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Biocontrol of *Salmonella* by bacteriophage application in seafood

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Abstract
Salmonella is a food-borne pathogen and reports of human cases of Salmonellosis are on the rise. *Salmonella* enterica is an important species with several serovars responsible for both enteric fever and gastrointestinal illness. A major challenge to the management of bacterial disease is the growing resistance to antimicrobials thus resulting in high morbidity and mortality. Eliminating this dangerous pathogen in food and preventing the spread of it would be a safer strategy for reducing the illness. One such eliminating process is through the application of lytic bacteriophage. Lytic bacteriophages are bacterial viruses that offer promise to combat bacterial diseases. As phages are non-toxic, do not impart any color, do not alter the texture or taste of the food, have no residual effect, and also maintain the original desired quality of food. Owed to all its desirable characteristics, bacteriophages are permitted for managing pathogenic bacteria and would be immensely useful to the pharmaceutical and food industries. In the present investigation, *Salmonella* phages having a broad host range capacity targeting many serovars of *Salmonella* enterica were applied to the seafood. The marked reduction in the *Salmonella* numbers in the spiked clam homogenate suggests its application value and its efficiency be a promising therapeutic biocontrol agent to combat *Salmonella*.

Keywords: *Salmonella*, bacteriophage, biocontrol, therapeutic

Introduction
National dietary guidelines have recommended for consumption of fish and other seafood regularly (World Health Organization, 2003) [21]. Seafood is a good source of high proteins, iodine, vitamin A and D, low in saturated fats, and rich in omega-3 essential fatty acids (Sidhu, 2003) [19]. Consumption of seafood plays a vital role in supplementing the protein along with the micronutrients, which prevents the scenario of nutrient deficiency of 166 million predictable cases in the low food security zones like Africa and Asian parts by 2030 (Golden *et al*., 2021) [7].

However, one should consume seafood that is devoid of pathogens. Seafood is more likely to get contaminated with pathogenic bacteria like *Salmonella* if it is harvested from a polluted water source. *Salmonella* is one of the common pathogens resulting in the outbreak of gastroenteritis or sporadic cases as reported by Jain *et al.* (2020) [9]. It causes zoonotic infection and it transfers infection from the animals to humans and also to humans transfer (Acha and Sczfres, 2001) [1]. *Salmonella* can easily cause infection in patients who are predisposed by human Immunodeficiency (HIV) resulting in virulent and severe extraintestinal disease (Angulo and Swerdlow, 1995) [3]. In 2005, the Food and Drug Administration (FDA) has issued nationwide alerts on contamination of *Salmonella* on two food items *viz*., orange juice and ice cream (CIDRAP, 2005) [6]. Zhang *et al.* (2007) [22] isolated the *Salmonella* Typhimurium from the outbreak-associated cake mix. It causes illnesses such as typhoid, paratyphoid, and salmonellosis. Over the last few years, the antibiotic resistance rate of S. Typhimurium has been increasing at an alarming rate, making it a global issue of increasing concern that might result in more severe health outcomes (Parisi *et al*., 2018; Jiu *et al*., 2020) [15]. The presence of *Salmonella* in seafood is considered an adulterant in both raw and cooked seafood products. All seafood should be devoid of *Salmonella* as it is a public health risk. Every shipment from the processor should be free from this pathogen, and if not the shipment is rejected. In recent years, frequent and abundant disease outbreaks by *Salmonella* spp. have been reported worldwide giving an alarming call to improve the prevention and control programmes (Popa and Popa, 2021) [17]. The control of this most dangerous foodborne pathogen like *Salmonella* is a challenging and highly specialized task. In view of the public health significance and the need for an alternative method to combat *Salmonella* in seafood,

the current study of 'Biocontrol of *Salmonella* by bacteriophage application in seafood' was undertaken with the following objectives *viz*.,

- Application of broad host range lytic Salmonella bacteriophage in seafood and
- Enumeration of bacterial load reduction in seafood upon bacteriophage application

Materials and Methods

Bacteriophage

The *Salmonella* bacteriophage showing the maximum host range and high titer were chosen for experimental studies on bacteriophage control of *Salmonella* contamination in seafood. The Phage 'R' was taken and was then propagated as previously described using specific host bacteria. The final concentration of phage 3×109 PFU/ml was taken and stored at 4 °C until use.

Food samples

The clam meat was chosen, to study the application spectrum of phages in seafood. The samples were purchased at the local fish market and screened for *Salmonella* contamination.
The screened samples were then kept at -80 °C until further usage.

Contamination procedure

An overnight culture of Multi-Drug Resistant -7 Salmonella (MDR-7) was taken to determine the bacterial load (CFU/ml) upon serial dilution. The desired number of bacterial cells (104CFU/ml) was then spiked to the seafood. The spiked seafood sample was incubated at 8 °C for 2h.

Phage treatment

The food samples that received aliquots of *Salmonella* Typhimurium (MDR-7) were treated with bacteriophage at a concentration of 109PFU/ml. Samples were then incubated at 8 °C for a total of 7 days.

Monitoring bacteria and phage counts

The bacterial viable counts (CFU/g) and phage concentrations (PFU/g) were initially determined immediately after the respective addition of bacteria and phage and monitored at 7h and 1, 2, 3, and 5days. For this purpose, 1gm of solid foods was homogenized in 9ml saline by using a stomacher lab blender for 2 to 3min. For quantitative determination of *Salmonella* cell counts, 100µl of homogenates or the liquid test samples were directly surface plated on the Xylose Lysine Desoxycholate (HiMedia, Mumbai). The plates were incubated for 24h at 37 °C until typical *Salmonella* colonies could be enumerated.

Results and Discussion

Selection of broad host ranged bacteriophage

Bacteriophage having a broad host range of 89.70% which was studied and confirmed previously was chosen as an ideal candidate to treat *Salmonella* in seafood. Bacteriophages having active and rapidly lytic activity *in vitro* were chosen to prevent septicemia and cerebritis or meningitis in chickens (Paul *et al*., 1998) [16]. Likewise, bacteriophages having a broad host range and high titer i.e., 'Phage R' were chosen in the current experimental study. Goode *et al.* (2003) [8] observed the eradication of phage-susceptible Salmonella strains. In the year 2007, Jones *et al.* [12] used bacteriophages as a disease control agent to protect plants with great potential to replace chemical control measures. In the present study, phage was used to control the foodborne pathogen in seafood in place of other laborious methods.

Pre-phage treatment

Before taking the seafood into the phage treatment, the seafood is subjected to screening for *Salmonella* initially. Later an experimental addition of the bacterial load should not be get added with the initial presence of bacteria. After an initial screening of *Salmonella* in the seafood, a known volume of a colony-forming unit (CFU) of the bacterial load in ml (CFU/ml) is added to the experimental seafood sample. Bacterial load upon serial dilution was determined in Table 1.

Phage treatment

Seafood was made to incubate along with *Salmonella* at a lower temperature for 5h. This incubation hour facilitates bacteria to adapt to the lower temperature. The phage concentration plaques forming the unit (PFU) upon serial dilution were determined in Table 2. The known volume of lytic bacteriophage load was added to the seafood. The great concern about the drug resistance of bacteria has stimulated new alternatives to combat bacterial threats using novel products such as bacteriophage and probiotics (Van Immerseel *et al.*, 2002; Andreatti Filho *et al*., 2003; Joerger, 2003) [20, 2, 11]. The potential of phages to control bacterial infections in cultured fish, in plants, and control cyanobacterial blooms has been studied (Nakai *et al*., 1999; Nakai and Park, 2002) [14, 13]. An oral toxicity study with rats receiving high doses of Listeria- phage Listex P100 did not reveal side effects (Carlton *et al*., 2005) [5]. A study in humans, with E. coli-specific phages, infected commensal E.coli strains as well as pathogenic strains and failed to show any adverse effects (Bruttin and Brussow, 2005) [4].

Post phage treatment-Enumeration of bacterial reduction upon phage application

The reduction of bacterial load upon phage application was enumerated and tabulated in Table 3. Ross *et al*. (2008) [18] have used two phages SSP5 and SSP6 against *Salmonella* Oranienburg in contaminated alfalfa seeds and could bring about 1 log10 CFU g− 1 reduction in the bacterial load. But the inhibitory effect on *Salmonella* lasted until 3h after which there was no effect. The *Salmonella* population was not affected by the 2nd dose of phageduction (Ross *et al*., 2008) In the current study, phage 'R' could bring about a maximum inhibitory effect on *Salmonella* with 1 log 10 CFU g−1 reduction. A bacterial reduction from 1,00,000 to 10,000 ensures its promising potential. Even though in the natural environment such a high bacterial load is not expected in seafood the low number of *Salmonella* when encountered would be lysed by this potent bacteriophage.

Table 1: Determination of MDR-7 bacterial load upon serial dilution

Dil^n Bact. (CFU/ml)	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
MDR-7	>300	>300	>300	>300	>300	100*	<30	<30	<30	<30

Table 2: Determination of phage concentration (PFU/ml) upon serial dilution

Dil^n Phage PFU/ml	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
R	>300	>300	>300	>300	77*	<30	<30	<30	<30	<30

*Indicates countable colonies (30-300)

Table 3: Enumeration of bacterial load reduction upon phage application

Days	Samples	
	Untreated (Control)	Treated
Day 1	3.0×10^5	3.0×10^5
Day 2	4.5×10^5	4.0×10^4
Day 3	5.1×10^5	1.0×10^4
Day 4	5.0×10^6	4.0×10^4
Day 5	4.8×10^5	3.0×10^4

Conclusion

The present study indicated an exemplary antibacterial efficiency of the bacteriophage by a noticeable reduction in the bacterial load in the seafood upon *Salmonella* phage application. No changes in seafood quality even after phage application exemplify its environmentally friendly approach to combating this food-borne pathogen. *Salmonella* phages in seafood are quite stable and can be an effective tactic for making the food safe. Phage-based quality control in the food dynamic process needs continuous evolvement of the phage preparation in order to effectively control the adapting pathogenic bacteria. It is expected that biocontrol of more food- borne bacterial pathogens may be addressed by phage application in years to come.

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