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Bio-Control of *E. coli* and *Salmonella* in Foods Using Bacteriophage to Improve Food Safety

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Abstract: *Escherichia coli* and *Salmonella* are considered major foodborne pathogens. In this study, the use of different bacteriophages to control bacterial contamination of foods was investigated. Specific bacteriophages for both *E. coli* and *S. enterica* were isolated and characterized. Five isolated phages showed a strong killing potential against tested strains were tested for their stability at 4°C and all phages showed good stability over the experimental period (10 days). The *in vitro* experiments showed that phage EC3 was able to reduce the numbers of *E. coli* to undetectable levels after 120 min of infection while SE1 phage reduced the numbers of *S. enterica* by 7 $\log_{10}$ after 180 min of infection. When applied to the surface of cucumber and eggs stored at 4°C, both phages reduced the numbers of *E. coli* and *S. enterica* to undetectable levels ($P < 0.005$) by day 5 of experiment. These results support the possibility to use phages to control pathogenic bacteria in foods.

Key words: Bacteriophage · Food Safety · *E. coli* · *Salmonella* · Bio-control

INTRODUCTION

Pathogenic bacteria are involved in public health problems, including risking human health. *Salmonella* and *E. coli* are two pathogens that can infect humans through contaminated food. *E. coli* can be found in cattle farms and can live in the intestines of most animals [1]. Consequently, meat can become contaminated accidentally with *E. coli* after slaughtering especially minced meat [2]. *E. coli* is also present on the cow's udders [3]. Therefore, milk and ground beef could be sources of *E. coli* infection. Contamination of food with antibiotic resistant bacteria such as *E. coli* and *Salmonella* presents a significant risk of illness [4]. Pathogenic bacteria constitute a serious risk for public health and they affect about 48 million people, resulting in 3,000 deaths annually in the U.S.A. alone according to the Center for Disease Control and Prevention (CDC) [5]. Different physical, chemical and biological methods have been used to control the presence of pathogenic bacteria in food processing chain, but their efficiency was quite low in most cases and new methodology must be developed to decrease the risk of foodborne illness. *E. coli* is a very important contamination index of water and food, indicating contamination with wastewater [6].

Human infection has been linked to the consumption of contaminated food. Health risks of human have increased due to the misuse of antibiotics in animal breeding that has led to the occurrence of antibiotic-resistant bacteria [7]. However, bacteria and their products can be used to kill or reduce the numbers of pathogenic bacteria. For example, probiotic bacteria that produce bacteriocins can be used to kill or inhibit the pathogenic bacteria [8]. Alternatively, purified Nisin (bacteriocins) can be added to food products to kill such pathogens. Any new methodology should have no adverse effect on human health or the quality of the food product [9]. Bacteriophages have become a focus of interest to control bacterial contamination of food products. Bacteriophages occur naturally in any environment where their specific hosts proliferate. Bacteriophages have several characteristics that make them attractive as therapeutic or agents of biocontrol, these include their effectiveness, their specificity, natural

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residence in the environment and the fact that they are self-replicating and self-limiting [10]. Each kind of bacterium has its own bacteriophage, which can be isolated wherever its particular host grows [11]. Bacteriophage therapy can be limited by its narrow host range but this can be overcome by the use of phage cocktail, a combination of several phages, which can infect different bacterial strains [12]. Since resistance to antibiotics has become a serious problem, the use of bacteriophage to combat bacterial infections has regained general interest [13]. Phages have been used to successfully reduce the numbers of *E. coli* O157:H7 both *in vitro* and *in vivo* [14, 15] and effectively used to reduce the numbers of *S.* Typhimurium in turkey deli meat and in chocolate milk [16]. Recently, phages applications for bio-control have been approved by the Food and Drug Administration's (FDA) as a safe food additive on ready-to-eat products [17, 18].

In this study, different phages specific to *E. coli* and *S. enterica* have been isolated. The effectiveness of isolated bacteriophages specific for *E. coli* and *S. enterica* in reducing populations on cucumber and eggs at 4 °C was investigated.

MATERIALS AND METHODS

Bacterial Strains and Growth Media: The bacterial strains used in this study were isolated as part of this research. *Salmonella* and *E. coli* strains were isolated and identified from different food samples by direct plating on Xylose lysine deoxycholate agar (XLD), MacConkey and Eosin methylene blue (EMB) selective media using standard techniques [14]. Five typical colonies of each bacteria were examined by Gram stain and wet mount. Oxidase, catalase and rapid tests (RapID One system, remel, USA) were performed for confirmation of the *Salmonella* and *E. coli* isolates. Strains were stored at -80 °C in Tryptic Soy Broth (TSB) with 20% glycerol.

Cucumber and Eggs Samples: Cucumber and eggs were purchased from a large retail outlet. The surface area of cucumber and eggs were sterilized with 70% ethanol to assure the killing of any possible bacteria before they were used in this study.

Bacteriophages Isolation and Amplification: Phages were isolated from sewage water and other environmental samples. The two phages, EC3 and SE1, were propagated on their host strains in Tryptic Soy Broth (TSB) by growing them to an OD_{600} nm ~0.3 before adding phage to a Multiplicity of Infection (MOI) of 1 [14]. The mixtures were then incubated in a shaking water bath (180 rpm) at 37 °C for about 3 h. Then, a few drops of chloroform ($CHCl_3$) were added to the flask and left overnight for complete lysis. Afterward, the phage culture was centrifuged for 20 min at 5500 × *g* to remove bacterial cells, followed by centrifugation at a high speed (15,000 × *g*) for 2 h to precipitate the phage. The phage pellets were kept overnight at 4 °C to allow the dissociation of phage which were suspended in phage buffer (1 mM Tris, pH 7.6, 0.1 mg/ ml gelatin, 4 mg/ ml, NaCl), yielding a final titer > 10^{10} PFU/ ml.

Bacteriophages Enumeration: Isolated bacteriophages were enumerated by making serial dilutions of the centrifuged samples. Each dilution of sample suspension was applied as a 10 µl spot, in triplicate onto prepared bacterial soft agar overlay lawns and allowed to absorb into the agar [14]. Plates were incubated for 24 h at 37 °C and the number of plaques counted to get the phage titer per ml for each sample.

Bacteriophages Lytic Profiles: Lytic activities of isolated bacteriophages were determined for 6 *E. coli* isolates and 7 *Salmonella* strains. The lytic activity was measured by dispensing 10 µl of each phage containing about $\log_{10}$ 7 to 8 PFU onto prepared bacterial lawns. After absorption into the overlay agar, the plates were then incubated for 24 h at 37°C. If $\geq$ 20 plaques were produced, the tested bacteria were regarded as being sensitive to the phages [15].

***In vitro* Bacterial Infections:** Bacterial infections were carried out in TSB, as previously described [19]. Infection experiments used shake flasks at 37 °C with agitation (180 rpm). All samples were carried out under sterile conditions. Once the culture had reached OD600nm ~0.3, phages were added at an MOI of ~1 for *Salmonella* phage (SE1) and *E. coli* phage (EC3) with rapid mixing. Samples were periodically taken to enumerate bacterial survivors (CFU/ ml) and total phage (PFU/ ml). All samples were tittered in triplicate.

The Stability of Phages at 4°C: The stability of 5 different *E. coli* and *S. enterica* phages at 4°C was investigated. The inoculated samples were incubated at 4°C. Samples were taken daily over 10 days to enumerate the numbers of phages (PFU/ ml) as described before.

Control of *Salmonella* on Eggs and *E. coli* on Cucumber Subject to Chilling: Sterile cucumber and eggs were inoculated with 6.5 and 6.1 log_{10} of *E. coli* and *Salmonella* respectively and treated with 7.1 (MOI of ~1) and 7.5 log_{10} (MOI of ~1.2) of phages respectively. Inoculated cucumber and eggs were treated with phage after 30 min of inoculation with bacteria and held at refrigeration temperature (4 °C) for 5 days [21]. Viable counts of bacterial and phage counts were performed at time zero and various time points.

Statistical Analysis: Single factor ANOVA was used to test the differences between phage-free control samples and samples treated with phages.

RESULTS AND DISCUSSION

Bacteriophages Selection: Selected bacteriophages were isolated from different environmental sources as described earlier. The isolated bacteriophages (*n*= 5) were selected on the basis of their host range and their ease of propagation. The results indicated that phage EC1, EC2 and EC3 were almost completely specific for *E. coli* while SE1 and SE2 were specific for *Salmonella*. Two phage isolates were selected for further investigation and for *in vitro* and bio-control experiments (SE1 and EC3). *E. coli* 12900, *E. coli* ZK126 and *E. coli* B were kindly provided by Dr. Elizabeth Kutter's phage biology lab, Evergreen, WA, USA.

Table 1: Host range of isolated phages

	E. coli 12900	*E. coli* ZK126	*E. coli* B	*E. coli* 1	*E. coli* 2	*S. enterica* 1	*S. enterica* 2
EC1	+	-	+	+	-	-	-
EC2	+	+	-	-	-	-	-
EC3	+	+	-	+	+	-	-
SE1	-	-	-	-	-	+	+
SE2	-	-	-	-	-	+	-

The Stability of Phages at 4°C: When phages incubated on the surface of cucumber (EC1, EC2 and EC3) and on eggs (SE1 and SE2) at 4 ºC, a slight decrease in their numbers was observed. The results indicated that all phages were stable at refrigeration temperature (4 °C). Bacteriophage EC3 and SE1 showed a better stability than others over the experimental period (10 days). The isolated bacteriophages can be stable for long time periods at 4 ºC and these results are consistent with other published results in which bacteriophages remained stable for a long period of time at low temperature [20, 21]. The low decline in phage numbers could be due to phage decay. Generally, bacteriophages are stable in different chemical and physical conditions [22] and those factors could play an important role in their replication dynamics [23].

Infection of Host Strains with Phage *in vitro*: *E. coli* and *S. enterica* strains were grown in TSB to an OD_{600} nm ~ 0.3 before they were infected with phages EC3 and SE1 under aerobic conditions with shaking at 37°C at MOI 1. Both bacteria and phage were enumerated over a 3 h period. The infection (Fig. 2 A and C) resulted in a significant reduction in *E. coli* and *S. enterica* counts

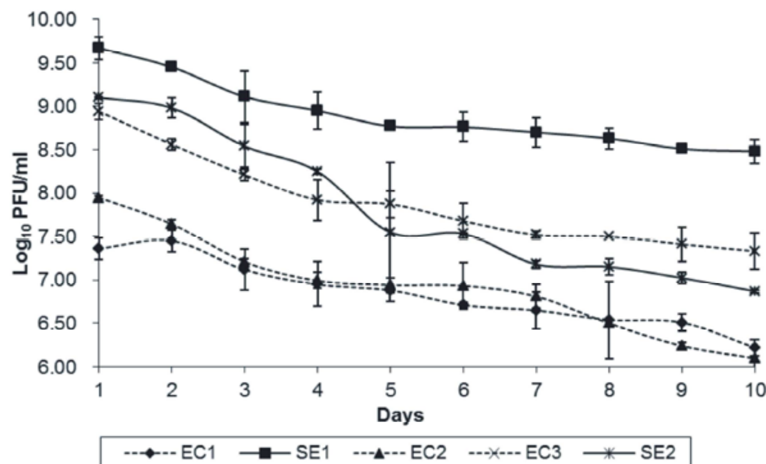

Fig. 1: Survival of phages on the surface of cucumber (EC1, EC2 and EC3) and on eggs (SE1 and SE2) at 4°C.

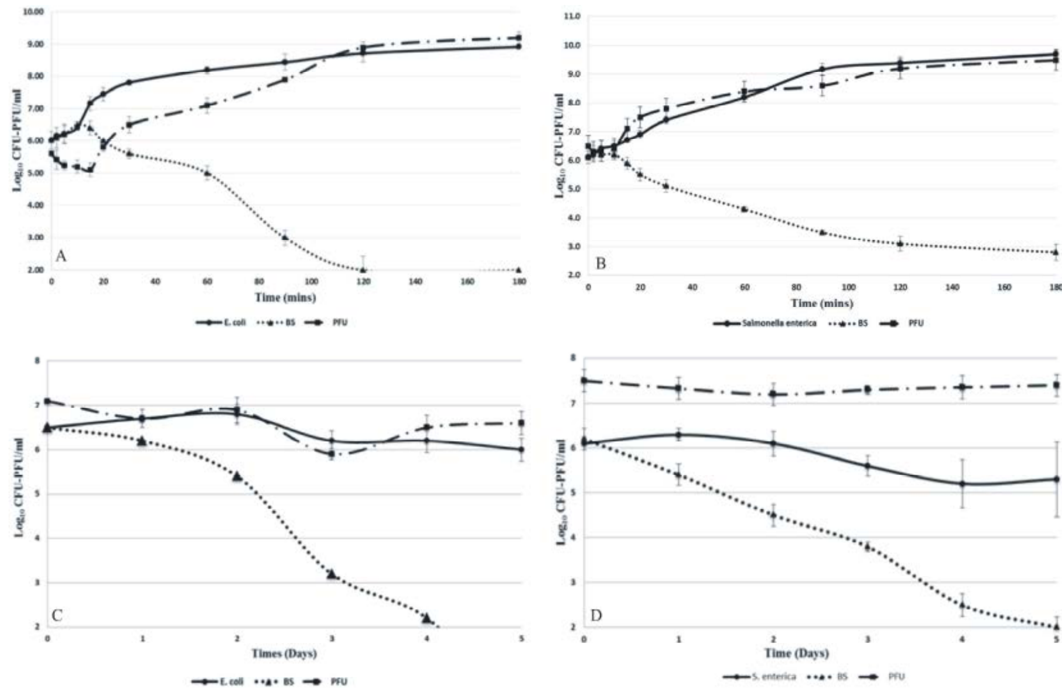

Fig. 2: Infection of *E. coli* 12900 and *S. enterica* with phages EC3 and SE1 respectively: A. Infection of *E. coli* 12900 with phage EC3 *in vitro.* B. Infection of *E. coli* 12900 with phage EC3 on cucumber. C. Infection of *S. enterica* 1 with phage SE1 *in vitro*. D. Infection of *S. enterica* 1 with phage SE1 on eggs

($P < 0.005$). The numbers of *E. coli* have decreased to undetectable levels after 120 min of infection while *S. enterica* have decreased by 7 $\log_{10}$ after 180 min of infection with SE1 phage. The bacteriophage numbers were increased slowly after 20 min. of infection. The numbers of phage EC3 were highly increased (3.6 $\log_{10}$ PFU/ ml) after 3 h of infection of *E. coli*. Alternatively, phage SE1 numbers were increased slightly by 3 $\log_{10}$ PFU/ ml when infected its bacterial host (*S. enterica*) at the end of experiment. The phage production of both phages has largely ceased after 120 min. of infection.

The conditions used in this study were used according to standard storage conditions and the application of phage may be applied during refrigeration storage to reduce both *E. coli* and *Salmonella* contamination in foods. A single bacteriophage has been chosen for each experiment to study the effectiveness of each phage to reduce its host. The results (Fig. 2 B and D) indicated that, the application of phages was very effective in reducing the numbers of *Salmonella* and *E. coli* of contaminated surface of cucumber and eggs to below the limit of detection. The inoculated cucumber and eggs with *E. coli* and *Salmonella* respectively were infected with phages at MOI of approximately 1. The samples were held at refrigeration temperature (4 °C) for 5 days and the results indicated that both phages were able to reduce the numbers of *E. coli* and *S. enterica* to undetectable levels ($P < 0.005$) after 4 days of infection. A very significant reduction ($P < 0.005$) in *E. coli* numbers (3.3 $\log_{10}$ PFU/ ml) was observed after 3 days of treatment with EC3 phage. Alternatively, a lower reduction rate (2.4 $\log_{10}$ PFU/ ml) in *S. enterica* numbers was observed in contaminated eggs when treated with SE1 phage. The application of high concentrations of phages to the contaminated surfaces of food increases the chance of killing host bacterial cells and increases food safety [24, 25].

The observed results in this study are in agreement with previous studies that have highlighted the effectiveness of phages specific for *Salmonella* to reduce the populations of *Salmonella* Enteritidis 3.5 log CFU on fresh-cut honeydew melons when stored at low temperatures [26]. The data are also in agreement with the data indicated that ECP-100 phage significantly reduced the numbers of *E. coli* O157:H7 in experimentally contaminated tomatoes, broccoli, spinach and ground beef [27]. These results may have significant practical implications during food storage at low temperature. For example, fresh vegetables and other foods may be

sprayed with phages during different postharvest and processing stages, e.g., before packaging and transport or during storage in the refrigerator.

The titers of both EC3 and SE1 phages were quite stable until the end of experiments and no increase in phage numbers was observed in bio-control experiment and this may due to the starvation conditions of bacteria under refrigeration storage indicating that phage replication can only happen if bacterial host cells are growing. In general, high titer of phage can reduce the numbers bacteria without replication [21] and this may due to lysis from without [28, 29]. Bacteriophages were used quite a lot as for controlling pathogenic bacteria and they could play an important role in controlling bacterial infections in foods and humans [30].

The work presented here indicated that pathogenic bacteria such as *E. coli* and *Salmonella* can be rapidly lysed by bacteriophage and possibly prevent the bacteria from surviving during processing and retail storage under refrigeration temperature. Previous results indicated that, the control of bacterial pathogens with antibiotics resulted in increasing the antibiotic resistant bacteria and other results indicated that phages were more effective than antibiotics [10, 31]. A combination of phage and antibiotics may improve the control of pathogenic bacteria [30]. To get a better reduction rate in a short time in commercial application, a cocktail of bacteriophages with appropriate specificity would be the most rational approach to reduce foodborne illness.

CONCLUSIONS

In conclusion, different lytic phages specific to *E. coli* and *Salmonella* have been isolated and studied in this study. Based on these observations, it was concluded that phage treatment can be used as a mean of bio-control for reducing the microbial load of *E. coli* and *Salmonella* bacteria in foods. Accordingly, phages can play a significant role in improving the safety of food products contaminated with different pathogenic bacteria, including *E. coli* and *Salmonella*.

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