Electronic Journal of Biotechnology 31 (2018) 57–60

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Short communication

# Encapsulation of specific *Salmonella* Enteritidis phage f3αSE on alginate-spheres as a method for protection and dosification

María José Soto [a], Julio Retamales [a], Humberto Palza [b], Roberto Bastías [a,*]

[a] Laboratorio de Microbiología, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile
[b] Departamento de Ingeniería Química y Biotecnología, Facultad de Ciencias Físicas y Matemáticas, Universidad de Chile, Santiago, Chile

ARTICLE INFO

*Article history:*
Received 30 June 2017
Accepted 17 November 2017
Available online 24 November 2017

*Keywords:*
Alginate encapsulation
Alginate
Bacteriophage encapsulation
Bacteriophage therapy
Bacteriophage
Flow
Hydrogel polymers
Phage protection
Phage survival
Phages
Sphere

ABSTRACT

*Background:* Bacteriophages have been proposed as an alternative to control pathogenic bacteria resistant to antibiotics. However, they are not extensively used due to different factors such as vulnerability under environmental conditions and the lack of efficient administration methods. A potential solution is the encapsulation of bacteriophages in hydrogel polymers to increase their viability and as a controlled release method. This work describes the use of alginate-$Ca^{+2}$ matrixes as mechanisms for protection and dosification of the phage f3αSE which has been successfully used to prevent infections produced by *Salmonella* Enteritidis.
*Results:* The viability of the pure phage is reduced in near 100% after 1-h incubation at pH 2 or 3. However, the encapsulated phage remains active in 80, 6% at pH 3, while no differences were observed at pH 2, 4 or 7. Exposition of f3αSE to different T° showed that the viability of this phage decreased with increased T° to near 15% at 60°C, while the encapsulated phage remains with 50% viability at same temperature. Finally, the encapsulation of phages showed to extend their presence for 100 h in the medium compared to non-encapsulated phages in a water flow system, which simulate automatic birdbath used in poultry industry, maintaining the phage concentration between $10^2$ and $10^4$ PFU/mL during 250 h.
*Conclusions:* Encapsulation in alginate-$Ca^{+2}$ spheres can be a good alternative to extend viability of phages and can be used as a phage method dosification method in water flow systems.



## 1. Introduction

*Salmonella enterica* subspecies *enterica* serotype Enteritidis (*S.* Enteritidis) is a bacterium frequently identified in human outbreak food-borne diseases associated with the consumption of contaminated avian meat or eggs [1]. In poultry farms, drinking water can be a major contamination source of *Salmonella* and generates continuous reinfection despite the disinfection processes that have been established [2]. Moreover, the emergence of antibiotic-resistant strains of *S.* Enteritidis promotes the development of new prevention and/or control strategies to reduce spread of this pathogen during pre-harvest production stage [3].

Bacteriophages or phages are viruses that infect bacteria and are the most abundant biological entities on Earth [4]. Lytic phages have been proposed as a potential therapeutic tool to control pathogenic bacteria because of the ability to kill its cell host. Besides, they can be very specific therefore they will not affect the normal microbiota, and are self-replicating at the site of infection then smaller or limited doses respect to conventional treatments may be required. Finally, they are effective against antibiotic resistant bacteria; however, they will not infect eukaryotic cells thus are harmless to humans and animals [5,6].

Several *Salmonella* phages with potential to control *S.* Enteritidis infections in poultry have been described [7,8,9]. Santander and Robeson [10] characterized three dsDNA phages against *S.* Enteritidis, and demonstrated that the phage f3αSE protects against the infection of this pathogen in a *Caenorhabditis elegans* model. Later Borie et al. [11] evaluated the potential of this phage to control infection produced by *S.* Enteritidis in poultry administrating the phage through both spray and drinking water. These results proved the potential of the phage f3αSE to control this pathogenic bacteria, however, raised the question about the viability of this phage to different environmental conditions and potential administration methods.

Increase viability of phages in the environment and develop optimal administration methods are key to effective phage treatment [12,13]. In this vein, the phages encapsulated with polysaccharide hydrogels has been proposed as an alternative [3,14]. The hydrogels generates a protective barrier depending on pH, further allowing a controlled release of phages. In this work we present the encapsulation of the phage f3αSE in alginate-$Ca^{+2}$ spheres showing the potential of this technique to increase the viability of this phage, and as a potential

* Corresponding author.
*E-mail address:* roberto.bastias@pucv.cl (R. Bastías).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2017.11.006

dosification method in water flow systems that simulate automatic birdbath used in poultry industry. This work contributes in the exploration of efficient methods for administration of phages in the context of phage therapy.

## 2. Material and methods

### 2.1. Bacteria and phage production

The phage f3αSE together with the bacterial host *Salmonella enterica* subsp. *enterica* ATCC 13076 (Thereafter *Salmonella* Enteritidis) were obtained from the personal collection of Dr. James Robeson (Microbiology Laboratory, PUCV). A bacterial culture in the early exponential phase (~$1 \times 10^7$ CFU/mL) grown in broth Luria-Bertini (LB) (10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl) at 37°C. For phage propagation, cultures were infected at a multiplicity of infection (MOI) of 0.1 and incubated overnight with shaking at 37°C. Subsequently, the culture was centrifuged at 10.000 rpm for 10 min, and the supernatant obtained was filtered (0.22 μm filter) and stored at 4°C. The phages were quantified by the standard double layer agar assay [15].

### 2.2. Phage encapsulation

To prepare encapsulated phages, a 2% solution of Sodium alginate (LOBA Chemie) was mixed with the f3αSE phage lysate and shaken for 15 min inside a laminar flow chamber. The mixture was extruded through a 20-mL syringe to a solution of 1.5% $CaCl_2$ and then shaken for 30 min until the alginate-$Ca^{+2}$ spheres were formed. After that, the spheres were removed and washed in deionized water and dried inside a laminar flow chamber. Each assay was performed with 1 g of alginate-$Ca^{+2}$ spheres which correspond to ~$1 \times 10^6$ phages. For tittering encapsulated phages, the spheres were dissolved in MBS medium according the procedure described by Ma et al. [3]. After that, phages were tittered using the standard double layer agar assay [15]. Stability of alginate-$Ca^{+2}$ spheres without phages was also evaluated.

### 2.3. pH and temperature assay

To determine the potential protective effect of alginate-$Ca^{+2}$ spheres over the phage f3αSE against different pHs, 100 μL of non-encapsulated phage at a concentration of $1 \times 10^7$ PFU/mL was added to 0.9 mL of saline solution (0.85% NaCl) equilibrated at the corresponding pHs (pH 2–7). Simultaneously, 1 g of alginate-$Ca^{+2}$ spheres equivalent to same amount of non-encapsulated phage was added to 10 mL of saline solution equilibrated to same pHs conditions. Samples were incubated for 1 h at 25°C in the corresponding solution. Same procedure was used to evaluate the protective effect of encapsulation against different temperatures, but in this case the saline solution was equilibrated at pH 7 and incubated at the corresponding temperatures (25, 40, 50 and 60°C). The percentage of viability of phages was determined comparing the titers of viable phages after incubation in each condition with the titers before the incubation in each condition. After the treatments the spheres were dissolved in MBS medium according the procedure described by Ma et al. [3], and then, the remaining viable phages were tittered by standard double layer agar assay [15].

### 2.4. Water flow system assay

A water flow system was designed to evaluate the encapsulation of phage f3αSE in alginate-$Ca^{+2}$ spheres as a potential method of dosification. The system was designed to resemble automatic birdbath present in poultry industry where a controlled and mechanized water flow system provides permanent water to birds. For this purpose, a separatory funnel of 250 mL was connected to a birdbath through a regulated flow hose. Each separatory funnel contained 200 mL of water, and the water flow was adjusted to 100 mL/d. After each sampling, same amount of fresh water was added to the funnel to refill the system. In this way, the alginate-$Ca^{+2}$ spheres were exposed to a permanent water flow resembling the automatized systems of the poultry industry. To evaluate the persistence of non-encapsulated phages in this system, 200 μL of phage f3αSE at a concentration of $1 \times 10^9$ UFP/mL was added for unique time to the 200 mL of water in the separatory funnel, which correspond to a total of $10^8$ phages in the system. Similarly, to evaluate the release of encapsulated phages in this system, 2 g of alginate-$Ca^{+2}$ spheres, equivalent to the same total amount of non-encapsulated phage, were added to the separatory funnel for unique time. In both cases samples of 300 μL were collected every 2 h for 1 d; afterwards, samples were collected every 24 h. All samples were collected from the birdbath and then tittered by the standard double layer agar assay [15]. Stability of alginate-$Ca^{+2}$ spheres without phages was also evaluated. Each experiment was performed by independent triplicates.

## 3. Results and discussion

Phages have been proposed as an alternative to control different pathogenic bacteria including *Salmonella* [4]. Some of the problems regarding this technology are the liability of phages to environmental conditions and the need to explore efficient methods for administration. Here, we show the encapsulation of the phage f3αSE in alginate-$Ca^{+2}$ spheres as a method of protection and dosification.

Phages were encapsulated at a concentration of $10^6$ PFU/g of alginate-$Ca^{+2}$ spheres having diameters 2.5–3 mm, and for each assay, 1 g of spheres was used (Fig. 1a). Alginate-$Ca^{+2}$ spheres were stable for several weeks in solution with and without phages, suggesting that phages do not alter the stability of spheres. Encapsulated phages and non-encapsulated phages were exposed for 1 h to different T° and pH (Fig. 1b and c). The results show the effect of increasing the temperature on the reduction of phage viability in agreement with general behavior of bacteriophages exposed to increased temperatures [16]. Noteworthy, although at low temperatures there is no differences between pure and encapsulated phages, at 60°C encapsulated phages remain more stable than non-encapsulated phages maintaining 50% of viable phages while pure phage has only 15% (Fig. 1b). To our knowledge, this is the first report about increasing phage resistance to temperature by encapsulation.

When the non-encapsulated phages were exposed to different pH for 1 h (Fig. 1c) the percentage of viable phages remains stable at pH 7 and 4. However at pH 3, only 1% of phages remain active, and at pH 2, no phage were detected (detection limit 50 PFU/mL). In the case of encapsulated phages, the percentage of viable phages remains stable at pH 4 and 7, similar to non-encapsulated phages. However, 80.6% of encapsulated phages remain viable at pH 3; although at pH 2, encapsulation did not offer protection. As in previous reports with different phages [14,16], these results show that phage f3αSE is very sensitive to very acidic pHs but alginate encapsulation could be a good alternative to increase the phage resistance to pHs as low as 3.

To evaluate the phage release from the alginate-$Ca^{+2}$ spheres, we designed a flow water system resembling birdbath used in poultry industry of 200 mL with a flow rate of 100 mL/d (Material and methods) at pH 7 and room temperature. This will allow us to determine if alginate-$Ca^{+2}$ spheres can extend the presence of phages in the water comparing to a unique dose of phages. The bacteriophage f3αSE non-encapsulated was initially added to a final amount of $1 \times 10^8$ total phages in the system. The results showed that concentration of phages was decreasing rapidly from $5 \times 10^6$ PFU/mL during the first hours to undetectable levels after 150 h (detection limit 50 PFU/mL) probably due to water exchange. On the contrary, when encapsulated phages were added in the same total amount to the system (2 g of alginate-$Ca^{+2}$ spheres), the concentration of phages in the water presents an increase during the first hours from $3.5 \times 10^2$

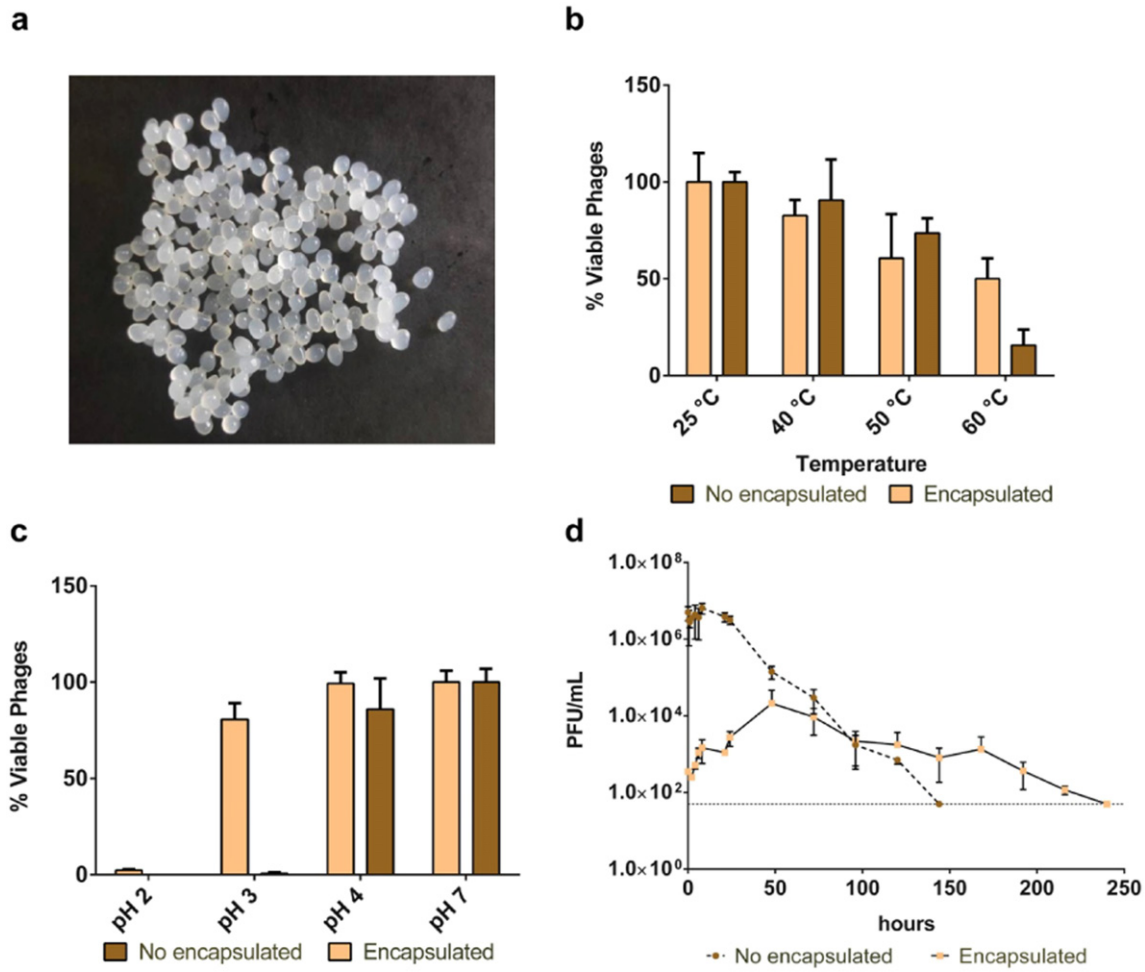


**Fig. 1.** Encapsulation of Bacteriophage f3αSE as a protection and dosification method. (a) Bacteriophage f3αSE encapsulated in alginate spheres. (b) Stability of bacteriophage f3αSE encapsulated and not encapsulated after incubation for 1 h in the corresponding temperatures. (c) Stability of bacteriophage f3αSE encapsulated and not encapsulated after incubation for 1 h at the corresponding pHs. (d) Release of bacteriophage f3αSE encapsulated or directly added in a water flow system. Dotted line represents the detection limit of 50 PFU/mL. Each experiment was performed in triplicates. For (b) and (c) the percentages were calculated based on titters before incubation in the corresponding condition.

to $2.1 \times 10^4$ PFU/mL at 48 h as a result of phage release from the spheres. After that, the concentration of phages was decreasing gradually until being undetectable after 250 h (Fig. 1c). In this case, alginate-$Ca^{+2}$ spheres without phages behave similarly to sphere with phages and remain stable in the water flow system for even two weeks, suggesting that phages are not altering alginate-$Ca^{+2}$ spheres stability in this condition neither.

These results showed that encapsulated phage can extend the presence of phages in the water flow system up to 100 h more than non-encapsulated phages, maintaining concentrations between $10^4$ and $10^2$ PFU/mL. Considering that in both cases (encapsulated phage and non-encapsulated phage) the total amount of phages was the same, the differences in phage concentration and persistence of phages in the medium, are probably due to the controlled release of phages from the alginate-$Ca^{+2}$ spheres. This control release is associated with swelling/dissolution/erosion processes in the alginate [17]. On the other hand, the encapsulation in a gel network protects the phage by reducing the diffusion rate of the protons into the bead matrix that depend on the structural properties of the gel [18]. The functional and physical properties of cation crosslinked alginate beads are dependent on the composition, sequential structure, and molecular size of the polymers [19]. For instance alginates containing a high content of guluronic acid have a more open pore structure and exhibited high diffusion rates [20]. The pore sizes created in the alginate matrices further change the swelling and shrinkage process during gelling. Moreover, the diffusion rate of small molecules is not only affected by the pore diameter but also by its number and length [21]. Although molecular weight affect the diffusion process, gel strength and other properties appeared to be unaffected by this variable beyond 200 kDa, so it seems that the chemical composition is more relevant [20]. Based on this, future works can explore different polysaccharide compositions or mixtures to determine if is possible to increase the protective effect of encapsulation, as has been tested with other phages [3], or if it is possible to increase concentration and persistence of phages in the medium. Similarly, it would be important to evaluate the protective effect and release of this phage under other environmental conditions, such as, intestinal tracts to explore new alternatives applications [3,22].

The results presented in this work show that encapsulation in alginate spheres can be a good alternative to protect the phage f3αSE against environmental conditions, and also can be seen as an alternative for dosification of phages in an aqueous medium.

## Conflict of interest

The authors declare no financial or commercial conflict of interest.

## Financial support

This work was partially supported by project Fondecyt 11140412.

## Acknowledgments

We thank Dr. James Robeson for kindly providing the phage f3αSE and the bacterial host to perform the experiments.

## References

[1] Fardsanei F, Dallal MMS, Douraghi M, et al. Genetic diversity and virulence genes of *Salmonella enterica* subspecies *enterica* serotype Enteritidis isolated from meats and eggs. Microb Pathog 2017;107:451–6. https://doi.org/10.1016/j.micpath.2017.04.026.

[2] Kollanoor Johny A, Darre MJ, Hoagland TA, et al. Antibacterial effect of trans-cinnamaldehyde on *Salmonella* Enteritidis and *Campylobacter jejuni* in chicken drinking water. J Appl Poultry Res 2008;17(4):490–7. https://doi.org/10.3382/japr.2008-00051.

[3] Ma YH, Islam GS, Wu Y, et al. Temporal distribution of encapsulated bacteriophages during passage through the chick gastrointestinal tract. Poult Sci 2016;95(12): 2911–20. https://doi.org/10.3382/ps/pew260.

[4] Doss J, Culbertson K, Hahn D, et al. A review of phage therapy against bacterial pathogens of aquatic and terrestrial organisms. Viruses 2017;9(3):50. https://doi.org/10.3390/v9030050.

[5] Sulakvelidze A, Alavidze Z, Morris JG. Bacteriophage therapy. Antimicrob Agents Chemother 2001;45(3):649–59. https://doi.org/10.1128/AAC.45.3.649-659.2001.

[6] Viertel TM, Ritter K, Horz HP. Viruses versus bacteria-novel approaches to phage therapy as a tool against multidrug-resistant pathogens. J Antimicrob Chemother 2014;69(9):2326–36. https://doi.org/10.1093/jac/dku173.

[7] Adhikari PA, Cosby DE, Cox NA, et al. Effect of dietary bacteriophage supplementation on internal organs, fecal excretion, and ileal immune response in laying hens challenged by *Salmonella* Enteritidis. Poult Sci 2017;96(9):3264–71.

[8] Sillankorva S, Pleteneva E, Shaburova O, et al. *Salmonella* Enteritidis bacteriophage candidates for phage therapy of poultry. J Appl Microbiol 2010;108(4):1175–86. https://doi.org/10.1111/j.1365-2672.2009.04549.x.

[9] Atterbury RJ, Van Bergen MA, Ortiz F, et al. Bacteriophage therapy to reduce *Salmonella* colonization of broiler chickens. Appl Environ Microbiol 2007;73(14):4543–9. https://doi.org/10.1128/AEM.00049-07.

[10] Santander J, Robeson J. Bacteriophage prophylaxis against *Salmonella enteritidis* and *Salmonella pullorum* using *Caenorhabditis elegans* as an assay system. Electron J Biotechnol 2004;7(2). https://doi.org/10.2225/vol7-issue2-fulltext-1.

[11] Borie C, Albala AB, Sánchez AP. Bacteriophage treatment reduces *Salmonella* colonization of infected chickens. Avian Dis 2008;52(1):64–7. https://doi.org/10.1637/8091-082007-Reg.

[12] Ly-Chatain MH. The factors affecting effectiveness of treatment in phages therapy. Front Microbiol 2014;5:51. https://doi.org/10.3389/fmicb.2014.00051.

[13] Nobrega FL, Costa AR, Kluskens LD, et al. Revisiting phage therapy: new applications for old resources. Trends Microbiol 2015;23(4):185–91. https://doi.org/10.1016/j.tim.2015.01.006.

[14] Dini C, Islan GA, de Urraza PJ, et al. Novel biopolymer matrices for microencapsulation of phages: enhanced protection against acidity and protease activity. Macromol Biosci 2012;12(9):1200–8. https://doi.org/10.1002/mabi.201200109.

[15] Kropinski AM, Mazzocco A, Waddell TE, et al. Enumeration of bacteriophages by double agar overlay plaque assay. Methods Mol Biol 2009;501:69–76. https://doi.org/10.1007/978-1-60327-164-6_7.

[16] Jun JW, Yun SK, Kim HJ, et al. Characterization and complete genome sequence of a novel N4-like bacteriophage, pSb-1 infecting *Shigella boydii*. Res Microbiol 2014; 165(8):671–8. https://doi.org/10.1016/j.resmic.2014.09.006.

[17] Ma Y, Pacan JC, Wang Q, et al. Enhanced alginate microspheres as means of oral delivery of bacteriophage for reducing *Staphylococcus aureus* intestinal carriage. Food Hydrocoll 2012;26(2):434–40. https://doi.org/10.1016/j.foodhyd.2010.11.017.

[18] Ma Y, Pacan JC, Wang Q, et al. Microencapsulation of bacteriophage Felix O1 into chitosan-alginate microspheres for oral delivery. Appl Environ Microbiol 2008; 74(15):4799–805. https://doi.org/10.1128/AEM.00246-08.

[19] Gombotz WR, Wee SF. Protein release from alginate matrices. Adv Drug Deliv Rev 2012;64:194–205. (supp). https://doi.org/10.1016/j.addr.2012.09.007.

[20] Goh CH, Heng PWS, Chan LW. Alginates as a useful natural polymer for microencapsulation and therapeutic applications. Carbohydr Polym 2012;88(1): 1–12. https://doi.org/10.1016/j.carbpol.2011.11.012.

[21] Hannoun BJM, Stephanopoulos G. Diffusion coefficients of glucose and ethanol in cell-free and cell-occupied calcium alginate membranes. Biotechnol Bioeng 1986; 28(6):829–35.

[22] Kim S, Jo A, Ahn J. Application of chitosan-alginate microspheres for the sustained release of bacteriophage in simulated gastrointestinal conditions. Int J Food Sci Technol 2015;50(4):913–8. https://doi.org/10.1111/ijfs.12736.