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Short Paper

Biocontrol of *Escherichia coli* and *Salmonella* in poultry meat using phage cocktail

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Abstract

Background: Despite advances in food management techniques, foodborne illness remains a major concern. Contamination of *Salmonella* and *Escherichia coli* pathogens, especially in the poultry sector, is responsible for salmonellosis and other gastrointestinal illness, leading to millions of deaths worldwide. Overuse of antibiotics and other chemical treatments have further increased the emergence of antibiotic resistant bacteria. **Aims:** This study aimed to study the efficacy of phages cocktail to reduce the load of *E. coli* and *Samlonella* spiked on poultry meat. **Methods:** In this study, a broad spectrum cocktail of phages was used to lyse *E. coli* and *Salmonella* spiked on chicken meat. **Results:** Based on the result of the CFU drop assay, phages like *E. coli* 153T 3ii and *Salmonella* 191(3) were selected. Phage concentration of 0.01 MOI showed a reduction in *E. coli* and *Salmonella* count to 6 h and 2 h, respectively. Further, phages were tested on the surface of chicken meat. *E. coli* showed a 90% reduction up to 4 h, whereas *Salmonella* showed a 90% reduction up to 6 h. When phages were treated in combination, a significant reduction of up to 12 h was found with *Salmonella* phage, showing better antimicrobial activity. **Conclusion:** The suitable concentration of a specific phage or phage cocktail can significantly reduce the bacterial count on chicken meat. Phage mediated biocontrol can be used as an alternative approach to eliminate enteric pathogens in the poultry industry.

Key words: Biocontrol, *Escherichia coli*, Phage, Poultry, *Salmonella*

Introduction

The worldwide poultry industry is one of the great sources of animal protein. Hence, globally the production and consumption of chicken meat have increased over the years. India records the largest chicken meat producer, followed by Brazil and China (Apostolakos and Piccirillo, 2018). Foodborne diseases are one of the major public concerns that affect developed and developing countries, risking public health and economy. Contamination of poultry meat can occur either in the production environment via air, through vertical or horizontal transmission, or during the slaughtering process (Bantawa *et al*., 2019). Hence microbial safety of poultry and its products is essential (Rouger *et al*., 2017). *Salmonella*, *Escherichia coli*, *Shigella*, *Campylobacter*, and *Listeria*, are the major pathogens that can infect humans through contaminated chicken meat. Among these pathogens, *Campylobacter* spp, *Salmonella* spp, and *E. coli* 0157:H7 are of high risk, and hence it is crucial to detect their presences even at a very low level (EFSA Panel on Animal Health and Welfare, 2012). Various technologies such as microbial inactivation, irradiation, heat treatment, high hydrostatic pressure, and antibiotic therapy are used as preventive measures to control the microbial biota. Even though various control methods are available to combat bacteria in the food processing chain, efficiency is quite low and has adverse effects on the organoleptic properties of food (Davis *et al*., 2018; Ramirez *et al*., 2019). In contrast to these technologies, there is a need for a biocontrol strategy to combat bacterial contamination (Kazi and Annapure, 2016). Phages are used for efficient biocontrol of *Listeria monocytogenes*, *Salmonella* Typhimurium and other non-typhoidal *Salmonella* spp, *E. coli* O157:H7, *Campylobacter* and other spoilage organisms in chicken meat, fruits, and in ready to eat products (Bardina *et al*., 2012; Sukumaran *et al*., 2015). The use of phage cocktails helps reduce poultry infected with more than one pathogen and can be successfully implemented in the poultry units (El-Dougdoug *et al*., 2019). The application of phage cocktails can prevent the emergence of phage

resistant mutants (Carvalho *et al*., 2010; Richards *et al*., 2019). The use of phage as a biocontrol agent is considered the natural and green technology because it targets only specific bacteria in various food products. In the present study, single and cocktail of phages (at low MOI) were used to control *E. coli* and *Salmonella* infection in poultry meat that showed a significant reduction in the bacterial count and its cell control count decreased over 12 h.

Materials and Methods

Ethics statement

The study does not involve any clinical trial hence no ethical consideration is required in this work.

Escherichia coli (ICMR153) and *Salmonella* Weltevreden (S191) stock cultures isolated from poultry were used for the study. A series of biochemical tests (Gram staining, oxidase, catalase, indole, methyl red, triple sugar iron agar, and urease) were used for phenotypic characterisation. Polymerase chain reaction was carried out by targeting *uid*A and *inv*A genes for *E. coli* and *Salmonella*, respectively.

Revival of bacteriophage

E. coli (153T 3ii) and *Salmonella* 191(3) phage previously isolated, characterised and maintained at Nitte University Centre for Science Education and Research were used for the study. Total of five *Salmonella* and ten *E. coli* phages were isolated from the effluents of the local commercial chicken market by enrichment method. Reference strains as bait to isolate these phages were used (Sonalika *et al*., 2020).

Escherichia coli (ICMR153) and *Salmonella* Weltevreden (S191) were grown to an exponential growth phase in nutrient broth, and 10 µL of phage stock was added. The tubes were incubated for 6 h at 30°C. The suspension was centrifuged, and the supernatant was filtered through a 0.22 µm filter to obtain the phage lysate. Obtained phage lysate was further used for phage titre determination.

Phage titre determination

Phage titre was determined by the overlay technique. Phages were enriched by adding 500 µL bacterial culture to 5 ml of nutrient broth and grown for approximately 3 h at 37°C. 1 ml of phage lysate was added and incubated for 4 h at 37°C in a shaking incubator. Further, cultures were centrifuged and filtered through a 0.22 µm syringe filter and used for spot assay. Enrichment of phages to high titres was essential to generate phage lysates of concentrations greater than 10^8 PFU/ml for further use.

Host susceptibility

Host susceptibility is an essential tool for determining the sensitivity of bacteria to the phage. In this technique bacterial lawn was made on a nutrient agar plate and was allowed to dry. 5 µL of phage lysates were spotted on the lawn and incubated at 37°C to observe the lysis.

Control of *E. coli* and *Salmonella* on poultry meat

Separate tests were performed for *E. coli* and *Salmonella* and then in combination. The fresh chicken meat was purchased from the local retail grocery shop. The meat was surface sterilized by UV treatment for 10 min to reduce the total bacterial load. Meat sample was distributed into four groups (25 g each of meat):

Group 1: Treatment (treated with bacteria and phage)
Group 2: Bacteria control (treated only with bacteria)
Group 3: Phage control (treated with phage)
Group 4: Broth control (treated with saline)

The chicken meat was macerated after infection with 10^5 cells/ml bacteria and 0.01 MOI of phage. Serial plating as well as overlay for the plaque of each dilution after every 0, 1, 2, 4, 6, 8, and 12 h was performed on the selective medium (Mac Conkey agar, Xylose-Lysine Deoxycholate agar). Plates were incubated at 37°C overnight, followed by counting to obtain CFU/ml and PFU/ml.

Statistical analysis

One-way ANOVA was used to determine the significance of the reduction of bacterial load due to the phage activity.

Results

Suspected pink colonies from eosin methylene blue (EMB) agar and green colonies with a black centre from hekton enteric agar (HEA) plate were used for biochemical and molecular characterisation. *E. coli* showed negative for Gram staining, citrate and oxidase test and positive for indole, catalase test. *Salmonella* showed positive for catalase and citrate test and negative for indole and oxidase test. Obtained isolates were further characterised by PCR.

In vitro activity of bacteriophages

When the mixture of both bacterial culture and phage were overlaid with soft agar, plaques or clear zone on the bacterial lawn was observed indicating the presence of bacteriophages. Phage lysate of concentration greater than 10^8 PFU/ml was generated. A high titre was achieved on enrichment broth obtained through lysis of the culture in the broth. Phage 153T 3ii was effective in lysing the *E. coli* among the other phages (Fig. 1). Phage host range was studied using twenty-eight *Salmonella* isolates besides the reference strains. For *Salmonella* spp phage 191(3) had a broader host range of 82.75% (Sonalika *et al*., 2020).

Control of *E. coli* and *Salmonella* on poultry meat

Meat infected with 10^5 CFU bacteria (*E. coli* and *Salmonella*) was treated with 0.01 MOI phage. The result showed that the specific phage was effective in reducing bacteria up to 4 h for *E. coli* and up to 6 h in the case of *Salmonella*. When *Salmonella* 191(3) and *E. coli* (153T

3ii) phage were used on meat spiked with *E. coli* and *Salmonella* combination, a reduction of both bacterial species was observed. One-way ANOVA was performed by comparing bacteria control with treatment. F ratio was found to be 94.00 with a p-value of 0.000011 which is considered statistically significant at P≤0.05 (Figs. 2-5).

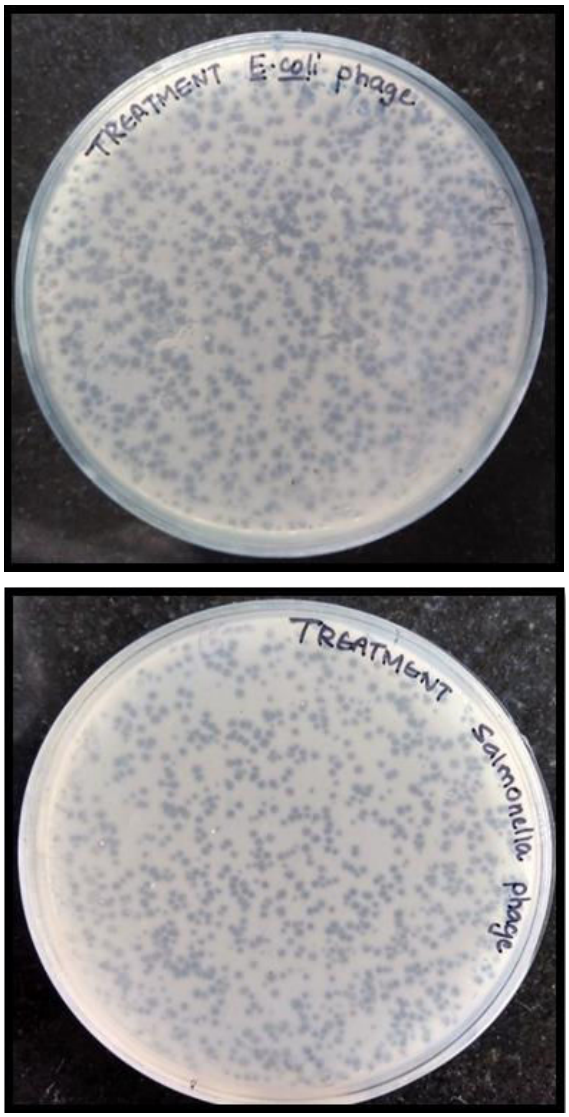

Fig. 1: Representative image of plates showing plaques

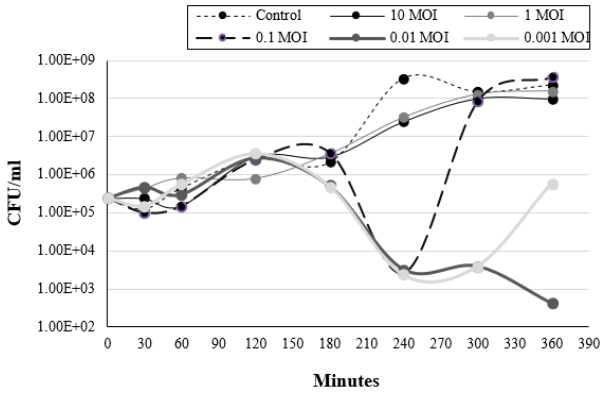

Fig. 2: Viable *E. coli* count at various time point intervals in presence of phage at different MOI

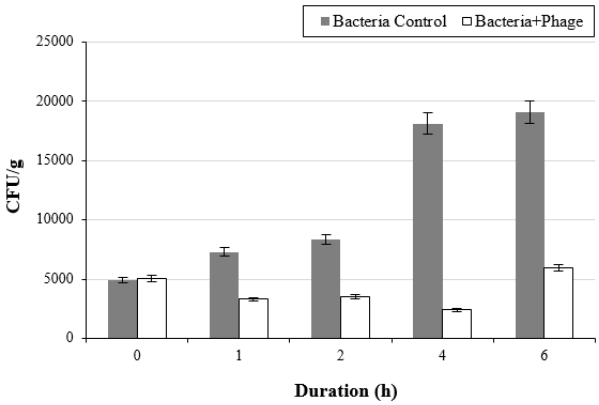

Fig. 3: Viable *E. coli* count on chicken meat at various time interval

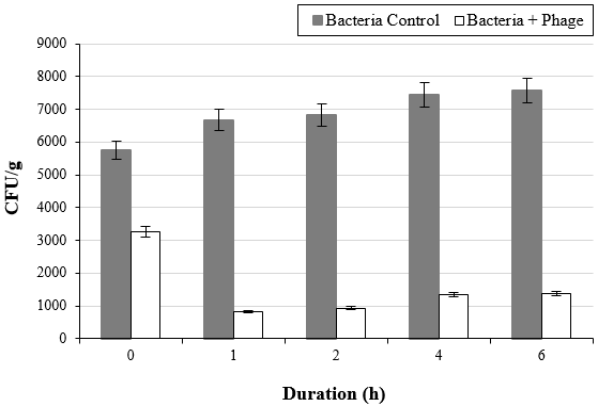

Fig. 4: Viable *Salmonella* count on chicken meat at various time interval

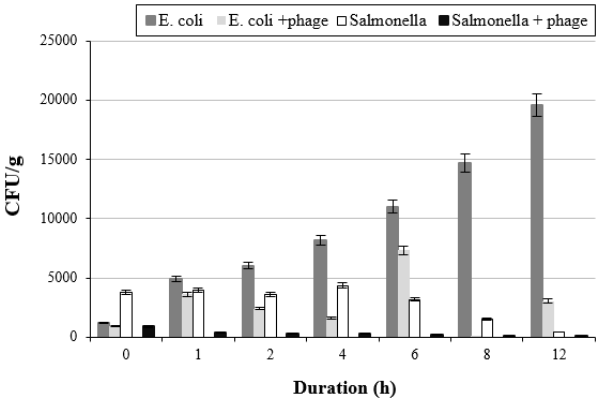

Fig. 5: *Salmonella* and *E. coli* count in combination on chicken meat at different time interval

Discussion

Microbiological safety of food is greatly influenced by multiple factors in the food chain. *Salmonella* and *E. coli* are frequently associated with most food spoilage. Despite all the traditional food treatments and antibiotic therapy, foodborne illness is still a major concern due to the development of multidrug resistant strains of many zoonotic pathogens. Due to the lack of effectivity therapy in eliminating pathogens, a need for alternative biocontrol measures to use in food (Abedon *et al*., 2017;

Canica *et al*., 2019). Phages have effective use in controlling bacteria in poultry and its products due to their target specificity, a broad range of host killing actions, and their ability to self-replicate inside hosts Duc *et al*., 2018; Ahmad *et al*., 2021). The application of phage in the meat industry can help to control pathogens and has the potential to replace other physical and chemical treatments. In this study, the isolated phages and bacteria from poultry sources were used. Host susceptibility towards different phages helps in determining the phage host better. *E. coli* 153T sii phage and *Salmonella* phage 191(3) were considered effective and used for *in vivo* study. *Salmonella* phage 191(3) and *E. coli* 153T sii phages at 0.01 MOI showed good ability to reduce viable count in 2 and 6 h in CFU drop assay. It is clear from this result that phages replicate inside the host and show antibacterial activity at applied concentration. Low MOI is favourable for the commercial feasibility of large-scale applications since it reduces the cost of preparation, purification, and application of phage (Hudson *et al*., 2015). Significant host reduction (2-3 log) was achieved when phage was used in raw and cooked meat compared to phage-free controls (Sukumaran *et al*., 2016). In this study, the lytic activity of bacteriophage was evaluated on chicken meat *in vivo*. Based on the CFU drop assay study results, used 0.01 MOI of phage on the chicken meat surface to reduce the surface contamination of bacteria. The result indicated that phage administration showed a 50% reduction within 1 h and more than 90% reduction at 4 h in the case of *E. coli* infected meat. In the case of *Salmonella*, more than 90% reduction was observed at 1 h, and it retained that level up to 6 h. On the other hand, in combination where cocktail of phages was used, *E. coli* showed a significant 50% reduction for 2 h, which was retained up to 8 h whereas *Salmonella* showed a 90% reduction up to 12 h; however, its cell control count decreased over 12 h. A study showed that phages infecting *S*. Enteritidis were able to prevent the colonisation of *S*. Enteritidis for a short duration (Lee *et al*., 2017).

A cocktail of three *E. coli* phages proved to be highly effective in reducing *E. coli* O157:H7 colonisation on meat (Ramirez *et al*., 2018). Phage application showed a significant reduction in the bacterial count of *Salmonella* and *Campylobacte*r while the contaminated sample was incubated with phages for a longer period, up to 48 h at 4°C (Carvalho *et al*., 2010; Sukumaran *et al*., 2016). In this study, we evaluated phage activity for a shorter period of time with temperature conditions maintained according to the poultry industry (4°C). Phage mediated reduction has been reported in many foods like *Listeria monocytogenes* specific phages reduced the bacteria by 2-4 log on melons (Lee *et al*., 2017; Ishaq *et al*., 2020), and *E. coli* specific phages were able to control the bacteria in contaminated beef by >94%, a cocktail of phages prevented *S. aureus* from contaminated milk (Titze *et al*., 2020). In our study, both cocktail and single phages effectively reduced the *E. coli* and *Salmonella* count on the surface of contaminated chicken meat.

Phage can be used as an alternative biocontrol approach in controlling foodborne pathogens on chicken meat. Phage production is cost-effective and will not be burdensome for large scale production. Replacing recalcitrant antibiotics with self-limiting phages will reduce antibiotic use and the dissemination of antimicrobial resistance. The use of phage in meat is approved in the USA, but its use is still limited in India. Thus, this study provides insight into preventive and control strategies against *Salmonella* and *E. coli* infection in the poultry industry.

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Conflict of interest

None to declare.

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