
Source	Type III sum of squares	df	Mean square	F	Sig.
Treatment	73.91	6	12.32	30.18	.000
Time	155.21	3	51.74	126.76	.000
Treatment * Time	4.63	18	.26	.63	.860
Error	22.86	56	.41		
Total	983.32	84			

significant difference and post hoc tests with $\alpha = 0.05$.

3. Results and discussion

Lytic Bacteriophages are a possible method to improve the biosecurity in poultry because they are natural predators of bacteria and replicate only on the targeted bacterium (Clokie et al., 2019). Bacteriophages, or phages, are viruses that can invade bacterial cells, and in the case of Lytic bacteriophages, it can disrupt the bacterial metabolism and cause the cell to lyse (Sulakvelidze et al., 2001). Likewise, LBA is a natural polyhydroxy acid and has been widely used in the food industry against foodborne pathogens (Cao et al. l., 2019). The application of different antimicrobials, including bacteriophages, will help to reduce or eliminate the bacterial pathogen from the meat surface.



[A, B, C] Groups lacking common superscript letters are different ($P<0.05$).

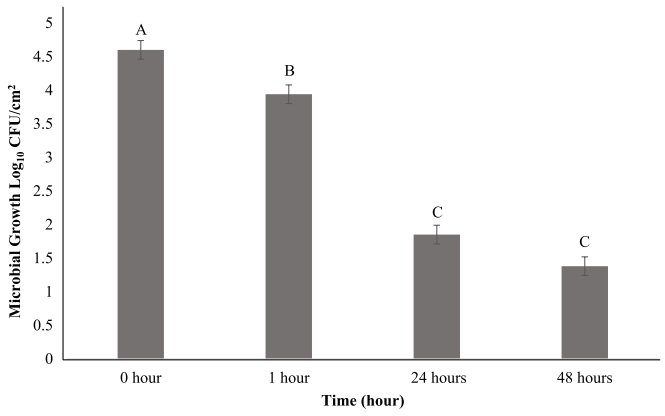
Fig. 2. Effect of different antimicrobial treatments on *Salmonella* Typhimurium counts on chicken breast across all sampling hours. Values are means of CFU/cm^2 with different treatments.

The application of Lytic bacteriophage (1% and 5%) on chicken breast results in a significant reduction ($P < 0.05$) of *S.* Typhimurium counts, however, there was no treatment and time interaction (Fig. 1, Table 1). Likewise, a significant reduction ($P < 0.05$) of *S.* Typhimurium was observed when 5% of phage and 20 mg/mL LBA were combinedly used on chicken breast (Fig. 1). Out of all seven different treatments, the highest log_{10} reduction was shown by 5% phage, which reduced the pathogen by 2.17 log_{10} CFU/cm^2 (Fig. 1) when compared with control. Interestingly, much higher bacterial reduction was achieved (2.42 log_{10}) from the application of 5% phage when compared that with DI water treatment. However, there were no significant differences between 1% and 5% phage application to raw chicken breasts. Since there were no treatment and time interactions, individual treatment effects were not analyzed for refrigeration time. However, *S.* Typhimurium was significantly reduced ($P < 0.05$) after refrigerating the raw chicken breast for 24 and 48 h (Fig. 2) (Table 2).

In the case of ground chicken, a higher concentration of bacteriophage (10^8 PFU/mL) can reduce *Salmonella* significantly irrespective of the holding times (30 min and 6 h) (Yeh et al., 2017). However, holding for six days or more, has a significant impact on bacterial reductions on chicken thighs when compared with the three days of holding time (Fiorentin et al., 2005).

The weight of the chicken samples (n = 28) was measured for each treatment because a significant decrease in weight due to the treatment process is undesirable since chicken is sold by weight and would result in a lower profit for sellers. The test results showed that the different treatments applied to the chicken samples did not have a significant impact on the post-treatment weight of the meat ($P = 0.703$). However, the time the treated chicken samples were left in the refrigerator did have a significant impact on the post-treatment weight ($P < 0.0001$) (Table 1). This is likely due to the evaporation and mass transfer exchange with the environment from the chicken breast as it was left uncovered in the refrigerator (Campanone et al., 2002). There was no significant difference in weight loss between 0 h and 1 h sampling times. However, there was a considerable difference in weight loss at 24 h and 48 h (Fig. 1). To our knowledge, there is no published literature on bacteriophage application and its effect on meat weight to compare this data.

The number of CFU/mL was calculated based on the plate counts at various dilutions of the sponged chicken portions. Both the treatment applied to the chicken portion and the amount of time between treatment and sampling had significant impacts on the number of *Salmonella* colonies that grew ($P < 0.0001$). There was no observed statistical interaction between time and treatment on CFU/mL ($P = 0.86$). When compared to the control (Group A), treatment groups B, E, and F did not show a significant difference in CFU/mL (Fig. 2). However, treatment



[A, B, C] Groups lacking common superscript letters are different ($P<0.05$).

Fig. 3. Effect of different antimicrobials on *Salmonella* Typhimurium counts on the chicken breast at individual sampling hours. Values are means of CFU/cm^2 at different hours.

with Lytic bacteriophage1% and 5% (Groups C and D) as well as Lytic bacteriophage 5% + LBA 20 mg/mL (Group G) showed a significant decrease in CFU/mL when compared to the control (Fig. 2). These three treatments were not significantly different from each other, so the results are inconclusive as to which dosage is the best. However, Lytic bacteriophage used at 5% showed higher numerical reductions. Fig. 3 shows that increasing the amount of time that the treatment is in contact with the chicken portions (up to 24 h) decreases the microbial growth (CFU/mL). Extending the treatment time beyond 24 h did not significantly change the amount of microbial growth (CFU/mL) present on the chicken portions.

More research is needed to evaluate the proper dosage of Lytic bacteriophage to substantially decrease *Salmonella* Typhimurium presence on poultry meat. Some of the studies that were conducted in the past showed similar microbial reduction regardless of different bacteriophage concentrations (Goode et al., 2003; Sharma et al., 2015; Yeh et al., 2017; Shebs-Maurine et al., 2020). However, the majority of the studies showed no difference in holding time. Interestingly, Spricigo et al. (2013) demonstrated that after seven days, bacteriophage cocktail reduced 2.2 $\log_{10}$ CFU/g of *Salmonella* Typhimurium on chicken breast. In contrast, *Salmonella* Enteritidis had the most significant reduction (1.4 $\log_{10}$ CFU/g) on day 5 of the study. Thus, foodborne microorganisms, cocktails of bacteriophage and its concentration holding time, and food matrices play a role in reducing pathogens.

4. Conclusion

These results demonstrate that the Lytic bacteriophage applied to raw chicken breast portions for at least 24 h effectively reduces the concentration of *Salmonella* on the surface of the chicken. We decided to study non-antibiotic treatments for reducing *Salmonella* concentrations because it has been demonstrated that common *Salmonella* strains that colonize poultry are resistant to at least some antibiotics (Akbar and Anal, 2013). Future research should be conducted to elucidate the minimal dosage of Lytic bacteriophage needed to significantly reduce the *Salmonella* concentration on raw chicken breasts. Further research could also be done on the impact of treatment on taste and shelf life in the refrigerator. Again, the ability of bacteriophage to reduce the number of foodborne pathogens on different food products depends on numerous variables, such as the concentration of bacteriophage, hurdle concept, combining different bacteriophages (making cocktail), holding time, and even the food matrices. This research has found that 5% of Lytic bacteriophage and the combination of 5% Lytic bacteriophage and LBA 20 mg/mL significantly reduced *Salmonella* Typhimurium ($P <$ 0.05) on raw chicken breast meat. The reduction of foodborne pathogens on the meat and meat products by the help of natural antibiotics, as discussed above, will help to reduce the number of foodborne infections. Both the phage treatments showed the least amount of quantifiable colony forming units (CFU), indicating that the bacteriophages are very potent to reduce foodborne pathogens in meat products.

Declaration of competing interest

The authors declare no conflict of interest associated with this study.

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