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

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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 bacterophages in foods contaminated with a variety of foodborne pathogens including *Campylobacter jejuni*[3,4], *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-ìm 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-ìm 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 ìl 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-ìm 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-ìm 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 ìl of the bacteriophage suspension with 25 ìl of 50% glutaraldehyde in 4% paraformaldeyde. A 5-ìl aliquot of this mixture was placed on a carbon Formvar-coated copper grid (Proscitech, 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*RI (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	Final phage titer
Heat (for 3 min)	(log PFU/ml)	(log PFU/ml)
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

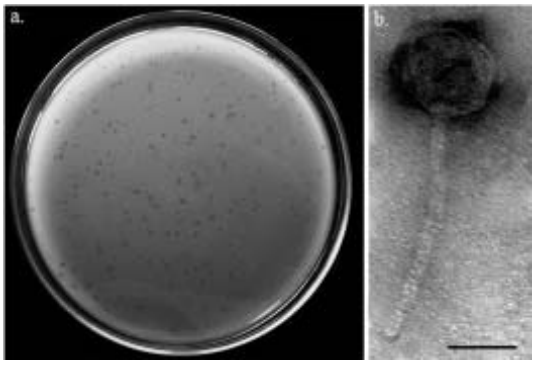

Fig. 1. Plaques on *S.* Typhimurium lawn (a) and transmission electron micrograph of bacteriophage ST1 (b). Bar = 50 nm

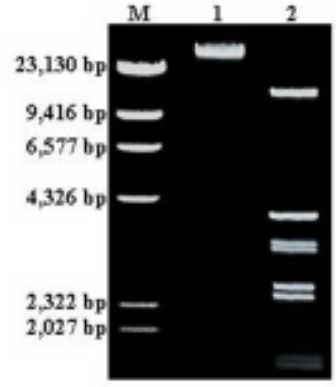

Fig. 2. Agarose gel electrophoresis of bacteriophage ST1 genome. M = » DNA digested with *Hin*dIII marker, 1 = uncut genome, 2 = cut genome with *Eco*RI

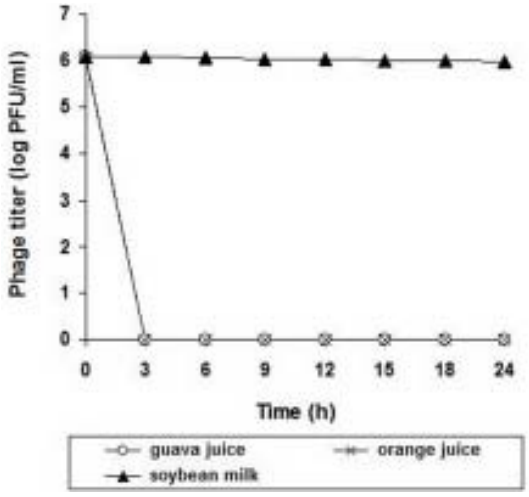

Fig. 3. Stability of bacteriophage ST1 in beverages

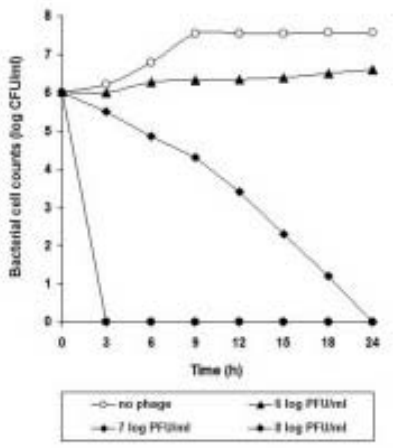

Fig. 4. The reduction of *S.* Typhimurium in soybean milk inoculated with different concentrations (10^6, 10^7 and 10^8 PFU/ml) of bacteriophage ST1

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 ÕSA012 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 ÕSPB 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 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[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.*[7,14]. 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. moncytogenase* 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. moncytogenase* 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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