LYTIC BACTERIOPHAGE FOR EFFICIENT BIOCONTROL OF *Salmonella* Typhimurium IN FOODS

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY MAJOR IN BIOTECHNOLOGY FACULTY OF SCIENCE UBON RATCHATHANI UNIVERSITY ACADEMIC YEAR 2014

UBON RATCHATHANI UNIVERSITY

THESIS APPROVAL

DOCTOR OR PHILOSOPHY

IN BIOTECHNOLOGY FACULTY OF SCIENCE

TITLE LYTIC BACTERIOPHAGE FOR EFFICIENT BIOCONTROL OF *Salmonella* Typhimurium IN FOODS

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ACADEMIC YEAR 2014

Acknowledgement

The thesis would not have been successful without a kind assistance from Asst. Prof. Dr. Parichat Phumkhachom and Assoc. Prof. Dr. Pongsak Rattanchaikunsopon who are a supervisor and a co-supervisor. They both kindly provided valuable advice and suggestions until the present work was completed. I highly appreciate their efforts and time. I would like to express my thanks to Asst. Prof. Dr. Prasongsom Punyauppa-path and Dr. Adithepchaikarn Pachanawan examiners who provided suggestions and recommendations on corrections of the thesis. My thanks go to Ms. Nual-anong Narkkong from the Faculty of Science of Mahasarakam University who willingly helped in the operation of the electron microscpoe in studying the morphology of bacteriophage. I am thankful to Ekkalak Somboon, Wanpen Thamniamton, Rungnapha Wannasutta and Chayanee Chimpalee who always gave encouragement and offered help when needed. I would like to thank the personnel of the Faculty of Science of Ubon Ratcathani University and the Program of Microbiology of the Faculty of Science of Ubon Ratchathani Rajabhat University. My thanks also go to all my colleagues for their constant help and support.

Last but not least, my special thanks go to my family that all the time show their concern, encouragement and support until the thesis has been completed.

ทนุก=นวม

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เรื่อง : ไลติกแบคเทอริโอเฟจเพื่อการควบคุมซัลโมเนลลา ไทฟิมิวเรียมในอาหาร
ผู้วิจัย : ตระหนัก สมเนตร
ชื่อปริญญา : วิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชา : เทคโนโลยีชีวภาพ
อาจารย์ที่ปรึกษา: ผู้ช่วยศาสตราจารย์ ดร.ปาริชาติ พุ่มขจร
คำสำคัญ : แบคเทอริโอเฟจ, ซัลโมเนลลา ไทฟิมิวเรียม, การควบคุมทางชีวภาพ, เครื่องดื่ม

การปนเปื้อนแบคทีเรียในอาหารเป็นปัญหาที่สำคัญทั่วโลกรวมทั้งประเทศไทย ในปัจจุบันนิยมใช้สารเคมีถนอมอาหารในการแก้ไขปัญหาดังกล่าว อย่างไรก็ตามความสามารถในการก่อปัญหาต่อสุขภาพของสารเคมีดังกล่าวกลายเป็นข้อกังวลจนทำให้มีงานวิจัยจำนวนมากเกิดขึ้นเพื่อหาสารยับยั้งการเจริญของจุลินทรีย์ที่ปลอดภัยมาใช้แทนสารเคมีถนอมอาหาร แบคเทอริโอเฟจได้รับความสนใจเป็นอย่างมากที่จะนำมาใช้เป็นสารยับยั้งการเจริญของจุลินทรีย์ที่ปลอดภัย ทั้งนี้เนื่องจากแบคเทอริโอเฟจหลายชนิดได้รับการรับรองจากองค์การอาหารและยาของสหรัฐอเมริกาให้สามารถใช้ในอาหารได้

การศึกษานี้มีวัตถุประสงค์เพื่อแยกแบคเทอริโอเฟจที่จำเพาะต่อ *Salmonella* Typhimurium ATCC 13311 จากน้ำที่เก็บจากเล้าหมู และเพื่อศึกษาคุณสมบัติบางประการของแบคทอริโอเฟจที่แยกได้ นอกจากนี้ยังได้ศึกษาความสามารถของแบคเทอริโอเฟจดังกล่าวในการควบคุม *S.* Typhimurium ATCC 13311 ในเครื่องดื่ม ในการศึกษานี้สามารถแยกไลติกแบคเทอริโอเฟจที่จำเพาะต่อ *S.* Typhimurium ATCC 13311 ได้ 1 ชนิด และให้ชื่อว่า แบคเทอริโอเฟจ ST1 แบคเทอริโอเฟจนี้มีความสามารถในการทำลายแบคทีเรียได้หลายสายพันธุ์นอกเหนือไปจาก *S.* Typhimurium ATCC 13311 ซึ่งได้แก่ *Shigella dysenteriae* (type1) DMST 2137 และ *Salmonella* Typhi DMST 5784 แบคเทอริโอเฟจทนต่อความร้อนที่อุณหภูมิ 50°C และ 60°C เป็นเวลา 3 นาทีได้ และทนต่อความเป็นกรดเป็นด่างได้ในช่วง pH ตั้งแต่ 5 ถึง 11 อนุภาคของแบคเทอริโอเฟจจำนวนประมาณ 80 เปอร์เซ็นต์ยึดเกาะที่ผิวของเซลล์เจ้าบ้านภายในเวลา 40 นาทีหลังจากผสมเซลล์เจ้าบ้านกับแบคเทอริโอเฟจ การศึกษาการเจริญของแบคเทอริโอเฟจพบว่าแบคเทอริโอเฟจมี latent period และ burst period เท่ากับ 70 นาที และ 330 นาที ตามลำดับ และมี burst size ประมาณ 172 PFU/infected cell แบคเทอริโอเฟจมีจีโนมเป็นแบบดีเอนเอสายคู่ และมีรูปร่างภายใต้กล้องจุลทรรศน์อิเล็คตรอนแบบส่องผ่านดังนี้ มีหัวรูปหลายเหลี่ยมขนาดเส้นผ่านศูนย์กลางประมาณ 70 นาโนเมตร และมีหางยาวแบบยืดหดไม่ได้ขนาดยาวประมาณ 150 นาโนเมตร กว้างประมาณ 7 นาโนเมตร แบคเทอริโอเฟจนี้จัดอยู่ในสกุล *Siphoviridae* สำหรับการทดลองในเครื่องดื่ม แบคเทอริโอเฟจ ST1 สามารถคงอยู่ได้ในนมถั่วเหลือง และในน้ำเฉาก๊วย แต่จะสูญหายไปในน้ำฝรั่งและน้ำส้ม เมื่อนำแบคเทอริโอเฟจและ *S.* Typhimurium ATCC 13311 (ปริมาณ 10^6 CFU/ml) ใส่ร่วมกันลงในนมถั่วเหลือง และในน้ำเฉาก้วยพบว่าแบคเทอริโอเฟจสามารถลดจำนวนของเซลล์แบคทีเรียได้ตามปริมาณของแบคเทอริโอเฟจที่ใส่ลงไปในเครื่องดื่ม โดยการใส่แบคเทอริโอเฟจจำนวนมาก (10^8 PFU/ml) ให้ผลการลด

จำนวนเซลล์แบคทีเรียได้ดีกว่าการใส่แบคเทอริโอเฟจจำนวนน้อย (10^6 และ 10^7 PFU/ml) การศึกษานี้แสดงให้เห็นถึงศักยภาพของแบคเทอริโอเฟจ ST1 ในการใช้เป็นสารควบคุมทางชีวภาพในการควบคุม *S.* Typhimurium ในเครื่องดื่ม

ABSTRACT

TITLE : LYTIC BACTERIOPHAGE FOR EFFICIENT BIOCONTROL OF *Salmonella* Typhimurium IN FOODS
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Food contamination with bacteria is a major problem worldwide, including Thailand. Presently, chemical food preservatives have been commonly used to solve this problem. However, their potential health risk has become a consumers' concern. Therefore, many research efforts have been made to find safe antimicrobial agents to replace chemical food preservatives. Bacteriophages have received increase attention as safe biocontrol agents because the several bacteriophages have been approved by the United State Food and Drug Administration (USFDA) to be able to use in foods.

The objectives of this study were to isolate a bacteriophage specific to *Salmonella* Typimurium ATCC 13311 from water collected from a swine lagoon and to study some of its characteristics. Furthermore, its ability to control *S.* Typhimurium ATCC 13311 in beverages was also investigated. In this study, a lytic bacteriophage, specific to *S.* Typhimurium ATCC 13311, was isolated and designated as bacteriophage ST1. It had a broad host range inactivating not only *S.* Typhimurium ATCC 13311, but also *Shigella dysenteriae* (type1) DMST 2137 and *Salmonella* Typhi DMST 5784. The bacteriophage was stable at 50 and 60°C for 3 min and over a wide pH range (5 to 11). Approximately 80% of bacteriophage particles adsorbed to the host cells within 40 min after infection.One-step growth kinetics of the bacteriophage showed that the latent and burst periods were 70 and 330 min, respectively, and the burst size was about 172 PFU/infected cell. Its genome was double stranded DNA. Transmission electron microscopy revealed that the bacteriophage had an isometric head of about 70 nm in diameter and a long noncontractile tail of about 150 nm long and 7 nm wide.

It was classified as a member of the family *Siphoviridae*. For experiments in beverages, bacteriophage ST1 was stable in soy bean milk and jelly grass drink but not in guava juice and orange juice. When applied bacteriophage ST1 together with *S.* Typhimurium ATCC 13311 (10^6 CFU/ml) in soy bean milk and jelly grass drink, the bacteriophage reduced the bacterial cells with a dose dependent pattern. The application of more bacteriophage (10^8 PFU/ml) was more effective than lower doses (10^6 and 10^7 PFU/ml). This study suggests that bacteriophage ST1 has a potential for being use as a biocontrol agent against *S.* Typhimurium in beverages.

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Abbreviations	Meaning
BHIB	Brain Heart Infusion Broth
bp	base pair
cfu	colony forming unit
DNA	Deoxyribonucleic acid
ds	double stranded
kb	kilobase
MOI	Multiplicity of Infection
PEG	Polyethylene glycol
pfu	plaque forming unit
rpm	revolutions per mininute
RNA	Ribonucleic acid
TAE	Tris Acetate
TBE	Tris Borate

CHAPTER 1
INTRODUCTION

1.1 Background and rational of study

Salmonellosis is one of the most common and widely distributed foodborne disease caused by consuming the contaminated food containing *Salmonella*. *Salmonella*, a member of Enterobacteriaceae, is composed of phenotypically and genotypically related bacteria. They are straight rod facultative gram negative anaerobes, usually motile with peritrichous flagella. The presence of *Salmonella* in food product presents a risk when these products are undercooked, mishandled or allowed to cross contaminate other food during food production or preparation. *Salmonella* infections are a major worldwide public health threat. Each year, there are an estimated 1.3 billion cases of non-typhoid salmonellosis that contribute towards more than three million deaths worldwide. In Thailand, the street foods that are prepared on the street especially soft drink such as orange juice, coconut juice, soy milk, jelly grass drink are frequently associated with diarrheal disease due to their improper handling and serving practices. *Salmonella* contamination of soft drink sold by street venders and hawkers has become a major health problem.

Presently, chemical food preservatives are commonly used to reduce or eliminate foodborne pathogens including *S.* Typhimurium contaminated in foods and beverages. However, their safety has become a major concern of consumers many health problems related to chemical food preservatives have been reported. Therefore, many research efforts have been made to find natural and safe antimicrobial agents to replace chemical food preservatives. Bacteriophages have received increase attention as natural and safe biocotrol agents because the United State Food and Drug Administration (USFDA) has approved some bacteriophages as "generally regarded as safe" for food products to control *Listeria monocytogenes* and *Escherichia coli* infections.

Bacteriophages are the most abundant organisms on earth which infect bacteria. These bacterial viruses have genetic material in the form of either DNA or RNA

(single or double-stranded), encapsidated by a protein coat. The lethal effect of bacteriophages on their bacterial hosts has been known since their discovery. There have been several reports showing the ability of bacteriophage to control bacteria in foods [1-4]. *Salmonella* reduction after application of bacteriophages has been demonstrated for many food products including cheddar cheese [5], honeydew melon slices [4], mustard seeds [2], chicken frankfurters [6], chicken skin [2], sprouting mung bean and alfalfa seeds [7]. These findings present potential of bacteriophages as biocontrol agents in foods.

1.2 Objective of thesis

The objectives of this study are

1.2.1 To isolate bacteriophage specific to *Salmonella* Typhimurium.

1.2.2 To partially characterize the isolated phage in some aspects.

Host range

One step growth kinetics

Morphology

Genome

1.2.3 To investigate the ability of *Salmonalla* phage to control *Salmonella* Typhimurium in food models.

1.3 Scope of the Thesis Research

The thesis research is divided into 3 major parts.

1.3.1 Isolation of a bacteriophage specific to *S.* Typhimurium.

1.3.2 Partial characterization of the isolated phage.

1.3.3 Examination of the ability of the isolated phage to control *S.* Typhimurium in food models.

1.4 Expected results

The bacteriophage from this study may be useful as a biocontrol agent for controlling *S.* Typhimurium in beverages.

CHAPTER 2
LITERATURE REVIEWS

2.1 *Salmonella*

2.1.1 Biology of *Salmonella*

Salmonella enterica is a rod shaped, flagellated, facultative anaerobic, Gram-negative bacterium belonging to the *Enterobacteriaceae* family. In 1885, pioneering American veterinary scientist, Daniel E. Salmon, discovered the first strain of *Salmonella* [8]. Up to 2400 serotypes of *Salmonella* have been found, which pose a great concern to the food industries concerned. Classification of *Salmonella* is made based on Kauffimann-White schemes on the presence of specific surface antigens as present in the envelop, cell wall and flagella known as Vi, O and H. O antigens type can identified by oligosaccharides which are associated with lipopolysaccharide. Although serological analyses such as slide agglutination tests are most often performed to identify isolates, bacteriophages may be utilized to gain valuable information during outbreaks of infection. *Salmonella* also contains extrachromosomal DNA, termed plasmids, which are important due to the presence of genes that confer pathogenicity and antibiotic resistances. Lately, a change of nomenclature was proposed in order to minimize improper medical treatments due to confusion between serotypes [9]. A new classification for *Salmonella* has been adopted based on DNA relatedness. This new nomenclature recognizes only two species: *Salmonella bongori* and *Salmonella enterica*. Analysis of recent incidence patterns in the United States has shown *S.* Typhimurium to be the most frequently isolated serotype in human salmonellosis cases followed closely by *S.* Enteritidis [10]. The cases of salmonellosis increase during the summer days when there are more conditions favorable to bacterial growth and spread. Children and infants are more vulnerable for the spread. According to Foodnet, between 1996 and 2008, 129.4 per 100,000 infants who were less than 12 months old were estimated to have contracted salmonellosis [11]. In the past, the children who were between one and ten years of age accounted for a higher incidence rate than average [12]. The reason for this may be due to reduced immune

system. Thus, children who had contracted with it required more attention than their adult counterparts. This, coupled with a child's potentially higher incidence of contact with the bacteria (e.g. from soil) [13], may account for higher rates of salmonellosis among 3 individuals less than ten years of age. Following recovery, individuals are protected against subsequent infections to some extent. Incidence rates among the elderly are marginally higher than in younger adults [10]; however, salmonellosis in the elderly tends to be more serious than in other adults. It has also been observed that large outbreaks tend to occur in establishments serving large numbers of people including hospitals, institutions, nursing homes and restaurants.

2.1.2 Salmonella Morphology and Characteristics

Salmonella belongs to the family of bacteria called *Enterobacteriaceae*, which is comprised of facultative anaerobic, Gram-negative, bacillus (or rods). *Salmonella* can grow under different environments. For their growth, sodium chloride is not essential. They can even grow in the presence of 0.4 to 4 %. Most *Salmonella* serotypes grow at temperature range of 5 to 47°C with optimum temperature of 35 to 37°C but some can grow at temperature as low as 2 to 4°C or as high as 54°C [14]. Structurally, most *Salmonella* possess long flagella which direct their movement, acting as a propeller for swimming. They also are covered with surface pili, which are short, hair-like structures that are involved in cellular attachment. Like other Gram-negative bacteria, the outer membrane of the cell wall is composed of various structurally and functionally important molecules. One of these molecules is lipopolysaccharide (LPS), which is an important virulence factor for Gram-negative bacteria. One portion of LPS, the O-specific polysaccharide tail, contains sugar variations which are used to identify different Salmonella types. These O or somatic antigens are heat stable and are exposed on the surface of the bacteria to the surface environment. Some capsulated Salmonella (*S.* Typhi and *S.* Paratyphi) also possess another surface polysaccharide, the Vi antigen, which is heat-labile and may provide the organism protection from phagocytosis [15].

Considering molecular relationships, Salmonella have two species, that is, *Salmonella enteric* and *Salmonell bongori* [16-18]. The genus *Salmonella* has a large number of named serovars, but most belong to *Salmonella enterica. S. enterica* can be divided into a number of subspecies and these can be divided into serovars, which

might display different phage types. *S. enterica* subspecies are: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI) [19]. *Salmonella bongori* is listed as subspecies V, even though this is a separate species [19-20]. The complete correct designation is, for example: *Salmonella enterica* subspecies *enterica* serovar Enteritidis, but this is usually abbreviated to *Salmonella* serovar Enteritidis (*S.* serovar Enteritidis) or simply *S.* Enteritidis [20, 21].

Subspecies I (enterica) includes nearly 1,400 serovars, some of which are commonly isolated from infected birds and mammals, including humans, and are 65 responsible for most Salmonella infections in humans; the other subspecies mainly colonize cold-blooded vertebrates (Rotger and Casadesús, 1999; Lucas and Lee, 2000). The serovars might be composed of several phage types. Isolates, which are pathogenic to man belong to subspecies I, but not all serovars, subspecies, or species are pathogenic. There are several virulence factors of Salmonella. Some may spread widely, while others seem to be present in a limited number of serovars or even strains (Fluit, 2005). Major causes of foodborne infection worldwide arise from some serotypes. However, most of the infections are not severe, most of which are confined to the intestinal tract. There may be a short-live diarrhea. However, some Salmonellae lead to fatal systematic diseases, for example typhoid fever and paratyphoid fever [21]. The comparison is demonstrated in table 1.

Table 1 Comparison of characteristics of *Salmonella* species. [22]

Characteristics	*Salmonella enterica* subsp.						*Salmonella bongori*
	enterica	***salamae***	***arizonae***	***diarizonae***	***houtenae***	***indica***	
Classification (roman numeral)	I	II	IIIa	IIIb	IV	VI	V
Usual habitat	Warm blooded animals	Warm blooded animals	Cold blooded animals & environment	Cold blooded animals & environment	Cold blooded animals & environment	Cold blooded animals & environment	Cold blooded animals & environment
Morphological characteristics							
Gram stain	-	-	-	-	-	-	-
Motility	+ (except pullorum & gallinarum)	+	+	+	+	+	+
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Size (width, μm)	0.7-1.5	0.7-1.5	0.7-1.5	0.7-1.5	0.7-1.5	0.7-1.5	0.7-1.5
Size (length, μm)	2-5	2-5	2-5	2-5	2-5	2-5	2-5
Colony morphologies							
Bismuth sulphite agar	Black colonies surrounded by a brown to black zone that casts a metallic sheen						
Eosin-methylene blue agar	Translucent amber to colorless colonies						
Hektoen enteric agar	Blue to blue-green colonies, mostly with black centers (H_2S producers)						
Salmonella-Shigella agar	Colorless colonies on a pink background						
Xylose lysine desoxycholate agar	Black-centered red colonies (H_2S producers)						

Table 1 Comparison of characteristics of *Salmonella* species. (Continued)

Characteristics	*Salmonella enterica* subsp.						*Salmonella bongori*
	enterica	*salamae*	*arizonae*	*diarizonae*	*houtenae*	*indica*	
Growth characteristics							
Optimum temperature (°C)	35-37	35-37	35-37	35-37	35-37	35-37	35-37
Optimum pH	6.5-7.5	6.5-7.5	6.5-7.5	6.5-7.5	6.5-7.5	6.5-7.5	6.5-7.5
Biochemical characteristics							
α-glutamyltransferase	d	+	-	+	+	+	+
β-Gluocuronidase	d	d	-	+	-	d	-
Dulcitol	+	+	-	-	-	d	+
Galacturonate	-	+	-	+	+	+	+
Gelatinase	-	+	+	+	+	+	-
Glucose	+	+	+	+	+	+	+
Hydrogen sulfide	+	+	+	+	+	+	+
Indole test	-	-	-	-	-	-	-
Lactose	-	-	-	+	-	+	d
Lysine decarboxylase	+	+	+	+	+	+	+
L(+)-tartrate	+	-	-	-	-	-	-
Malonate	-	+	+	+	-	-	-
Murate	+	+	+	-	-	+	+
Ortho-nitrophenyl-β-D-Galactopyranoside test	-	-	+	+	-	d	+
Phage O1 susceptible	+	+	-	+	-	+	d
Potassium cyanide broth	-	-	-	-	-	-	-
Salicine	-	-	-	+	-	+	d
Sorbitol	+	+	+	+	+	-	+

Table 1 Comparison of characteristics of *Salmonella* species. (Continued)

Characteristics	*Salmonella enterica* subsp.						*Salmonella bongori*
	enterica	*salamae*	*arizonae*	*diarizonae*	*houtenae*	*indica*	
Biochemical characteristics							
Urease	-	-	-	-	-	-	-
Voger-Proskauer test	-	-	-	-	-	-	-

Note: +, more than 90% positive reactions; -, less than 10% positive reactions; d, different reactions given by different serovar

2.1.3 Salmonellosis

Non-typhoidal salmonellosis is a disease which can be spread through the food we eat in both developed and developing countries. Its spread is enhanced by a wide range of animal reservoir and an animal and food product distribution for commercial purposes. The disease in question causes a particular concern for socio-economic consequences [23, 24].

Salmonellosis can be found worldwide [25, 26]. However, the disease is most commonly found in the place where there is an intensive animal husbandry. This disease has been almost eradicated in domestic animals and humans in some countries; however, it is still prevalent among the wild animals. The distribution of serovars is different from place to place. Some like enteritidis and Salmonella ser are present across the globe, whereas others are restricted to certain geographic areas. In the U.S., the most common serotypes isolated from humans in 2002 were, in descending order: *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* ser. Newport, *Salmonella* ser. Heidelberg, *Salmonella* ser. Javiana, *Salmonella* ser. Montevideo, *Salmonella* ser. Muenchen, *Salmonella* ser. Oranienburg and *Salmonella* ser. Saintpaul. In 2002, the most common serovars from clinically ill animals reported to the CDC and the National Veterinary Services Laboratory (NVSL) were, in descending order: *Salmonella* Typhimurium, *Salmonella* Newport, *Salmonella* ser. Agona, *Salmonella* Heidelberg, *Salmonella* ser. Derby, *Salmonella* ser. Anatum, *Salmonella* ser. Choleraesuis, *Salmonella* Montevideo, *Salmonella* ser. Kentucky, *Salmonella* ser. Senftenberg and *Salmonella* ser. Dublin.

Salmonella spp. are mainly transmitted by the fecal-oral route. They are carried asymptomatically in the intestines or gall bladder of many animals, and are continuously or intermittently shed in the feces. They can also be carried latently in the mesenteric lymph nodes or tonsils; these bacteria are not shed, but can become reactivated after stress or immunosuppression. Fomites and mechanical vectors (insects) can spread *Salmonella*. Vertical transmission occurs in birds, with contamination of the vitelline membrane, albumen and possibly the yolk of eggs. *Salmonella* spp. can also be transmitted in utero in mammals. Animals' infection can arise from contaminated feed, drinking water or close contact with other animals which are infected. Salmonella can be spread to livestock through birds and rodents.

Meat-eating humans can be infected through their consumption of meats, eggs and other animal products not thoroughly cooked. The cats that feed on infected birds can be infected with Salmonella. People who eat contaminated foods such as meat or eggs can also be infected. Besides, they can get infection directly or indirectly through animal feces and contaminated food or water. Directly transmitted human infections are most often acquired from the feces of reptiles, chicks and ducklings. Livestock, dogs, cats, adult poultry and cage birds can also be involved.

As for the human cases, salmonellosis differs from a self-limiting gastroenteritis to septicemia. Host factors and the virulence of the strain contribute to the spread of the disease. All serovars can cause all forms of salmonellosis. Salmonellosis which is derived from reptiles is often severe and may be life-threatening thanks to septicemia and meningitis. Salmonellosis acquired from the reptiles are found in the children who are under 10 years of age and people who are immunocompromised. The symptoms of gastroenteritis include nausea, vomiting, cramping abdominal pain and diarrhea. Others may be headache, fever, chills and myalgia. Deaths are rare except in very young, very old, debilitated or immunocompromised persons. Enteric fevers are a severe form of systemic salmonellosis. Although most cases are caused by *S.* Typhi, a human pathogen, other species can also cause this syndrome. Gastrointestinal disease may be the first sign, but it usually resolves before the systemic signs appear. The symptoms of enteric fever are non-specific and may include fever, anorexia, headache, lethargy, myalgias and constipation. This disease can be fatal, due to meningitis or septicemia, if not treated quickly. Focal infections such as septic arthritis, abscesses, endocarditis or pneumonia are occasionally seen. Many tissues and organs can be affected. Reiter's syndrome may be a sequela in some cases of gastroenteritis. This syndrome is characterized by mild to severe arthritis, nonbacterial urethritis or cervicitis, conjunctivitis and small, painless, superficial mucocutaneous ulcers. Reiter's syndrome occurs in approximately 2% of cases of salmonellosis. It is also seen after other enteric infections. Reiter's syndrome usually resolves in 3 to 4 months, but approximately half of all patients experience transient relapses for several years. Chronic arthritis can occur in some cases.

2.1.4 Communicability and transmissibility of salmonellosis in humans

Although salmonellae are present everywhere, their main source is the intestinal tract of infected domestic and wild animals and humans [27]. Contaminated food and water is mainly responsible for Salmonella infection worldwide [25, 28]. Salmonella is present in the faeces of both infected animals and healthy ones. When the infected animals are culled, it is possible for the contamination to occur. Another factor which may lead to the infection is the consumers are unable to handle the raw food properly; as a result an infection results. Other significant sources of human salmonellosis are shellfish harvested from water contaminated with faecal materials and also contaminated fresh fruits and vegetables [28]. Water contaminated with sewage is always a hazard as it was responsible for a massive outbreak of *S.* Typhimurium in 1971 in the US [29]. Person to person spread of salmonellosis is possible particularly in hospitals [27].

2.1.5 Epidemiological aspects of human salmonellosis

Out of 2200 serovars of Salmonella, only some which belong to subspecies I can cause human and animal diseases. There may be changes in the distribution of the serovars with the unknown reasons [30]. Salmonellosis is more commonly found among the children, elderly and people having chronic diseases. Based on the data available in many countries, there is an increased incidence of a steady and significant increase in salmonellosis during the past 20-30 years. In Canada, during the period 1983 and 1992 a total of 89,760 Salmonella strains from humans were reported to the National Laboratory for Enteric Pathogens [31]. Furthermore, there were 2,180 reported outbreaks associated with 10,065 cases during the 10-year period. Likewise, in the United states, approximately 25,000 cases of Salmonella infection were reported annually in the 1970s to the Centre for Disease Control (CDC) with continual increase to more than 50,000 reported infections in 1985. In 1995, 41,222 infections were reported to the CDC [27]. The major recent change in the epidemiology of Salmonella has been the emergence and increase of *Salmonella enteritidis* in industrialized nations and *Salmonella* Typhimurium definitive phage type DT 104 in the United Kingdom, the United States and Canada [32-34]. An important feature of this increase has been the spread of a strain of *Salmonella* Typhimurium DT 104 with multi-resistance patterns characterized by resistance to ampicillin, chloramphenicol, streptomycin,

sulfonamides and tetracycline (R-type ACSSuT) [25, 32, 35]. Recent studies have shown that infection with R-type ACSSuT was associated with higher hospitalization and fatality rates than other *Salmonella* serovars. Studies have identified farm animals as the source of human infection and consumption of improperly handled red meat as a risk factor for Salmonellosis [27, 32].

2.1.6 *Salmonella* Typhimurium

Salmonella Typhimurium has been recognized to be the most common human pathogen linked to the environment and the food chain [36]. The largest outbreak of waterborne salmonellosis was due to this sevorar. Between 1985 and 1991, it was *S.* Typhimurium which was the most prevalent pathogen associated with food borne salmonellosis in the United States or more than 20% of the total Salmonella isolates. Importantly, *S.* Typhimurium R-type becomes drug resistant. Furthermore, *S.* Typhimurium isolated from cattle before 1986 did not have this R-type compared to 13% of the isolates obtained between 1986 and 1991, and 64% of the isolates obtained in 1992 and 1995 [27]. In addition, a high number of isolates from either human or cattle sources with the R-type ACSSuT that were phage typed were found to be phage type 104 [27]. In Canada, *S.* Typhimurium has been the most common serovar isolated between 1983 and 1992. *S.* Typhimurium was associated with 1,622 human cases reported from 222 outbreaks [31]. This serovar accounted for the highest number of Salmonella isolates implicated in recent food borne diseases from 1992 and 1993, representing 20.396 of the total Salmonella [31]. Thirty-seven percent of *S.* Typhimurium isolates from humans were found to be *S.* Typhimurium DT 104. In Europe, the increase in prevalence of *S.* Typhimurium in foodborne disease continued to be significant [37]. In Spain the frequency of the isolation of *S.* Typhimurium has increased over the last few year from 13 % in 1988 to 25.1 % in 1994 [38]. There has been continual increases in the isolation of *S.* Typhimurium DT 104 in the United Kingdom from 259 in 1990 to 2,873 in 1994 and 3,837 in 1995 [30, 32]. Moreover, *S.* Typhimurium DT 104 is the second most prevalent *Salmonella* in humans after *S.* Enteritidis, although a few years ago *S.* Typhimurium Phage type 204 was predominant in the United Kingdom. [32]

2.1.7 Foodborne outbreaks caused by *Salmonella* spp.

Foodborne illness is a major international health problem [39, 40]. Yearly, food borne diseases are responsible for the illness of millions of people. It is believed that there are more cases which are not reported. Salmonella is believed to be the main culprit involved in foodborne bacterial outbreaks and diseases [41]. They cause an estimated 1.4 million cases of foodborne disease each year in the United States alone.

In June 2006, the British Broadcasting Corporation (BBC) reported that the Cadbury chocolate manufacturer withdrew a number of products when products contaminated with *Salmonella* caused up to 56 cases of salmonellosis. The problems had been traced to a leaking pipe at a Cadbury plant in Herefordshire in January 2006, though the announcement was not made until June

The U.S. Government reported that 16.3% of all chickens were contaminated with *Salmonella* in 2005, and in the late 1990s as many as 20% were contaminated. In the mid to late twentieth century, *Salmonella enterica* serovar Enteritidis was a common contaminant of eggs. This is much less common now with the advent of hygiene measures in egg production and the vaccination of laying hens to prevent *Salmonella* colonization. Many different *Salmonella* serovars also cause severe diseases in animals other than human beings.

In EU 2006, twenty-two Member states (MS) and three non-MS reported a total of 3,131 foodborne outbreaks of human salmonellosis, which constituted 53.9% of the total number of reported outbreaks in the EU and in the reporting non-MS (Table 2).

Table 2 *Salmonella* serovars reported for foodborne outbreaks, 2006 [42]

Serrovars	Outbreaks		Human cases		
	Number	%Total	Number	Number Admitted	Death
Salmonella spp.	1,188	37.9	1,197	192	6
S. Enteritidis	1,729	55.2	13,853	2,714	14
S. Typhimurium	129	4.1	1,088	149	3
S. group D	26	0.8	207	63	0
S. group B	12	0.4	98	0	0
S. group C	6	0.2	24	0	0
S. Infantis	5	0.2	48	9	0
S. Hadar	4	0.1	33	1	0
S. Kentuckey	4	0.1	8	2	0
S. Paratyphi B	4	0.1	25	1	0
S. Virchow	4	0.1	138	2	0
S. Abony	2	0.1	6	2	0
S.. Ajiobo	2	0.1	161	13	0
S. Bovismrbificans	2	0.1	4	1	0
S. Give	2	0.1	55	0	0
S. Moutevideo	2	0.1	52	5	0
S. Muenchen	2	0.1	34	0	0
S. Newport	2	0.1	59	7	0
S. Paratyphi A	2	0.1	8	0	0
S. Saintpaul	2	0.1	12	1	0
S. Stanley	2	0.1	95	23	0
Total	3,131	100	22,705	3,185	23

In 2006, Germany, Austria, Slovakia, Spain and Poland accounted for 78.0% of the *Salmonella* outbreaks, reporting 908, 453, 452, 338 and 292 outbreaks respectively. Germany reported 330 general and 578 household outbreaks, involving 4,851 persons of which seven died. The majority of *Salmonella* outbreaks in Austria were small household outbreaks (83.9%), with 2 – 8 cases. In total, the non-MS, Norway, Romania and Switzerland reported 14 general and 6 household outbreaks caused by *Salmonella*. *S.* Enteritidis was the predominant *Salmonella* serovar associated with outbreaks (Table 2) and accounted for 29.8% of all reported outbreaks, 47.0% of all hospitalizations and 25.5% of all deaths in 2006. For 37.9% of the outbreaks caused by *Salmonella,* no serovar was specified. In outbreaks caused by *S.* group D, *S.* Enteritidis or *S.* Stanley involving more than 25 human cases, relatively large proportions of cases required hospitalization (30.4%, 19.6% and 24.2%,

respectively). In two *S.* Enteritidis outbreaks in Hungary and France, three out of four cases required hospitalization. Slovakia reported 451 *S.* Enteritidis outbreaks affecting 1,849 persons and Spain reported *S.* Enteritidis as the cause of 100 general and 63 household outbreaks, involving 1,724 persons and causing one death [42].

Phage type data were provided for 25.2% of all *S.* Enteritidis outbreaks. Phage type information was only provided for a subset of the outbreaks reported by Austria, Belgium, Germany, Slovakia and the United Kingdom. The five most commonly reported phage types were *S.* Enteritidis PT4, PT8, PT21, PT6 and PT6a; accounting for 120, 110, 92, 32 and 23 outbreaks respectively.

For the 129 outbreaks caused by *S.* Typhimurium, phage types were provided for 20.2%. Phage type information was reported for the majority of *S.* Typhimurium outbreaks in Austria (25 outbreaks) and Norway (1) and from Slovakia (1), Sweden (1) and the United Kingdom (2). Nine different phage types were reported, and the most common phagetypes were DT104 (7 outbreaks), DT120 (4) and DT193 (3) [42].

In February 2007, the U.S. Food and Drug Administration (FDA) issued a warning to consumers not to eat certain jars of Peter Pan peanut butter or Great Value peanut butter due to risk of contamination with *Salmonella* Tennessee

In March 2007, around 150 people were diagnosed with salmonella-poisoning after eating tainted food at a governor's reception in Krasnoyarsk, Russia. Over 1,500 people attended the ball on March 1 and fell ill as a consequence of ingesting salmonella-tainted sandwiches

In December 2007, about 150 people were sickened by salmonella-tainted chocolate cake produced by a major bakery chain in Singapore

In Sweden, 51 domestic cases with *Salmonella* Stanley were reported in July and August 2007. Domestic cases of this serotype are unusual in Sweden. The majority of cases were adults. An outbreak investigation was initiated in July involving the Swedish Institute for Infectious Disease Control, the Swedish Food Safety Authority and the county medical officers. An Enter-net alert was issued but did not reveal anything out of the ordinary in other countries. The case control study performed pointed strongly towards alfalfa sprouts. The cases had eaten alfalfa sprouts from various food stores or restaurants throughout Sweden.

Most of the product was traced to a large scale sprout producer who had imported alfalfa seeds through a wholesaler in Denmark from an Italian seed producer. The same seeds had also been sold to other sprout growers in Sweden. There were no longer any sprouts or seeds of the implicated batches in the grower's stock but samples (unpasteurized seeds) were taken from another bag of seeds of the same batch and brand and tested positive for *Salmonella* but for another serotype, *S.* Mbandaka. An alert was issued through the Rapid Alert System for Food and Feed (RASFF) on 31 August 2007 and the sprouts were withdrawn from the Swedish market. The grower had heat treated the seeds before sprouting but it did not seem to have been efficient. Later, four cases with *S.* Mbandaka from May and June were recognized to be infected with *S.* Mbandaka having the same molecular typing patterns as the *S.* Mbandaka isolated from the sprouts and two of the cases remembered eating sprouts.

In Norway, an alert was raised when four domestic cases with *Salmonella* Weltevreden were reported in October 2007. Domestic cases of this serotype are unusual in Norway. An outbreak investigation was initiated involving the Norwegian Institute of Public Health (FHI), the Norwegian Food Safety Authority (NFSA), and the municipal medical officers and an urgent inquiry was sent to the former Enter-net network through ECDC. In response to the inquiry, 19, 19 and 8 cases were reported in Norway, Denmark and Finland respectively. The demographic characteristics were comparable: the cases were adults and predominantly female.

On 23 October 2007, a *Salmonella* isolate obtained from a major Danish alfalfa sprout producer was serotyped as *S.* Weltevreden. The Danish food authorities issued an alert through RASFF on the same day. The isolate was later shown to have the same molecular typing patterns as the isolates from the case-patients from Denmark, Norway and Finland. *S.* Weltevreden was also verified in the sprouts sold in Finland and Norway.

As of July 8, 2008, from April 10, 2008, the rare Saintpaul serotype of *Salmonella enterica* caused at least 1017 cases of salmonellosis food poisoning in 41 states throughout the United States, the District of Columbia, and Canada. As of July 2008, the U.S. Food and Drug Administration suspects that the contaminated food product is a common ingredient in fresh salsa, such as raw tomato, fresh jalapeño pepper, fresh serrano pepper, and fresh cilantro. It is the largest reported salmonellosis

outbreak in the United States since 1985. New Mexico and Texas have been proportionally the hardest hit by far, with 49.7 and 16.1 reported cases per million, respectively. The greatest numbers of reported cases have occurred in Texas (384 reported cases), New Mexico (98), Illinois (100), and Arizona (49). There have been at least 203 reported hospitalizations linked to the outbreak, it has caused at least one death, and it may have been a contributing factor in at least one additional death. The Center for Disease Control and Prevention (CDC) maintains that "it is likely many more illnesses have occurred than those reported." If applying a previous CDC estimated ratio of non-reported salmonellosis cases to reported cases (38.6:1), one would arrive at an estimated 40,273 illnesses from this outbreak.

2.2 Bacteriophages

2.2.1 History and Morphology of Bacteriophage

Bacteriophages are viruses that infect bacteria. The name bacteriophage is derived from bacteria and Greek *phagein* "to eat". The history of bacteriophages started in 1915 when F. W. Twort observed a "glassy transformation" within a layer of bacterial cocci, which could be induced in colonies of normal appearance after inoculating with substance of such "glassy" colonies [43]. Two years later, F. d`Herelle described independently a similar phenomenon that was "antagonistic" to bacteria and that resulted in lysis in liquid culture and death in discrete patches, that he called plaques [44]. He was interested in their biological nature and claimed the idea of an organized infection agent that is an obligate intracellular parasite. It was also d`Herelle who proposed this culture as a therapeutic agent in the preantibiotic era. Early studies dealt with the use of phages for the control of epidemics but the interest in phage therapy diminished after the invention of antibiotics in the forties. However, the extensive investigation is on into phage therapy particularly in the former republics of former Soviet Union. Now, focus is again on the antimicrobial agent. This is because there is more resistance to antibiotics. Furthermore, phages could be a model organism for molecular biology and tools for application.

Bacteriophages structure can include a head, tail, tail fiber, and contractile sheath that are composed of proteins. All phages have a chromosome encased in a capsid that is composed of phage-encoded proteins. For many phage types, the capsid is attached

to a tail structure that is also made from phage-encoded proteins. The nucleic acid inside the coating, called the phage genome in a bacteriophage, encodes most of the gene products needed for making more phage. The DNA can be linear or circular, double stranded or linear or circular, single stranded. The DNA molecule is packed in the head, which can be round or hexagonal. T4 and P1 contain a linear double-stranded DNA genome enclosed in a capsid and attached to a tail (Figure 1A). The T4 genome is 172 kb, while P1 is a smaller phage with a genome of 90 kb. The T4 capsid is an elongated icosahedron. T4 has a very elaborate tail structure including a collar at the base of the head and a rigid tail core surrounded by a contractile sheath. The core and sheath are attached to a hexagonal base plate. Also attached to the tail plate are tail pins and six kinked tail fibers. λ also has an icosahedral capsid, a tail with a contractile sheath, a base plate, and tail fibers. P1 contains a linear double-stranded DNA genome of 48.5 kb, a capsid, and a tail (Figure 1B). The finished capsid is again shaped like an icosahedron whereas the tail is a thin flexible tube that ends in a small conical part and a single tail fiber. M13 contains a circular single-stranded DNA genome of 6407 nucleotides surrounded by five phage-encoded proteins (Figure 1C).

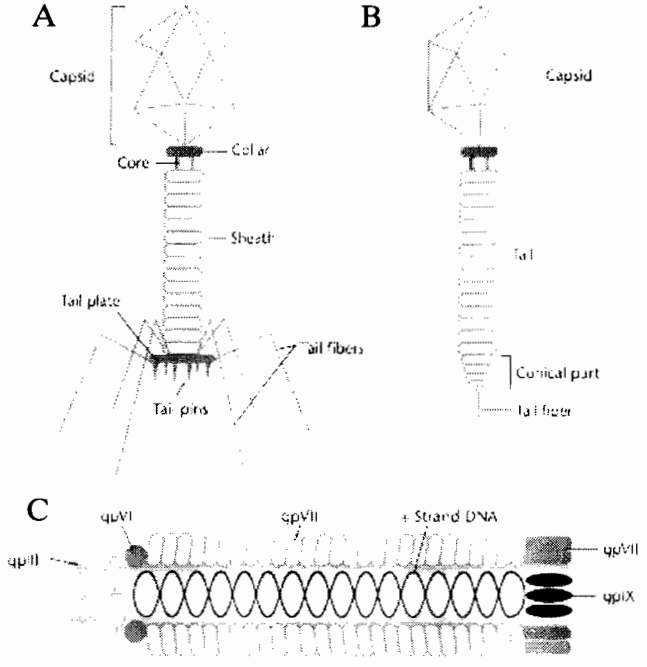

Figure 1 The structures of bacteriophage T4 (A), λ (B) and M13 (C) [45]

The phage genome which is covered by the protective coating comprises phage-encoded proteins. For bacteriophages to reproduce, a host cell is required. Bacteriophages use the tails to attach on to the surface of a host bacterial cell and then inject their DNA inside the cell cytoplasm. If it is a lytic bacteriophage, the bacterial cell will produce a large number of copies of bacteriophage DNA and the bacteriophage proteins. Following assembly of the protein and bacteriophage DNA to produce mature bacteriophages, the bacterial cell will lyse, releasing the bacteriophages in to environment. They in turn will attach other bacterial cells. Phages are extremely host-specific, able only to infect specific species or even strains, with a few exceptions such as Listeria phage A511, which can infect and kill bacteria within an entire genus [46]. Bacteriophages that lyse a variety of indigenous bacteria, including *Vibrio* spp. *Pseudomonas* spp., *Cytophaga* spp., *Agrobacterium* spp., *Photobacterium* spp., and various nonmarine contaminants particularly members of Enterobacteriaceae have been isolated from marine environment. Enrichments using seawater, sediments, fish and shellfish, have been successful used as source of bacteriophages [47].

2.2.2 Phage replication and life cycle

Bacteriophage multiplication process is divided into (i) the attachment or adsorption stage where the phage particle attaches to the host cell, (ii) penetration stage of the phage into the bacterium, (iii) biosynthesis or intracellular multiplication of the viral components, (iv) maturation stage, (v) release of phage progeny from the host cell [48].

Adsorption or phage attachment to the host is the initial step of the infectious cycle of a bacteriophage. During adsorption, the phage attaches to a complementary receptor site of the host cell membrane, forming weak bonds [48].

After absorption comes a penetration process in which the phage injects in genetic material inside the bacterium. Before an enzyme is penetrated, lysozyme is released from the phage's tail to overcome a part of the bacterial cell wall. After that the tail sheath reduces in size, pushing through the cell wall and plasma membrane. As regards penetration in some bacteriophages, ions like magnesium or calcium are needed [49].

Biosynthesis of viral components begins after the entry of the phage DNA into the host cytoplasm. After this stage, there is a synthesis of viral nucleic and proteins. Protein synthesis in the host bacteria ends when phage causes degradation of host DNA. There are an interference of phage protein with transcription and repression of translation [50-55]. Bacteriophages utilize the host's nucleotides and enzymes to synthesize copies of its DNA before synthesis of its macromolecules. Thereafter, synthesis of enzymes and capsid proteins are produced from mRNA transcribed from phage DNA. The host's ribosomes, enzymes and amino acids are used for the translation process. These early produced proteins are presumed to be enzymes and are essential for phage multiplication. It is argued that the bacterial host contains information in its genetic material and that phage DNA elicits specific inducers of the new proteins or removes inhibitors against preformed proteins [56]. These processes are mediated by mRNA polymerase together with other genes.

In maturation process, a step-by-step assemblage of phage DNA and capsid structure occurs to produce complete bacteriophage units or matured phage [48]. Matured phages inside the host cell are released upon lysis of the cell membrane subsequently infect other susceptible host cells [48]. Young describes two mechanisms and regulations involved in phage-induced lysis: (1) the normal release of progeny virions from a phage-encoded enzyme which degrades the murein (e-lysozyme or R-transglycosylases) and, (2) the release of progeny without the participation of phage-enconded enzymes [57].

Depending on the life cycle, phages can either be lytic (virulent) phage or lysogenic (temperate) phage.

Lytic phage

The life cycle of lytic phage begins when there is the interaction between the virion and the host cell surface receptor molecules. After phage being absorbed into molecules, the cell wall is penetrable. The nucleic acid enters the cell and the capsid is outside the cell. Inside the host occur several steps which include gene expression, genome replication and morphogenesis – i.e. the formation of the capsids (and tails) and the packaging of the genomes into the capsids (Figure 2). Phages are reproduced very quickly, forming new virion particles and this reproduction phase ends with the lysis of bacteria. With the host lysis, hundreds of new phages are released from each

infected bacteria [58]. The number of new phages produced, or progeny, depends on the species and conditions, nevertheless each "parent" phage is able to produce in average 50 - 200 "daughter" phages per lytic cycle [59]. Lytic phage infection results in clear plaques on the respective host bacterial lawns.

Lysogenic phage

Phages with lysogenic growth integrate their prophage into the chromosome or other replicon of the host bacteria. This results in a so called lysogenic infection where the phage genome is passed, through cell division, to all daughter cells (Figure 2). Phages with this type of growth cycle form turbid plaques on the susceptible host bacterial lawns. When a bacterial strain is lysogenised with a phage, it becomes resistant to infection by other related phages that share the same immunity group profile or same repressor specificity [60]. Some phages represent their prophages as plasmids that are replicated and that are allocated to the bacterial daughter cells in strict accord with bacterial replication and division. Occasionally, there is a decrease in the level of repressor concentrations. When that happens, the transcription is activated. This leads to the formation of the phage progeny and cell lysis. The virulent mutants of temperate phages do not respond to the presence of a specific cell receptor [61].

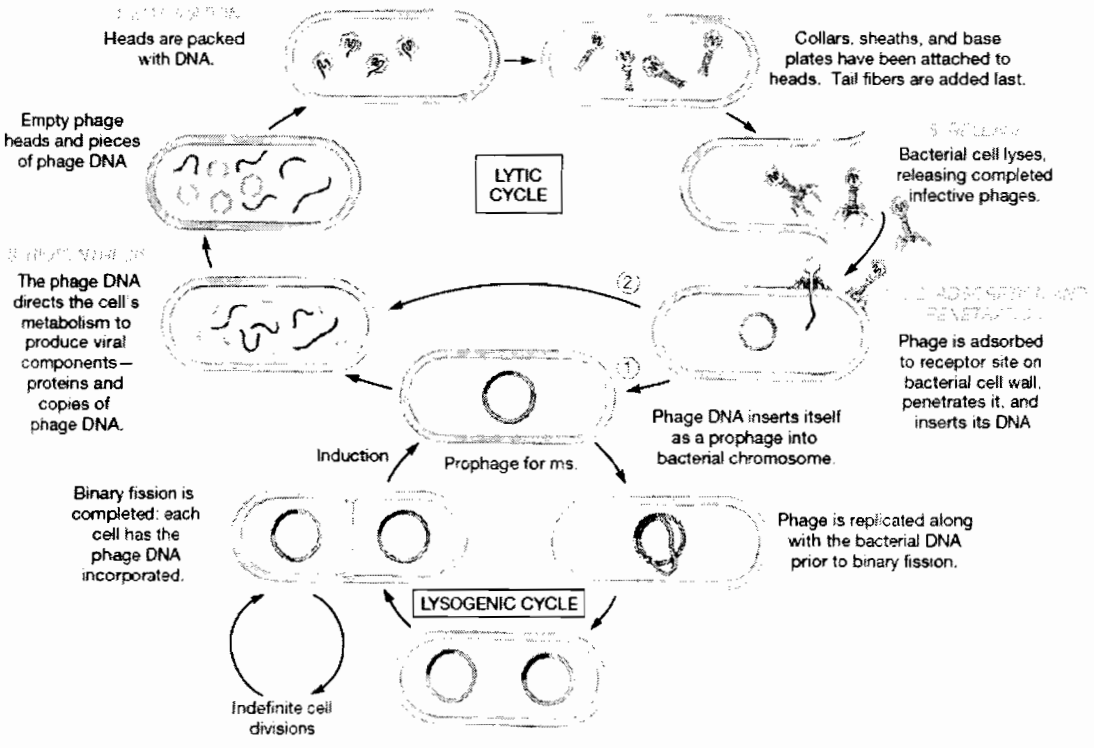

Figure 2 Bacteriophage life cycle [62]

2.2.3 Phage classification and Nomenclature

Initially there was a design for a classification system for bacteriophage in the 1930s by using the different host specifications of various bacteriophages. With electron microscope available in the 1940s and 1950s, classification of bacteriophages was made based on morphotype [63]. They have since been further classified with respect to molecular characteristics as techniques to identify DNA or RNA type and replication strategies have developed [64-65]. The current typing system identifies at least 13 distinct groups of bacteriophage [65] including those infecting Archaea. These are very diverse structurally and genetically and those bacteriophage infecting Archaea will not be discussed further. Of those bacteriophage examined by electron microscopy, about 96% belong to order *Caudovirales*.

The International Committee on the Taxonomy of Viruses (ICTV) used virion morphology and nucleic acid composition as a basis for the classification of phages into 13 families. Over 95% of all phages described in the literature belong to the order of the *Caudovirales* or tailed dsDNA phages. The three main families comprising the *Caudovirales* are distinguished by their very distinct tail morphologies: 60% of the

characterized phages are *Siphoviridae*, with long, flexible tails; 25% are *Myoviridae*, with double-layered, contractile tails; and 15% are *Podoviridae* with short, stubby tails (Figure 3)

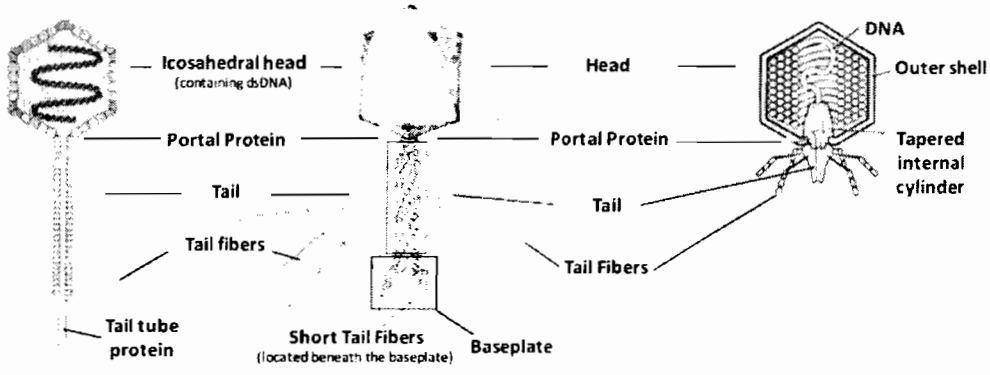

Figure 3 Basic morphology of the three families of the *Caudovirales*. From left to right, schematic drawings of a *Siphovirus* (long, non-contractile tail), *Myovirus* (long, contractile tail) and *Podovirus* (short tail) are shown. [66]

According to the ICTV system, bacteriophages are currently classified into one order, *Caudovirales* which consists of three phylogenetically related families. In addition, there are 17 families or floating genera some of which presently await classification as indicated in table 3 below. Phage virions can be tailed, polyhedral, filamentous, and pleomorphic and most of them contain double-stranded DNA (Table 3 and Figure 4). About 5568 bacterial viruses have been examined by electron microscopy since 1959 when negative staining was introduced. At least 5360 (96.2%) of these are tailed and 208 (3.7%) are polyhedral, filamentous or pleomorphic [67].

Table 3 Phage families, their classification and basic properties [68]

Shape	Nucleic acid	Virus group	Particulars	Examples
Tailed	DNA, 2, L	*Myoviridae*	tail contractile	T4
		Siphoviridae	tail long, noncontractile	λ
		Podoviridae	tail short	T7
Polyhedral	DNA, 1, C	*Microviridae*	conspicuous capsomers	ϕX 174
	2, C, S	*Corticovidae*	complex capsid lipids	PM2
	2, L	*Tectiviridae*	inner lipid vessicle, pseudotail	PRD1
	2, L	SHI. group*	inner lipid vessicle	SH1
	2, C	STV1. group*	turret-shaped	ST1V
	RNA 1, L	*Leviviridae*	protrusions	MS2
	2, L, seg	*Cystoviridae*	envelope, lipids	ϕ6
Filamentous	DNA, 1, C	*Inoviridae*	a. long filaments	fd
			b. short filaments	MVL1
	2, L	*Lipothrixviridae*	envelope, lipids	TTV1
	2, L	*Rudiviridae*	TMV-like	SIRV-1
Pleomorphic	DNA, 2, C, S	*Plasmaviridae*	envelope, lipids, no capsid	L2
	2, C, S	*Fuselloviridae*	same, lemon-shaped	SSV1
	2, L, S	*Salterprovirus*	same, lemon-shaped	His1
	2, C, S	*Guttaviridae*	droplet-shaped	SNDV
	2, L	*Ampullaviridae**	bottle-shaped	ABV
	2, C	*Bicaudaviridae**	two-tailed, growth cycle	ATV
	2, L	*Globuloviridae**	paramyxovirus-like	PSV

Note: *C* Circular: L linear: *S* superhelical: seg segmented: 1 single-stranded: 2 double-stranded

* Awaiting classification

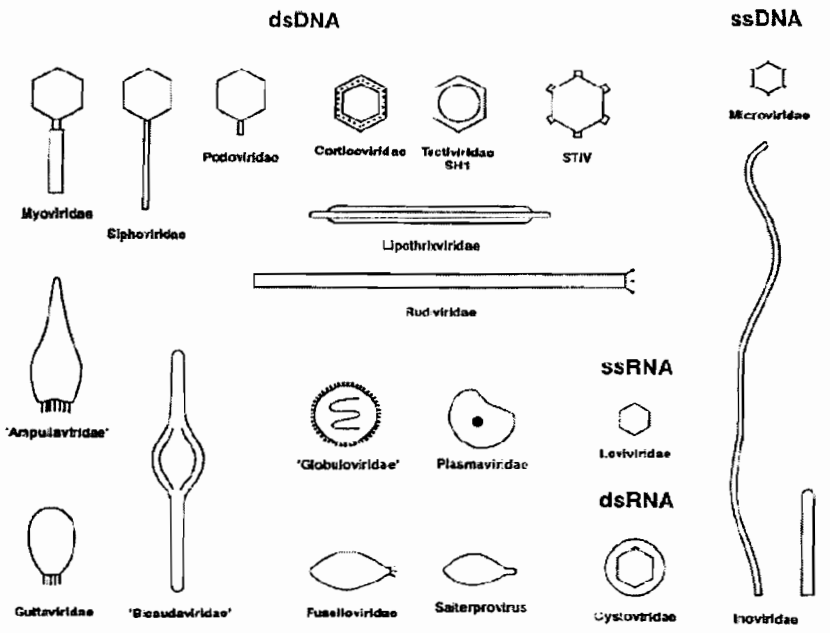

Figure 4 Basic bacteriophage morphotypes [67]

2.2.3.1 Tailed phages

The largest group of bacterial viruses are tailed phages whose particles feature a head of cubic symmetry and helical tails. Phage tails are helical in shape or have stacked disks. Besides, they have terminal structures such as base plates, spikes or fibers (Ackermann, 2005). The tails have fixed dimensions, are proteinic in nature and are composed of subunits which form transverse striations [67].

The linear, double-stranded DNA composition of tailed phages is usually a reflection of their bacterial host although some DNAs, such as those of coliphage T4, contain unusual bases, for example, 5-hydroxymethylcytosine [69].

Tailed phages are considered the most diverse and widespread of all viral groups because their properties are highly wide-ranging. Some of these differences include DNA content and composition, host range, physiology, serology and the nature of constitutive proteins [70]. Despite these differences, morphological, physiological and physiochemical properties reveal that this class of phages make up a monophyletic evolutionary group.

Myoviridae, *Siphoviridae* and *Podoviridae* are the components of the tailed phages. A different tail structure accounts for a distribution. *Myoviridae*

has contractile tails with a sheath and central tube. Myoviruses have larger capsids and more DNA as compared to their tailed counterparts. *Siphoviridae* which have long, noncontractile tails represent about 61% of tailed phages. *Podoviridae* posess short, noncontractile tails [70].

2.2.3.2 Polyhedral, Filamentous, and Pleomorphic phages

Polyhedral, filamentous, and pleomorphic (PFP) phages include about 208 viruses, contributing to 3.7% of total examined population [67]. This tailless group consists of families whose basic properties differ and appear to represent many independent lines of descent [69].

Microviridae (Polyhedral, ssDNA) have small, unenveloped virions containing circular ssDNA and are icosahedral in shape. DNA replication occurs via the rolling-circle model as a double-stranded replicative form. Enterobacteria, *Bdellovibrio, Chlamydia* and *Spiroplasma* are some of the different hosts infected by *Microviridae* [70].

Some families of tailless phages have as few as one member which is fully characterized. An example is *Corticoviridae* (polyhedral, dsDNA), phages which possess a protein capsid with an internal phospholipoprotein. Maritime phage PM2, a lytic phage is the only member with a complete description in this family [71]. There is little evidence on two similar phages which are isolated from sea water. Likewise, *Fusselloviridae* contains one member, SSVI. Its existence can be triggered by mytomycin C and UV light. *Fusselloviridae* have the shape of a spindle and short spikes at one end. Their outer covering contains two hydrophobic proteins and lipids. They are freed from their host by extrusion [69].

Interesting and unique features are also found in other groups. *Tectiviridae* (Polyhedral, dsDNA) have a rigid protein capsid containing a thick, flexible, lipoprotein vesicle which has the ability to change into a tail-like tube of approximately 60 nm long. This tube serves the same function as tails of tailed phages in that it acts as a vehicle for nucleic acid ejection following the phages adsorption to their respective host or treatment with chloroform (Ackermann, 2005). Some of the bacterial groups which form tectiviruses hosts are enterobacteria, *Pseudomonas, Thermus, Vibrio, Bacillus, Acinetobacter*, and *Alicyclobacillus.* Tectiviruses of *Bacillus* have apical spikes [69].

The family *Leviviridae* (polyhedral, ssRNA) is divided into two genera based on serology and genome structure. Their genome is made up of four partially overlapping genes. In addition, their RNA serves as mRNA and is thus positive-stranded. Morphologically, these viruses are non-enveloped. Many of the known leviviruses are plasmid-specific coliphages that adsorb to F or sex pili [70].

The cystoviruses (polyhedral, dsRNA) have lipid-containing envelopes which enfold the icosahedral capsids. They contain a dodecahedral RNA polymerase complex and three molecules of dsRNA [72]. During the infection process, the envelope is lost and the capsid passes through the spaces between the cell wall and the cytoplasmic membrane. Cystoviruses are highly host specific in that they only infect *Pseudomonas syringae* [69].

DNA replication in members of the *Inoviridae* family (filamentous, ssDNA) occurs via a rolling-circle mechanism in a double-stranded state. This group consists of two genera: *Inovirus* and *Plectovirus* which have different host ranges. Members of *Plectovirus* are short, straight rods and they only infect mycoplasmas. Viral particles of the *Inovirus* genus are long, rigid or flexible filaments whose length gives an indication of genome size [73]. Unlike plectoviruses, they infect a number of hosts namely, Clostridia, Propionibacteria, the genus *Thermus,* enterobacteria and their relatives. Progeny inoviruses are released forcibly from the host cells and no lysis takes place, thus phages may be produced for an indefinite period [69, 70].

Progeny Lipothrixviruses (filamentous, dsDNA) are released by lysis. Virions of the *Lipothrixviridae* family have a rod-like shape, a lipoprotein envelope and a nucleosome-like core. Examples of their hosts are *Acidianus, Sulfolobus* and *Thermoproteus,* all of which are thermophilic archaebacteria. Peng [74] reported that this family as well as *Rudiviridae* (filamentous, dsDNA) share similarities in their genomes, which implies that they form a superfamily. Rudiviruses were isolated from the thermophile *Sulfolobus*. This family has two viruses whose lengths differ. Their viral particles are straight, non-enveloped rigid rods with fixation structures on one end [74].

Budding may be used as another mechanism to release progeny viruses. That virions contain an envelope and a thick nucleoprotein granule.

The infection process sees the fusion of the envelope with the host membrane. After that budding will occur [69].

Guttaviridae (pleomorphic, dsDNA) Virion particles are droplet-shaped with a distinct beehive-like structure and thin fibers at its pointed end. An example is SNDV (*Sulfolobus neozealandicus* droplet-shaped virus) which was found in a *Sulfolobus* isolate from New Zealand. Genome size is approximately 20 kbp and its DNA is only cleaved by a few restriction enzymes such as *Dpn*I. Host range is limited to a few *Sulfolobus* strains [75]. New phages of archaebacteria were recently isolated and they have yet to be classified by the ICTV.

Sulfolobus turreted icosahedral virus (STIV, polyhedral ssDNA) is an archael virus isolated from an acidic hot spring. It has apical turret-shaped protrusions on its capsid and is the only known member of this family. It has been speculated that the turret-like structures may function in host recognition and/or attachment. It has also been suggested that the nucleic acid might be transferred via the central channel in each turret [76]. *Sulfolobus solfataricus,* a hyperthermophilic archaeon is the only host [77].

SH1 (polyhedral, dsDNA) virions contain lipid components in their structure and a distinct proteinaceous outer layer [78]. The genome is linear, 31 kbp and is unique in that it does not share any similarities with any of the published sequences. They are lytic phages and infect halobacteria of the genera, *Halorubrum* and *Haloarcula hispanica* [72]. Another family which is also unique in that no significant matches are found between its genes and those in public databases is *Globuloviridae* (pleomorphic, dsDNA). Virions are about 100 nm in diameter, spherical with an envelope and a nucleoprotein core [79].

Ampullaviridae (Pleomorphic dsDNA) members possess a unique bottle-shaped structure and a funnel-shaped core. Its broad end has thin filaments and the pointed end possibly plays a role in adsorption and translocation of DNA into the host [80].

There is a striking feature of independent morphological development outside the host as observed in *Bicaudaviridae*. Viral particles which are lemon-shaped have long tails at each end after being extruded from the host. The process takes place at 75-90 °C., which is close to the temperature of the natural

habitat without a host. This extracellular morphogenesis is a way to survive in severe environments [81].

2.2.3.3 Phage Nomenclature

It is impossible to separate nomenclature from classification. The ICTV employs the terms based on Latin for order, family, and genus names. As for the families, the suffix, -virdae is used. However, Latin is not used for species epithets. Phage T4 remains the same, for instance. The family names have served as a very helpful purpose. '*Siphoviridae*' appears better than 'phages with long, noncontractile tails.

2.2.4 Use of Bacteriophage

Recently, bacteria have become more resistant to antimicrobial agents. That regains an attention to phage therapy and a re-evaluation of the biological therapeutic alternative for getting rid of pathogenic bacteria. Using phages for the therapeutic goals is quite simple. As for phages, there have been several advantages over antimicrobial agents, for example, specificity against a host or host range not affecting the normal micro flora.

2.2.4.1 Food industry

There are many challenges for the food industry to keep products safe and free of pathogenic microorganism. Each year owing to food borne pathogens present in the foods consumed by humans, there are reports of illness, and even deaths. More than 200 different types of food borne agents are known. They include viruses, bacteria and parasites that can be conducive to illness. For some years there are increased uses and field research on natural antimicrobial agents thanks to the consumers changes in the use of antibiotics and synthetic preservatives and the bacterial resistance.

Nowadays ready-to-eat foods are easily available. It is a great challenge for the food production technologies. As a result, it is important to come up with new strategies to cope with the changing scenario. The main aim to have new technologies is to prevent food borne pathogen present in the products. Without strict safety measure enforce, there can be a spread of microbial contaminants, which can play a key role in causing illnesses.

So, recently phages have been studied and applied to food products of animal and plant origin.

Animals such as poultry, fish and livestock have been infected with phages in order to decrease the population of pathogenic bacteria and attempt to minimize animal disease [82-85]. The vast majority of studies reported in this occur in poultry industry where the animals and products (meat,eggs) get frequently contaminated with *E. coli,* Salmonella and Campylobacter. Many of these studies report successful reduction of these pathogens load with different phage [82, 86-90]. For instance, phage treatment of chickens and calves has shown to protect the animals against septicemia and meningitis caused by *E.coli* and to reduce morbility of the treated animals compared to control experiments [88, 91, 92]. Phage also showed to delay the appearance of this bacterium and by this mean lengthened the animal life period [91]. Chicken treated with *Campylobacter jejuni* phages showed a decrease in the amount of his pathogen in the chicken skin and caecal content and resulted in a delay of *C. jejuni* colonization. The same observation has also been described with Salmonella phage [93] and decrease of this pathogens' recovery from the carcass rinsing waters due to phage application [85].

Plants pathogens are rarely dangerous to humans; however, they can cause a product loss. Plant pathogens such as Rastonia can be the cause of bacterial wilt and have an impact on a variety of products like potto, tobacco, tomato, banana and soybean. Phage research which is aimed to protect plants, vegetable and fruits from decay and spread of bacteria diseases is conducted on horticulture and agriculture.

The main products to which phages have been applied to are: geranium [94, 95], tobacco [96], tomato ([97, 98], potato [99], mush room [100], sprouts [2], peach [101] and apple [102]. Environment factors such as sunlight irradiation, temperature, desiccation can affect phage application. For example, in handling the phage population decrease caused by UV irradiation exposure, phage application to tomato fields should always be carried out in the early- and mid-morning, early afternoon and late evening. It is possible to form phage formulas by combining phage with other components. Phage formulas which are not protected are reduced by dissecation after 60 days and by fluorescent light after 2 weeks. However

the factors in question had little impact on phage mixed with skim milk, which indicates that phage formula have a high potential as plant disease control agents [95, 98, 103].

The vast research carried out with phage and some food product of plant and animal origin has led to the fabrication of phage or phage-based product. Today, in animal production, animal and plant products, the application of three four product is allowed. Two of phage products, LISTEX™ 100PM and LMP-2, target *Listeria monocytogenes* present in cheese, meat and fish products and both have been approved by the US Food and Drug Administration (FDA). Additionally, LISTEX™ 100PM has also received the Generally Recognized as Safe (GRAS) status by the FDA and by the United State Department of Agricuture (USDA), to be incorporate in food production processes. Another phage product named BacWASH™, has been issued a No Objective Letter for Use, by the USDA, and it targets *Salmonella* and *E.coli* O157:H7. BacWash™ phage was especially created to be used on animal prior to slaughter and it can be applied as a wash, mist, or sprayed directly to the live animal. The Environmental Protection Agency (EPA) approved, in 2005, the product AgriPhage™ for control purpose of *Xanthomonas campestris* pv. vesicatoria and *Pseudomonas syringae* pv. in tomato, and pepper plant among others. This product can decrease bacteria spot and speck and it can be used on farms and at the pre-harvest level, diluted prior dispersal and can be applied to crops by different mean, such as drip irrigation or using ground or aerial spray equipment.

2.2.4.2 Phage therapy

Bacteria can be distinguished by bacteriophages on the basis of the particular phage or phages. The phages can get rid of bacteria and besides they are not dangerous to humans. There are efforts to use them to treat bacterial infections. However, there are factors on a variety of phage-specific strains of bacteria within a species and a rapidity of phage resistance.

A long history of phage can be traced back after the discovery of phages. D'Herelle saw the therapeutic potential of phages. He first treated dysentery patients and succeeded [104]. In 1921, it was Joseph Maisin who used phages treat staphylococci induced skin diseases. However, following the discovery of the first antibiotics in the western nations, phage therapy was no longer in use. Only certain

countries in the former Soviet Union still developed phages for a therapeutic objective [104]. Nowadays, in the wake of a discovery that pathogenic bacteria became resistant to antibiotics, many view phage therapy as an alternative to treat bacterial infections. This kind of therapy has many advantages over antibiotics: phages are specific to certain bacteria and can be isolated and investigated rapidly. It is possible to prevent an emergence of phage resistant bacteria by means of phage cocktails [105]. Phages are not toxic and can be used to treat surface infections with negative effects. On the other hand, the application of phages orally or intravenously is still highly discussed, because little is known about the reaction of the immune system to the phages, the clearance of the phages in the body and the actual effects on the infection. But not only whole phages are used today. Some attempts showing that isolated endolysins, the enzymes which helps the phage to free the progeny phages after a complete life cycle, can be used to treat infections of Gram positive bacteria [106]. Jado used a phage encoded murein hydrolase to treat *Streptococcus pneumoniae* infected mice [107]. They suggest that phage lysins protect animals from bacterial infections due to their results that the used murein hydrolase rescued the infected mice in their study.

The use of bacteriophage as biocontrol agents in aquaculture is possible. Experiments using phage PPp W-4 and PPpW-3, isolated and experimentally used to control *Pseudomonas plecoglossicid*a infections in fish ayu (*Plecoglossus altivelis*) Temminck and Schlegel showed significantly lower mortalities in phage-treated fish groups compared to those of untreated controls [108]. Imbeault demonstrated that counts of *Aeromonas salmonicida* (etiological agent of Furunculosis) decreased by six log units in 3 d after the introduction of a phage HER 110, at a multiplicity of infection factor of 1 [109]. Bacteriophage of *Vibrio harveyi* was reported and the survival of white shrimp *Penaeus monodon Fabricius* larvae to be 80% versus 25 % observed in the controls [110]. Furthermore, in vitro and in vivo challenges with bacteriophages isolated from aquatic environments have demonstrated the potential of reducing mortalities, in yellow tail (*Seriola quinqueradiata,* Temminck & Schlegel) and Ayu fish (*Plecoglossus altivelis*), abalone (*Haliotis discus hannai* Ino), loaches (*Misgurnus anguillicaudatus* Cantor), brook trout (*Salvelinus fontinalis* Mitchill) and eastern oysters (*Crassostrea virginica* Gmelin) [109, 111-114]. Other virulent bacteriophages that have been isolated and are of great

importance to the aquaculture industry include; phages that infect *Chondrococcus columnaris* or *Flavobacterium columnare* [115], phage PLgY of *Lactococuss garvieae* isolated from diseased yellowtail [116], phages against *Yersinia ruckeri* [117] and phages Aeh1 and Aeh2 of *Aeromonas hydophila* have been isolated [118]. Advantages of phages therapy include: (1) they are specific and therefore cannot infect other microbial cell organisms that are ecologically important, (2) bacteriophage switch to a dormant state soon after all host cells are destroyed and hence will disperse harmlessly, (3) human patients who are allergic to antibiotics are treated with phages with no side effects and are safe because they do not attack human and animal cells, (4) phages can be administered in various routes, (5) phages produce exponentially, hence, a single dose can be sufficient to treat an infection, (6) when resistant strains of the host develop, the phage has capabilities of mutating in step with the evolving bacteria, (7) production of bacteriophages is simple and inexpensive, (8) bacteriophages are ubiquitous, [119, 120, 104, 105, 121, 122, 123]. Disadvantages in using bacteriophages include; (1) their specificity implies that the causative bacterial pathogens have to be identified prior to their administration and the lytic spectrum may be limited to one subtype bacterial pathogen, (2) low or no efficacy have been reported in certain cases but may be attributed to insufficient diagnosis of the disease and phage-dose, together with improper delivery mechanisms, (3) phage administration requires a neutralized environment which is rarely found in the digestive system of animals due to presence of gastric secretions, (4) the lytic life-cycle of bacteriophages described in in vitro environments may not be maintained under normal physiological conditions found in the body, and instead may revert to a lysogenic cycle, (5) the existence of bacteriophage as a prophage may lead to propagated resistance of the bacteria towards antibiotics [124, 119, 120, 104, 105, 125, 121, 122, 126, 123].

CHAPTER 3
METHODOLOGY

3.1 Isolation of bacteriophage

The bacterium used as a host strain for the isolation of bacteriophage was *Salmonella* Typhimurium ATCC 13311. It was obtained from the DMST Culture Collection, Bangkok, Thailand, and maintained at the laboratory of the Department of Biological Science, Faculty of Science, Ubon Ratchathani University. The bacterium was cultured in Brain Heart Infusion (BHI) broth at 37°C and kept as a glycerol (20% v/v) stock at -20°C until use. Water samples used as sources of bacteriophage specific to *S.* Typhimurium were collected from swine lagoon in Ubon Ratchathani province, Thailand. The samples were centrifuged at 4,500 rpm to precipitate cell debris for 15 minutes. The supernatant was filtered through 0.45 μm pore size syringe filters. Five milliliters of 2X BHI was added to 5 ml of filtrate along with 100 μl of logarithmic-phase cells of bacterial host strain grown in BHI broth. The mixture was mixed thoroughly and incubated in an incubator at 37°C for 24 h. At the end of incubation period, the suspension was centrifuged at 4,500 rpm for 15 minutes and the supernatant was filtered through 0.45 μm pore size syringe filters. The bacteriophage suspension was maintained at 4°C and tested for the presence of bacteriophage activity against its host by spot test method. Briefly, 4 ml of BHI sloppy agar was seeded with 0.1 ml of the bacterial host culture, mixed gently, and poured onto a BHI agar plate. After solidification, 10 μl of bacteriophage suspension was spotted onto the lawn of *S.* Typhimurium. After drying at room temperature, the plate was incubated at 37°C overnight. A clear zone appearing on the plate, resulting from the bacterial cell lysis, indicated the presence of bacteriophage in the tested bacteriophage suspension. In all cases, positive tests were confirmed by plaque assay using the double layer agar plate method as described by Lu et al. [127].

3.2 Characterization studies

3.2.1 Enumeration of bacteriophage

Bacteriophage titer was determined as plague forming unit (PFU)/ml using the double layer agar plate method. Briefly, after ten fold dilution of bacteriophage containing sample with BHI broth, 0.1 ml of each bacteriophage dilution and 0.1 ml of actively growing host cell (10^8 colony forming unit, CFU/ml) were added to a tube containing 4 ml of sloppy BHI agar (50°C). The mixture was overlaid onto the surface of a Nutrient Agar (NA) plate and incubated overnight at 37°C before enumeration of lysis plagues.

3.2.2 Host range determination

The spot test method as mentioned earlier was used to examine bacteriophage lysis activity against various strains of bacteria. Eighteen bacterial strains used for the determination of bacteriophage host range were *Bacillus cereus* ATCC 11778, *Bacillus subtilis* ATCC 6633, *Enterobacter aerogenes* ATCC 13048, *Escherichia coli* ESBL$^+$ *Escherichia coli* UBU, *Klebsiella pneumoniae* ATCC 27736, *Proteus vulgaris* ATCC 29905, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* (Imipenem resistant) , *Salmonella* Typhi DMST 22842, *Salmonella* Typhi DMST 5784, *Shigella dysenteriae* ATCC , *Shigella dysentariae* (type1) DMST 2137, *Staphylococcus aureus* UBU, *Staphylococcus aureus* (MRSA) , *Staphylococcus epidermidis* ATCC 12228, *Vibrio vulnificus* DMST 21245, *Vibrio cholerae* non O1 non O139 DMST 2873.

3.2.3 Bacteriophage adsorption

A host strain culture (OD600 = 0.3 or $1x10^8$ CFU/ml) in 25 ml of BHI broth was infected by 2.5 ml of bacteriophage suspension to give a multiplicity of infection (MOI) of 0.01, and incubated at 37°C. Aliquots of 3 ml were take at 0, 10, 20, 30 and 40 min after infection and immediately filtered through a 0.45 μm pore size syringe filters. Filtrates were tittered for unabsorbed bacteriophage by the double layer agar plate method as mentioned earlier. BHI broth containing only bacteriophage ($1x10^7$ pfu/ml) was used as control.

Percent adsorption of bacteriophage was calculated as [(control titer-residual titer)/(control titer)] x 100%.

3.2.4 One step growth curve

Forty-five ml of BHI was inoculated with host strain and incubated for 4 h at 37°C to reach the log phase (OD600 = 0.3 or $1x10^8$ CFU/ml) and then infected with the bacteriophage at a multiplicity of infection (MOI) of 0.1. After 40 minutes of incubation, the infected bacterial culture was centrifuged for 15 min at 5,000 rpm. The supernatant was carefully decanted, and the infected pellet was thoroughly resuspended in 10 ml of fresh and warm BHI broth (37°C). Aliquots of 3 ml were taken at 0, 10, 20, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330 and 360 minutes for bacteriophage enumeration by using the double layer agar plate method. The latent period was defined as the interval between adsorption of the bacteriophage to the host cell and host cell lysis. The burst size was determined as the number of progeny bacteriophage particles produced by a single host cell.

3.2.5 Stability of bacteriophage

To examine pH stability of bacteriophage, 0.1 ml of bacteriophage was incubated for 24 h at 37°C in 0.9 ml of BHI broth with pH varying from 2-13. The titer of bacteriophage residue was determined by the double layer agar plate method. To determine the thermal stability of bacteriophage, 5 ml of sterile water was preheated to a desirable temperature, ranging from 50, 60, 70 and 80 °C. 0.5 ml of bacteriophage solution ($1x10^6$ PFU/ml in water) was added to the tube. After heating for 3 minutes, the tube was placed immediately in an ice water bath. Samples were subjected to bacteriophage titer determination by using the double layer agar plate method.

3.2.6 Bacteriohage purification

One hundred μl of overnight culture of host strain and 100 μl of bacteriophage suspension were added to 4.5 ml of sloppy BHI agar. After mixing, the mixture was poured rapidly onto a BHI agar plate. The plate was swirled to ensure that the mixture spread over the surface. After drying, the plate was incubated at 37°C overnight. A single plaque was picked from the lawn and inoculated into the tube containing 100 μl of overnight culture of the same host strain. The infected bacterial culture was subjected to single plaque isolation as mentioned above for 3 more times. At the end of the experiment, a single plaque containing purified bacteriophage was obtained.

3.2.7 Bacteriophage propagation

The purified bacteriophage was added to 5 ml of log phase bacterial host culture (10^8 CFU/ml) at the MOI of 0.01. After incubation at 37°C with shaking (180 rpm) for 6 h, the infected culture was centrifuged at 7000 rpm for 20 min to remove bacterial cell debris. The clear supernatant was kept as bacteriophage stock and stored at 4°C.

3.2.8 Bacteriophage concentration

To prepare a high titer bacteriophage stock, bacteriophage was propagated in 500 ml culture using the method mentioned above. Phage-containing supernatant was centrifuged at 7000 rpm for 20 min and the supernatant was filtered through 0.45 μm membrane to remove the remaining bacterial cells. The filtrate was stirred slowly at 4°C overnight in the presence of polyethylene glycol 8000 (PEG 8000) at the final concentration of 10% before being centrifuged at 28500 rpm for 1 h at 4°C. The bacteriophage pellet was resuspended in 1 ml of STE Buffer.

3.2.9 Electron microscopy

The morphology of bacteriophage ST1 was examined by transmission electron microscopy. Twenty five μl of the bacteriophage suspension was mixed with 25 μl of 50% glutaraldehyde in 4% paraformaldehyde. Five μl of the mixture was dropped on a carbon Formvar-coated copper grid and left at room temperature for 5 min. After being negatively stained with 2% (w/v) phosphotungstic acid, the bacteriophage was examined with a JEM-1230 transmission electron microscope (JEOL, Tokyo, Japan) at different magnitudes. The bacteriophage size was determined from the average of five independent measurements.

3.2.10 Bacteriophage genome analysis by restriction endonuclease

Bacteriophage genome was extracted by using PureLink Viral RNA/DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. It was cut with *Eco*RI at 37°C for 16 h. After digestion, the sample was heated for 10 min at 70°C to inactivate the enzyme activity. Electrophoresis of the digested genome was carried out on 0.8% agarose gel. Gel was stained with ethidium bromide and photographed under an UV transilluminator.

3.3 The ability of bacteriophage ST1 to control *S.* Typhimurium in beverages

3.3.1 Stability of bacteriophage ST1 in beverages

The stability of bacteriophage ST1 in beverages was examined in four different beverages including orange juice, guava juice, soymilk, and grass jelly drink. The beverages were all purchased from local retailers and allowed to adjust to room temperature prior to inoculation. Bacteriophage filtrates were added to each beverage (final concentration of approximately 10^6 PFU/ml). All trials were kept at 37°C throughout the experiment, and samples for plaque assays were withdrawn after 0, 3, 6, 9 and 24 hour.

3.3.2 Effect of bacteriophage concentration on the reduction of *S.* Typhimurium in soybean milk and in jelly grass drink

The effect of the concentration of bacteriophage ST1 on the reduction of *S.* Typhimurium was examined in soybean milk and in jelly grass drink. For each beverage, 4 sets of treatment were prepared. All of them were separately inoculated with *S.* Typhimurium at the final concentration of 10^6 CFU/ml. The bacteriophage suspension was added to 3 of 4 sets of treatment at different concentration which were 10^6, 10^7 and 10^8 PFU/ml. The other set of treatment without bacteriophage was used as a control. All treatments were incubated at 37°C and their samples were collected at 0, 3, 6, 9, and 24 h for the determination of bacterial and bacteriophage counts. The determination of bacterial concentration in each beverage sample was performed by plating on Xylose lysine deoxycholate (XLD) agar, a selective medium for *S.* Typhimurium. For the determination of bacteriophage titer in each beverage sample, the sample was filtered through a 0.45 μm pore size syringe filter and the filtrate was subjected to bacteriophage titer determination by using the double layer agar plate method as mentioned earlier.

CHAPTER 4
RESULTS

4.1 Bacteriophage isolation

A filtrate prepared from swine lagoon was found to produce clear zone on the lawn of *S.* Typhimurium ATCC 13311 by spot test (Figure 5) moreover, the filtrate also produced plaques by plaque assay, indicating that it contained a lytic bacteriophage. The plaques were small with an average diameter of 1 mm and did not have a halo surrounding the plaques (Figure 6), and the isolated bacteriophage was designated ST1.

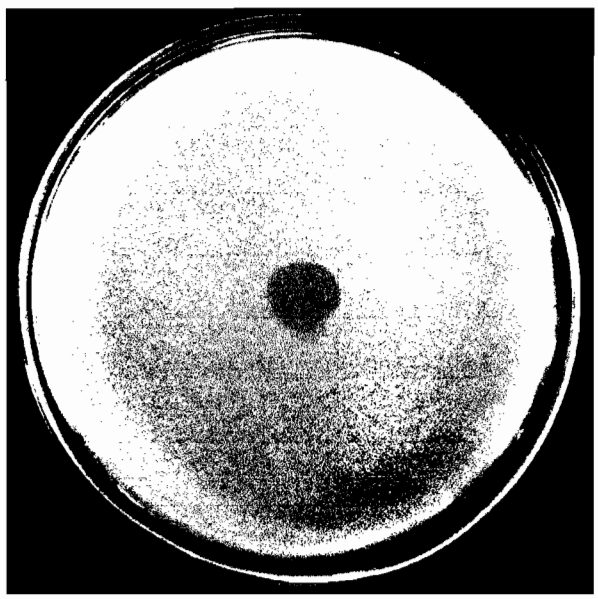

Figure 5 Clear zone on *S.* Typhimurium lawn produced by bacteriophage ST1

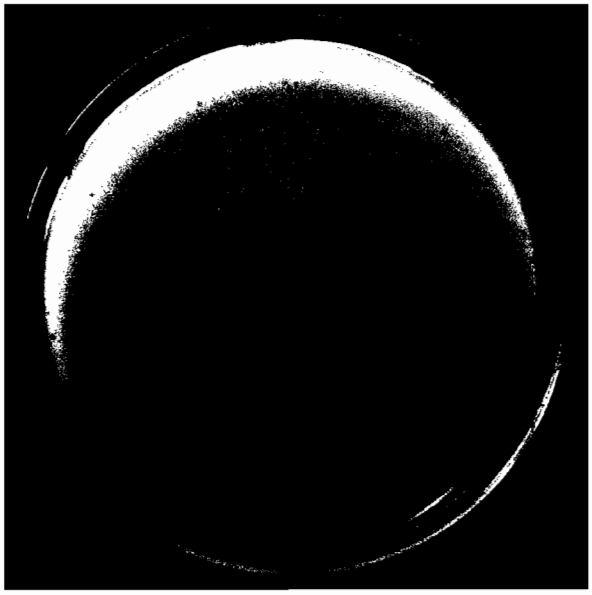

Figure 6 Plaques on a lawn of *S.* Typhimurium produced by bacteriophage ST1

4.2 Characterization studies

4.2.1 Bacteriophage host range

Specificity of bacteriophage ST1 to other bacterial strains was examined by the spot test method. Of all 18 bacterial strains used in this experiment, only *Shigella dysentariae* (type1) DMST 2137 and *Salmonella* Typhi DMST 5784 were susceptible to the bacteriophage as shown in Table 4. On the other hand, the rest of the tested bacterial strains used in this study were not sensitive to the bacteriophage. The results indicated that bacteriophage ST1 had a board host range.

Table 4 Host range specificity study of bacteriophage ST1

Bacteria strain[a]	Spot test[b]
Bacillus cereus ATCC 11778	-
Bacillus subtilis ATCC 6633	-
Enterobacter aerogenes ATCC 13048	-
Escherichia coli ESBL$^+$	-
Escherichia coli UBU	-
Klebsiella pneumonia ATCC 27736	-
*Proteus vulgaris*ATCC 29905	-
Pseudomonas aeruginosa ATCC 27853	-
Pseudomonas aeruginosa (Imipenem resistant)	-
Salmonella Typhi DMST 22842	-
Salmonella Typhi DMST 5784	+
Shigella dysenteriae ATCC 29026	-
Shigella dysentariae (type1) DMST 2137	+
Staphylococcus aureus UBU	-
Staphylococcus aureus (MRSA)	-
Staphylococcus epidermidis ATCC 12228	-
Vibrio vulnificus DMST 21245	-
Vibrio cholerae non O1 non O139 DMST 2873	-

Note: [a]American Type Culture Collection (ATCC); Department of Management Science and Technology (DMST) and UBU, Culture Collection of Biological Science Department, Faculty of Science, UbonRatchathani University.
[b], + clear zone; - no clear zone.

4.2.2 Bacteriophage adsorption

The adsorption rates of bacteriophage ST1 in BHI broth are shown in Figure 7. About 70% and 80% of bacteriophage ST1 particles adsorbed onto its host cells within 20 and 40 minutes, respectively. After that, the bacteriophage particles adsorbed onto the host cells at a relatively slow rate.

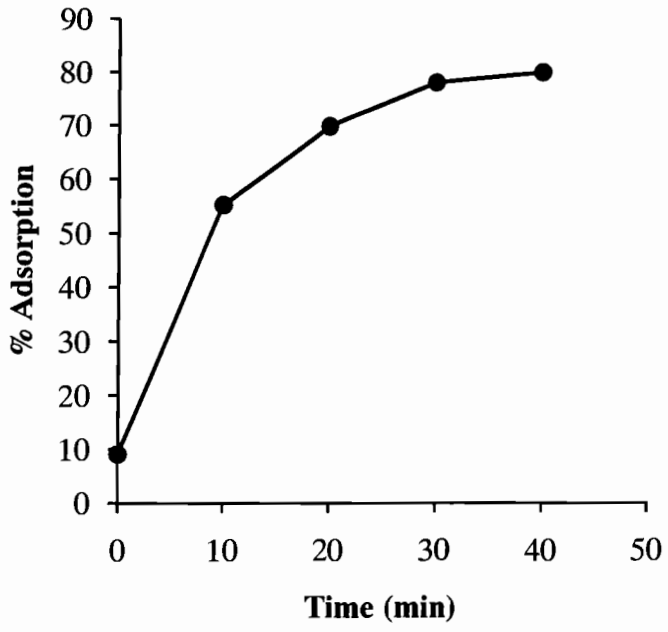

Figure 7 Adsorption curve of bacteriophage ST1 on *S.* Typhimurium in BHI broth at 37 °C

4.2.3 Thermal and pH sensitivity

Thermal sensitivity of bacteriophage ST1 was investigated by thermal treatments at 50, 60, 70 and 80°C for 3 minutes (Table 2). No significant change of bacteriophage titer was observed when the bacteriophage was treated at 50°C and 60°C. The bacteriophage titer decreased from about 6 log PFU/ml to about 5 and 4 log PFU/ml after heat treatment at 70 and 80°C, respectively.

The pH sensitivity of bacteriophage ST1 was also investigated by incubating the bacteriophage overnight in BHI broth at pH ranging from 2 to 13. The bacteriophage maintained its infectivity when incubated in a pH range of 5 to 11. In contrast, the bacteriophage lost its infectivity completely at pH 4 or below as well as at pH 12 or above. The bacteriophage was found to have highest stability at pH 9 (Table 5).

Table 5 Sensitivity of bacteriophage ST1 to temperature and pH

Treatment	Initial phage concentration (PFU/ml)	Final phage concentration (PFU/ml)
Heat (for 3 min)		
50 °C	6.16	6.14
60 °C	6.13	6.13
70 °C	6.13	5.09
80 °C	6.19	3.98
pH (for 24 h)		
2	6.01	-
3	6.10	-
4	6.08	-
5	6.12	5.32
6	6.14	5.39
7	6.09	5.47
8	6.03	5.71
9	6.02	5.79
10	6.03	5.46
11	5.91	4.94
12	6.01	-
13	5.06	-

Note: - = undetectable

4.2.4 One step growth kinetics

The infection cycle of bacteriophage ST1 was analyzed from the one step growth curve. The curve showed that the bacteriophage ST1 has a latent period of 70 min, the burst period of 330 min and a burst size of about 172 PFU per infected cell (Figure 8).

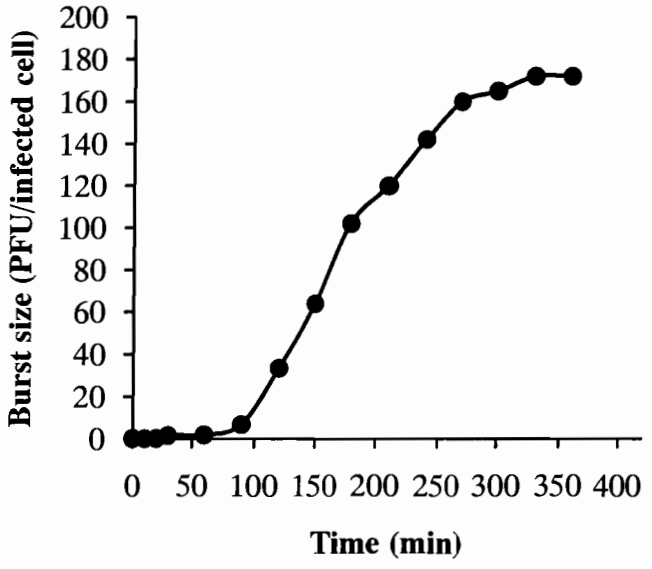

Figure 8 One step growth curve of bacteriophage ST1 in BHI broth at 37°C using *S.* Typhimurium as the host

4.2.5 Transmission electron microscopy

The ultrastructure of bacteriophage ST1 by transmission electron microscopy revealed that the bacteriophage had an isometric head of about 70 nm in diameter with a noncontractile tail of 150 nm long and 7 nm wide without collar or tail appendages (Figure 9).

Figure 9 An electron micrograph of bacteriophage ST1 (bar = 50 nm).

4.2.6 Restriction endonuclease pattern of bacteriophage genome

The genome of bacteriophage ST1 was subjected to restriction analysis by digestion with *Eco*RI and separated by agarose gel electrophoresis. Restriction endonuclease pattern of bacteriophage ST1 genome digested with *Eco*RI indicated that it was double stranded DNA (Figure 10). According to the Internationals Committee on Taxonomy of Viruses [128] the bacteriophage ST1 can be classified into the family *Siphoviridae.*

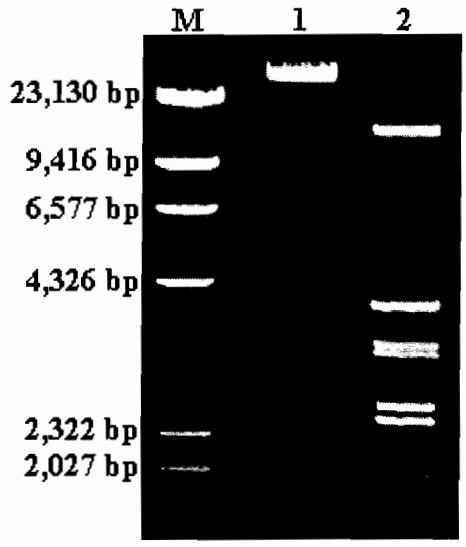

Figure 10 Agarose gel electrophoresis of bacteriophage ST1 genome: M = λ DNA Digested with *Hind*III marker; lane1 = uncut genome; lane 2 = cut genome with *Eco*RI.

4.3 The ability of bacteriophage ST1 to control *S.* Typhimurium in beverages

4.3.1 Stability of bacteriophage ST1 in beverages

The stability of bacteriophage ST1 was investigated in 4 different beverages (soybean milk, jelly grass drink, guava juice and orange juice). The titers of the bacteriophage added to the beverages were monitored within the incubation period of 24 hours. As shown in figure 11, in soybean milk and jelly grass drink, no significant change of bacteriophage titer was found throughout the observation period. On the other hand, the decrease of bacteriophage titer was observed in guava juice and orange juice. In both beverages, the titer drastically decreased from about 6 PFU/ml to undetectable level within 3 h. Since bacteriophage ST1 was stable in soybean milk and jelly grass drink, both beverages were selected to used for further experiments.

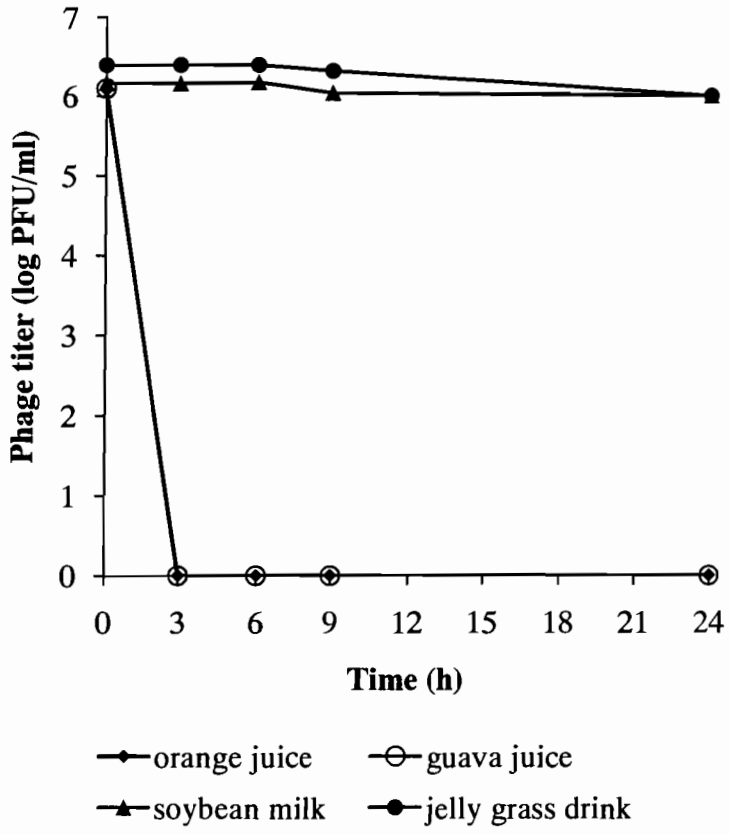

Figure 11 Stability of bacteriophage ST1 in different 4 beverages.

4.3.2 Effect of bacteriophage concentration on the reduction of *S.* Typhimurium in soybean milk

The reduction of *S.* Typhimurium in soybean milk was found to be dependent on bacteriophage concentration (Figure 12). Complete elimination of the bacterium was found only when the bacteriophage concentrations used were 10^7 and 10^8 PFU/ml, but not 10^6 PFU/ml. Furthermore, Figure 12 also showed that the time required for complete elimination of *S.* Typhimurium in soybean milk could be lowered from 24 h to 3 h when the concentration of the phage was increased from 10^7 PFU/ml to 10^8 PFU/ml.

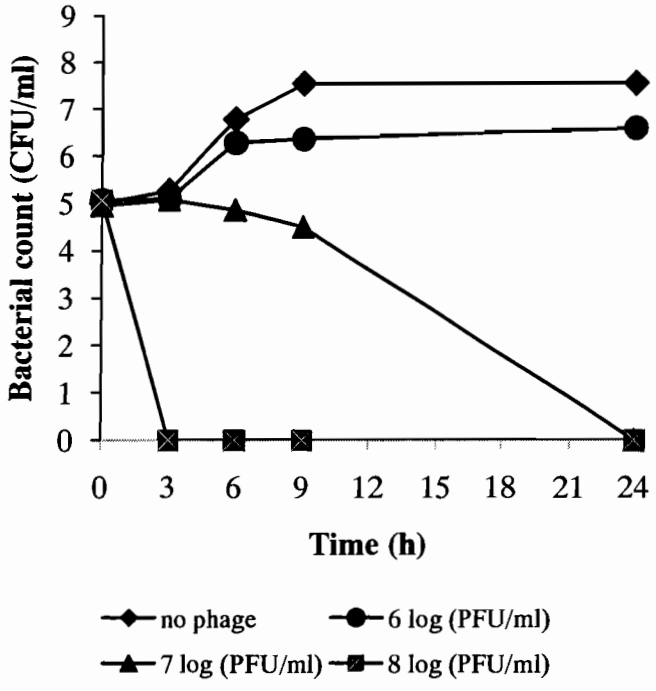

Figure 12 Effect of bacteriophage ST1 on the growth of *S.* Typhimurium in soybean milk at various bacteriophage concentrations.

4.3.3 Effect of bacteriophage concentration on *S.* Typhimurium reduction in jelly grass drink

The reduction of *S.* Typhimurium in jelly grass drink was found to be dependent on the concentration of bacteriophage ST1 (Figure 13). Complete elimination of the bacterium was found at 24 h and 3 h after bacteriophage inoculation when 10^7 and 10^8 PFU/ml of the bacteriophage were used, respectively.

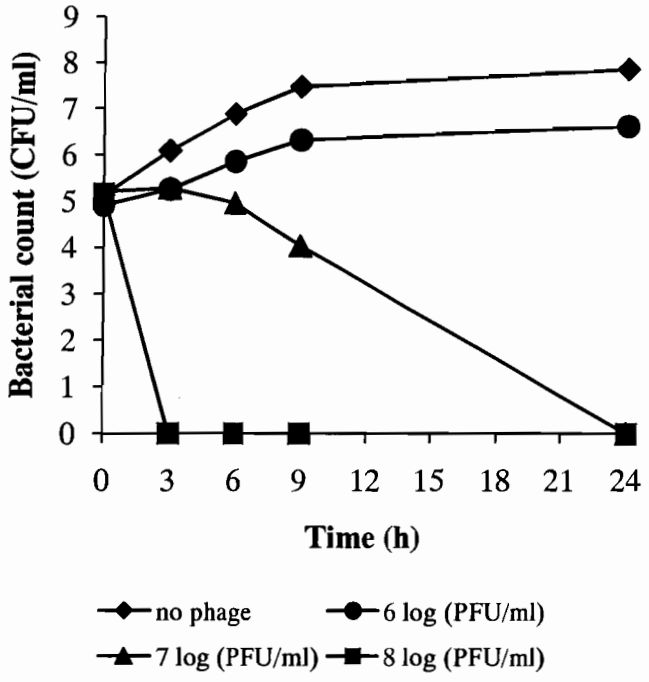

Figure 13 Effect of bacteriophage ST1 on the growth of *S.* Typhimurium in jelly grass drink at various bacteriophage concentrations.

CHAPTER 5
DISCUSSION AND CONCLUSION

Salmonella Typhimurium (*S.* Typhimurium) bacteria are classified as one of the most dangerous microorganisms causing serious intestinal disorders of humans. It has been found in all types of foods such as meats, vegetables, fruits and beverages. Generally, the contamination of the bacterium in foods can occurs at any step of food production due to the mishandling, improper sanitation and inadequate hygiene practices. Street foods and beverages are examples of consumed products commonly found to be contaminated with foodborne pathogenic bacteria including *S.* Typhimurium. The disease caused by *S.* Typhimurium infection is characterized by gastrointestinal disorders manifested predominantly by diarrhea and abdominal cramps. Annually, millions of cases of *S.* Typhimurium associated disease occur worldwide. The disease not only affects people's health and well being, but also has an economic impact on individuals and countries. Substantial economic losses are documented annually worldwide due to clinical treatment costs and lost working hours.

At present the reduction or elimination of contaminated foodborne pathogens including *S.* Typhimurium in foods is commonly achieved by using chemical food preservatives. Chemical food preservatives are substances that are added to many different types of food to keep them fresh and inhibit microbe growth. There are generally three kinds of preservatives: antimicrobials that slow down the growth of microorganisms, antioxidants that inhibit the oxidation of air by lipids and fats, and chemicals that inhibit the natural development of processes of enzymes that occur in foods after they have been harvested. Recently, the health risk of chemical food preservatives has become a major concern of consumers around the world because many health problems related to chemical food preservatives have been reported. Some chemical food preservatives have induced allergic reactions while others have been linked to cancer, hypersensitivity, toxic reactions, ADHD, and lung disease.

Chemical food preservatives and cancer:

There are concerns of certain types of food preservatives being linked to cancer, particularly nitrites and butylated hydroxyanisole (BHA). Nitrites prevent the growth of botulism causing bacteria and serve as a color enhancement for processed meat and fish. Nitrites react with amines and amides and create nitrosamines and nitrosamides, which have been identified as carcinogens. BHA is a phenolic antioxidant that prevents the oils and fats from becoming rancid. High levels of BHA cause tumors in some laboratory animals.

Chemical food preservatives and hypersensitivity:

Sulfites act as antioxidants and prevent light-colored produce from losing their color and prevent bacteria growth in wine. Sulfites have been shown to cause hypersensitivity in some individuals. Some people such as those with asthma or many allergies have allergic reactions to sulfites. The most common reported symptom of an allergic reaction to sulfates is having difficulty breathing. Other common issues include hives, stomach pain and anaphylactic shock.

Chemical food preservatives and toxic Reactions:

Butylated hydroxtoluene (BHT) is a phenolic antioxidant that prevents food fats and oils from rotting. The substance may cause toxic reactions with other substances. An example is it increased toxicity in animal studies when consumed after the consumption of carcinogens and mutagens.

Chemical food preservatives and Attention Deficit Hyperactivity Disorder (ADHD):

According to The Medical News, preservatives may contribute to the development of ADHD in children. Many parents and educators believe that food and beverages containing preservatives have negative consequences on the behavior of some children. The results of a clinical trial where preservatives were eliminated from the diet of children with ADHD showed that should be a standard treatment procedure of the disorder. Research also showed that children that were not typically hyperactive had significant levels of hyperactivity after the consumption of foods containing preservatives.

Chemical food preservatives and lung disease:

According to an observational study that took place in 2006, individuals who consume cured meats containing nitrites on a regular basis are more likely to develop lung disease symptoms. Nitrites are added meat to prevent oils and fats from being coming rancid, improve the color of the meat and retain the flavor. The results of animal studies reveal that nitrites can create reactive types of nitrogen that may cause negative effects on the lungs such as modifications in the structure of the organ.

Since chemical food preservatives do not meet increasing consumers' demand for natural food products, the exploration of natural and safe antimicrobial agents to replace chemical food preservatives is receiving increase attention. Bacteriophages fit in the class of natural antimicrobial. Their safety and effectiveness in controlling bacterial pathogens in agro-food industry has led to the development of different phage products already approved by United State Food and Drug Administration (USFDA) and United State Department of Agriculture (USDA). Examples of bacteriophage products approved to have generally recognized as safe (GRAS) status are ListShield produced by Intralytix to kill *Listeria monocytogenes* (approved in 2006), LISTEX produced by Micreos to kill *L. monocytogenes* (approved in 2006) and EcoShield produced by Intralytix to kill *Eschrichia coli* O157:H7 (approved in 2011).

Phages offer advantages as biocontrol agents for several reasons:

They have high specificity to target their host determined by bacterial cell wall receptors, leaving untouched the remaining microbiota. High specificity to hosts is a property that favors phages over other antimicrobials that can cause microbiota collateral damage.

They have self-replication and self-limiting ability, meaning that low or single dosages of phages will multiply as long as there is still a host present, multiplying their overall antimicrobial impact.

As bacteria develop phage defense mechanisms for their survival, phages continuously adapt to these altered host systems.

They have low inherent toxicity since they consist mostly of nucleic acids and proteins.

Phages are relatively cheap and easy to isolate and propagate.

They can generally withstand food processing environmental stresses (including food physiochemical conditions).

They have proved to have prolonged shelf life.

They are reported to lyse hosts at temperatures as low as 1°C [129, 130], limiting growth of pathogenic and spoilage bacteria on refrigerated foods (specially psychrotrophic bacteria); once the foods are taken to room temperature, phages can further control their proliferation [131].

Phages are readily abundant in foods and have been isolated from a wide variety of raw products (e.g., beef, chicken) [132, 133], processed food (e.g., pies, biscuit dough, and roast turkey) [134], fermented products (e.g., cheese, yoghurt) [135], and seafood (e.g., mussels and oysters) [133, 136]. This suggests that phages are daily consumed by humans; therefore, they are considered to be safe for the use in foods. The use of phages to promote food safety can be basically done at four different stages along the food chain (Figure 14). Several bacteriophages have been used to control bacteria in foods [1-4]. *Salmonella* reduction after application of bacteriophages has been demonstrated for cheddar cheese [5], honeydew melon slices [4], mustard seeds [2], chicken frankfurters [6], chicken skin [2], and sprouting mung bean and alfalfa seeds [7].

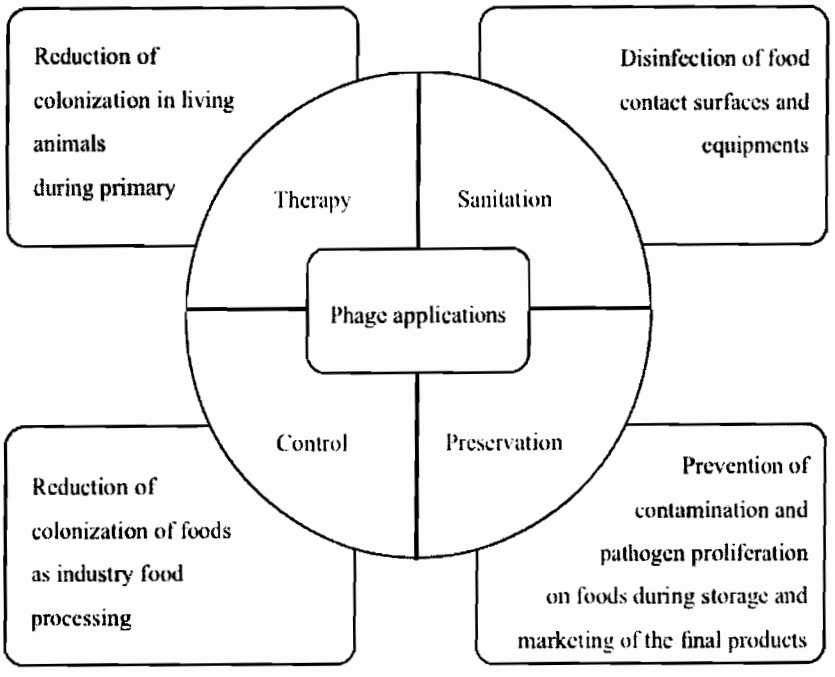

Figure 14 Feasible applications of phages along the food chain towards an increased food safety [adapted from Greer (2005)] [101]

In this study, a lytic bacteriophages specific to *S.* Typhimurium ATCC 13311 was isolated from water collected from a swine lagoon. Although *S.* Typhimurium ATCC 13311 is a clinical strain isolated from faeces of a patient suffering from food poisoning, it is not unusual to find its specific bacteriophage in different place from which the bacterial host was isolated.This is because it has been known for a long time that bacteriophages are wide spread in the environment. They can be found in air, soils, water reservoirs, foods, household and hospital wastewater and so on. Several previous works reported the discovery of bacteriophages from places outside from where their specific hosts exit. Bacteriophage KSP20 and KSP100 specific to *Serratia marcescens* SM8 originally from specimens of patients were isolated from environmental water (river and sewage) [137]. Bacteriophage Aeh1 specific to *Aeromonas hydrophila* A3 originally from frog with red leg disease was isolated from sewage [118] and bacteriophage ϕSA012 specific to *Staphylococcus aureus* originally from mastitic cow's milk was isolated from wastewater treatment plant [138].

Bacteriophage host range is one of the parameters needed to be considered when a bacteriophage is selected to be used as a biocontrol agent. A bacteriophage with a broad host range may not be suitable for biocontrol use because it has a tendency to be virulent to beneficial normal flora residing in human body. Therefore, before use, it has to be ensured that it is safe and cause no side effect relating to the loss of normal flora. On the other hand, a bacteriophage with a narrow inhibitory spectrum sometimes causes limitation in its use. This problem can be overcome by using a cocktail or a combination of several bacteriophages. The bacteriophage isolated in this study had a broad host range. They inhibited not only its specific host, *S.* Typhimurium ATCC 13311, but also *S. dysentariae* (type1) DMST 2137 and *S.* Typhi DMST 5784. Several *Salmonella* bacteriophages were found to possess the same characteristics. Examples of these bacteriophage are bacteriophage AV-05 specific to *E. coli* O157:H7 and *C. freundii* ([139], Bacteriophage SFP1 specific to *S.* Typhimurium, *S.* Enteritidis, *S.* Dublin and *E. coli* O157:H7 [140], Bacteriophage ϕst1 specific to *S.* Typhimurium and *S.* Hadar [141], and *Salmonella* phage PVP-SE1 infecting both *Salmonella* and *E. coli* [142].

The burst size of ST1 was 172 particles per cell, with a latent period of 70 min. Similar large burst size and average latent period were also found in other bacteriophages such as *Salmonella* Enteritidis PT160 phage FGCSSa1 (139 particles per cell/50 min) [143], *Salmonella* Typhi phage STP B (112 particles per cell/20 min) [144], *Shigella boydii* bacteriophage ϕSboM-AG3 (152 particles per cell/ 23 min) [145], *Acinetobacter baumannii* phage AP22 (240 particles per cell/40 min) [146] and *Lactiobacillus plantarum* phage ϕLPN014 (135 particles per cell/ 30 min) [147]. These burst sizes are considered large when compared to *Salmonella* Typhimurium phage ϕst1 (22 particles per cell/ 40 min) [141] and *Salmonella* Enteritidis phage vB_SenS-Ent1 (35 particles per cell/ 25 min) [148]. The variations in latent period and burst size of different phage isolates could be due to the differences in medium, host cell, pH and temperature [149-152].

Several studies have demonstrated that thermal and pH stability of bacteriophages varied depending on types of bacteriophage; therefore, it is of interest to investigate the stability of bacteriophage ST1 in a wide temperature and pH range. These results demonstrate that bacteriophage ST1 is stable in a broad pH range (5-11) and at a

temperature up to 60°C for at least 3 min. Having these characteristics is very useful for designing the most suitable condition for the use of bacteriophage ST1 as a biocontrol agent in foods.

In order to classify bacteriophage ST1, information on its genome and morphology are required. Digestion of the genome of bacteriophage ST1 by the restriction enzyme *Eco*RI suggests that its genome was double stranded DNA. By using TEM, the bacteriophage was found to have an isometric head with a noncontractile tail. According to the International Committee on Taxonomy of Viruses, tailed bacteriophage with double stranded DNA are classified in the the *Caudovirales* order. This order contains three families, namely, the *Myoviridae* (with long, contractile tail), the *Siphoviridae* (with long, noncontractile tail), and the *Podoviridae* (with short tail). Based on its genomic and morphological characteristics, the bacteriophage ST1 was tentatively classified as a member of *Siphoviridae* family. Besides our bacteriophage, several *Salmonella* bacteriophages have been found to be members of the family *Siphoviridae* such as bacteriophage VB_SenS-ENT1 [148], PSPu-95 [153] and SPN3UB [154]. However, *Salmonella* bacteriophage are not restricted to the family *Siphoviridae*, many of them were classified as member of family *Myoviridae* and *Podoviridae* such as ϕ151 (Myophage) [83], ϕFSH19 (Myophage) [155], UAB_Phi20 (Podophage) [156], SPN9CC (Podophage) [157].

Bacteriophage ST1 was stable in soybean milk and jelly grass drink over 24 h of observation period. However, it lost infectivity in guava juice and orange juice within 3 h. Stability of bacteriophages has been reported not only in beverages but also on solid foods. Carlton et al. reported no significant change of *Listeria* bacteriophage P100 titer on the surface of cheese after 6 days [158]. This was also observed for *Salmonella* and *Campylobacter jujeni* bacteriophages on chicken skin for at least 48 h [159].

The decrease of bacteriophage ST1 titer in guava juice (having pH of 2.8) and orange juice (having pH of 3.4) is probably due to a low tolerance of the bacteriophage in acid. This explanation is supported by the results obtained from pH stability study showing the loss of infectivity of bacteriophage ST1 at pH 4 or less. The influence of food acidity on bacteriophage survival was also reported by Leverentz et al. [3, 4]. They observed a rapid decrease of *Listeria* bacteriophage concentrations on apple

slices as well as for *Salmonella* bacteriophage counts on honeydew melon and apple slices. These results suggested that pH of foods is an important factor to be considered for selection of bacteriophages to use as biocontrol agents in foods.

Our results show that the reduction of *S.* Typhimurium in soybean milk by bacteriophage ST 1 was dose dependent. The application of higher phage concentration resulted in greater inactivation. This is in accordance with the results of other studies showing that higher phage concentrations yielded better results of antibacterial activity. Guenther et al. demonstrated that the application of more *Listeria* bacteriophage A511 particles (3×10^8 PFU/g) was more effective than lower doses in the reduction of *L. moncytogenase* WSLC1001 cells on hot dog and cabbage and in chocolate milk [1]. The dose dependent inactivation of bacteria by phage was also found by Carlton et al. who studied the antibacterial activity of *Listeria* bacteriophage P100 against *L. moncytogenase* applied on the surface of soft cheese [158]. Our data suggest that for complete eradication of *S.* Typhimurium, the concentration of bacteriophage ST1 should not be less than 10^8 PFU/ml. However, this number has to be optimized for individual food systems. We found that the concentration of the phage was changed from 10^8 PFU/ml to 5×10^9 PFU/ml when food system was changed from soybean milk to chicken skin (data not shown).

As mentioned in several papers, in order to obtain the same result of bacterial inhibition, more bacteriophage particles are required for solid foods than for liquid foods. This is because in liquid foods bacteriophages can diffuse almost freely and contact with the host cells more easily.

In conclusion, Bacteriophage ST1, a virulent siphophage, was shown to have ability to inactivate *S.* Typhimurium ATCC 13311 both in vitro and in beverage.

In soybean milk, its antibacterial efficiency was dependent on bacteriophaege concentration. Our data suggest that bacteriophage ST1 has a potential for being use as a biocontrol agent in foods. However, at this point, we are just beginning to exploit the potential of the bacteriophage for control bacteria in foods. Since the antibacterial activity of the bacteriophage is dependent on many factors such as contaminated bacterial concentration, food system and temperature, in order to use the bacteriophage effectively, these factors are required to be studied and optimized for each individual condition.

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APPENDICES

APPENDIX A
CULTURE MEDIA

1. CULTURE MEDIA

1.1 Brain Heart Infusion (BHI) Agar 1.5%

Brain Heart Infusion Broth	37.0	g/L
Agar	15.0	g/L

Components are added into distilled water and mixed thoroughly. Medium is sterilized by autoclaving at 121°C for 15 min.

1.2 Brain Heart Infusion (BHI) Sloppy Agar 0.4%

Brain Heart Infusion Broth	37.0	g/L
Agar	4.0	g/L

Components are added into distilled water and mixed thoroughly. Medium is sterilized by autoclaving at 121°C for 15 min.

1.3 Brain Heart Infusion (BHI) broth

Brain Heart Infusion Broth	37.0	g/L

Components are added into distilled water and mixed thoroughly. Medium is sterilized by autoclaving at 121°C for 15 min

1.4 Xylose Lysine Deoxycholate (XLD) Agar

Yeast extract	3.0	g/L
L-Lysine	5.0	g/L
Lactose	7.5	g/L
Sucrose	7.5	g/L
Xylose	3.5	g/L
Sodium chloride	5.0	g/L
Sodium deoxycholate	2.5	g/L
Sodium thiosulphate	6.8	g/L
Ferric ammonium citrate	0.8	g/L
Phenol red	0.08	g/L
Agar	15.0	g/L

Suspend 56.68 grams in 1000 ml distilled water. Heat with frequent agitation until the medium boils. Do not autoclave or overheat. Transfer immediately to a water bath at 50°C. After cooling, pour into sterile Petri plates

APPENDIX B

REAGENTS AND SOLUTIONS

1. REAGENTS AND SOLUTIONS

1.1 Gram's Iodine Solution

Iodine	1.0	g
Potassium iodide	2.0	g
Distilled water	300	ml

Ingredients are grinded in a mortar and dissolved by adding water slowly. The prepared solution is mixed well by stirring.

1.2 Crystal Violet Solution

5 g/L crystal violet is dissolved in sufficient water and the solution is mixed well by stirring.

1.3 Safranin Solution

Safranin	0.25	g
Alcohol	10	ml
Distilled water	100	ml

Safranin is dissolved in the alcohol. Water is added and the solution is filtered through paper.

2. BUFFERS AND STOCK SOLUTIONS

2.1 50 X TAE

242 g of Tris base is dissolved in deionised water. 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) are added. Volume is adjusted to 1000 ml with deionised water.

2.2 1 X TAE

20 ml of 50X TAE buffer is taken and 980 ml of deionised water is added to obtain 1X TAE buffer.

2.3 10X STE Buffer

1 M NaCI

200 mM Tris-HC1 (pH 7 5)

100 mM EDTA

10X Alkaline Buffer (per 50 ml)

3 ml of 5.0 M NaOH

2 ml of 0.5 M EDTA

45 ml of deionized water

2.4 Ethidium Bromide Stock Solution (10 mg/ml)

1 g of ethidium bromide is dissolved in 100 ml of deionised water by stirring on a magnetic stirrer to dissolve the dye completely. Solution is transferred to a dark bottle and stored at room temperature.

2.5 Gel-loading Dye (6X)

2 ml of 10xTBE, 6 ml of glycerol is mixed in a falcon and the volume is adjusted to 20 ml with sterile deionised water. Bromophenol blue is added until the adequate colour is obtained.

2.6 20 % Glycerol Stock

20 ml glycerol and 80 ml Nutrient Broth are autoclaved separately at 121°C for 15 min and then mixed aseptically.

APPENDIX C
PUBLICATION

JOURNAL OF PURE AND APPLIED MICROBIOLOGY, APRIL 2014. Vol. 8[2], p. 1311

Potential of Virulent Bacteriophage as a Biocontrol Agent Against *Salmonella* Typhimurium in Beverages

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(Received: 21 January 2014; accepted: 13 March 2014)

A virulent bacteriophage, specific to pathogenic bacteria *Salmonella* Typhimurium ATCC 13311, was isolated from water collected from a swine lagoon. The bacteriophage, designated ST1, had a broad host range and was stable at the temperature up to 60°C for 3 min and over a wide pH range (5 to 11). Its genome was double stranded DNA. Transmission electron microscopy revealed that the bacteriophage had an isometric head of 70 nm in diameter and a long noncontractile tail of 150 nm long and 7 nm wide. It was classified as a member of the family *Siphoviridae*. Bacteriophage ST1 was stable in soybean milk but not in guava juice and orange juice. When applied together with *S.* Typhimurium (10^6 CFU/ml) in soybean milk, the bacteriophage reduced the bacterial cells with a dose dependent pattern. The application of more bacteriophage (10^8 PFU/ml) was more effective than lower doses (10^6 and 10^7 PFU/ml). This study suggests that bacteriophage ST1 has a potential for being use as a biocontrol agent against *S.* Typhimurium in beverages.

Key words: Bacteriophage, Beverages, Biocontrol, *Salmonella* Typhimurium.

Salmonella are gram negative bacilli belonging to the family Enterobacteriaceae. They are non-spore forming, motile, facultative anaerobes. Based on somatic, flagellar and capsular antigen types, over 2,000 serotypes of *Salmonella* have been classified. Among these, *Salmonella enterica* subsp. *enterica* serovar Typhimurium (hereafter as *S.* Typhimurium) is a common causative agent of foodborne salmonellosis[1]. This disease has become a major global public health threat. Each year, there are an estimated 1.3 billion cases of salmonellosis that contribute towards more than three million deaths worldwide. In Thailand, street beverages such as guava juice, orange juice and soybean milk are frequently associated with *S.* Typhimurium contamination due to mishandling and inadequate hygiene during food preparation distribution and selling. Therefore, they are among most common causes of salmonellosis in Thailand.

Presently, chemical food preservatives are commonly used to reduce or eliminate foodborne pathogens including *S.* Typhimurium contaminated in foods and beverages. However, their health risks have become a major concern of consumers because many health problems related to chemical food preservatives have been reported. Therefore, many research efforts have been made to find natural and safe antimicrobial agents to replace chemical food preservatives. Bacteriophages have received increase attention as natural and safe biocotrol agents because the United State Food and Drug Administration (USFDA) have awarded some bacteriophages the "generally regarded as safe (GRAS)" status for application in foods to control *Listeria monocytogenes* and *Escherichia coli*[2].

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Bacteriophages, the most abundant organisms on earth, are considered as natural antimicrobial agents. Generally, their inhibitory effects are extremely specific to bacterial hosts. This property makes bacteriophages become candidates for use as biocontrol agents in foods because they cause no harm on bacteria necessary in food production, especially in the case of fermented foods, and on beneficial bacteria residing in the body of consumers. A number of research works have been conducted to investigate antimicrobial activity of bacteriophages in foods contaminated with a variety of foodborne pathogens including *Campylobacter jejuni*[1-4], *Escherichia coli* O157:H7[5,6], *Salmonella* spp.[7-12], and *Listeria monocytogenes*[11, 13-17]. *Salmonella* reduction after application of bacteriophages has been observed for many food products including honeydew melon slices[7], cheddar cheese[8], chicken frankfurters[9], mustard seeds[10], chicken skin[10], apple slices[11], sprouting mung bean[12] and alfalfa seeds[12]. These findings suggest the possibility of using bacteriophages as biocontrol agents in foods. Recent advances in research concerning the use of bacteriophages against undesired bacteria in food systems have been summarized by Mahony *et al.*[2].

This study aims to isolate a bacteriophage specific to *Salmonella* Typimurium and to study some of its characteristics including host range, pH and thermal stability, morphology and genome. Moreover, its ability to control *S.* Typhimurium in beverages was also investigated. The bacteriophage from this study may be useful as a biocontrol agent for controlling *S.* Typhimurium in beverages.

MATERIALS AND METHODS

The bacterial strain used as a host strain for the bacteriophage isolation was *Salmonella* Typhimurium ATCC 13311. It was obtained from the DMST Culture Collection, Bangkok, Thailand. The bacterium was cultured in Brain Heart Infusion (BHI) broth at 37°C and kept as glycerol (20% v/v) stock at-20°C until use.

Water samples used as sources of bacteriophage specific to *S.* Typhimurium were collected from swine lagoon in Ubon Ratchathani province, Thailand. For bacteriophage isolation, each water sample of about 10 ml was prepared as follows. The sample was centrifuged at 4,500 xg for 15 min to precipitate cell debris. The supernatant was filtered through a 0.45-im pore-size membrane filter. The filtrate of 4.9 ml and 0.1 ml of log phase *S.* Typhimurium culture were added to 5 ml of double strength BHI (2X BHI) broth. The mixture was mixed thoroughly and incubated at 37°C for 24 h. At the end of incubation period, the suspension was centrifuged at 4,500 xg for 15 min and the supernatant was filtered through a 0.45-im pore-size membrane filter. The resulting filtrate, called tested filtrate, was examined for the presence of bacteriophage activity against its specific host by spot test method. Briefly, a log phase culture of *S.* Typhimurium was mixed gently with BHI sloppy agar (0.4% agar) and plated as a thin top layer on a BHI agar plate (1.2% agar). The plate was left at room temperature for at least 30 min to allow the top agar to solidify. A 10 il aliquot of the tested filtrate was spotted onto the top agar. After incubation at 37°C for 24 h, the presence or absence of a lysis zone was observed and recorded. In all cases, positive tests were confirmed by plaque assay.

Plaque assay

Plaque assay was performed by using the double layer agar plate method as described by Lu *et al.*[18] with some modifications. Briefly, a bacteriophage containing sample was subjected to ten-fold dilution with BHI broth. Each bacteriophage dilution (0.1 ml) along with an equal volume of the log phase host cell (10^8 colony forming unit (CFU)/ml) were added to a tube containing 4.8 ml of sloppy BHI agar (pre-warmed to 50°C). The mixture was mixed thoroughly and overlaid onto the surface of a BHI agar plate. After incubation at 37°C for 24 h, lysis plaques on host bacterial lawn were observed. For calculation of bacteriophage titer, plaques were counted in the plate containing 50-300 plaques and expressed as plaque forming unit per milliliter (PFU/ml).

Bacteriophage purification

For bacteriophage purification, 100 µl of 24 h culture of *S.* Typhimurium and 100 µl of the tested filtrate were added to 4.8 ml of sloppy BHI agar. After mixing, the mixture was poured onto a BHI agar plate. The plate was swirled to ensure that the mixture spread evenly over the plate. After

drying the plate was incubated at 37°C for 24 h. A single plaque was picked from the bacterial lawn and inoculated into the tube containing 100 µl of log phase culture of *S.* Typhimurium. After incubated at 37°C for 24 h, the bacteriophage-host mixture was centrifuged at 4,500 xg for 10 min and filtered through a 0.45-im pore-size membrane filter. The filtrate was subjected to the plaque assay method as mentioned above. Three repeated rounds of single plaque isolation and re-inoculation were performed. The bacteriophage was eluted from the final resulting plate by adding 5 ml of SM buffer (50 mM Tris-HCl, pH 7.5, 99 mM NaCl, 8 mM $MgSO_4$, 0.01% gelatin) on top of the plate and incubated at room temperature for 4 h with shaking. The bacteriophage containing buffer retrieved from the plate was centrifuged at 4,500 xg for 10 min and filtered through a 0.45-im pore-size membrane filter. The resulting filtrate was called bacteriophage suspension.

Host range determination

The bacteriophage host range was determined by using the spot test method, as described above, to examine the lytic activity of the bacteriophage against eighteen bacterial strains listed in Table 1.

Thermal and pH stability test

To determine the thermal stability of bacteriophage, 5 ml of BHI broth was preheated to a desirable temperature, ranging from 50 to 80°C. Then, the bacteriophage suspension was inoculated into each of the preheated BHI broth to obtain a final concentration of 10^6 PFU/ml. After heating at the assigned temperatures for 3 min, the samples were placed in an ice bath. Residual titers of all samples were determined by using the double layer agar plate method.

For the study of pH stability of bacteriophage, the BHI broth was pre-adjusted to a wide range of pH values (pH 2-13) with 1 N HCl and 1 N NaOH. Then, the bacteriophage suspension was inoculated to obtain a final concentration of 10^6 PFU/ml and incubated overnight at 37 °C. The samples were withdrawn at time intervals and the bacteriophage titers were determined using the double layer agar plate method.

Bacteriophage morphology study

The morphology of bacteriophage ST1 was examined by transmission electron microscopy. Bacteriophage particles were fixed by mixing 25 il of the bacteriophage suspension with 25 il of 50% glutaraldehyde in 4% paraformaldeyde. A 5-il aliquot of this mixture was placed on a carbon Formvar-coated copper grid (Prosciteeh, Brisbane, Queenland, Australia) and allowed to adsorb for 5 min at room temperature. The bacteriophage was negatively stained with 2% (w/v) phosphotungstic acid for 1 min then inspected with a JEM-1230 transmission electron microscope (JEOL, Tokyo, Japan) at different magnitudes. The bacteriophage size was determined from the average of five independent measurements.

Bacteriophage genome analysis

Bacteriophage genome was extracted by using PureLink Viral RNA/DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. The purified genome was tested for sensitivity to restriction enzyme *Eco*R1 (Sigma-Aldrich, St. Louis, MO, USA), according to the supplier's recommendations. Electrophoresis of the digested genome was carried out on 0.8% agarose gel. Gel was stained with ethidium bromide and photographed under an UV transilluminator.

Stability of bacteriophage in beverages

Bacteriophage suspension at the titer of 10^8 PFU/ml was diluted 1:100 in 30 ml of 3 different beverages including guava juice (pH 2.8), orangejuice (pH 3.4), and soybean milk (pH 6.7). The beverages were all purchased from a local street food vendor and allowed to adjust to room temperature prior to inoculation. All trials were kept at 37°C throughout the experiment, and samples for plaque assay were withdrawn at 0, 3, 6, 9 and 24 h after bacteriophage inoculation.

Effect of bacteriophage concentration on S. Typhimurium reduction in soybean milk

Four sets of 30 ml of soybean milk were separately inoculated with *S.* Typhimurium at the final concentration of 10^6 CFU/ml. The bacteriophage suspension was added to 3 of 4 sets of soybean milk at different concentrations which were 10^6, 10^7 and 10^8 PFU/ml. The set of soybean milk without bacteriophage was used as a control. All treatments were incubated at 37°C and their samples were collected at 0, 3, 6, 9, and 24 h after bacteriophage inoculation for the determination of bacterial counts. The determination of bacterial concentration in the

beverage was performed by plating on Xylose lysine deoxycholate (XLD) agar, a selective medium for *S.* Typhimurium.

RESULTS

A bacteriophage was isolated from the swine lagoon by the double layer plaque assay using *S.* Typhimurium ATCC 13311 as a host strain. The bacteriophage produced clear plaques on the lawn of the host strain, indicating that it was a virulent (or lytic) bacteriophage. The plaques were small with an average diameter of 1 mm (Fig. 1a), and the isolated bacteriophage was designated ST1.

Specificity of bacteriophage ST1 to the other bacterial strains was examined by the spot test method. Of all 18 bacterial strains used in this experiment, only *Shigella dysentariae* (type1) DMST 2137 and *Salmonella* Typhi DMST 5784 were susceptible to the bacteriophage as shown in Table 1. On the other hand, the rest of the tested bacterial strains used in this study were not sensitive to the bacteriophage. Since the lytic activity of bacteriophage ST1 was not limited only to its specific host *S.* Typhimurium ATCC 13311, it could be considered as a bacteriophage with a board host range.

Table 1. Host range specificity of bacteriophage ST1

Bacteria strain[a]	Spot test[b]
Bacillus cereus ATCC 11778	-
Bacillus subtilis ATCC 6633	-
Enterobacter aerogenes ATCC 13048	-
Escherichia coli ESBL	-
Escherichia coli UBU	-
Klebsiella pneumoniae ATCC 27736	-
Proteus vulgaris ATCC 29905	-
Pseudomonas aeruginosa ATCC 27853	-
Pseudomonas aeruginosa (Imipenem resistant)	-
Salmonella Typhi DMST 22842	-
Salmonella Typhi DMST 5784	+
Shigella dysenteriae ATCC 29026	-
Shigella dysentariae (type1) DMST 2137	+
Staphylococcus aureus UBU	-
Staphylococcus aureus (MRSA)	-
Staphylococcus epidermidis ATCC 12228	-
Vibrio vulnificus DMST 21245	-
Vibrio cholerae non O1 non O139 DMST 2873	-

[a]American Type Culture Collection (ATCC); Department of Management Science and Technology (DMST); Culture Collection of Ubon RatchathaniUniversity (UBU)
[b]+ clear zone; - no clear zone.

Thermal stability of bacteriophage ST1 was investigated by testing its survival under different temperatures (50, 60, 70 and 80°C) for 3 min. No significant change of bacteriophage titer was observed when the bacteriophage was treated at 50 and 60°C. However, the bacteriophage titer was found to drop from about 6 log PFU/ml to about 5 and 4 log PFU/ml after heating the bacteriophage at 70 and 80°C, respectively. No completely elimination of the bacteriophage was observed in all temperature treatments (Table 2).

The pH stability of bacteriophage ST1 was investigated by incubating the bacteriophage for 24 h at pH ranging from 2 to 13. The bacteriophage maintained its infectivity when incubated at pH ranging from 5 to 11. In contrast, the bacteriophage lost its infectivity completely at pH 4 or below as well as at pH 12 or above (Table 2).

The ultrastructure of bacteriophage ST1

Table 2. Stability of bacteriophage ST1 to temperature and pH

Treatment	Initial phage titer (log PFU/ml)	Final phage titer (log PFU/ml)
Heat (for 3 min)		
50°C	6.16	6.14
60°C	6.13	6.13
70°C	6.13	5.09
80°C	6.19	3.98
pH (for 24 h)		
2	6.01	ud
3	6.10	ud
4	6.08	ud
5	6.12	5.32
6	6.14	5.39
7	6.09	5.47
8	6.03	5.71
9	6.02	5.79
10	6.03	5.46
11	5.91	4.94
12	6.01	ud
13	6.06	ud

ud = undetectable

by transmission electron microscopy revealed that the bacteriophage had an isometric head of about 70 nm with a noncontractile tail of 150 nm long and 7 nm wide. No collar and tailed appendages were observed (Fig. 1b)

The genome of bacteriophage ST1 was subjected to restriction analysis by digestion with *Eco*RI and separated by agarose gel electrophoresis. The restriction pattern shown in Fig. 2 clearly demonstrated that the bacteriophage genome was digested by the restriction enzyme *Eco*RI.

The stability of bacteriophage ST1 in beverages was studied by monitoring the survival of the bacteriophage in 3 different beverages (soybean milk, coconut juice and orange juice) after bacteriophage inoculation for 24 h (Fig. 3). In soybean milk, no significant change of bacteriophage titer was found throughout the observation period. On the other hand, the decrease of bacteriophage titer was obviously noticed in guava juice and orange juice. In both beverages, the bacteriophage titer drastically decreased from about 6 PFU/ml to an undetectable level within 3 h.

Since the bacteriophage was stable in soybean milk, the beverage was selected to use in this experiment to determine whether or not the bacteriophage concentration affected the reduction of *S.* Typhimurium in the beverage. The reduction of *S.* Typhimurium in soybean milk was found to be dependent on bacteriophage

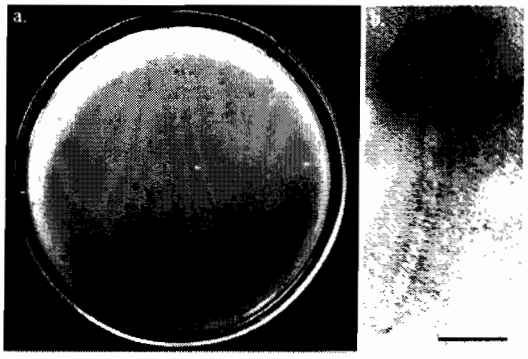

Fig. 1. Plaques on *S.* Typhimurium lawn (a) and transmission electron micrograph of bacteriophage ST1 (b). Bar = 50 nm

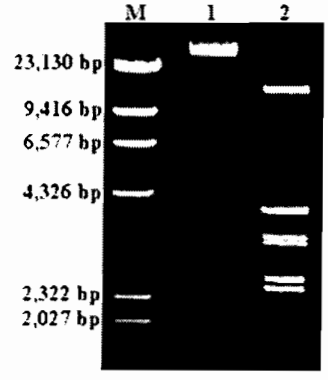

Fig. 2. Agarose gel electrophoresis of bacteriophage ST1 genome. M = λ DNA digested with *Hin*dIII marker, 1 = uncut genome, 2 = cut genome with *Eco*RI

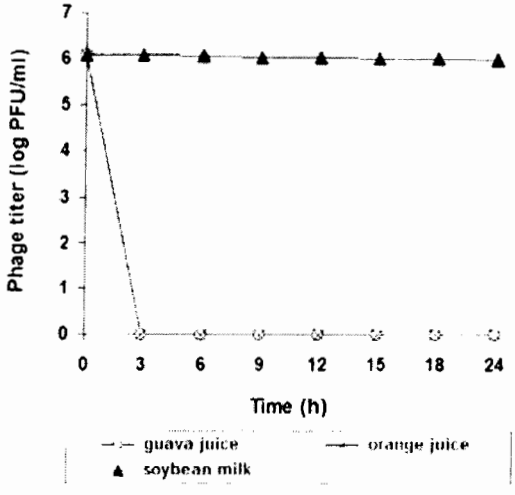

Fig. 3. Stability of bacteriophage ST1 in beverages

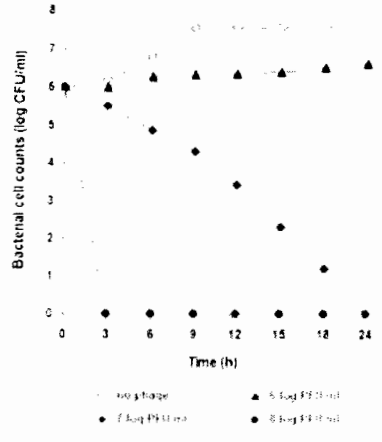

Fig. 4. The reduction of *S.* Typhimurium in soybean milk inoculated with different concentrations (10^6, 10^7 and 10^8 PFU/ml) of bacteriophage ST1

concentration (Fig. 4). Complete elimination of the bacterium was found only when the bacteriophage concentrations used were 10^7 and 10^8 PFU/ml, but not 10^6 PFU/ml. Furthermore, Fig. 4 also shows that the time required for complete elimination of *S.* Typhimurium in soybean milk could be lowered from 24 h to 3 h when the concentration of the bacteriophage was increased from 10^7 PFU/ml to 10^8 PFU/ml.

DISCUSSION

S. Typhimurium is one of the most dangerous microorganisms causing serious intestinal disorders of humans. It can be found in all types of foods such as meats, vegetables, fruits and beverages. The reduction or elimination of contaminated foodborne pathogens including *S.* Typhimurium in foods is commonly achieved by using chemical food preservatives. Concerns on the health risks of chemical preservatives have lead to the search of natural and safe antimicrobial agents. Since some bacteriophages have been approved by the USFDA as safe for use in foods, they are considered to be potential candidates for use as biocontrol agents in foods. Our study demonstrates the usefulness of the virulent bacteriophage ST1 for biocontrol of *Salmonella Typhimurium*in in beverages.

In this study, a virulent bacteriophages specific to *S.* Typhimurium ATCC 13311 was isolated from water collected from a swine lagoon. Although *S.* Typhimurium ATCC 13311 is a clinical strain isolated from faeces of a patient suffering from food poisoning, it is not unusual to find its specific bacteriophage in different place from which the bacterial host was isolated. This is because it has been known for a long time that bacteriophages are wide spread in the environment. They can be found in air, soils, water reservoirs, foods, household and hospital wastewater and so on. Similar finding was reported by McLaughlin and King who isolated a bacteriophage specific to *S.* Typhimurium ATCC 13311 from swine lagoon wastewater[19]. In addition, several bacteriophages were also isolated from places outside from where their specific hosts exist. Examples of such bacteriophages are as follows. Bacteriophage PR04-1 specific to *S.* Typhimurium ATCC 14028 originally from animal tissue was isolated from swine lagoon wastewater[19]. Bacteriophage ÔSA012 specific to *Staphylococcus aureus* originally from raw milk of cows suffering from mastitis was isolated from sewage influent[20]. Bacteriophage Kpn5 specific to *Klebsiella pneumonia* strain B5055, a mouse clinical strain, was isolated from sewage influent[21].

Bacteriophage host range is one of the parameters needed to be considered when a bacteriophage is selected to be used as a biocontrol agent. A bacteriophage with a broad host range may not be suitable for biocontrol use because it has a tendency to be virulent to beneficial normal flora residing in human body. Therefore, before use, it has to be ensured that it is safe and cause no side effect relating to the loss of normal flora. On the other hand, a bacteriophage with a narrow inhibitory spectrum sometimes causes limitation in its use. This problem can be overcome by using a cocktail or a combination of several bacteriophages. The bacteriophage isolated in this study had a broad host range. They inhibited not only its specific host, *S.* Typhimurium ATCC 13311, but also *S. dysentariae* (type1) DMST 2137 and *S.* Typhi DMST 5784. From previous researches, some *Salmonella* bacteriophages possessed a broad host range while others had an extremely narrow host range. Examples of broad host range *Salmonella* bacteriophages are Bacteriophage FGCSSa1[22] and Bacteriophage PPST1[23]. Bacteriophage FGCSSa1 was specific to several species of *Salmonella* including *S.* Typhimurium PT150 NZRM 1891, *S.* Typhimurium LT2, *S.* Typhimurium PT 12A, *S.* Saintpaul NZRM 423, *S.* Enteritidis PT4 NZRM 352, and *S.* Enteritidis PT 9a NZRM 3484[22]. Bacteriophage ÔSPB is an example of *Salmonella* bacteriophage having an extremely narrow host range[24]. Its lytic activity was specific only to *S.* Paratyphi B ATCC 8759.

Several studies have demonstrated that thermal and pH stability of bacteriophages varied depending on types of bacteriophage; therefore, it is of interest to investigate the stability of bacteriophage ST1 in a wide temperature and pH range. These results demonstrate that bacteriophage ST1 is stable in a broad pH range (5-11) and at a temperature up to 60°C for at least 3 min. Having these characteristics is very useful for designing the most suitable condition for the use of bacteriophage ST1 as a biocontrol agent in foods and beverages.

In order to classify bacteriophage ST1, information on its genome and morphology are required. Digestion of the genome of bacteriophage ST1 by the restriction enzyme *Eco*RI suggests that its genome was double stranded DNA. By using TEM, the bacteriophage was found to have an isometric head with a noncontractile tail. According to the International Committee on Taxonomy of Viruses, tailed bacteriophage with double stranded DNA are classified in the the *Caudovirales* order. This order contains three families, namely, the *Myoviridae* (with long, contractile tail), the *Siphoviridae* (with long, noncontractile tail), and the *Podoviridae* (with short tail). Based on its genomic and morphological characteristics, the bacteriophage ST1 was tentatively classified as a member of *Siphoviridae* family. Besides our bacteriophage, several *Salmonella* bacteriophages have been found to be members of the family *Siphoviridae* such as bacteriophage SETP3[25], bacteriophage SE2[26] and bacteriophage SPN3UB[27]. However, *Salmonella* bacteriophages are not restricted to the family *Siphoviridae*, many of them were classified as members in the families *Myoviridae* and *Podoviridae* such as bacteriophage SETP2 (myophage)[25], bacteriophage Felix O1 (myophage) [28], bacteriophage SETP1 (podophage) [25], bacteriophage P22 (podophage) [29], and bacteriophage epsilon 34 (podophage) [30].

Bacteriophage ST1 was stable in soy bean milk and jelly grass drink over 24 h of observation period. However, it lost infectivity in guava juice and orange juice within 3 h. Stability of bacteriophages has been reported not only in beverages but also on solid foods. Carlton *et al.* reported no significant change of *Listeria* bacteriophage P100 titer on the surface of cheese after 6 days[16]. This was also observed for *Salmonella* and *Campylobacter jujeni* bacteriophages on chicken skin for at least 48 h[3].

The decrease of bacteriophage ST1 titer in guava juice (having pH of 2.8) and orange juice (having pH of 3.4) is probably due to a low tolerance of the bacteriophage in acid. This explanation is supported by the results obtained from pH stability study showing the loss of infectivity of bacteriophage ST1 at pH 4 or less. The influence of food acidity on bacteriophage survival was also reported by Leverentz *et al.*[34]. They observed a rapid decrease of *Listeria* bacteriophage concentrations on apple slices as well as for *Salmonella* bacteriophage counts on honeydew melon and apple slices. These results suggested that pH of foods is an important factor to be considered for selection of bacteriophages to use as biocontrol agents in foods.

Our results show that the reduction of *S.* Typhimurium in soybean milk by bacteriophage ST1 was dose dependent. The application of higher phage concentration resulted in greater inactivation. This is in accordance with the results of other studies showing that higher phage concentrations yielded better results of antibacterial activity. Guenther *et al.* demonstrated that the application of more *Listeria* bacteriophage A511 particles (3×10^8 PFU/g) was more effective than lower doses in the reduction of *L. monocytogenase* WSLC1001 cells on hot dog and cabbage and in chocolate milk[17]. The dose dependent inactivation of bacteria by phage was also found by Carlton *et al.*[16] who studied the antibacterial activity of *Listeria* bacteriophage P100 against *L. monocytogenase* applied on the surface of soft cheese. Our data suggest that for complete eradication of *S.* Typhimurium, the concentration of bacteriophage ST1 should not be less than 10^8 PFU/ml. However, this number has to be optimized for individual food systems. Guenther *et al.* [17] mentioned that the successful phage infection and subsequent killing of specific host cells is strongly dependent on environmental conditions and characteristics of foods. For example, in general more bacteriophage particles are required for solid foods than for liquid foods in order to obtain the same result of bacterial inhibition. This is because in liquid foods bacteriophages can diffuse almost freely and contact with the host cells more easily.

In conclusion, Bacteriophage ST1, a virulent siphophage, was shown to have ability to inactivate *S.* Typhimurium ATCC 13311 both in vitro and in beverage. In soy bean milk, its antibacterial efficiency was dependent on bacteriophage concentration. Our data suggest that bacteriophage ST1 has a potential for being use as a biocontrol agent in foods. However, at this point, we are just beginning to exploit the potential of the bacteriophage for control bacteria in foods. Since the antibacterial activity of the bacteriophage is

dependent on many factors such as concentration of contaminated bacteria, type of food, food matrix and temperature, in order to use the bacteriophage effectively, these factors are required to be studied and optimized for each individual condition.

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