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Evaluation of the use of encapsulated SE-P47 phage in the control of *Salmonella* in milk

Gamze Koçer Alaşalvar [1, *], Zeliha Yıldırım [2],

[1] *Niğde Ömer Halisdemir University, Bor Vocational School, Food Processing Department, 51240, Niğde Türkiye*
[2] *Niğde Ömer Halisdemir University, Engineering Faculty, Food Engineering Department, 51240, Niğde, Türkiye*

Abstract

This study evaluated the potential of the SE-P47 phage, encapsulated by the extrusion method using alginate, as a biopreservative agent against *Salmonella* Enteritidis in milk. Initially, phage release from capsules was determined in whole (3.5% fat) and semi-skimmed (1.5% fat) UHT milk samples at 25°C. The average phage titers detected in whole milk after 5 and 240 minutes were 6.18 and 6.16 log PFU/g, respectively, with a release rate of 66.78%. In semi-skimmed milk, a phage titer of 7.02 log PFU/g was obtained at 5 minutes, while at 240 minutes, the phage titer reached an average of 7.38 log PFU/g with a 75% release rate. These findings indicate that higher fat content negatively affects phage release. For lytic activity assays, semi-skimmed milk was selected due to its higher release levels. At 4°C, bacterial reductions ranged between 0.45-1.22 log CFU/mL at MOI 1000 and 0.91-1.18 log CFU/mL at MOI 100000. At 25°C, bacterial counts decreased between 2.01 and 3.27 log CFU/mL at MOI 100000, while a higher reduction of 4.03 log CFU/mL was achieved at MOI 1000 after 12 hours. Greater lytic activity at 25°C compared to 4°C was attributed to enhanced phage replication promoted by bacterial growth. In conclusion, the encapsulated phage was shown to exhibit significant inhibitory activity against *S.* Enteritidis in milk. The existing lytic efficacy has the potential to be enhanced through studies aimed at improving phage release in milk or other food systems.

Keywords: Bacteriophage, Biopreservative, Encapsulation, Milk, *Salmonella* Enteritidis.

1 Introduction

Bacteriophages, or phages, are bacteria-specific viruses that can infect bacteria. Each phage recognizes and binds to particular bacterial species or even strains. Lytic phages cause lysis and subsequent death of their bacterial hosts. Due to their high specificity and ability to selectively target pathogenic or spoilage microorganisms without affecting beneficial microflora, phages offer significant potential for application in food safety and preservation [1, 2].

The phage-based approaches are becoming increasingly popular as a strategy to combat pathogenic bacteria in the food sector. Phages have demonstrated efficacy in controlling pathogens across diverse food systems, including meat and poultry, dairy products, fresh produce, and convenience foods [3, 4]. However, there are several challenges regarding the ability of phages to maintain their effectiveness within the food matrix. The complex composition of the food matrix (e.g., pH, fat, salt, and protein content), fluctuations in temperature, and processing treatments such as pasteurization may adversely affect phage viability and stability. Encapsulation technology can be used to eliminate these negativities [5]. Phage encapsulation refers to the entrapment of phage particles within a matrix. The advantages of encapsulation include prolonging phage shelf life, enhancing resistance to various physicochemical conditions (e.g., pH, temperature, salt), enabling controlled and sustained release within the food matrix, expanding the potential applications of phages, and providing ease of use, transport, and storage [6, 7].

At present, various methods are available for the encapsulation of different active compounds. The extrusion method is an ionotropic gelation-based technique frequently employed for phage encapsulation. This method relies on the principle of forcing a mixture of phage suspension and a polymeric matrix (e.g., alginate, carrageenan, chitosan, and whey protein) through a nozzle/orifice of defined diameter. As a result of this process, small beads (micro- or macro-capsules) are formed [8–10]. Encapsulated phages can be applied to food surfaces, mixed into foods, or integrated into food packaging as biopreservatives [11–14]. However, research remains limited regarding the use of phage encapsulated by various techniques as biopreservatives in foods. In particular, liquid products such as milk serve as an ideal model for such applications due to their suitability for homogeneous distribution. Moreover, milk possesses high water activity, rich nutrient content, and an almost neutral pH, all of which provide favourable conditions for microbial growth. Therefore, the use of encapsulated phages in skimmed milk has been explored as a biocontrol strategy against a variety of pathogens, including *Salmonella* spp., *Escherichia coli*, *Listeria monocytogenes*, and spoilage microorganisms such as *Pseudomonas fluorescens* [11, 12].

This study aims to examine the release and evaluate the efficacy as a bioprotective agent of the *S.* Enteritidis-specific phage SE-P47, encapsulated in alginate coating material via

* Corresponding author, e-mail: gkoceralasalvar@ohu.edu.tr (G. Koçer Alaşalvar)
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the extrusion method, when applied in milk. Our study represents the first attempt to evaluate the use of encapsulated SE-P47 phage in a food system, employing both whole and semi-skimmed milk as model food matrices.

2 Material and methods

2.1 Preparation of microbial cultures

The *S.* Enteritidis-specific SE-P47 phage and its host bacterium, *S.* Enteritidis METU-S1-411 were achieved from the culture collection of the Department of Food Engineering, Niğde Ömer Halisdemir University. The SE-P47 phage was isolated and characterized in our previous studies. It was determined to have lytic activity against *S.* Enteritidis, *S.* Typhimurium, and some other *S. enterica* serovars [15, 16]. Brain Heart Infusion (BHI) (LabM, Lancashire, England) and Luria-Bertani (LB) medium (LabM, Lancashire, England) were used for the development of bacterial cultures and phage, respectively.

2.2 Production and concentration of high-titer phage suspension

High concentration phage suspension was prepared to be used in the encapsulation process. Briefly, 4 mL of molten LB soft agar (0.7%, w/v agar) supplemented with 100 µL of phage stock and 300 µL of active host culture were mixed thoroughly (Reax top, Heidolph, Germany) and spread into sterile Petri dishes. Following agar solidification, incubation was conducted at 37 °C for 24 h to allow bacterial growth. Phages were recovered by adding 2 mL of sodium chloride–magnesium sulfate buffer (SM, 50 mM Tris-HCl, 99 mM NaCl, 8 mM $MgSO_4$, 0.01% (w/v) gelatin, pH 7.5) to each plate, gently scraping the soft agar layer, and collecting into sterile centrifuge tubes. The suspension was shaken at 25°C and 120 rpm for 2 h, after which chloroform (50 µL/mL) was added. Afterward, the samples were centrifuged at 7000 × g for 15 min at 4°C, and the resulting supernatants were passed through sterile 0.45 µm filters to eliminate remaining bacterial residues [16]. The high-titer phage suspension was stored at 4°C.

2.3 Phage encapsulation using extrusion technique

SE-P47 phage was encapsulated using the extrusion technique under optimum conditions determined in our previous study [17]. Firstly, a sodium alginate solution prepared at a concentration of 1.5% (w/v) was sterilized at 121°C for 15 min. Under aseptic conditions, the alginate solution was mixed with the phage suspension (initial titer 10^{10} PFU/mL) at a phage-to-alginate ratio of 1:2 and homogenized at 1000 rpm for 5 min. The resulting mixture was extruded through a syringe needle (26 G, 0.45 × 13 mm) into a 0.125 M calcium chloride solution ($CaCl_2$/alginate ratio 1:4) via a peristaltic pump (BT600-2J, Longer Pump, China) and droplet control set. Droplet formation was carried out from a height of 17 cm at a flow rate of 0.75 mL/min. The $CaCl_2$ solution was continuously stirred at 200 rpm during bead formation and stirred for an additional 30 min to ensure gelation after extrusion was completed. The obtained alginate beads were separated using Whatman No. 4 filter paper, washed with sterile distilled water, and transferred to sterile Petri dishes. The beads were stored at 4°C for 24 h and then allowed to air-dry for 1 h inside a laminar flow cabinet.

2.4 Release of encapsulated bacteriophages in milk

To investigate phage release from alginate beads, ultra-high temperature (UHT) sterilized whole milk (3.5% fat) and semi-skimmed milk (1.5% fat) (İçim, Ak Gıda, Istanbul) purchased from the markets in Niğde, Turkey were used. In the experiments, 0.1 g of alginate beads were added to tubes containing 0.9 mL of milk and incubated at 37°C with constant agitation (100 rpm) for up to 240 min. During incubation, samples were collected at 5, 15, 30, 60, 90, 120, 150, 180, 210, and 240 min intervals, and phage titers were determined by the double-layer agar method. For this, 100 µL aliquots from serially diluted phage samples were mixed with 300 µL of host culture in molten LB soft agar (45-50°C) and poured over solidified LB agar plates. After incubation at 37°C for 24 h, plaques were counted, and phage titers were expressed as plaque-forming units (PFU) [18]. In the control group, phage-containing capsules were dissolved in microsphere broken solution (MBS, 50 mM sodium citrate, 0.2 M sodium bicarbonate, 50 mM Tris-HCl, pH 7.5), and titers were determined using the same procedure.

2.5 Determination of lytic activity of encapsulated phage against S. Enteritidis in milk

The lytic activity of encapsulated SE-P47 phage against *S.* Enteritidis METU-S1-411 was assessed in semi-skimmed UHT milk (depending on phage release results). The experiments were conducted at two different multiplicities of infection (MOI) 1000 and 100000. For this purpose, 1 g of encapsulated phage (10^8 log PFU/g) was added to 5 mL of milk inoculated with *S.* Enteritidis and mixed at room temperature. Milk samples containing *S.* Enteritidis without encapsulated phage served as positive control, while those containing neither *S.* Enteritidis nor phage served as the negative control. All samples were incubated separately at 4°C and 25°C for 24 h. At 0, 1, 4, 8, 12, and 24 h, bacterial counts were determined using Rappaport Vassiliadis Soy selective medium. Following incubation at 37 °C for 24 h, the number of colonies was enumerated, and the data were given as colony forming units (CFU) [5].

2.6 Statistical analysis

Statistical analyses of phage release and lytic activity data were performed using MINITAB 17 software. Analysis of variance (ANOVA) was employed to determine significant differences among mean values. Tukey's test was applied for multiple comparisons. All statistical evaluations were performed at a 95% confidence level ($p<0.05$).

3 Results and discussions

3.1 Release of bacteriophages from capsules in milk

The number of phages released from alginate capsules into the milk matrix and converted to the free form is a critical factor for exhibiting lytic activity against the target pathogenic bacteria. Therefore, in this study, the time-dependent release profile of the encapsulated SE-P47 phage from alginate beads was investigated in full-fat and semi-

skimmed UHT milk at 25°C (Figure 1). The titer of the encapsulated SE-P47 phage, determined by dissolving the beads in MBS, was found to be 9.84 log PFU/g on average and was considered as the control group.

In whole milk samples, phage titers obtained after 5 and 240 minutes were 6.18 and 6.16 log PFU/g, corresponding to a 66.78% release. In semi-skimmed milk, 5 minutes of incubation resulted in a 71.31% release (7.02 log PFU/g), while after 240 minutes, the release reached 75%, with an average phage titer of 7.38 log PFU/g. The increase observed at 240 minutes was found to be statistically significant compared to other time points ($p<0.05$). These findings indicate that as the fat content of milk increases, the rate of phage release decreases.

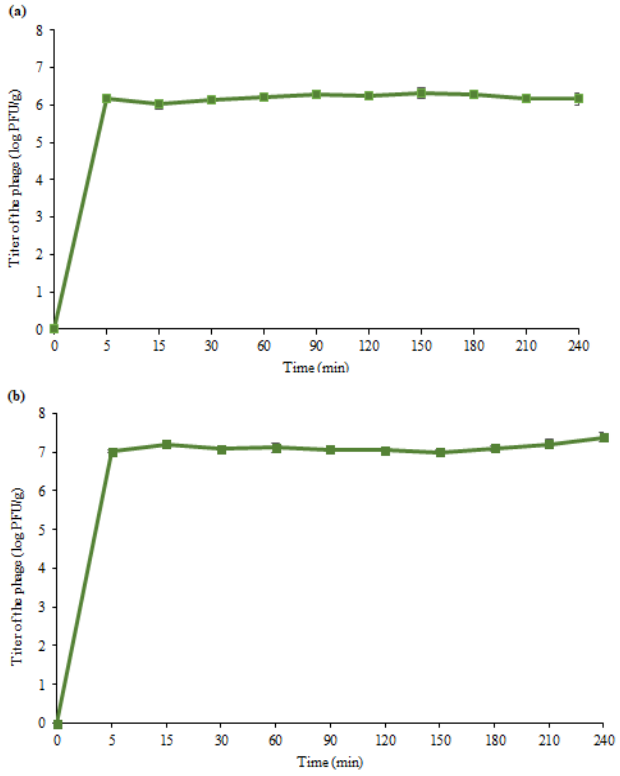

Figure 1. Phage release from alginate beads in whole (a) and semi-skimmed (b) milk (25°C)

Encapsulation plays an essential role in maintaining phage viability until they reach the target pathogen. However, for bacteriophages to function effectively as bioprotective agents, they must be released from the capsules into the food matrix, remain viable, and successfully infect the target bacteria. In our study, high phage viability and stable release were observed during the 4-h incubation period, suggesting that alginate beads provide a suitable environment for phage stability. Schubert et al. [19] reported that *E. coli* K5 phage, which was encapsulated using a spray-drying method with a mixture of sodium alginate, milk protein, succinic acid and calcium hydrogen phosphate dihydrate, failed to maintain its stability when applied to both skimmed (0.3% fat) and whole (3.5% fat) milk samples. In a study evaluating the stability of probiotic bacteria loaded into alginate microcapsules in milk at 4°C [20], a 100-fold decrease in the viability of *Bifidobacterium longum* Bb-46 occurred after 12 days, while *B. lactis* Bb-12 retained its activity.

Overall, studies investigating the survival or release behaviour of encapsulated phages in milk and other food matrices remain limited, with most existing research focusing on encapsulated probiotic microorganisms. The findings in our study, contribute valuable insight into the release dynamics of encapsulated phages in milk systems and highlight the potential of alginate encapsulation for maintaining phage stability and functionality as bioprotective agents in dairy products.

3.2 Lytic activity of encapsulated bacteriophage against S. Enteritidis in milk

Salmonella is among the major foodborne pathogen posing a significant risk to public health through milk and dairy products [21]. Therefore, demonstrating the applicability of the encapsulated phage in the control of *Salmonella* in milk is of great importance. Semi-skimmed milk was selected for assessing the antibacterial effect of the encapsulated SE-P47 phage against *S.* Enteritidis, since a higher release rate had previously been observed in this matrix (Figure 1b.). During storage, the phage titers determined in milk samples inoculated with *S.* Enteritidis and treated with encapsulated phages were 7.90 and 8.46 log PFU/g at 4°C and 25°C, respectively. The higher phage viability observed in milk samples compared to those in the release study is attributed to phage replication resulting from the presence of its host bacterium.

The changes in bacterial counts at MOI of 1000 and 100000 over a 24-hour period at different temperatures are presented in Figures 2 and 3. At 4°C, the reductions in bacterial counts ranged between 0.45-1.22 log CFU/mL for MOI 1000 and 0.91-1.18 log CFU/mL for MOI 100000. At 25°C, reductions of 0.85-4.03 log CFU/mL and 2.01-3.27 log CFU/mL were observed for MOI 1000 and 100000, respectively. In all cases, a statistically significant difference was observed in the number of *Salmonella* in phage-treated samples compared to the control samples ($p<0.05$).

The lytic activity of encapsulated phages in milk was higher at 25°C than at 4°C for both MOI values. This observation aligns with previous studies reporting reduced antimicrobial performance of phages at refrigeration temperatures. In the study of Choi et al. [12], T4 phage encapsulated with maltodextrin and trehalose using a spray dryer achieved a 4.66 log reduction in *E. coli* K12 artificially inoculated into skim milk after 24 h of storage at 25°C. At the lower storage temperature of 4°C, the bacterial inactivation efficiency of the phage microcapsules was reduced compared to that observed at 25°C. Similarly, researchers evaluating liquid phage formulations in milk, sausage, and lettuce observed lower phage activity at 4°C

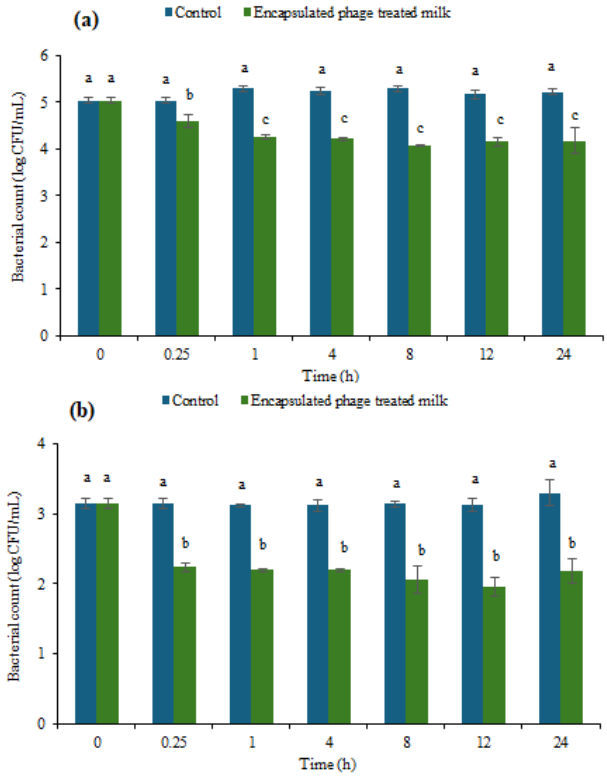

Figure 2. Lytic activity of encapsulated phage against *S.* Enteritidis in milk at MOI of 1000 (a) and 100000 (b) at 4°C. [a-c]Different letters indicates significant ($p<0.05$) differences between samples.

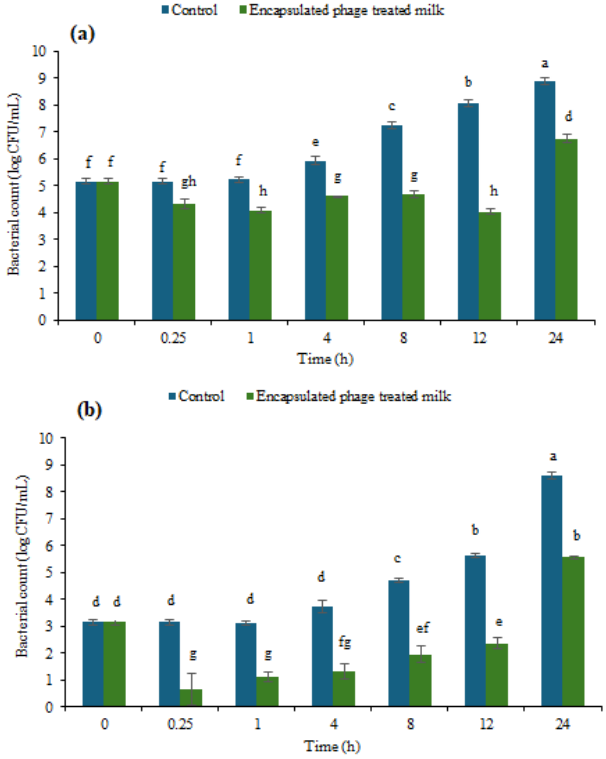

Figure 3. Lytic activity of encapsulated phage against *S.* Enteritidis in milk at MOI of 1000 (a) and 100000 (b) at 25°C. [a-h]Different letters indicates significant ($p<0.05$) differences between samples.

compared to 28°C [22]. Generally, low temperatures are known to decrease phage-induced bacterial lysis rates and extend the latent period primarily due to suppressed bacterial metabolism and growth under such conditions [12]. Since phage activity depends on metabolically active host cells, the restriction of bacterial growth at lower temperatures consequently leads to reduced phage replication and lytic performance.

In a study evaluating the antibacterial effect of the encapsulated phage formulation, phage-containing powders were applied by sprinkling onto raw chicken meat pieces and sunflower sprouts that had been inoculated with *S.* Enteritidis or *S.* Typhimurium at a level of 10^5 log CFU (MOI 100). The food samples were preserved at 4°C for 4 days. While the bacterial counts in untreated samples (control) continued to increase during storage, chickens treated with encapsulated phage resulted in bacterial reductions of 0.57 log CFU/cm² for *S.* Enteritidis and 1.78 log CFU/cm² for *S.* Typhimurium after 4 days of storage. In sunflower sprouts, reductions of 0.86 and 1.2 log CFU/g for *S.* Enteritidis and *S.* Typhimurium were detected, respectively [5]. However, the inhibitory effect of the powdered phage formulation against *S.* Enteritidis after 4 days was less pronounced than that observed in our study after 1 day of storage at the same temperature (MOI 1000). The higher inhibition observed in our study is attributed to the strong lytic activity of the phage used and the higher MOI value applied.

In the study of Li et al. [11], *Salmonella*-specific phage T156, encapsulated via the extrusion technique, was applied to skim milk and lettuce samples inoculated with *S.* Typhimurium and subsequently incubated for 12 hours at various temperatures. In the milk samples, application of the phage at an MOI of 1000 resulted in reductions of 0.65 and 4.15 log CFU/mL in *S.* Typhimurium counts at 4°C and 25°C, respectively. Increasing the MOI to 10000 resulted in greater reductions, with decreases of 0.91 and 4.73 log CFU/mL at 4°C and 25°C, respectively. In lettuce, a similar trend was observed, with decreases of 0.52 and 2.49 log CFU/mL at an MOI of 1000 and 0.74 and 3.18 log CFU/mL at an MOI of 10000 at 4°C and 25°C, respectively. Although the microencapsulated phages in this study were applied after dissolving the MBS and resuspending them in SM buffer, the bacterial log reduction achieved in skimmed milk at 4°C was lower than that observed in our study.

In our study, the semi-skimmed milk used as a model food matrix differs from those employed in previous research. Although the findings obtained are generally comparable to earlier studies, variations in the results are expected depending on factors such as the specific phage used, encapsulation conditions, and the characteristics of the food sample. At 4°C, the reductions in bacterial counts observed in semi-skimmed milk treated with encapsulated phages were found to be like or higher than those reported in studies using skim milk; however, the reductions observed at 25°C were relatively lower compared to previous findings

[11, 12]. Although the nutritional components of foods (proteins, lipids, sugars, and inorganic salts) provide a suitable environment for bacterial growth, the lipid fraction may negatively affect phage infection by interfering with or limiting interactions between bacteria and phages [21, 23]. Phage adsorption, the initial step of the lytic cycle, is likely impeded by milk components that induce agglutination of bacterial cells [24]. Therefore, the ability of phages to disperse throughout the food matrix plays a crucial role in determining their lytic activity. In the present study, the presence of fat globules in semi-skimmed milk is considered to reduce the efficiency of phage infection. This observation is consistent with the findings indicating that high fat content in milk adversely affects the release of phages from the encapsulating matrix.

4 Conclusions

In this study, the release profile and antibacterial activity of *S.* Enteritidis-specific phage SE-P47 encapsulated within alginate beads in milk were evaluated. The results demonstrated that the phage exhibited higher rates of release in semi-skimmed milk compared to whole milk, resulting in more effective lytic activity. Furthermore, the antibacterial efficacy of the encapsulated phage was found to be temperature-dependent, and the metabolic activity of the host cells supported phage lytic activity, leading to more pronounced lysis at 25°C. As a result, the encapsulation technology enabled high release and survival of the SE-P47 phage in milk. Furthermore, the application of encapsulated phage has been shown to have strong potential for use in biocontrol interventions in food matrices susceptible to microbial growth, such as milk.

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Conflict of interest

The authors declare that there is no conflict of interest.

Similarity rate (iThenticate): 13%

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