Isolation, characterization, and application of a novel *Salmonella* phage

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Dedicated

To

The experiences I never expected, and
the paths that were redirected.

To

The friends and family, I found along
the way.

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Moumita Dutta 11-10-2023
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Declaration

I hereby declare that the research work embodied in the thesis entitled "**Isolation, characterization, and application of a novel *Salmonella* phage**" is carried out by me at **ICMR- National Institute of Cholera and Enteric Diseases, Kolkata, India under the supervision of Dr. Moumita Dutta, Scientist-D, Division of Electron Microscopy.** This work is original and not submitted in part or full for any degree or diploma to this or any other university. All ideas and references are duly acknowledged.

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Thesis Title: Isolation, characterization, and application of a novel *Salmonella* phage

ABSTRACT

Enteric bacteria *Salmonella* is the causative agent for gastroenteropathy and enteric (typhoid) fever. The population of multi-drug-resistant (MDR) bacteria has considerably expanded over the past several years due to the overuse and misuse of antibiotics; consequently, developing an alternative antibacterial therapy to treat MDR bacterial strains is of utmost importance. Thus, there has been a recent, remarkable surge in phage research involving isolation, characterization, and application.

In this study, an enteric *Salmonella* bacteriophage STWB21 was isolated from a lake water sample, Kolkata, and found to be a novel lytic phage with promising potential against the host bacteria *Salmonella Typhi*. In addition, the phage STWB21 was also able to infect *S. Paratyphi*, *S. Typhimurium*, *S. Enteritidis*, and a few other bacterial species such as *Sh. flexneri 2a*, *Sh. flexneri 3a*, and *ETEC*. The phage morphology study using a transmission electron microscope revealed that the phage STWB21 belongs to the *Siphoviridae* family with an icosahedral head and a long flexible non-contractile tail. Phage stability was analyzed under various environmental conditions. Phage STWB21 was found relatively stable under a wide range of pH (4-11) and temperatures (4°C-40°C) for both typhoidal and non-typhoidal *Salmonella strains*. A one-step growth curve of bacteriophage was performed to assess the population kinetics of this isolated lytic phage. The latent period and burst size of phage STWB21 against *S. Typhi* were 25 min and 161 plaque-forming units per cell. The whole genome sequencing study revealed that phage STWB21 contained a dsDNA of 112,834 bp in length with a GC content of 40.37%. The existence of lytic genes and the absence of any lysogeny or toxin genes were also confirmed by genomic analysis. Furthermore, phylogenetic analysis revealed that the phage STWB21 cluster together with T5-like *Salmonella* phages. A detailed proteomic characterization identified 19 proteins in phage STWB21 by high-resolution Nano LC-MS/MS, which provided insight into the structural architecture of the phage. The structural models of the morphogenesis proteins were predicted using deep learning and homology-based methods. An *in-vitro* assay was performed to evaluate the capability of this phage for therapeutic purposes. Since *Salmonella* is a foodborne pathogen, it was found that bacteriophage STWB21 treatment significantly reduced biofilm not only on a 96-well microplate but also on food samples. The antibiofilm activity of phage STWB21 was also evaluated against *S.* Typhi, and *S.* Enteritidis alone and in comparison, with antibiotic cephalosporin. In both cases, a significant reduction was observed in the bacterial population of *S.* Typhi biofilm. The prophylactic and therapeutic efficacy of phage STWB21 was studied in a preclinical mouse model of *S.* Typhi infection. After introducing phage treatment to the infected mice, the phage showed reduced colonization in the liver and spleen in both the treatment and prevention groups. Overall, this thesis work showed phage STWB21 has a promising ability as a biocontrol agent of *Salmonella spp*. and proposes its application in food industries and for therapeutic purposes.

Moumita Dutta 11-10-2023

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Payel Mondal.

Payel Mondal

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List of Abbreviations

x g	gravitational force (multiples of gravity)
ºC	Degree Celsius
approx.	Approximately
APS	Ammonium per sulphate
ATCC	The American Type Culture Collection
bp	base pair
CFU	colony forming unit
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
Ds	Double-stranded
e.g.	for example
EM	Electron Microscope
EDTA	Ethylene diamine tetra acetic acid
ESI-MS	Electrospray ionization mass spectrometry
et al.	"*et alia*" and others
gm	Gram
h	Hour
HEA	Hektoen enteric agar
ICTV	International Committee on Taxonomy of Viruses
i.e.	"*id est*" that is
kbp	kilo base pairs
Kv	Kilo volt
kDa	Kilo Dalton
LB	Luria-Bertani
LD_{50}	lethal dose 50%
LPS	Lipopolysaccharide
µm	Micrometer
µg	microgram
µL	microlitre
Mb	million base pairs

MDR	Multidrug resistance
Mm	Millimeter
min	minute
mL	milliliter
M	Molar
mM	milli-Molar
Mv	Milli volt
MOI	multiplicity of infection
MS	mass spectrometry
MS/MS	tandem mass spectrometry
N	Normal
NTS	Non-typhoidal *Salmonella*
nm	nanometre
OD	optical density
ORF	open reading frame
PAGE	Polyacrylamide gel electrophoresis
PDB	Protein Data Bank
PFU	Plaque-forming unit
RPM	Rotations Per Minute
RT	Room temperature
RTE	Ready-to-Eat
SDS	Sodium dodecyl sulphate
Sec	Second
SEM	Scanning electron microscope
sp.	species
spp.	species pluralis (multiple species)
subsp.	subspecies
TAE	Tris-Acetate-EDTA
TEM	Transmission Electron Microscope
TEMED	N, N, N’, N’-tetramethylethylenediamine
TSA	Tryptic Soy Agar
TS	Typhoidal *Salmonella*
TSB	Tryptic Soy Broth

UK	United Kingdom
USA	United States of America
UV	ultraviolet
V	volt
v/v	volume/volume
w/v	weight/volume

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Chapter 1

Introduction & Objective of the work

Chapter 1

1.1 Introduction

Enteric bacteria such as *Salmonella*, *Shigella*, and *E. coli* are the principal agents that cause food-borne gastroenteritis, typhoid fever, and diarrhea in people and continue to be serious global health challenges. Among them, *Salmonella* is very different from others. It is a Gram-negative, flagellated, rod-shaped bacteria. More than 2600 distinct serovars of the bacterium *Salmonella enterica* made up this diversified species (Achtman et al., 2012). It is divided into two main groups: (a) Typhoidal *Salmonella*, and (b) Non-typhoidal *Salmonella*. *Salmonella* may infect a broad range of hosts, including humans and different warm-blooded animals One of the most frequent causes of diarrhea in the world is salmonellosis. Salmonellosis-related mortality from typhoidal and nontyphoidal causes included 178,000 and 90,300, respectively, in 2015 (Wang et al., 2016). They are primarily responsible for a variety of disease syndromes including gastroenteritis, enteric fever, and bacteremia. It is a common foodborne pathogen that is primarily present in poultry, eggs, and dairy products (Silva et al., 2012). It may be transferred by human feces, contaminated food, water, and person-to-person contact. The fecal-oral route is the most prevalent way of transmission (Mogasale VV et al., 2018). In the last few years, antibiotics have been used more often to treat bacterial illnesses, which has led to an increase in the emergence of multi-drug-resistant bacteria. Therefore, bacteriophages have been suggested as an alternative biocontrol agent for bacterial infections, and most of the bacteriophages were lytic. Bacteriophages are bacterial viruses that are highly specific and effective in killing their targeted host bacteria. The mechanism of killing bacteria and developing bacterial resistance in the case of phage and antibiotics is fundamentally different (Sulakvelidze A et al., 2001; Lu TK et al.,2011; Merril CR et al., 2003). The advantage of phage usage in the prevention of bacterial infections is due to their strict host specificity, high bactericidal efficiency, safety, low cost of production, and ease of extraction and preservation (Miedzybrodzki et al., 2008; Haq et al., 2012). Usually, *Salmonella* bacteriophages are isolated from the habitat of host bacteria, i.e., food, soil, feces contents, and sewage water. The lytic bacteriophages occupy the three families of Caudovirales: *Myoviridae*, *Siphoviridae*, and *Podoviridae*. *Salmonella* phages were also reported to be infective over a wider range of serovar hosts. The present study endeavors to isolate and characterize a lytic phage from a water sample to control and reduce the presence of *Salmonella spp.*

1.2 Aims

The major aims of this project were to isolate and characterize a novel polyvalent bacteriophage effective against different typhoidal and non-typhoidal *Salmonella* serovars with strong potential to be used in phage therapy. To achieve this, a series of experiments were designed under each objective as follows:

1. Isolation and characterization of a *Salmonella* phage STWB21.
2. Genomic and proteomic study of phage STWB21 followed by prediction of the structure of significant phage proteins.
3. *In-vitro* antibiofilm activity of phage STWB21 and its application in food samples.
4. *In-vivo* experiments to test the safety and efficacy of the phage in a mouse model.

1.3 Thesis outline

Section 1.2 identified the four areas of investigation designed to address the aims of the project. **Chapter Two** provides a review of the literature in order to introduce the major concepts relevant to the project. After a brief introduction to the epidemiology of *Salmonella spp.* bacteria in the worldwide discovery of bacteriophages, their classification, and modes of replication, the review describes the different areas of phage biocontrol research with a specific focus on food-based applications and therapeutic applications. The review concludes with an overview of the major features of the organisms to be targeted in the current study, *Typhoidal Salmonella spp.* and *Non-typhoidal Salmonella spp*.

This is followed, in **Chapter Three**, by a description of the methods undertaken in the current study.

Chapter Four outlines the isolation and biological characterization of bacteriophage required for use in subsequent studies of phage biocontrol. The characterized phage STWB21 was tested for its ability to control *Salmonella spp.* under different conditions.

Chapter Five describes the isolation and exploration of phage genomes will help define the origins, genetic diversity, and evolutionary mechanisms of phages and contribute to a better understanding of the broader biology of microbial populations and how their genomic characteristics contribute to observable features.

Chapter Six discusses the proteomics analysis of the phage STWB21 described in order to investigate and identify the relevant phage structural peptides using LC-ESI-MS/MS. The models of their structural proteins generated by AlphaFold.

In **Chapter Seven**, the use of phage therapy to control S. Typhi biofilm *in vitro* studies is discussed in order to determine various aspects of this therapy for more widespread application.

Chapter Eight describes the effectiveness of the phage STWB21 in preventing *Salmonella* Typhi invasion into the liver and spleen tissue in a mouse model of salmonellosis.

Chapter Nine outlines the isolation and characterization of another novel non-typhoidal bacteriophage SEWB23. It was required for use in subsequent studies of phage biocontrol**.** It evaluated the efficacy of two *Salmonella* phages STWB21 and SEWB23 cocktail in reducing concentrations of *S.* Typhi and *S.* Enteritidis in contaminated chicken and biofilm under laboratory conditions.

Finally, **Chapter Ten** presents a summary of the major findings of this thesis work as well as a general discussion of the broader topic of phage-mediated bacterial infection control and the scope for further research.

Chapter 2

Literature Review

Chapter 2

2.1. The *Salmonella* Bacteria

Salmonella is one of the members of the *Enterobacteriaceae* family. They are facultative anaerobes, rod-shaped, non-spore-forming, and negative for Gram stain and oxidase tests (Figure 2.1). They are also motile due to the presence of peritrichous flagella (Giannella RA 1996). *Salmonella*, among other genera in the Enterobacteriaceae family, is found in the gastrointestinal systems of several mammals, birds, and reptiles. It may also survive outside of such living organisms. The genus is named after an American bacteriologist, D. E. Salmon. After the discovery and isolation of the bacteria, it was known as "bacillus choleraesuis" (now known as *Salmonella* Choleraes) from porcine intestines by his research assistant Theobald Smith in 1884 (Agbaje et al., 2011). Based on the differences in their 16S rRNA sequence analysis, the genus *Salmonella* is classified into two species, *Salmonella enterica* (type species) and *Salmonella bongori* (Table 2.1). *S. enterica* can be further classified into six subspecies based on their genomic relatedness and biochemical properties. The subspecies are denoted with roman numerals: I, *S. enterica* subsp. enterica; II, *S. enterica* subsp. salamae; IIIa, S. enteric subsp. arizonae; IIIb, *S. enterica* subsp. diarizonae; IV, *S. enterica* subsp. houtenae; and VI, *S. enterica* subsp. indica. Among all the subspecies of *Salmonella*, *S.* enteric subsp. *enterica* (I) is found predominantly in mammals and contributes to approximately 99% of *Salmonella* infections in humans and warm-blooded animals.

Table 2.1: The nomenclature of *Salmonella* used at the CDC

Position of taxonomy	**Nomenclature**
Genus (in italics)	• *Salmonella*
Species (in italics)	• *enterica* (subspecies I, II, IIIa, IIIb, IV, and VI) • *bongori* (formerly subspecies V)
Serotype (Word not italicized, first letter capitalized)	The name of a serotype should be preceded by the word “serotype” or “ser.”, the first time it’s mentioned in a text. • Subspecies I serotype are named, those in subspecies II to IV, VI, and *S. bongori* are designated by antigenic formulae. • Subspecies II, IV, VI, and *S. bongori* members retain their names if named before 1966

Along with phylogenetic classification, Kauffman and White proposed a method for distinguishing *Salmonella* serotypes based on three primary antigenic determining factors: flagellar (H), capsule (K), and somatic (O) antigens (Brenner et al., 2000).

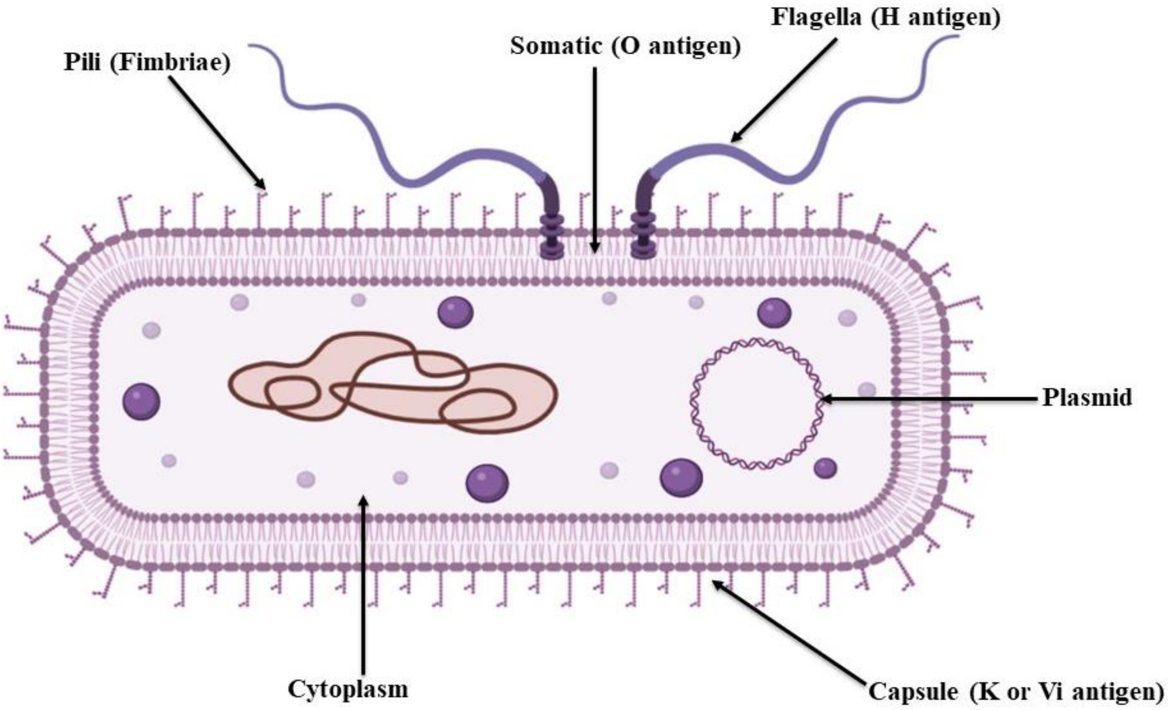


Figure 2.1: Schematic illustration of the structure of *Salmonella* using Biorender software.

The lipopolysaccharides that are present on the outer membrane are in their heat-stable form, which is the O antigen. Some *Salmonellae* serotypes express one or more O antigen classes. The immune system of the host is triggered by H antigens embodied in bacterial flagella. Two distinct genomic regions that code to produce flagella are present in most of the *Salmonella* serovars. The reason they are referred to as diphasic (phase I and II) bacteria is due to the fact they have the unusual capacity to express a single of these proteins at a time. The immunological characteristics of a serotype are determined by phase I H antigens, whereas phase II antigens are common by multiple serotypes.

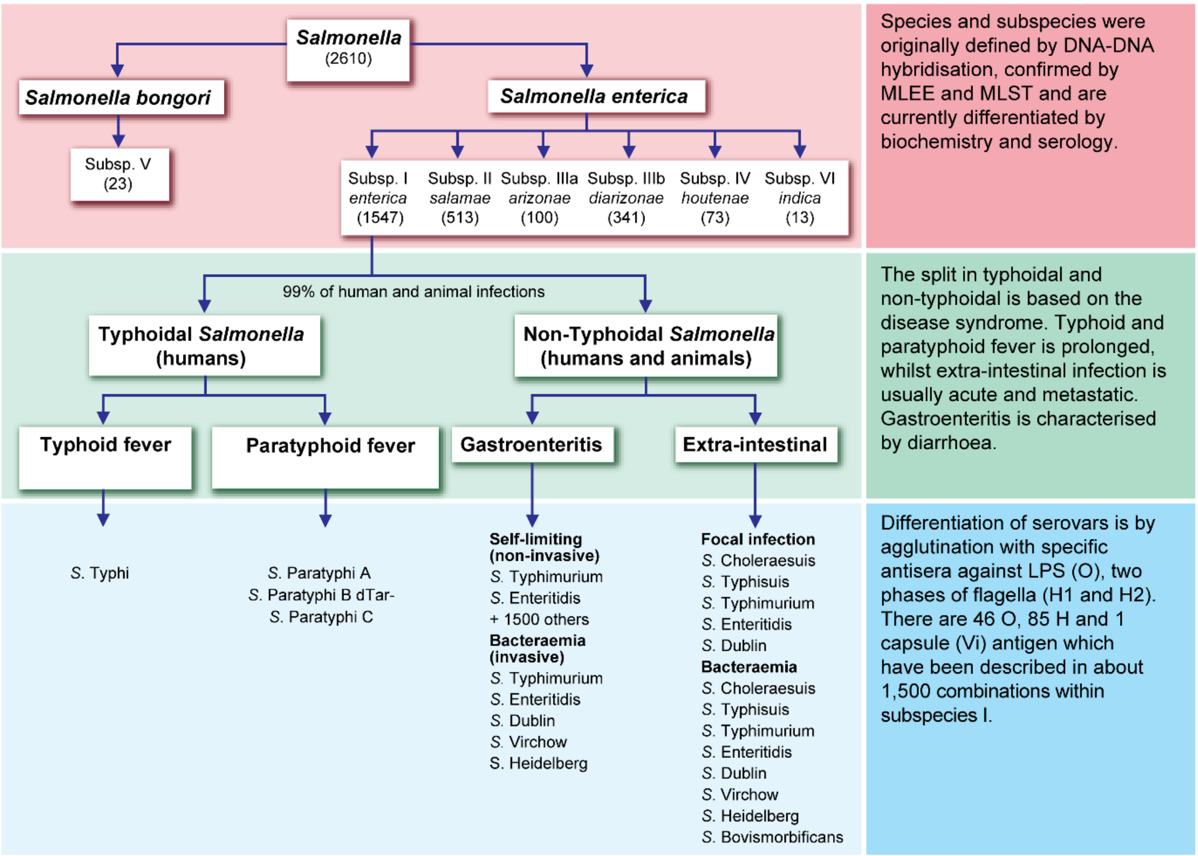


Figure 2.2: Classification of *Salmonella spp*. extracted from Achtman et al., 2012.

2.1.1 Salmonellosis: Routes of Transmission

Incidence of salmonellosis is defined as the detection of *Salmonella* in humans, animals, or a group of animal products or in the environment that can be explicitly linked to identified human, animal, or animal feed. *Salmonella* may cause a variety of illness syndromes, including enteritis, septicemia, and abortion. Meningitis is an uncommon consequence that affects fewer than 1% of cases of clinical salmonellosis. Most *Salmonella*-related illnesses have limited host-specificity. Most of the *Salmonella* infections, also called Salmonellosis, are among the biggest threats to public health in many nations worldwide. They are caused by the consumption of foods and water contaminated with *Salmonella*. Many different foods have been found to be contaminated with *Salmonella* spp., serving as carriers of this pathogen in outbreaks. These foods include milk, eggs, poultry beef, pork, raw meat, milk, fish, shrimp, frog legs, tomatoes, cantaloupes, leafy greens, and yeast (Todd, 2014). Salmonellosis can be classified into two major categories: typhoidal and non-typhoidal salmonellosis (Figure 2.2). *Salmonella* is mostly found in the intestines of people and other animals, but it has also been found in insects and reptiles. *Salmonella* infections can come from a variety of sources, but the most significant ones include eggs, meat, dairy products, vegetables, and water (Brenner et al., 2000). Eggs and meat (chicken, pork) continue to be the most significant sources, although Pires et al. highlighted that vegetables and fruits should not be overlooked (Shi et al., 2015). The illness has been associated with exposure to chicken and other birds, which are known reservoirs of the bacterium. Additionally, infections have been documented following interaction with animals. The animal frequently shows no symptoms. It is possible to transmit from person to person. Serotypes can infect any warm-blooded mammal, whereas others are host-specific. As a result, *S.* Typhi only infects human, is transmitted from person to person via the fecal-oral route, and have no significant animal reservoirs (Figure 2.3). The prevalence of illnesses in both people and animals is influenced by around 50 serovars (Ford et al., 2016).

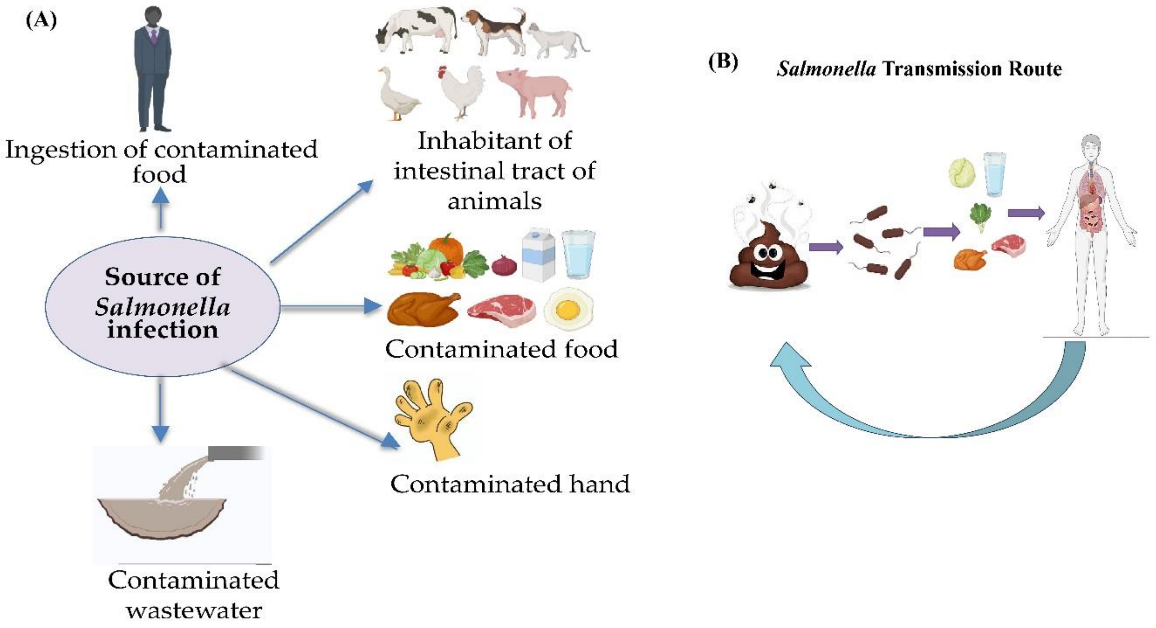


Figure 2.3: Transmission of *Salmonella spp.* using Biorender software.

2.1.1.1 Non-Typhoidal Salmonellosis (NTS)

Non-typhoidal salmonellosis (NTS) is mostly an intestinal infection (enteritis) characterized by fever, diarrhea, malaise, nausea, and vomiting (Coburn et al., 2007). Salmonellosis is thought to be associated with about 400 deaths per year, 15,000 hospitalizations, and an estimated 1.4 million people infections in the United States alone (Akil et al., 2014). NTS is responsible for three million deaths and 1.3 billion instances of gastroenteritis worldwide (Piya et al., 2015). India has a low number of data and reports on NTS infections, but Asia is the region where these cases of NTS-associated diarrhea are most common (Kumar et al., 2022). *Salmonella spp*. induced acute enteritis is self-limiting, and significant systemic sequelae may lead to challenges for infected people. The severity of the infection is influenced by the serovars involved and other host-specific variables.

2.1.1.2 Typhoidal Salmonellosis (TS)

Typhoidal disease (or typhoid fever) is a systemic illness often caused by *S. enterica subsp*. enterica serovar Typhi (*S*. Typhi) in humans (Coburn et al., 2007). In terms of severity and duration, paratyphoid fever is a different form of typhoidal salmonellosis that is very similar to typhoid fever. *Salmonella* Paratyphi A, B, and C are the causative agents of the symptoms resembling typhoid fever. The term "enteric fevers" has been used to describe both typhoid fever and paratyphoid fever (Bhan et al., 2005). *Salmonella* bacteria adhere to cells in the jejunum causing Typhoidal Salmonellosis. It infiltrates the host system by macrophage endocytosis in the subserosa moves into the mesenteric lymph nodes and multiplies and spreads via the lymphatic and circulatory systems. The spleen, liver, bone marrow, bile ducts, skin, and Peyer's patches are all addressed during the second stage of infection (Kayser et al., 2005). Infected people often exhibit fever, headache, nausea, cramping in the stomach, vomiting, leucopenia, bradycardia, swelling of the spleen, abdominal roseola, and diarrhea. Other symptoms, such as arthritis, may appear 3 to 4 weeks after the beginning of symptoms in severe or chronic conditions.

The diagnosis of typhoid Salmonellosis is mostly dependent on different techniques:

1. Isolation of the organism from the stool. 2. Using specialized antisera. The titer of agglutinating antibodies against the O and H antigens will be determined in the subsequent procedure, which is often referred to as the WIDAL test. Antibiotics are often recommended for therapy since the condition causes generalized sepsis (Kayser et al., 2005).

2.1.2 *Salmonella* Virulence Factors

A range of virulence factors mediates the capacity of *Salmonella* to infiltrate gut epithelia and avoid host immune responses, both of which are important characteristics underlying the production of gastrointestinal illness and survival inside the host (Mohler et al., 2009). The genes that encode these virulence factors are contained in *Salmonella* pathogenicity islands (SPI), which can be found on the chromosome or plasmid (Knodler et al., 2005). In the literature to date, 23 SPIs have been identified. However, further research is needed to fully understand the roles played by the genes found on these islands. Only five (SPI1–SPI5) are present in all *Salmonella enterica* serovars out of these twenty-three (Hurley et al., 2014).

These genes produce the effector proteins that *Salmonella* needs to invade and survive inside cells, as well as the type III secretion system that enables these proteins to pass the plasma membrane and enter mammalian cells (Coburn et al., 2007; Fierer 2001). The functions of SPIs include gut epithelial invasion, cytokine secretion stimulation, ileal fluid secretion stimulation, intracellular growth by altering the surrounding environment, enterocolitis in its complete form, and systemic salmonellosis (Coburn et al., 2007; Fierer 2001; Hurley et al., 2014).

2.1.3 Host specificity

Salmonella serovars have developed a variety of preferences for specific hosts. The reason for this predilection for distinct hosts is believed to be related to variations or specifics in how the organism adheres to surface molecules on the mucosal surface of the gastrointestinal system (Bäumler et al., 1998). Some serovars are termed “host-adapted” when they selectively infect a particular host (Bäumler et al., 1998). These serovars may be observed in their respective hosts, such as the serovars Dublin in cattle and Choleraesuis in swine (Uzzau et al., 2000; Coburn et al., 2007). Although infections with host-adapted serovars generally occur for a specific host, they can sporadically infect different hosts. For instance, Dublin and Choleraesuis can cause serious sickness in pigs and cattle, respectively, they may also spread a lesser form of the illness to humans and other mammalian species (Wray and Sojka, 1977; Nnalue 1991). Other host-adapted serovars, including serovars Gallinarum/Pullorum in poultry and Typhi/Paratyphi in humans, are associated with the emergence of illness in a single organism and referred to as “host un-adapted”. In contrast, a few serovars, such as Typhimurium, Enteritidis, and Newport, are referred to as "host un-adapted" since they may infect a variety of hosts (Uzzau et al., 2000).

2.1.4 *Salmonella* Outbreaks

2.1.4.1 Foods Associated with Salmonella Outbreaks

Salmonella serovar identification is crucial, but it is also important to take into consideration the association between a certain serovar and a given diet in order to develop intervention techniques that are specifically tailored to that product. Many different types of foods can become contaminated with *Salmonella* as shown in Table 2.2 (Crum-Cianflone 2008; CDC 2011; Jackson et al., 2013). Until two decades ago, reported *Salmonella* outbreaks predominantly involved chicken, turkey, pork, meat, and eggs. The traditionally common foods in which *Salmonella* outbreaks occur, such as chicken, beef, turkey, pork, and eggs, were still a prominent component of *Salmonella* outbreaks. However, the number of outbreaks of *Salmonella* infection associated with fresh produce has recently experienced a marked increase. Compared with 2008, the number of outbreaks associated with fresh produce increased almost 4.3-fold in 2017. The major sources of outbreaks from fresh produce include alfalfa sprouts, cantaloupes, mangoes, cucumbers, bean sprouts, and papayas. The most unexpected finding was the occurrence of *Salmonella* outbreaks even in dried foods, such as peanut butter, nut butter, seeds, and pistachios since 2007. As the consumption of fresh fruits and vegetables is becoming increasingly popular owing to the trend for healthy foods and diets, the impact of *Salmonella* outbreaks from fresh produce will increase. More importantly, most fresh produce undergoes minimal processing (cutting, peeling, and slicing); often, fresh produce is usually consumed raw. After contamination during processing, it is hard to kill or reduce *Salmonella* contamination in fresh produce prior to consumption. Thus, the contamination of *Salmonella* in fresh produce should be controlled at the early stages of production. Between 1998 and 2008, the most common food item implicated in 1,491 human *Salmonella* outbreaks were eggs (28%), followed by chicken (16%), pork (9%), beef (8%), fruit (8%), and turkey (7%) (Jackson et al., 2013). Therefore, a new intervention strategy should be developed to manage the new trends in *Salmonella* outbreaks.

Table 2.2: List of selected outbreak investigations linked to foods by CDC

Year	Food	Bacterial source
2023	Raw Sprouted Nut Butter Spreads	*Salmonella* Paratyphi B variant L (+) tartrate (+)
	Cucumbers Frozen	*Salmonella* Poona
	Pork	*Salmonella* I 4,[5],12: i: - and *Salmonella* Infantis
	Raw, Frozen, Stuffed Chicken Entrees	*Salmonella* Enteritidis
	Raw Tuna	*Salmonella* Paratyphi B variant L (+) tartrat (+) and *Salmonella* Weltevreden
2022	Alfalfa Sprouts	*Salmonella* Typhimurium
	Fish	*Salmonella* Litchfield
	Peanut Butter	*Salmonella* Senftenberg
	Salami Sticks	*Salmonella* I 4,[5],12: i: -

2021	Seafood	*Salmonella* Thompson
	Onions	*Salmonella* Oranienburg
	Italian-Style Meats	*Salmonella* Infections
	Prepackaged Salads	*Salmonella* Typhimurium
	Frozen Cooked Shrimp	*Salmonella* Weltevreden
	Raw Frozen Breaded Stuffed Chicken Products	*Salmonella* Enteritidis
	Cashew Brie	*Salmonella* Infections
	Ground Turkey	*Salmonella* Hadar
2020	Wood Ear Mushrooms	*Salmonella* Stanley
	Peaches	*Salmonella* Enteritidis
	Onions	*Salmonella* Newport
2019	Cut Fruit	*Salmonella* Javiana
	Ground Beef	*Salmonella* Dublin
	Papayas	*Salmonella* Uganda
	Frozen Raw Tuna	*Salmonella* Newport
	Kawaran Brand Tahini	*Salmonella* Concord
	Pre-Cut Melon	*Salmonella* Carrau
	Butterball Brand Ground Turkey	*Salmonella* Schwarzengrund
2018	Raw Chicken Products	*Salmonella* Infantis
	Chicken	*Salmonella* I 4,[5],12: i:-
	Raw Sprouts [Español]	*Salmonella* Montevideo
	Kratom	*Salmonella* I 4,[5],12: b:-
	Chicken Salad	*Salmonella* Typhimurium
	Dried Coconut	*Salmonella* Typhimurium

	Shell Eggs	*Salmonella* Braenderup
	Pre-Cut Melon	*Salmonella* Adelaide
	Kellogg's Honey Smacks Cereal	*Salmonella* Mbandaka
	Gravel Ridge Farms Shell Eggs	*Salmonella* Enteritidis
	Raw Turkey Products	*Salmonella* Infections
	Hy-vee Spring Pasta Salad	*Salmonella* Sandiego
	Ground Beef	*Salmonella* Newport
2017	Maradol Papayas [Español]	*Salmonella* Newport, *Salmonella* Infantis, *Salmonella* Urbana, *Salmonella* Anatum, *Salmonella* Thompson, *Salmonella* Kiambu, *Salmonella* Agona, and *Salmonella* Gaminara
2016	Shell Eggs	*Salmonella* Oranienburg
	Organic Shake & Meal Products	*Salmonella* Virchow
	Alfalfa Sprouts	*Salmonella* Muenchen and *Salmonella* Kentucky
	Pistachios	*Salmonella* Montevideo
	Alfalfa Sprouts	*Salmonella* Reading and *Salmonella* Abony
2015	Raw Sprouted Nut Butter Spreads	*Salmonella* Paratyphi B variant L (+) tartrate (+)
	Frozen Raw Tuna	*Salmonella* Paratyphi B Variant L (+) tartrate (+) and *Salmonella* Weltevreden
	Raw, Frozen, Stuffed Chicken Entrees	*Salmonella* Enteritidis
	Raw, Frozen, Stuffed Chicken Entrees	*Salmonella* Enteritidis
	Pork	*Salmonella* I 4,[5],12: i:- and *Salmonella* Infantis
	Cucumbers	*Salmonella* Poona
	Cucumbers	*Salmonella* Newport

2014	Raw Cashew Cheese	*Salmonella* Stanley
	Organic Sprouted Chia Powder	*Salmonella* Newport, *Salmonella* Hartford, and *Salmonella* Oranienburg
	Chicken	*Salmonella* Heidelberg
	Bean Sprouts	*Salmonella* Enteritidis
	Clinical and Teaching Microbiology Laboratory Exposure	*Salmonella* Typhimurium
2013	Live Poultry	*Salmonella* Typhimurium
	Live Poultry	*Salmonella* Infantis, *Salmonella* Lille, *Salmonella* Newport, and *Salmonella* Mbandaka
	Small Turtles	*Salmonella* Sandiego, *Salmonella* Pomona, and *Salmonella* Poona
2012	Peanut Butter	*Salmonella* Bredeney
	Mexican Style Restaurant Chain	*Salmonella* Enteritidis
	Raw Scraped Ground Tuna Product	*Salmonella* Bareilly and *Salmonella* Nchanga
	Ground Beef	*Salmonella* Enteritidis
	Cantaloupe	*Salmonella* Typhimurium and *Salmonella* Newport
	Mangoes	*Salmonella* Braenderup

2.1.4.2 Salmonella in poultry production and processing

Salmonella contamination in poultry has the potential to spread to humans and result in illnesses (Kimura et al., 2004; USDA-FSIS, 2011). Indeed, the possibility of contracting this disease in humans is significantly influenced by the presence of *Salmonella* in poultry and avian products (Bryan and Doyle, 1995). In poultry farms, intestinal colonization takes place due to intensive rearing practices, and contamination of body parts is facilitated by their close proximity (Bryan and Doyle, 1995). It is possible for feed to be contaminated because it might combine heat-treated products with raw materials, especially in untouched feed. *Salmonella* present in the feces can persist in these conditions for a few days after fecal shedding and cause contamination of the soil and litter (Bryan and Doyle, 1995). Another source of contamination in poultry facilities is the outer surface of the bird, particularly the feathers and skin (Corry and Atabay, 2001). Bird contamination may also spread during transportation from farms to industrial facilities. The CDC has included below their chosen epidemic investigations that involve animals (Table 2.3).

Table 2.3: List of selected outbreak investigations linked to animals by CDC

Year	Animal	Bacterial source
2023	Backyard Poultry	*Salmonella* infections
2022	Pet Bearded Dragons	*Salmonella* infections
	Small Turtles	*Salmonella* Stanley
	Backyard Poultry	*Salmonella* Infections
	Pet Bearded Dragons	*Salmonella* Uganda
2021	Backyard Poultry	*Salmonella* Infections
	Wild Songbirds	*Salmonella* Typhimurium
	Small Turtles	*Salmonella* Typhimurium
2020	Pet Bearded Dragons	*Salmonella* Muenster
	Pet Hedgehogs	*Salmonella* Typhimurium
	Backyard Poultry	*Salmonella* Infections
	Small Pet Turtles	*Salmonella* Typhimurium
2019	Pet Turtles	*Salmonella* Oranienburg
	Pet Hedgehogs	*Salmonella* Typhimurium
	Pig Ear Dog Treats	*Salmonella* I 4,[5],12: i: -
	Backyard Poultry	*Salmonella* Infections
2018	Live Poultry in Backyard Flocks	*Salmonella* Infections
	Pet Guinea Pigs	*Salmonella* Enteritidis

2017	Pet Turtles [Español]	*Salmonella* Agbeni
	Live Poultry	*Salmonella* Infections
2016	Dairy Calves	*Salmonella* Heidelberg
	Live Poultry	*Salmonella* Enteritidis, *Salmonella* Muenster, *Salmonella* Hadar, *Salmonella* Indiana, *Salmonella* Mbandaka, *Salmonella* Infantis, and *Salmonella* Braenderup
2015	Small Turtles	*Salmonella* Sandiego, and *Salmonella* Poona
	Live Poultry	*Salmonella* Enteritidis, *Salmonella* Hadar, *Salmonella* Indiana,and *Salmonella* Muenchen
	Pet Crested Geckos	*Salmonella* Muenchen
2014	Frozen Feeder Rodents	*Salmonella* Typhimurium
	Live Poultry	*Salmonella* Infantis and *Salmonella* Newport
	Pet Bearded Dragons	*Salmonella* Cotham
2013	Live Poultry	*Salmonella* Typhimurium
	Live Poultry	*Salmonella* Infantis, *Salmonella* Lille, *Salmonella* Newport, and *Salmonella* Mbandaka
	Small Turtles	*Salmonella* Sandiego, *Salmonella* Pomona, and *Salmonella* Poona
2012	Hedgehogs	*Salmonella* Typhimurium
	Live Poultry	*Salmonella* Hadar, *Salmonella* Montevideo, *Salmonella* Infantis, *Salmonella* Newport, and *Salmonella* Lille
	Small Turtles	*Salmonella* Sandiego, *Salmonella* Pomona, and *Salmonella* Poona

2.1.5 Government Initiatives Globally for the Reduction of *Salmonella spp.*

Since the 1950s, antibiotics have served a significant role in cattle and poultry production (Castanon, 2007). In order to prevent disease, they are often administered at subtherapeutic levels, while treating a condition, at therapeutic levels. The use of antibiotics in food animals may be able to lower the frequency of foodborne pathogens in foods (Singer et al., 2007); however, this advantage is severely threatened by the rise of antibiotic-resistant bacteria. The U.S. Department of Agriculture Food Safety and Inspection Service has taken action to limit *Salmonella*. In order to lower the frequency and incidence of foodborne diseases, including *Salmonella*, the Pathogen Reduction; Hazard Analysis, and Critical Control Point (HACCP) systems Final Rule established the standards applicable to meat and poultry establishments (UDSA-FSIS, 1996). It is now possible for inspected establishments to examine their procedures using baseline data from studies on *Salmonella* done between 1997 and 2000 (USDA-FSIS, 2005). In 2010, the Food Safety and Inspection Service (FSIS) published the third version of the compliance guideline for reducing *Salmonella* and *Campylobacter* in chicken (USDAFSIS, 2010). This guideline includes advice for the industry to adhere to FSIS standards for potential food safety risks. Additionally, starting in July 2011, revised performance criteria for *Salmonella* in young chicken and turkey slaughter operations cut the allowable limit of *Salmonella* infection at processing plants from 20% to 7.5% of carcasses (USDA-FSIS, 2011). However, there is a lack of information from studies conducted in hospitals and communities in India. A literature review reveals a lack of community-based and hospital-based studies in the last decade, indicating a significant gap in understanding the incidence of typhoid (Mukhopadhyay et al., 2019).

2.1.6 *Salmonella* Interventions in the Poultry Industry

The meat and poultry industries must comply with microbiological performance standards and the HACCP final rule's zero tolerance for visible fecal contamination on carcasses. Other enteric infections may be less prevalent as a result of interventions aimed against *Salmonella* (USDA-FSIS, 1996). Antimicrobial compounds have been used in rinses and spray washes as part of techniques for reducing pathogen loads.

Major intervention tactics used in the poultry business may be categorized into four groups, according to Stopforth et al. (2007): (i) scalding, which involves immersing carcasses in a bath of hot water between 42°C (107.6°F) and 60°C (140°F); (ii) rinses/spray washes, which involve applying antimicrobial solutions post-picking, post-evisceration, pre- and post-chilling; (iii) online reprocessing, deluging and/or spraying of antimicrobial solutions to ensure that visible contamination and (iv) cooling of carcasses by immersion in a cold bath containing antibacterial treatments. According to Narendran (2003), antimicrobial agents should be non-toxic, not interfere with sensory properties, inexpensive, and simple to use. Food-grade chemical and biological antimicrobial agents that are permitted for use in meat, poultry, and egg products were listed by the Food Safety and Inspection Service (FSIS) in its Directive 7120.1 Rev 11 (USDA-FSIS, 2012). According to Ricke et al. (2005), ozonated or electrolyzed water, trisodium phosphate, cetylpyridinium chloride, and organic acids (such as lactic or acetic acid) are some of the common chemical agents used to decontaminate beef and poultry carcasses. The USDA-FSIS (2012) lists bacteriophage preparations for target bacteria as additional acceptable and safe additives for chicken products. Both a bacteriophage preparation of six lytic phages against Listeria monocytogenes that may be used on ready-to-eat (RTE) chicken products and a bacteriophage preparation that targets *Salmonella* should be administered to the feathers of live fowl before slaughter. Following de-feathering and evisceration, two potential sources of cross-contamination, online carcass spray washes are often used in commercial operations (Berrang et al., 2009). Food Safety Inspection Service Directive 6420.2 mandates a "zero tolerance" threshold for visible fecal contamination on poultry carcasses prior to chilling (USDA-FSIS, 2004). According to Smith et al. (2005), cross-infection from contaminated to clean carcasses by touch or through the chilling water caused an equilibration of contamination (uniform CFU counts) in grill carcasses during immersion chilling. Antimicrobials were also suggested to prevent cross-contamination by killing germs in the water during the chilling process (Smith et al., 2005). In addition to using antimicrobials in rinses, washes, or solutions, other methods for sanitizing beef and poultry products have included steam pasteurization or steam vacuum treatments, cutting of contaminated regions, and -irradiation or electron beam irradiation (Ricke et al., 2005). Although applying irradiation may not be possible due to concerns about using irradiation on food, steam pasteurization or steam vacuum is most likely to be used on cattle carcasses.

2.1.7 Antibiotic Resistance in *Salmonella*

Antibiotics are often used as medicinal agents and growth enhancers in animal production on farms (Joerger, 2003). The rise of antibiotic-resistant foodborne pathogens is a significant global public health problem as shown in Figure 2.4. These bacteria may be transmitted to people through food (Witte, 1998; White et al., 2002). *Salmonella* serovars that are non-typhoid resistant have emerged as a result of the widespread use of antibiotics in animals (Witte, 1998). *Salmonella spp.* is highly resistant to at least one antimicrobial agent on chicken products, according to studies by Manie et al. (1998) and Antunes et al. (2003), and this antibiotic-resistant spectrum is frequently evolving.

The CDC, in collaboration with the U.S. Food and Drug Administration (FDA) and the U.S. Department of Agriculture (USDA), launched the National Antibiotic Resistance Monitoring System (NARMS) to monitor antibiotic resistance among enteric bacteria isolated from people. *S.* Newport (31.4%) was the most prevalent among the ceftriaxone-resistant non-typhoidal *Salmonella* isolates, whereas *S.* Enteritidis (55.1%) was the most prevalent serovar resistant to nalidixic acid, per CDC (2012a).

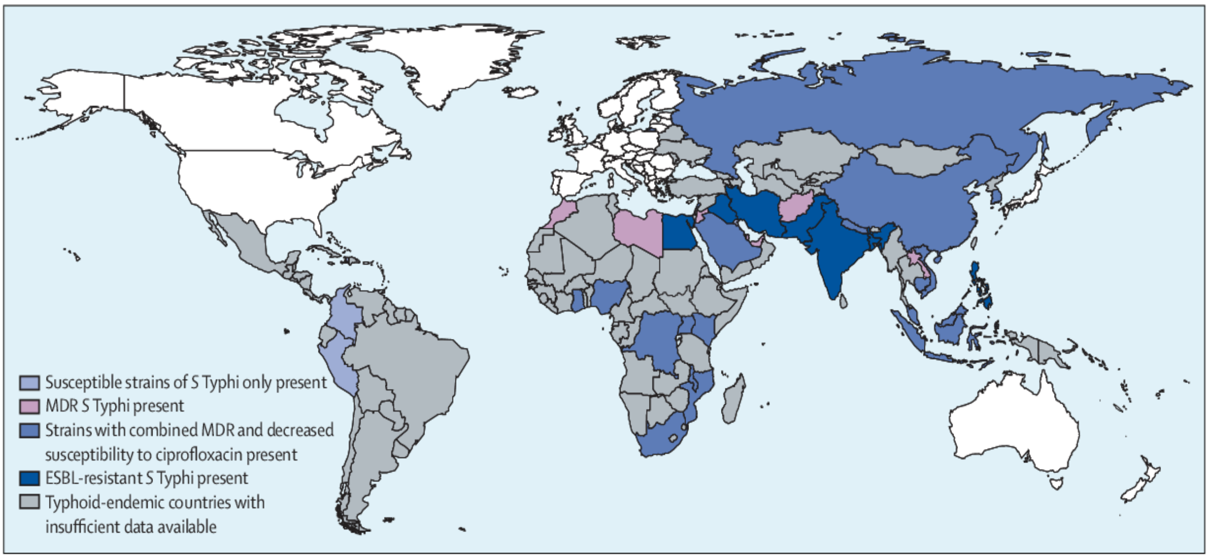


Figure 2.4: Worldwide distribution of antimicrobial drug resistance in *Salmonella enterica* serovar Typhi. Extracted from Crump et al., 2015.

Enteric fever is widespread in India, where it is one of the leading differential diagnoses for fever of unknown causes. Aside from the morbidity and mortality associated with enteric fever, the disease's empiric and proper treatment continue to increase antibiotic resistance (AMR). A first-line antibiotic used for the treatment of salmonellosis, along with ceftriaxone and trimethoprim-sulfamethoxazole, is nalidixic acid, an elementary quinolone. Resistance to this antimicrobial corresponds with a decreased sensitivity to ciprofloxacin (CDC, 2012a; CDC, 2012b) and is considered Multidrug-resistant typhoid fever (MDRTF) (Zaki and Karande, 2011). Multidrug-resistant typhoid originated roughly 20 years ago and has expanded globally, increasing the risk of disease-related death (Gupta, 1994, Saha et al., 1999). This multidrug-resistant typhoid fever contributes to the healthcare burden in underdeveloped nations. As a result, an effective strategy for controlling multidrug-resistant typhoid is necessary. Bacteriophages have been thought to be life-saving since the advent of MDRTF. Now, antibiotics appear to be ineffective in treating MDRTF. Bacteriophages, which can evolve along their hosts and are strong predators of bacteria, will undoubtedly provide an advantage in treating bacterial infections.

2.2. General introduction of bacteriophage

2.2.1 Discovery

Bacteriophages (phages) are bacteria-infecting viruses. British physician Ernest Hankin initially noted the effects of phage in 1859 after observing the bactericidal properties of Ganges River water purified to remove *Vibrio cholerae* (Sharp, 2001). However, Frederick Twort (1915) and Felix D'Herelle (1917), two more Scientists, are often given recognition for the discovery of phages. They separately identified phages as filterable, transmissible agents capable of bacterial lysis (Sharp, 2001). Twort chose not to pursue his discoveries, but D'Herelle carried on working with bacteriophages (a name he developed that means "bacteria eater"). He became the first proponent of "phage therapy”, which he felt would hold the key to curing bacterial infections, as soon as he saw the potential for phages to be utilized as therapeutic agents. After thoroughly analyzing these viruses, including their multiplication and adaption, D'Herelle suggested they may be used as an anti-bacterial medication. D'Herelle was a highly contentious person, and the scientific community found it difficult to accept his theories and findings since they cast doubt on the existence of bacteriophages and the idea of phage treatment. After a pre-antibiotic era, bacteriophages were neglected as important therapeutic agents in the West, mostly because of the relatively simple use of antibiotics. In certain nations, including Georgia (a former USSR republic), bacteriophages are still frequently identified and used to treat various ailments. This includes both research and clinical usage (Chanishvili et al., 2012). The study of bacteriophages then concentrated on a variety of model phages that mostly infected *E. coli*. These investigations served as the foundation for contemporary molecular biology, identifying the genetic components of life through the use of phages and demonstrating that an amino acid is coded for by three nucleotides. They also made it possible to identify restriction enzymes (Clokie et al., 2009). Only a few phages were extensively examined over many years.

The recent renaissance in phage biology has been brought on by increased awareness of the prevalence of phages in all bacterial-dominated environments (as demonstrated by electron and epifluorescent microscopy, as well as from molecular studies), as well as in the genomes of bacteria as a result of whole genome sequencing projects. Because of this complicated past, scientists only have a fragmented understanding of phage biology, but there is enough data to show that phages control a substantial amount of bacterial and archaeal biology.

2.2.2 Bacteriophage Ecology

The discovery of a significant number of phage particles in the seas established phage ecology as a necessary section of Marine Microbiology. Phages are prevalent in surface and marine waters all over the planet. Phages have been found in surface waters, deep oceans, ice, and sediments. The study of marine phages leads to the notion that phages are mainly unknown biological gems in the biosphere. The total number of phages on Earth was predicted to be between 10^{30} and 10^{32}. Phages, together with their host bacteria, may be found even in the most adverse habitats on the planet (Kutter and Sulakvelidze, 2004). Breitbart et al. (2002) estimated that there are 400 to 7000 different species of phages in 100 liters of seawater. He also proposed that the human gut harbors hundreds of distinct species of phages. The phage-host relationship influences phage ecology.

2.2.3 Evolution of Bacteriophages

Bacteriophages are estimated to number more than 10^{30} on the planet, making them the most abundant and diversified living entity (Ashelford et al., 2003). Because phages are extremely specific to the host bacteria, the environment allows them to multiply quickly (Brabban et al., 2005). In coastal seawater, there are usually 10^{7} tailed phage particles per millimeter (Wommack and Colwell, 2000). When compared to the total number of bacteria in the biosphere, phages are five to 10 times more abundant. Tailed phages are thought to be extremely old. Phages develop swiftly and continuously by exchanging DNA through recombination. In addition to tailed phages, several varieties of phages exist in nature. Lifestyle, structure, and genetic material all differ from one another. Despite differences in physiological and biochemical features and function, there is evidence that some of these phage classifications share a common ancestor with other phages and eukaryotic viruses (Hendrix, 2002).

2.2.4 Phage Basic Structure

Bacteriophages are composed of proteins that encapsulate a DNA or RNA genome and may have structures that are either simple or elaborate (Figure 2.5). Nineteen families are currently recognized by the ICTV (International Committee on Taxonomy of Viruses) that infect bacteria and archaea. Generally, encountered phages belong to the *Caudovirales* order. *Caudovirales* are group I viruses as they have double-stranded DNA (dsDNA) genomes. The virus particles have a distinct shape; each virion has an icosahedral head that contains the viral genome and is attached to a flexible tail by a connector protein.

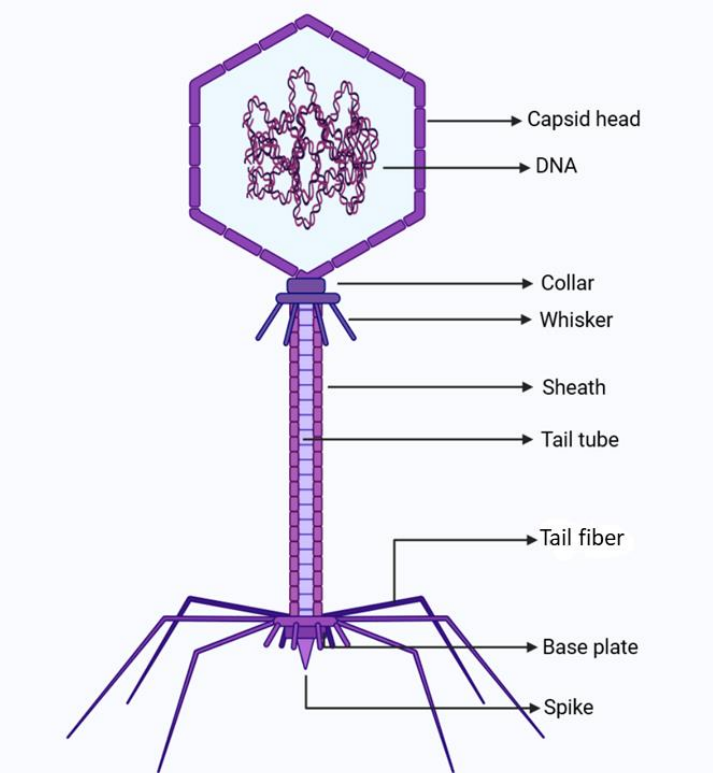


Figure 2.5: Phage structure

2.2.5 Bacteriophage Taxonomy and Classification

The classification of phages is based on morphology, nucleic acid type, and physicochemical characteristics of the virion, which are increasingly supplemented by genomics (Ackermann, 2011). In 1967, Bradley established six essential morphotypes of phages based on their gross morphology and the type of nucleic acid they contained. These characteristics correspond to the current phage families *Myoviridae, Siphoviridae, Podoviridae Microviridae, Leviviridae,* and *Inoviridae* respectively as shown in Figure 2.6 (Ackermann & Prangishvili, 2012). In accordance with the VIIIth report of the International Committee on Taxonomy of Viruses (ICTV), phages are currently divided into one order and ten families (Ackermann, 2011).

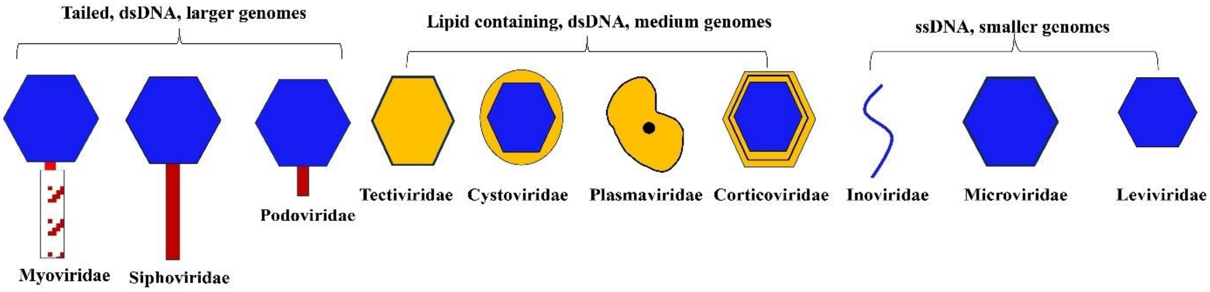


Figure 2.6: Overview of bacteriophage families. The figure is partially based upon those used in Ackermann, Hyman, and Abedon. Note that virion particles are not drawn to scale.

2.2.5.1 Classification of tailed phages

Over 96% of phages have tails, and all of them are members of the Caudovirales order, which is made up of three large families that are phylogenetically connected: *Myoviridae*, *Siphoviridae,* and *Podoviridae* (Figure 2.7). All of the phages in the three families have double-stranded (ds) linear DNA and icosahedral heads, but members of the distinct families have varied morphologies, particularly in terms of their tails.

According to Ackermann (2011), *Myoviridae* phages have contractile tails, whereas *Siphoviridae* phages have long, non-contractile tails, and *Podoviridae* phages have short tails respectively. Bacteriophages have tail fibers attached to the bottom of the tail. Other 4% of the reported bacteriophages lack genes for tail proteins, and these bacteriophages are polyhedral, filamentous, or pleomorphic. (Ackermann, 2007).

Myoviruses are inclined to be larger than other groups and include some of the largest and most highly evolved tailed phages; their tails are made up of a neck, a contractile sheath, and a central tube. They are typically lytic and have relatively broad host ranges.

Siphoviruses are often found in saltwater, generally have a wide range of potential hosts, and many can integrate into the host genome. They constitute the largest viral family, with nearly 3600 descriptions, or 57.3% of all viruses, which makes them quite possibly the most common-tailed phage. The tail of the *siphoviridae* phages is straightforward, noncontractile, flexible, or stiff tubes.

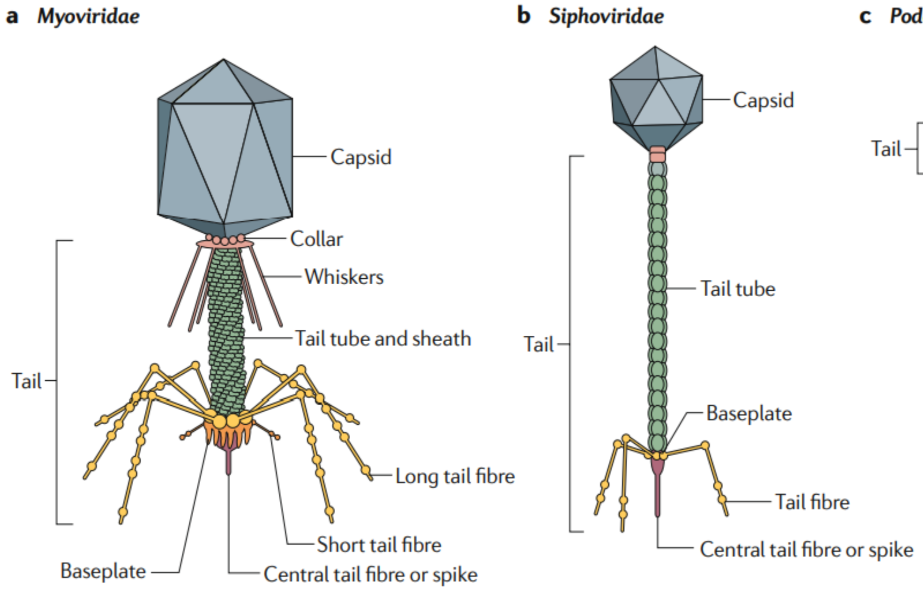


Figure 2.7: Representative structures of tailed phages belonging to the Caudovirale family. (a) *Myoviridae* bacteriophage (long contractile tail), (b) *Siphoviridae* bacteriophage (long non-contractile tail), and (c) *Podoviridae* bacteriophage (short tail). This figure is extracted from Nobrega et al., 2018.

Podoviruses are also frequently lytic, have extremely limited host ranges, and are less frequently recovered from saltwater (Suttle, 2005; Ackermann, 2009; Ackermann & Prangishvili, 2012). The remaining phages with polyhedral, filamentous, or pleomorphic morphologies are grouped into 7 families and distinguished by significant variations in nucleic acid composition and structure. In the Caudovirales - the tailed phages, include double-stranded (ds) DNA or RNA. Four of the families have lipids in their virions, and two of them have lipoprotein envelopes (Ackermann, 2007; 2011). In Caudovirales, the genetic material accounts for 50% of the total mass of the phage particle, while the protein accounts for the remaining 50%. Viruses are often composed of several copies of a single protein or two. Each corner is composed of pentamers, while each side is constructed of hexamers.

2.2.5.2 Membrane containing phages

Bacteriophages are members of the *Tectiviridae* and *Corticoviridae* families and are composed of non-tailed icosahedral virions with an internal lipid membrane and either a circular or linear double-stranded genome. The presence of the trimeric main capsid proteins, which have a double β-barrel shape, distinguishes these two families of phages (Benson et al. 1999). The major capsid protein structure was studied structurally, and it was discovered that it has structural similarities to the major capsid protein of adenovirus and possesses an N-terminal -helix that interacts with the membrane lipid. The phages in these two families lack any tail that may be used to transfer their genome to Gram-negative host bacteria. In order to implant its genome, the membrane of this phage changes into a proteolipid tube and pierces the host cell membrane (Peralta et al. 2013). The icosahedral phage capsid is encased in a lipid membrane in phages that belong to the *Cystoviridae* family (Vidaver et Al., 1973). Another family of viruses that incorporate membranes is the *Plasmaviridae*. There is no capsid structure present in the phages that fall under this classification. According to Greenberg and Rottem (1979), the genome of these phages is encased in a proteinaceous, lipid-containing vesicle.

2.2.5.3 Bacteriophages with small icosahedral capsids

Bacteriophages belonging to the *Microviridae* family have a tiny icosahedral capsid structure around their ssDNA genome. The eukaryotic ssDNA virus and the capsid structure are both formed on protein folds that feature a -barrel shape. The transport of the phage's DNA to the host cell is facilitated by the proteins. The *Bullavirinae* and *Gokushovirinae* subfamilies of the *Microviridae* phage are further subdivided. In contrast to *Gokushovirinae*, which has a protrusion that extends along the three-fold icosahedral axis of the phage capsid, *Bullavirinae* has a pentameric main spike protein at the end of the capsid vertex (Chipman et al. 1998).

2.2.5.4 Phages with filamentous morphology

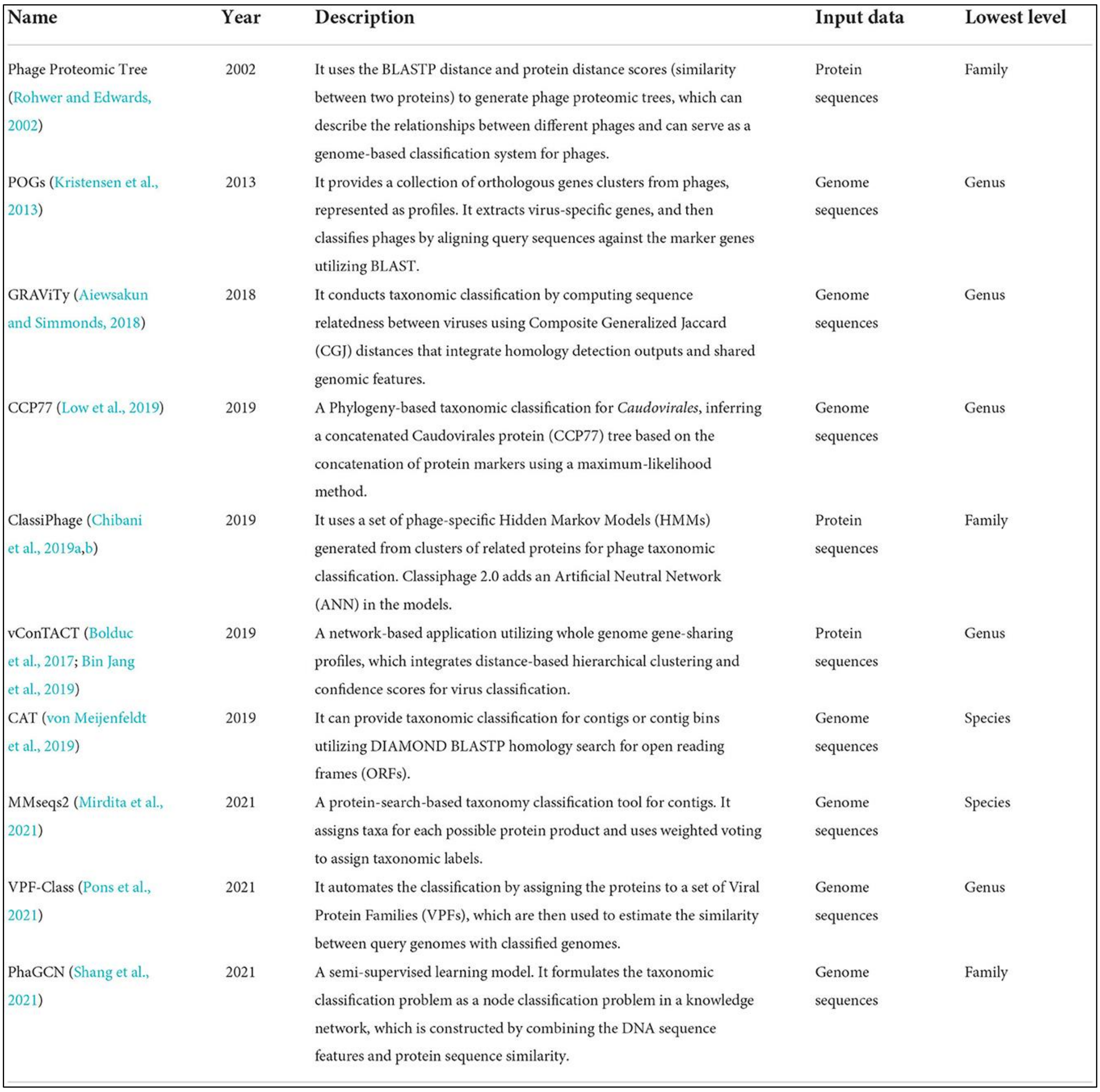
This class includes *Inoviridae* phages. The morphology of phages in the *Inoviridae* family varies significantly. The ssDNA genome of these phage particles is encircled by thousands of copies of the main capsid proteins, which are continuously constructed and expelled from the bacterial host cell (Rakonjac et al. 2017). A lengthy helix and an N-terminal signal peptide make up the main capsid protein (MCP) of this family of phages (Xu et al. 2019). The signal peptide is cleaved before the assembly of the phage proteins into a long, cylindrical spiral shell with a C-terminus directly attached to the ssDNA genome (Russel and Model 1981).

2.2.6 Approaches for Phage Taxonomic Classification

Many taxonomic techniques for phages may produce categorization findings in many ranks, including order, family, and genus. For the following reasons, we concentrate on evaluating the performance of several tools at the family level in this evaluation. First, the ICTV taxonomy is constantly changing, which has a big impact on the overall number of genera. For instance, the 2016 edition of the ICTV database has 735 genera. Nevertheless, in 2020 there were 2,224 genera. The term "ground truth" might be difficult to define because of the revision of the genus-level taxonomy.

Additionally, the development of reference and test sets is challenging since many uncommon genera only have one phage. Second, since there are fewer similarities across classes and more numerous sequences within each class, categorization at higher taxonomic levels is typically simpler than at lower ranks. As a result, categorization at the order or above is not as difficult as classification by family. The bulk of all phage sequences belongs to the Caudoviricetes class of tailed phages, which can be categorized using nearly all of the methods outlined above. In this study, we thus concentrate on the classification of the families under Caudoviricetes.

Following the historical sequence, Figure 2.8 provides a summary of the phage taxonomy categorization techniques. It contains a brief summary, publication year, the kind of input data needed, and the lowest anticipated level for each tool. The bulk of these tools perform nucleotide- or protein-level similarity between a query virus and the reference database-based phage taxonomy categorization based on sequence comparison. The created reference database, the alignment method, and the application of these alignments vary amongst the comparison-based methods. The two types of alignments—pairwise sequence alignment and hidden Markov model (HMM)-based profile alignments—are frequently employed. To create viral protein families and use them as marker genes, numerous techniques are available. Using all phage genomes typically uses more memory than utilizing markers. Newly sequenced phages with novel genes, however, might not be linked to any flag gene families and hence cannot be classified into a known class. On the other hand, freshly sequenced phages with unique genes might not correspond to any marker gene families and hence cannot be classified into a recognized class. The categorization of phages has also been used in learning-based methods. The sequence patterns in the phage genomes of various families may be automatically inferred by learning models, which then employ the learned characteristics for automated categorization. Below is an explanation of these tools in more depth.



Name	Year	Description	Input data	Lowest level
Phage Proteomic Tree (Rohwer and Edwards, 2002)	2002	It uses the BLASTP distance and protein distance scores (similarity between two proteins) to generate phage proteomic trees, which can describe the relationships between different phages and can serve as a genome-based classification system for phages.	Protein sequences	Family
POGs (Kristensen et al., 2013)	2013	It provides a collection of orthologous genes clusters from phages, represented as profiles. It extracts virus-specific genes, and then classifies phages by aligning query sequences against the marker genes utilizing BLAST.	Genome sequences	Genus
GRAViTy (Aiewsakun and Simmonds, 2018)	2018	It conducts taxonomic classification by computing sequence relatedness between viruses using Composite Generalized Jaccard (CGJ) distances that integrate homology detection outputs and shared genomic features.	Genome sequences	Genus
CCP77 (Low et al., 2019)	2019	A Phylogeny-based taxonomic classification for *Caudovirales*, inferring a concatenated Caudovirales protein (CCP77) tree based on the concatenation of protein markers using a maximum-likelihood method.	Genome sequences	Genus
ClassiPhage (Chibani et al., 2019a,b)	2019	It uses a set of phage-specific Hidden Markov Models (HMMs) generated from clusters of related proteins for phage taxonomic classification. Classiphage 2.0 adds an Artificial Neutral Network (ANN) in the models.	Protein sequences	Family
vConTACT (Bolduc et al., 2017; Bin Jang et al., 2019)	2019	A network-based application utilizing whole genome gene-sharing profiles, which integrates distance-based hierarchical clustering and confidence scores for virus classification.	Protein sequences	Genus
CAT (von Meijenfeldt et al., 2019)	2019	It can provide taxonomic classification for contigs or contig bins utilizing DIAMOND BLASTP homology search for open reading frames (ORFs).	Genome sequences	Species
MMseqs2 (Mirdita et al., 2021)	2021	A protein-search-based taxonomy classification tool for contigs. It assigns taxa for each possible protein product and uses weighted voting to assign taxonomic labels.	Genome sequences	Species
VPF-Class (Pons et al., 2021)	2021	It automates the classification by assigning the proteins to a set of Viral Protein Families (VPFs), which are then used to estimate the similarity between query genomes with classified genomes.	Genome sequences	Genus
PhaGCN (Shang et al., 2021)	2021	A semi-supervised learning model. It formulates the taxonomic classification problem as a node classification problem in a knowledge network, which is constructed by combining the DNA sequence features and protein sequence similarity.	Genome sequences	Family

Figure 2.8: Overview of bioinformatic approaches used for phage taxonomic classification. Extracted from Zhu et al., 2022.

2.2.7 Receptors-Phage Relation

Gram-negative phages have been the subject of several studies on the interaction between them as well as their bacterial hosts, whereas Gram-positive phages have received far less attention (Mahony and van Sinderen 2015). The receptor binding proteins (RBPs), also known as tail fiber proteins, are the primary determinants of phage specificity for all phages. Because of their stability, affinity for certain carbohydrate-binding proteins, and specific binding nature, RBPs have been used as therapeutic agents to decrease bacterial colonization (Simpson et al. 2016, Waseh et al. 2010). RBPs are also being used as diagnostic tools for several bacterial genera and are being developed for this purpose. According to phage morphology and the distinct processes by which phages adhere to bacteria, RBPs differ structurally between different phages. For instance, myoviruses like T4 bind to *Escherichia coli* using both long and short-tail fibers, nevertheless siphoviruses like the *Lactococcus lactis* phage TP901-1 have RBPs located within a large structure called a baseplate. Additionally, RBPs have been found on tail spikes, such as in the *Bacillus subtilis* phage SPP1 (Table 2.4) (Veesler et al. 2012; Simpson et al. 2016).

Table 2.4: List of well-studied phages that infect Gram-negative bacteria and Gram-positive bacteria, along with their known receptors to which phages attach. Adapted from Dowah & Clockie, 2018.

Host	Phage	Phage family	Name of the receptors on the host	References
E. coli	Lambda	*Siphoviridae*	Protein LamB	Chatterjee and Rothenberg (2012)
E. coli	T5	*Siphoviridae*	Protein FhuA	Mahony and van Sinderen (2012)
Salmonella	ES18	*Siphoviridae*	Protein FhuA	Killmann et al. (2002)
S. thermophilus	OBJ	*Siphoviridae*	Glucosamine/ribose	Quiberoni et al. (2000)
S. thermophilus	CYM	*Siphoviridae*	Glucosamine/rhamnose	Quiberoni et al. (2000)
L. lactis	TP901-1	*Siphoviridae*	Saccharide	Mahony and van Sinderen (2012)
L. lactis	Tuc2009	Siphoviridae	Saccharide	Mahony and van Sinderen (2012)
Bacillus subtilis	SPP1	*Siphoviridae*	Protein YueB	São-José et al. (2004)
S. aureus	W	*Siphoviridae*	N-acetylglucosamine (GlcNAc) glycoepitope on wall teichoic acids	Xia et al. (2011)
S. aureus	ϕSLT	*Siphoviridae*	Poly(glycerophosphate) moiety of lipoteichoic acid	Kaneko et al. (2009)

2.2.8 Bacteriophage Replication Cycles

The type and unique features of the receptor on the surface of the host cell determine the way the phages are absorbed. Phages are primarily divided into virulent and temperate phages based on their life cycles. The lytic life cycle is followed by virulent phages. However, under certain circumstances, temperate phages can occasionally switch from the lysogenic to the lytic cycle. The two main proteins employed by lytic phages to kill their host cells are holin and lysine.

Phages have two different life cycles that depend on their bacterial hosts for replication. The decision to switch between lysis and lysogeny may be influenced by peptide-based viral communication (Erez et al. 2017) or host repressor genes that are part of a quorum-sensing system (Silpe and Bassler 2019).

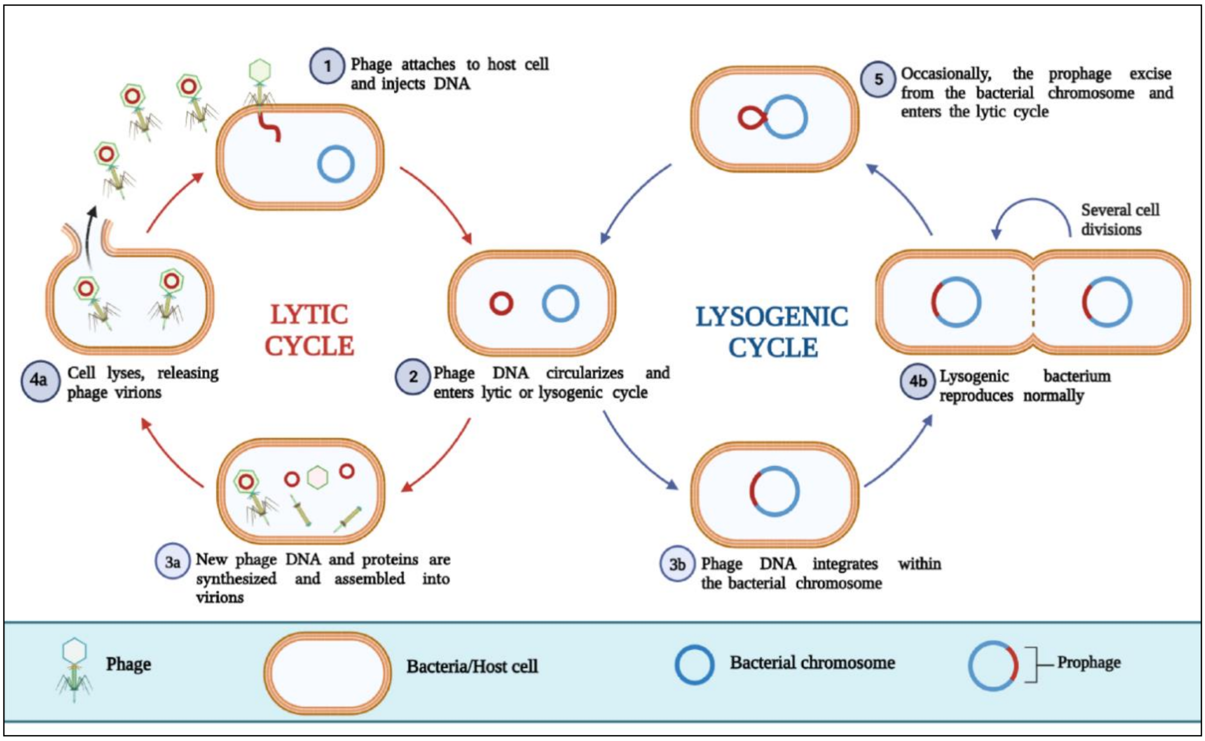


Figure 2.9: Phage replication cycle using Biorender software.

2.2.8.1 Bacteriophage Lytic Cycle

In the lytic cycle, the phages infect their bacterial hosts, reproduce, and then release their mature progeny after lysing the bacterial cells. Lytic phages are a source of prospective new treatments and have indeed long been used to treat bacterial illnesses (Czaplewski et al. 2016). On the host bacterial lawns, lytic infection results in obvious plaques. According to Weinbauer

(2004), the process generally entails the following steps: nucleic acid contact, adsorption, injection, or penetration; nucleic acid replication and expression; virion assembly; release of new phage particles; and transmission (Weinbauer, 2004). Initially, phages bind to certain receptor proteins on the bacterial host surface or surface structures, such as flagella, pili, bacterial capsules, and various lipopolysaccharide components as shown in Figure 2.10 (Weinbauer, 2004). The cell wall of the bacterium can be reached by certain phages by breaking down capsule-like substances on the bacterial surface using enzymes (Skurnik & Strauch, 2006). Only certain locations on the surface of bacterial cells can be used by a certain phage or collection of phages for adsorption (Ackermann & DuBow, 1987). As a result of this phenomenon, it may be concluded that the host ranges of most known phages are rather constrained (Ackermann & DuBow, 1987).

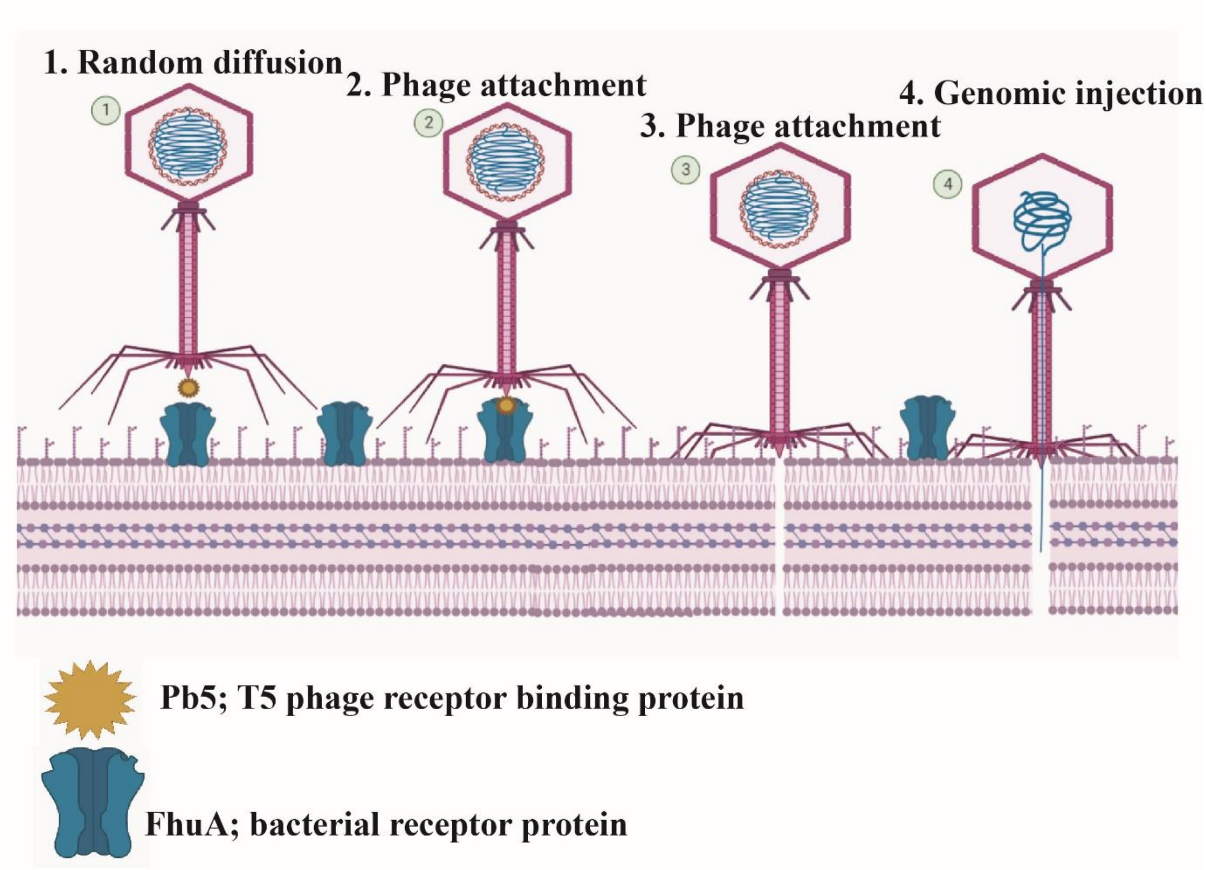


Figure 2.10: Schematic diagram of the DNA injection mechanism of T5 bacteriophage using Biorender software.

After adsorption, phages employ the enzymes in their tails or capsids to break down the bacterial cell wall so that their nucleic acid may be injected into the cytoplasm of the host cell, while the capsid stays outside the cell. The phage genome then directs and utilizes host cell processes and resources to finish its own gene expression, genome replication, production of capsid proteins, and assembly of the genome and capsid into full virions. Finally, the host cell releases the offspring phages. Two types of enzymes are involved in the release process in tailed phages: peptidoglycan hydrolases (endolysins), which degrade peptidoglycan in the cell wall, and holins, which permeabilize the plasma membrane and enable the endolysins to degrade the peptidoglycan (Ackermann, 1999; Weinbauer, 2004). The latent period of phage infection is the interval from adsorption to host cell lysis (Abedon, 1989). The eclipse period refers to the stage of the latent period prior to the assembly of the genome and capsid into adult phages. The burst size (Abedon, 1989; Ackermann, 1999; Weinbauer, 2004) is the number of virions discharged per cell into the extracellular environment.

The use of lytic phages as therapeutic or preventative agents is predicated on their capacity to eradicate bacteria (Almeida et al., 2009). A unique type of lytic cycle called chronic infection, on the other hand, allows phages to be discharged via budding or extrusion without causing host cell death (Ackermann & DuBow, 1987; Ackermann & Prangishvili, 2012). The extracellular stages of all the phages conclude with either the inactivation of the phage or a fresh infection (Weinbauer, 2004).

2.2.8.2 Bacteriophage Lysogenic Cycle

In the lysogenic cycle, the procedures for attachment and penetration are the same as those in the lytic cycle (Figure 2.9). However, after entering the host cell, the phage genome fuses with the host's and transforms into a latent prophage. The phage genome integrates into a host cellular replicon, such as the chromosome, a plasmid, another phage genome, or existing as a self-replicating plasmid, at this relatively quiescent stage after being injected into the host cell. The phage genome is known as a prophage at this point. Along with the host cell, it multiplies and is transferred to daughter cells (Williamson et al., 2001; Dalmasso et al., 2014). The prophages may remain stable for several generations before triggering the lytic lifecycle by induction due to environmental or physiological factors. More than 90% of phages are temperate (Freifelder., 1983).

After phage infection, a "lysogenic decision" is taken on whether to create a prophage state (Ackermann & DuBow, 1987). Prophages can occur naturally or can be brought on by a variety of physical or chemical agents, including UV radiation, mitomycin C, hydrogen peroxide, polyaromatic hydrocarbons, pollution, changes in temperature, salinity, and nutrient concentration, as well as antibiotics (Fortier & Sekulovic, 2013), mitomycin C, hydrogen peroxide, and other similar substances (Williamson et al., 2001; Weinbauer, 2004).

Lysogeny is a strategy that phages use to overcome challenging environmental and biological conditions, such as extremes in water temperature and low host density during nutrient starvation. On the other hand, lysogenic bacteria gain specific benefits from their relationship with phages, such as increased metabolic capacity, antibiotic resistance, toxin production, and promoting bacterial evolution. The most frequent outcome is homo immunity, which provides protection against superinfection by the same or similar phage strains. These effects might be brought about by unidentified processes, the conversion process, which involves the expression of prophage genes in lysogens, or the prophage's modulation of the expression of the bacterial host genes (Williamson et al., 2001; Fortier & Sekulovic, 2013).

2.2.8.3 Pseudolysogeny

The term "pseudolysogeny" (also known as "false lysogen" or "phage-carrier state") refers to a phage-host cell interaction in which the phage nucleic acid neither forms a long-lasting and stable relationship (i.e., lysogeny) nor triggers a lytic response, but merely resides inside the cell in an inactive state. In this instance, the phage nucleic acid does not integrate into the host cellular replicons, and it does not duplicate and segregate into all offspring cells evenly after host reproduction. As a result, a high host abundance coexists with a continual generation of the phage (Ackermann, 1987). Similar to lysogeny, pseudo-lysogeny cannot be initiated to the lytic cycle by chemical or physical stimuli (Baess, 1971; Wommack & Colwell, 2000). According to Ripp & Miller (1998), pseudo lysogeny is a genuine adaptation technique for environmental phages to employ to survive times of famine of their host species. It occurs when starving bacterial cells dwell in an unsteady relationship with infectious viruses. The phage cannot begin either a true lysogenic or lytic stage under the circumstances since the host cells are unable to supply enough energy. The development of real lysogeny or the replication and expression of the viral genome, however, both depend on the capacity of the host cell to access more nutrients, at the point where the phage gains the energy required to facilitate gene expression (Ripp & Miller, 1997). This causes the host cell to lyse and the virion to develop (Ripp & Miller, 1997). It is still unclear how pseudolysogeny is regulated and what impact it has on the host (Weinbauer, 2004). Another reasonable theory suggests that pseudo-lysogeny is an evolutionary step toward a more stable lysogenic state or a quick reaction to environmental changes (Wommack & Colwell, 2000).

2.2.9 Factors Affecting the Effectiveness of Phage Use against Pathogenic Bacteria

The efficiency of phage therapies against harmful bacteria relies on a number of variables, including the bacteriophage/bacteria ratio, physicochemical variables (pH, temperature), phage neutralization, or phage resistance (Figure 2.11). Additionally, neither *in vivo* data from one phage nor *in vitro* data from another phage can be easily related to the *in vivo* environment. The phage adsorption rate, burst size, latent duration, and initial phage dosage are crucial variables that influence phage therapy (Payne et al., 2000). The rate at which phage particles are removed from the body via the reticuloendothelial system or by phage-neutralizing antibody events is another crucial characteristic.

2.2.9.1 Phages/Bacteria Ratio

According to Gill (2010), two separate methods—one passive and the other active—have been used to study the effectiveness of bacteriophages against pathogenic bacteria. In the passive strategy, the bacteriophages are introduced at a level sufficient to ensure that all target bacteria are infected and lysed in a short amount of time. However, a modest number of phages must be added for active biocontrol to be effective. In this instance, phage replication through several generations is assumed to be necessary for bacterial eradication. The metabolic and physico-chemical properties of the system (for example, viscosity) may make it harder for newly reproduced phages to enter the target bacterium. According to appearances, the passive approach is more effective than the active one. In virology, the term MOI (multiplicity of infection), which describes the number of viruses introduced per cell during infection, is used to explain the bacteriophages/bacteria ratio. Only fluid systems containing a substantial number of host cells are MOI-compatible. The MOI ranges from 0.01 to 100 are traditionally used in phage studies against bacteria conducted *in vitro* and *in vivo*.

2.2.9.2 Phage Administration

The simplicity of administration is another advantage of phage usage. Phages may be administered orally, topically, intravenously, intraperitoneally, or intravenously. Using the pathogenic bacterial culture, phage preparation and generation may be carried out. In this instance, it must regulate the toxin or bacterium culture remnants that may be the cause of the inflammatory event (Gill and Hyman, 2010).

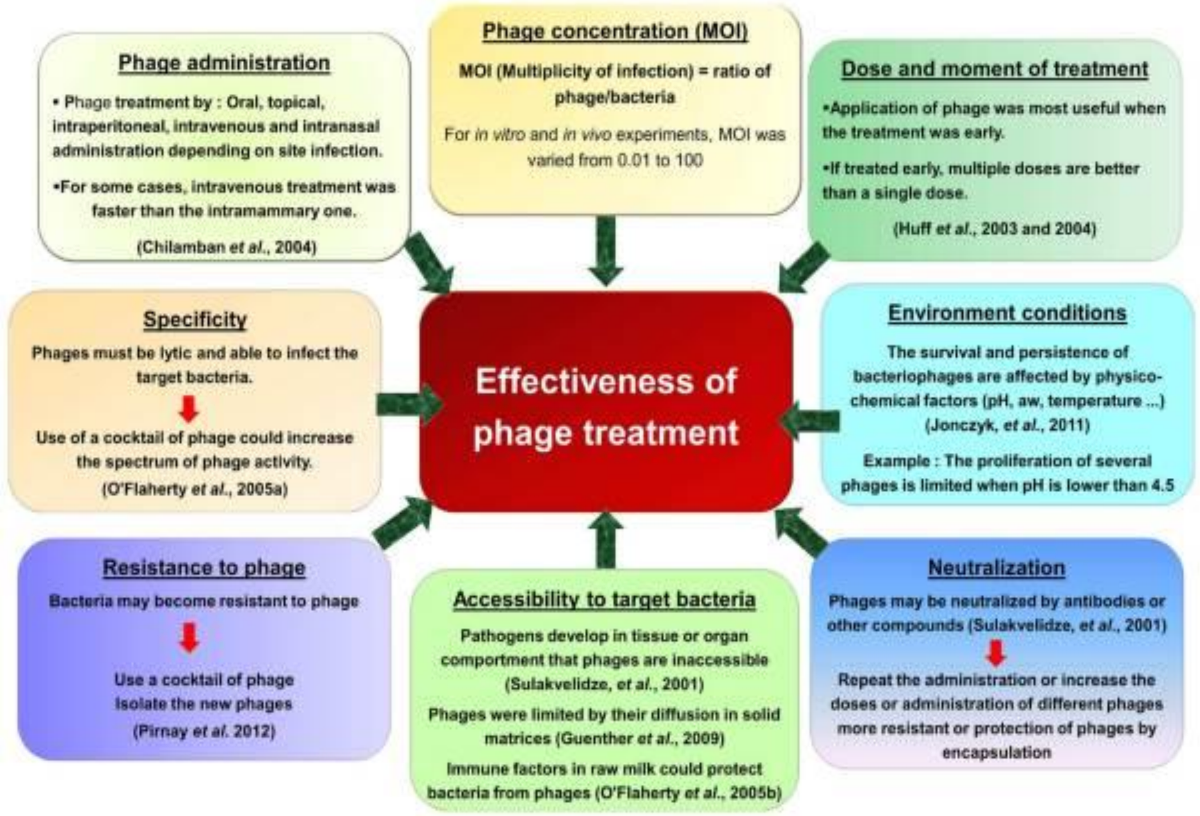


Figure 2.11: Factors affecting the effectiveness of phage use against pathogenic bacteria. Extracted from Ly-Chatain, 2014.

2.2.9.3 Host Specificity

One of the main benefits of bacteriophage is its host specificity. This characteristic renders them harmless to both eukaryotic and prokaryotic cells outside of their host range. In fact, there is growing evidence that suggests our internal microbial inhabitants have a big influence on our overall health. In contrast, because of its more pervasive, wide-ranging influence and severe side effects, antibiotic medication can have a considerable negative impact on our typical microbiome. The efficacy of phage therapy would appear to be indicated by the capacity of the bacteriophage to infect a variety of hosts since this would increase the likelihood that phages will come into contact with a bacterial cell that is amenable to phage treatment. It is necessary for the phage population to survive (Koskella and Meaden 2013). The use of a phage cocktail is a more effective therapy since bacteriophages used for therapeutic purposes are not always able to infect a range of bacterial types.

2.2.9.4 Accessibility to Target Bacteria

Bacteriophage diffusion may be inhibited or promoted based on the matrix's structure and composition as well as the surrounding environment, as suggested by Marco et al., 2010. Bacteriophage diffusion in a solid medium may be constrained, resulting in less phage adsorption on bacteria and, as a result, reduced phage infection capability. For instance, Guenther et al. (2009) demonstrated that the dispersion of bacteriophages in solid food matrices including hot dogs, smoked salmon, and shellfish restricted their utilization.

2.2.9.5 Protection of Phages

Microencapsulation, which is defined as a technology of packaging solids, liquids, or gaseous materials in miniature capsules that can release their contents at controlled rates under specific conditions, is one of the ways to protect the bacteriophage at the site of infection and during the journey to this site. Probiotic bacteria have been microencapsulated to increase their viability during processing and to transport them specifically to the gastrointestinal system. Viral microencapsulation has been investigated as a potent adjuvant technique to trigger certain immune responses through mucosal pathways. It has been reported that oral microencapsulated bacteriophages have been developed to treat gastroenteritis in cattle (Ma et al., 2008; Dini et al., 2012).

2.2.9.6 Neutralization

Although there are not many articles on the topic, many researchers have researched how phages travel to better understand the accessibility of phages to bacteria. According to certain researchers, phages enter the circulation of lab animals (after a single oral dosage) within 2 to 4 hours and are discovered in the internal organs (liver, spleen, kidney, etc.) in around 10 hours. The persistence of injected phages has also been studied, and the results show that phages may survive in the human body for quite an extended period, up to several days (Bogovazova et al., 1991, 1992).

Although the phage circulates efficiently in the blood and in other organs, a few authors suggested that antibodies may neutralize the phage, preventing it from effectively lysing the target microorganisms. Colostrum supplementation in milk infected with phage inhibited the lysis of starter cultures of *Lactobacillus lactis*, as demonstrated by Geller et al. in 1998. The duration that the antibodies will stay in the body, though, is unknown.

2.2.9.7 Environmental Conditions

Physico-chemical variables (pH, ions, temperature, etc.; Jonczyk et al., 2011) have an impact on the survival and persistence of bacteriophages. Regarding outside variables, the phage population is usually steady. The acidity of the environment is a significant factor affecting phage stability. Wine-specific phages belonging to the lactic acid bacteria *Leuconostoc oenos* have been observed, according to Davis et al. (1985). pH values below 3.5 and SO_2 at a total concentration of 50 mg/L served as the activity's limiting factors. Lu et al. (2003) revealed how phages may survive in an acidic environment like sauerkraut. After 60 and even 100 days, they discovered 24 phage isolates in sauerkraut-containing fermentation tanks (pH 3.5). From previous studies, it has been demonstrated that phage aggregation is influenced by hydrogen ion concentration. According to Olson et al. (2004), Nasser and Oman (1999), Yates et al. (1985), and Hurst et al. (1980), temperature has a significant impact on the capacity of bacteriophages to survive. It is crucial for attachment, penetration, multiplication, and the duration of the latent phase (in the case of lysogenic phages). Fewer phages can participate in the multiplication phase because less of their genetic material enters bacterial host cells when temperatures are lower than ideal. Furthermore, the most crucial element affecting bacteriophage activity is the storage temperature.

2.2.9.8 Dose and Moment of Treatment

The choice between a single dosage and multiple doses is a significant aspect that can alter the efficacy of phage therapy. Numerous studies have demonstrated that many doses are preferable to a single dosage. In one study by Huff et al. (2003a, b), it was discovered that treating chickens with severe *E. coli* respiratory infections helped to alleviate symptoms. The use of bacteriophage was most beneficial right away after the hens were introduced to the bacterium, and if treated right away, many doses were preferable to a single dosage.

2.2.10 Importance of Phage Genome and Proteome Characterization

A significant increase in the number of completely sequenced phage genomes can be attributed to the interest in phage treatment. Currently, the NCBI genome database has 12127 distinct phage genomes. These phage genomes are mostly dsDNA, with a tiny proportion also being ssDNA, dsRNA, and ssRNA. Prevalent phage genomes often fall into one of three sizes: less than 10 kbp, 30–50 kbp, and 100–200 kbp (Hatfull, 2008). For several reasons, phage characterization must include annotation of their genomes. Firstly, it enables an improved understanding of phage infection at the genomic level by locating genes involved in host identification, phage genome replication, structural protein expression, and bacterial lysis. Additionally, new information about the evolution of phages can be provided through comparative genomics. Identifying undesirable genes, such as those that give antibiotic resistance, lysogeny, toxins, and allergies, is necessary as a crucial safety review during phage commercialization. Despite a significant increase in the number of phage genomes that are accessible and recent advances in bioinformatic analysis, insights on the characterization of whole phage genome sequences are still lacking. As a result, relatively vast quantities of putative genes with unknown functions may be found in the majority of reported phage genomes. Even in the case of T4 phage, one of the most well-characterized phage genomes, nearly fifty percent of the T4 genes have been characterized (Miller et al., 2003). A more comprehensive characterization of phages, both biologically and in terms of safety, might be provided by additional annotation of the large unknown regions, which would also prevent the spreading of undesirable genes.

Proteomic characterization of bacteriophage is important for the usage of engineered phages that help to kill the bacteria and it could be the next step to confront antibiotic resistance. Engineered phages are a new source of antimicrobials and an approach to delivering vaccine antigens and medications, as well as precisely detecting and controlling bacteria. For instance, the modular structure of endolysins can be used to create chimeric proteins with the necessary properties by replacing and recombining the functional domains of different species. Other phage lytic proteins like virion-associated peptidoglycan hydrolases (VAPGHs) may also benefit from this method. Additionally, it could be helpful for non-phage origins such as bacteriocins and autolysins. The specificity of such modified proteins would be a major benefit when used as antibacterials, for more bactericidal activity, with a small risk of resistance development, and strong efficacy against persister cells, multidrug-resistant strains, and biofilms (Aslam et al., 2021). To better understand the defense mechanism of bacteriophage, a proteome analysis is crucial. The development of more effective therapeutic uses for phages may be facilitated by a better understanding of phage-host interaction gained through a proteomic analysis.

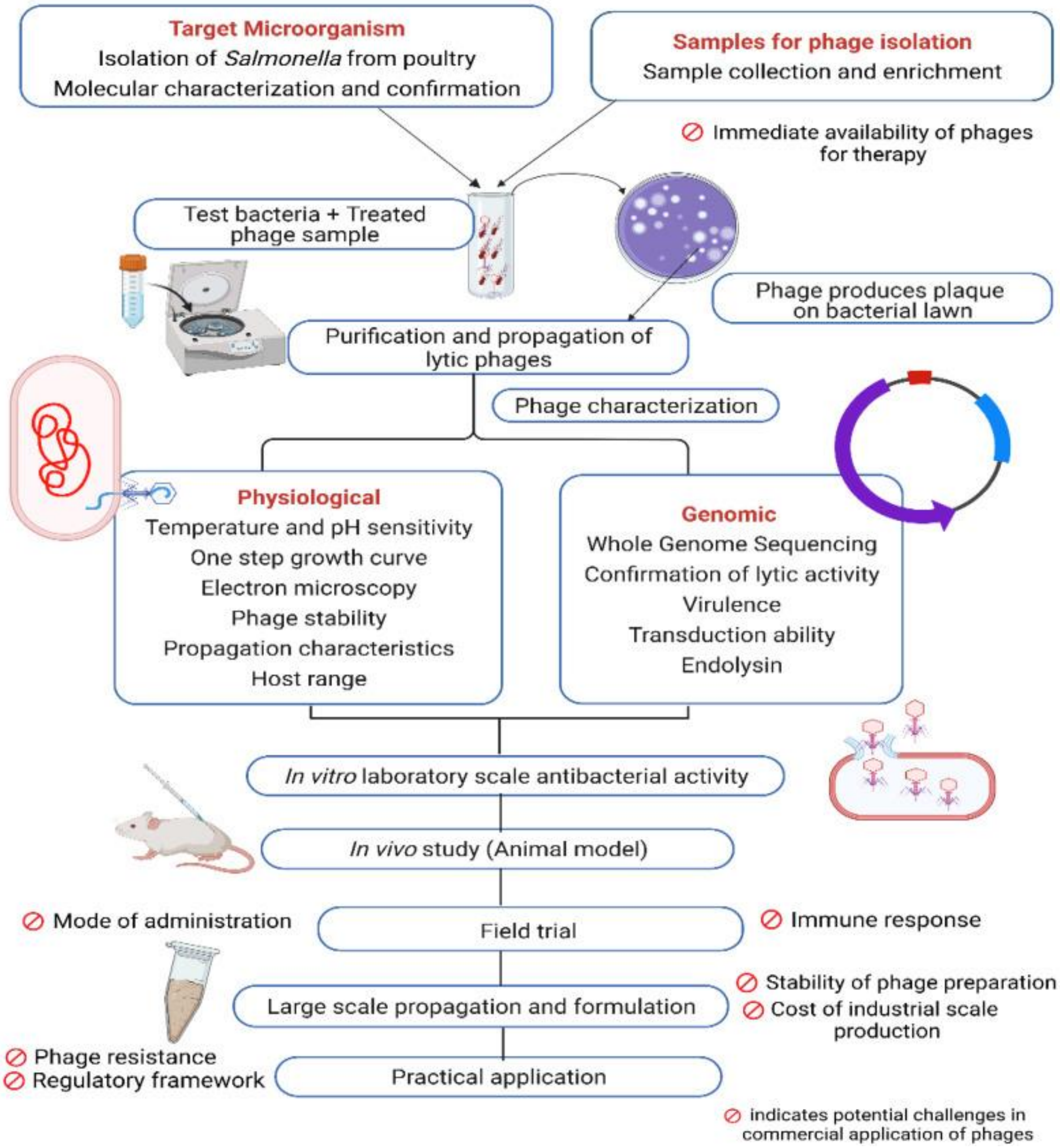


Figure 2.12: Steps in the phage application process with potential challenges in the poultry industry. Extracted from Khan & Rahman (2018).

2.2.11 Field and Lab Animal Trials of Bacteriophages in Infectious Diseases

In order to use phage as a potential biocontrol agent and implement it *in vivo*, physiological, genomic, and proteomic characterization of the phage is essential (Figure 2.12). Phage treatment has developed over the past few years in response to the crisis that antibiotic resistance is causing, and more studies describing its success have been published. However, the therapeutic efficacy of phage treatment has not been explicitly demonstrated by a successful clinical trial that adhered to the most recent criteria of evidence-based medicine. Therefore, to construct a trial with a higher likelihood of success, it is crucial to critically analyze the data obtained from experimental research in animals (Górski et al., 2020). In addition to physiological characterization, phage genomic and proteomic characterization is crucial for employing it as a possible biocontrol agent and applying it *in vivo*.

Poultry is the most commonly used model in phage treatment research. Combining two bacteriophages that are lytic for *Campylobacter jejuni* was found to be quite effective on broilers. It was discovered that the type and number of bacteriophages utilized had an effect on the effectiveness of phage treatment (Loc et al., 2005). Wagenaar et al. (2005) found that prophylactic oral treatment of a combination of phages (phage strains 69 and 71) in chickens showed a delayed *C. jejuni* colonization of the gastrointestinal tract and resulted in this level of colonization within 1 week. Additionally, infection with *E. coli* is a prevalent issue among sheep farmers. Bach et al. reported bacteriophages and their potential utilization in the defense of sheep against *E. coli* infection (Bach et al., 2009).

Mice are another frequent model implemented in phage therapy studies. Studies from various groups that used BALB/c mice as an infection model and oral administration of phages for therapy showed promising outcomes and phages were shown to cure *Salmonella enterica* serotype Enteritidis infection more effectively (Dallal et al., 2019).

Table 2.5: Studies on phage therapy according to organ system, bacteria, and animal model.

System	Bacteria	Animal Model	References
CNS	*Escherichia coli*	Rats	Pouillot et al., 2012
GI	*Escherichia coli*	Calves	Smith and Huggins, 1983; Smith et al., 1987
GI	*Escherichia coli*	Mice	Galtier et al., 2016
GI	*Escherichia coli*	Pigs	Smith and Huggins, 1983; Jamalludeen et al., 2009
GI	*Escherichia coli*	Sheep	Smith and Huggins, 1983; Raya et al., 2006
GI	*Salmonella enterica*	Chicken	Colom et al., 2015; Tie et al., 2018
GI	*Salmonella enterica*	Mice	Dallal et al., 2019

System	Bacteria	Animal Model	References
GI	*Salmonella enterica*	Quails	Ahmadi et al., 2016
GI	*Vibrio cholerae*	Mice	Jaiswal et al., 2014
GI	*Vibrio cholerae*	Rabbits	Bhandare et al., 2019
GI	*Vibrio parahaemolyticus*	Mice	Jun et al., 2014
Keratitis	*Pseudomonas aeruginosa*	Mice	Furusawa et al., 2016

2.2.12 History of Phage Therapy

Salmonella Gallinarum-related avian typhosis was the primary focus of D'Herelle's early field investigations. D'Herelle evaluated the efficacy of his phage formulations on himself by swallowing and injecting them prior to undertaking human trials. Cholera and bacillary dysentery were the subjects of his initial human trials. A few instances of bubonic plague were successfully treated by him using Yersinia phage. The interest in phage treatment research later grew once these case reports were published in a well-read journal (Summers, 2001). Phage therapy has a wide variety of uses, from treating wound infections to treating respiratory and gastrointestinal illnesses. However, several tests had inconsistent outcomes. The lack of sufficient scientific rigor in experimental design was just one of several elements that led to this contradiction. Few known instances of rigorous investigations, such as double-blind trials, were conducted while being in line with the norms of the period. Another problem was the incomplete, still-controversial understanding of phage biology. As a result, it's possible that temperate phages were utilized, which don't always result in cell lysis, and that techniques utilized to "stabilize" and proliferate phages may have lowered the viability of preparations (Summers, 2001).

Phage therapy is the direct delivery of virulent phages to a patient with the goal of lysing the bacterial pathogen producing a clinically significant illness (Viertel et al., 2014). The first findings on the efficacy of phage treatment were received with considerable, though transient, excitement. Phage therapy involves using bacteriophages to cure illnesses caused by harmful bacteria. Phage therapy was effectively used in the 1920s and 1930s to treat several illnesses prior to the widespread use of antibiotics (Potera, 2013). Phage treatment was widely rejected by much of Western medicine in the 1940s because of a lack of knowledge of the biological nature of phages, historical medical restrictions, and the development of a broader range of antibiotics (Lin et al., 2017). Phage treatment research was continued, but not completed entirely. Phage treatment gradually increased in popularity in nations like Georgia (a former Soviet Union member) and Poland. The literature suggested widespread and generally effective use of phage treatment across a variety of medical professions, despite the fact that a significant portion of it included methodological flaws (Chanishvili; 2012). The George Eliava Institute of Bacteriophage, Microbiology, and Virology, established in 1923 by the Georgian microbiologist, and the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, established in Wroclaw, Poland, in 1952, are two of the institutions that have been involved in phage therapy the longest period.

Table 2.6: Timeline of major events in the history of research on phages, phage therapy, and antibiotics.

Years	Discovery Milestone
1896	Hankin observes antibacterial activity against *Vibrio cholerae* from Indian river water.
1898	Gamaleya “Ferments that can destroy bacteria.”
1915	**Bacteriophage was discovered as a mysterious organism that was smaller than a bacterium and capable of eradicating bacterial cells.**
1917	Bacteriophages were discovered to combat bacteria that produced the fatal dysentery type of diarrhea. Diseases like cholera and dysentery were still being treated with phage therapy.
1919	Birth of phage therapy. Dysentery cases are cured using phage plaques.
1920s	Expansion of phage therapy trials: cholera, dysentery, bubonic plague, conjunctivitis, skin infections.
1929	Discovery of penicillin by Flemming.
1930	***Salmonella* phage was recognized by Felix O1.**
1933-34	Critical review of phage therapy trials: lack of controls, low efficacy, possible confounders, low purity of phage lysates.
1937-39	Ellis & Delbrück Broad characterization of the phage life cycle: adsorption, growth within the host, lysis.

1940	Ruska’s First EM image of a phage.
1942	Introduction of penicillin.
1940s-1970s	The golden age of antimicrobials over 40 antibacterial compounds were discovered and introduced.
1977	Sanger’s first phage genome was sequenced.
1983-86	Phage therapy against antibiotic-resistant bacterial strains, in humans and multiple animal models. Infections including septicemia, and meningitis.
1990 onwards	As an alternative to antibiotics, bacteriophage reemerged to treat certain bacterial infections.
1992-1996	Resurgence of phage therapy studies with animal models.
2000	The number of new antibiotics decreases. Start of the dry pipeline phenomenon.
2006	First commercial phage-based biocontrol product. For use against Listeria monocytogenes.
2009	The first phase I/II controlled clinical trial of phage therapy. Chronic otitis due to MDR-Pseudomonas aeruginosa.
2015	Phagoburn trial. Multicenter phase I/II. Wound infections in burned patients.
2017	Successful personalized intravenous phage therapy. Acinetobacter baumannii septicemia.

2.2.13 Phage Therapy for Pathogens

The use of phages to decrease infections in food animals has several benefits. Phage has been used to prevent or diminish different foodborne pathogens both at the pre-harvest and post-harvest stages (Figure 2.12).

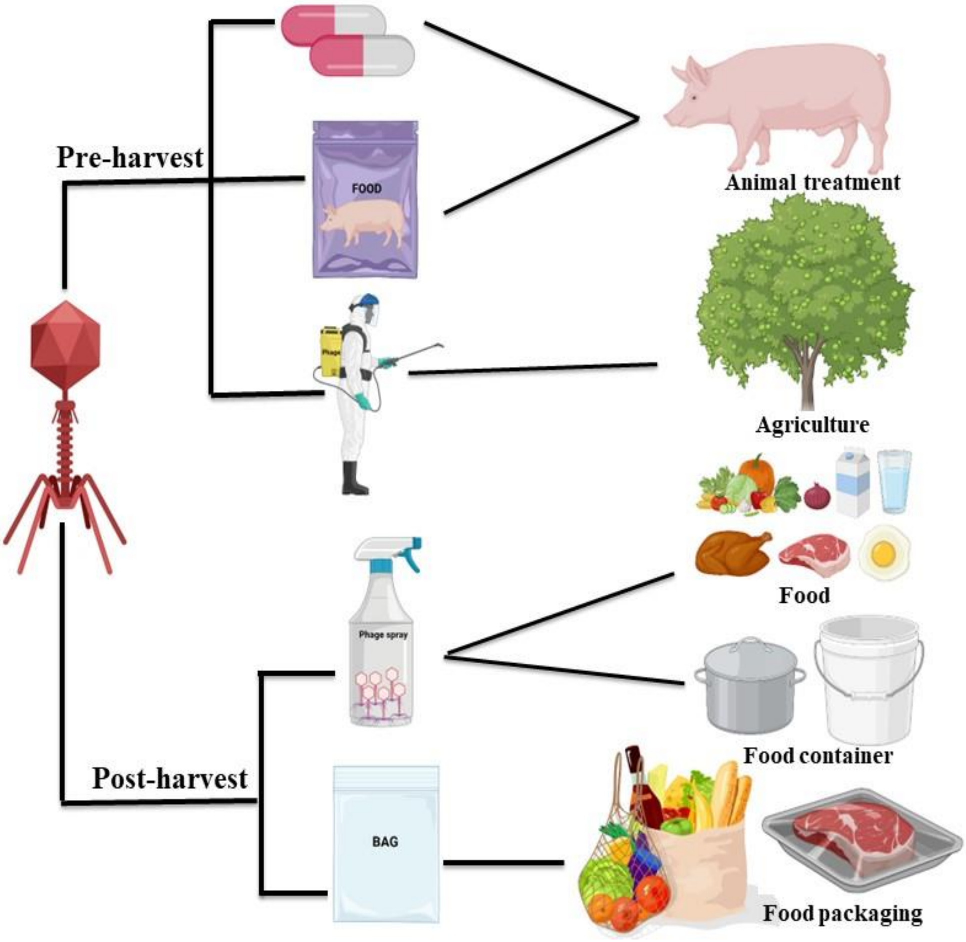


Figure 2.13: Graphical representation of pre and post-harvest phage applications using Biorender software.

2.2.13.1 Pre-harvest Phage Applications

The effectiveness of phage-based treatments against the most common foodborne diseases in swine, poultry, cattle, and other animals has been well-researched during the pre-harvest stage. The animal gastrointestinal (GI) tract is the primary target location in the investigations indicated above since it is the important colonization site for many major foodborne pathogens. However, the *in vivo* efficacy of phage applications is less predictable than that of *in vitro* research because of the complicated physiological and microbiological environment in the GI tract.

The primary concern is the presence of stomach acid and certain proteolytic enzymes during the oral administration of phage (Dabrowska et al., 2005, Northrop, 1964). Due to the denaturation of phage structural proteins, which are crucial for the preservation of phage genetic material and identification of the bacterium host, phage survival is significantly reduced in extremely acidic conditions (Koo et al., 2001; Zhang et al., 2015). Protection strategies like buffering and microencapsulation are frequently used to overcome these issues. Wall et al. demonstrated the effectiveness of a microencapsulated phage cocktail to lower experimentally infected small pigs with *Salmonella* Typhimurium (Wall et al., 2010). Chicken stomachs are smaller than those of other animals therefore phage solutions including antiacid may be sufficient for use in chickens. *Salmonella* Enteritidis and *Salmonella* Typhimurium in grill chickens were effectively reduced by *Salmonella* phages suspended in phosphate-buffered saline with 30% $CaCO_3$, as previously reported (Atterbury et al., 2007).

Additionally, phages have also been sprayed into the air to target germs on poultry feathers, cattle hides, and farming environments. According to a prior study, aerosol-sprayed phage therapy substantially reduced the prevalence of *Salmonella* Enteritidis infection in young chicks by over 30% (Borie et al., 2008).

In addition to phage delivery methods, choosing appropriate treatment time points is crucial to the success of pre-harvest phage therapy. According to several investigations, phage therapy in animals temporarily reduced the percentage of foodborne microorganisms present (Berchieri et al., 1991; Loc Carrillo et al., 2005).

Moreover, Phage-resistant bacteria have been reported to arise in several trials, particularly those that only used one phage treatment (Loc Carrillo et al., 2005; Atterbury et al., 2007). Phage-resistant bacteria may also temporarily reduce the amount of the targeted bacterium. Furthermore, if the administration strategy employs several treatments, microorganisms with phage resistance can compromise the effectiveness of further phage application. Pre-harvest phage treatment is typically recommended as being more successful when used prior to animal slaughter due to the incidence of bacterial regrowth and phage resistance.

2.2.13.2 Post-harvest Phage Applications

Phage treatments administered at the post-harvest stage are often anticipated to be more successful than those applied pre-harvest. The post-harvest environment is less dynamic and complex than the GI tract of live animals, but it is still important to consider the intrinsic qualities of foods (such as composition, pH, and water content) and processing-related factors (such as temperature, chlorinated water, and UV) when developing the post-harvest phage applications (Zhang et al., 2015).

Applications of phages have been investigated in a range of food sources, including meat, dairy products, fresh vegetables, and processed meals. In fresh honeydew melon slices incubated at 5°C, 10°C, and 20°C, the introduction of a *Salmonella* phage cocktail resulted in a considerable decrease of *Salmonella* Enteritidis by 2.5 log to 3.5 log. On apple slices, however, the identical phage cocktail failed to reduce the number of germs. The key factor that most likely contributed to the lower effectiveness was the higher pH of apple slices (Leverentz et al., 2001). On the other hand, a temperature influence on the effectiveness of phage treatment in ground beef was found by Hong and colleagues (Hong et al., 2014). After being treated for 24 hours at room temperature (24°C), the three-phage cocktail utilized in the study reduced the levels of ETEC O157:H7 by 1.97 log CFU/ml. In contrast, under refrigeration (4°C) for 24 hours, a decrease of just 0.48 log CFU/mL was observed. This was most likely caused by the inhibition of bacterial and phage activity at lower-than-optimal growth temperatures. Other research also assessed the capacity of phages to eliminate bacterial contamination in surroundings and food processing equipment.

2.2.14 Application of Bacteriophages

2.2.14.1 Food Industry

Phages can be used in the food industry at many stages (Harda et al., 2018) directly on animals or plants to reduce the risk of bacterial infection and illness, in food processing plants to avoid the formation of bacterial biofilms, or directly on food to preserve the product. It has been proposed that bacteriophages be utilized as biocontrol agents in food to avoid foodborne illnesses. This curiosity fuels studies of food safety and animal rehabilitation. Phages are commonly employed as biological control agents in the food sector to combat harmful microorganisms. Several major *Salmonella* phage products, such as SalmoFreshTM (Intralytix, Inc., Baltimore, United States), SalmonelexTM (Micreos BV, Wageningen, Netherlands), Phage Guard S^{TM} (Micros Food Safety), SalmoPro® (Phagelux) and BioTector® (CheilJedang Corporation, Seoul, South Korea), have been widely used in meat products (Goodridge et al., 2018; LeLièvre et al., 2019; Lewis and Hill, 2020). Other phage products against different bacteria are mentioned in Table 2.7.

Table 2.7: List of some FDA-approved phage-based products

Agency name	Product	Bacteria
Intralytix, Inc.,	ListShield^{TM}	*L. monocytogenes*
FINK TEC GMBH	EcoShield^{TM}	*E. coli*
Micros Food Safety	Phage Guard Listex^{TM}	*L. monocytogenes*
Intralytix, Inc.,	Shiga Shield^{TM}	*Shigella* spp
Phagelux	Agri Phage^{TM}	Xanthomonas campestris pv Vesicatoria *Pseudomonas syringae* pv tomato
Passport Food Safety Solution	Finalyse®	*E. coli*

2.2.14.2 In animals

Bacteriophages as therapeutic agents have been studied on cattle, sheep, and poultry to treat *Escherichia coli O157:H7*, *Salmonella,* and *Campylobacter* (Berchieri et al., 1991; Carrillo et al., 2005; Fiorentin et al., 2005a; Toro et al., 2005; Wagenaar et al., 2005; Sheng et al., 2006; Andreatti Filho et al., 2007; Atterbury et al., 2007; Borie et al., 2008; Callaway et al., 2008; Ricci and Piddock, 2010). The majority of animal phage therapy studies have been on Campylobacter and *Salmonella* colonization in poultry, as well as the *E. coli O157* colonization of ruminants, both of which are important reservoirs for the respective pathogens.

2.2.14.3 In Human Infections

Since the early 20th century, phages have been employed to treat bacillary dysentery and cholera. Phage research is now gaining attention from doctors and academics as a result of the growing issue of antibiotic resistance. The number of research studies on phage therapy has increased during the past few years. Phage therapy is also radically evolving in terms of form, ranging from fecal phage filtrate transplantation to single/cocktail phage therapy, phage lytic enzyme therapy, and more. The benefits of phages, such as their high host specificity and minimal resistance, certainly provide an essential new approach to resolving the problem of bacterial drug resistance. With phage therapy, 1307 patients with multidrug-resistant bacterial illnesses were successfully treated in a Polish research facility, with 85.9% of the patients improving or completely cured (Żaczek et al., 2020). A previous report also described that it is possible to successfully treat refractory *Clostridium difficile* infection with fecal filtrate made by phage extraction from healthy human feces (Ott et al., 2017). Phages and phage lytic enzyme therapy has been the subject of 41 clinical trials, as shown in Table 2.8 of the trials.

Table 2.8: Clinical therapeutic trials involving the use of phage or phage lytic enzymes extracted from Xu et al., 2022

NCT number	Registry date	Conditions	Interventions	Phases	Locations
NCT00663091	22-04-2008	Venous leg ulcers	Phages cocktail	Phase 1	United States
NCT00937274	10-07-2009	Diarrhea	Phages cocktail	Not applicable	Banglades h
NCT00945087	23-07-2009	Bacterial infections	Single phage/phages cocktail/ Phage lytic enzymes	Not applicable	Poland
NCT01617122	12-06-2012	Primary immune deficiency diseases	Single phage	Not applicable	United States
NCT01746654	11-12-2012	Infectious disease/bacterial infections	Phage lytic enzymes	Phase 1/phase 2	Singapore
NCT01818206	26-03-2013	Cystic fibrosis	Phages cocktail	Not applicable	France
NCT01855048	16-05-2013	Healthy volunteers	Phage lytic enzymes	Phase 1	Korea
NCT02116010	16-04-2014	Wound infection	Phages cocktail	Phase 1/phase 2	Belgium
NCT02439359	08-05-2015	Bloodstream infections	Phage lytic enzymes		United States

NCT02664740	27-01-2016	Diabetes/diabetic foot	Phages cocktail	Phase 1/phase 2	France
NCT02757755	02-05-2016	Healthy volunteers	Phages cocktail	Phase 1	United States
NCT02840955	21-07-2016	Atopic dermatitis	Phage lytic enzymes	Not applicable	Netherlands
NCT03089697	24-03-2017	Antibacterial agents	Phage lytic enzymes	Phase 2	Korea
NCT03140085	04-05-2017	Urinary tract infections	Phages cocktail	Phase 2/phase 3	Georgia
NCT03163446	23-05-2017	Antibacterial agents	Phage lytic enzymes	Phase 2	United States
NCT03269617	01-09-2017	Gastrointestinal disorder	Phages cocktail	Not applicable	United States
NCT03808103	17-01-2019	Crohn disease	Phages cocktail	Phase 1/phase 2	United States
NCT04191148	09-12-2019	Urinary tract infections	Phages cocktail	Phase 1	United States
NCT04287478	27-02-2020	Urinary tract infections	Single phage/phages cocktail	Phase 1/phase 2	United States
NCT04289948	28-02-2020	Diabetes/diabetic foot	Phages cocktail	Phase 1/phase 2	United Kingdom
NCT04323475	26-03-2020	Wound infection	Phages cocktail	Phase 1	Australia
NCT04325685	27-03-2020	Trauma injury/brain injuries, Abdominal sepsis	Phages cocktail	Not applicable	Russian
NCT04596319	20-10-2020	Cystic fibrosis/lung infection	Phages cocktail	Phase 1/phase 2	United States

NCT04650607	2-12-2020	Prosthetic joint infection/bone and joint infection/implant infection	Single phage/phages cocktail	Not applicable	France
NCT04682964	24-12-2020	Acute tonsillitis	Single phage	Phase 3	Uzbekistan
NCT04684641	24-12-2020	Cystic fibrosis	Single phage	Phase 1/phase 2	United States
NCT04724603	12-01-2021	Bone and joint infection/prosthetic joint infection	Single phage/phages cocktail	Not applicable	France
NCT04737876	04-02-2021	Healthy volunteers	Phages cocktail	Phase 1	United States
NCT04787250	08-03-2021	Prosthetic joint infection	Single phage/phage cocktail	Phase 1/phase 2	United States
NCT04815798	25-03-2021	Pressure ulcer	Phages cocktail	Phase 1/phase 2	United Kingdom
NCT05010577	18-08-2021	Chronic *Pseudomonas aeruginosa* infection /cystic fibrosis	Phages cocktail	Phase 1/phase 2	Israel
NCT05177107	04-01-2022	Osteomyelitis/diabetic foot osteomyelitis	Single phage/phages cocktail	Phase 2	United States
NCT05182749	10-01-2022	Shigellosis	Phages cocktail	Phase 1/phase 2	United States
NCT05184764	11-01-2022	Bacteremia/*Staphylococcus aureus/Staphylococcusaureus* bacteremia /bacteremia due to *Staphylococcus aureus*	Phages cocktail	Phase 1/phase 2	United States

NCT05240300	15-02-2022	Atopic dermatitis	Single phage/phages cocktail	Phase 1/phase 2	Israel
NCT05269121	07-03-2022	Prosthetic joint infection/bacterial infections	Single phage/phages cocktail	Phase 1/phase 2	United States
NCT05272566	09-03-2022	Feeding patterns/microbial colonization	Fecal phages transfer	Not applicable	Denmark
NCT05272579	09-03-2022	Necrotizing enterocolitis/microbial substitution	Fecal phages transfer	Early phase 1	Denmark
NCT05277350	14-03-2022	*E. coli* infections/blood stream infection	Phages cocktail	Phase 1	Denmark

2.2.15 Controlling *Salmonella* infections in poultry and poultry products using phages

Salmonella phage therapy in the poultry business has two primary objectives. First, phage therapy reduces the damage that bacterial infections cause to the health and productivity of animals. Second, phage-based biocontrol is regarded as a potent method for reducing the incidence of human foodborne diseases. Phage titer, manner of administration, and length of administration are the main variables that affect the efficacy of phages as a means of therapy (Atterbury et al., 2007). Phage cocktails can be delivered in a variety of ways, including spraying on eggs, adding the phage solution directly to infected items, and orally ingesting the mixture after combining it with water or as a feed supplement. Therefore, using bacteriophages as an intervention technique has promise for reducing *Salmonella* transmission both horizontally and vertically. A practical method for reducing the horizontal spread of *Salmonella* through eggs might be to spray phages as an aerosol during the transfer of eggs from incubators to hatchers (Henriques et al., 2013; Sonalika et al., 2020). P22 and Felix-O1 are two of the most well-known *Salmonella* phages that were found. A recognized effective option for therapeutic and diagnostic employs is Felix-O1, a broad-spectrum lytic phage that can lyse a variety of *Salmonella* serotypes. Despite the fact that phage therapy has been shown to reduce the number of bacteria after small-scale investigations, a sufficient safety evaluation is still required before its industrial-scale deployment. There will be more information accessible on the security and effectiveness of phage treatment when additional research is conducted. In a phage therapy trial involving 34,680 broiler chickens on a farm where there had previously been a *Salmonella* outbreak, no significant differences in mortality, productivity, or gut microbiota were found between the phage-treated group and the untreated control group, demonstrating the safety of the phage preparation. By using 16s rRNA gene amplicon sequencing in this instance, the impact of phage therapy on the gut microbiota was assessed. Up to this point, it is the largest experiment to assess the security and effectiveness of *Salmonella* phages in a commercial context.

2.2.16 Advantages and Challenges of Phage Therapy

2.2.16.1 Advantages

Phage therapy provides a variety of benefits over antibiotic treatments. Typically, phages are quite specific to a particular bacterial species or strain. Therefore, phage treatment will only have a small influence on the natural microflora and cause negligible disturbance to the normal intestinal flora in animals (Loc-Carrillo & Abedon, 2011). The broad-spectrum action of many antibiotics, in contrast, results in the non-specific death of both infections and members of the normal flora (Loc-Carrillo & Abedon, 2011). This increases the possibility of changing the natural flora's composition and may also result in secondary or superinfections (Loc-Carrillo & Abedon, 2011; Golkar et al., 2014).

Since there have been no major adverse effects associated with or after phage treatments in people, phage therapy is thought to be safer than antibiotics (Sulakvelidze et al., 2001). The release of endotoxins from bacteria when they are lysed by phages inside the human body, however, has occasionally been described as having some modest adverse effects (such as fever) (Gunawardhana, 2009). In contrast, the use of antibiotics frequently results in adverse reactions, such as gastrointestinal problems, allergies (which can occasionally be deadly), and secondary infections (Sulakvelidze et al., 2001; Gunawardhana, 2009; Golkar et al., 2014).

During phage treatment, bacteria can evolve resistance to phages, but it is often easy to identify and purify a new phage with lytic activity against the virus-resistant bacterial variants within a few days or weeks. This is because phages outnumber bacteria in the environment ten to one and are co-evolving with their host bacteria (Morrison & Rainnie, 2004; Almeida et al., 2009). In contrast, the process of developing a new antibiotic often takes a very long time and costs a lot of money in order to prove its efficacy, safety, and compliance with quality standards (Huys et al., 2013; Golkar et al., 2014). According to Golkar et al. (2014) and Viertel et al. (2014), the issues with resistance may be solved by using phages or phage enzymes that have been created or by combining a variety of phages with various bacterial receptors. Furthermore, certain phages may employ receptors that are linked to virulence in order to attach to bacteria, which may result in the loss of phage-resistant bacteria or some of their virulence (Smith & Huggins, 1983; Skurnik & Strauch, 2006; Capparelli et al., 2010a).

2.2.16.2 Challenges

Despite these benefits, the use of phage treatment in humans, food, or animal products is not yet recognized (Dahl et al., 2004). For phage treatment to be properly applied, it is necessary to take into consideration the obvious complexity of phage-bacteria interactions (Roach and Debarbieux 2017). The defense system contributes to and effects on resistance generation to phages in clinical settings remain unknown, if they are disregarded, this might be a barrier to the development of effective phage-based therapeutics.

Utilizing phages against a wide range of bacterial variations is challenging due to specificity and limited host ranges of phages. In this case, the nature of the infection and verification of phage susceptibility is essential before administering phage treatment. Phage cocktails, which are a combination of phages, can, however, get over this restriction (Sulakvelidze et al., 2001; Loc-Carrillo & Abedon, 2011; Lu & Koeris, 2011).

According to Almeida et al. (2009), host defensive mechanisms, such as antibody neutralization, can quickly remove phage particles from the body, reducing the number of phages to reach the infection sites. These issues might be solved by choosing phage mutants that can stay in the bloodstream of the body (Merril et al., 1996) or by repeatedly administering the same phage to the organism (Barrow & Soothill, 1997).

Some lytic and temperate phages may function as vectors for delivering genes to bacteria that can impart antibiotic resistance and encode pathogenicity (Almeida et al., 2009). According to Almeida et al. (2009), this may increase virulence and cause non-pathogenic strains to become pathogenic. The creation of genetically engineered mutant phages and careful phage selection are also potential solutions to this problem (Matsuzaki et al., 2005; Skurnik & Srauch, 2006; Loc-Carrillo & Abedon, 2011). Phage genome sequencing to prevent the spread of virulence genes is another potential solution.

Chapter 3

Materials & Methods

Chapter 3

3.1 Equipments

Table 3.1: Equipments used in the current investigation

Equipment	**Manufacturer**	**Purpose**
Agarose gel electrophoresis apparatus	ATTO (AE-6111), Japan	Gel electrophoresis
Autoclave	Daihan Labtech Co., Ltd (LabTech), Korea	Sterilization
Centrifuge	Thermo SCIENTIFIC (SORVALL LEGEND X1R)	Centrifugation of solutions
Finnpipette micropipettes	Thermo Fisher Scientific, Scoresby, VIC, Australia	Dispensing liquids
Drying oven (MOV-112)	Sanyo Electric Co., Ltd, Japan	Evaporation, sterilization, and incubating temperature-sensitive experiments
Embedding oven (MK111)	Agar SCIENTIFIC, UK	To orientate a histological specimen accurately and precisely into a block of paraffin wax
Gel Doc Go Imaging System	Bio-Rad, USA	Capturing images of gels
Hydrophilic Treatment device (HDT-400)	JEOL DATUM, Japan	To increase the hydrophilicity of the surface of TEM grids
Laminar Flow Cabinet (BSL-2)	Nippon Medical & Chemical Instruments Co., Ltd, Japan	Drying plates, sterile work
Light microscope (Primo Star)	ZEISS, Germany	Visualizing fine detail of an object
Microcentrifuge	Eppendorf, Germany	Centrifugation of solutions

Mini-Sub cell GT electrophoresis gel tank	Bio-Rad, USA	Agarose gel electrophoresis
Nanodrop (µCuvette G1.0)	Eppendorf, Germany	To measure DNA, RNA, or protein concentrations
pH meter	EUTECH INSTRUMENT (Thermo Fisher Scientific), USA	Measures acidity/alkalinity
PowerPac Mini power supply	Bio-Rad	Agarose gel electrophoresis
Refrigerator	Sanyo Trading Co., Ltd., Japan	To store samples and specimens in cold conditions
Scanning electron microscope (FEI Quanta 200)	FEI, Netherlands	Obtaining information about the surface's topography and composition
Shaker incubator (BS-11)	JEIO TECH, Korea	Mixing cultures
SDS- PAGE apparatus Mini-PROTEAN® 3	Bio-Rad, USA	Polyacrylamide gel electrophoresis
Sputter coater (SC7640)	Polaron, UK	To deposit a thin coating on a specimen to be viewed in SEM
Transmission Electron Microscope (FEI Tecnai 12 BioTwin)	FEI, Netherlands	To view specimens through which electrons can pass generating a projection image
Vortex mixer (G-560E)	SCIENTIFIC INDUSTRIES INC., Japan	Mixing solutions
Water bath	Labec, Marrickville, NSW, Australia	Incubation of cultures
Weighing machine	SCIENTECH, USA	To determine the weight
Ultra centrifuge (Sorvall WX100)	Thermo Fisher Scientific, Japan	Centrifugation
Ultramicrotome (LEICA ULTRA CUT)	LEICA, Germany	Cutting thin sections of cells and tissues for visualization under TEM
UV/VIS Spectrophotometer (Optizen 2120UV PLUS)	Mecasys Co., Ltd, Japan	To quantify the amount of DNA or protein in a sample

3.2 Chemical reagents & Miscellaneous items

3.2.1 Chemical reagents

Table 3.2: List of reagents

Reagents	Company	Country
Acrylamide	Sigma-Aldrich	USA
Aetone AR	Merck	India
Agar 100 resin kit	Agar Scientific	UK
Agarose (Medium EEO)	Himedia	India
Ammonium acetate	Merck	India
Ammonium sulphite	Qualigens	India
Amyl acetate	Qualigens	India
Ammonium per sulphate	Sigma-Aldrich	USA
Bacto agar	Becton Dickison and company	USA
Bromophenol blue	Sigma-Aldrich	USA
Beta marcaptoethanol	Sigma-Aldrich	USA
Cacodylic acid, sodium salt	Ted Pella	USA
Coomassie brilliant blue	Sigma-Aldrich	USA
Chloroform	Qualigens	India
Dimethyl sulfoxide (DMSO)	Merck	India
Dithiothreitol (DTT)	SRL	India
EDTA	Qualigens	India
Eosin	HiMedia	India
Ethanol	Bengal Chemicals and pharmaceuticals	India
Ethidium bromide	Himedia	India
Formaldehyde	Bengal Chemicals and pharmaceuticals	India
Glacial acetic acid	Qualigens	India
Glutaraldehyde	Sigma-Aldrich	USA
Glycerol	Merck	India
Glycine	Merck	India
Gram crystal violet	HiMedia	India
Hematoxylene	HiMedia	India
Hexamethyldisilazane	Sigma-Aldrich	USA

Hydrochloric acid	Merck	India
Lead citrate	Polaron	UK
Luria Bertani Broth	Himedia	India
Methanol	Merck	India
Magnesium chloride	Himedia	India
N, N’ bisacrylamide	Sigma-Aldrich	USA
Nutrient broth	Himedia	India
Nutrient agar	Himedia	India
Osmium tetraoxide	Agar Scientific Ltd.	USA
Potassium bromide	Merck	India
Phloxine-B	Sigma-Aldrich	USA
Sodium acetate	Himedia	India
Sodium cacodylate	Sigma-Aldrich	USA
Sodium carbonate	Merck	India
Sodium chloride	Himedia	India
Sodium hydroxide	Merck	India
Sucrose	Himedia	India
SDS	SRL	India
Tris-base	Himedia	India
Tris-HCL	Himedia	India
Tryptone soya broth	Himedia	India
TEMED	Himedia	India
Uranyl acetate	BDH chemicals	England
Whatman no. 1 filter paper	Merck	India

3.2.2 Miscellaneous items

Table 3.3: List of Miscellaneous items

Items	**Company**	**Country**
Carbon rods	Bio-Rad	USA
Copper grids (300 mesh)	EMS	USA
Coverslip	BLUE STAR	India
Nickel thin bar grids (300 mesh)	Agar Scientific	UK

Mica sheets	Pelco	USA
Tungsten wire	Pelco	USA
Platinum wire	Pelco	USA
Specimen mount, Al,1/2” Slotted Head, 1/8” Pin	TED PELLA	USA
Slide	BLUE STAR	India

3.3 Chemicals and buffers

3.3.1 Enzymes and Commercial Kit

Table 3.4: List of Enzymes and commercial kit

Enzyme	Manufacturer	Purpose
1. DNase I (10 mg/mL)	Invitrogen	Digestion of phage DNA
2. EcoRI	Promega	RE digestion of phage DNA
3. EcoRV	Himedia	RE digestion of phage DNA
4. MluI	Himedia	RE digestion of phage DNA
5. BglII	Himedia	RE digestion of phage DNA
6. PstI	Promega	RE digestion of phage DNA
7. HindIII	Promega	RE digestion of phage DNA
8. BamHI	Himedia	RE digestion of phage DNA
9. 6X DNA Loading dye	Himedia	Agarose gel electrophoresis
10. High-range DNA Ladder (250-25kb)	Himedia	Agarose gel electrophoresis
11. Prestained Protein Ladder (11-245kDa)	Himedia	Polyacrylamide gel electrophoresis
12. 5X protein Loading Buffer	Himedia	Polyacrylamide gel electrophoresis
13. DNA isolation kit	Norgen	Agarose gel electrophoresis

3.3.2 Compositions of buffers

All chemicals used in the preparation of buffers were of analytical reagent (AR) grade and were prepared with distilled water/ Milli-Q water (Millipore). For general-purpose uses, buffers and solutions were prepared according to Sambrook and Russell (2001). Stock solutions were appropriately diluted for use in experiments.

3.3.2.1 Tris-$MgCl_2$ (0.1M-0.01M) buffer:

Tris HCl 13.22 gms

Tris base 1.92 gms

$MgCl_2$ 2.033 gms

Volume made up to 1000 ml with distilled water, pH was adjusted to 7.4.

3.3.2.2 Phosphate-buffered saline (PBS)

3.2 mM Na_2HPO_4,

0.5 mM KH_2PO_4,

1.3 mM KCl, 135 mM NaCl,

Volume made up to 1000 ml with distilled water, pH 7.2.

3.3.2.3 TAE Buffer (50X)

2 M Tris base,

6.5 M EDTA disodium salt,

Volume made up to 1000 ml with distilled water, pH 8.0.

3.3.2.4 Tris-Glycine electrophoresis buffer

Tris Base 3gms (25 mM)

Glycine 14.4gms (250 mM)

SDS 1gm (0.1%, w/v)

Volume made up to 1000 ml with distilled water, pH 8.3.

3.3.2.5 Tris-HCl electrophoresis buffer

Acrylamide 14.6gms (29.2%)

Bis acrylamide 4gms (0.8%)

Volume made up to 500 ml with distilled water, pH 8.8.

3.3.2.6 Tris-HCl electrophoresis buffer

Tris HCL 6.057gms (0.5M)

SDS 0.4 gm (0.4%)

Volume made up to 100 ml with distilled water, pH 6.8.

3.3.2.7 SM buffer:

Sodium chloride (NaCl) 5.8 gms

$MgSO_4$, $7H_2O$ 2 gms

Tris-Cl (1M, pH-7.5) 50 ml

Double distilled water 1 L

To prepare 1 L of SM buffer, 5.8 gms NaCl, and 2 gms $MgSO_4$, $7H_2O$ was dissolved in 800 ml of double distilled water. Thereafter Tris-Cl was added and the volume was adjusted up to 1 L. It was sterilized by autoclaving at 15 lbs/atm pressure. It was stored at room temperature.

3.4 Microbiological Media

3.4.1 Luria agar (LA)

Bacto tryptone (Difco) 10.0 gms

BactoYeast extract (Difco) 5.0 gms

Sodium Chloride (SRL) 10.0 gms

Agar (Difco) 15.0 gms

All the ingredients were dissolved in 1000 ml of distilled water followed by the addition of agar. The pH of the media was auto-adjusted to 7.2. The media was sterilized by autoclaving at 15 lbs for 15 minutes and a 20 ml portion of the media was poured onto a 90 mm plate.

3.4.2 Hektoen Enteric Agar (HEA)

Proteose peptone 12.0 gms

Yeast extract 3.0 gms

Lactose 12.0 gms

Sucrose 12.0 gms

Salicin 2.0 gms

Bile salts mixture 9.0 gms

Sodium chloride 5.0 gms

Sodium thiosulphate 5.0 gms

Ferric ammonium citrate 1.50 gms

Acid Fuchsin 0.10 gms

Bromothymol blue 0.065 gms

Agar 15.0 gms

Because of the presence of some heat-labile ingredients in HEA media, it was not sterilized by using an autoclave at 121°C instead it was boiled until totally dissolved.

3.4.3 Soft agar

Luria Bertani broth 25 gms

Agar (bacteriological grade) 1 %

Double distilled water 1 L

25 gms Luria Bertani Broth and 1% agar were mixed in 1 L double distilled water and the mixture was heated until the agar melted. 3 ml of the molten soft agar was poured into small test tubes in each. Then each tube was plugged with non-absorbent cotton and put into an autoclave for sterilization at 15 lbs/atm pressure. After sterilization, it was stored at 4ºC.

3.4.4 Tryptic Soya Broth (TSB)

Tryptone Soya broth 30 gms

Double distilled water 1000 ml

30 gms of Tryptone Soya broth was suspended in 1000 ml of double distilled water. It was then autoclaved (15 lbs/atm pressure) and stored at 4ºC.

3.4.5 L Broth, pH 7.2

Bacto tryptone (Difco) 10.0 gms

BactoYeast extract (Difco) 5.0 gms

Sodium Chloride (SRL) 10.0 gms

All the ingredients were dissolved in 1000 ml of distilled water. pH of the media was auto-adjusted to 7.2. The media was sterilized by autoclaving at 15 lbs for 15 minutes.

3.5 Chemical preparation:

3.5.1 Fixative & hardening agent

3.5.1.1 Sodium Cacodylate (0.4 M)

Sodium Cacodylate 42.8 gms

Double distilled water 500 ml

42.8 gms of sodium cacodylate was added to 200 ml of distilled water and mixed thoroughly. Then the volume was adjusted up to 500 ml and it was then stored at 4ºC.

3.5.1.2 Cacodylate buffered glutaraldehyde (3%)

0.4 (M) sodium cacodylate 25 ml

25% glutaraldehyde 12 ml

Doubled distilled water 63 ml

In a 100 ml lab bottle, 25 ml of 0.4M sodium cacodylate and 12 ml of 25% glutaraldehyde were added. The volume was adjusted up to 100 ml.

3.5.1.3 Sodium Cacodylate (0.2 M)

Sodium Cacodylate 21.4 gms

Double distilled water 500 ml

21.4 gms of sodium cacodylate was added to 200 ml of distilled water and mixed thoroughly. Then the volume was adjusted up to 500 ml and it was then stored at 4ºC.

3.5.1.4 Formaldehyde solution (10%)

100 ml formalin (38%-40% formaldehyde)

900 ml distilled water

4.5 gms sodium phosphate (monobasic)

3.6 gms sodium hydroxide

Reagents should be combined, and the pH of the solution should be between 6.8 and 7.2.

3.5.1.5 Agar 100 resin

Agar 100 resin 6 gms

Dodecenyl succinic anhydride (DSA) 2.5 gms

Methyl nadic anhydride (MNA) 3.25 gms

N- benzyl dimethyl amine (BDMA) 0.2 gm

6 gms of Agar 100 Resin was mixed with 2.25 gms dodecenyl succinic anhydride and methyl nadic anhydride. Next benzyl di-methyl amine (BDMA) was added. All the above-mentioned components were mixed well in a magnetic stirrer and kept overnight at 60ºC.

3.5.2 Staining and destaining solutions

3.5.2.1 Osmium tetroxide (2%)

Osmium tetroxide 250 mg

Double distilled water 12.5 ml

250 mg of osmium tetroxide was dissolved into 12.5 ml double distilled water and stored in a black bottle (to avoid light) at room temperature.

3.5.2.2 Working osmium tetroxide [1% OsO4]

2% Osmium tetroxide 2 ml

0.2M Sodium Cacodylate 2 ml

2% osmium tetroxide was dissolved into 2 ml of sodium cacodylate and mixed properly.

3.5.2.3 Preparation of negative staining solution

2% Uranyl acetate was prepared in double distilled water (pH 4.2) and stored in dark-colored bottles at room temperature.

3.5.2.4 Preparation of Lead citrate:

Lead citrate 0.2 gms

Double distilled water 100 ml

0.2 gms of lead citrate was added to 100 ml of double distilled water and mixed properly. After that, 1N NaOH solution was added drop by drop until the pH reached 12.

3.5.2.5 Haematoxylin Stain

Ammonium sulphate 50 gms

Absolute Ethyl alcohol 25ml

Haematoxylin 2.5 gms

Sodium iodate 1.25 gms

Glacial acetic acid 20-40 ml

50.0 g of aluminum ammonium sulfate was added to 800 ml of double-distilled water and stirred to mix properly. Next, 1.2 g of hematoxylin crystals was added to absolute ethyl alcohol and mixed until it dissolved. Following that, the two solutions were heated for 3 minutes until they became yellow or red. Then 1.25 gms sodium iodate was mixed and boiled for 3 minutes until it became deep purple. It was then filtered and stored in a dark bottle at room temperature. Before every use, 20-40 ml of glacial acetic acid was added and it was kept in a dark place.

3.5.2.6 Coomassie Blue Stain

50% (v/v) methanol

10% (v/v) glacial acetic acid

0.1% (w/v) Coomassie brilliant blue R

3.5.2.7 Eosin Stain

Eosin yellow 1 gm

Phloxine B 0.1 gm

Distilled water 40 ml

Propan-2-ol 760 ml

Glacial acetic acid 4 ml1 gm of eosin and 0.1 gm of phloxine B were added to 40 ml of double-distilled water. It was mixed properly. Then 760 ml of propane-2-ol was added into it. Finally, 4 ml of acetic acid was added to the mixture and mixed properly.

3.5.2.8 Fixative for protein gel

50% (v/v) methanol

5% (v/v) glacial acetic acid

45% (v/v) water

3.5.2.9 Destaining solution for protein gel

10% (v/v) methanol

5% (v/v) glacial acetic acid

3.5.3 Glue solution for TEM grid preparation

Strips of cello tape were immersed in amyl acetate and left overnight.

3.5.4 Gel for polyacrylamide gel preparation

Table 3.5: Gel preparation for polyacrylamide gel preparation

Resolving gel	Stacking gel
Acrylamide: Bisacrylamide solution 4.2 ml	Acrylamide: Bisacrylamide 1.67 ml
Tris-Glycine buffer (pH 8.8) 2.5 ml	Tris-Glycine buffer (pH 6.8) 0.75 ml
H_2O 3.33 ml	H_2O 6.33ml
APS (20%) 23 µl	APS (20%) 10 µl
TEMED 5.5 ml	TEMED 7 µl

3.5.5 Sucrose solutions

Table 3.6: Sucrose gradients

40%	30%	20%	10%
Sucrose 8 gms	Sucrose 6 gms	Sucrose 4 gms	Sucrose 2 gms
Double distilled water 20 ml	Double distilled water 20 ml	Double distilled water 20 ml	Double distilled water 20 ml

10 ml water was taken and 8 gms of sucrose was added and mixed properly. Then the volume was made up to 20 ml. Then the solution was autoclaved (15lbs/atm pressure) and stored at 4ºC.

3.6 Bacterial cultures

3.6.1 Storage and Maintenance of Bacterial Cultures

In order to maintain the survival and growth of microorganisms, pure cultures are often sub-cultured and regularly moved onto or into a new medium in regular laboratory practices. For the sake of preventing contamination, the transfer is always handled under aseptic settings. It becomes challenging to sustain a large number of pure cultures successfully for an extended period of time because frequent subculturing takes time. Additionally, there is a chance of contamination and genetic alterations. As a result, certain contemporary techniques that do not require repeated subculturing are now replacing them. These techniques include refrigeration, the paraffin process, cryopreservation, and lyophilization. Stab cultures at room temperature are used for *Enterobacteriaceae*.

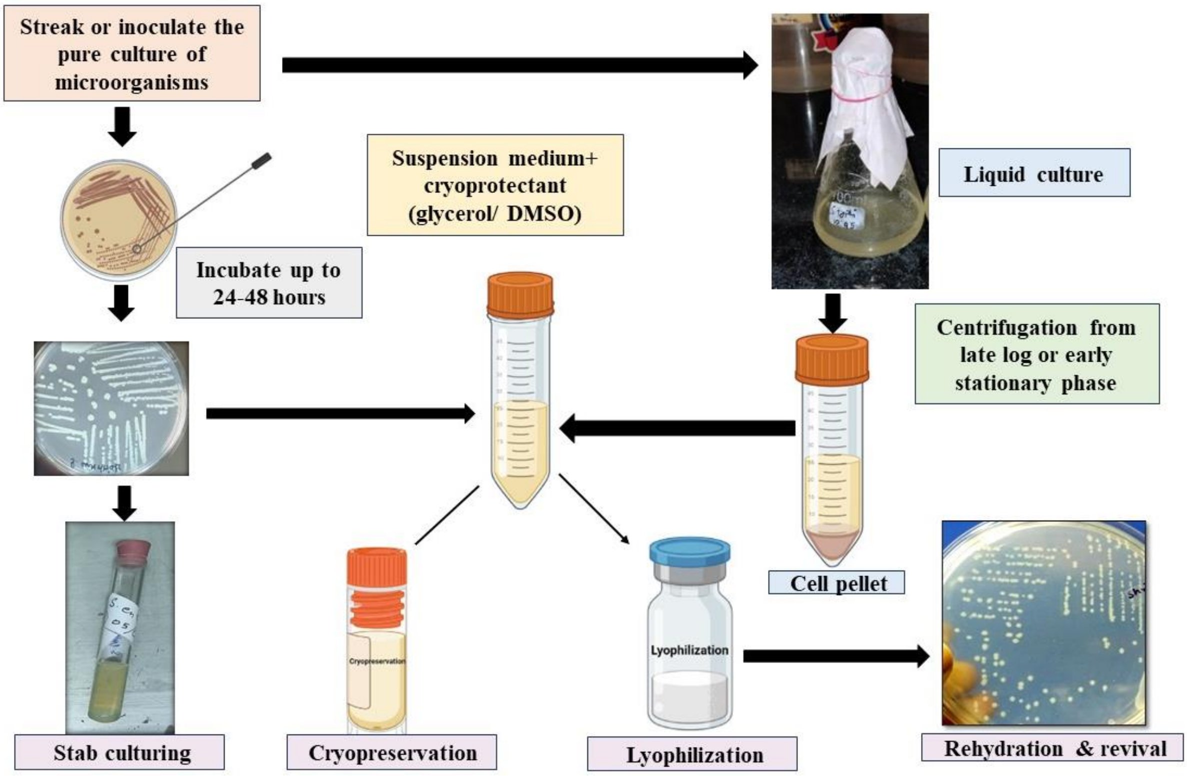


Figure 3.1: Maintenance and storage of bacterial cultures.

3.6.2 Bacterial Strains

The *Salmonella* bacterial strains used in this study were collected from the Division of Bacteriology, ICMR-NICED, Kolkata. The bacterial strains were grown under aerobic conditions in Luria-Bertani (LB) broth at 37°C following institutional standard biosafety guidelines.

3.6.3 Bacterial growth curve determination

Turbidimetric determination is useful for plotting the growth curves of bacteria in broth or liquid media. At first, a single colony of *S.* Typhi bacteria was taken with an inoculation loop and it was then added to 5 ml nutrient broth in a culture flask and kept in a shaker incubator overnight. The next day a fresh culture media was prepared by adding 1 ml of overnight culture and 40 ml of nutrient broth. The wavelength of the spectrophotometer was set to 600 nm. 1 ml of uninoculated sterile media was added to a clean cuvette and the machine was blanked by setting it to 0 ABS with this sample. Immediately after inoculating, 1 ml of the inoculated sample was taken into a clean cuvette. It was placed in the blank spectrophotometer, and the OD reading was recorded. The previous step was repeated at 30-minute intervals until the absorbance no longer increased.

3.6.4 Colony forming unit (CFU) determination

To determine the concentration of bacteria in the culture condition, a colony-forming unit calculation was carried out and it was determined by serial dilution. In brief, the broth was serially diluted with the bacterial culture. An L-shaped spreader was used to evenly distribute the 100 µl of serially diluted bacterial culture on the agar plates. Overnight, the plates were incubated in a 37°C incubator. The following day, colonies were counted.

3.6.5 Bacterial OMV isolation

The OMV isolation method was followed as described and adapted by Schild et al. In a nutshell, *S.* Typhi cells were plated on LB agar and left to incubate for an entire night at 37°C. A single colony was inoculated into 6 ml of LB broth the next day, where it was cultured for around 8 hours at 37°C with aeration in a shaker incubator. This culture was then sub-cultured at a ratio of 1:100 in 600 ml of LB broth, and it was incubated at 37°C with aeration overnight. For the purpose of pelleting bacterial cells, cultures were chilled in an ice-water bath for around 10 minutes. The OMV-containing supernatants were filtered using a 0.22 µm filter system (Millipore) to get rid of any residual bacterial cells. Using a fixed angle rotor TH-685, OMVs were pelleted from culture filtrates by centrifugation at 25,000 rpm for 3.5 hours at 4°C. OMV pellets from a single 600 ml culture were resuspended in a total of 0.5 ml of phosphate-buffered saline, supernatants removed by decanting, and then kept in aliquots at -80°C.

3.7 Isolation of bacteriophage from the lake water sample

3.7.1 Lake water sample

The water sample was collected from lake water in the outskirt area about 18 km from Kolkata, West Bengal, India in search of phage isolation. The 30 ml aliquots of the sample were centrifuged at 4,000 rpm for 10 minutes. The supernatants were pooled and filtered (0.22µm filter) and stored at 4°C until use.

3.7.2 Isolation of bacteriophages and preparation of stocks

25 ml of water sample was mixed with 25 ml of 10X phage broth media and 5 ml of log-phase *Salmonella* Typhi culture and incubated at 37°C for 24 h under shaking conditions at 100 rpm. The residual bacterial cells were removed by centrifugation at 10,000 rpm for 10 min at 4°C and the supernatant was filtered with a 0.22µm membrane filter.

3.7.3 Spot assay to determine the presence of bacteriophage in the solution

A spot test is a quick way to determine whether a phage sample can infect a bacterium by placing a small drop or “spot” of phage onto a plate inoculated with the bacterium. This test will determine whether phage was present or not.

A double agar plate was prepared by mixing soft agar and bacterial culture and then the mixture was poured onto agar plates and it was allowed to solidify. Then, 10 µl of the sample was taken and spotted on the double-layered agar plate. Then the plates were kept in an incubator at 37°C overnight. The next day, the results were observed.

3.7.4 Plaque assays to determine the concentration of bacteriophage

A single colony was inoculated in a conical flask containing 20 ml nutrient broth by an inoculation loop. From the phage stock solution, different dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7},10^{-8}) were prepared. 100µl of these dilutions were then added to 250µl early log phase bacterial culture in fresh microcentrifuge tubes. Sugar tubes containing soft agar were liquefied and the content of microcentrifuge tubes was mixed with them and poured into Petri plates. The plates were tilted immediately to all sides so that the mixture could be spread uniformly throughout the plates. Then it was allowed to solidify. After that, solidified plates were kept in an incubator at 37°C overnight.

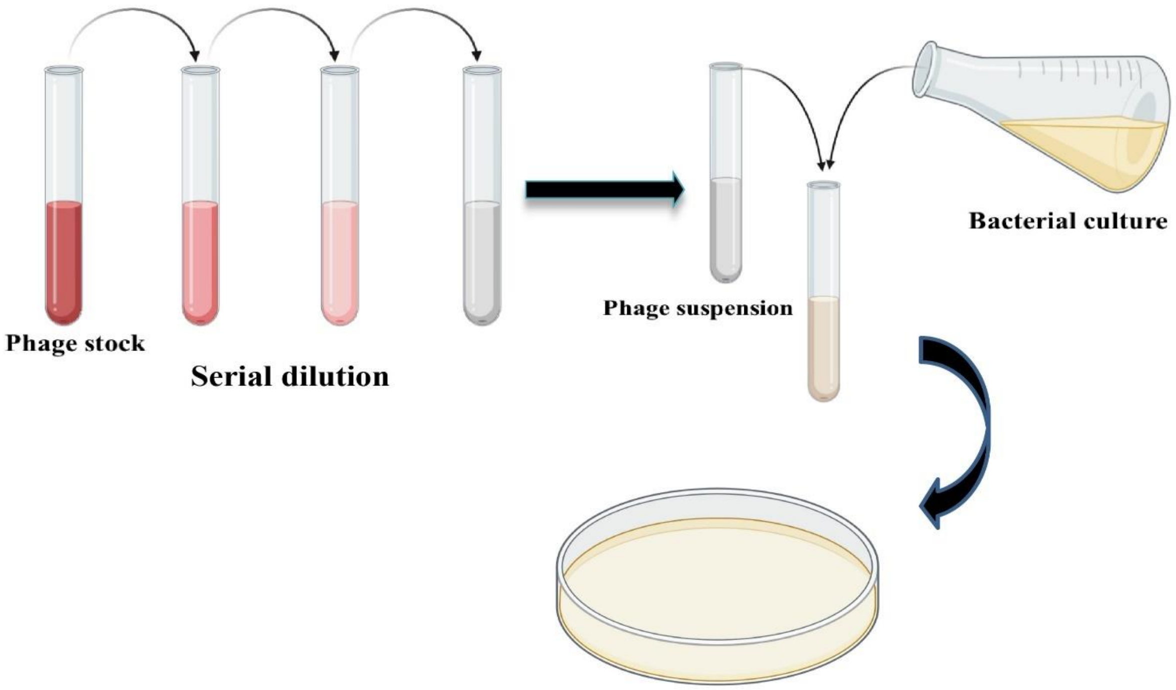


Figure 3.2: Plaque assay method

3.7.5 Preparation of high-titer bacteriophage stocks

The stock phage solution was diluted 3 folds so that the phage concentration became 3×10^{12} pfu/ml. 25µl of this phage was mixed with 250µl of this bacterial culture (log phase) in each of the tubes and incubated at 37°C for 10 minutes. 3 ml of soft agar was added to each of the tubes and plated on nutrient agar plates. The plate was incubated for 2 hours for confluent lysis. Just after confluent lysis 1 ml of Tris-$MgCl_2$ buffer was added to each plate and soft agar was scrapped off and collected. Then it was centrifuged at 10,000 rpm for 10 minutes at 4°C and the supernatant containing phage was collected.

3.7.6 Concentration of high titer phage

Phage solution was centrifuged by ultracentrifugation at 25,000 rpm for 1.30 hours at 4°C in a fixed angle rotor TH-685. The bacteriophage pellet was resuspended in 1.5 ml Tris-$MgCl_2$ buffer. Then it was again centrifuged at low speed (10,000 rpm for 10 minutes at 4°C) to pellet down all bacterial debris and the supernatant containing phage was collected in a microcentrifuge tube.

3.7.7 Purification of high titer phage

Zone centrifugation in sucrose gradients is used to purify the sample. At first, the sucrose gradient was prepared by layering 0.7 ml of different concentrations (40%,30%,20%, and 10%) of chilled sucrose solution in succession in centrifuge tubes. Then, concentrated phage suspension was loaded at the top of the sucrose gradient. After that, it was centrifuged in a swing bucket rotor TH-660 for 2 hours at 30,000 rpm. Then the supernatant was discarded and the pellet formed at the bottom was resuspended into the Tris-$MgCl_2$ buffer. Next, the resuspended solution was collected followed by overnight dialysis and finally stored at 4°C.

3.8 Biological investigation using TEM & SEM

3.8.1 Transmission Electron Microscopy

3.8.1.1 Preparation of carbon coating of grids

25x75 mm sheet of mica was cleaved with a razor blade to expose a clean surface. The mica sheet was placed on a JEOL 400 vacuum evaporator. A small triangular filter paper was cut and placed next to the mica for visualization of carbon deposition. A pointed graphite rod connected to the flat end of another graphite rod was placed inside the vacuum evaporator. The apparatus was evacuated to 10^{-4} torr and the current was increased until the carbon rod became white hot. Carbon was evaporated until the deposit on the mica was of the required thickness. This was indicated by a light brown color on the exposed part of the filter paper. A drop of adhesive solution (glue tapes dissolved in amyl acetate solution) was placed on each grid and allowed to dry. The grids were placed on a wire mesh under the distilled water; with forceps mica carbon film uppermost was pushed, slowly into the water at an angle of 35° to 40°. Carbon film spread from mica and floated on the water. The wire mesh with the grids on it was lifted slowly so that the grids were covered with carbon films. The grids were then air-dried (Sommerville & Scheer, 1987).

Carbon-coated grids can be made hydrophilic by subjecting them to ionized gases. Carbon-coated grids were placed on a glass slide and kept in the glow discharge machine HDT-400 hydrophilic treatment device. The chamber was evacuated and the glow discharge was maintained for 100 seconds. The grids were used within 30 minutes of the procedure.

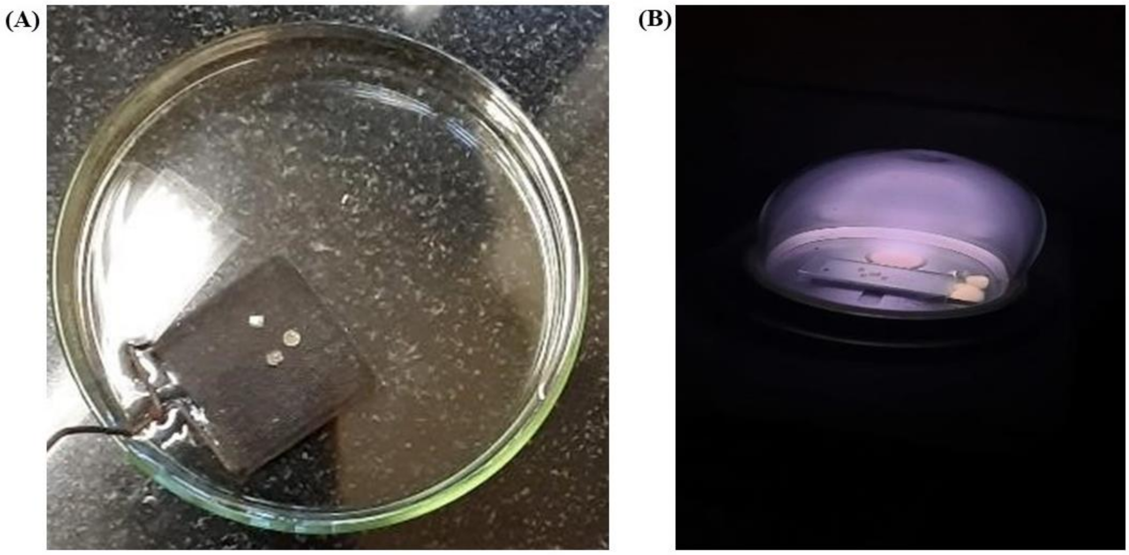


Figure 3.3: Grid preparation steps before negative staining

3.8.1.2 Negative Staining

Negative staining relies on the principle of staining the background rather than the sample which gives better visual information about the sample. A 5µl of the sample was applied to a carbon-coated copper grid and negatively stained with 2% (w/v) uranyl acetate for 30 s and excess liquid was blotted off (Czajkowski et al., 2015). After air-drying the grids were visualized with FEI Tecnai 12 BioTwin Transmission Electron Microscope (TEM) operating at 80-120 kV using 100 µm C2 aperture and 50 µm objective aperture.

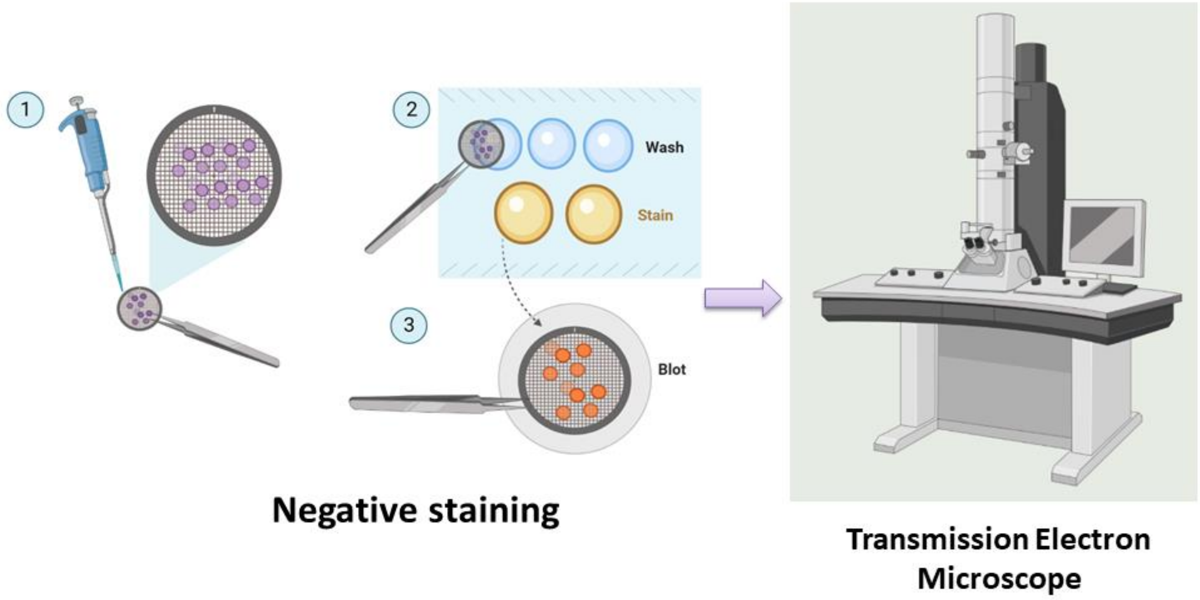


Figure 3.4: Negative staining method

3.8.2 Scanning Electron Microscopy

3.8.2.1 Sample Preparation for Scanning Electron Microscope (SEM)

The Scanning Electron Microscope (SEM) is used largely to investigate the surface topology of objects, in contrast to the Transmission Electron Microscope (TEM), which is frequently used to examine the inside structural complexity of cells. The goal of specimen preparation for SEM is to produce an object devoid of fluid for observation under a vacuum yet with the same shape and surface characteristics as the living condition.

Therefore, in brief, the specimens that will be studied using a scanning electron microscope (SEM) are first fixed, then gradually dehydrated using an ascending ethanol or acetone series, and finally dried using a technique called critical point drying (CPD). Following the process, cells were fixed for 4 hours at room temperature in 2.5% buffered glutaraldehyde. After fixation, the sample was gradually dehydrated with a series of ethanol gradients by letting cells sit in ethanol concentrations of 30%, 50%, 70%, 90%, and 100% for 10 minutes.

The sample was subsequently dried using a critical point dryer or a chemical method. The solvent portion of the cells was replaced with a transitional fluid like carbon dioxide (CO_2) during the CPD drying process. The dried sample was then fixed using conductive adhesive tape on the specimen stub. After that, a sputter coater device was used to apply a thin conducting coating of Au on the specimen to reduce damage.

3.8.2.2 Imaging by Scanning Electron Microscope (SEM)

Images of the sample were taken by FEI Quanta 200 SEM (FEI Netherlands), using a secondary electron (SE) or ET detector. Images were taken at various magnifications at 5-15 kV range with a spot size of 3.5.

3.9 Biological Characterization of Phage

3.9.1 Host range of bacteriophage isolates

Host range assay was performed to evaluate the lytic spectrum of the isolated bacteriophages to the susceptible bacterial strains. It was determined by spotting 10µl of phage lysate (10^{10} PFU/ml) on each agar plate with different bacterial strains. The plates were incubated at 37°C overnight and examined for clear zones that indicate the susceptibility of the bacteria.

3.9.2 Efficiency of plating assay (EOP)

EOP is a measure of the effectiveness of certain cells that can sustain viral replication while being infected by viruses. The plaque count to virions in the inoculum ratio essentially determines this. By comparing the titer on a test strain with the titer on the isolation strain, it was possible to estimate the virulence of the phage. An EOP assay of the obtained phage was then conducted by the double-layer agar method to quantify the lytic activity of the phage.

EOP = Titer test/Titer isolation

3.9.3 Thermal Stability

The thermal stability experiment was carried out to assess the capacity of bacteriophage thermal stability. A thermal stability test of the phage was carried out at pH 7.0 in order to investigate the heat-resistant capability of the phage. The phage was incubated at different temperatures (4°C, 25°C, 37°C, 50°C and 70°C respectively) for 1 hour. After the incubation, the phage titer was determined by the soft-agar overlay method (Yuan et al., 2015).

3.9.4 pH Stability

The pH stability of the phage was examined by pre-incubating the phage suspensions at a range of pH (pH 3–13) at 37°C for 1 hour (Jamal et al. 2015). Then the surviving phages were immediately counted by the double-layer agar method. The survival at any pH was expressed as a percentage of maximum survival.

3.9.5 UV Stability

In order to assess the stability of the phage under UV light, phage suspension viability was checked after exposure to UV light at various time points of 5, 10, 15, and 20 sec, as described elsewhere (Dutta M & Ghosh AN; 2007). After that, the soft agar overlay technique was used to test the bacteriophage survival rate. Each assay was performed in triplicate.

3.9.6 Adsorption assay & One-step growth curves of bacteriophage isolates

The adsorption assay was conducted as described previously with some modifications (Lu et al., 2003). Briefly, the bacterial culture (1 ml) was infected with a phage suspension at an MOI of 0.1 and incubated for 20 minutes at 37°C. After infection, aliquots were taken at different time intervals (0, 5, 10, 15, and 20 minutes) and immediately diluted in Tris-$MgCl_2$ buffer, followed by centrifugation at 8,000 rpm for 5 minutes. Then the supernatant un-adsorbed phage titers were estimated by the double-layer agar method.

In addition, a one-step growth curve of bacteriophage was performed to assess the growth kinetics of the phage. It was performed according to the method described previously (Bloch et al., 2013). At an interval of 10 minutes, the exponentially growing bacterial culture (20 ml) was harvested and the pellet was resuspended in around 1 ml Luria broth followed by the addition of phage. The mixture was incubated for adsorption at 37°C for 5 minutes. The mixture was collected and immediately plated by the double-layer agar method. Burst size was calculated as the ratio of the final count of liberated phage particles to the initial count of infected bacterial cells during the latent period.

3.9.7 Infection

Bacterial cell

At a MOI of 10, the isolated phage was introduced into exponentially developing *Salmonella* Typhi cells. At a temperature of 37°C, the phages were allowed to adsorb to the bacteria. The mixture was treated with an equivalent amount of a fixative containing 2.5% glutaraldehyde. Samples were prepared after adsorption had been allowed for 5 to 15 minutes at intervals of 1 minute. For each sample, negatively stained grids were prepared, which were then examined under an electron microscope. Ultra-thin sectioning of the samples after the 10 minutes of incubation was carried out.

Bacterial OMV

Salmonella Typhi OMVs were infected with phage at 37°C. Using electron microscopy, the interactions between phages and OMVs were studied by negative staining. 1µl of *S.* Typhi OMVs at a concentration of 8.4 mg/ml (protein content) was added to 100µl of phage at a titer of 10^{10} PFU/ml in phosphate-buffered saline and allowed to incubate for 10 minutes and 1 hour at room temperature.

3.10 Genetic characterization of bacteriophage isolates

3.10.1 Extraction of bacteriophage nucleic acid

DNA was extracted from the high-titer stocks of phage using a phage DNA isolation kit (Norgen Biotek Corp., Canada) according to the manufacturer's instructions. Briefly, 1 ml of highly concentrated phage (1×10^{10} pfu/ml) lysate was transferred into a microcentrifuge tube. Then, 10µl of RNase-free DNase I was added and it was incubated at room temperature for 15 minutes. After that, 500µl of lysis buffer B was added and it was subjected to vigorous vortex for 10 seconds followed by incubation at 65°C for 15 minutes. Next, 320µl of isopropanol was added to the lysate and it was again subjected to vortex for 10 seconds. Then, 650µl of phage lysate was then transferred to a spin column attached to collection tubes and it was centrifuged at 8000 rpm for 1 minute. The flowthrough was discarded and it was reassembled. This process was repeated for the remaining phage lysate. After that, 400µl of wash solution A was added to the spin column and it was centrifuged at 8000 rpm for 1 minute. Then the flowthrough was discarded and the spin column was reassembled with a collection tube. This step was performed three times. Thereafter, the column was again centrifuged at 10000 rpm for 2 minutes to dry the resin the collection tube was discarded and the spin column was attached to an elution tube. Finally, 75µl of elution buffer B was added to the spin column and it was centrifuged at 8000 rpm for 1 minute. The concentration of DNA was determined using a UV-vis spectrophotometer at 260 and 280 nm. The DNA sample in the elution tube was stored in the refrigerator at -20°C.

3.10.2 Nuclease digestion of bacteriophage DNA

The purity and concentration of the DNA were determined using a spectrophotometer. Then the DNA was digested by seven different restriction enzymes namely: EcoRI, EcoRV, MluI, BglII, PstI, BamHI, and HindIII. Incubation temperatures and reaction buffers were used as directed by the manufacturer. Reactions were stopped by heating the samples at 65°C for 15 minutes followed by rapid cooling. Restriction fragments were separated by electrophoresis on a 1% agarose gel.

Table 3.7: List of Restriction Endonucleases used in the present work

Sl. No.	Enzymes	Sequence
1.	EcoRI	G ↓AATTC
2.	EcoRV	G↓ATATC
3.	MluI	A↓CGCGT
4.	BglII	A↓GATCT
5.	PstI	CTGCA↓G
6.	BamHI	G↓GATCC
7.	HindIII	A↓AGCTT

3.10.3 Agarose gel electrophoresis

Agarose solution (1%) was prepared in the electrophoresis buffer (1X TAE) and poured at room temperature in a horizontal slab gel apparatus, 16×12 cm in size and 1.5 mm in thickness. DNA samples were mixed with glycerol-containing loading buffer and layered into the wells in submerged conditions. The best resolution was obtained by electrophoresis at low voltage (1.5 V/cm). After the run, the gels were stained with ethidium bromide (0.5µg/ml), and the DNA bands were visualized by placing the gel over a UV transilluminator.

3.10.4 Genome Sequencing

Purified genomic DNA of phage STWB21 was then sequenced by using an Illumina Platform at Xcelris (Ahmedabad, India). Reads were trimmed with Bioedit (version 7.1) and assembled with CLC Genomics Workbench v.6.0.5 with the reads map back option.

Qualitative and quantitative analysis of DNA

The quality of genomic DNA was checked on 0.8% agarose gel (loaded 3µl) for the single intact band. The gel was run at 110 V for 30 minutes. 1 µl of each sample was used for determining concentration using a Qubit® 2.0 Fluorometer.

Preparation of library:

The paired-end sequencing library was prepared from sample DNA using the *Truseq Nano DNA Library prep kit*. The library preparation process was initiated with 200 ng/DNA. The gDNA was mechanically sheared into smaller fragments by covaries followed by the continuous step of end-repair where an ‘A’ is added to the 3’ ends making the DNA fragments ready for adapter ligation. Following this step, platform-specific adapters are ligated to both ends of the DNA fragments. These adapters contain sequences essential for binding dual-barcoded libraries to a flow cell for sequencing, allowing for PCR amplification of adapter-ligated fragments, and binding standard Illumina sequencing primers. To ensure maximum yields from limited amounts of starting material, a high-fidelity amplification step was performed using HiFi PCR Master Mix.

Quantity and quality check (QC) of the library on Bioanalyzer:

The amplified library was analyzed in Bioanalyzer 2100 (Agilent Technologies) using a high-sensitivity (HS) DNA chip as per the manufacturer's instructions.

Cluster Generation and Sequencing:

After obtaining the Qubit concentration for the library and the mean peak size from the Bioanalyser profile, the library will be loaded onto the Illumina platform for cluster generation and sequencing. Paired-end sequencing allows the template fragments to be sequenced in both the forward and reverse directions. The library molecules will bind to complementary adapter oligos on paired-end flow cells. The adapters are designed to allow selective cleavage of the forward strands after re-synthesis of the reverse strand during sequencing. The copied reverse strand is then used to sequence from the opposite end of the fragment.

3.10.5 Genomic characterization, visualization, and comparison

The putative ORFs were predicted and annotated by GeneMark.hmm version 3.25 and further confirmed by the Rapid Annotation Subsystem Technology toolkit (RAST) (McNair et al., 2018) server, and tRNAs carried by the phage genomes were detected using the protein ARAGORN (Laslett and Canback, 2004) and tRNAscan-SE (Lowe and Eddy, 1997). Based on the sequence similarity between each predicted protein and the non-redundant protein sequences database (NCBI-nr) of the National Centre for Biotechnology Information (NCBI), BLASTp analysis (90% coverage and 95% identity) was performed to identify putative functions for each predicted protein. Visualization of genome alignments of phages with closely related Siphoviridae *Salmonella* phages was performed using the Easyfig application. The CG view software was used to generate a circular visualization of gene distribution.

3.10.6 Phylogenetic analysis

To construct the phylogenetic tree, amino acid sequences of the terminase large subunits and major capsid protein were selected and obtained from the NCBI Genbank database. Additionally, the terminase large subunits are usually considered genetic markers (Lee et al., 2021) for the order Caudovirales and the major capsid protein is a highly conserved protein (Oh et al., 2014). The two phylogenetic trees were constructed with the default pipeline “ONE CLICK” at Phylogeny.fr (Dereeper et al., 2008).

3.11 Phage proteomics and structure determination

3.11.1 Polyacrylamide gel electrophoresis

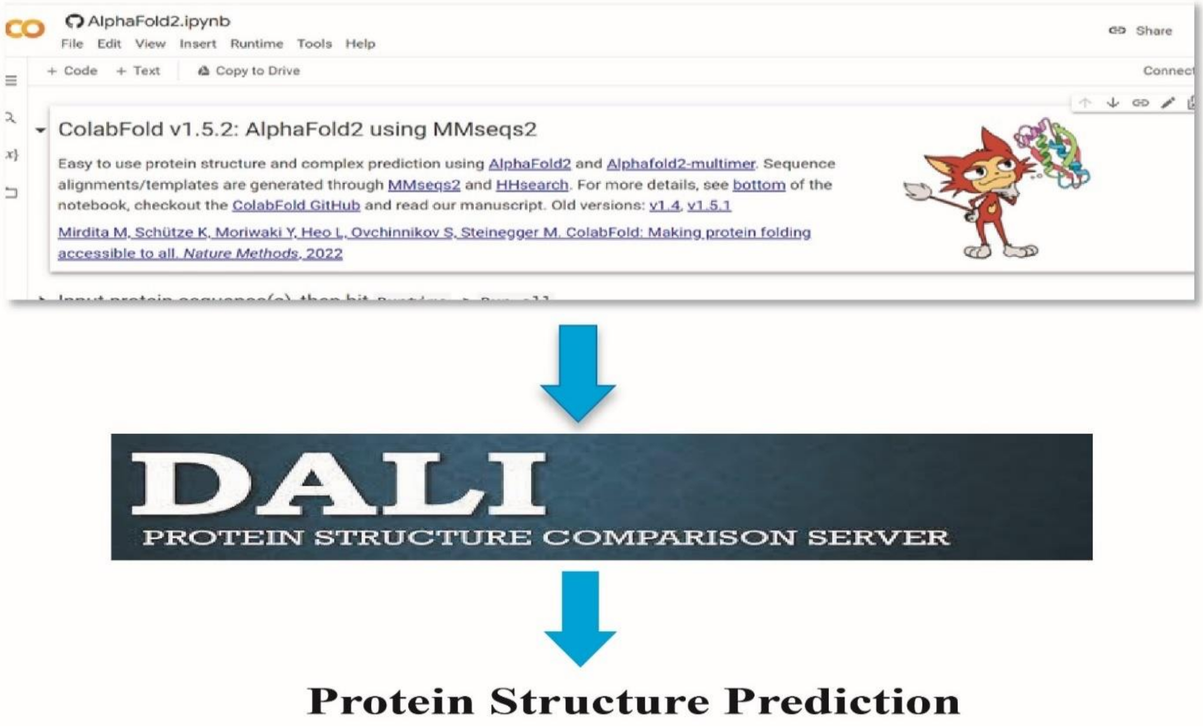
To analyze the phage protein profile, the phage solution was boiled for 5 minutes and the structural proteins were extracted. The denatured proteins were separated by using 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) with Mini-PROTEAN TGX Precast Gels (Bio-Rad, United States). Polyacrylamide gel containing 5% stacking gel and 12.5% resolving gel was prepared from a stock solution of acrylamide/N, N-bisacrylamide (29:0.2). The gel was 15×12 cm in size and 1 mm thick and the stacking gel was 2-3 cm in height. Purified phage (10^{11} pfu/ml) was dissociated in a 5X protein loading buffer. The sample was heated at 90 –100°C for 5 minutes, cooled, and loaded in 12.5% SDS-PAGE for 2.5 hours at 100V. For visualization of the protein after electrophoresis, the gel was stained with Coomassie Brilliant Blue R (0.1% w/v) for 10 minutes. Destaining was performed by immersing the gel in a destaining solution for 1 h. Medium proteins were visualized by staining with Coomassie Brilliant Blue.

3.11.2 Proteomic analysis by LC-ESI-MS/MS

Protein extraction was prepared as described in the SDS-PAGE assay. A total of 100µg of protein was extracted and lyophilized. The 20µg lyophilized protein was re-dissolved in 1.5 ml ice-cold 1 mM HCl (13 ng/µl trypsin prepared) and 100µl aliquots stored at −20°C for further protein analysis. Protein in-solution digestion was performed at C-CAMP (NCBS, India) according to the previously described method (Wiese et al., 2007). Proteomic analysis was performed on 1,200, 1D nano-LC (Agilent Technologies, San Diego) that was coupled to Nanomate Triversa (Advion) and LTQ – Orbitrap Discovery (Thermo Fisher Scientific, United States). Nano LC-ESI-MS/MS spectra were searched using a PEAKS engine against phage genomes. For protein identification, the following parameters were used. Peptide mass Error tolerance = 10.0 ppm, fragment mass error tolerance = 0.6 Da, enzyme = trypsin, missed cleavage = 2, fixed modification: carbamidomethyl (C), variable modifications: oxidation (M), deamidation (NQ).

3.11.3 Protein structure prediction

Recently, AlphaFold2 has been made accessible via notebooks with a user-friendly interface. In this work, the ColabFold notebook was utilized, for quick multiple sequence alignment generation MMseqs2 and AlphaFold2 for structure prediction (Mirdita M et al., 2021) were used. Additionally, the modeling of homo- or hetero-complexes is possible with this ColabFold. Then additional predictions were carried out using each domain sequence as input after identifying the various domains. The Dali server was used to find the PDB's closest structural homologs using the final projected domain structures (Holm L; 2020). Finally, using ChimeraX, the Views of the domain 3D structures were generated (Pettersen EF, 2021).



ColabFold v1.5.2: AlphaFold2 using MMseqs2

Easy to use protein structure and complex prediction using AlphaFold2 and Alphafold2-multimer. Sequence alignments/templates are generated through MMseqs2 and HHsearch. For more details, see bottom of the notebook, checkout the ColabFold GitHub and read our manuscript. Old versions: v1.4, v1.5.1

Mirdita M, Schütze K, Moriwaki Y, Heo L, Ovchinnikov S, Steinegger M. ColabFold: Making protein folding accessible to all. *Nature Methods*, 2022

Figure 3.5: Structural homolog identification procedure

3.12 Bacteriophage application *in vitro*

3.12.1 Biofilm formation assay and effect of phage against biofilm

On glass coverslip

The biofilm degradation activity of newly isolated phage on *S.* Typhi was accessed following a previously discussed method with some modifications (Cerca et al., 2006; Nickerson et al., 2017; Mallick et al., 2021). A 10µl of an overnight culture of *S.* Typhi was dispensed into a Petri dish containing glass coverslips (22 mm × 22 mm) and incubated at 37°C for 24 h to form the biofilm. Two sets of Petri dishes were incubated for biofilm formation. One set was treated with buffer after 24 hours of incubation and the other set was treated with phage STWB21 overnight at 37°C.

3.12.2 Application of bacteriophage in food items for controlling *Salmonella* Typhi

On onion & tomato

The fresh onion bulb and tomato were chosen due to their association with foodborne illness outbreaks and variability in their surface structure. On the day of the experiment, fresh onion and tomato were purchased. Before the experiment, the onion and tomato were washed thoroughly for 5 minutes under running tap water (room temperature) followed by 70% alcohol to remove any soil or organic matter and any microflora present. After that, the cleaned onion bulb was placed in the center of a sterile Petri dish. Using a sterile scalpel, the outer membrane was removed. Next, the remaining scales of the onion and the tomato were cut into 3 × 3 cm pieces. Further, these onion and tomato pieces were inoculated with 200μl of *S.* Typhi at 10^8 CFU/ml and incubated at 37°C. After overnight incubation, the onion and tomato were treated with 20μl of purified phage solution (1.5×10^{10} PFU/ml) for 4 hours.

In milk

To study the removal and degradation of biofilm in milk by newly isolated phage, we used pasteurized milk as the culture medium for the *S.* Typhi biofilm. We purchased commercial sterile ultra-heat-temperature (UHT) treated milk from local retail. A logarithmic-growth-phase culture of *S.* Typhi at a final concentration of 10^6 CFU/ml was added to 1 ml of milk in two different sets of Petri dishes containing glass coverslips (22 mm × 22 mm) to form the biofilm when incubated at 37°C for 24 hours. After overnight incubation, one set of Petri dishes was treated with 40μl of purified phage solution (1.5×10^{10} PFU/ml) for 4 hours.

3.12.3 Microtiter plate-based assay of biofilm

To evaluate the anti-biofilm efficacy of phage, biofilm was developed in 96-well plates in accordance with the previously described method with some modifications (Cerca et al., 2006). Briefly, the *S.* Typhi strain was grown overnight at 37°C and was diluted to 1:100 in a fresh LB medium. Then 200μl of the diluted culture was added to 96-well plates and was placed in an incubator at 37°C for 24 hours without agitation. After incubation, the supernatant was removed from the well plate. To examine the effect of phage on biofilm, phage stock was added into the biofilm at different concentrations (MOI 0.1 and 0.01) and incubated at 37°C for 4 and 24 hours, respectively. Thereafter, the wells were rinsed with 1X PBS thrice and allowed to air-dry. The air-dried plate was then stained with crystal violet (0.1%, w/v) for 30 minutes. The optical densities of the biofilm were measured on the microplate reader at the absorbance of 595 nm (iMark Microplate Reader S/N 21673).

3.13 Bacteriophage application *in vivo*

3.13.1 Selection of Animals

BALB/c mice were collected from the NICED animal house facility to investigate the effect of phage STWB21 on *Salmonella* Typhi. Six-week-old, disease-free, healthy, active female and male BALB/c mice were chosen for the experimental purpose with animals weighing in the range of 22 ± 2 gms. Mice were fed sterile food and water *ad libitum*. Animals were kept for 10 days before experimentation to acclimatize to laboratory conditions. The animals were housed and the entire experiment was carried out in the animal house of ICMR-NICED.

3.13.2 Ethical statement

The animal experiment protocol was reviewed and conducted following the standard operating procedure as outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India. The Institutional Animal Ethical Committee (IAEC) of NICED approved the animal experimental protocol with Registration No. 68/GO/Rebi/S/1999/ CPCSEA Valid 17/7/2024, Approval No. PRO/186/ - Nov2022-25.

3.13.3 Determination of LD_{50}

To determine the LD_{50} of *Salmonella* Typhi (Kol-551 strain), a total of thirty mice were distributed into five groups of six mice in each group and intraperitoneally injected with different *Salmonella* doses (from 1×10^4 to 3×10^7 colony-forming units/group). Six mice per group were kept in cages and challenged with the above-mentioned inoculum of the *Salmonella* strain. Mice were closely monitored and deaths of mice were noted during 7 days. The experiment was done in triplicates.

3.13.4 Efficacy of bacteriophage in challenged BALB/c mice

To compare the efficiency of prophylactic versus therapeutic application of phage on *S.* Typhi shedding, BALB/c mice were divided into five (5) groups and each group consisted of six (6) animals (Figure 3.5). The doses were fixed and prepared in PBS and administered. Mice from Group I received only PBS as a control, Group II animals were administered by intraperitoneal injection (i.p.) with *S.* Typhi (4.5×10^8 CFU) diluted in PBS. Group III animals were orally administered with only STWB21 phage (1.5×10^{10} PFU) to check any lethal effect of phage on mice. Group IV mice represent the prevention group and Group V mice represent the treatment group. All five groups were kept in hygienic conditions with a continuous supply of food and water for 14 days. A significant observation was made and results were recorded (Table 3.8).

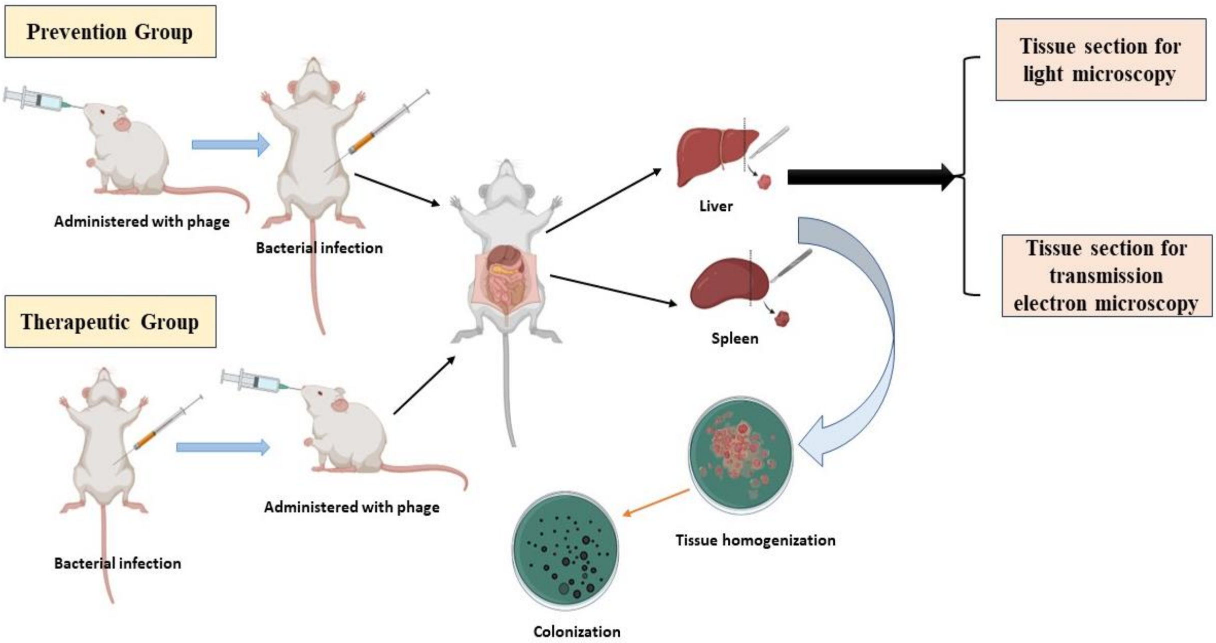


Figure 3.6: Graphical representation of the animal model experiment

Table 3.8: Group-wise distribution of mice with intraperitoneal administration of bacteria and oral administration of phage

Group	Administration with various inducing agents
Group I (Receiving no treatment)	Mice + PBS
Group II (Infected with bacteria)	Mice + *S.* Typhi bacteria
Group III (Receiving only phage)	Mice + STWB21
Group IV (Prevention group)	Mice + STWB21 phage before 1hour infection + *S.* Typhi
Group V (Treatment group)	Mice + *S.* Typhi + STWB21 phage after 1hour infection

3.13.5 Enumerations of *Salmonella* and phages from mice tissues

Mice tissues (liver, and spleen) were collected on day two post-infection when the most prominent gut inflammation occurred. Tissues were immediately weighed and homogenized using a bead-beating machine with 1.0 mm diameter silica beads (Biospec Products, Bartlesville, United States). *Salmonella* Typhi colony-forming unit (CFU)/gm tissues were determined by a serial dilution technique on the HEA agar.

3.13.6 Preparation and imaging of tissue sections by different microscopy

3.13.6.1 Ultramicrotomy

Tissue fixation

For transmission electron microscopy (TEM) analysis, tissues were cut into 1 mm x 1 mm pieces and were dissolved in the 900µl 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer with pH 7.0 for 2 days immediately after isolation of the organ. From the whole organ, a small piece was cut and washed in 0.1 M sodium cacodylate buffer for 10 minutes. The rest of the organ was kept in 0.1M sodium cacodylate buffer for further use (Glauert,1975).

Dehydration

The fixed tissues were then dehydrated in a graded series of ethanol for different time intervals as described below:

1. In 50% ethanol for 15 minutes
2. In 70% ethanol for 10 minutes × 2
3. In 90% ethanol for 15 minutes × 2
4. In 50% ethanol for 15 minutes × 3

Samples were constantly rotated in a rotator (Agar Scientific)

Intermediated infiltration

Ethanol is not miscible with epoxy resin (the embedding medium) therefore an intermediate infiltration is necessary. The dehydrated tissue cubes were transferred in propylene oxide and kept for 20 minutes. This was repeated thrice.

Infiltration

The tissue was embedded in agar100 resin. Then it was brought down to room temperature and 0.35g of BDMA (N-benzyl dimethyl amine) was mixed slowly avoiding any bubble formation. After that, the tissue was kept in 30% resin (a mixture of 1-part resin: and 2-part propylene oxide) overnight. The tissue was kept in 70% resin (2-part resin: 1 part propylene oxide) overnight. The next day it was centrifuged and kept in pure resin for 3 hours.

Embedding & Polymerization

It was then embedded in Agar 100 resin in “BEEM” capsules. A small piece of paper showing specimen numbers was put in each capsule. Capsules were kept at 60°C overnight
The capsules were incubated at 60°C overnight for polymerization.

Semithin sectioning

The face of the resin blocks was trimmed down to a small trapezium with the help of a razor blade. A glass knife was then prepared from 6.5 mm thick Belgium glass strips in an LKB Bromma 7800 knife maker, set properly to the ultramicrotome (LKB Nova). The knife speed was adjusted to 2 mm/sec and the knife angle was set at 5°.

Ultrathin Sectioning

The size of the block was reduced further to 1 mm. The blocks were then cut with a glass knife placed at an angle of 5°, a speed of 2mm per sec in an LKB ultramicrotome. The semi-thin section was observed under an optical microscope to check the location of the sample. Subsequent ultrathin sections were cut with a diamond knife. The sections colored gold to silver were then picked on a grid, and precleaned in chloroform. The ultrathin silver-colored sections had a size of 60-90nm. An uncoated grid was then submerged in the water in the trough and placed directly under the sections to be collected. Slowly, the grid was lifted parallel to the water surface to collect the sections. The sections floated on the small droplet of water on the grid. The other side of the grid was touched to the edge of the filter paper, permitting the water to soak into the filter paper (Bozzola & Russel, 1999).

Section Staining

The grid containing the section was stained with 2% uranyl acetate for 30 minutes. It was thoroughly washed with double distilled water and stained again with 0.2% lead citrate for 5 minutes. It was washed thoroughly with double distilled water and the liquid was removed by placing the grid on the filter paper.

3.13.6.2 Processing of mice tissue for histological studies

At indicated time points, mice were euthanized and intestinal organs were carefully removed and fixed with 10% buffered formalin and embedded in paraffin (Sarichai et al., 2020).

Tissue preparation

Fixation employs chemicals to cross-link proteins in a way that keeps the tissue's structure intact and protects against deterioration. Neutral Buffered Formalin is a popular option for this stage despite the availability of a number of specialized fixatives. The fixation stage is essential to the rest of the histologic staining process because it hardens the sample and facilitates sectioning by preserving the chemical composition of the tissue. Tissue fixed with 10% formalin buffer.

Dehydration

Further, it was fixed in alcohol gradients for dehydration (70%- 90%-100%) each for two times and 30 minutes. Dehydrating a sample is accomplished by adding ethanol. It removed the sample and further dehydrated the tissue to prepare it for future light microscopy. After the application of ethanol and after the completion of tissue dehydration, the ethanol is removed using xylene twice for 30 minutes each and then into chloroform once for 30 minutes.

Embedding

The embedding was done at liquid paraffin at 60°C through successive three changes each of 30 minutes duration. After trimming excess embedding material from the paraffin block, they were mounted for 3 µm sectioning in a microtome. The sections were then floated in cool water and were placed (mounted) on the adhesive (albumin: glycerol =50:50, v/v) coated glass slides. The slide was then dipped into the same temperature hot water bath maintained at 55°C for once. Then keep the slide in a hot air oven at 60°C for 1 hour de-paraffinization. Then the procedure down to the water was followed where slides were passed through xylene and alcohol gradients in reverse direction to the water.

Sectioning

The specimen is mounted on a microtome and sliced into slices during sectioning. For staining and placing on a microscope slide for evaluation, a thickness of 4-6 µm is ideal. Semithin sections (1µm) were cut with a glass knife. The sections were collected in a trough filled with water. The sections were collected on a slide.

H&E staining

As the name suggests, it involves applying two stains in sequence. A basic dye called hematoxylin stains acidic materials. The resultant color is purple/blue, and the structures that this dye targets are known as basophilic. DNA in cell nuclei, RNA in ribosomes, and the rough endoplasmic reticulum are examples of basophilic structures. Eosin is an acidic dye that is used as a counterstain following hematoxylin and targets fundamental structures. The resultant color is a pinkish-red tint, and eosinophilic structures are those that draw eosin. An illustration of an eosinophilic structure is the cytoplasm.

Sample visualization

The stained sections were observed under a JENOPTIK GRYPHAX digital microscope.

3.13.7 Statistical analyses

Data were expressed as mean ± standard deviation (SD). Three technical and three biological repeats were carried out, as needed. GraphPad Prism version 5 was used to carry out all the statistical analyses. Statistical significance was assessed using two-way ANOVA (*$P < 0.05$; **$P < 0.01$).

3.14 Phage cocktail preparation to control the *Salmonella spp*. in food samples

3.14.1 Phage cocktail preparation

Two bacteriophages were tested for stability and virulence against *Salmonella* strains using the overlay, host range, morphology, and EOP tests prior to the formulation of a phage cocktail for *in vitro* investigations. For the phage cocktail, we employed the newly isolated phage and a previously isolated *Salmonella* phage. The phage cocktail utilized in this study consisted of a 1:1 combination of both phages, with a final concentration of 3.7×10^{10} PFU/ml ($1. 5 \times 10^{10}$ PFU/ml of each phage). The lysates of phages were kept at 4°C. At the indicated concentrations, cephalosporin antibiotic was utilized. The chicken pieces were immediately weighed and homogenized using a bead-beating machine (Biospec Products, Bartlesville, United States) and 1.0 mm diameter silica beads. A serial dilution method on HEA agar was used to calculate the colony-forming unit (CFU)/gm tissues of *Salmonella* Typhi.

3.14.2 Phage cocktail assay in chicken meat

Chicken breasts that were purchased from a nearby store and sliced into pieces aseptically in the laboratory. Before inoculation, the pieces of chicken breast were exposed to UV light for 30 minutes in a safety cabinet (15 minutes on each side) to lower the number of studied bacteria. Using a sterile scalpel, chicken breasts were cut aseptically into small pieces 2 cm × 2 cm squares (about 1 g), and placed into four different Petri dishes. These chicken pieces were also given a 40 µl *S*. Typhi inoculation at 10^8 CFU/ml, followed by a 1-hour incubation at 37 °C. This was followed by adding 40 µl of each phage and a phage cocktail (10^{12} PFU/ml) and for the controls, the same volume of Tris-$MgCl_2$ buffer was used instead of the phage suspension. Then all samples were incubated for 6 hours at 37°C.

3.14.3 Assessment of biofilm biomass by crystal violet staining after treatment with phage lysates and phage cocktail

Biofilm cell biomass after phage infection was determined according to the protocols described by Cerca et al. with some modifications. For biofilm cell culture, *S.* Typhi and *S.* Enteritidis were grown at 37°C in a 96-well polystyrene plate with LB medium for 24 hours without agitation. After the incubation period, the liquid medium containing planktonic cells was removed and the biofilm was washed with 1 ml of distilled H_2O. In the next step, phage lysate or phage cocktail was added to each well, except for controls, to the final 10^{10} PFU/well titer. Antibiotic cephalosporin was added to the following concentration 12.5μg/ml. Then the plates were incubated at 37°C for 4 hours. After incubation with phage lysate, phage cocktail, or antibiotic solution, the liquid medium was removed, and surface-attached cells were treated with 1 ml of crystal violet solution (0.1%, w/v). Plates were incubated in the dark for 20 minutes at 25°C for air drying. After that, crystal violet was carefully removed and biofilms were washed twice with 1 ml of distilled H_2O. Then, biofilms were fixed by additional incubation at 37°C for 20 minutes. Afterward, crystal violet was dissolved by the addition of 200μl of DMSO, and the absorbance was measured in a plate reader at 595 nm (iMark Microplate Reader S/N 21673) in order to assess biofilm biomass.

Chapter 4

Isolation and Biological Characterization of a *Salmonella* phage STWB21

Chapter 4

4.1. Introduction

Salmonella is a gram-negative, facultatively anaerobic, rod-shaped, mobile bacterium that is most well-known for its capacity to spread a variety of illnesses in both humans and animals, including salmonellosis, typhoid fever, septicaemia, and fowl typhoid. More than 2600 *Salmonella enterica* serotypes have been discovered, accounting for around 99% of the pathogenic strains of *Salmonella*, based on changes in somatic and flagellar antigens (Khan & Rahmann, 2022). *Salmonella* may be obtained from food, drink, and environmental sources and is a member of the Enterobacteriaceae family. In the intestines of many different species, including dogs, livestock, and birds, it is quite common (Jajere, 2019). *Salmonella* may spread from animals to people via fecal-oral routes, food intake, water tainted with animal waste, and direct contact with *Salmonella*-infected animals.

Moreover, gram-negative bacteria, *Salmonella* produce outer membrane vesicles (OMVs), which are spherical structures 10 to 300 nm in diameter that pinch off from the cell surface and contain a variety of molecules found in the periplasm and outer membrane, as well as occasionally in the inner membrane and cytoplasm (Lee et. al., 2007, Choi et. al., 2011). Proteins, phospholipids, lipopolysaccharides (LPSs), lipoproteins, and DNA are among the many components of OMV. They have a broad range of functions in bacterial life. They serve as a secretion system that releases their substance into the surroundings. By using OMVs, a variety of bacterial species have been demonstrated to produce virulence factors and toxins in a host. (Horstman and Kuehn 2000; Wai et al. 2003). OMVs have been demonstrated to play a role in several processes over the decade, including the development of biofilm, destruction of rival microbes, secretion of bacterial proteins, the transmission of virulence and signaling elements, bacterial self-defense, cell-to-cell communication, bacterial pathogenicity, and infection. Recent research has revealed that OMVs can shield bacteria against several antibiotic classes, physical stress, chemical stress, and antimicrobial peptides (Kulkarni et al.,2014; Schaar et al., 2011).

Over the last few decades, improper use of antibiotics has resulted in the emergence and spread of microorganisms that are resistant to antibiotics, making drugs useless for curing diseases.

Additionally, by means of horizontal gene transfer pathways, resistant determinants can be passed to other pathogens, fostering resistance in environmental pathogens (Agyare et al., 2019). MDR *Salmonella* bacteria have significant rates of morbidity and mortality due to their resistance to ampicillin, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, and tetracycline (Crump JA et al., 2011; Sjölund-Karlsson M et al., 2011 Azmatullah et al., 2015, Shrestha et al., 2016, Hendriksen et al., 2011, Samajpati S et al., 2021). MDR *Salmonella* infections make conventional chemotherapeutic therapies even more challenging. One strategy that has gained popularity recently for attaining this aim is bacteriophage therapy.

Bacteriophages are common in the microbiome and important components of many bacterial ecosystems. Bacteriophages are specific to the host in which they proliferate. The contact of a phage with host surface receptors to begin infection of the bacterial host is a crucial stage in the life cycle of a phage (Feiner et al., 2015). Host-specific bacteriophages have been promoted as a flexible and affordable method of controlling zoonotic bacteria (Sulakvelidze & Barrow, 2005). Phages that infect Gram-negative bacteria usually bind the outer membrane or one of its constituents to identify their host. It is obvious that these blebs might be crucial in the interaction between bacteria and phage because OMVs are made up of parts of the Gram-negative outer membrane. In contrast to antibiotics, phages provide unique advantages. They prevent the dysbiosis of commensal gut flora (dysbiosis) that is frequently brought on by broad-spectrum antibiotics since they only reproduce on the targeted group of bacteria. Additionally, they automatically self-limit their replication to only occur till the targeted bacteria are present.

The use of *Salmonella* phages as biocontrol agents has lately gained popularity. Because the *Salmonella* genus is so diverse, it is a massive task to study lytic *Salmonella* phages that target distinct serovars. Several studies have shown the isolation and effective use of phages for *Salmonella* control (Tang et al., 2019; Abhisingha et al., 2020; Li et al., 2020; Phothaworn et al., 2020). *Salmonella* serotyping is critical for illness prevention and control. *Salmonella* serovars differ in their host ranges and pathogenicity (Fierer and Guiney, 2001). Although *Salmonella* Enteritidis and *Salmonella* Typhimurium are among the most frequent serotypes, new (previously uncommon) serovars have developed in recent years. Lin et al. (2020) detected 156 *Salmonella* isolates, with *S.* Albany (41.7%), *S.* Schwarzengrund (20.5%), *S.* Kentucky (12.8%), and *S.* Tennessee (5.1%) being the most often isolated serovars. S. Newport (56.8%) was the most prevalent serovar in samples taken from 10 irrigation ponds in produce farms over a 2-year period (Li et al., 2014); moreover, *S.* Corvallis is resistant to a wide variety of antibiotics (Ma et al., 2020). A large number of *Salmonella* phages have been reported to have *S.* Enteritidis or *S.* Typhimurium as hosts (Majtanova et al., 2011; Petsong et al., 2019). Only a few phages have been demonstrated to lyse minor *Salmonella* serovars. The main limitation of utilizing phages as a biological control for *Salmonella* is their limited host range since most isolated phages are exclusive to *S.* Enteritidis or *S.* Typhimurium. Although genetic engineering might be used to broaden the host range, it would necessitate sophisticated technologies (Lin et al., 2012; Kilcher and Loessner, 2019). As a result, new *Salmonella* phages targeted on diverse serovars with strong lytic capacity are still required.

Furthermore, it has been proposed that phages isolated from one country may not have the ability to lyse bacteria in other regions due to their defense mechanisms and the high diversity of *Salmonella* (Hagens and Loessner, 2009); thus, continuous provision of new phages that focus on different serovars and possess a high lytic capacity is required for practical application.

4.2 Chapter aims:

The major aims of this chapter were to:

i. Isolate and purify novel *Salmonella* phage from lake water.
ii. Biological characterization of the phage isolates to determine their potential for use as biocontrol agents.
iii. Visualization of the interaction of phage STWB21 and bacterial outer membrane vesicle by TEM.
iv. Visualization of phage and host bacteria interaction by TEM and SEM.

4.3. Results

4.3.1 Biological Study of *S.* Typhi bacteria

4.3.1.1 Morphology study of *Salmonella* bacteria

Salmonella Typhi is a gram-negative, rod-shaped, flagellated bacterium. The transmission electron micrograph of *S.* Typhi is shown in Figure 4.1A. The electron micrograph containing the ultrathin section of *S.* Typhi is shown in Figure 4.1B. The image showed the intact double membrane around the periphery of the bacterial cell. In Figure 4.1C, the *Salmonella* Typhi bacterium was imaged right at the point where it was undergoing the process of cell division, resulting in the formation of two separate organisms.

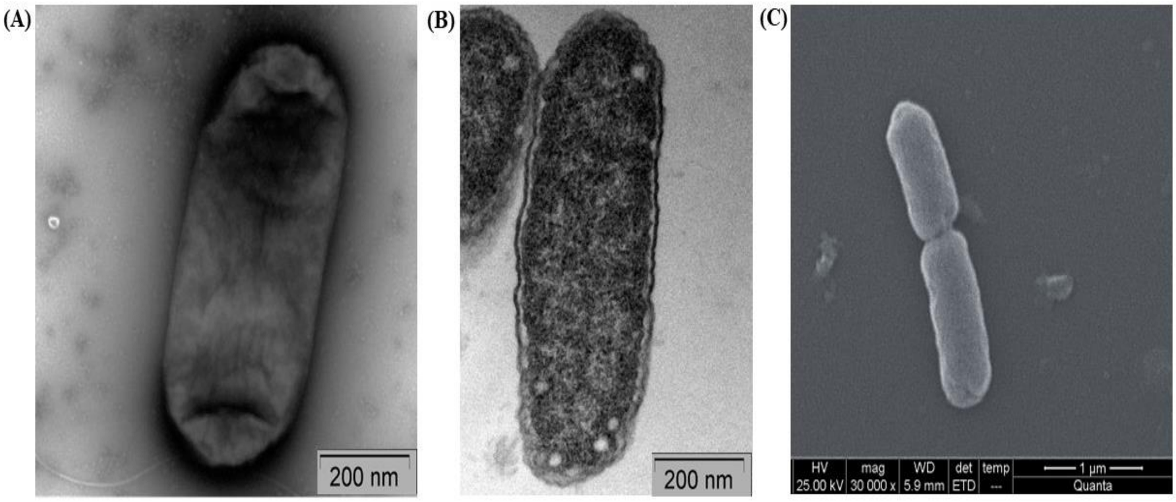


Figure 4.1 Electron micrographs of *S.* Typhi. (A) Negative staining of *Salmonella* Typhi, (B) Ultrathin section image of *Salmonella* Typhi. The double membrane of gram-negative bacteria is clearly visible. (C) Cell division of *Salmonella* Typhi visualized under scanning electron microscope.

4.3.1.2 Bacterial Growth Curve Determination

Three separate phases, the lag phase (1-3 hours), the log or exponential phase (4-8 hours), and the stationary phase (8–10 hours) were seen in the growth curve of *S.* Typhi, which was comparable to the growth curve seen in most bacterial populations. The bacterial sample was collected every 1 hour up to 14 hours. The OD_{600} nm values at each time point were measured.

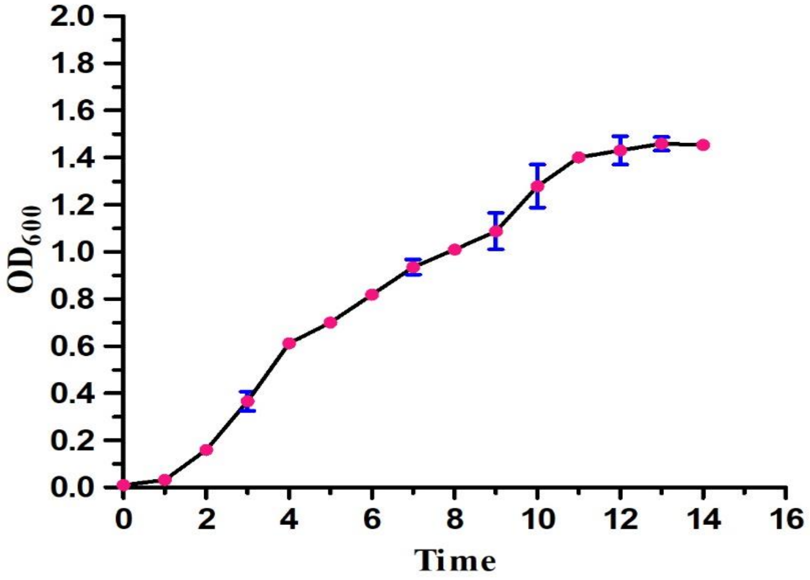


Figure 4.2 Growth curve of *S.* Typhi bacteria.

4.3.1.3 Bacterial Colony Forming Unit (CFU)

Colony-forming units are known as CFUs. The number of viable bacteria in a sample is estimated using this unit. It is necessary to culture the microorganisms before counting by CFUs, or colony-forming units.

CFU/ml = (Number of colonies × dilution factor) / volume of culture plate.

$= (450\times 10^5)/0.1 \quad = 4.5\times10^8$ CFU/ml

As shown in Figure 4.3, the CFU of *S.* Typhi bacteria is 4.5×10^8 CFU/ml.

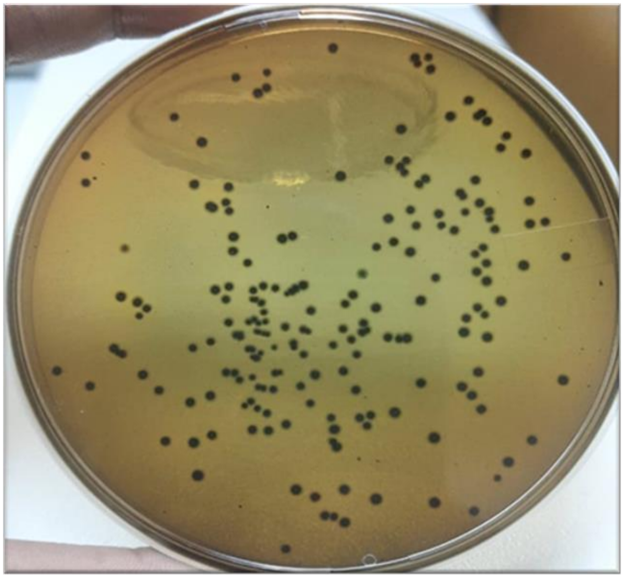


Figure 4.3 *S.* Typhi bacterial colony.

4.3.2 Biological study of bacteriophage STWB21

4.3.2.1 Spot assay to determine the presence of bacteriophage

The phage was isolated from a lake water sample in the outskirts of the Kolkata area. According to revised phage naming guidelines, the recently discovered bacteriophage was given the name STWB21 (Adriaenssens and Rodney Brister 2017). After isolation, ultracentrifugation and sucrose step gradient centrifugation were done to prepare the concentrated and purified phage stock. The purified phage sample was then spotted on an agar plate at different concentrations containing a lawn culture of *Salmonella* Typhi. After overnight incubation, the clear zones of lysis were seen in Figure 4.4. That the isolated bacteriophage could infect and kill the *S.* Typhi strain at different concentrations was further supported by this observation.

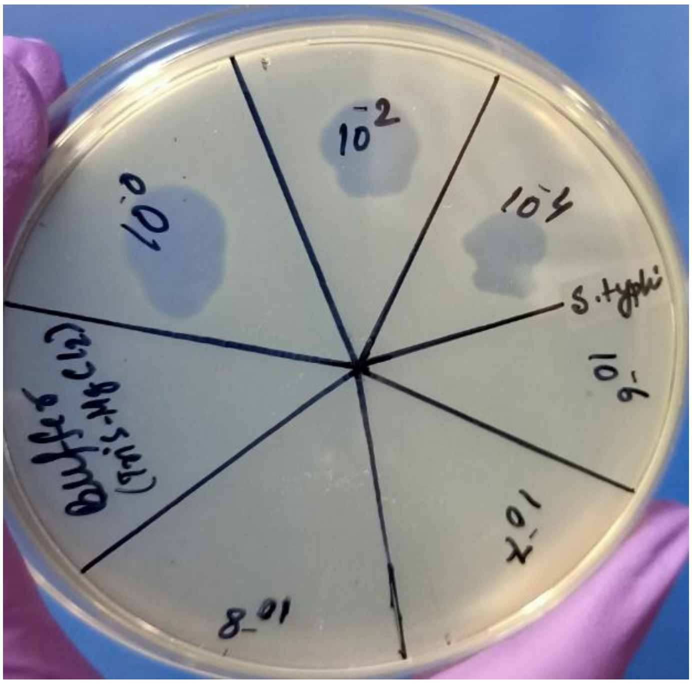


Figure 4.4. Spot assay of bacteriophage at different concentrations. 10 µl of different phage dilutions in Tris-MgCl$_2$ buffer (only Tris-MgCl$_2$ buffer, undiluted 10^{-0}, 10^{-2}, 10^{-4}, 10^{-6}, 10^{-7}, and 10^{-8}) were spotted on the *S.* Typhi lawn, and clear zones were observed in undiluted 10^{-0}, 10^{-2}, 10^{-4} at 18 h after the incubation at 37°C.

4.3.2.2 Plaque Assay

STWB21 could infect and lyse its host in a short time. STWB21 showed clear and transparent plaques on *S.* Typhi bacterial lawn which were measured around 1 mm diameter after overnight incubation. The phage-infected agar plate showing plaque morphology is represented below in Figure 4.5.

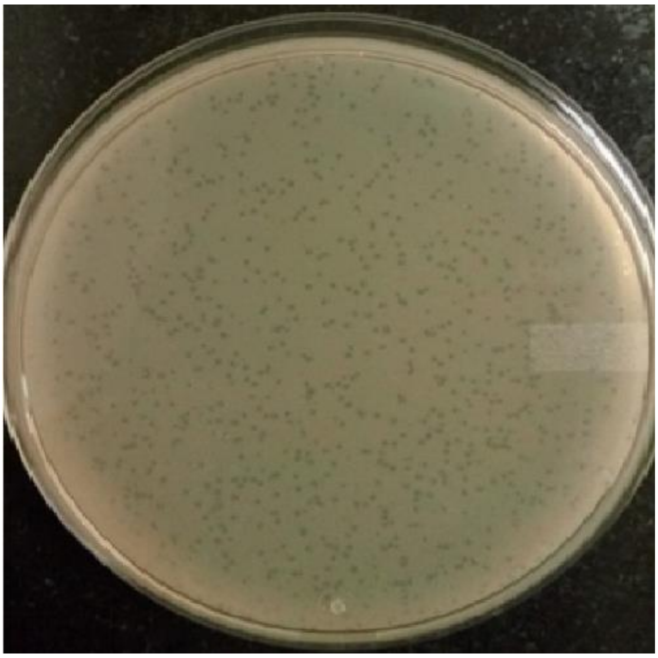


Figure 4.5. Plaque morphology of phage STWB21.

4.3.2.3 Phage morphology

Transmission Electron Microscopic study of phage STWB21 revealed a structure comprising an icosahedral head with an estimated diameter of 65 ± 3 nm (n = 20) and a long flexible, non-contractile tail of 113 ± 6 nm in length (n = 20) approximately (Figure 4.6). Phage STWB21 was subsequently identified as a member of the *Siphoviridae* family within the Caudovirales order due to its morphological characteristics and ICTV criteria (Fauquet and Fargette, 2005).

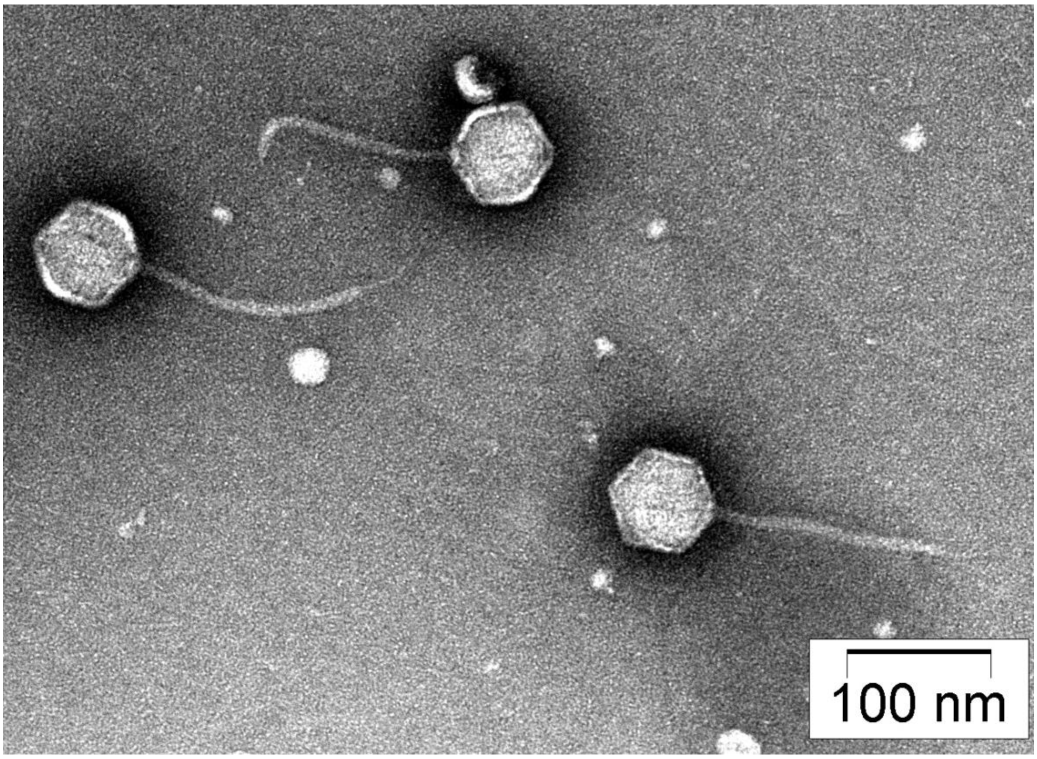


Figure 4.6. Transmission electron microscopic morphological study of phage STWB21.

4.3.2.4 Host Range Determination

The host spectrum of phage STWB21 exhibited specific lytic activity against the prevalent typhoidal and non-typhoidal *Salmonella* spp., *Sh. flexneri 2a*, *Sh. flexneri 3a* and ETEC (Table 3.1). Phage STWB21 was found to be a novel polyvalent phage with high lytic activity against both typhoidal and non-typhoidal *Salmonella*. However, the phage did not show infectivity against some other species: *Sh. flexneri6*, *Sh. boydii* and *V. Cholerae 01* which were used in this study. During infection of the prevalent *Salmonella* serovars, phage STWB21 formed clear plaques against *S.* Typhi, *S.* Paratyphi, *S.* Typhimurium, and *S.* Enteritidis. The higher EOP values were obtained for the phage-sensitive *Salmonella* strains compared to the *Shigella spp*. and *ETEC* strains.

Table 4.1: Host range analysis of phage STWB21 on different strains.

Strains	**No. of strains used**	**No. of strains susceptible**
1. *S.* Typhi	**4**	**4**
2. *S.* Paratyphi	**3**	**3**
3. *S.* Enteritidis	**1**	**1**
4. *S.* Typhimurium	**1**	**1**
5. ETEC	**3**	**2**
6. Sh. flexneri 2a	**1**	**1**
7. Sh. flexneri 3a	**1**	**1**
8. Sh. flexneri 6	**1**	**0**
9. Sh. boydii	**1**	**0**
10. V. cholerae O1	**1**	**0**

4.3.2.5 Efficiency of Plating

To determine the ability of phage to infect different types of bacterial cells, the efficiency of the plating (EOP) experiment was carried out (Table 4.2). STWB21 provided the highest EOP value for *S.* Typhi Kol 551. When compared to the *Shigella* strains, the EOP values for additional typhoidal *Salmonella* strains such as *Salmonella* Paratyphi, *Salmonella* Typhimurium, and *Salmonella* Enteritidis strains, were similarly on the higher side. Furthermore, the results of this investigation also showed that the phage STWB21 preferred *Salmonella* Typhi (Kol 551) as its host strain.

Table 4.2: EOP value of phage STWB21 on different strains.
*** Strains were used in this study.**

Strains	**Strain name**	**E. O. P**
***1. S.* Typhi**	(KOL 551) *	1
***2. S.* Typhi**	(KOL 553)	0.07
***3. S.* Typhi**	(C6953)	0.33
***4. S.* Typhi**	(K432)	0.06
***5. S.* Paratyphi**	(KOL 534) *	0.51
***6. S.* Paratyphi**	(KOL 539)	0.30
***7. S.* Paratyphi**	(KOL 520)	0.34
***8. S.* Enteritidis**	(520833) *	0.73
9. S.* Typhi*murium	(PH-94) *	0.81
10. ETEC	(IDH07942)	0.05
11. ETEC	(IDH H10407)	0.0002
12. ETEC	(IDH H10404)	-
13. Sh. flexneri 2a	(2457T)	0.016
14. Sh. flexneri 3a	(UB811)	0.004
15. Sh. flexneri 6	(UB812)	-
16. Sh. boydii	(NK02379)	-
17. V. cholerae O1	(MAK757)	-

4.3.2.6 Thermal Stability Test

Phages that are thermostable have a greater chance of surviving in various situations when the temperature may increase significantly. The thermal stability of the phage STWB21 was analyzed on different typhoidal and non-typhoidal *Salmonella spp.* The phage STWB21 remained stable up to 40°C for 1 h, but significantly decreased and lost its lytic activity at 50°C against all the typhoidal and non-typhoidal *Salmonella* strains used in this study (Figure 4.7).

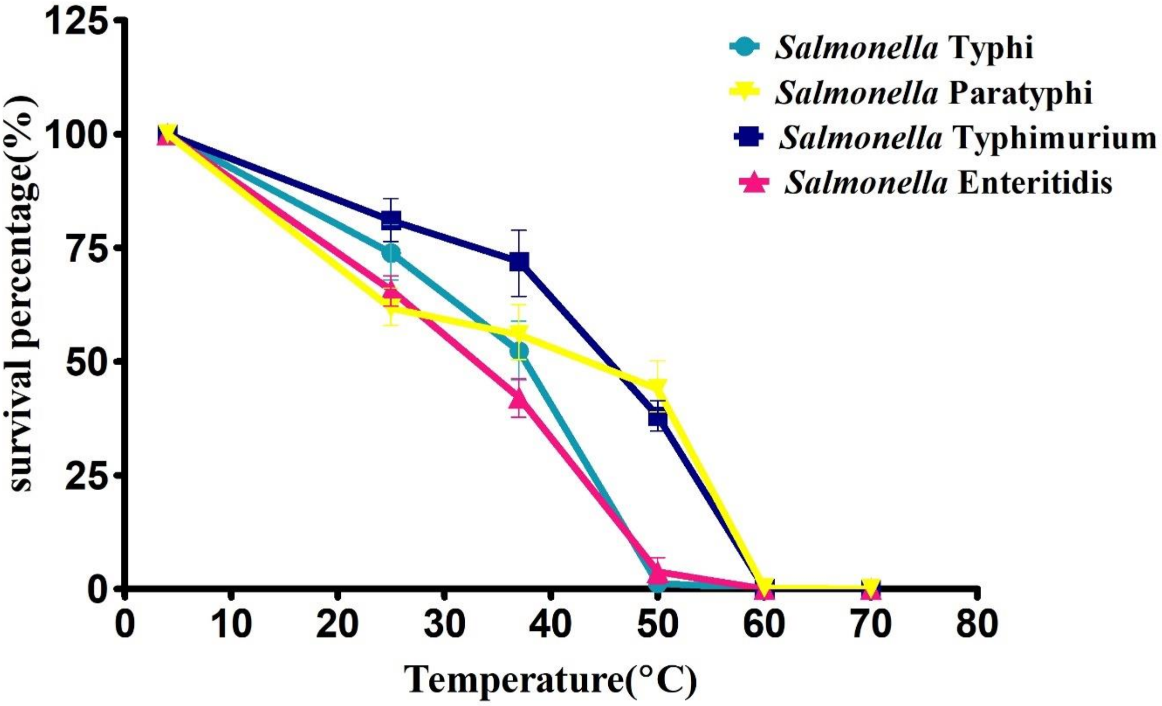


Figure 4.7: Thermal stability of phage STWB21.

4.3.2.7 pH Stability Test

The stability of phage STWB21 was evaluated at pH levels ranging from 3 to 13 for the typhoidal and non-typhoidal *Salmonella* strains. The phage STWB21 showed the highest activity at pH 7.0 and notable stability in acidic and alkaline conditions over a wide pH range (4–11) after 1 h incubation against all the typhoidal and non-typhoidal strains used in this study (Figure 4.8). Phage viability began to decline below pH 11. At pH 3 and pH 13, phage STWB21 was totally rendered inactive.

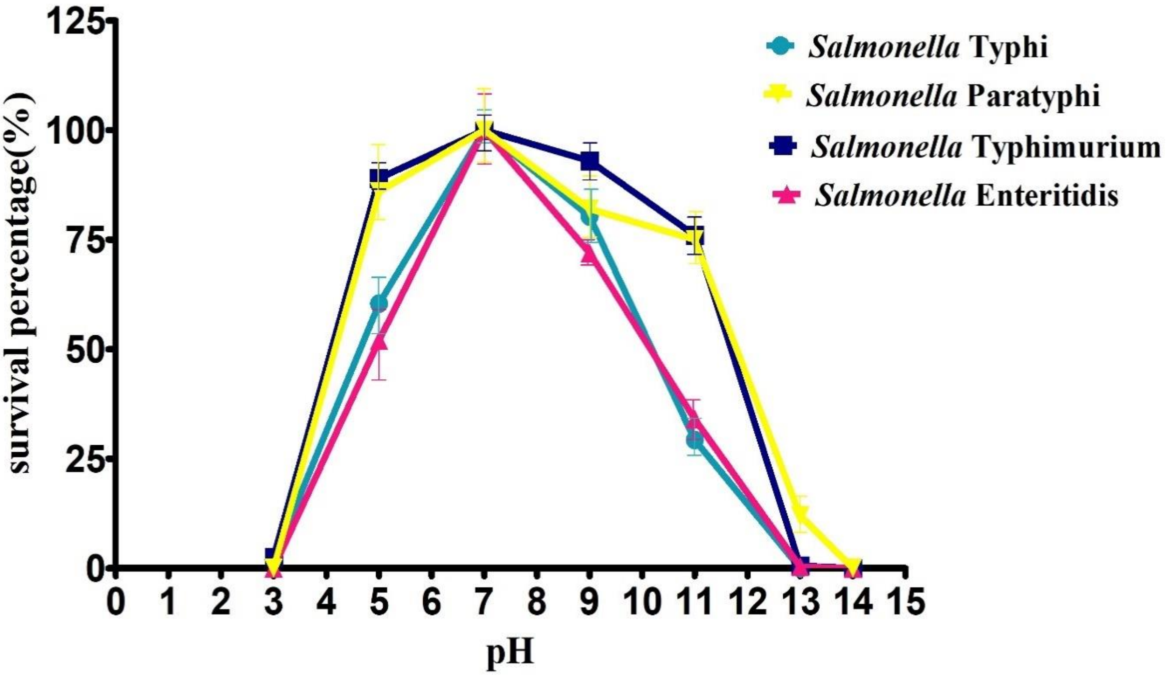


Figure 4.8: pH stability of phage STWB21.

4.3.2.8 UV Stability Test

Ultraviolet light is highly mutagenic. It produces some serious physiological and genetic effects that result in the death of the cell. UV inactivation of phages was found to obey the first-order reaction kinetics. When exposed to UV light, the phage STWB21 was completely inactivated within 10 seconds against all the typhoidal and non-typhoidal *Salmonella* strains used in this study (Figure 4.9). Therefore, UV has the same devastating effect on the phage STWB21.

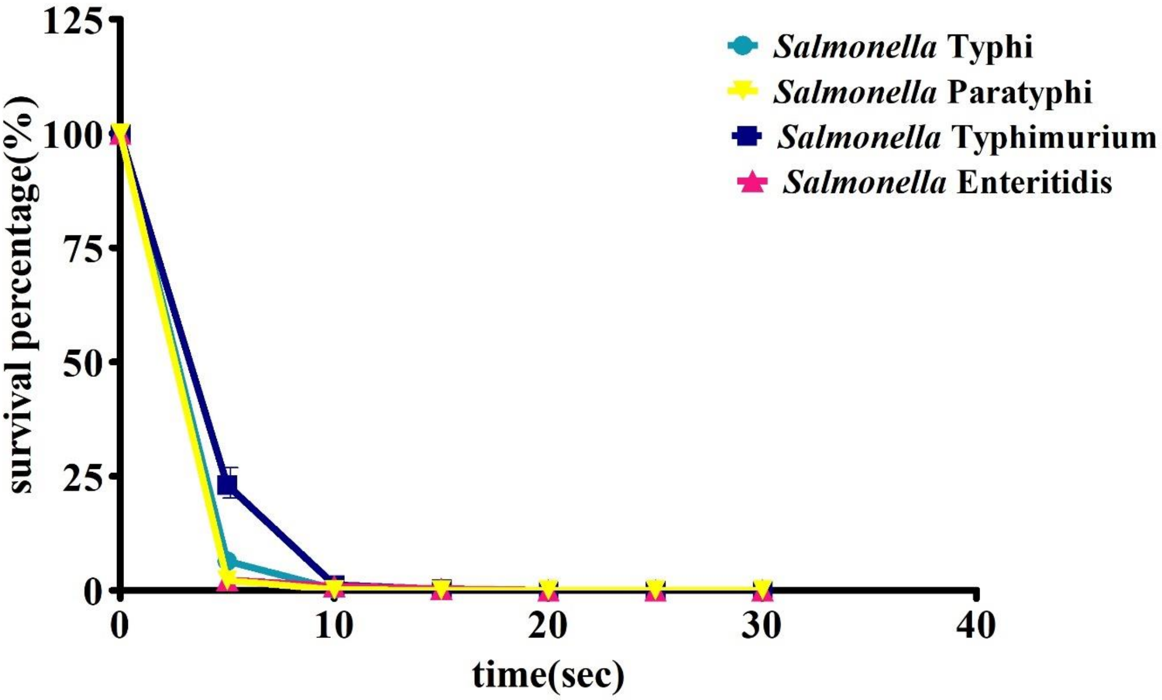


Figure 4.9: Inactivation kinetics of phage STWB21 stability after UV irradiation for different time periods.

4.3.2.9 One-step growth assay and Adsorption rate

Intracellular lytic development and the growth kinetics of phage STWB21 were analyzed in a one-step growth assay on different typhoidal and non-typhoidal *Salmonella* species including host bacteria (Figure 4.10). At a MOI of 0.1, the phage STWB21 one-step growth curve was performed. The latent phase and the burst size have been calculated from this investigation. The latent period/burst size for *S.* Typhi, *S.* Paratyphi, *S.* Typhimurium, and *S.* Enteritidis was found to be 25 min/101, 55 min/53, 50 min/163, 35 min/224 pfu, respectively.

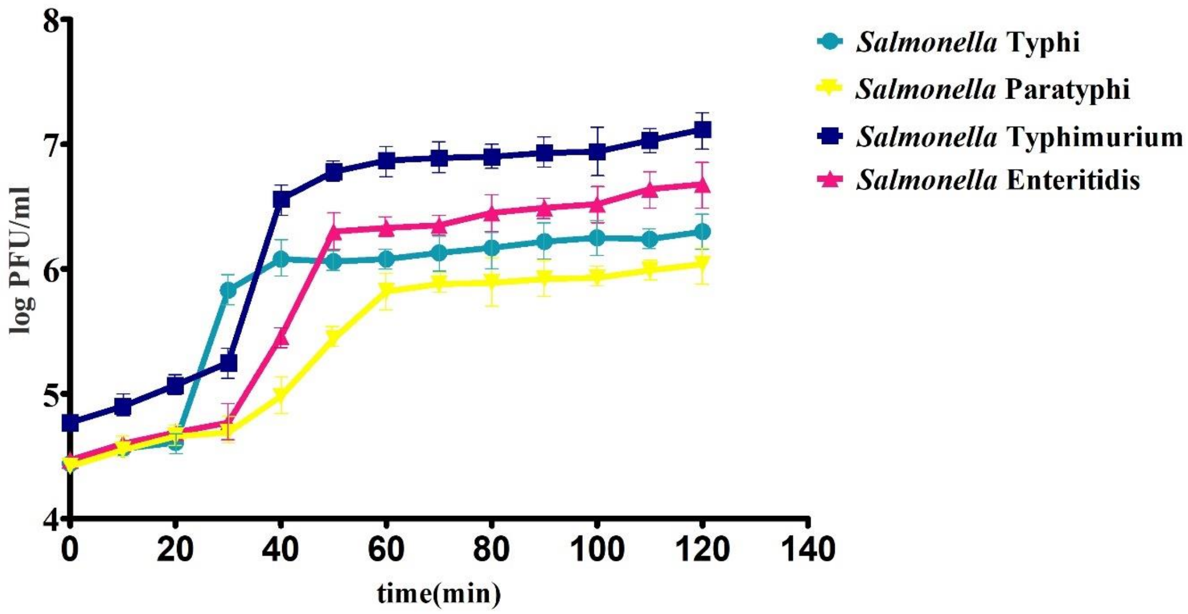


Figure 4.10: One-step growth curve of bacteriophage STWB21 against *S.* Typhi, *S.* Paratyphi, *S.* Typhimurium, and *S.* Enteritidis.

4.3.2.10 Adsorption rate

To determine how quickly the phage particle was adsorbed, a phage adsorption experiment was carried out. Additionally, rapid adsorption occurred for different typhoidal and non-typhoidal *Salmonella* species in the first 5 min, followed by a slower adsorption phase thereafter (Figure 4.11).

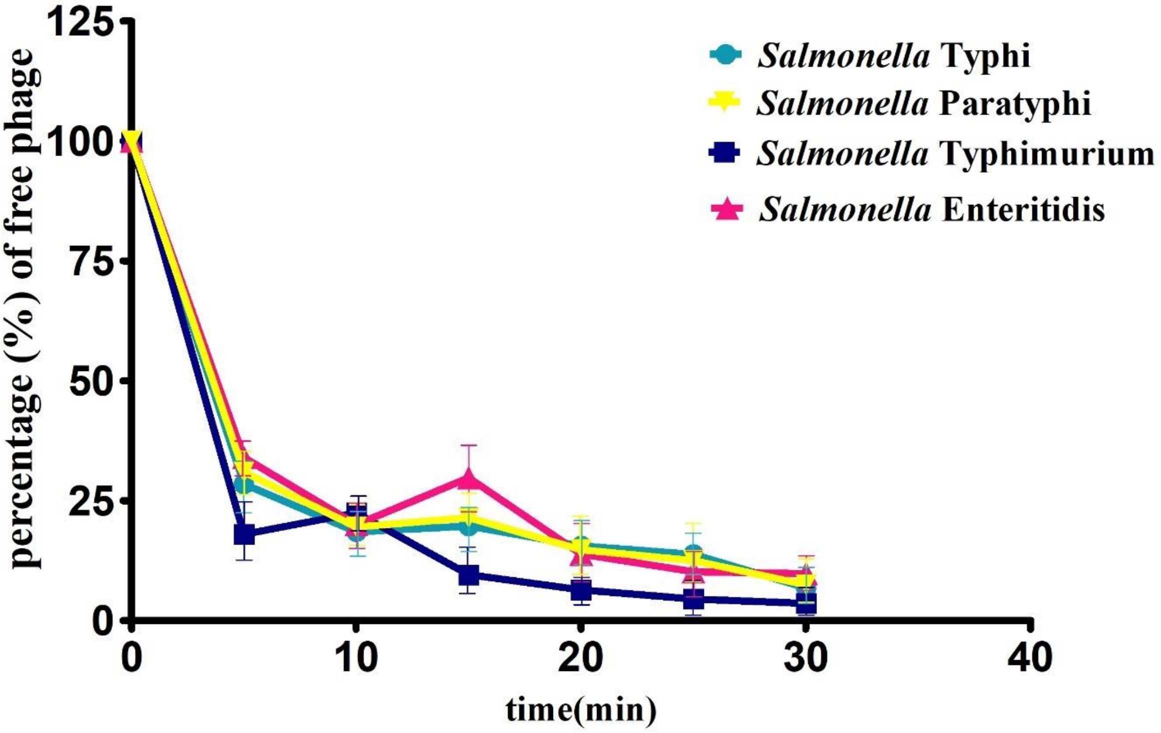


Figure 4.11: Adsorption kinetics of bacteriophage STWB21 against *S*. Typhi, *S*. Paratyphi, *S*. Typhimurium, and *S*. Enteritidis.

4.3.3 Interaction of outer membrane vesicles of *Salmonella* Typhi with bacteriophage STWB21

Transmission electron microscopy showed that STWB21 bacteriophages interacted with OMVs. The negatively stained samples revealed the outer membrane structure as a bright ring enclosing the vesicle lumen that appeared darker in Figure 4.12A. The phage STWB21 used its tail apparatus to adhere to the outer membrane vesicle surface (Figure 4.12B) after 1hr incubation and the bacterial OMV was found to be deformed in shape after 4hr incubation at 37°C (Figure 4.12C).

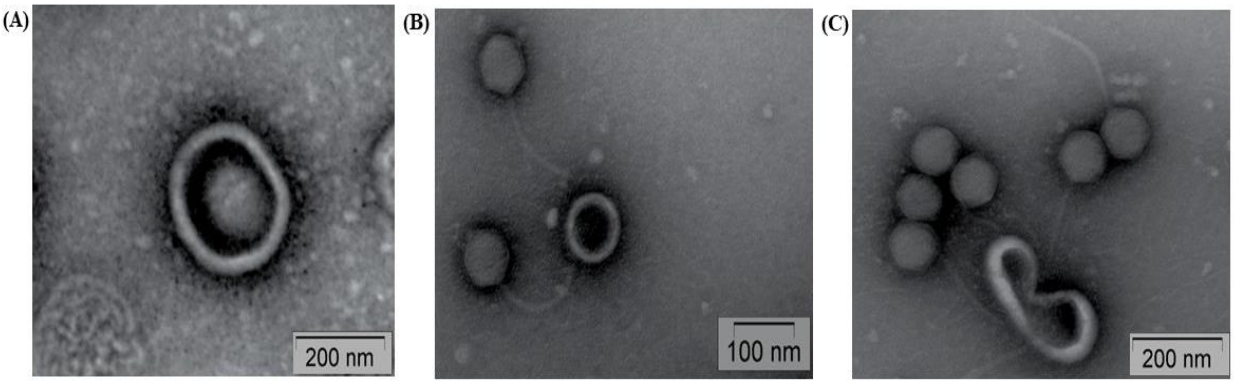


Figure 4.12: Bacterial omv and phage attachment. (A) *S.* Typhi bacterial omv, (B) Phage attachment on bacterial omv after 1hr of incubation, (C) Phage degraded bacterial omv after 4hr of incubation.

4.3.4 Visualization of phage-host attachment

The phage STWB21 was introduced into the exponentially growing bacterial cells, and after 15 minutes of incubation, the phage-infected bacterial cells were visualized using the transmission electron microscope and scanning electron microscope in Figure 4.13A and Figure 4.13B respectively. The micrograph showed several phages with their intact tails attached to bacteria.

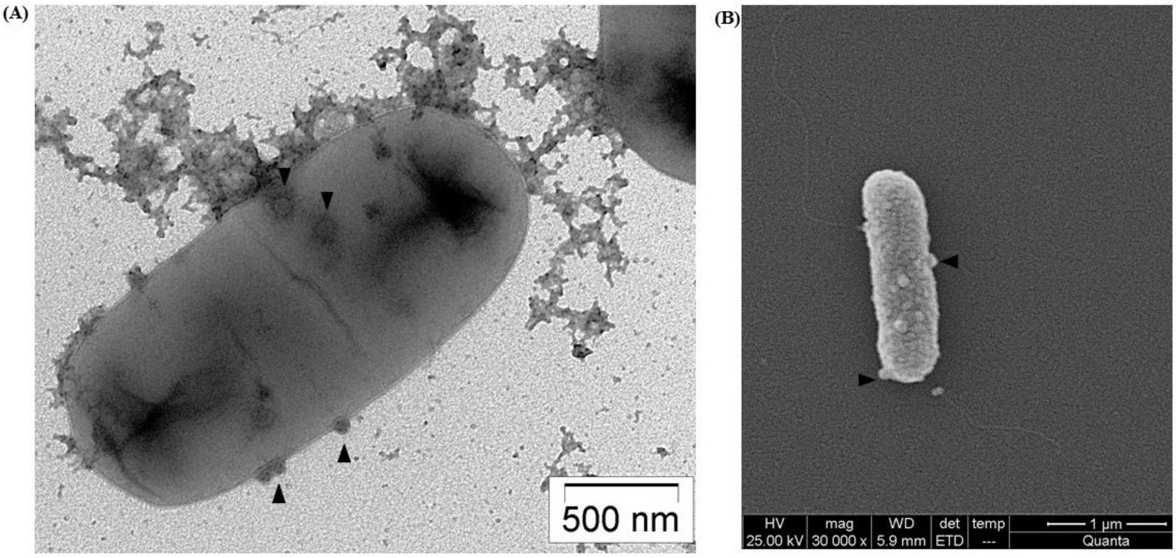


Figure 4.13: Bacteria and phage attachment. (A) Transmission electron micrograph of phage attachment on bacteria after 15 mins of infection (B) Scanning electron micrograph of phage attachment on bacteria after 15 mins of infection. The black arrow indicates the phage attachment to bacteria.

4.4 Discussion

Salmonella genus is considered one of the biggest public threats due to its expanded reservoir (WHO, 2017). In addition, antibiotic resistance amidst bacterial infection has become a global burden (Tacconelli et al., 2018). To overcome this situation an alternative approach to treat bacterial infection is a pressing priority. Bacteriophages especially lytic bacteriophages can be effective against specific bacterial infections due to their ability to infect and kill specific host bacteria. Therefore, an upsurge in research on bacteriophage isolation, characterization, and effectiveness against pathogenic bacteria has reflected the awareness worldwide.

Effective phage therapy requires specific conditions such as a large collection of bacteriophages, the use of obligately lytic phages rather than the temperate phage, host range, and screening of phage genomes to confirm the absence of toxin genes (Weber-Dabrowska et al., 2016). Furthermore, it is advantageous to use phages that adsorb efficiently on host cells, propagate rapidly with a relatively high burst size and may eliminate target bacteria in a relatively short time. Additionally, the one-step growth curve of phage STWB21 suggested a latent period (25 min) and a large burst size of 161 pfu per cell.

In this study, lytic bacteriophage STWB21 was isolated from the lake water sample in the outskirts of the Kolkata area using a strain of *S.* Typhi as the host bacterium. Phage STWB21 was found to be effective against many other strains of typhoidal and non-typhoidal *Salmonella* which indicates the high specificity of STWB21 against *Salmonella* (Abdelsattar et al., 2021). On the other hand, the ability of phage STWB21 to lyse *Sh. flexneri 2a*, *Sh. flexneri 3*, and *ETEC* indicate that the phage can infect more than one bacterial species. Thus, phage STWB21 is a "polyvalent phage", as this quality has been reported before for other *Salmonella* phages (Parra and Robeson, 2016). In addition, to the best of our knowledge, this is the first *Salmonella* phage active against typhoidal strains as well as non-typhoidal strains.

Resistance to heat and acid–base properties are the foundations of phage for use in bio-control applications (Jończyk-Matysiak et al., 2019). Different foods like fruits, vegetables, pasteurized milk, or chicken breast are usually stored at 4°C or at ambient temperature (25°C). According to our results, the phage STWB21 was stable at 4°C to <50°C, and the stability of phage STWB21 at ambient temperature without losing viability for a month, implied its suitability for large-scale applications. The phage STWB21 is relatively stable within the range of pH between 4 and 11, which is congenial with the range (pH 5.5–7.0) of many foods. The phage stability in different pH ranges also provides valuable information to take into account when considering oral delivery of this phage during treatment. Therefore, their ability to survive at different temperatures and pH conditions suggests that phage STWB21 is tolerant to heat and extreme pH conditions and it could be useful in different applications.

4.5 Chapter Summary

Salmonella phage STWB21 was isolated from a lake water sample and found to be a novel lytic phage with promising potential against the host bacteria *Salmonella* typhi. However, some polyvalence was observed in their broad host range. In addition to *S.* Typhi, the phage STWB21 was able to infect *S.* Paratyphi, *S.* Typhi*murium*, *S.* Enteritidis, and a few other bacterial species such as *Sh. flexneri 2a*, *Sh. flexneri 3a*, and *ETEC*. The newly isolated phage STWB21 belongs to the *Siphoviridae* family with an icosahedral head and a long flexible non-contractile tail. Phage STWB21 is relatively stable under a wide range of pH (4–11) and temperatures (4°C–50°C) for different *Salmonella* serovars. The latent period and burst size of phage STWB21 against S. Typhi were 25 min and 161 plaque-forming units per cell.

Chapter 5

Genomic Characterization of phage STWB21

Chapter 5

5.1 Introduction

The size of a phage genome varies significantly, ranging from 3,300 nucleotide ssRNA viruses in *E. coli* to over 500 kbp genomes in Bacillus megaterium phage G (Hatfull & Hendrix.; 2011). The *Podoviridae*, *Siphoviridae*, and *Myoviridae* have the shortest dsDNA-tailed phage genomes at 11.5 kbp (e.g., *Mycoplasma* phage P1), 21kbp (e.g., *Lactococcus* phage c2), and 30 kbp (e.g., *Pasteurella* phage F108) respectively (Tu et al., 2001; Lubbers et al., 1995; Chen et. al., 2018). However, there are broad size variations among them as well. In broad terms, these genomes are packaged at similar densities inside their capsids, and the size of the capsid varies with different genome sizes. The amount of DNA packaged into a given capsid affects the capacity of the virion to infect and too little or too much DNA might cause the virion to become unstable. Due to evolutionary pressures to accommodate packaging and virion stability, DNA may either gain or lose some of its strands. The selection of genome size is significant in bacteriophage evolution because it provides a distinct mechanism for DNA acquisition and loss. Instead of being selected for immediate value, newly acquired DNA serves as an accumulation of genetic information for potential future use (Hatfull & Hendrix, 2011). This is a significant divergence from bacterial genome evolution, in which cell membrane and cell wall flexibility do not impose any evident limit on genome size.

In the 1950s, bacteriophage T5 was one of the first enterobacterial phages that infect enterobacteria *Escherichia coli*. It is one of the phages from the T5-like virus genus of the *Siphoviridae* family that has been most thoroughly studied. Since McCorquodale and Warner (1988) reviewed the research on bacteriophage T5 and related phages, not much progress has been documented. Only one strand of the linear, double-stranded DNA genome gets nicked at specific locations. These nicks may be removed using DNA ligase (Jacquemin-Sablon and Richardson, 1970) and are not necessary for phage replication (Rogers et al., 1980). These interruptions may be categorized into two categories: large nicks and minor nicks (Scheible et al., 1977). The big nicks occur at five distinct places in 80–90% of the population. Despite the discovery of at least four T5-induced endonucleases that are capable of nicking double-stranded DNA, the cause and function of these nicks remain unexplained (Rogers and Rhoades, 1976).

A two-step transmission method is used by T5 phages to infect their hosts: The remaining sequence enters the host only after the expression of the first-step transfer (FST) genes; the first-step transfer (FST) sequence, also known as the left terminal repeat, is initially injected into the host. Both the two-step transfer injection and the formation of concatemers during phage DNA replication may be significantly impacted by the presence of several host function inhibitors, direct repeats, inverted repeats, and palindromes in the big terminal repetition (Heusterspreute et al., 1987). Several enzymes involved in nucleotide metabolism are likewise activated by T5 (McCorquodale and Warner, 1988). Furthermore, many restriction segments from T5 DNA cannot be directly cloned (McCorquodale, 1999), and many T5 regions remain unknown. The bacteriophage T5 genome was eventually significantly sequenced (Wang et al., 2005), and new members of this bacteriophage group were also characterized (Kim and Ryu, 2011; Golomidova et al., 2015). Most of the T5 bacteriophages appear to share an identical genomic structure in which pre-early, early, and late sections may be identified based on the time of transcription throughout the infectious cycle (reviewed by Davison, 2015).

Previous studies showed several T5-like bacteriophages are possible candidates for use against several foodborne diseases, including *Salmonella* enterica serovar Typhimurium (Kim and Ryu, 2011; Piya et al., 2015). coli O157:H7 strains (Niu et al., 2012; Hong et al., 2014), with encouraging *in vivo* results.

5.2 Chapter aims

The major aims of this chapter were to:

i. Isolation of the genomic DNA from *Salmonella* phage STWB21.
ii. Genome comparison with other *Salmonella* phages.
iii. Visualization of the genome structure of phage STWB21 using CG view software.
iv. Understand the evolutionary relationship of phage STWB21 with other T4 and T5 phages.

5.3. Results

5.3.1 Restriction profile of phage DNA

Bacteriophage genomic DNA was isolated using a phage DNA isolation kit. The phage genome was isolated and the measured concentration was 112µg/ml. STWB21 genome was cut with restriction enzymes EcoRI, EcoRV, MluI, BglII, PstI, BamHI, and HindIII. The digestion pattern of phage genomic DNA is shown in Figure 5.1. This finding led to the hypothesis that the genome of the phage STWB21 is a double-stranded DNA virus.

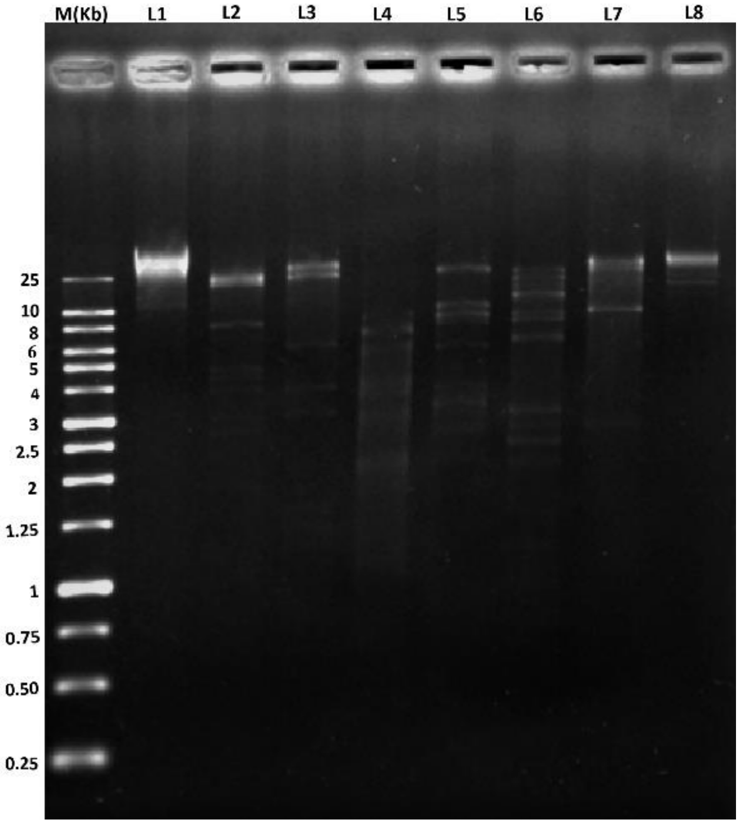
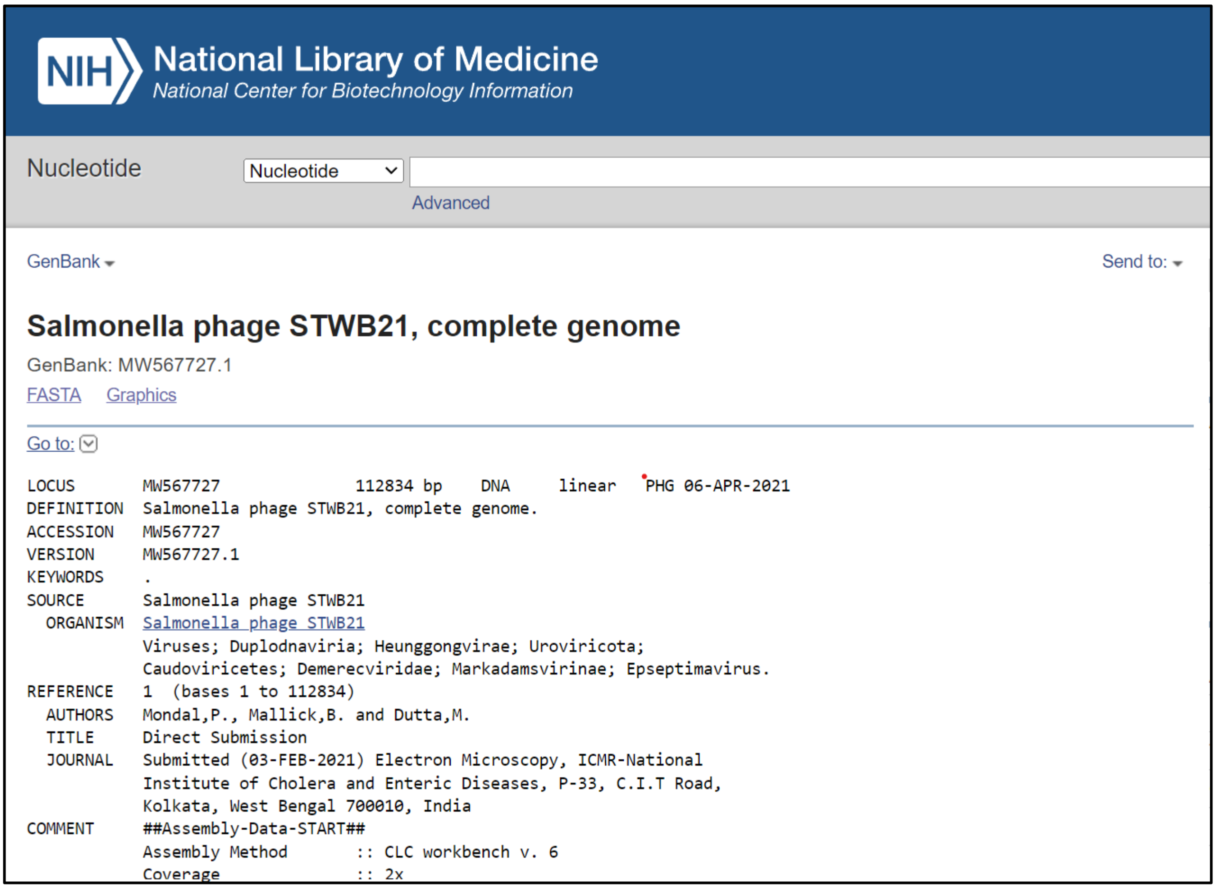


Figure 5.1: Restriction fragment analysis of the phage STWB21 genomic DNA. Digestion pattern with EcoRI (L2), EcoRV (L3), MluI (L4), BglII (L5), PstI (L6), BamHI (L7), and HindIII (L8). Control: Genomic DNA (L1). M, Marker.

5.3.2 General features of the STWB21 genome

The complete genome sequence of phage STWB21 has been deposited in NCBI GenBank under accession no. MW567727 (Figure 5.2).



Salmonella phage STWB21, complete genome

GenBank: MW567727.1

```
LOCUS       MW567727              112834 bp    DNA     linear   PHG 06-APR-2021
DEFINITION  Salmonella phage STWB21, complete genome.
ACCESSION   MW567727
VERSION     MW567727.1
KEYWORDS    .
SOURCE      Salmonella phage STWB21
  ORGANISM  Salmonella phage STWB21
            Viruses; Duplodnaviria; Heunggongvirae; Uroviricota;
            Caudoviricetes; Demerecviridae; Markadamsvirinae; Epseptimavirus.
REFERENCE   1  (bases 1 to 112834)
  AUTHORS   Mondal,P., Mallick,B. and Dutta,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-FEB-2021) Electron Microscopy, ICMR-National
            Institute of Cholera and Enteric Diseases, P-33, C.I.T Road,
            Kolkata, West Bengal 700010, India
COMMENT     ##Assembly-Data-START##
            Assembly Method       :: CLC workbench v. 6
            Coverage              :: 2x
```

Figure 5.2: The genome sequence data was deposited in GenBank and the NCBI accession number is MW567727

The complete genome length of phage STWB21 is 112,834 bp with a 40.37% GC content very similar to the *Salmonella* phages of the T5 virus family: *Salmonella* phage S124, Seafire, Gec_vB_N3, S133, Seabear, Stitch (Table 5.1). Thus, according to Megablast results, STWB21 was enumerated as a member of the T5 virus genus, *Siphoviridae* subfamily. Phage STWB21 genome was predicted to encode 166 putative open reading frames (47 on the complementary strand and 119 on the direct strand) with 152 ATG (91.07%), 10 GTG (6.02%) and 5 TTG (2.99%) as initiation codons. After genome analysis, 22 tRNA encoding genes were found to be present in phage STWB21 which is considered as a characteristic of a virulent phage. Closely related *Siphoviridae Salmonella* phages were also reported to carry a similarly large number of tRNA-encoding genes.

Table 5.1: List of reported *Salmonella* phages from the *Siphoviridae* family

Phage name	Similarity	Length	GC content	tRNA	Host	Taxonomic genera	Accession number	Reference
STWB21	**-**	**112834**	**40.39%**	**22**	***S.* Typhi**	**T5 virus**	**MW567727**	**Present study**
S124	92.69%	112564	40.12%	28	*S. enterica subsp.*	T5 virus	NC_048013	NCBI Database (Unpublished)
Gec_vB_N3	92.40%	109645	40.08%	17	*S.* Enteritidis	T5 virus	MW006478	Makalatia K et. al., 2020
S133	92.28%	110926	40.06%	29	*S. enterica subsp.*	T5 virus	NC_048011	NCBI Database (Unpublished)
Sea bear	92.08%	112472	40.38%	25	*S.* Typhimurium	T5 virus	MK728824	Patil K et. al., 2019
Stitch	91.90%	123475	40.31%	30	*S.* Typhimurium	T5 virus	KM236244	Grover M J et al., 2015

Phage STWB21 whole genome sequence was compared against the nucleotide sequence database in NCBI using BLASTn, Phage STWB21(MW567727) showed high homology with six bacteriophages: phage S124 (NC_048013), Seafire (NC_048110), Gec_vB_N3 (MW006478), S133 (NC_048011), Seabear (MK728824) and Stitch (KM236244). The detailed genomic features of the above-mentioned phages are listed in Table 5.1. The genomic sequence of STWB21 is 92.6% identical to that of S124, 92.6% to Seafire, 92.4% to Gec_vB_N3, 92.2% to S133, 92% to Seabear and 91.9% to Stitch as shown in Table 5.1.

5.3.3 Genome comparison with other *Salmonella* phages

To investigate genomic architecture and the similarity with other genomes, we compared phage STWB21 genomic synteny with other related *Siphoviridae Salmonella* phages: S124, Seafire, Gec_vB_N3, S133, Seabear, Stitch by using the Easyfig genome comparison tool (Figure 5.3). Multiple alignments of phage STWB21 with the other six relative phages have shown that the gene inventories of these six closely related phages were highly similar. As indicated in Figure 5.3, the genomes of the phages contained a block of clustered genes encoding predicted structural and functional proteins. In some cases, they were arranged differently and even oriented in opposite directions.

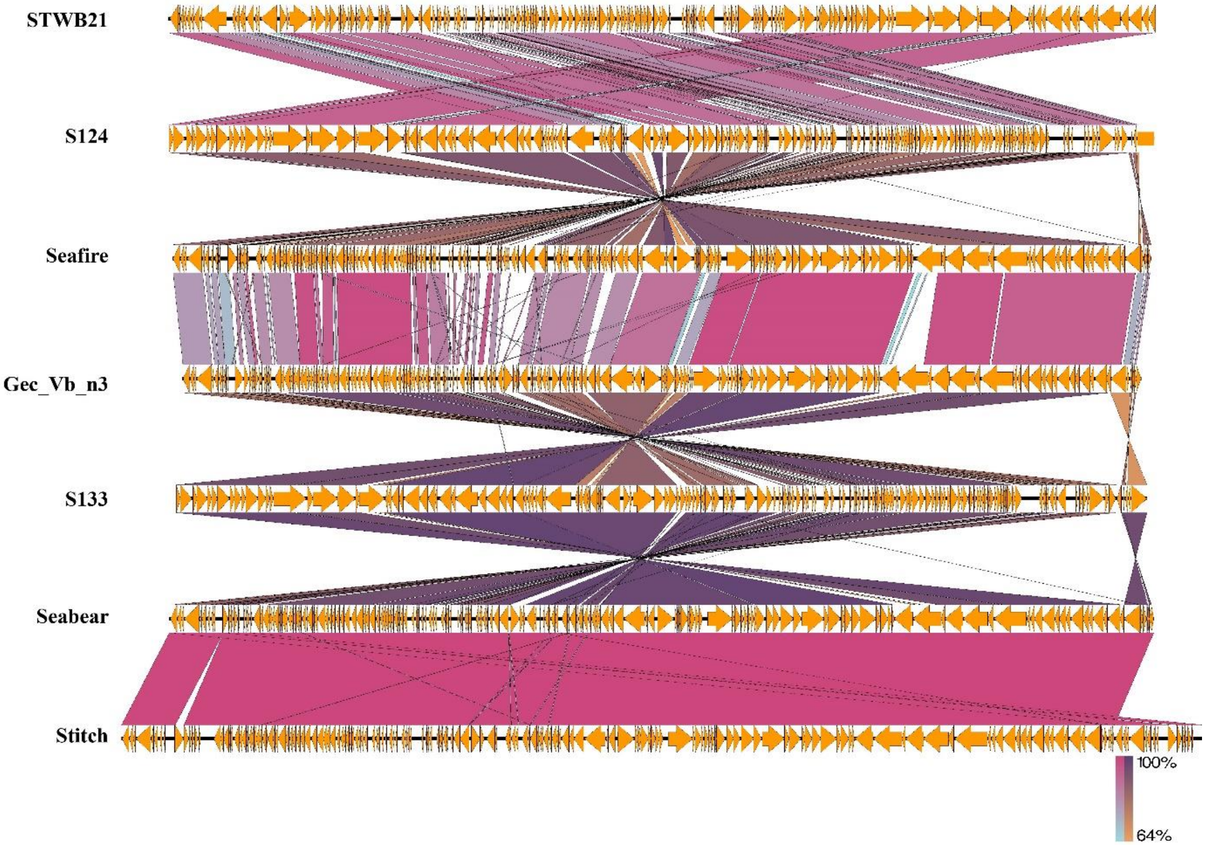


Figure 5.3: Schematic genomic comparison of the phage STWB21 with other six homologous *Salmonella* phages. Homologous ORFs or genes are present in yellow, and the percentages of amino acid identities are shown in different colors.

5.3.4 Visualization of genome structure using CG view software

A Java program called CG View was created in 2005 as a means of producing high-quality, navigable maps of circular genomes. CGView provides a unique XML (Extensible Markup Language) input format for specifying the details of a map, which the software subsequently renders in graphical form. Figure 5.4 displays the contents of a sample XML file and the accompanying map produced by CGView. The ORFs of STWB21 were broadly scattered across the genome.

Description of phage modules

The annotated proteins of STWB21 were categorized into four different genetic modules, which enabled the following: DNA replication/modification/transcriptional regulations, lysis, DNA packaging, and morphogenesis.

DNA replication/modification/transcriptional regulation module proteins

STWB21 encodes at least 12 genes co-localized as a distinct DNA modification module and were anticipated to play role in phage DNA metabolism, including a DNA helicase (ORF 160), a helicase-primase (ORF 163-ORF 164), a DNA polymerase I (ORF 162), a transcriptional factor (ORF 165), DNA ligase (ORF 1), transcriptional regulator/methyltransferase (ORF 3), HNH homing endonuclease (ORF 21), ribonuclease (ORF 27), exonuclease (ORF 156), recombinase (ORF 157). The high presence of DNA metabolism-associated genes in the STWB21 genome might reduce the dependence of phage on bacteria (Peng and Yuan, 2018).

Two-component host lysis module proteins

A classic eubacterial phage lysis cassette compromises two-component cell lysis proteins, a holin (ORF 79) and lysozyme (ORF 80) were present in the STWB21 genome. During the burst step of the phage life cycle, these genes are crucial to effect host-cell lysis. Pore-forming enzyme holin permeabilizes the cytoplasmic membrane and cell wall degrading protein lysozyme degrades the bacterial cell wall (Wang et al., 2000). According to previous reports, holin have usually two or three transmembrane (Bläsi et al., 1999) domains but the genome analysis revealed that the holin of STWB21 has one transmembrane domain group (TMHMM-2.0). Besides, genomic analysis of phage STWB21 was studied for the presence of phage lysogeny factors and toxin genes. The presence of the lysis gene and the absence of lysogeny-related genes in the STWB21 genome indicate that the phage is a potent lytic phage.

DNA packaging module proteins

In the DNA packaging modules, only ORF 130 was predicted which encodes the large subunit of terminase in the STWB21 genome and displayed 99% similarity with other *Salmonella* phage S124. Previous studies suggested that the large subunit of terminase is very conserved among the related phages (Burroughs et al., 2007). The junction of the DNA packaging module and the morphogenesis module is constituted by portal protein.

Phage morphogenesis module proteins

The arrangements of 22 genes encoding STWB21 phage structural proteins were scattered in the complementary strand of the genome and followed the gene orders of *Siphovirus*. These 22 ORFs mainly encoded proteins like portal protein (ORF 133, ORF 134), tail fiber protein (ORF 135, ORF 151, ORF 152), major capsid protein (ORF 137), capsid and scaffold protein (ORF 138), tail length-tape measure protein (ORF 145), tail completion protein (ORF 166). As previously described, tail fiber proteins are subject to horizontal gene transfer between phages in a constant manner (Greive et al., 2016). During infection, tail length tape measure protein DNA is transited to the bacterial cell by the tape-measure protein (Mahony et al., 2016).

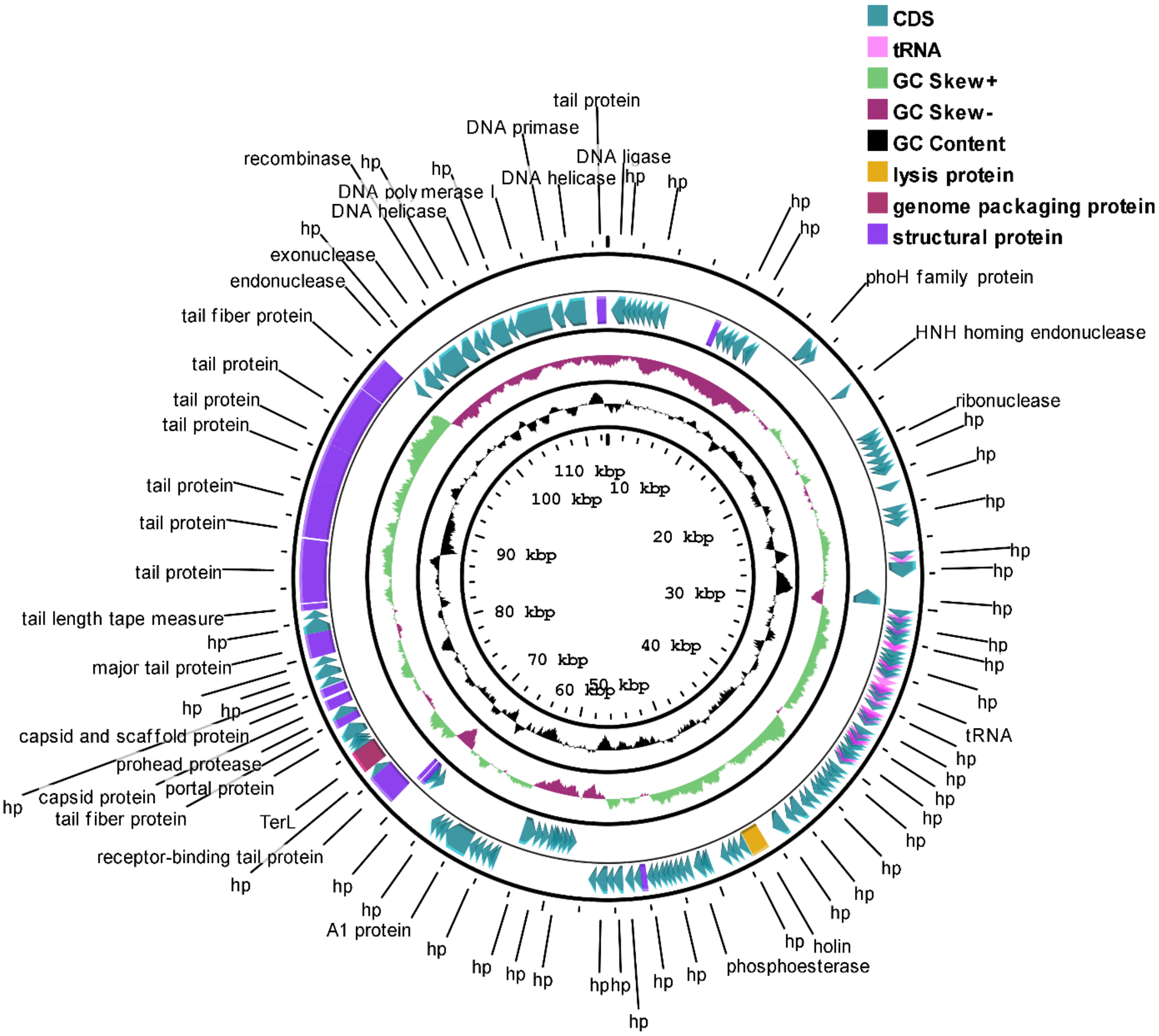


Figure 5.4: Genome structure of phage STWB21 prepared using CGView. The outer ring denotes the STWB21 genome and ORFs. The inner rings show G+C content and G+C skew, where peaks represent the positive (outward) and negative (inward) deviation from the mean G+C content and G+C skew, respectively.

Table 5.2. Features of the open reading frames (ORFs) of bacteriophage STWB21 and homology to a protein database.

	CDS position	Function	Blast hit
ORF 1	1195..221	DNA ligase	PHAGE_Salmon_S124_NC_048013
ORF 2	1388..1188	HP	PHAGE_Salmon_S131_NC_048009
ORF 3	1782..1474	Transcriptional factor (Methyl transferase)	PHAGE_Salmon_S124_NC_048013
ORF 4	2129..1833	HP	PHAGE_Salmon_S124_NC_048013
ORF 5	2577..2167	putative D3 protein	PHAGE_Salmon_bobsandoy-_MT074464
ORF 6	2939..2685	DNA helicase	PHAGE_Salmon_S147_NC_048012
ORF 7	3636..2932	D2 protein	PHAGE_Salmon_bobsandoy-_MT074464
ORF 8	3938..3705	HP	PHAGE_Salmon_vBSenS3_154_ MT004791
ORF 9	6711..3922	replication origin binding protein	PHAGE_Salmon_STG2_NC_048089
ORF 10	7742..7332	HNH endonuclease	PHAGE_Salmon_STG2_NC_048089
ORF 11	8182..7753	HP	PHAGE_Escher_fp01_NC_048731
ORF 12	8690..8184	Exonuclease	PHAGE_Salmon_S132_NC_048010
ORF 13	8862..8677	capsid maturation protease	PHAGE_Salmon_faergetype_NC_048867
ORF 14	9779..8979	recombination protein	PHAGE_Salmon_S132_NC_048010
ORF 15	10189..9986	HP	PHAGE_Salmon_rokbiter_NC_048868
ORF 16	10480..10199	HP	PHAGE_Salmon_ende_75_MT074454
ORF 17	12453..10579	anaerobic ribonucleoside-triphosphate reductase	PHAGE_Salmon_Sw2_NC_048062
ORF 18	12806..13558	PhoH family protein	PHAGE_Salmon_enterica_ABBNMR010000014
ORF 19	13560..13802	HP	PHAGE_Salmon_S126_NC_048008

ORF 20	13866..16259	Ribonucleoside diphosphate reductase I alpha chain	PHAGE_Salmon_vB_SenS-3_MT004791
ORF 21	16341..16838	HNH homing endonuclease	PHAGE_Salmon_vB_SenS-SB13_ NC_048781
ORF 22	16835..17980	Ribonucleotide reductase of class Ia beta subunit	PHAGE_Salmon_S124_NC_048013
ORF 23	17980..18516	Dihydrofolate reductase	PHAGE_Salmon_S124_NC_048013
ORF 24	18613..19367	Thymidylate synthase	PHAGE_Salmon_rokbiter_NC_048868
ORF 25	19460..19714	HP	PHAGE_Salmon_S133_NC_048005
ORF 26	19714..19983	HP	PHAGE_Salmon_sent65_MT653145
ORF 27	19983..20459	Ribonuclease	PHAGE_Salmon_S132_NC_048010
ORF 28	20536..20814	HP	PHAGE_Salmon_S124_NC_048013
ORF 29	20899..21414	HP	PHAGE_Salmon_S124_NC_048013
ORF 30	21476..21691	HP	PHAGE_Salmon_faergetype_NC_048867
ORF 31	21733..21939	HP	PHAGE_Salmon_S124_NC_048013
ORF 32	21965..22666	Metallopeptidase	PHAGE_Salmon_S124_NC_048013
ORF 33	22737..22919	Hp	PHAGE_Salmon_S126_NC_048008
ORF 34	22974..23612	Tail fiber protein	PHAGE_Salmon_Stitch_NC_027297
ORF 35	24054..24371	Hp	PHAGE_Salmon_vB_SenS_SB13_NC_048781
ORF 36	24377..24826	Cell wall hydrolyse	PHAGE_Salmon_S124_NC_048013
ORF 37	24895..25065	HP	PHAGE_Salmon_SE11_NC_048786
ORF 38	25065..25508	YqeY protein domain-containing protein	PHAGE_Salmon_falkor_MT074467
ORF 39	26674..27183	HP	PHAGE_Salmon_S124_NC_048013
ORF 40	27300..28244	HP	PHAGE_Salmon_OSY_STA_NC_048808

ORF 41	28980..28564	cAMP-dependent protein kinase catalytic subunit	PHAGE_Salmon_Sepoy_NC_048760
ORF 42	29995..29003	HP	PHAGE_Salmon_Sepoy_NC_048760
ORF 43	30261..30557	HP	PHAGE_Salmon_S126_NC_048008
ORF 44	30803..30988	HP	PHAGE_Salmon_S132_NC_048010
ORF 45	30990..31322	HP	PHAGE_Salmon_STG2_NC_048089
ORF 46	31508..31630	HP	PHAGE_Salmon_Stitch_NC_027297
ORF 47	31644..31850	HP HOT59_gp160	PHAGE_Salmon_S113_ NC_048005
ORF 48	31941..32216	HP	PHAGE_Salmon_Stitch_ NC_027297
ORF 49	32703..32975	HP HOT61_gp148	PHAGE_Salmon_S116_NC_048007
ORF 50	33026..33250	HP	PHAGE_Salmon_S113_NC_048005
ORF 51	33247..33519	HP	PHAGE_Salmon_S124_NC_048013
ORF 52	33714..33899	HP	PHAGE Salmon_Stitch_ NC_027297
ORF 53	34003..34161	HP HOS12_gp103	PHAGE_Salmon_SP01_NC_047859
ORF 54	35148..35312	HP	PHAGE_Salmon_falkor_MT074467
ORF 55	35411..35728	HP	PHAGE_Salmon_Stitch_NC_027297
ORF 56	35961..36314	HP BOW73_gp180	PHAGE_Salmon_100268_sal2_NC_031902
ORF 57	36773..36991	HP	PHAGE_Salmon_S116_NC_048007
ORF 58	37097..37270	HP	PHAGE_Salmon_Smaug_MT074461
ORF 59	37720..37899	HP	PHAGE_Salmon_Stitch_ NC_027297
ORF 60	38115..38315	HP	PHAGE_Salmon_ vB_SenS_SB9_ MK867835
ORF 61	38578..38799	HP	PHAGE_Salmon_Stitch_ NC_027297
ORF 62	38792..38956	HP	PHAGE_Salmon_Stitch_ NC_027297
ORF 63	39116..39409	HP	PHAGE_Salmon_faergetype_ NC_048867
ORF 64	39409..39609	HP	PHAGE_Salmon_Stitch_ NC_027297
ORF 65	39801..39974	HP	PHAGE_Salmon_vB_SenS-3_MT004791
ORF 66	39952..40320	Pyruvate formate lyase	PHAGE_Salmon_S113_NC_048005

ORF 67	40402..40689	HP	PHAGE_Salmon_S113_NC_048005
ORF 68	40754..40939	HP	PHAGE_Salmon_faergetype_NC_048867
ORF 69	40998..41393	HP	PHAGE_Salmon_faergetype_NC_048867
ORF 70	41470..41742	HP	PHAGE_Salmon_rokbiter_NC_048868
ORF 71	41742..42041	HP	PHAGE_Salmon_atrejo_NC_048872
ORF 72	42034..42453	HP	PHAGE_Salmon_vb Sens-3_MT004791
ORF 73	42697..42981	HP	PHAGE_Salmon_STG2_NC_048089
ORF 74	43036..43449	HP	PHAGE_Salmon_S124_NC_048013
ORF 75	43598..44296	HP	PHAGE_Salmon_rokbiter_NC_048868
ORF 76	44253..44702	HP	PHAGE_Salmon_faergetype_NC_048867
ORF 77	44986..45738	HP	PHAGE_Salmon_OSY_STA_NC_048808
ORF 78	45751..46350	ATP-dependent Clp protease	PHAGE_Salmon_rokbiter_NC_048868
ORF 79	46507..47163	Holin	PHAGE Salmon_Stitch_ NC_027297
ORF 80	47160..47573	Lysozyme/endolysin	PHAGE Salmon_Stitch_ NC_027297
ORF 81	47649..48065	HP	PHAGE_Salmon_faergetype_NC_048867
ORF 82	48139..48549	HP	PHAGE_Salmon_faergetype_NC_048867
ORF 83	48542..48832	Thioredoxin	PHAGE_Salmon_S124_NC_048013
ORF 84	48935..49315	HP	PHAGE_Salmon_S124_NC_048013
ORF 85	49315..50178	Serine threonine phosphatase	PHAGE_Salmon_S124_NC_048013
ORF 86	50178..50384	HP	PHAGE_Salmon_S113_ NC_048005
ORF 87	50384..50482	HP	PHAGE Salmon_ vB_SenS_SB6_ MK809530
ORF 88	50492..51115	Phosphoesterase	PHAGE Salmon_ vB_SenS_ SB6_ MK809530
ORF 89	51277..51708	HP	PHAGE_Salmon_S124_NC_048013

ORF 90	51787..52038	HP	PHAGE_Salmon_S126_NC_048008
ORF 91	52038..52445	HP	PHAGE_Salmon_Sw2_NC_048062
ORF 92	52442..52723	HP	PHAGE_Salmon_S124_NC_048013
ORF 93	52720..52965	HP	PHAGE_Salmon_1_23_NC_048149
ORF 94	52955..53284	HP	PHAGE_Salmon_1_23_NC_048149
ORF 95	53385..53582	HP	PHAGE_Salmon_1_23_NC_048149
ORF 96	53579..54046	Hp	PHAGE_Salmon_OSY_STA_NC_048808
ORF 97	54021..54371	Capsid and scaffold protein	PHAGE_Salmon_bobsandoy-_MT074464
ORF 98	54382..54804	HP HOT59 gp107_	PHAGE_Salmon_S113_ NC_048005
ORF 99	54826..55374	HP	PHAGE_Salmon_S132_NC_048010
ORF 100	55480..56136	HP	PHAGE_Salmon_vB_SenS_SB13_NC_048781
ORF 101	56136..56444	HP	PHAGE_Salmon_Seafire_NC_048110
ORF 102	56441..57169	HP	PHAGE_Salmon_1_23_NC_048149
ORF 103	57295..57435	HP	PHAGE_Salmon_Seafire_NC_048110
ORF 104	58946..58707	HP	PHAGE_Salmon_2_3_NC_048809
ORF 105	59286..59074	Methyl transferase	PHAGE_Salmon_OSY_STA_NC_048808
ORF 106	59627..59283	Hp	PHAGE_Salmon_S124_NC_048013
ORF 107	59873..59751	Hp	PHAGE_Salmon_bobsandoy-_MT074464
ORF 108	60082..59870	HP	PHAGE Salmon_Stitch_ NC_027297
ORF 109	60363..60136	HP	PHAGE_Salmon_SH9_NC_047999
ORF 110	61016..60471	HP	PHAGE_Salmon_STG2_NC_048089

ORF 111	61339..61031	HP	PHAGE_Salmon_SH9_NC_047999
ORF 112	62395..61397	HP	PHAGE_Salmon_S114_NC_048006
ORF 113	63487..63645	HP	PHAGE_Salmon_Seafire_NC_048110
ORF 114	63645..63851	HP	PHAGE_Salmon_S114_NC_048006
ORF 115	64023..64274	HP	PHAGE_Salmon_S126_NC_048008
ORF 116	64374..64790	A2 protein	PHAGE_Salmon_S113_NC_048005
ORF 117	64841..65065	HP	PHAGE_Salmon_vB_SenS_SB13_NC_048781
ORF 118	65175..66842	A1 protein	PHAGE_Salmon_S124_NC_048006
ORF 119	66896..67156	HP	PHAGE_Salmon_SH9_NC_047999
ORF 120	67169..67447	HP	PHAGE_Salmon_bux_MT074460
ORF 121	67481..67873	HP	PHAGE_Salmon_fuchur_NC_048869
ORF 122	67957..68691	Deoxynucleoside-5-monophosphatase	PHAGE Salmon_Stitch_ NC_027297
ORF 123	69064..68849	HP	PHAGE Salmon_Stitch_ NC_027297
ORF 124	69493..69299	HP	PHAGE_Salmon_fuchur_NC_048869
ORF 125	69597..69493	HP	PHAGE_Salmon_S116_NC_048007
ORF 126	69984..69670	Receptor-blocking tail protein	PHAGE_Salmon_S124_NC_048006
ORF 127	70325..70059	Receptor-blocking tail protein	PHAGE Salmon_Stitch_ NC_027297
ORF 128	70409..72190	Receptor-binding tail protein	PHAGE_Salmon_fuchur_NC_048869

ORF 129	72201..72683	HP	PHAGE_Salmon_S124_NC_048013
ORF 130	72683..73999	Terminase large subunit	PHAGE_Salmon_S124_NC_048013
ORF 131	74114..74287	HP	PHAGE_Salmon_SP3_NC_048001
ORF 132	74373..74513	Hp	PHAGE_Salmon_S124_NC_048013
ORF 133	74513..74731	Portal protein	PHAGE_Salmon_S131_NC_048009
ORF 134	74739..75623	Portal protein	PHAGE_Salmon_S124_NC_048013
ORF 135	75620..76102	Tail fiber protein	PHAGE_Salmon_Sw2_NC_048062
ORF 136	76106..76738	Prohead protease	PHAGE_Salmon_S147_NC_048012
ORF 137	76756..77370	Major capsid protein	PHAGE_Salmon_S124_NC_048013
ORF 138	77549..78064	Capsid and scaffold protein	PHAGE_Salmon_S124_NC_048013
ORF 139	78124..78636	HP	PHAGE_Salmon_S124_NC_048013
ORF 140	78636..79403	HP	PHAGE_Salmon_S124_NC_048013
ORF 141	79407..79892	HP	PHAGE_Salmon_S124_NC_048013
ORF 142	79919..81328	Major tail protein	PHAGE_Salmon_S124_NC_048013
ORF 143	81333..82229	HP	PHAGE_Salmon_S124_NC_048013
ORF 144	82226..82630	HP	PHAGE_Salmon_S124_NC_048013
ORF 145	82692..83060	Tail length tape-measure protein	PHAGE_Salmon_S124_NC_048013
ORF 146	83141..86821	Tail protein	PHAGE_Salmon_S124_NC_048013

ORF 147	86930..87540	Tail protein	PHAGE_Salmon_S124_NC_048013
ORF 148	87541..90390	Tail protein	PHAGE_Salmon_SH9_NC_047999
ORF 149	90391..92448	Tail protein	PHAGE_Salmon_S124_NC_048013
ORF 150	92454..92876	Tail protein	PHAGE_Salmon_S124_NC_048013
ORF 151	92876..96112	L-shaped tail fiber	PHAGE_Salmon_Stitch_NC_027297
ORF 152	96153..98243	Tail fiber protein	PHAGE_Salmon_vb Sens-3_MT004791
ORF 153	98534..98280	HP	PHAGE_Salmon_S131_NC_048009
ORF 154	99830..98995	Endonuclease	PHAGE_Salmon_Sw2_NC_048062
ORF 155	100312..99830	D 14 protein	PHAGE_Salmon_Sw2_NC_048062
ORF 156	102153..100315	Exonuclease	PHAGE_Salmon_Polluks_MT074456
ORF 157	103111..102134	Recombinase	PHAGE_Salmon_SH9_NC_047999
ORF 158	103924..103151	HP	PHAGE_Salmon_SH9_NC_047999
ORF 159	104201..103917	HP	PHAGE_Salmon_S114_NC_048006
ORF 160	105774..104422	DNA helicase	PHAGE Salmon_ vB_SenS_SB9_ MK867835
ORF 161	106268..105771	HP	PHAGE_Salmon_fuchur_NC_048869
ORF 162	108828..106261	DNA polymerase I	PHAGE_Salmon_atrejo_NC_048872
ORF 163	109781..108891	DNA primase	PHAGE_Salmon_S124_NC_048013
ORF 164	111301..109778	DNA helicase	PHAGE_Salmon_S124_NC_048013

ORF 165	112100..111333	Transcriptional factor	PHAGE_Salmon_S124_NC_048013
ORF 166	112737..112093	Tail completion protein	PHAGE_Salmon_S124_NC_048013

5.3.5 Phylogenetic analysis

To further understand the phylogenetic relationship of STWB21 to other *Salmonella* phages the two different types of phylogenetic trees were generated. As shown in Figures 5.5 A, and B these trees were generated based on the terminase large subunit (TerL) and the major capsid protein, respectively. The tree showed that the phage STWB21 clustered together with T5-like *Salmonella* phages, such as S124, Stitch, SH9, S113, Sea bear, SE3, and Sepoy. Therefore, the investigated phage was considered a new species of the T5 genus.

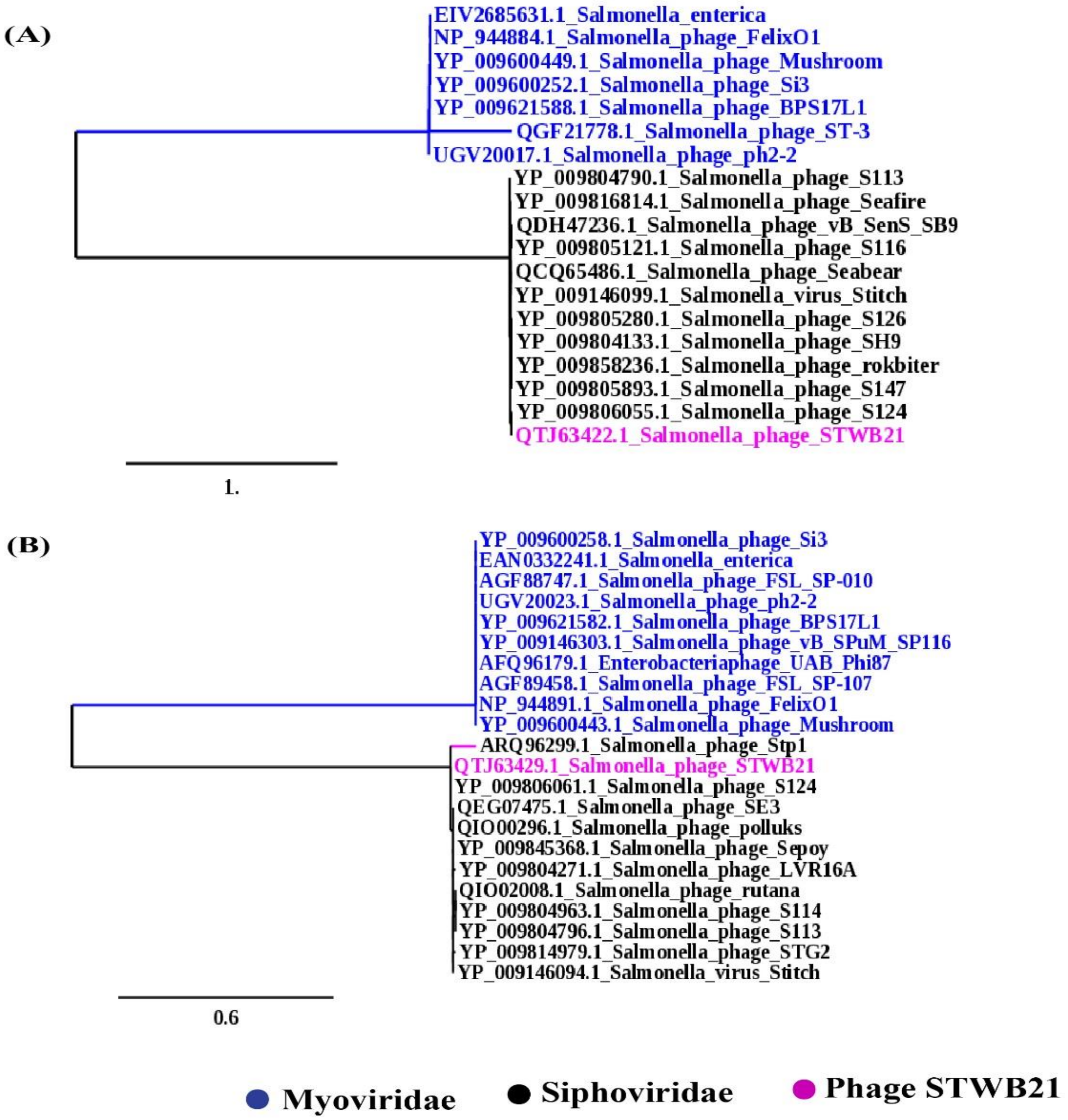


Figure 5.5: Phylogenetic analysis a) large terminase unit, b) major capsid proteins of *Salmonella* phage STWB21 with phages belonging to T4 and T5 family constructed using "One Click" at phylogeny.

5.4 Discussion

Bacteriophages possess an important role in evolutionary biology since they belong to a large percentage of all living things in the biosphere. Because phages have very small genomes, it would appear simple to understand the genetic diversity of the phage population, understand their origins, and identify the evolutionary processes that have shaped the population. However, over the past three years, the frequency of phage genome characterization decreased, largely due to the necessity to switch from sequencing well-known and well-characterized bacteriophages to isolating and analyzing novel isolates.

The current state of bacteriophage genomics demonstrates that the genetic diversity of a population is very substantial, that phages have been vigorously evolving for billions of years through horizontal genetic exchange, and that their genomes are thus pervasively mosaic in their architectures. However, we have only scratched the surface, and the next several years of phage genome investigation promise to be very illuminating.

Bacteriophage T5 is a member of a broad family of lytic *Siphoviridae* that infect Gram-negative bacteria. The low-resolution structure of T5 revealed the T=13 shape of the capsid, the peculiar trimeric organization of the tail tube, and the assembly pathway of the capsid. Although important structural proteins of T5 have been identified in these investigations, most of the genes encoding morphogenesis proteins have yet to be identified.

In our study, detailed genomic and proteomic analyses of phage STWB21 revealed valuable information concerning its biology and showed a modular organization that is very similar to other T5-like *Salmonella* bacteriophages S124, Gec_vB_N3, S133, Seabear, Stitch (Grover et al., 2015; Patil et al., 2019; Necel et al., 2020). Moreover, genomic analyses indicated that the phage STWB21 genome does not encode any toxin gene, antibiotic resistance gene, phage lysogeny factors, or pathogen-related genes, suggesting that the phage STWB21 may be considered a reliable phage therapy candidate with no side effects.

Bacteriophage STWB21 forms clear small plaques without any halo. In addition, the presence of host lysis proteins (holin and lysozyme) in its genome confirms that phage STWB21 is a virulent lytic phage. *Salmonella* phages vB_Sen-TO17 and vB_Sen-E22 reported by other groups were also confirmed to be lytic based on similar observations (Kosznik-Kwaśnicka et al., 2020).

The phage STWB21 harbors 22 tRNA genes corresponding to 14 different amino acids and has a significantly different codon usage than its host. Moreover, tRNA genes are usually considered indispensable information-related and housekeeping genes which are least susceptible to lateral gene transfer (Grover et al., 2015). In a previous study on *Siphoviridae* phages, it was found that there is no clear correlation between the number of tRNAs and burst size or latent period (Ding et al., 2020). Our result also supports the previous study as phage STWB21 has 22 tRNA but showed different latent periods and burst sizes for typhoidal and nontyphoidal *Salmonella* spp. The phylogenetic analysis led to phage STWB21 clustering with T5-like phages rather than with T4-like phages.

5.5 Chapter Summary

Genome analysis is rapidly being recognized as an essential requirement for ensuring the safety of phage preparation. Phage STWB21 contained a dsDNA of 112,834 bp in length, and the GC content was 40.37%. The detailed genomic studies of the phage STWB21 provided important details about its biology and demonstrated a modular structure that is closely related to other T5-like *Salmonella* bacteriophages S124, Gec_vB_N3, S133, Seabear, Stitch. Also, genomic analysis confirmed the presence of lytic genes and the absence of any lysogeny or toxin genes.

Chapter 6

Proteomic Characterization of phage STWB21

Chapter 6

6.1 Introduction

Bacteriophages are regarded as one of the most significant biocontrol agents for combating multidrug-resistant bacteria. Phages include two types of proteins: phage structural (such as phage virion proteins) and phage non-structural proteins (such as phage non-virion proteins). Non-structural proteins are produced by viral genes that take part in viral gene expression but do not bind to viral particles (Tan et al., 2018). Structural proteins are required to create mature and contagious virus particles including shell proteins, envelope proteins, virus particle enzymes, etc. Among these, the shell proteins are the proteins that make up the structure of the virus capsid and serve as a barrier between bacteriophages and the viral nucleic acid, among other things. The major purpose of envelope proteins, which make up the viral envelope structure, is to act as a viral surface antigen, which preserves the viral structure and takes part in virus budding, and other activities. Phage virion proteins (PVPs) adhere to the surface of the host and implant genetic elements into the host cell. As structural proteins and non-structural proteins have clearly different functions, identifying them correctly will help to further understand bacteriophage genetics and to develop antimicrobial agents (Lekunberri et al.,2017). Several experimental approaches, including protein arrays, gel electrophoresis, and mass spectrometry, have been employed to identify PVPs and non-PVPs.

Proteins are linear unbranched polymers with unique three-dimensional structures, known as the "native state." They have four levels of organization: primary, secondary, tertiary, and quaternary. The primary structure is determined by the amino acid sequence. The secondary structure is characterized by α-helices and β-sheets local conformations. The tertiary structure is known as the three-dimensional structure of an entire polypeptide chain and the three-dimensional arrangement of the subunits of a multisubunit protein is referred to as quaternary structure. Understanding the protein structure is crucial for any drug design (Gutnik et al., 2023).

Due to the availability of protein sequences in databases, current mass spectrometry (MS) methods allow sensitive and accurate protein identification. This chapter provides a brief introduction to MS techniques used in the identification of phage structural proteins and concentrates on an electron spray peptide ionization (ESI-MS/MS) strategy to identify the phage structural proteome comprehensively and methodically. Such studies allow for the experimental investigation of structural proteins and the confirmation of genome-based gene predictions (Lavigne R et al.,2009; Yuan Y et al., 2017).

Using cryo-electron microscopy, single-particle analysis, and X-ray diffraction of crystallized materials, the 3D protein structures have mostly been solved (Dubach and Guskov, 2020). Protein prediction is difficult, and several techniques are used, including as comparative modelling, threading, ab initio, and machine learning (Kuhlman & Bradley, 2019; Gutnik et al., 2023). New methods currently outperform older ones as a result of recent developments in end-to-end machine learning. A popular deep-learning method called AlphaFold2 (AlphaFold, AF2) from Alphabet-Google DeepMind uses experimentally confirmed PDB structures, protein primary sequences, and multiple sequence alignment to estimate protein distance and torsion distribution. AlphaFold predictions are crucial in biological and medical research, particularly in virology, which studies viruses, and their harmful proteins. Additionally, phages and their proteins that are harmful to bacteria can be used to treat bacterial infection in humans, animals and plants could be predicted by the AlphaFold (Brives & Pourraz, 2020). By simply using the amino acid sequence of the target protein and a deep learning-based algorithm called AlphaFold, which was recently published with its source code and model parameters (Jumper et al., 2021), it is now possible to predict protein structure with remarkably high accuracy.

To predict the phage structural proteins in this investigation, we evaluated the newly developed NN-based AlphaFold2 modeling approach. The predicted structures were then compared with the experimental structures obtained from the PDB.

6.2 Chapter aims

The major aims of this chapter were to:

i. Isolate and analysis of structural proteins from *Salmonella* phage STWB21.
ii. Proteomic analysis by Nano LC-MS/MS.
iii. The bioinformatics analyses including the models of some structural proteins of phage STWB21 generated by AlphaFold with high confidence scores.

6.3 Results

6.3.1 Analysis of structural proteins of phage STWB21 by SDS-page

SDS-PAGE analysis of phage STWB21 showed the presence of structural proteins ranging from 10 to 150 kDa. There are only two major bands that appeared at around 36 and 61 kDa while the visible minor bands appeared at 17, 41, 48, 74, 120, and 132 kDa (Figure 6.1).

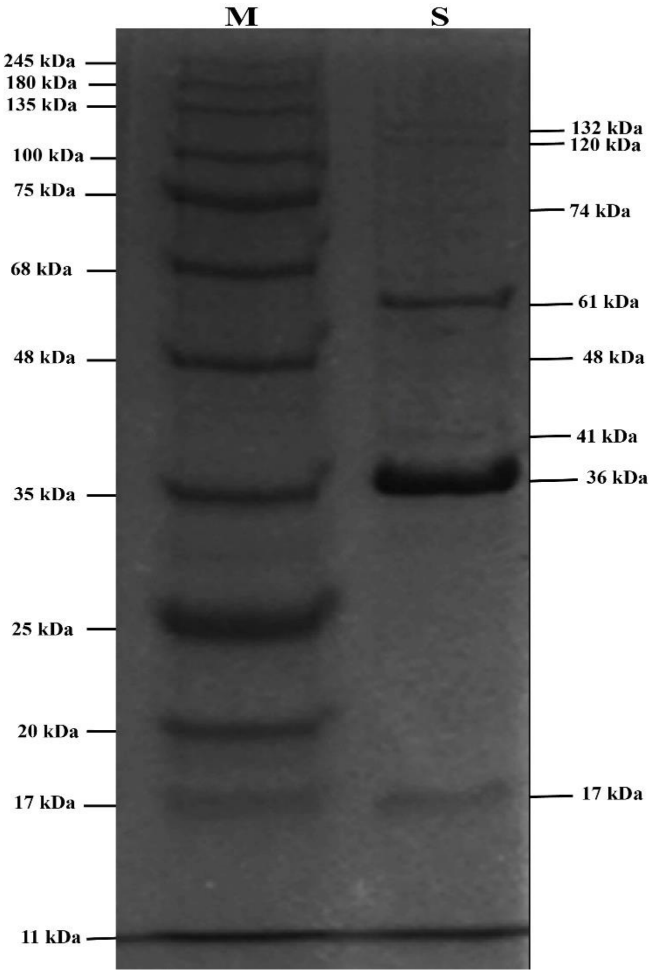


Figure 6.1: The SDS-PAGE analysis of phage STWB21 structural proteins on 12.5% gel staining with Coomassie brilliant blue. M, a standard marker of molecular weight (kDa).

6.3.2 Proteomic features

LC-MS/MS-based methods are more accurate than other methods because the phage peptides may be quickly identified without the need for further methods. A thorough proteomic study utilizing trypsin proteolysis and liquid chromatography-tandem mass spectrometry was carried out in order to identify the whole proteome.

To identify the structural proteins in phage STWB21, purified phage particles were analyzed by high-resolution Nano LC–MS/MS. A detailed proteomic characterization identified 19 proteins in phage STWB21 (Table 2). The structural proteins identified in phage STWB21 were six tail proteins, three tail fiber proteins, one prohead protease protein, one receptor-binding tail protein, one major capsid protein, one portal protein, and one capsid and scaffold protein. Interestingly, DNA metabolism module-related genes (A1 protein) and host lysis module-related genes (lysozyme) were also identified. It is believed that the expression of the A1 gene in the T5 phage is required for the second step transfer of DNA.

Table 6.1: Identification of the phage STWB21 proteins by LC_MS/MS

ORF	Protein	Molecular mass (Da)	No. of peptides	Sequence coverage (%)
1. ORF146	Tail protein	132066	35	40
2. ORF151	L-shaped tail fiber	114851	31	38
3. ORF142	Major tail protein	50322	21	57
4. ORF148	Tail protein	107179	20	30
5. ORF135	Tail fiber protein	16967	16	82
6. ORF152	Tail fiber protein	75110	8	20
7. ORF139	Hypothetical protein	19405	8	71
8. ORF118	A1 protein	61470	9	22
9. ORF149	Tail protein	74758	5	9
10. ORF97	Capsid and scaffold protein	18742	9	42
11. ORF137	Major capsid protein	23016	6	11

12. ORF143	Hypothetical protein	34437	6	34
13. ORF134	Portal protein	32976	6	28
14. ORF150	Tail protein	15508	5	49
15. ORF136	Prohead protease	23372	6	38
16. ORF128	Receptor binding tail protein	64149	2	6
17. ORF112	Hypothetical protein	36314	4	21
18. ORF80	Lysozyme	15236	3	27
19. ORF147	Tail protein	22658	2	5

6.3.3 Protein Structure Prediction

To compare and structurally align the predicted models, a TM-align analysis was carried out.

6.3.3.1 Phage head protein

Tailed dsDNA bacteriophages (phages) are widespread and highly diverse in the biosphere (Hendrix et al., 1999). The large (90-nm) icosahedral capsid of the T5 bacteriophage is made up of 775 copies of the major capsid protein (mcp), as well as portal, protease, and decorating proteins (Huet et al., 2019). The bacteriophage T5 mcp has an HK97-fold variation at high plasticity that enables accurate assembly of a massive macromolecule as well as the adaptability required to interact with other proteins and packaged DNA. An angular mature capsid is produced as a result of a tightly controlled assembly process that includes several intermediates, including a round thick-walled precursor prohead that expands during the packaging of viral DNA.

Capsid and Scaffold protein

Along with the primary capsid protein, other proteins such as a scaffolding protein, a portal, and occasionally a maturation protease are crucial for the assembly and operation of tailed dsDNA phage capsids. The scaffolding protein is crucial for controlling proper head formation, and its absence results in abnormal structures. In Figure 6.2, the STWB21 capsid and scaffold protein (ORF 97) model showed a structural similarity with the F chain of bacteriophage T5 non-decorated head protein (PDB ID: 6OMA), with an RMSD value of 2.9.

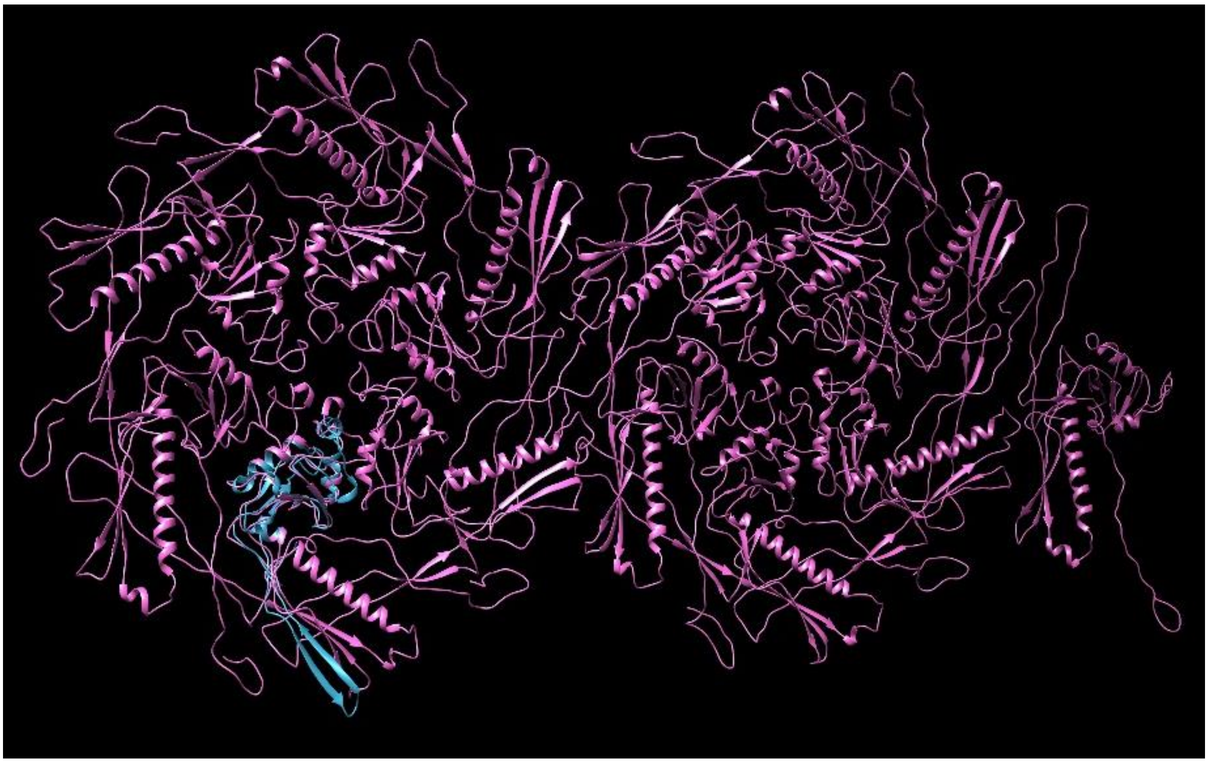


Figure 6.2: Structural alignment of the STWB21 capsid and scaffold protein model (cyan color) to the non-decorated head of the phage T5 electron microscopy structure (PDB ID: F chain of 6OMA, pink).

Portal protein

The presence of a single gateway complex, through which DNA is packaged and driven out, is a crucial component found in all bacteriophages. During assembly process of dsDNA bacteriophages with tails, the portal protein complex performs a unique role in capsid assembly, including DNA packaging, as well as portal ring assembly (Dedeo et al., 2019). In some bacteriophages, it serves as the nucleator for procapsid assembly (Murialdo et al., 1978). Portal proteins serve as DNA sensors for all bacteriophages, allowing the packaging and release of the genome in addition to controlling procapsid formation. Therefore, the portal complex is, indispensable as it plays a direct role in every step of phage assembly (Huet et al., 2010). The predicted model of the portal protein (ORF 134) of STWB21 was compared with the B chain of the non-decorated head of the bacteriophage G20C portal protein (PDB ID: 4ZJN) with an RMSD value of 4.9 (Figure 6.3).

Figure 6.3: Structural alignment of the STWB21 portal protein model (cyan color) to the non-decorated head of the bacteriophage G20C portal protein X-ray crystal structure (PDB ID: B chain of 4ZJN, pink).

Prohead proteases

The phage capsid includes a dodecameric portal complex, scaffolding domain, prohead proteases, and decoration proteins (Huet et al., 2019). Two processes are involved in the assembly of the heads of double-stranded DNA bacteriophages: first, capsid proteins generate a prohead (or procapsid) around a scaffold, and then, the prohead goes through a maturation phase to grow into the final, mature head (Dokland, 1999). The prohead protease gene is always found in the middle of the genes encoding the portal protein and the major capsid protein in phages (Liu & Mushegian, 2004). Prohead protease helps in the proteolytic cleavage and maturation process of the head during the development of the capsid of double-stranded DNA (dsDNA) bacteriophages (Cheng et al., 2004). In this study, the predicted model of the prohead proteases protein (ORF 136) of STWB21 was compared with the D chain of the T4 capsid assembly protease protein (PDB ID: 5JBL) with an RMSD value of 2.9 (Figure 6.4).

Figure 6.4: Structural alignment of the STWB21 prohead protease model (cyan color) to T4 capsid assembly protease X-ray crystal structure (PDB ID: D chain of 5JBL, pink).

6.3.3.2 Phage tail protein

The T5 tail is a flexible noncontractile tail tube 160 nm long, with a collar structure serving as a baseplate for three L-shaped fibers and a cone ending in a central fiber. It contains a multifunctional protein pb2, which plays the role of tape measure protein (TMP) and is likely involved in the channel through which the phage genome crosses the host envelope. The receptor binding protein (RBP) ensures the binding of T5 phage to the *E. coli* outer membrane protein FhuA, but its exact location remains uncertain.

Major tail protein

Long phage tails can either be noncontractile, as in the *Siphoviridae* (such as phage T5), or contractile, as in the *Myoviridae* (such as phage T4). The major tail protein (MTP), which is present in many copies throughout the tube of noncontractile tails, makes up most of the structure. The STWB21 major tail protein (ORF 142) model showed a structural similarity with the B chain of the major tail tube protein of bacteriophage T5 (PDB ID: 5NGJ), with an RMSD value of 3.2 (Figure 6.5).

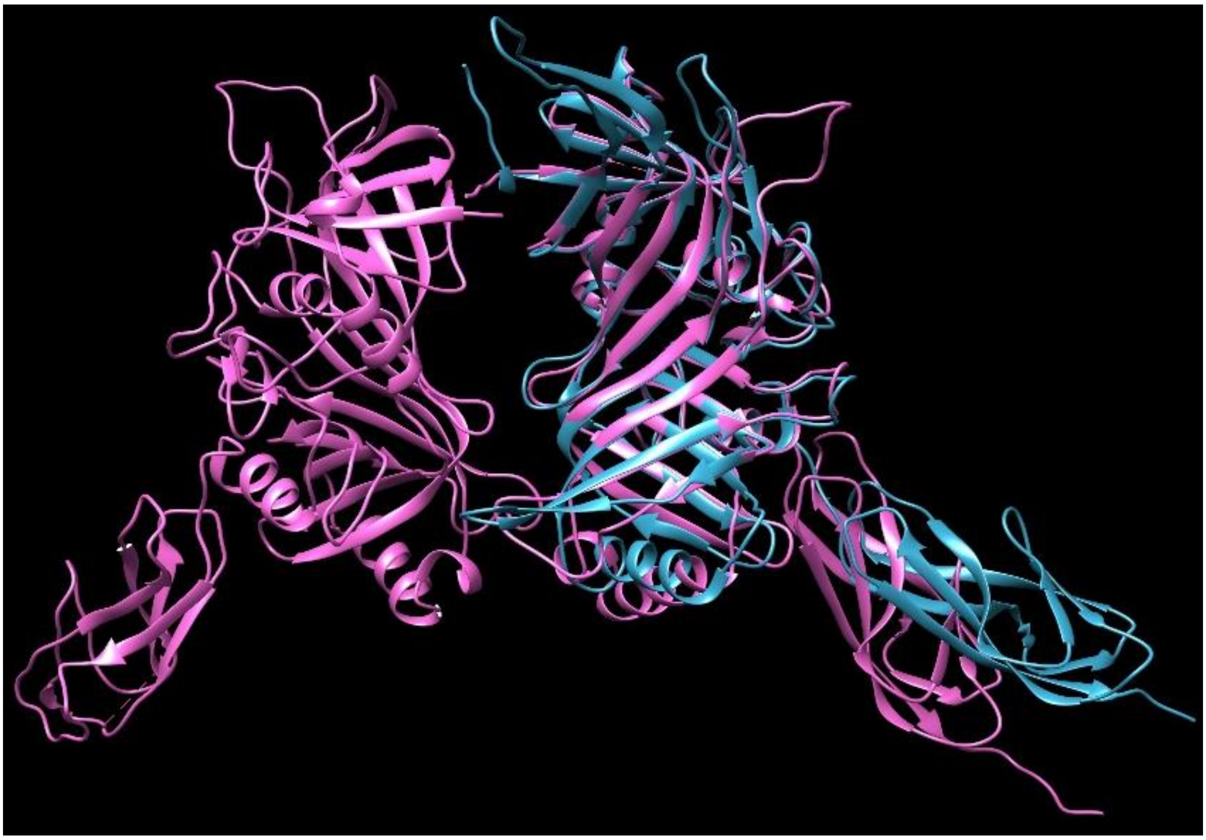


Figure 6.5: Structural alignment of the STWB21 major tail protein model (cyan color) to major tail tube protein of bacteriophage T5 X-ray crystal structure (PDB ID: B chain of 5NGJ, pink).

Tail protein

More than 95% of all bacteriophages belong to the order Caudovirales, often known as tailed bacteriophages. They all share a proteinaceous capsid with double-stranded DNA and a tail that helps in attachment, digestion, and penetration of the cell wall as well as genome ejection. The Caudovirales bacteriophages have three different functions for their tails: adsorption, cell wall perforation, and genome transport. The long and flexible tail of the *Siphoviridae* is assembled in a highly coordinated and controlled way, starting with the proteins that comprise the distal tail tip complex. The STWB21 tail protein (ORF 147) model showed a structural similarity with the A chain of the distal tail protein of the T5 bacteriophage (PDB ID: 4JMQ), with an RMSD value of 3.6 (Figure 6.6).

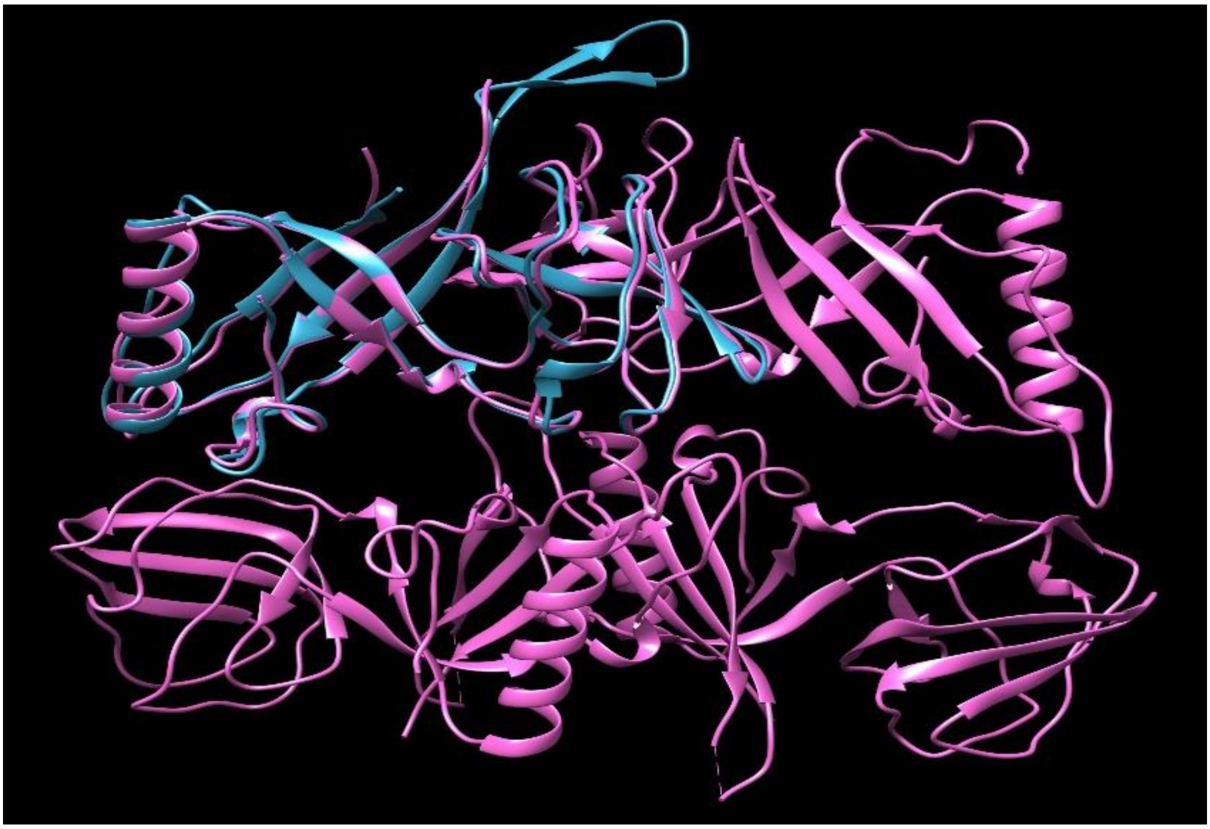


Figure 6.6: Structural alignment of the STWB21 T5 distal tail protein model (cyan color) to the distal tail protein of bacteriophage T5 X-ray crystal structure (PDB ID: A chain of 4JMQ, pink).

6.4 Discussion

Bacteriophage T5, which belongs to the *Siphoviridae* family, is composed of an icosahedral capsid with a large double-stranded DNA (dsDNA) molecule connected to a long, flexible noncontractile tail (Zivanovic et al., 2014). The studied phage STWB21 is also a T5-like bacteriophage that infects the Gram-negative bacteria *Salmonella*. Following LC-MS/MS analysis, 19 structural and functional proteins were identified such as Major capsid protein, Capsid and scaffold protein, Portal protein (ORF 134), Prohead protease (ORF 136), Tail protein (ORF 146, ORF 147, ORF 148, ORF 149, ORF 150), L-shaped tail fiber (ORF 151), Major tail protein (ORF 142), Tail fiber protein (ORF 152), Receptor binding tail protein (ORF 128), Hypothetical protein (ORF 112, ORF 139, ORF 143), A1 protein (ORF 118), Lysozyme (ORF 80).

Most of the bacteriophages have a tail, which facilitates host recognition, cell wall penetration, and viral DNA channeling from the capsid to the infected bacterium cytoplasm (Linares et al., 2023). Phage STWB21 tail consists of a flexible noncontractile tail tube 113 nm in length. According to earlier research, the distal tail tip of the T5 phage is made up of three long L-shaped fibers (LTF), a cone that ends in a central fiber, and a collar structure that acts as a baseplate for the LTFs (Kaliman et al., 1995). The majority of other *Siphoviridae* have a hexameric organization. However, the T5 tail tube has an unusual 3-fold symmetry and is made up of a stack of trimeric rings of the tail tube protein (TTP) pb6 (Effantin et al., 2006). The multifunctional and oligomeric protein pb2, which serves as the tape measure protein (TMP), is in the central core of the tail tube (Zivanovic et al., 2014). Using LC-MS/MS analysis, 10 different tail proteins of phage STWB21 were identified such as Receptor binding tail protein (ORF 128), Tail protein (ORF 146, ORF 147, ORF 149, and ORF 150). These proteins are mostly associated with phage-host interaction and infection mechanisms.

The structural biology area has developed several methods (including cryo-electron microscopy, nuclear magnetic resonance, and X-ray crystallography) over the past few decades. With the aid of significant technological advancements that have considerably advanced the area of structural biology, the "resolution revolution" in cryo-EM began about ten years ago (Kühlbrandt 2014). As these techniques developed over time, it became easier for us to understand key molecular details of critical biological processes. The effectiveness of all these methods varies depending on access to equipment/facility, the nature of the sample, and expertise. Additionally, these methods are time-consuming and costly. Therefore, the development of machine learning structure prediction algorithms like AlphaFold is an alternate and quicker method that is currently being used by the scientific community to gain a quick concept of the structure of various proteins. By utilizing evolutionary information from multiple-sequence alignments of homologs, AlphaFold2 generates a three-dimensional (3D) structure of a protein from the amino acid sequence (Zhang and Skolnick 2004). With the help of AlphaFold, we have been able to predict the 3D protein structure of phage STWB21 with remarkable accuracy using only the targeted amino acid sequences in this study.

In this study five structurally relevant phage STWB21 proteins were selected and their structures were predicted using the novel neural network-based method AlphFold2 such as capsid and scaffold protein (ORF 97), portal protein (ORF 134), prohead proteases (ORF 136), major tail protein (ORF 142) and another tail protein (ORF 147).

6.5 Chapter Summary

Protein structure and function are frequently predicted using homology, or evolutionary relationships. Protein structure modelling is a crucial job that supports both basic and practical virology research. The continued development and enhancement of AlphaFold ensure that it will continue to make a substantial contribution to antiviral research in the future. The development of new drugs as well as research into viral pathogens and the mechanics of viral infection can both benefit from the application of the AlphaFold deep-learning algorithm, which has been shown to be a highly accurate prediction tool. In this study, several structural and genetic features of phage STWB21 have been revealed that lead to a deeper understanding of the phage structure and its assembly.

Chapter 7

Assess the capacity of the characterized phage STWB21 to control the Salmonella biofilm

Chapter 7

7.1 Introduction

In the natural environment, biofilms are the most predominant form of microbial life. A large number of bacteria can develop as single planktonic cells or as communities within a biofilm (Flemming and Wuertz, 2019). By establishing symbiotic relationships with other species, such as marine invertebrate larvae settling (Nelson et al., 2020), decomposing harmful substances in the soil (Ahmad et al., 2017), and supporting plant development (Liu et al., 2013), biofilms provide a stable home for bacteria. Biofilms are surface-attached clusters of cells surrounded by extracellular polymeric substances (EPSs) such as proteins, exopolysaccharides, and nucleic acids secreted by the cells themselves (Costerton et al., 1999; Branda et al., 2005; Hall-Stoodley and Stoodley, 2009; Flemming, 2016). These structures usually develop as a result of perceived unfavorable environmental cues such as pH, temperature, oxygen, and changes in nutrient supply (O'Toole and Kolter, 1998; O'Toole et al., 2000a; O'Toole et al., 2000b). They develop and mature in this niche, at that time some cells detach and return to planktonic growth to continue the process (Khatoon et al., 2018). Inside a biofilm, bacteria are shielded against a range of threats, including UV radiation (Espeland and Wetzel, 2001), host defense systems, and antibiotics (Mah and O'Toole, 2001; Stewart and Costerton, 2001). Additionally, bacteria within a biofilm are more prone to resistant to antibiotics compared to those in planktonic bacterial cells (Hall and Mah, 2017). As a result, biofilms play a critical role in the leading cause of chronic infections in the environment, industry, and medicine. Therefore, the biofilm-mediated infection has become a public health concern. For treatment purposes, newly synthesized drugs and combinational therapy have been developed but satisfactory results are yet to be achieved.

Food-borne illnesses caused by bacterial biofilms on the matrix of food or manufacturing equipment can manifest as intoxications or infections. Toxins, for instance, can be released by biofilm found in food processing plants. They can then contaminate a food matrix, producing individual or many (such as, in the circumstance of an outbreak) intoxications. Therefore, the food-borne disease has a significant influence on public health and is economically significant. In any situation, the existence of biofilms in a food manufacturing facility endangers human health. The bacterial species comprising this three-dimensional living structure determine the threat level. Water, milk, and other liquid pipelines, pasteurizer plates, reverse osmosis membranes, tables, employee gloves, animal carcasses, contact surfaces, storage silos for raw materials and additives, dispensing tubing, packing material, and other locations for biofilm development vary depending on the factory type (Camargo et al., 2017). Additionally, the benefits of consuming fresh vegetables for health and nutrition have been established and they are essential parts of a balanced diet for humans. This has caused considerable changes in lifestyles, a significant increase in the demand for fresh produce, and a significant shift in consumption patterns (Abadias et al., 2008; Tang et al., 2012). According to Mukherjee et al. (2006), while still in the fields, vegetables can get infected with bacteria that might cause human illnesses.

Salmonella is one of the commonly known bacterial species that form biofilms (Done et al., 2015). It has also been reported that bacterial biofilm can significantly contribute to pathogenesis by creating resistance against the defense system of the body (Bai et al., 2021). The capability of *Salmonella spp.* to form biofilm is generally connected with their pathogenicity (Vestby et al., 2020). *Salmonella* adheres to and forms biofilms on a variety of abiotic and biotic surfaces (Merino et al., 2019; Moraes et al., 2019). *Salmonella* adhesion is the first stage in biofilm development; depending on environmental circumstances, cells can adhere to a surface in minutes or hours (Sadekuzzaman et al., 2015). Besides their composition, the EPS of the *Salmonella* biofilm matrix has been characterized based on their function. Structural EPS is the largest and most important category in this categorization for disinfectant resistance. Polymers like as cellulose, curli, colanic acid, and proteinaceous O-antigen are used to make structural EPS (Maruzani et al., 2018). Bacteria in biofilms are also resistant to antibiotics, lasting 10 to 1,000 times longer than planktonic cells (Hoiby et al., 2010; Mah, 2012). Studies have also revealed the presence of non-dividing, metabolically inactive persister cells, which are identical to the rest of the bacterial cells and are resistant to a variety of antibiotics within the biofilm (Lewis, 2005). Antibiotics are incapable of damaging bacterial cells because of the biofilm's matrix (Cerca et al. 2006; Jefferson et al., 2005). As a result, the treatment process becomes very difficult.

Lytic bacteriophages have some unique properties that allow the removal of biofilms effectively. There are several reports on the anti-biofilm properties of lytic phages both *in vitro* and in clinical infection (Azeredo and Sutherland, 2008; Doub, 2020). According to Harper et al. (2014), a phage produces a depolymerase enzyme that can destroy the EPS matrix of the biofilm. Phage application to prevent food-borne infectious illnesses has recently been proposed (Greer GG., 2005; Hudson et al., 2005). Despite significant advancements in hygiene and sanitation in food processing, many infections continue to occur. *Salmonella* spp. (Modi et al., 2003), *Listeria monocytogenes* (Guenther et al.,2009), *Campylobacter jejuni* (Goode et al., 2003), and *E. coli O157:H7* (Sharma et al., 2009) have all been employed to cause food-borne illnesses. The *L. monocytogenes*-specific phage Listex P100 (Bren L., 2007), which was authorized as a food preservative by the US Food and Drug Administration, recently received the classification of generally recognized as safe (GRAS). Therefore, researchers have been inspired to examine the development of phages for biocontrol.

7.2 Chapter aims

The major aims of this chapter were to:

i. To investigate the lytic activity of the phage STWB21 in the formation and degradation of *S.* Typhi biofilms on the surface (coverslip).
ii. In order to determine *Salmonella* adhesion and pathogenicity, this study investigated the contamination of two essential vegetables (onion and tomato) and pasteurized milk.

7.3 Results

7.3.1 Biofilm degradation assay and scanning electron microscopy analysis

In coverslip

In the LB medium at 37°C under static conditions for 24 hours, *S.* Typhi produced a biofilm on the coverslip (Figure 7.1A & 7.1B). Due to the early formation of extracellular polymeric molecules, at 24 hours of development, greater-density cell agglomerations were observed and the cell boundaries were not clearly delineated in certain locations.

Micrographs of the surfaces that had been exposed to the bacteriophages revealed altered morphology in most of the cells as a result of the lytic activity of the bacteriophages, and some unaltered cells, probably as a result of the development of phage-resistant cells or the incapacity of the bacteriophage to infect the cell and reach its receptor (Figure 7.1C & 7.1D).

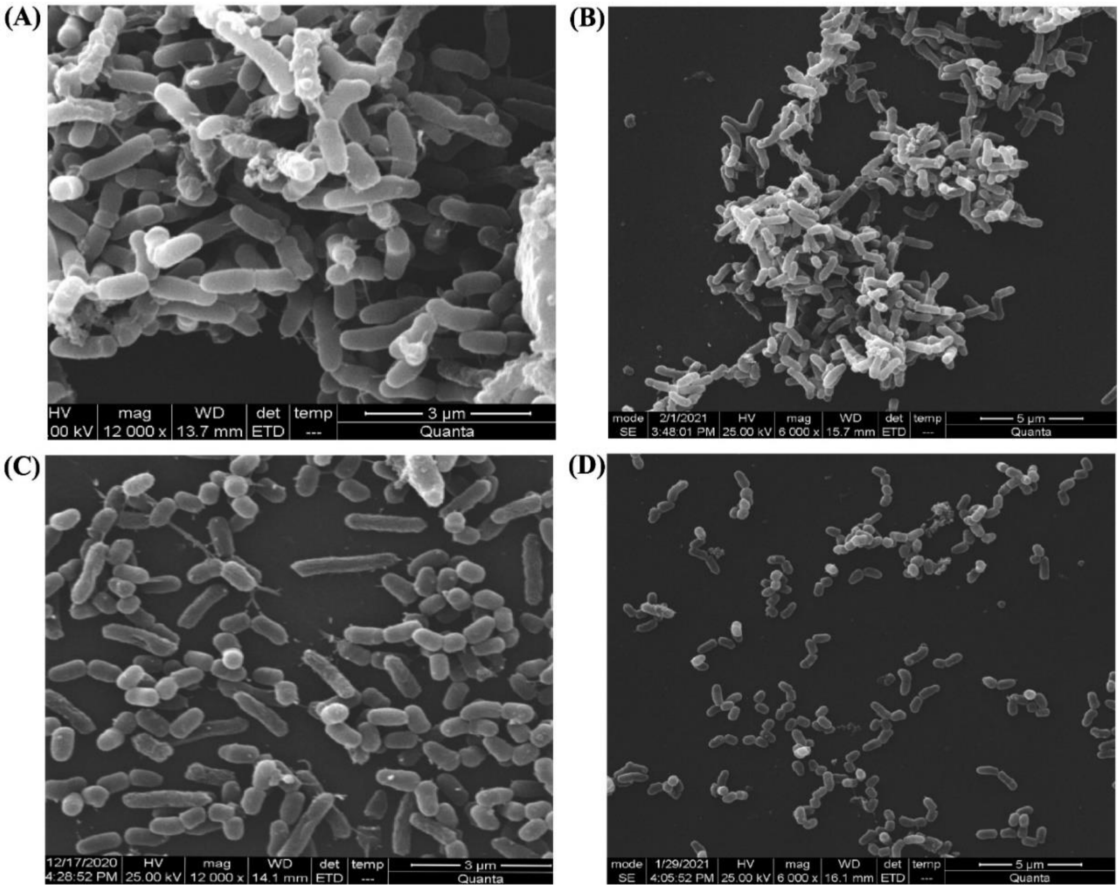


Figure 7.1: Scanning electron microscopy of *Salmonella* Typhi biofilm grown on the coverslip under static conditions at 37°C for 24 hours. Control untreated: (A) High magnification (12000X) and (B) Low magnification (6000X). Treatment with phage: (C) High magnification (12000X) and (D) Low magnification (6000X).

On onion & tomato

Using SEM, the biofilms developed on onion (Figure 7.2A) and tomato (Figure 7.3A) were observed both before and after phage treatment. Untreated onions and tomatoes displayed extensive biofilms that were extremely dense and heavily colonized by adherent cells.

Treating the biofilms with phages reduced the cell density to the extent that only a small number of cells were visible in the prominent areas in Figure 7.2B for onion and Figure 7.3B for tomato.

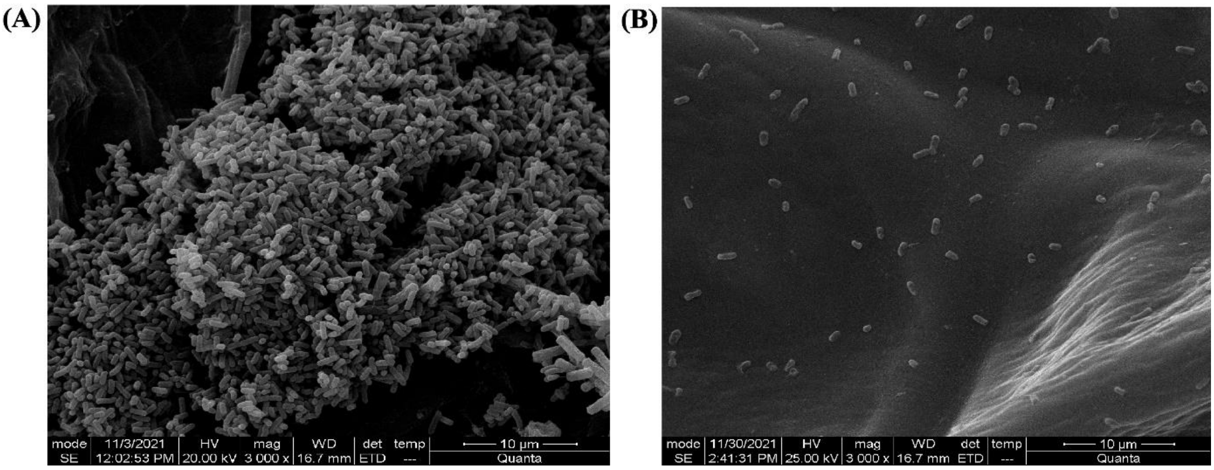


Figure 7.2: Scanning electron microscopy of *Salmonella* Typhi biofilm grown on the red onion under static conditions at 37°C for 24 hours. (A) Control untreated (magnification 6000X). (B) Treatment with phage (magnification 6000X).

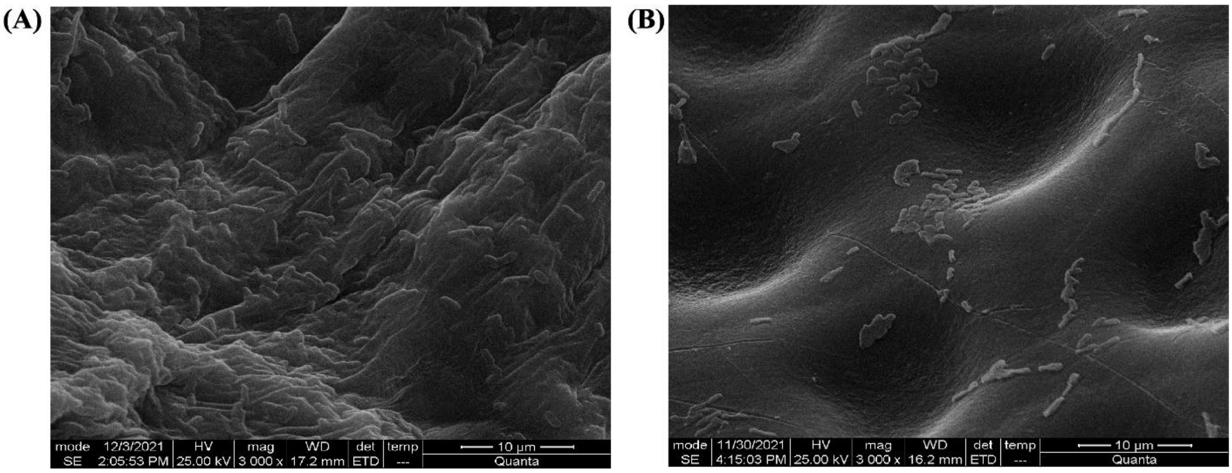


Figure 7.3: Scanning electron microscopy to show the effect of phage on biofilms grown on tomato at 37 °C for 24 h. (A) Biofilm on tomato surface (control). (B) Biofilm on tomato surface after phage treatment (magnification 3000X).

In milk

By using SEM, the bacterial biofilm of *S.* Typhi microstructure in the milk medium was examined. Diffuse extracellular material was observed in the biofilms that were developed in milk as a result of the aggregation of bacterial cells (Figure 7.4A). After treatment with the STWB21 phage, a substantial decrease in the bacterial population of the *S.* Typhi biofilm was observed (Figure 7.4B).

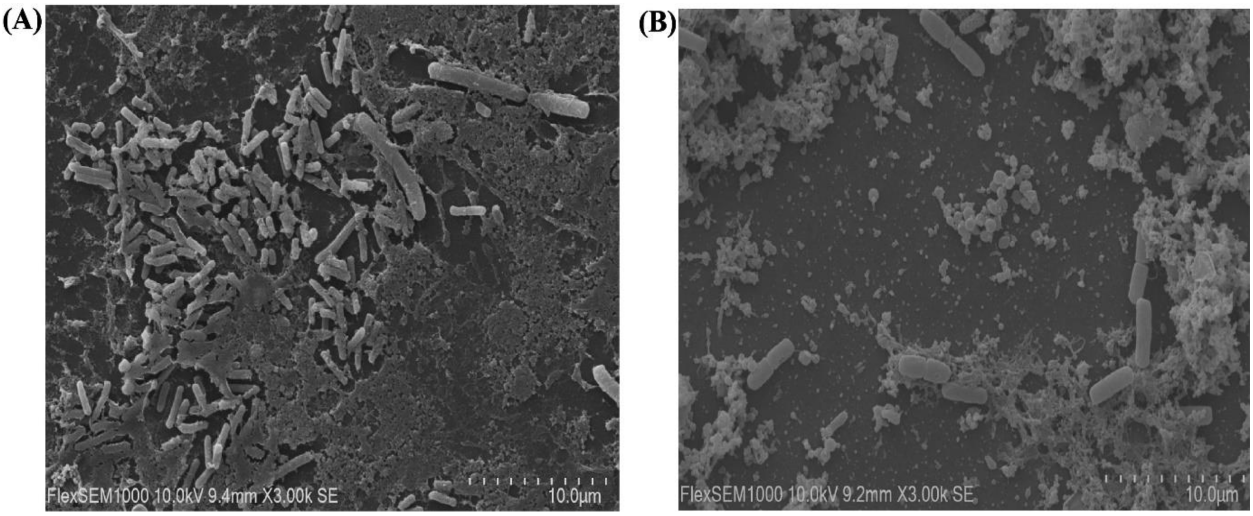


Figure 7.4: Scanning electron microscopy images: (A) Biofilm of *S.* Typhi in milk. (B) Biofilm in milk after phage treatment (magnification 3000X).

7.3.2 Quantification of *Salmonella* biofilm in a microtiter plate to determine the optimum contact time for phage treatment

In order to determine the optimum contact time for phage treatment, preliminary experiments were conducted using phage STWB21 treatment of *S.* Typhi in a microtiter plate (Figure 7.5). Phage-treated glass and controls were incubated at ambient temperature for between 10 min The mature biofilm of *S.* Typhi strain was significantly reduced by STWB21 phage. As shown in Figure 7.5 biofilm formation was significantly decreased ($p < 0.001$) by phage STWB21 at different concentrations (MOI 0.1 and 0.01). After 4 h, phage STWB21 removed 31% ($p < 0.001$) and 36% ($p < 0.001$) of the biofilm biomass at MOIs 0.1 and 0.01 respectively, when compared to the control. However, after 24 hours of treatment, the above-mentioned values changed to 68% ($p < 0.001$) and 37% ($p < 0.001$) respectively.

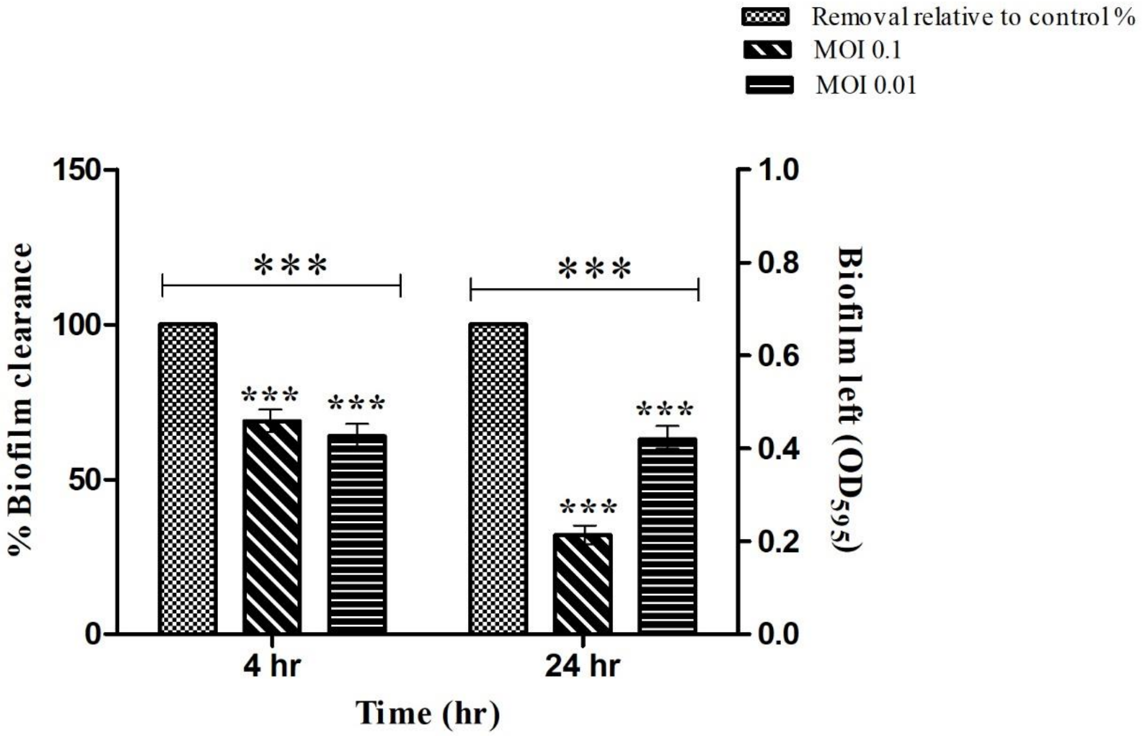


Figure: 7.5: Quantification of *Salmonella* biofilm in Microtiter Plate. A 24h biofilm of S. Typhi formed in 96-well plates was treated with phage STWB21 at MOI 0.1 and 0.01 concentrations both at 4h and 24h time points.

7.4 Discussion

Biofilms are extremely different and unique to both the organism that makes them as well as the specific environment in which they are developing. This makes *in vitro* characterization of biofilms challenging and necessitates the establishment of laboratory settings that closely resemble the natural environment being researched. Because the microenvironment in which the bacteria infect or proliferate may be impossible to mimic, the resemblance of the natural environment is likewise highly difficult. It is interesting to note that many of the signals that determine the biofilm's constituent parts appear to come from the media and support matrix (Prouty & Gunn, 2003).

In October 2021, food poisoning outbreak news came out from California, New York, North Carolina, Pennsylvania, and Texas among adults due to the consumption of imported whole red, white, and yellow onions. Although there are published reports controlling the biofilm on coverslips, fruits, vegetables, and pasteurized milk using bacteriophages (Isoken, 2015; Amrutha et al., 2017; Wang et al., 2018). However, no reports are available yet using phages that can control *Salmonella* spp. biofilm formation on the red onion.

Salmonella may develop slowly in milk and can persist at storage temperatures below 10°C. Recent research, including the suppression of *P. lactis* (Tanaka et al., 2018), *S.* Typhimurium (Phongtang et al., 2018), and *E. coli O157: H7* (Grygorcewicz et al., 2020, have demonstrated the use of phage as an efficient control agent against bacteria in milk. In this investigation, after 24 hours of incubation, *Salmonella* levels remained optimum in the absence of phage, but phage-treated bacteria gradually declined without promoting secondary bacterial development. This bacteriolytic capacity of the phage STWB21 in the milk was observed by the decrease in viable bacterial cells after three hours of incubation. These results support the use of bacteriophages as an alternative, antibiotic-free strategy to reduce bacterial contamination in milk.

In this study, we evaluated the ability of lytic phage STWB21 to remove biofilm from glass coverslips, red onion, tomato, and pasteurized milk. After treatment with phage STWB21, the number of bacteria in the biofilm decreased significantly. Moreover, biofilms were considerably degraded and disrupted and did not show any microbial regrowth. Therefore, anti-biofilm results support that phage STWB21 can be used as a “biological disinfectant” capable of controlling *Salmonella* biofilm infections.

7.5 Chapter Summary

Salmonella is a foodborne pathogen, the phage STWB21 was applied to treat a 24-hour biofilm formed in coverslip, red onion, tomato, and milk under laboratory conditions. A significant reduction was observed in the bacterial population of *S.* Typhi biofilm. This study revealed that bacteriophages STWB21 could be useful in disrupting, inactivating, and destroying the viability of cells attached to biofilms. As a result, it could be a beneficial method for limiting *S.* Typhi proliferation in biofilms. Indeed, bacteriophages could be an essential part of an ideal, environmentally friendly biocontrol intervention approach to manage biofilm-mediated infections in many industries.

Chapter 8

Investigate the capacity of the characterized phage STWB21 to control the Salmonella spp. in mice model

Chapter 8

8.1 Introduction

Salmonella enterica, a gram-negative bacterium that belongs to the Enterobacteriaceae family, is generally divided into typhoidal *Salmonella* (TS) and non-typhoidal *Salmonella* (NTS) serotypes based on clinical symptoms (Feasey et al.,2012). A potentially fatal multisystemic infection, typhoid fever or enteric fever, is primarily produced by the human-adapted pathogen *Salmonella enterica* serovar Typhi and significantly contributes to global morbidity and mortality in low-and middle-income countries (Als et al., 2018). *Salmonella* Typhi is a common foodborne pathogen that is primarily present in poultry, eggs, and dairy products (Silva et al., 2011). It may be transferred by human feces, contaminated food, water, and person-to-person contact. The fecal-oral route is the most prevalent way of transmission (Mogasale VV et al., 2018). The risk of morbidity and death associated with typhoid fever can be decreased by treating the condition with appropriate antibiotics.

During acute infection of *S.* Typhi shedding, the dosage and duration are important determinants in antibiotic therapy (Gibani et al., 2019). According to the WHO priority list of pathogens, *Salmonella* belongs to the highest priority pathogens group that needed attention to search for new antibiotics. Fluoroquinolone, ampicillin, trimethoprim-sulfamethoxazole, and chloramphenicol made up the conventional typhoid fever treatment regimens (Crump et al., 2011; Sjölund-Karlsson et al., 2011). Nevertheless, most of the bacterial strains are now found to be resistant due to the development of multidrug-resistance mechanisms. The percentage of typhoid fever isolates that were nalidixic acid-resistant was reported to be over 60% in Kolkata (Azmatullah et al., 2015). As a result of this, third-generation cephalosporins are now being used more often. Moreover, first- and second-line antibiotics and both fluoroquinolones and third-generation cephalosporins-resistant *Salmonella* strains have been reported in Nepal and Eastern India (Shrestha et al., 2016, Hendriksen et al., 2011, Samajpati et al., 2021). The World Health Organization (WHO) has given an estimate that by 2050 drug-resistant infections will kill millions of people every year (de Kraker ME et al., 2016). The increase in treatment costs due to the unavailability of a suitable antibiotic will create a huge economic burden and may push millions of people into acute poverty (Zhen et al., 2019). This is minacious to mankind and requires to be handled with an unconventional treatment strategy such as phage therapy (Shrestha et al., 2016; Wain et al., 2003).

Bacteriophages are bacterial viruses, abundant in nature, highly specific, and effective in killing their targeted host bacteria (Fernandez et al., 2019). The mechanism of killing bacteria and developing bacterial resistance in the case of phage and antibiotics is fundamentally different (Sulakvelidze et al., 2001; Lu et al.,2011; Merril et al., 2003). The advantage of phage usage in the prevention of bacterial infections is due to their strict host specificity, high bactericidal efficiency, safety, low cost of production, and ease of extraction and preservation (Miedzybrodzki et al., 2007; Haq et al., 2012). However, the therapeutic efficacy of phage treatment has been explicitly demonstrated by only a handful of successful clinical trials that adhered to the current norms of evidence-based medicine (Schooley et al., 2018). Therefore, to conduct a trial with a greater possibility of success, it is crucial to analyze carefully the data obtained from experimental trials in animals.

In vivo investigation using laboratory animals is an essential component of research on determining the efficacy and safety of novel isolated bacteriophages (Cieślik et al., 2021). An accurate assessment of the mechanism of phage therapy in living organisms can be preferred using animal models such as mouse, rat, and rabbit models because of their genetic closeness to humans. These models also provide information on the immune response (including any potential interactions with immune system cells like phagocytes), the gut microbiota, and infected tissue, as well as the extent of safety, tolerability, and observation of any possible adverse effects of the preparation being used. (Hsu et al., 2019, Brix et al., 2020).

8.2 Chapter aims

1. To develop a model for *Salmonella* infection while optimizing the LD_{50} for the *Salmonella* Typhi strain.
2. To determine the treatment regimen that would be most effective in getting rid of *Salmonella* in the mice model and to convert the knowledge acquired on the phage activity *in vitro* into a possible use *in vivo*.
3. To investigate the bacterial proliferation and systemic tissue inflammation in *S.* Typhi-infected mice treated with a single oral administration of phages that includes direct visualization of histopathology and ultrathin section microscopy images from the liver and spleen sections.

8.3 Results

8.3.1 Phage propagation

After overnight incubation, the plaques of phage on the HEA plate with *S.* Typhi bacterial lawn were measured approximately 1 mm in diameter. It indicates the presence of *S.* Typhi-specific phage STWB21 (Figure 8.1).

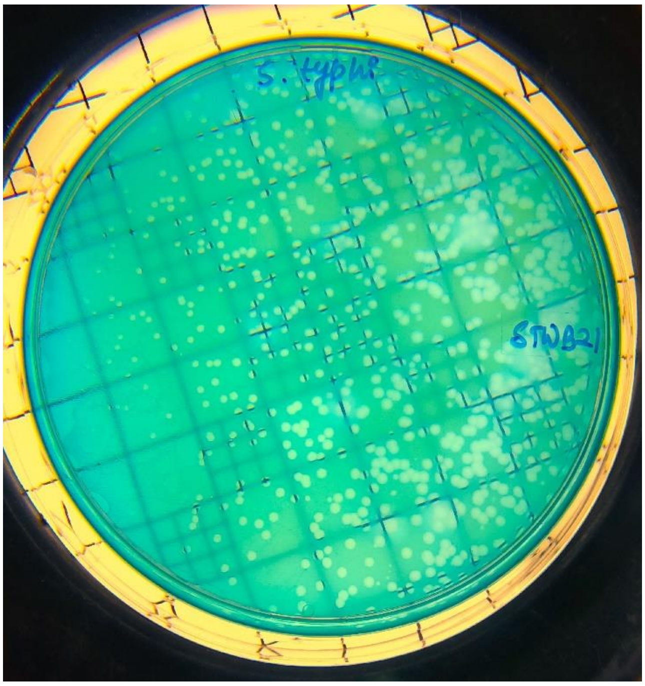


Figure 8.1: Plaques of STWB21 against *S.* Typhi on *HEA* plate

8.3.2 Determination of LD_{50}

All infected mice in the LD_{50} determination study developed illness starting at the post-challenge. Only a few mice in groups 1, 2, and 3 were still sick at the end of the experiment, and death was nil or minimal. However, significant deaths occurred in groups 4 and 5. From the post-challenge day onwards, these mice began to perish, and by the conclusion of the trial, every mouse that had survived was gravely sick. Figure 8.2 shows the mortality of the mice on the fifth day following infection. The mean fatal dosage was determined to be 3 × 10^7 CFU/mice based on the observation.

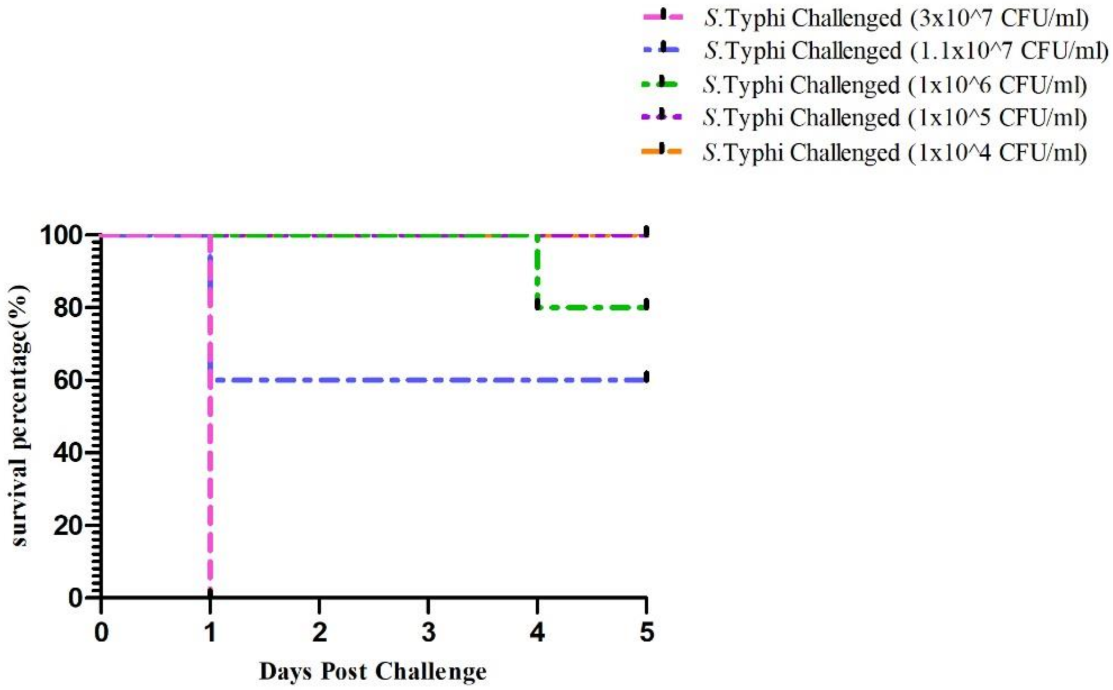


Figure 8.2: Determination of the Mean lethal dose (LD_{50}) of *S.* Typhi host strain in BALB/c mice n=6; each group was intraperitoneally (i.p) infected with serially diluted bacterial suspensions of *S.* Typhi. The percentage of survival was determined for 7 days.

8.3.3 Oral treatment with phage STWB21 significantly reduced *Salmonella* proliferation in mice

The significant effectiveness of phage STWB21 against the *S.* Typhi infection was examined in a mouse model. The effect of phage indicated decreased colonization in both the prevention group (or prophylactic group) and the treatment group (or therapeutic group) as shown in Figure 8.3A & Figure 8.3B. Additionally, after observing 4 days, the survival rate of the prevention group is almost 66% (Figure 8.3C) in comparison with the treated group which is almost 33% (Figure 8.3D). However, the viability of the phage control group was similar to the PBS control group, indicating the safety of phage STWB21.

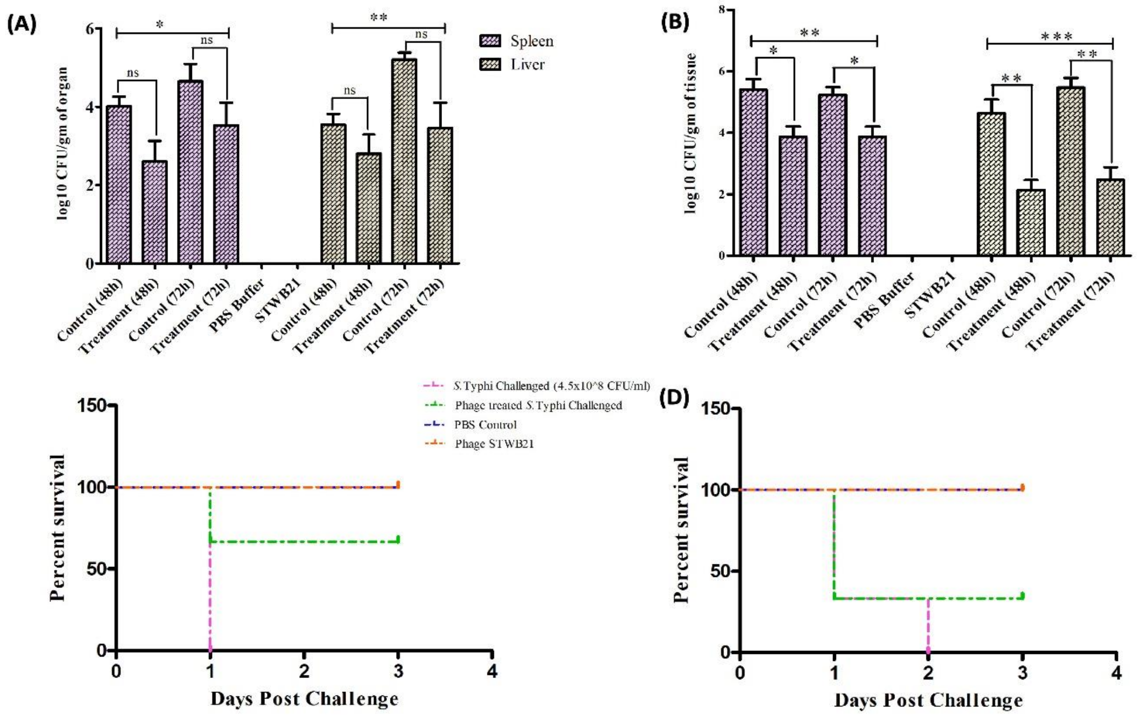


Figure 8.3: (A) Colonization of prophylactic treatment. (B) colonization of therapeutic treatment. (C) Impact of single dose phage treatment on *S.* Typhi before infection (prophylactic treatment) and survival rates of BALB/c mice. (D) Impact of single dose phage treatment on *S.* Typhi after infection (therapeutic treatment) and survival rates of BALB/c mice. A statistically significant difference (*P < 0.05) was observed. Error bars represent the standard error of the mean (SEM) of three independent replicates and data were analyzed using GraphPad Prism 5.0.

8.3.4 Comparison of preventive versus therapeutic phage treatment using light microscopy and transmission electron microscopy

A comparative study was carried out to evaluate the effectiveness of phage STWB21 treatment prophylactically and therapeutically. Figure 8.4 & Figure 8.5, showed the light microscopy images of histopathological sections and electron microscopic images of the stained ultrathin sections of mice liver tissue respectively. In Figure 8.4A, the liver section of normal mice showed normal histology with central vein and hepatocytes arranged in the hepatic cords, and in Figure 8.5A, a round nucleus (NU), normal mitochondria (MT), and endoplasmic reticulum (ER) were observed. However, in infected mice, pyknotic nuclei (PN) with the appeared nucleolus, mitochondria (M), and fragmented rough endoplasmic reticulum (RER), liver necrosis, and abscess were observed with hepatomegaly in Figures 8.4B & 8.5B. Transmission electron micrographs of both the preventive group and therapeutic group (Figures 8.5C & 8.5D) showed an elevation in lysosomes and mitochondria levels and an increase in their density too. Interestingly, the preventive group showed minimal damage rather than the therapeutic group.

The spleen is a large secondary lymphoid organ and it is composed of two compartments: red pulp and white pulp. Figure 8.6, showed the histopathological sections of the spleen tissue. A clear distinction between the red and white pulp, lymphoid follicles, and marginal zones was observed in the spleen of normal mice in Figures 8.6A & 8.7A.

However, the *S.* Typhi-infected spleen in Figures 8.6B & 8.7B showed severe congestion and enlarged red pulp, splenomegaly, inflamed central artery, lymphoid follicle hyperplasia, and eventually disruption of white pulp structure. But in the preventive group (Figures 8.6C & 8.7C) and therapeutic group (Figures 8.6D & 8.7D), significant changes were observed. In the therapeutic group, the white pulp structure was eventually disrupted but not as severe as in the infected group. In contrast, the preventive group showed less structural disorganization compared to the therapeutic group.

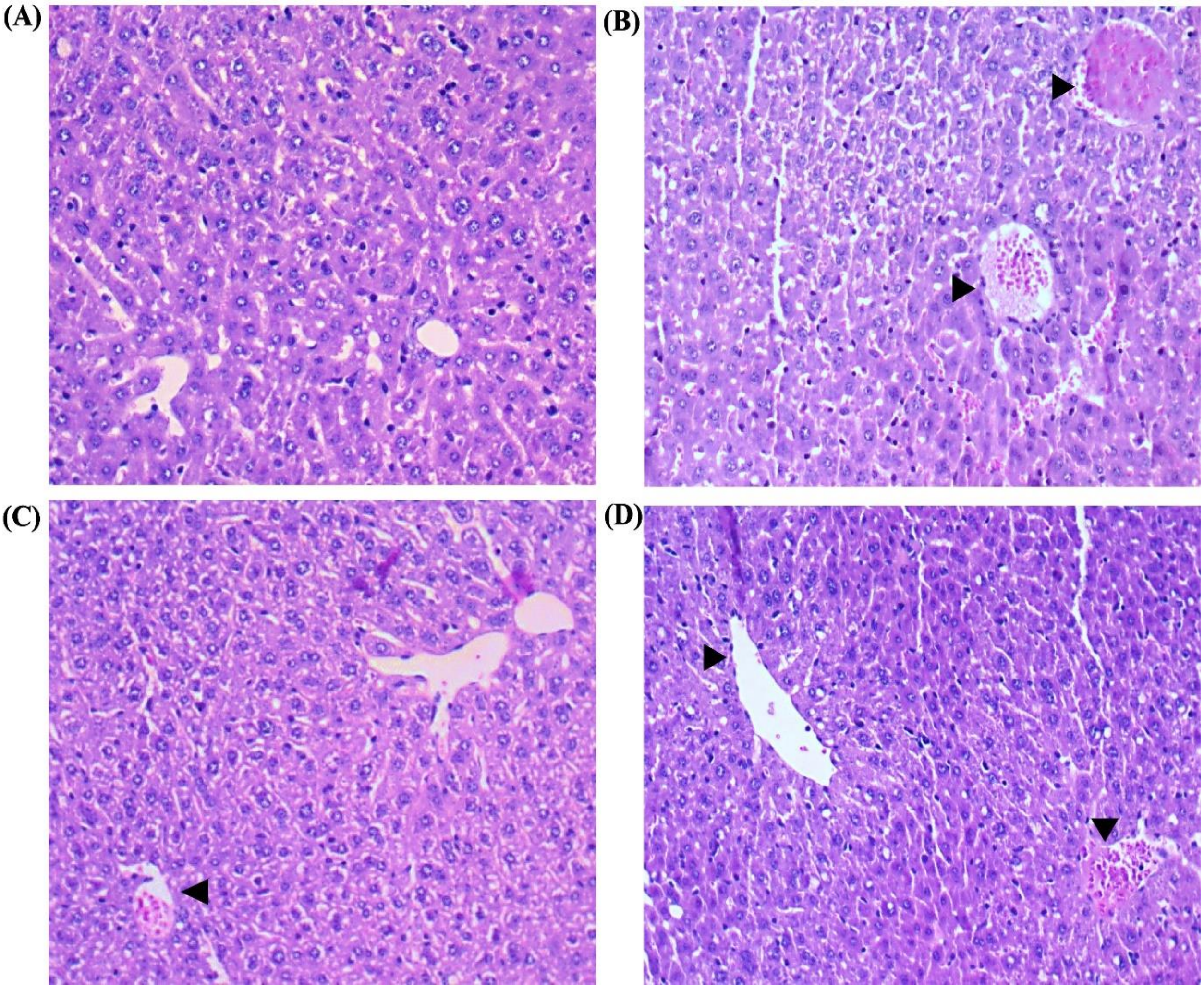


Figure 8.4: H&E staining sections of liver from normal, infected, and treated mice. Sections were stained with hematoxylin and eosin. Magnification, X10. (A) Normal: without any infection or treatment. Central venule, hepatic sinusoids, plates of hepatic cells, and portal areas were seen. (B) Infected: mice liver at 48 h post-challenge. The central venule was inflamed. (C) Liver histopathological sections of Prophylactic treatment grouped mice. (D) Liver histopathological sections of Therapeutic treatment grouped mice.

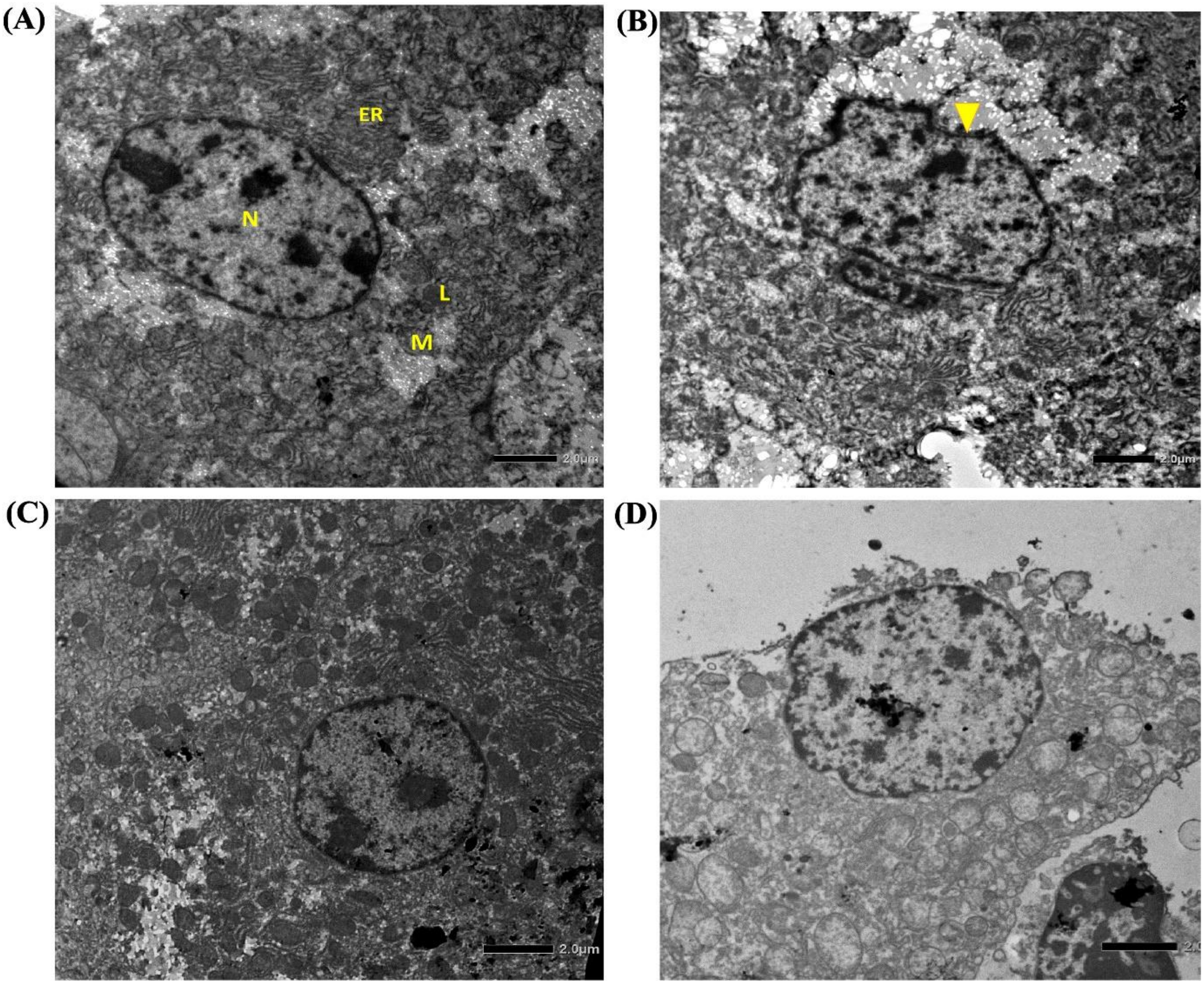


Figure 8.5: Transmission electron micrograph of mice liver sections. Magnification, X 1500. (A) Control: mouse liver section showing normal hepatic architecture (M: Mitochondria, N: Nucleus, L: Lysosome, ER: Endoplasmic reticulum). (B) Infected: mice liver at 48 h post-challenge. The central venule was inflamed. (C) Prophylactic treatment grouped mice. (D) Therapeutic treatment grouped mice.

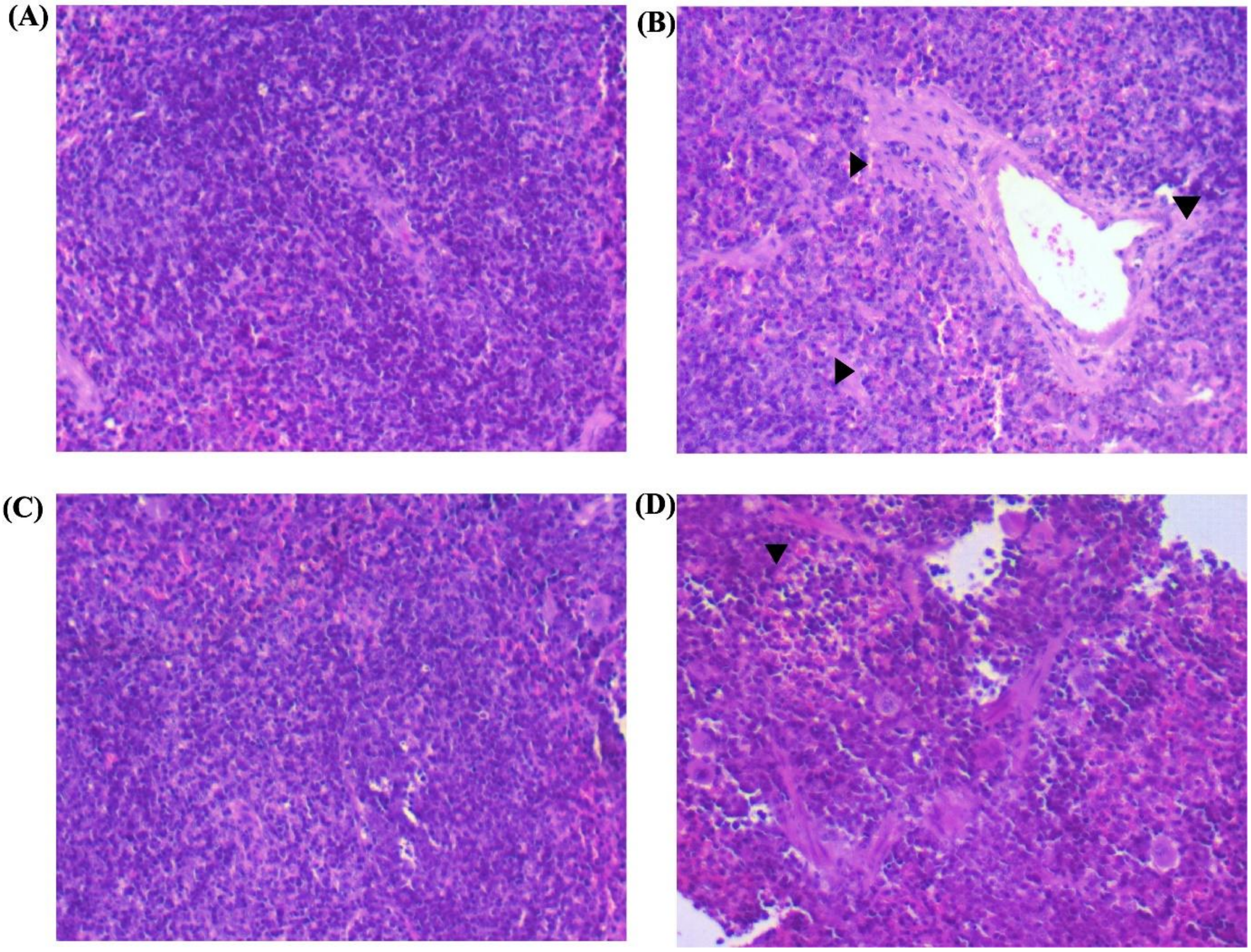


Figure 8.6: Images from the light microscope of mice spleen histopathological sections stained with hematoxylin and eosin. Magnification, X10. (A) Control: without any treatment. Red pulp, white pulp, plates of hepatic cells, and portal areas were visible. (B) Infected: mice spleen at 48 h post-challenge. The central venule was inflamed. (C) Spleen histopathological sections of preventive grouped mice. (D) Spleen histopathological sections of therapeutic treatment grouped mice.

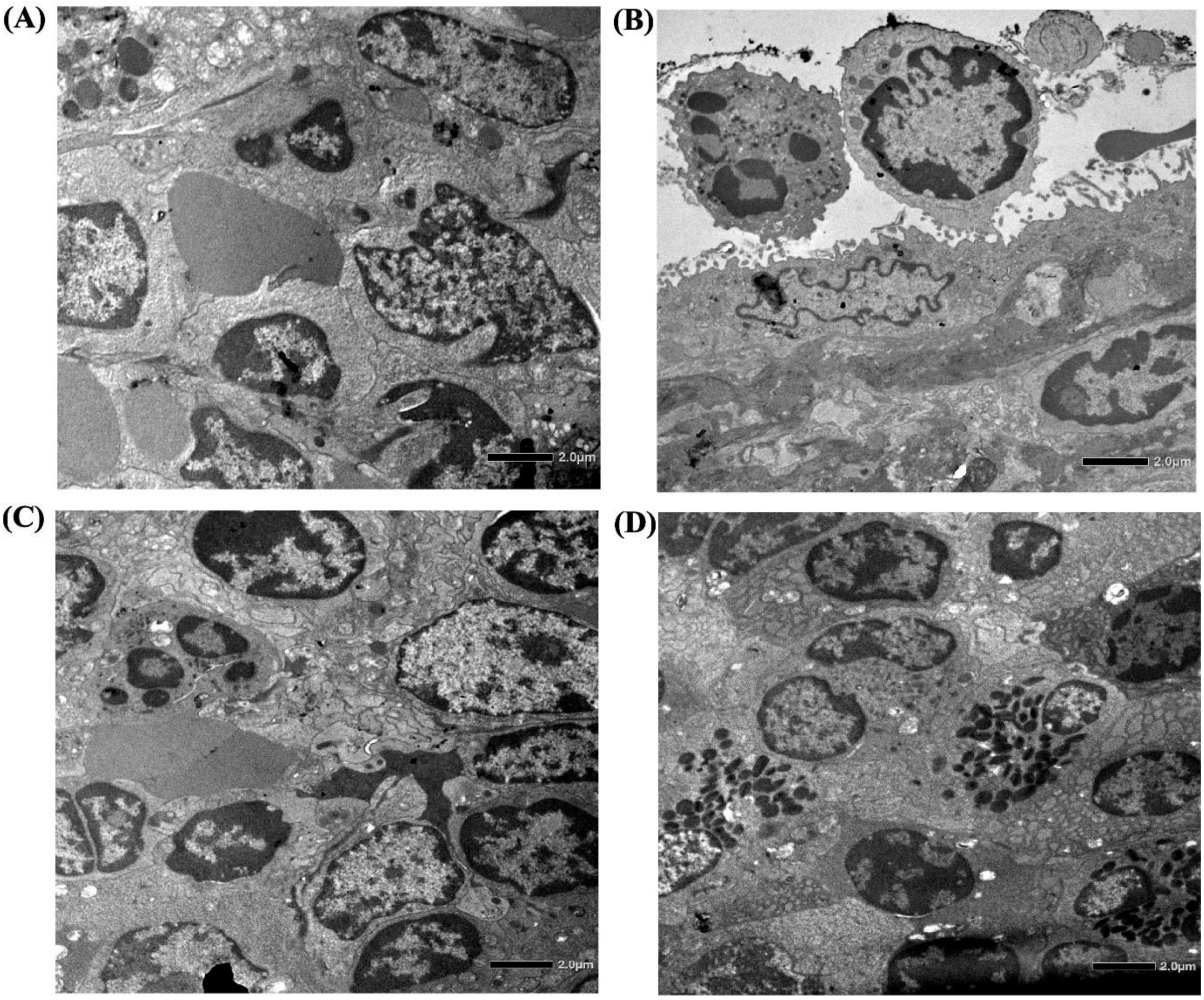


Figure 8.7: Transmission electron micrograph of mice spleen sections. Magnification, X 1500. (A) Control: without any treatment. Central venule, hepatic sinusoids, plates of hepatic cells, and portal areas were seen. (B) Infected: mice liver at 48 h post-challenge. The central venule was inflamed. (C) Prophylactic treatment grouped mice. (D) Therapeutic treatment grouped mice.

8.4 Discussion

There are over 2500 identified serotypes of *S. enterica* and they differ significantly in terms of pathogenicity. Among them, *S.* Typhi is one of the major *Salmonella enterica* serovars that cause enteric fever or salmonellosis in humans. Additionally, it can produce biofilm, which is pathogenic and develops antibiotic-resistant bacteria. Antimicrobial medicines are mostly used to treat and prevent salmonellosis in humans and animals, although some countries also use them to promote the growth of food animals (Woolhouse et al., 2015). In hospitals, communities, and livestock settings, their indiscriminate usage has facilitated the development of bacterial resistance. Either through the expansion of the resistant bacteria or through the transfer of resistance genes from animal to human, AMR can spread from animals to humans and vice versa (Argudín et al., 2017). Additionally, the development and spread of multidrug-resistant bacteria have provided a strong motivation to explore effective prophylactic and therapeutic means of eradication of bacterial infections. In this regard, the concept of phage therapy for controlling bacterial infections has been widely accepted.

With a population estimated to be $>10^{30}$, phages are the most prevalent biological species on earth (Hendrix, 2002). Because of this, they can be investigated using several bacterial models and infect a wide range of bacterial species. For therapeutic purposes, tailed bacteriophages (dsDNA viruses) are recommended and they belong to a single order of Caudovirales (Mehmood et al., 2023). They typically contain a tail with or without tail fibers and a head or capsid with a dsDNA. During bacterial attachment, the phage uses its tail, tail fibers, or both to create precise interactions with the surface (Mehmood et al., 2023).

Studies from previous groups that used BALB/c mice as an infection model and oral administration of phages for therapy showed promising outcomes and phages were shown to cure bacterial infections more effectively (Kumar et al., 2022). Based on other reported studies, the phage STWB21 was also administered orally along with saline. A single dose of phage STWB21 at MOI 1 was given 60 min before and after infecting mice with *S.* Typhi bacteria resulting in the rescue of 66% and 33% of the mice, respectively. This was a remarkable result compared to the control group, which caused the death of all infected mice. The effect of phage STWB21 only and PBS buffer showed no side effects on the health of the experimental mice groups. Thus, phage rescue experiments could be conducted without bias.

In this study, the most important finding was that the use of phage STWB21 has reduced the number of *Salmonella* Typhi colonies in mice in preventive as well as therapeutic treatment. These findings further suggest that phage STWB21 can reduce the adversity of salmonellosis and control the transmission of the bacteria to the environment.

The current study also showed that the prophylactic administration of phage STWB21 decreased the colonization of a foodborne intestinal pathogen *S.* Typhi and represents a potential strategy to reduce the spreading of *Salmonella* Typhi during the outbreak. Previous studies from other groups reported that prophylactic bacteriophage administration is more effective than therapeutic administration in phage therapy using a single phage or phage cocktail (Ahmadi et al., 2016; Lamy-Besnier et al., 2021).

8.5 Chapter Summary

In this study, it was reported that the phage STWB21 can control *Salmonella* invasion both prophylactically and therapeutically in mice. Additionally, the preventive and therapeutic benefits of STWB21 treatment were compared on mice against lethal *S.* Typhi infection without producing any negative effects, emphasizing the clinical importance of this phage to *S.* Typhi-induced illnesses. Phage treatment significantly improved the survival percentage of infected mice. This study also demonstrated that oral administration of phage treatment could be beneficial in both preventive and therapeutic treatment of salmonellosis caused by *S. Typhi*. Here, it was described a strategy for the creation of an efficient therapeutic that uses phages to prevent infection in animals. Altogether the result showed that the phage treatment could control tissue inflammation in mice before and after *Salmonella* infection. This phage has strong potential to be a therapeutic candidate.

Chapter 9

Application of phage cocktail to control the *Salmonella* spp. in food sample

Chapter 9

9.1.1 Introduction

A member of the Enterobacteriaceae family and a gram-negative, facultative, intracellular, rod-shaped bacteria, *Salmonella enterica* is one of the most common and deadly foodborne pathogens in the world (WHO, 2017). More than 2,600 serovars of *Salmonella enterica* exist, and most of them are pathogenic (Porwollik et al., 2004; Gal-Mor, 2019). Among these, *S.* Typhi is host-specific, whereas *S.* Typhimurium and *S.* Enteritidis are generalists. Non-typhoidal *Salmonella* species (NTS), such as *S.* Enteritidis and *S.* Typhimurium, mostly cause gastrointestinal infections that can result in hospitalization and even death (Musyoka et al., 2018).

Salmonellosis is caused by NTS, which are highly common cause of acquired foodborne illness in people worldwide (Esmael et al., 2021b). Salmonellosis is spread among people via the oral-fecal route by eating contaminated food that is either of animal origin (meat, poultry, eggs, milk), or green vegetables that have been exposed to manure (Youssef et al., 2021). Diarrhoea, fever, vomiting, stomach cramps, and many other symptoms that appear one or two days after infection and can last for a week are among the signs of infection (Wilson and Wilson, 2021). Even while salmonellosis symptoms are modest and patients often recover without treatment, it occasionally causes severe dehydration that can result in hospitalization and even death in young and immunocompromised individuals (Musyoka et al., 2018).

Salmonella has been found to be asymptomatically present in the gastrointestinal tracts of chickens or to cause signs of enteric illness (Pandey et al., 2021). As a result, the illness goes unnoticed, and its manifestation is a result of people eating contaminated food (Foley et al., 2008). According to Atterbury et al. (2007), NTS-infected animals can pass the illness to other animals directly or through contaminated food regimens, the environment, or both of them. Two factors affect NTS infections: its wide host range and the prevalence of multi-drug resistance (MDR).

MDR refers to the bacterial manifestation of antimicrobial resistance to at least one agent in three or more antimicrobial categories. Several antimicrobial resistance genes that provide an MDR characteristic against more than one antibiotic are encoded by a few *Salmonella* strains. The failure of antibiotic therapy has recently increased due to the development of MDR *Salmonella* serovars (Medeiros et al., 2011; Agyare et al., 2019). Low-dose antibiotic use has been widespread in the poultry industry for many years (Moore and Evenson, 1946), not just for prophylactic or therapeutic reasons but also to promote growth (Waibel et al., 1954; Libby and Schaible, 1955), and this improper use consequently leads to the development of MDR. The employed antibiotics could not completely eradicate the gut bacterium, and some resilient strains could endure and develop resistance. Eventually, the antibiotic-resistant bacteria spread their genes to other vulnerable bacteria.

Salmonella biofilm formation in food articles and industrial facilities

Salmonella typically coexists with sessile multicellular surface-associated forms known as biofilms in both food-related products and factories, in addition to free planktonic cells. *Salmonella* virulence is attributed to the development of biofilms because bacteria in complex biofilm communities are more resistant to antibiotics, leading to a chronic *Salmonella* infection (Gonzalez-Escobedo et al., 2011; Gonzalez-Escobedo and Gunn, 2013; Zeineldin et al., 2023). *Salmonella* load in food items and biofilms is mostly controlled by employing standard control measures such as utilizing specific preservatives and heat treatment in liquid food (Pérez-Daz et al., 2008; Neetoo and Mahomoodally, 2014; Chylkova et al., 2017; Musyoka et al., 2018). Despite the fact that these strategies are employed, the worry about the unfavorable side effects that the chemical stabilizers cause is disappointing (Pawlowska et al., 2012). Important nutrients are also lost as a result of heat treatment. Additionally, non-specific antibacterial action, long-term environmental stability, and the predominance of MDR bacteria all work against the use of antibiotics in food items (Medeiros et al., 2011; Agyare et al., 2019).

Importance of phage application on biofilm in food

Bacteriophages are considered desirable antibacterial agents because of their characteristics as obligate parasites, capable of self-replication, and host-specific. As a unique natural method for controlling bacteria in food items and to ease their biofilms as well, bacteriophages have attracted considerably more interest than antibiotics (Goodridge and Bisha, 2011; Matsuzaki et al., 2014; Lin et al., 2017; Esmael et al., 2021b). Additionally, human bacteriophage infections have not been reported (Kutter et al., 2010; Keen et al., 2015) and phages are not harmful to eukaryotic cells (Górski et al., 2020). As a result, phage therapy appears like a strong contender for antibacterial control in food. The results of several investigations (O'Flynn et al., 2006; Spricigo et al., 2013; Bao et al., 2015; Huang et al., 2018a; Esmael et al., 2021b; Alharbi et al., 2023) shown that phages could successfully be controlling the foodborne bacteria in various food components.

Additionally, the FDA has designated some phages as Generally Recognised as Safe (GRAS), making them commercially available and useful in combating *Salmonella* and their biofilms in food products (Goodridge and Bisha, 2011; Sukumaran et al., 2015; Moye et al., 2018). Examples of these phages include Armament, Salmonelex, and SalmoFresh. The evolution of phage-resistant bacteria is one of the problems thus manufacturing cocktails composed of many phages with the largest range of antibacterial activity seems to be a useful strategy.

Additionally, bacteriophage cocktails have been proven successful at removing *Salmonella* directly from food, in addition to their usage in cleaning food preparation surfaces. For instance, the *Salmonella*-specific cocktail reduced the levels of *Salmonella* on experimentally contaminated chicken parts when used alone, and this impact improved when the phage was used in combination with traditional chemical sanitizers (Sukumaran et al., 2016). When applied to the surface of the chicken breast fillets or when the fillets were dipped into a container having the phage solution, the bacteriophage cocktail drastically decreased the populations of a variety of *Salmonella* species.

9.2 Chapter aims

i. In order to prepare a phage cocktail, isolate and identify lytic bacteriophage specific for *Salmonella* Enteritidis.
ii. To evaluate the efficacy of phage cocktail as a zoonotic *Salmonella* control approach in raw chicken breast meat.
iii. To assess the efficiency of single phage, the phage cocktail, and the antibiotic treatment against biofilms of the *S.* Typhi and *S.* Enteritidis strains.

9.3 Results

9.3.2 Basic characterization of a newly isolated *Salmonella enteritidis* phage

9.3.1.1 Isolation and Screening of Salmonella Phage

For the preparation of the phage cocktail, another phage was isolated from the water samples using *S.* Enteritidis as a host. This newly isolated bacteriophage was given the name SEWB23. The plaques of SEWB23 were transparent and clear and no turbid plaques were observed (Figure 9.1). Phage titer after propagation ranged from 6.2 × 10^{12} PFU/ml.

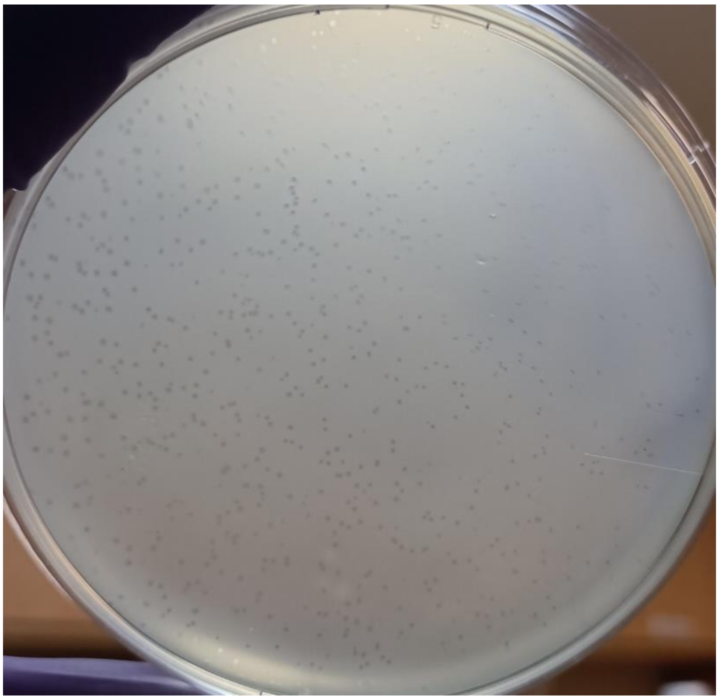


Figure 9.1: Plaque morphology of SEWB23 bacteriophage on nutrient agar plate

9.3.1.2 Host Range & EOP assay

Spot tests revealed that phage SEWB23 had a broader host range. To confirm its host range, an EOP assay was done for phage SEWB23 (Table 9.1). A wide range of lytic activity was demonstrated by phage SEWB23. All typhoidal and non-typhoidal *Salmonella* strains, *Sh. flexneri* and *E. coli* were all infected with a low to high efficiency (0.02 to 1.0). The majority of non-typhoidal strains were infected by this phage with high efficiency (0.3 to 1), however, the EOP values were only moderate (0.1 to 0.2) for some typhoidal strains.

Table 9.1: Host range analysis of phage SEWB23 on different strains.

Strains	**Strain name**	**Infectivity**	**E. O. P**
***1. S.* Enteritidis**	**(520833) ***	**(+) ve**	**1**
***2. S.* Typhi**	**(KOL 551)**	**(+) ve**	**0.262**
***3. S.* Paratyphi**	**(KOL 534)**	**(+) ve**	**0.140**
4. S.* Typhi*murium	**(PH-94)**	**(+) ve**	**0.370**
5. ETEC	**(IDH07942)**	**(+) ve**	**0.025**
6. Sh. flexneri 2a	**(2457T)**	**(+) ve**	**0.170**
7. Sh. flexneri 3a	**(UB811)**	**(+) ve**	**0.162**
8. Sh. flexneri 6	**(UB812)**	**(-) ve**	**-**
9. Sh. boydii	**(NK02379)**	**(-) ve**	**-**
10. V. cholerae O1	**(MAK757)**	**(-) ve**	**-**

+ = lysis; – = no lysis.

9.3.1.3 Phage morphology

Transmission electron microscopy (TEM) analysis of the purified phage SEWB23 indicated that it had a long, flexible, non-contractile tail that measured approximately 121± 6 nm in length (n = 20) and an icosahedral head with an estimated diameter of 64± 3 nm (n = 20). According to the International Committee on Taxonomy of Viruses (ICTV) guidelines for phage structure, the phage SEWB23 may belong to the Caudovirales subfamily *Siphoviridae*.

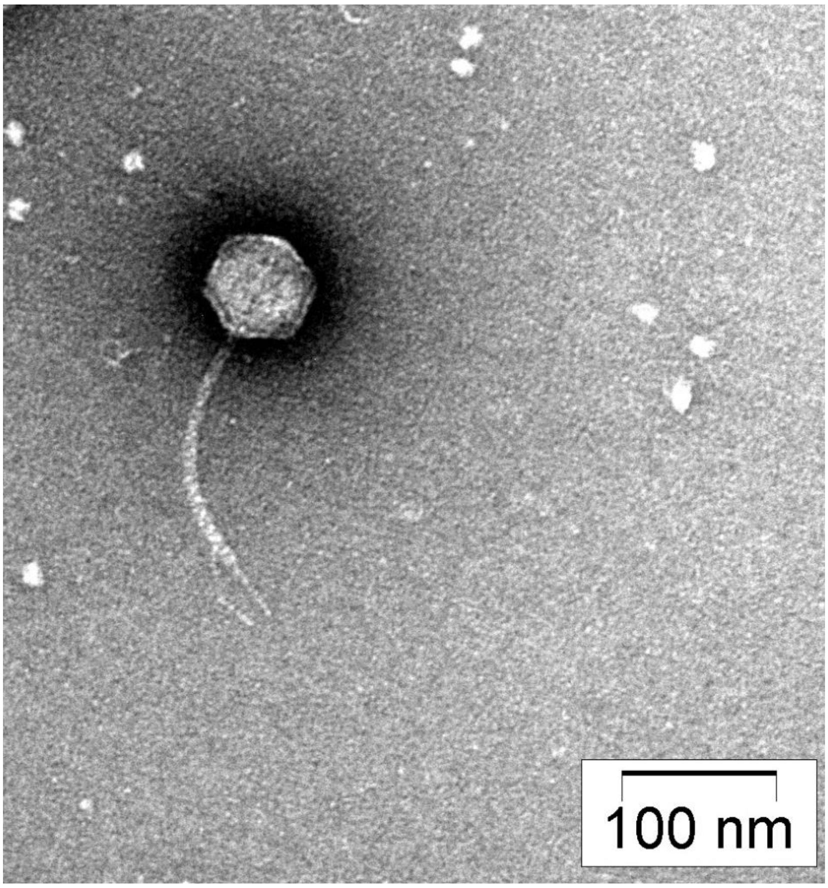


Figure 9.2: Morphology of SEWB23 bacteriophage using TEM

9.3.1.4 Analysis of structural proteins of phage SEWB23 by SDS-page

SDS-PAGE analysis of phage SEWB23 showed the presence of structural proteins ranging from 10 to 150 kDa (Figure 9.2). There are only two major bands that appeared at around 35 and 61 kDa while the visible minor bands appeared at 10, 12, 16, 18, 40, 48, 61, 75, 132, 135, and 180 kDa.

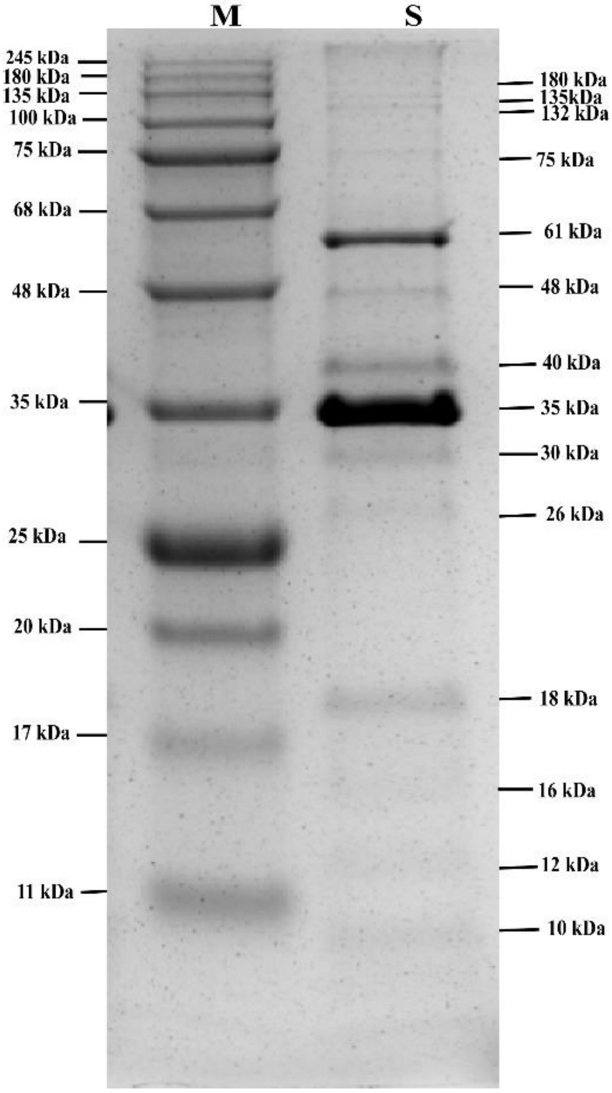


Figure 9.3: The SDS-PAGE analysis of phage SEWB23 structural proteins on 12.5% gel staining with Coomassie brilliant blue. M, a standard marker of molecular weight (kDa).

9.3.2 *Salmonella* reduction in chicken meat using individual phages and phage cocktail

In the untreated positive control, the *Salmonella* count is 10 log CFU/gm. The treatment of phage STWB21, phage SEWB23, and their cocktail on the chicken breasts reduced the bacterial load of approximately 4 log CFU, 3 log CFU, and 5 log CFU respectively (Figure 9.4). Biofilm reduction by phage STWB21, SEWB23, and phage cocktail infection were statistically significant ($p < 0.001$) compared with untreated control.

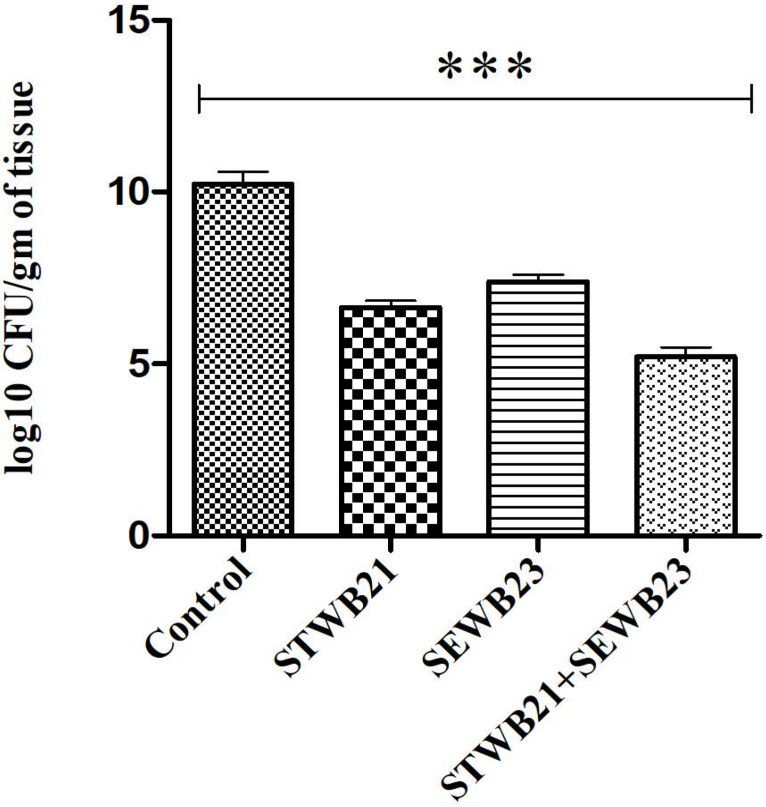


Figure 9.4: Effects of individual phages and phage cocktail on the growth of *S.* Typhi bacterial cells on the chicken breast. One-way ANOVA was performed by comparing bacteria control with treatment (*p< 0.001).**

9.3.3 Effect of phage cocktail and antibiotics on *S.* Typhi and *S.* Enteritidis biofilm

In a 96-well microplate at 37°C, the efficacy of the phage cocktail, consisting of a 1:1 mixture of the phages STWB21 and SEWB23, was assessed in comparison to that of each individual phage and antibiotic. By using crystal violet staining in 96-well plates, the effect of the phages STWB21, SEWB23, their cocktail, and the antibiotic cephalosporin in the degradation of biofilm was determined. Both the bacterial strains *S.* Typhi and *S.* Enteritidis were susceptible to the antibiotic cephalosporin. For the studied phage titers of single-phage lysates, the cocktail, and the antibiotic against *S.* Typhi and *S.* Enteritidis, biofilm biomass degradation as compared to the control was highly significant ($p < 0.0001$). On the 96-well plates, treatment with the phage STWB21, SEWB23, phage cocktail, and antibiotic cephalosporin decreased the *S.* Typhi bacterial population by approximately 38%, 37%, 44%, and 41%, respectively. For the *S.* Enteritidis bacterial strains, it was altered by 43%, 62%, 68%, and 66% respectively (Figure 9.5).

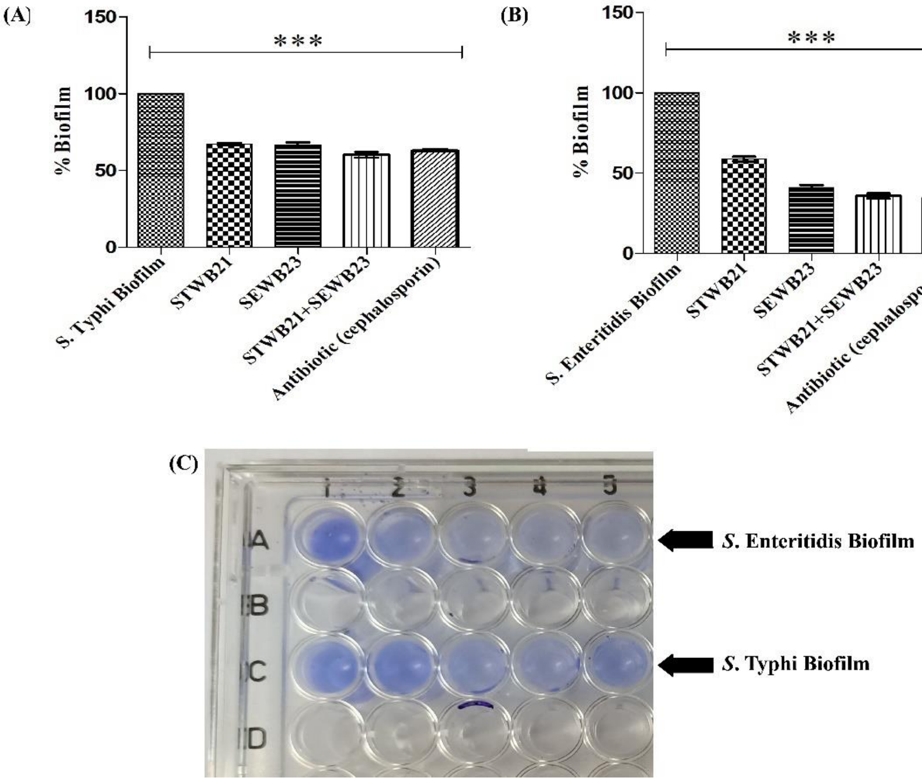


Figure 9.5: Percentage of the antibiofilm activity of phage (STWB21, SEWB23), phage cocktail, and antibiotic cephalosporin against the bacterial biofilm: (A) On *Salmonella* Typhi biofilm; (B) On *Salmonella* Enteritidis biofilm. One-way ANOVA was used to detect significant differences between control and test samples. *** $p < 0.001$. (C) Crystal violet assay on biofilms in a microtiter plate (1: Control bacteria, 2: STWB21 phage, 3: SEWB23 phage, 4: Phage cocktail, 5: Antibiotic cephalosporin).

9.4 Discussion

The eradication of pathogens, controlling the growth of bacteria resistant to antibiotics, and the prolongation of food shelf life all require the development of novel microbiological control methods that are relevant to food matrices. Because of their stability in abiotic environments, lack of toxicity, and specificity in antimicrobial action, lytic phages are an option that may be used to disinfect food or throughout the food production process. The fact that some of these preparations, which include no preservatives or chemicals and are permitted for use in organic food, have received Kosher- and Halal certifications and demonstrate the safety of bacteriophage-based bio-controllers (Moye et al., 2018).

A typhoidal *Salmonella* phage STWB21 and a nontyphoidal *Salmonella* phage SEWB23 were used for this investigation. They were chosen for their large viral progeny and rapid impact on *Salmonella* Typhi and *Salmonella* Enteritidis cultures and they cover the most common *Salmonella* serovars that impact the food and veterinary industries (particularly poultry), making them ideal for assessing the host range. The SEWB23 phage exhibited distinct and clear plaques.

While examined under an electron microscope, the bacteriophages STWB21 and SEWB23 were found to belong to the *Siphoviridae* family and had an icosahedral head and a long tail. 88% of all recognized phages, according to the International Committee on Taxonomy of Viruses (ICTV), are members of the order Caudovirales, which includes the family *Siphoviridae* (Li M et al., 2021). The developed phage cocktail has the required host range coverage and has effectively shown efficacy in considerably eradicating the tested bacterial cultures *in vitro* compared to individual phage treatments.

In order to prevent foodborne pathogens in chicken meat, phage can be employed as an alternate biocontrol strategy. Phage production is cost-effective and will not be challenging in large-scale production. Antibiotic use and the spread of antimicrobial resistance can be decreased by using self-limiting phages for refractory drugs. Phage usage in meat is permitted in the US, although it is still rarely permitted in India. As a result, this study sheds light on preventative and controlling measures for *Salmonella* infections in the poultry industry.

The biofilm investigation has demonstrated that a phage cocktail can infect *Salmonella* biofilm and has the capacity to reduce tested *S.* Typhi and *S.* Enteritidis strains. In 96-well microplates, it was found that phage cocktail treatment eradicated the post-treated biofilm of *S.* Typhi and

S. Enteritidis by 44% and 61% respectively. According to earlier studies, the components of the biofilm, the age of the biofilm, the efficacy of the phage, and the duration of the therapy are all factors that influence the way biofilms can be eliminated effectively (Corbin et al., 2001; Sharma et al., 2005). The results of this investigation demonstrated the theory that the use of a phage cocktail could decrease the population of *S*. Typhi and *S.* Enteritidis in food types and lower the levels of biofilms and it could be used as an alternative to antibiotics.

9.5 Chapter Summary

The broad host-spectrum, *Siphoviridae* phage SEWB23 was isolated which exhibits a potent lytic effect on *S.* Enteritidis. Our results suggested that the phage STWB21, phage SEWB23, and their combination may be useful in reducing *Salmonella* spp. bacteria viability in chicken breast meat. As a result, the phage cocktail has the potential to be a bactericidal agent for the biocontrol of *Salmonella* spp. in raw chicken breast meat and may be used in the future by a variety of poultry sectors. The findings also showed that the phage cocktail reduced the *Salmonella* Enteritidis bacterial biofilms more efficiently than *Salmonella* Typhi bacterial biofilms on a 96-well microplate. Furthermore, the result suggested that this phage cocktail is an ideal substitute for antibiotics to control *Salmonella* and can reduce the biofilms that are resistant to conventional approaches.

Chapter 10

General Discussion, Conclusion & Future Prospect

Chapter 10

10.1 General Discussion

Salmonella is a type of bacterium that can cause a wide range of diseases in both humans and animals. It is a Gram-negative, rod-shaped, facultatively anaerobic, mobile microbe belonging to the family Enterobacteriaceae. *Salmonella* is responsible for diseases like salmonellosis, septicemia, and typhoid fever (Kurtz et al., 2017). *S. enterica* serotype Typhi causes typhoid fever. Additionally, it can spread through asymptomatic people who have previously carried the infection, which is still a significant public health concern in underdeveloped nations (John et al., 2016). *Salmonella* infections caused by consuming contaminated food items continue to pose a serious threat to human health, with most cases leading to moderate to severe intestinal gastroenteritis and even fatalities in certain situations (Sockett and Roberts, 1991; Majowicz et al., 2010). *Salmonella spp*. are frequently referred to as environmental persisters and can create complex biofilms, which are surface-associated communities, in both industrial and food-related environments (Stapels et al., 2018; Steenackers et al., 2012). *Salmonella* biofilm was found as a bacterial reservoir in a food processing facility, and it caused many outbreaks of foodborne illnesses (Corcoran et al., 2014).

Antibiotics were originally thought to be an effective way to reduce the *Salmonella* burden in both farms and industry because most traditional techniques had a limited impact on the disease. Later, it was found that prolonged and frequent use of antibiotics gave rise to resistant bacteria. The Nordic Pig Production Committee (NCPP) in Denmark and the European Union (EU) both forbade the use of antibiotics in 1995 and 1999, respectively (Casewell et al., 2003). As a novel strategy, phage-based approaches have been suggested as revolutionary methods for preserving pathogens in food products and reducing biofilms (Matsuzaki et al., 2014; Goodridge & Bisha, 2011; Doub, 2020). The largest source of bacteriophage-infected microbiota is found in human and animal bodies. According to reports, phages are easily found in foods, animals, and healthy people (Hyman et al., 2012; Kutter et al., 2010). Thus, the use of phages appears to be a secure prerequisite for biologically controlling infections.

Bacteriophages are referred to as "smart antimicrobials" since they specifically target their host pathogenic bacteria without having an impact on the commensal flora (Fernebro, 2019). Consequently, they are considered a promising alternative to conventional antibacterial treatments. In fact, phage biocontrol is now highly in demand since it is natural, secure, and regarded as a green technology that is ecologically benign (Kutateladze & Adamia, 2010). Bacteriophage therapy has shown encouraging results in both *in vitro* and *in vivo* models, reducing the need for antibiotics. Indeed, phage therapy provides several benefits over antibiotics, including host-specificity, high diversity, and abundance, the ability to function in combination with other phages to develop a phage cocktail, and low-cost preparations.

The usefulness of phage therapy is affected by the host range in several ways. A single-species host range is preferable since it prevents the phage from eradicating other species and preserves the remainder of the host's microbiome. Therefore, the phage should have a narrow spectrum of bacterial species as hosts. Since most phages are usually specific to a bacterial strain or species, often to a genus, a key drawback of adopting phage-based biocontrol techniques is their limited antibacterial range. The development of a phage-based biocontrol agent is the most appropriate method for infecting many significant target microorganisms.

Bacteriophage survival and persistence, which are highly dependent on physicochemical variables like pH and temperature, affect the efficacy of phage therapies against illnesses. For instance, oral delivery of *Salmonella* bacteriophages may impair their viability by exposing them to the acidic environment of the stomach and gizzard (Gigante and Atterbury, 2019). When compared with the previously described *Salmonella* phages (Abdelsattar et al., 2021; Dallal et al., 2019), STWB21 also showed similar heat and pH stability (temperature: up to 50°C, pH: 4-12) for both the typhoidal and non-typhoidal strains. The results of the host range and physio-chemical characteristics together revealed that STWB21 has the potential to be a novel candidate for therapy medicines against typhoidal and non-typhoidal strains.

A previous study described that T5 genes are organized in three clusters (Wang et al., 2005). The first cluster consists of pre-early genes that encode proteins that are thought to function in the inhibition of inactivating host functions. The second cluster is made up of early genes that code for proteins that are involved in DNA replication, recombination, repair, and transcription, as well as proteins that are involved in phage lytic activities and genes that code for tRNA. Lastly, the third cluster, which includes late genes, predominantly encodes proteins for phage structural proteins and proteins involved in phage morphogenesis. During infection, T5 phage attaches to bacteria in two steps: first, through reversible binding to the lipopolysaccharide (LPS) O-antigen mediated by the L-shaped tail fibers; second, irreversible step, in which the T5-receptor-binding protein (Gp5) binds to the host's outer membrane iron-ferrichrome transporter FhuA; which causes a lytic infection cycle (Heller, K., & Braun 1979). Similarly, bacteriophage STWB21 infects Gram-negative bacteria *Salmonella* Typhi and belongs to the

T5-like *Siphoviridae* family that infects Gram-negative bacteria. The genome of the studied phage is 112,834 bp in length, and it contains a total of 166 predicted proteins with 152 ATG (91.07%), 10 GTG (6.02%), and 5 TTG (2.99%) as initiation codons and 22 tRNA coding genes. Mass spectrometric methods have identified 19 phage-particle-associated proteins. The genome of this studied phage also encoded the early, pre-early, and late genes like the T5 phage. Also, genomic analysis confirmed the presence of lytic genes and the absence of any lysogeny or toxin genes.

Proteomics approaches using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) instrument is used for the investigation and detection of harmful bacterial strains based on diagnostic peptides (Calo-Mata et al., 2016; Pfrunder et al., 2016). Additionally, bacteriophage proteins are also identified and detected using LC-ESI-MS/MS methods. In this study, the structural proteins of STWB21 were characterized. Following the LC-MS/MS nineteen different proteins were identified. Among them fifteen structural proteins, one functional protein, and three hypothetical proteins. Five structurally relevant phage structural proteins were selected and their structure was predicted using AlphaFold2. Then the predicted structures were aligned with the experimental structure collected from the PDB.

In both food products and industrial facilities, *Salmonella* commonly exists in both free planktonic cells and sessile multicellular surface-associated forms known as biofilms. *Salmonella* pathogenicity is attributed to the development of biofilms because bacteria in these communities are more resistant to antibiotics, leading to a chronic infection with *Salmonella* (Gonzalez-Escobedo et al., 2011; Gonzalez-Escobedo and Gunn, 2013; Zeineldin et al., 2023). Standard control procedures like preservatives and heat treatment are used to control *Salmonella* load and reduce biofilms in food products, but concerns about side effects, nutrient degradation, and antibiotic use are discouraged due to their non-specific antibacterial activity (Medeiros et al., 2011; Pérez-Díaz et al., 2008; Neetoo and Mahomoodally, 2014; Chylkova et al., 2017; Musyoka et al., 2018). In contrast, bacteriophages are considered desirable antibacterial agents because of their characteristics as obligate parasites, capable of self-replication, and host-specific. Lytic phages are effective biocontrol and phage therapy tools (Chan et al., 2013). Phage treatment, which uses lytic bacteriophages to treat patients with bacterial infections, is regaining popularity in the West after being largely abandoned in the 1940s as a result of the rising global anti-microbial resistance (AMR) (Lin et al., 2017). In this study, the antibiofilm activity of lytic phage STWB21 was evaluated. The phage STWB21 was used to treat on 24-hour formed biofilm that had developed in coverslip, red onion, tomato, and milk in a controlled laboratory environment. The bacterial population of the *S*. Typhi biofilm was found to have significantly decreased. This research showed that the viability of cells connected to biofilms could be disrupted, rendered reduced, and destroyed using bacteriophages STWB21. As a result, it might be a useful technique for reducing *S.* Typhi proliferation in biofilms. Therefore, bacteriophages might be a crucial component of a perfect, beneficial biocontrol interventional approach to manage biofilm-mediated infections in a variety of applications.

A newer technique for treating bacterial biofilm is a phage cocktail, which consists of two or more bacteriophages. Plage cocktail successfully overcomes the drawbacks of monophage therapy and enhances therapeutic effects. Due to the ongoing emergence of phage-resistant host bacterial mutants, there is now an increasing need for phage cocktails. Therefore, the presence of many types of phages in phage cocktails may prevent or delay the emergence of phage-resistant bacteria (Domingo-Calap and Delgado-Martnez 2018). Furthermore, numerous phage strains may complement one another by supplying crucial antibacterial components that another strain might lack. In this study, a phage cocktail was prepared using another lytic *Salmonella* Enteritidis phage SEWB23 along with the studied phage STWB21. The phage cocktail was used to reduce the bacterial population on artificially infected chicken breast and biofilm in a 96-microtiter well plate. It was found that the phage cocktail reduced the bacterial population of *S.* Typhi and *S.* Enteritidis more effectively than the individual phages. Additionally, it showed a similar antimicrobial activity to antibiotics. Therefore, the phage cocktail could be used as a substitute for antibiotics.

To prevent or reduce the transmission of foodborne infections in agricultural animals, phage therapy has been demonstrated to be quite effective. *In vivo* studies are essential to evaluate the safety of phage therapy more accurately as well as to understand the effects of oral phage treatments administered to animals at high concentrations. The murine infection model is frequently used in studies on the ability of phages to combat *Salmonella* (Kumiska-Bajor et al., 2015). In controlling colonization of all studied *Salmonella* strains, prophylactic was significantly more successful than remedial or co-infection with phage and bacteria (Nale et al., 2021). This was evident when treatment regimens were compared. This finding is consistent with research on phage therapy employing other organisms, such as *Pseudomonas aeruginosa* and *C. difficile*, in larvae and treatment with *Salmonella* phage in quals (Beeton et al., 2015; Ahmadi et al., 2016; Nale et al., 2016a). Similarly, our current study also demonstrates that the prophylactic administration of phage STWB21 reduced the colonization of *Salmonella* Typhi in the liver and spleen tissue of mice and could be a feasible method to prevent the spread of *Salmonella* Typhi during the outbreak.

10.2 Conclusion

Nowadays, bacteria are becoming more resistant to almost all available antibiotics. Novel approaches should be developed since antibiotics are stable molecules that allow selective pressure enabling bacteria to adapt so they can survive. An effective alternate solution to this problem is bacteriophage. Phages have a variety of possible uses, including phage therapy, phage display, phage-derived enzymes, and use as bacterial predators. In this work, a lytic phage named STWB21 was isolated from lake water and described in terms of its usefulness as a polyvalent and effective phage. Typhoidal and non-typhoidal *Salmonella* spp.*, Shigella spp.*, and *E. coli* were all effectively infected by STWB21 due to its polyvalent host spectrum. As revealed in the morphological study, STWB21 belongs to the Caudovirale order, the family of *Siphoviridae*. Additionally, it demonstrated great reproductive fecundity, quick adsorption, a short eclipse and latent time, large burst size, and pH and temperature stability across a broad range. The potential safety of STWB21 was predicated on the presence of lysis genes and the absence of genes related to virulence, allergenicity, lysogenicity, and antibiotic resistance. Furthermore, this confirmed the lytic nature of the phage STWB21. According to genome study and phylogenetic analysis, this bacteriophage is a novel phage of a T5-like genus. Understanding the relationship between phages that are closely related and the relationship between structure and function was facilitated by identifying the phage proteins. Here, based on certain peptides, the STWB21 phage proteins were analyzed and detected using a nano LC-MS/MS approach. Notably, STWB21 demonstrated remarkable efficiency by maintaining long-term control of *S.* Typhi with the lowest MOI. Consequently, STWB21 is an effective, economical, and promising biocontrol agent to be used for the management of key foodborne pathogens. For the simultaneous and effective biocontrol of *S.* Typhi, it would also be ideal to use STWB21 during the processing and preservation phases of foods that have different matrices. As an antibiofilm agent, the combination of the *Salmonella* phages STWB21 and SEWB23 was found to be more effective than either bacteriophage alone or the antibiotic. Although this isolated phage was extremely stable under challenging physiological settings, it can be employed as a therapeutic agent to treat salmonellosis. The use of an antacid, prior to the oral administration of the phage STWB21 can boost its effectiveness during phage therapy. Interestingly, phages STWB21 and SEWB23 are attractive not only from a therapeutic standpoint, but also from a perspective of possible applications in industries including agriculture, food safety, and food manufacturing. Therefore, this study suggested that phage STWB21 alone or in a cocktail has strong potential to be considered as a viable alternative to the present antibiotics.

10.3 Future Prospect

All the findings from this investigation indicated that the phage STWB21 would be an excellent substitute medication for treating salmonellosis. Phage STWB21 could eventually be used as a potential therapeutic alternative to antibiotics. Phage therapy could accomplish more than simply treating an illness by reducing its causes and preventing subsequent problems but this concept requires further research and confirmation. In this study, phages STWB21, SEWB23, and a phage cocktail were developed to provide an effective method for reducing *Salmonella* colonization to improve food safety. Hence, the application of phage STWB21 is congruent with the One Health approach because it may be administered in various settings (such as food and animals), avoiding the overuse or misuse of antibiotics.

Chapter 11

References

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Chapter 12

Appendix

Appendix

A.1 Publications:

A.1.1 Publications arising from this thesis

Mondal P, Mallick B, Dutta M and Dutta S. (2022). Isolation, characterization, and application of a novel polyvalent lytic phage STWB21 against typhoidal and nontyphoidal *Salmonella* spp. *Front. Microbiol.* 13:980025. doi: 10.3389/fmicb.2022.980025.

Mondal P, Halder P, Mallick B, Bhaumik S, Koley H, Dutta S, and Dutta M. (2023). Controlling the bacterial load of *Salmonella* Typhi in an experimental mouse model by a lytic *Salmonella* phage STWB21: a phage therapy approach. *BMC Microbiology*. (Manuscript accepted).

A.1.2 Publications arising from another project

Mallick B, **Mondal P** and Dutta M. (2021). Morphological, biological, and genomic characterization of a newly isolated lytic phage Sfk20 infecting Shigella flexneri, Shigella sonnei, and Shigella dysenteriae1. Sci. Rep. 11:19313. doi: 10.1038/s41598-021-98910-z.

A.2 Presentations:

A.2.1 Oral presentation

'Physicochemical and Structural Characterization of newly Isolated Enteric Bacteriophage'- 12th Asia-Pacific Microscopy Conference (Hyderabad, India. 3rd-7th February 2020).

A.2.2 Poster presentations

'Physicochemical and Structural Characterization of a novel Salmonella bacteriophage' -16th Asian Conference on Diarrhoeal Disease and Nutrition 2022 (Kolkata, India. 11th-13th November 2022).

'Isolation, characterization, and application of novel lytic Salmonella phage STWB21'- International Conference on Electron Microscopy & XLI Annual Meeting of Electron Microscope Society of India (New Delhi, India. 8th -10th February 2023).

Application of a novel polyvalent lytic phage STWB21 as a food preservative and for therapeutic purposes to control Salmonella Typhi*' (Abstract accepted for poster presentation)*- 25th Biennial Evergreen International Phage Meeting (The Evergreen State College in Olympia, Washington. 6th - 11th August 2023).

'Application of a novel polyvalent lytic phage STWB21 as a food preservative and for therapeutic purposes to control Salmonella Typhi*'*- 4th International Conference on Bacteriophage Research and Antimicrobial Resistance (Chennai, India. 28th - 30th September 2023).

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Isolation, characterization, and application of a novel polyvalent lytic phage STWB21 against typhoidal and nontyphoidal *Salmonella* spp.

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Salmonella is one of the common causal agents of bacterial gastroenteritis-related morbidity and mortality among children below 5years and the elderly populations. Salmonellosis in humans is caused mainly by consuming contaminated food originating from animals. The genus *Salmonella* has several serovars, and many of them are recently reported to be resistant to multiple drugs. Therefore, isolation of lytic *Salmonella* bacteriophages in search of bactericidal activity has received importance. In this study, a *Salmonella* phage STWB21 was isolated from a lake water sample and found to be a novel lytic phage with promising potential against the host bacteria *Salmonella typhi*. However, some polyvalence was observed in their broad host range. In addition to *S. typhi,* the phage STWB21 was able to infect *S. paratyphi, S. typhimurium, S. enteritidis,* and a few other bacterial species such as *Sh. flexneri 2a, Sh. flexneri 3a,* and *ETEC*. The newly isolated phage STWB21 belongs to the *Siphoviridae* family with an icosahedral head and a long flexible non-contractile tail. Phage STWB21 is relatively stable under a wide range of pH (4–11) and temperatures (4°C–50°C) for different *Salmonella* serovars. The latent period and burst size of phage STWB21 against *S. typhi* were 25min and 161 plaque-forming units per cell. Since *Salmonella* is a foodborne pathogen, the phage STWB21 was applied to treat a 24h biofilm formed in onion and milk under laboratory conditions. A significant reduction was observed in the bacterial population of *S. typhi* biofilm in both cases. Phage STWB21 contained a dsDNA of 112,834bp in length, and the GC content was 40.37%. Also, genomic analysis confirmed the presence of lytic genes and the absence of any lysogeny or toxin genes. Overall, the present study reveals phage STWB21 has a promising ability to be used as a biocontrol agent of *Salmonella* spp. and proposes its application in food industries.

KEYWORDS

***Salmonella*, lytic, bacteriophage, biofilm, onion**

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Introduction

Salmonella, a member of the family *Enterobacteriaceae,* is the causative agent for gastro enteropathy and enteric (typhoid) fever (Reeves et al., 1989). Antibiotic resistant *Salmonella* spp. responsible for community-acquired infections are in the WHO high priority list of pathogens (Tacconelli et al., 2018). According to a recent report, *Salmonella* causes 115 million cases of an acquired bacterial food-borne illness named Salmonellosis and 370 thousand deaths every year globally (Seif et al., 2018). *Salmonella enterica* is a diverse species of bacteria consisting of more than 2,600 different serovars and it is one of the serious pathogens to human health as well as in animals (Grimont and Weill, 2007; Gal-Mor et al., 2014). *Salmonella enterica* spp. is divided into two main groups, typhoidal *Salmonella* (TS) and non-typhoidal *Salmonella* (NTS). Typhoidal *Salmonella* being highly adapted to the human host causes life-threatening enteric fever whereas gastroenteritis, and bacteremia are caused by non-typhoidal *Salmonella* (Gal-Mor et al., 2014). Non-typhoidal *Salmonella* which is mostly zoonotic can manifest disease in two forms invasive and non-invasive (Hohmann, 2001). Also, different food sources like vegetables, fruits, milk, chicken, beef or the consumption of raw or undercooked foods, etc. were identified as vehicles of *Salmonella*-associated foodborne outbreaks in the past decade (Carrasco et al., 2012; Devleesschauwer et al., 2015).

Both in nature and disease state, some bacteria create a surface-associated community known as biofilm for long-term survival in hostile environments. Additionally, bacteria within a biofilm are more prone to resistance to antibiotics compared to those in planktonic bacterial cells (Hall and Mah, 2017). Therefore, the biofilm-mediated infection has become a public health concern. For treatment purposes, newly synthesized drugs and combinational therapy have been developed but satisfactory results are yet to be achieved. *Salmonella* is one of the commonly known bacterial species that form biofilms (Done et al., 2015). It has also been reported that bacterial biofilm can significantly contribute to pathogenesis by creating resistance against the body's defense system (Bai et al., 2021). In fact, the capability of *Salmonella* spp. to form biofilm is generally connected with their pathogenicity (Vestby et al., 2020). Bacteriophages have some unique properties that allow the treatment of biofilms effectively. There are several reports on the anti-biofilm properties of lytic phages both *in vitro* and in clinical infection (Azeredo and Sutherland, 2008; Doub, 2020).

Bacteriophages are naturally-occurring bacterial viruses, that can specifically infect and lyse a bacterial cell without affecting the host microflora (Housby and Mann, 2009). The specific antibacterial activities of lytic phages hint at their applications as alternatives to antibiotic therapy for humans and animals (Thung et al., 2017). However, some polyvalence is predominately observed among phages of Enterobacteriaceae at the beginning of the last century (Parra and Robeson, 2016). Additionally, the polyvalent phages are able to infect a broad host spectrum of bacteria from either different genera or species (Hamdi et al., 2017). Furthermore, it has been hypothesized that phages isolated from one region might not infect bacteria in other regions due to the broad diversity and defense mechanisms of enteric bacteria. Therefore, a continuous search of new phages focussing on different serovars with high lytic ability is certainly required for practical application (Kropinski et al., 2009). In phage-bacterial interactions, physicochemical factors such as temperature and pH play an essential role. Therapeutic phages are generally selected based on large burst size, short latent period, high adsorption rate, broad host range, strong antimicrobial property, and lytic capacity for further applications (Nilsson, 2019).

In this study, we aimed to isolate and characterize a novel polyvalent bacteriophage STWB21 with infectivity against different typhoidal and non-typhoidal *Salmonella* serovars for its potential use in phage therapy. We investigated the microbiological and morphological properties, genomic and proteomic analysis, and lytic activity of phage against biofilms under controlled conditions *in vitro*.

Materials and methods

Bacterial strains

The *Salmonella* bacterial strains used in this study were isolated from the patient's blood sample at the Division of Bacteriology, ICMR-NICED, India. The bacterial strains were grown under aerobic conditions in Luria-Bertani (LB) broth at 37°C following institutional standard biosafety guidelines.

Isolation and purification of bacteriophage

The water sample was collected from lake water in the outskirt area about 18 km from Kolkata, West Bengal, India in search of phage isolation. Briefly, 25 ml of water sample was mixed with 25 ml of 10X phage broth media and 5 ml of log-phase *Salmonella typhi* culture and incubated at 37°C for 24 h under shaking conditions at 100 rpm. The residual bacterial cells were removed by centrifugation at 10,000 rpm for 10 min at 4°C and the supernatant was filtered with a 0.22 μm membrane filter. To confirm the presence of the phage in the filtrate, a spot assay was performed on *Salmonella typhi* using the double-layer agar method (Sambrook and Russell, 2001; Kutter, 2009). Then the single plaque was picked up three times by plaque assay to obtain the purified phage plaque. After proliferation, the phage was purified and concentrated by ultracentrifugation (25,000 rpm, 1.5 h, 4°C) and sucrose step-gradient ultracentrifugation (30,000 rpm, 2 h, 4°C) respectively (Dutta and Ghosh, 2007).

Host range and efficiency of plating

Host range assay was performed to evaluate the lytic spectrum of the obtained bacteriophages to the susceptible bacterial strains.

It was determined by spotting 10 µl of phage lysate (10^{10} PFU/ml) on each agar plate with different bacterial strains. The plates were incubated at 37°C overnight and examined for clear zones that indicate the susceptibility of the bacteria. Further, an EOP assay of the obtained phage was then conducted by the double-layer agar method to quantify the lytic activity of phage STWB21.

Transmission electron microscopy

3 µl purified phage lysate (10^{10} pfu/ml) was applied to a carbon-coated copper grid and negatively stained with 2% (w/v) uranyl acetate for 30 s and excess liquid was blotted off (Czajkowski et al., 2015). After air-drying the grids were visualized with FEI Tecnai 12 BioTwin Transmission Electron Microscope operating at 100 kV.

Stability

A thermal stability test of phage STWB21 was carried out at pH 7.0 in order to investigate the heat-resistant capability of the phage. The phage was incubated at different temperatures (4°C, 25°C, 37°C, 50°C and 70°C respectively) for 1 h. After the incubation, the phage titer was determined by the soft-agar overlay method (Yuan et al., 2015). The pH stability of phage STWB21 was examined by pre-incubating the phage suspensions at a range of pH (pH 3–13) at 37°C for 1 h. Then the surviving phages were immediately counted by the double-layer agar method. Each assay was performed in triplicate.

Adsorption assay and one-step growth assay

The adsorption assay was conducted as described previously with some modifications (Lu et al., 2003). Briefly, the bacterial culture (1 ml) was infected with a phage suspension at an MOI of 0.1 and incubated for 20 min at 37°C. After infection, aliquots were taken at different time intervals (0, 5, 10, 15, and 20 min) and immediately diluted in Tris-$MgCl_2$ buffer, followed by centrifugation at 8,000 rpm for 5 min. Then the supernatant un-adsorbed phage titers were estimated by the double-layer agar method. In addition, a one-step growth curve of bacteriophage STWB21 was performed to access the growth kinetics of phage STWB21. It was performed according to the method described previously (Bloch et al., 2013). At an interval of 10 min, the exponentially growing bacterial culture (20 ml) was harvested and the pellet was resuspended in around 1 ml Luria broth followed by the addition of phage. The mixture was incubated for adsorption at 37°C for 5 min. The mixture was collected and immediately plated by the double-layer agar method. Burst size was calculated as the ratio of the final count of liberated phage particles to the initial count of infected bacterial cells during the latent period.

Biofilm formation assay and effect of phage against biofilm

On glass coverslip

The biofilm degradation activity of newly isolated phage STWB21 on *S. typhi* was accessed following a previously discussed method with some modifications (Cerca et al., 2006; Nickerson et al., 2017; Mallick et al., 2021). A 10 µl of an overnight culture of *S. typhi* was dispensed into a Petri dish containing glass coverslips (22 mm × 22 mm) and incubated at 37°C for 24 h to form the biofilm. Two sets of Petri dishes were incubated for biofilm formation. One set was treated with buffer after 24 h incubation and the other set was treated with phage STWB21 overnight at 37°C.

Application of bacteriophage in food items for controlling *Salmonella typhi*

On onion

The fresh onion bulb was chosen due to its association with foodborne illness outbreaks and variability in its surface structure. On the day of the experiment, fresh onion was purchased. Before an experiment, the onion was washed thoroughly for 5 min under running tap water (room temperature) followed by 70% alcohol to remove any soil or organic matter and any microflora present. After that, the cleaned onion bulb was placed in the center of a sterile Petri dish. Using a sterile scalpel, outer membranes were removed and the remaining scales of the onion were cut into 3 × 3 cm pieces. Further, these onion pieces were inoculated with 200 µl of *S. typhi* at 10^8 CFU/ml and incubated at 37°C. After overnight incubation, the onion was treated with 20 µl of purified phage solution (1.5×10^{10} PFU/ml) for 4 h.

In milk

To study the removal and degradation of biofilm in milk by phage STWB21, we used pasteurized milk as the culture medium for the *S. typhi* biofilm. We purchased commercial sterile ultra-heat-temperature (UHT) treated milk from local retail. A logarithmic-growth-phase culture of *S. typhi* at a final concentration of 10^6 CFU/ml was added to 1 ml of milk in two different sets of Petri dish containing glass coverslips (22 mm × 22 mm) to form the biofilm when incubated at 37°C for 24 h. After overnight incubation, one set of Petri dishes was treated with 40 µl of purified phage solution (1.5×10^{10} PFU/ml) for 4 h.

Characterization of biofilm using scanning electron microscopy analysis

Biofilm samples of control and phage-treated groups were prepared for SEM analysis as described by Jahid et al. with some modifications (Jahid et al., 2013). Briefly, samples were fixed with 2.5% glutaraldehyde (Sigma-Aldrich, United States) in 0.1 M sodium cacodylate buffer at 4°C overnight. After that, the samples were serially dehydrated with ethanol (30%, 50%, 70%, and 90% for 10 min each, and 100% two times for 15 min each) and then successively treated with 100% hexamethyldisilazane (Sigma-Aldrich, United States) for 1 h. The dehydrated samples were sputter-coated with gold and visualized on FEI Quanta 200 SEM (FEI, Netherlands).

Microtiter plate-based assay of biofilm

To evaluate the anti-biofilm efficacy of phage STWB21, biofilm was developed in 96-well plates in accordance with the previously described method with some modifications (Cerca et al., 2006). Briefly, the *S. typhi* strain was grown overnight at 37°C and was diluted to 1:100 in a fresh LB medium. Then 200 μl of the diluted culture was added to 96-well plates and was placed in an incubator at 37°C for 24 h without agitation. After incubation, the supernatant was removed from the well plate. To examine the effect of phage STWB21 on biofilm, phage stock was added into the biofilm at different concentrations (MOI 0.1 and 0.01) and incubated at 37°C for 4 and 24 h, respectively. Thereafter, the wells were rinsed with 1X PBS thrice and allowed to air-dry. The air-dried plate was then stained with crystal violet (0.1%, w/v) for 30 min. The optical densities of the biofilm were measured on the microplate reader at the absorbance of 595 nm (iMark Microplate Reader S/N 21673).

SDS page analysis

To analyze the phage STWB21 protein profile, phage solution was boiled for 5 min and the structural proteins were extracted. The denatured proteins of phage STWB21 were separated by using 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) with Mini-PROTEAN TGX Precast Gels (Bio-Rad, United States). After electrophoresis, proteins were visualized by staining with Coomassie Brilliant Blue.

Proteomic analysis

Protein extraction was prepared as described in the SDS-PAGE assay. A total of 100 μg of protein was extracted and lyophilized. The 20 μg lyophilized protein was re-dissolved in 1.5 ml ice-cold 1 mM HCl (13 ng/μl trypsin prepared) and 100 μl aliquots stored at −20°C for further protein analysis. Protein in-solution digestion was performed at C-CAMP (NCBS, India) according to the previously described method (Wiese et al., 2007). Proteomic analysis was performed on 1,200, 1D nano-LC (Agilent Technologies, San Diego) that was coupled to Nanomate Triversa (Advion) and LTQ – Orbitrap Discovery (Thermo Fisher Scientific, United States). Nano LC-ESI-MS/MS spectra were searched using a PEAKS engine against phage genomes. For protein identification, the following parameters were used. Peptide mass Error tolerance = 10.0 ppm, fragment mass error tolerance = 0.6 Da, enzyme = trypsin, missed cleavage = 2, fixed modification: carbamidomethyl (C), variable modifications: oxidation (M), deamidation (NQ).

Genomic DNA extraction and restriction digestion

DNA was extracted from the high-titer stocks of *Salmonella* phage STWB21 using a phage DNA isolation kit (Norgen Biotek Corp., Canada) according to the manufacturer's instructions. The purity and concentration of the DNA were determined using a spectrophotometer. Then the DNA was digested by seven restriction enzymes namely: EcoRI, EcoRV, MluI, BglII, PstI, BamHI, and HindIII. Restriction fragments were separated by electrophoresis on 1.0% agarose gel and stained with ethidium bromide. DNA molecular weight marker (high range DNA ladder, HiMedia; ranging from 250 bp to 25 kbp) was used for the size determination of DNA fragments.

Genomic characterization of phage STWB21

Purified genomic DNA of phage STWB21 was then sequenced by using an Illumina Platform at Xcelris (Ahmedabad, India). Reads were trimmed with Bioedit (version 7.1) and assembled with CLC Genomics Workbench v.6.0.5 with the reads map back option. The putative ORFs were predicted and annotated by GeneMark.hmm version 3.25[1] and further confirmed by the RAST (McNair et al., 2018) server and tRNAs carried by the phage genomes were detected using the protein ARAGORN (Laslett and Canback, 2004) and tRNAscan-SE (Lowe and Eddy, 1997).[2] Visualization of genome alignments of phage STWB21 with closely related *Siphoviridae Salmonella* phages was performed using the Easyfig application.[3]

To construct the phylogenetic tree, amino acid sequences of the terminase large subunits (ORF 130) and major capsid protein (ORF137) were selected and obtained from the NCBI Genbank database.[4] Additionally, the terminase large subunits are usually considered as genetic marker (Lee et al., 2021) for the order Caudovirales and the major capsid protein is a highly conserved protein (Oh et al., 2014). The two phylogenetic trees were constructed with the default pipeline "ONE CLICK" at Phylogeny.fr (Dereeper et al., 2008).

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[1] http://exon.gatech.edu/GeneMark/index.html

[2] http://lowelab.ucsc.edu/cgi-bin/tRNAscan-SE2.cgi

[3] http://mjsull.github.io/Easyfig/files.html

[4] https://www.ncbi.nlm.nih.gov

Statistical analyses

The statistical analysis was performed by *t*-test using GraphPad Prism (version 5, GraphPad Software, United States) software. All the values were tabulated as mean ± SD, and a significant difference between variations denoted by value of p were estimated using two-way ANOVA.

Results

Isolation, purification, and host range determination

The phage STWB21 was isolated from a lake water sample in the outskirts of Kolkata area. The host spectrum of phage STWB21 exhibited specific lytic activity against the prevalent typhoidal and non-typhoidal *Salmonella* spp., *Sh. flexneri* 2a, *Sh. flexneri 3a and ETEC* (Table 1). Phage STWB21 was found to be a novel polyvalent phage with high lytic activity against both typhoidal and non-typhoidal *Salmonella*. However, the phage did not show infectivity against some other species: *Sh. flexneri6, Sh. boydii* and *V. Cholerae 01* were used in this study. During infecting the prevalent *Salmonella* serovars, phage STWB21 formed clear plaques against *S. typhi, S. paratyphi, S. typhimurium* and *S. enteritidis*. The higher EOP values were obtained for the phage-sensitive *Salmonella* strains compared to the *Shigella* spp. and *ETEC* strains (Supplementary Table S1).

Virion morphology

Plaques of phage STWB21 on *S. typhi* bacterial lawn were measured around 1 mm diameter after overnight incubation (Figure 1A). Transmission Electron Microscopic study of phage STWB21 revealed a structure comprising an icosahedral head with an estimated diameter of 65 ± 3 nm (n = 20) and a long flexible, non-contractile tail of 113 ± 6 nm in length (*n* = 20) approximately (Figure 1B). Therefore, taking into account the morphological characteristics and criteria of ICTV (Fauquet and Fargette, 2005), phage STWB21 was classified within the *Caudovirales* order as a member of the *Siphoviridae* family (Ackermann, 2007).

Phage stability test

The thermal stability of the phage STWB21 was analyzed on different typhoidal and non-typhoidal *Salmonella* spp. The phage STWB21 was remained stable up to 40°C for 1 h, but significantly decreased and lost its lytic activity at 50°C against the typhoidal and non-typhoidal host strains (Figure 2A). On the other hand, phage STWB21 showed the highest activity at pH 7.0 and notable stability in acidic and alkaline conditions over a wide pH range (4–11) after 1 h incubation (Figure 2B).

TABLE 1 Host range analysis of phage STWB21.

Strains	No. of strains used	No. of strains susceptible
1. *S. typhi*	4	4
2. *S. paratyphi*	3	3
3. *S. enteritidis*	1	1
4. *S. typhimurium*	1	1
5. *ETEC*	3	2
6. *Sh. flexneri 2a*	1	1
7. *Sh. flexneri 3a*	1	1
8. *Sh. flexneri 6*	1	0
9. *Sh. boydii*	1	0
10. *V. cholerae O1*	1	0

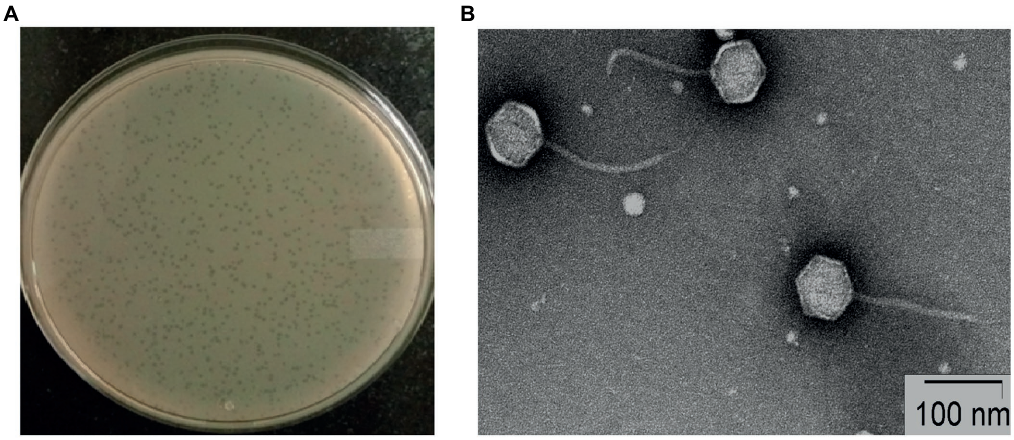

FIGURE 1
Morphology of bacteriophage STWB21. **(A)** Plaque morphology of phage STWB21, **(B)** transmission electron microscopic morphological study of phage STWB21.

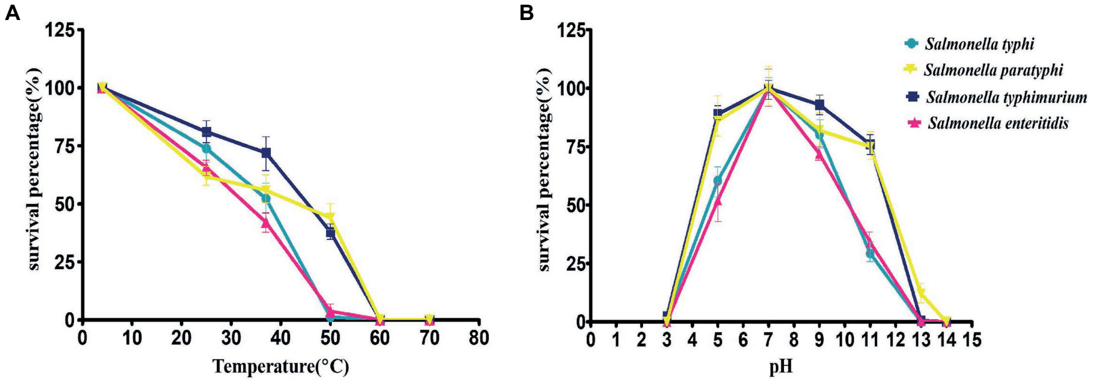

FIGURE 2
Biological characteristics of phage STWB21. **(A)** Thermal stability, **(B)** pH stability. Given values are the mean of three determinations.

Adsorption rate and one-step growth assay

Intracellular lytic development and the growth kinetics of phage STWB21 were analyzed in one-step growth assay on different typhoidal and non-typhoidal *Salmonella* species including host bacteria (Figure 3A). The latent period/burst size for *S. typhi, S. paratyphi, S. typhimurium* and *S. enteritidis* was found to be 25 min/101, 55 min/53, 50 min/163, 35 min/224 pfu, respectively, (Supplementary Table S2). Additionally, rapid adsorption was occurred for different typhoidal and non-typhoidal *Salmonella* species in first 5 min, followed by a slower adsorption phase thereafter (Figure 3B).

Biofilm degradation assay and scanning electron microscopy analysis in coverslip, onion, and milk

Salmonella typhi culture was kept at 37°C in static condition to form bacterial biofilm of 24 h and treated with phage STWB21 to examine its ability to degrade biofilm. The experiment was done in coverslip, onion and milk. Scanning electron microscope analysis revealed a clear indication of *Salmonella typhi* biofilm formation on coverslip, onion and milk (Figures 4A,C,E) respectively. Once the biofilm was treated with phage STWB21 the bacterial population in biofilm was notably decreased on coverslip, onion and milk (Figures 4B,D,F) respectively.

Quantification of *Salmonella* biofilm in microtiter plate

The mature biofilm of *S. typhi* strain was significantly reduced by STWB21 phage. As shown in Figure 5, biofilm formation was significantly decreased ($p < 0.001$) by phage STWB21 at different concentrations (MOI 0.1 and 0.01). After 4 h, phage STWB21 removed 31% ($p < 0.001$) and 36% ($p < 0.001$) of the biofilm biomass at MOIs 0.1 and 0.01 respectively, when compared to control. However, after 24 h treatment the above-mentioned values changed to 68% ($p < 0.001$) and 37% ($p < 0.001$) respectively.

Analysis of structural proteins of phage STWB21 by SDS-page

SDS-PAGE analysis of phage STWB21 showed the presence of structural proteins ranging from 10 to 150 kDa. There are only two major bands that appeared at around 36 and 61 kDa while the visible minor bands appeared at 17, 41, 48, 74, 120 and 132 kDa (Figure 6).

Proteomic features

To identify the structural proteins in phage STWB21, purified phage particles were analyzed by high-resolution Nano LC–MS/MS. A detailed proteomic characterization identified 19 proteins in phage STWB21 (Table 2). The structural proteins that were identified in phage STWB21 are six tail proteins, three tail fiber proteins, one prohead protease protein, one receptor-binding tail protein, one major capsid protein, one portal protein, and one capsid and scaffold protein. Interestingly, DNA metabolism module-related gene (A1 protein) and host lysis module-related gene (lysozyme) were identified. It is believed that the expression of A1 gene in T5 phage is required for the second step transfer of DNA.

Restriction profile of phage DNA

Phage STWB21 genome was cut with restriction enzymes EcoRI, EcoRV, MluI, BglII, PstI, BamHI, and HindIII

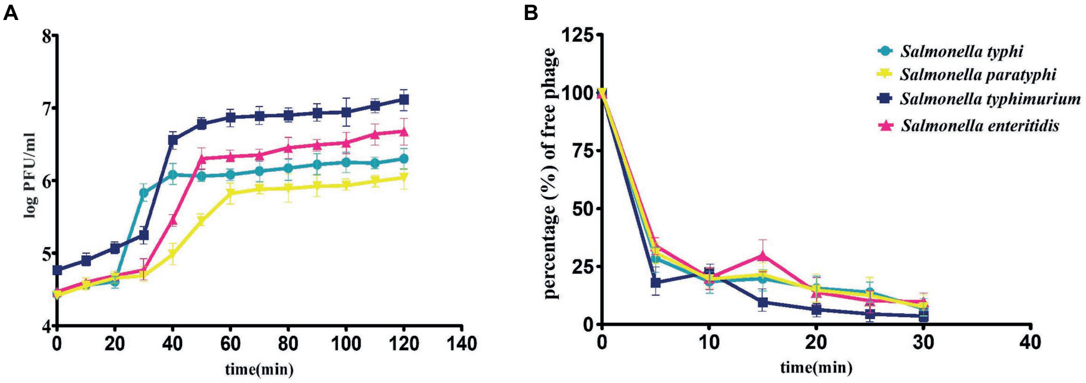

FIGURE 3
(A) One-step growth curve, **(B)** adsorption assay of bacteriophage STWB21 against *S. typhi, S. paratyphi, S. typhimurium* and *S. enteritidis*. Given values are the mean of three determinations.

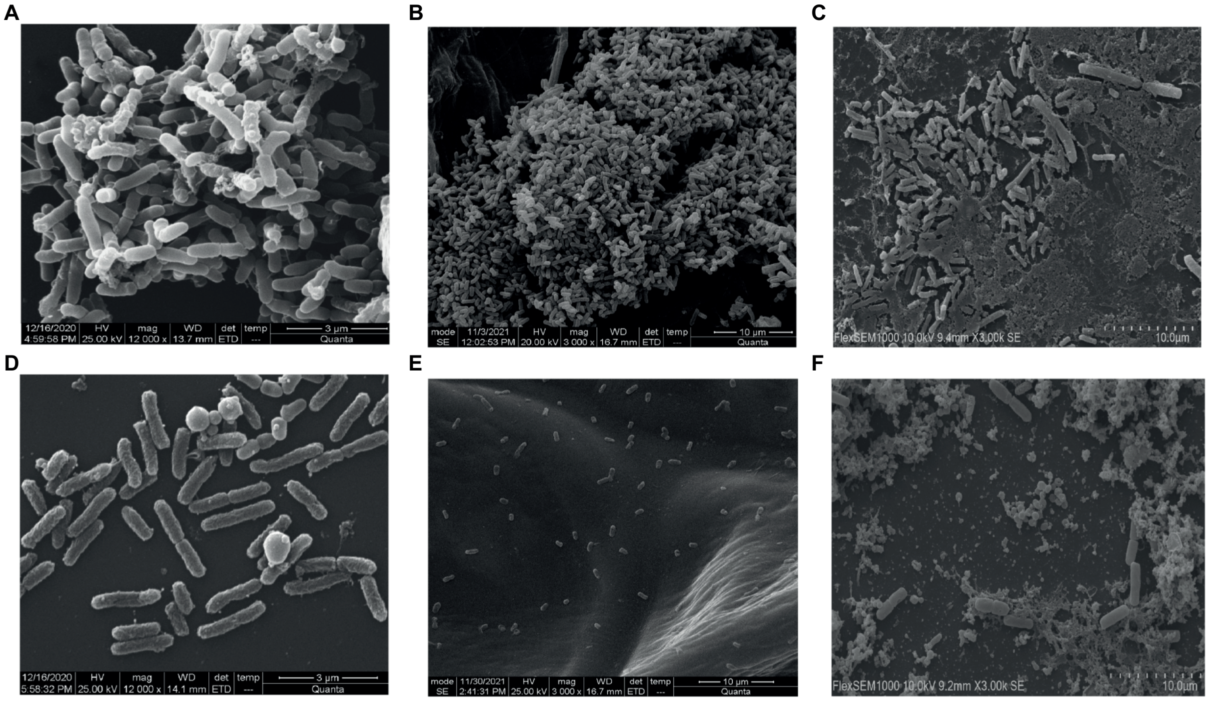

FIGURE 4
Scanning electron micrographs of *Salmonella typhi* biofilm formation and degradation assay. **(A, C, E)** show 24h biofilm on coverslip, onion and in milk, respectively. **(B, D, F)** reveal the effect of bacteriophage STWB21 on biofilms formed on coverslip, onion and in milk, respectively.

(Supplementary Figure S1). Digestion of STWB21 genetic material with restriction enzymes confirmed that it is a double-stranded DNA virus.

General features of the STWB21 genome

The complete genome length of phage STWB21 is 112,834 bp with a 40.37% GC content very similar to the *Salmonella* phages of T5 virus family: *Salmonella* phage S124, Seafire, Gec_vB_N3, S133, Seabear, Stitch (Table 3). Thus, according to Megablast results, STWB21 was enumerated as a member of the T5 virus genus, *Siphoviridae* subfamily.

Phage STWB21 genome is predicted to encode 166 putative open reading frames (47 on the complementary strand and 119 on the direct strand) with 152 ATG (91.07%), 10 GTG (6.02%) and 5 TTG (2.99%) as initiation codons. After genome analysis, 22 tRNA encoding genes were found to be present in phage STWB21 and it

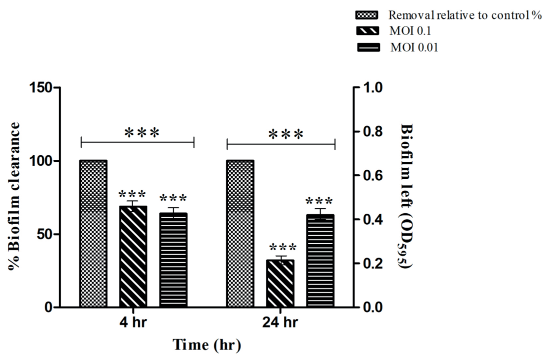

FIGURE 5
Quantification of *Salmonella* biofilm in Microtiter Plate. A 24h biofilm of *S. typhi* formed in 96-well plates was treated with phage STWB21 at m.o.i 0.1 and 0.01 concentrations both at 4h and 24h time points. All the values were tabulated as mean ± SD and a significant difference between variations was denoted by asterisks (***$p<0.001$) using two-way ANOVA.

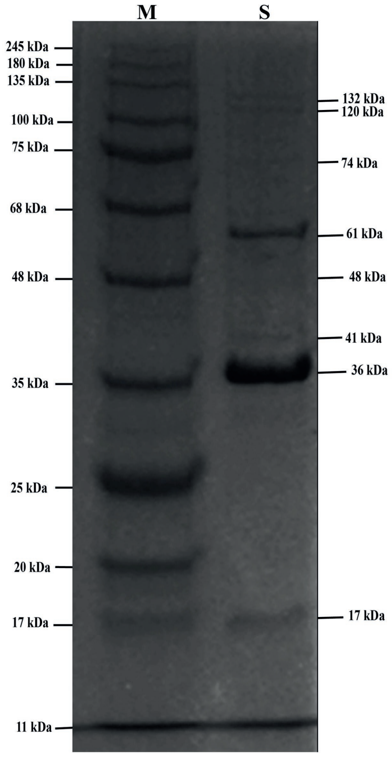

FIGURE 6
The SDS-PAGE analysis of phage STWB21 structural proteins on 12.5% gel staining with Coomassie brilliant blue. M, a standard marker of molecular weight (kDa).

seems to be a characteristic of a virulent phage. Closely related *Siphoviridae Salmonella* phages were also reported to carry a similarly large number of tRNA encoding genes (Table 3).

Phage STWB21 whole genome sequence was compared against the nucleotide sequence database in NCBI using BLASTn, Phage STWB21(MW567727) showed high homology with six bacteriophages: phage S124 (NC_048013), Seafire (NC_048110), Gec_vB_N3 (MW006478), S133 (NC_048011), Seabear (MK728824) and Stitch (KM236244). The detailed genomic features of the above-mentioned phages were listed in Table 3. The genomic sequence of STWB21 is 92.6% identical to that of S124, 92.6% to Seafire, 92.4% to Gec_vB_N3, 92.2% to S133, 92% to Seabear and 91.9% to Stitch as shown in Table 3.

By using the Easyfig genome comparison tool, we have compared phage STWB21 genomic synteny with other related *Siphoviridae Salmonella* phages: S124, Seafire, Gec_vB_N3, S133, Seabear, Stitch (Figure 7). Multiple alignment of phage STWB21 with other six relative phages have shown that the gene inventories of these six closely related phages are highly similar. As indicated in Figure 7, the genomes of the phages contain a block of clustered genes encoding predicted structural and functional proteins. In some cases, they were arranged differently and even oriented in opposite directions.

As shown in Figure 8, the ORFs of STWB21 were broadly scattered across the genome and the gene annotation of predicted ORFs is listed in Supplementary Table S3.

Description of phage modules

The annotated proteins of STWB21 can be categorized into four different genetic modules, which enable the following: DNA replication/modification/transcriptional regulations, lysis, DNA packaging and morphogenesis.

DNA replication/modification/transcriptional regulation module proteins

STWB21 encodes at least 12 genes co-localized as a distinct DNA modification module and were anticipated to play role in phage DNA metabolism, including a DNA helicase (ORF 160), a helicase-primase (ORF 163-ORF 164), a DNA polymerase I (ORF 162), a transcriptional factor (ORF 165), DNA ligase (ORF 1), transcriptional regulator/Methyltransferase (ORF 3), HNH homing endonuclease (ORF 21), ribonuclease (ORF 27), exonuclease (ORF 156), recombinase (ORF 157). The highly presence of DNA metabolism-associated genes in STWB21 genome might reduce the dependence of phage on bacteria (Peng and Yuan, 2018).

Two-component host lysis module proteins

A classic eubacterial phage lysis cassette compromises two-component cell lysis proteins, a holin (ORF 79) and lysozyme (ORF 80) are present in STWB21 genome. During the burst step of phage life cycle, these genes are crucial to effect host-cell lysis. Pore-forming enzyme holin permeabilizes the cytoplasmic membrane and cell wall degrading protein lysozyme degrades the bacterial cell wall (Wang et al., 2000). According to previous reports, holin have usually two or three transmembrane (Bläsi et al., 1999) domains but the genome analysis revealed that the holin of STWB21 has one transmembrane domain group (TMHMM-2.0). Besides, genomic analysis of phage STWB21 was

TABLE 2 Identification of the phage STWB21 proteins by LC_MS/MS.

ORF	Protein	Molecular mass (Da)	No. of peptides	Sequence coverage (%)
1. ORF146	Tail protein	132,066	35	40
2. ORF151	L-shaped tail fiber	114,851	31	38
3. ORF142	Major tail protein	50,322	21	57
4. ORF148	Tail protein	107,179	20	30
5. ORF135	Tail fiber protein	16,967	16	82
6. ORF152	Tail fiber protein	75,110	8	20
7. ORF139	Hypothetical protein	19,405	8	71
8. ORF118	A1 protein	61,470	9	22
9. ORF149	Tail protein	74,758	5	9
10. ORF97	Capsid and scaffold protein	18,742	9	42
11. ORF137	Major capsid protein	23,016	6	11
12. ORF143	Hypothetical protein	34,437	6	34
13. ORF134	Portal protein	32,976	6	28
14. ORF150	Tail protein	15,508	5	49
15. ORF136	Prohead protease	23,372	6	38
16. ORF128	Receptor binding tail protein	64,149	2	6
17. ORF112	Hypothetical protein	36,314	4	21
18. ORF80	Lysozyme	15,236	3	27
19. ORF147	Tail protein	22,658	2	5

TABLE 3 List of reported *Salmonella* phages from *Siphoviridae* family.

Phage name	Similarity	Length	GC content	tRNA	Host	Taxonomic genera	Accession number	Reference
STWB21	–	112,834	40.39%	22	*S. typhi*	T5 virus	MW567727	Present study
S124	92.69%	112,564	40.12%	28	*S. enterica* subsp.	T5 virus	NC_048013	NCBI Database (Unpublished)
Seafire	92.68%	111,851	40.01%	27	*S. enteritidis*	T5 virus	NC_048110	Hartman et al. 2019
Gec_vB_N3	92.40%	109,645	40.08%	17	*S. enteritidis*	T5 virus	MW006478	Makalatia et al. 2020
S133	92.28%	110,926	40.06%	29	*S. enterica* subsp.	T5 virus	NC_048011	NCBI Database (Unpublished)
Sea bear	92.08%	112,472	40.38%	25	*S. typhimurium*	T5 virus	MK728824	Patil et al. 2019
Stitch	91.90%	123,475	40.31%	30	*S. typhimurium*	T5 virus	KM236244	Grover et al. 2015

studied for the presence of phage lysogeny factors and toxin genes. The presence of the lysis gene and the absence of lysogeny-related genes in the STWB21 genome clearly indicates that the phage is a potent lytic phage.

DNA packaging module proteins

In the DNA packaging modules, only ORF 130 was predicted which encodes the large subunit of terminase in the STWB21 genome and displayed 99% similarity with other *Salmonella* phage S124. Previous studies suggested that the large subunit of terminase is very conserved among the related phages (Burroughs et al., 2007). The junction of the DNA packaging module and the morphogenesis module is constituted by portal protein.

Phage morphogenesis module proteins

The arrangements of 22 genes encoding STWB21 phage structural proteins were scattered in the complementary strand of the genome and followed the gene orders of *Siphovirus*. These 22 ORFs mainly encode proteins like portal protein (ORF 133, ORF 134), tail fiber protein (ORF 135, ORF 151, ORF 152), major capsid protein (ORF 137), capsid and scaffold protein (ORF 138), tail length-tape measure protein (ORF 145), tail completion protein (ORF 166). As previously described, tail fiber proteins are subject to horizontal gene transfer between phages in a constant manner (Greive et al., 2016). During infection, tail length tape measure protein DNA is transited to the bacterial cell by the tape measure protein (Mahony et al., 2016).

Phylogenetic analysis

To further understand the phylogenetic relationship of STWB21 to other *Salmonella* phages, two different types of phylogenetic trees were generated as shown in Figures 9A,B based

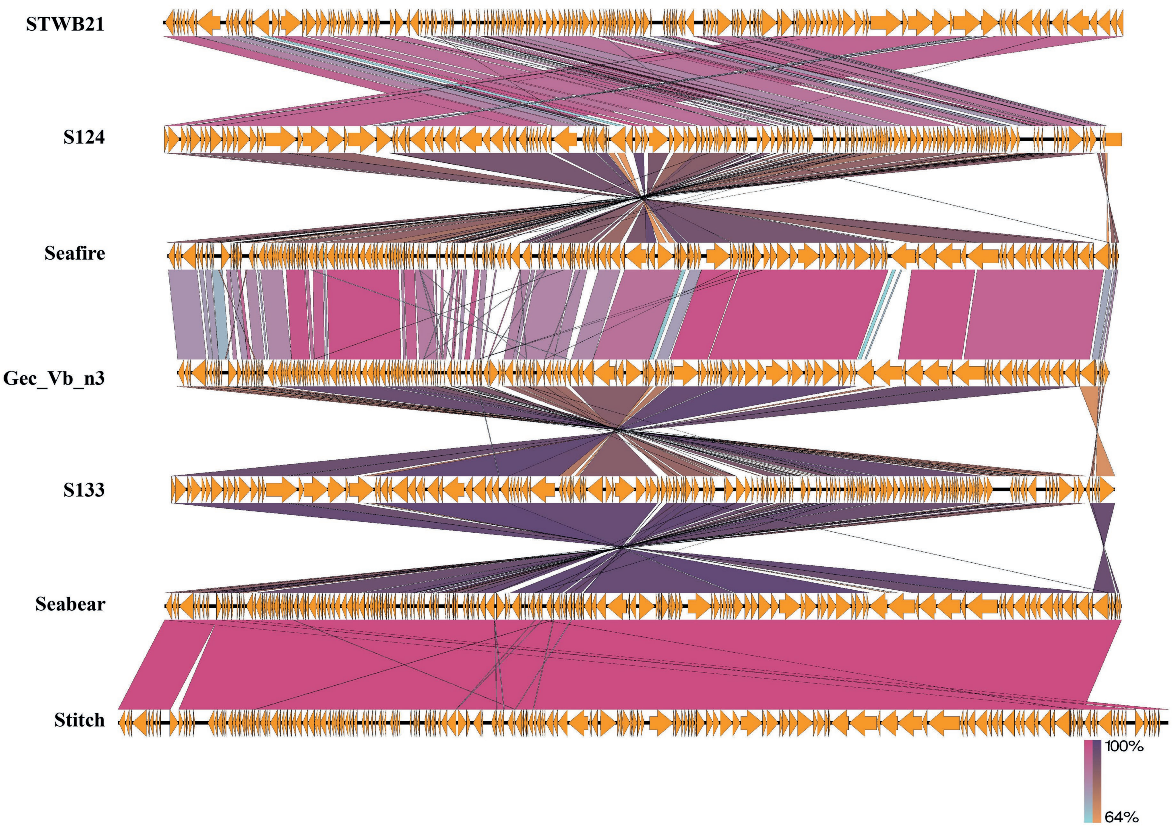

FIGURE 7
Genomic comparison of the phage STWB21 with other six homologous *Salmonella* phages using Easyfig application. Homologous ORFs or genes are present in yellow, and the percentages of amino acid identities are shown in different colors. Arrows indicate ORFs with either rightward or leftward direction.

on the terminase large subunit (TerL) and the major capsid protein, respectively. The tree showed that the phage STWB21 cluster together with T5-like *Salmonella* phages, such as S124, Stitch, SH9, S113, Sea bear, SE3 and Sepoy. Therefore, the investigated phage was considered as a new species of the T5 genus.

Discussion

Salmonella genus is considered one of the biggest public threats due to its expanded reservoir (WHO, 2017). In addition, antibiotic resistance amidst bacterial infection has become a global burden (Tacconelli et al., 2018). To overcome this situation an alternative approach to treat bacterial infection is a pressing priority. Bacteriophages especially lytic bacteriophages can be effective against specific bacterial infections due to their ability to infect and kill specific host bacteria. Therefore, an upsurge in researches on bacteriophage isolation, characterization and effectiveness against pathogenic bacteria has reflected the awareness worldwide. Effective phage therapy requires specific conditions such as a large collection of bacteriophages, the use of obligately lytic phages rather than the temperate phage, host range, and screening of phage genomes to confirm the absence of toxin genes (Weber-Dabrowska et al., 2016). Furthermore, it is advantageous to use phages that adsorb efficiently on host cells, propagate rapidly with a relatively high burst size and may eliminate target bacteria in a relatively short time. Here, we reported the isolation and characterization of phage STWB21 and evaluated its application in food samples.

In this study, we have characterized a lytic bacteriophage STWB21 isolated from the lake water sample in the outskirts of Kolkata area using a strain of *S. typhi* as the host bacterium. Phage STWB21 was found to be effective against many other strains of typhoidal and non-typhoidal *Salmonella* which indicates the high specificity of STWB21 against *Salmonella* (Abdelsattar et al., 2021). On the other hand, the ability of phage STWB21 to lyse *Sh. flexneri 2a*, *Sh. flexneri 3*, and *ETEC* indicate that the phage can infect more than one bacterial species. Thus, phage STWB21 is a "polyvalent phage," as this quality has been reported before for other *Salmonella* phages (Parra and Robeson, 2016). In addition, to the best of our knowledge, this is the first *Salmonella* phage active against typhoidal strains as well as non-typhoidal strains.

Resistance to heat and acid–base properties are the foundations of phage used in bio-control applications (Jończyk-Matysiak et al., 2019). Different foods like fruits, vegetables,

FIGURE 8
Genome structure of phage STWB21 prepared using CGView. The outer ring denotes the STWB21 genome and ORFs. The inner rings show G+C content and G+C skew, where peaks represent the positive (outward) and negative (inward) deviation from the mean G+C content and G+C skew, respectively.

pasteurized milk or chicken breast are usually stored at 4°C or at ambient temperature (25°C). According to our results, the phage STWB21 was stable at 4°C to <50°C, and the stability of phage STWB21 at ambient temperature without losing viability for a month, implied its suitability for large-scale applications. The phage STWB21 is relatively stable within the range of pH between 4 and 11, which is congenial with the range (pH 5.5–7.0) of many foods. Therefore, their ability to survive at different temperature and pH conditions suggest that phage STWB21 is tolerant to heat and extreme pH conditions and it could be used in different food industries.

During October 2021, food poisoning outbreak news came out from California, New York, North Carolina, Pennsylvania and Texas among adults due to the consumption of imported whole red, white and yellow onions. There are published reports controlling the biofilm on coverslips, fruits, vegetables and pasteurized milk using bacteriophages (Isoken, 2015; Amrutha et al., 2017; Wang et al., 2018). However, no reports are available yet using phages that can control *Salmonella* spp. biofilm formation on the red onion. In this study, we evaluated the ability of lytic phage STWB21 to remove biofilm from glass coverslips, red onion and pasteurized milk. After treatment with phage STWB21, the number of bacteria in the biofilm was decreased significantly. Moreover, biofilms were considerably degraded, and disrupted and did not show any microbial regrowth. Therefore, anti-biofilm results support that phage STWB21 can be used as a "biological disinfectant" capable of controlling *Salmonella* infections.

In our study, detailed genomic and proteomic analyses of phage STWB21 revealed valuable information concerning its biology and showed a modular organization that is very similar to other T5-like *Salmonella* bacteriophages S124, Gec_vB_N3, S133, Seabear, Stitch (Grover et al., 2015; Patil et al., 2019; Necel et al., 2020). Following LC–MS/MS analysis, 17 structural proteins and 2 functional proteins have been identified. Moreover, genomic and proteomic analyses indicated that the phage STWB21 genome does not encode any toxin gene, antibiotic resistance gene, phage lysogeny factors or pathogen-related genes, suggesting that the phage STWB21 may be considered as a reliable phage therapy candidate with no side effects. Bacteriophage STWB21 forms clear

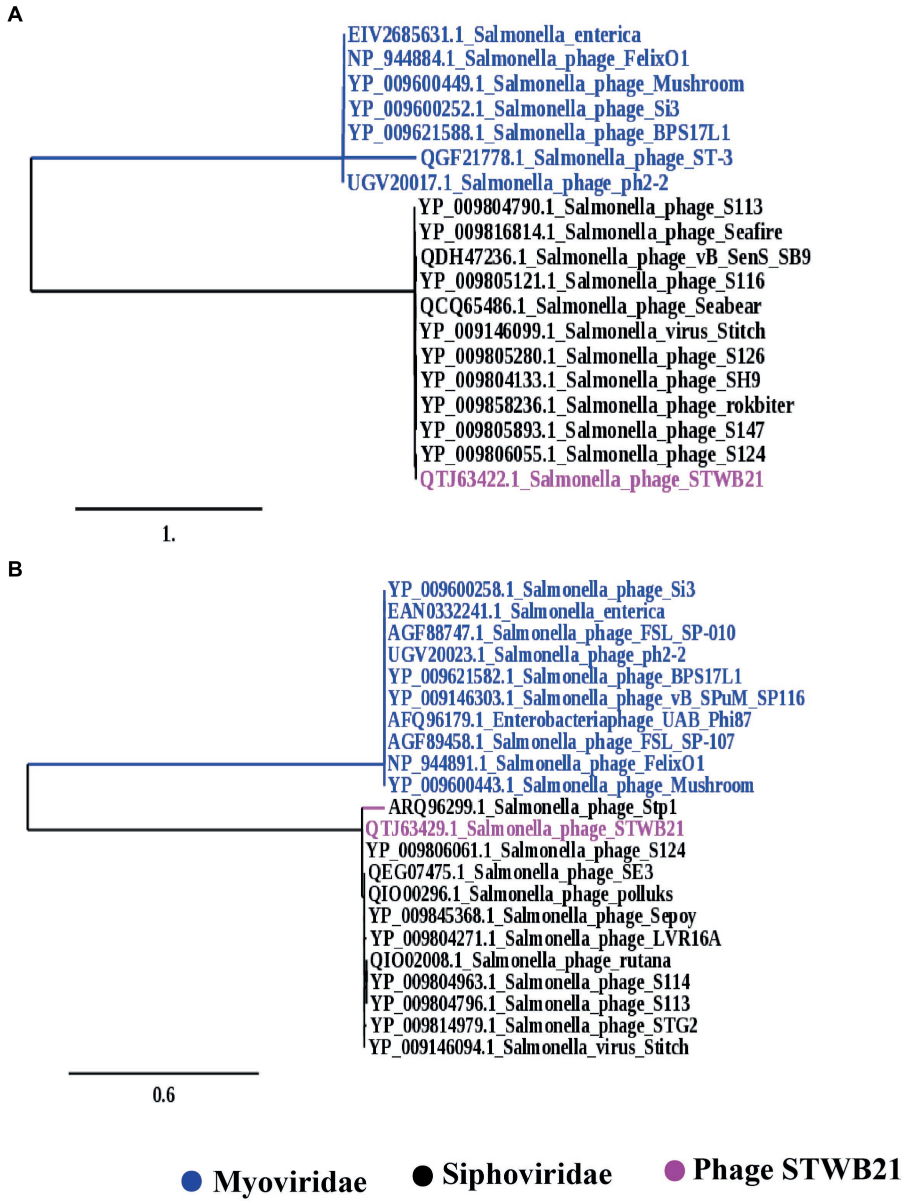

FIGURE 9
Phylogenetic analysis **(A)** large terminase unit, **(B)** major capsid proteins of *Salmonella* phage STWB21 with phages belonging to T4 and T5 family constructed using "One Click" at phylogeny.

small plaques without any halo as shown in Figure 1A. In addition, the presence of host lysis proteins (holin and lysozyme) in its genome confirms that phage STWB21 is a virulent lytic phage. *Salmonella* phages vB_Sen-TO17 and vB_Sen-E22 were also confirmed to be lytic in nature based on similar observations (Kwaśnicka et al., 2020).

The phage STWB21 harbors 22 tRNA genes corresponding to 14 different amino acids and has a significantly different codon usage than its host. Moreover, tRNA genes are usually considered as indispensable information-related and housekeeping genes which are least susceptible to lateral gene transfer (Grover et al., 2015). In a previous study on *Siphoviridae* phages, it was found that there is no clear correlation between the number of tRNAs and burst size or latent period (Ding et al., 2020). Our result also supports the previous study as phage STWB21 has 22 tRNA but showed different latent periods and burst sizes for typhoidal and nontyphoidal *Salmonella* spp. The phylogenetic analysis led to phage STWB21 clustering with T5-like phages rather than with T4-like phages.

Conclusion and future work

This study elucidated the environmental isolation of polyvalent *Salmonella* bacteriophage STWB21 shown to be efficacious against a wide host range spectrum of human pathogenic enteric bacteria. STWB21 exhibited robust pH and thermal stability as well as biofilm degradation activity. Thus, these observations place phage STWB21 among candidate phages as an effective biocontrol agent. Our future studies on in-vivo phage application to control *Salmonella* infection will be conducted to understand more about the antimicrobial property of phage STWB21.

Data availability statement

The complete genome sequence of phage STWB21 has been deposited in GenBank under accession no. MW567727.

Author contributions

PM and MD conceived and designed the experiments. PM carried out the main body of research, performed the experiments and bioinformatics analysis, and wrote the manuscript. BM contributed in performing the phage characterization experiment. SD provided the typhoidal *Salmonella* strains and reviewed the manuscript. MD supervised the work and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.980025/full#supplementary-material

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Morphological, biological, and genomic characterization of a newly isolated lytic phage Sfk20 infecting *Shigella flexneri*, *Shigella sonnei*, and *Shigella dysenteriae1*

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Shigellosis, caused by *Shigella* bacterial spp., is one of the leading causes of diarrheal morbidity and mortality. An increasing prevalence of multidrug-resistant Shigella species has revived the importance of bacteriophages as an alternative therapy to antibiotics. In this study, a novel bacteriophage, Sfk20, has been isolated from water bodies of a diarrheal outbreak area in Kolkata (India) with lytic activity against many *Shigella* spp. Phage Sfk20 showed a latent period of 20 min and a large burst size of 123 pfu per infected cell in a one-step growth analysis. Phage-host interaction and lytic activity confirmed by phage attachment, intracellular phage development, and bacterial cell burst using ultrathin sectioning and TEM analysis. The genomic analysis revealed that the double-stranded DNA genome of Sfk20 contains 164,878 bp with 35.62% G + C content and 241 ORFs. Results suggested phage Sfk20 to include as a member of the T4 myoviridae bacteriophage group. Phage Sfk20 has shown anti-biofilm potential against *Shigella* species. The results of this study imply that Sfk20 has good possibilities to be used as a biocontrol agent.

Shigella species, one of the most common causes of diarrheal diseases in developing and underdeveloped countries, affects mostly children below 5years[1,2]. Annually 188 million *Shigella* infection causes around 164,000 deaths worldwide[3]. *Shigella* transmitted primarily through the fecal–oral route and a very low number (10–100) of bacteria is sufficient to cause the infection[2]. In developing countries, outbreaks happen mainly due to contaminated food and water, poor hygiene, malnutrition, and lack of awareness[4]. *Shigella* spp. is gram-negative, non-motile, non-spore-forming, rod-shaped bacteria belonging to the family Enterobacteriaceae. There are four different serogroups: *S. flexneri*, *S. sonnei*, *S. boydii* and *S. dysenteriae1* that causes the outbreaks. Among them, *S. flexneri* and *S. dysenteriae*1 were the causative agents of diarrheal cases predominantly in the developing world[5]. *Shigella* dysentery used to be controlled by antibiotic therapy. Recently, overuse and misuse of antibiotics have increased the number of multidrug-resistant strains of pathogenic bacteria that include *Shigella*[6]. Although there is some progress in vaccine development but an effective vaccine for *Shigella* spp. is yet to achieve[7]. The World Health Organization (WHO) has included *Shigella* in the list of priority pathogens in search of new antibiotics[8].

Bacteriophages are bacterial viruses that infect specific host bacterial strains without affecting other bacteria. They are abundantly spread in all ecosystems wherever bacteria present[9] and survive through replicating inside the host cell by controlling its cellular components and releasing mature phage particles by host cell lysis[10]. Phage virulence was found to increase in the presence of human cells than in laboratory bacterial culture[11]. Lytic bacteriophages can infect and kill bacteria within a short period. Therefore, lytic phages are useful biocontrol agents and provide a potential alternative treatment to conventional antibiotic therapy to treat bacterial diseases[12]. The first bacteriophage was isolated at almost the same time but independently by Twort[13] and Felix d'Herelle[14]. Immediately after the discovery, this phage was used to treat children suffering from severe *S. dysenteriae* infection[15]. So far, around 78 lytic Shigella phages have been isolated from environmental samples and

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