SHORT COMMUNICATION

Reducing *Salmonella enterica* serovar Enteritidis contamination in food: lytic bacteriophages in a homemade mayonnaise-like matrix

Reducción de la contaminación por <u>Salmonella</u> <u>enterica</u> serovar Enteritidis en alimentos: bacteriófagos líticos en una matriz similar a la mayonesa casera

Redução da contaminação por <u>Salmonella</u> <u>enterica</u> serovar Enteritidis em alimentos: bacteriófagos líticos em uma matriz caseira semelhante à maionese

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Abstract

Background: *Salmonella enterica* serovar Enteritidis (SE) is one of the major causes of food-borne disease worldwide, mainly associated with the consumption of poultry products, such as eggs. Several control methods have been implemented in the egg production process, but they have not effectively reduced the outbreaks. Therefore, the use of bacteriophages for the biocontrol of food-borne pathogens is gaining increasing acceptance. **Objective:** To evaluate a bacteriophage cocktail's effectiveness in reducing SE counts in an experimentally contaminated mayonnaise-like matrix. **Methods:** Homemade mayonnaise was contaminated with SE (10^3 CFU/ml) with equal volume to a matrix (1:1) treated with a bacteriophage cocktail (five phages, MOI 10^5), and stored at 21 °C for 24 and 72 h. Bacterial counts were performed to evaluate the bio-controlling activity of the cocktail and compared with a contaminated but not treated group. **Results:** Significant reductions (up to 3.75 $\log_{10}$ CFU/ml) were observed in the bacteriophage-treated groups (p<0.0001). **Conclusions:** These results demonstrate the effectiveness of bacteriophages as biocontrol agents for *Salmonella* Enteritidis in a raw-egg-derivative foodstuff. Further studies are needed to prove the reduction in an undiluted homemade mayonnaise.

Keywords: *Bio-control; eggs; food-borne disease; food-borne pathogens; food safety; foodstuff; lytic bacteriophage; mayonnaise; raw food; <u>Salmonella</u> <u>enterica</u>.*

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Resumen

Antecedentes: La *Salmonella enterica,* serovar Enteritidis (SE), es una de las principales causas de enfermedades transmitidas por alimentos en todo el mundo, asociadas principalmente al consumo de productos avícolas tales como los huevos. Diferentes métodos de control se han ensayado en el proceso de producción de huevos, pero no han sido capaces de reducir eficazmente los brotes de salmonelosis en las personas. Por esta razón, el uso de bacteriófagos para el control biológico de patógenos transmitidos por los alimentos está ganando cada vez más aceptación. **Objetivo:** Evaluar la eficacia de un cóctel de bacteriófagos para reducir los recuentos de SE en una matriz similar a la de mayonesa contaminada experimentalmente. **Método:** La mayonesa casera fue contaminada con SE (10^3 UFC/ml) en igual volumen que la matriz (1:1), tratada con una mezcla de bacteriófagos (cinco fagos, MOI 10^5), y almacenada a 21 °C por 24 y 72 h. Se realizaron recuentos bacterianos para evaluar la actividad biocontroladora de la mezcla y compararlos con un grupo contaminado, pero no tratado. **Resultados:** Se observaron reducciones significativas (hasta 3,75 $\log_{10}$ CFU/ml) en los grupos tratados con bacteriófagos (p<0,0001). **Conclusiones:** Estos resultados demuestran la efectividad del uso de bacteriófagos como agentes de biocontrol de *Salmonella* Enteritidis en un alimento crudo derivado del huevo. Sin embargo, se necesita realizar más estudios para comprobar la reducción en mayonesa casera no diluida.

Palabras clave: *alimentos crudos; bacteriófagos líticos; biocontrol; enfermedad transmitida por alimentos; huevos; inocuidad alimentaria; mayonesa; patógenos transmitidos por alimentos; productos alimenticios; Salmonella enterica.*

Resumo

Antecedentes: *Salmonella enterica* serovar Enteritidis (SE) é uma das principais causas de doenças transmitidas por alimentos em todo o mundo, principalmente associada ao consumo de produtos derivados de aves, como ovos. Diferentes métodos de controle foram implementados no processo de produção de ovos, mas não foram capazes de reduzir efetivamente os surtos nas pessoas. Por esse motivo, o uso de bacteriófagos para o controle biológico de patógenos de origem alimentar está ganhando crescente aceitação. **Objetivo:** Avaliar a eficácia de um coquetel de bacteriófagos na redução da contagem de SE em uma matriz experimentalmente semelhante a maionese contaminada. **Método:** A maionese caseira foi contaminada com SE (10^3 UFC/ml) no mesmo volume da matriz (1:1), tratada com uma mistura de bacteriófagos (cinco fagos, MOI 10^5) e armazenada a 21 °C por 24 e 72 h. As contagens bacterianas foram realizadas para avaliar a atividade de biocontrole da mistura e comparadas com um grupo contaminado, mas não tratado. **Resultados:** Reduções significativas (até 3,75 $\log_{10}$ UFC/ml) foram observadas nos grupos tratados com bacteriófagos (p<0,0001). **Conclusões:** Esses resultados demonstram a eficácia do uso de bacteriófagos como agentes de biocontrole de Salmonella Enteritidis em alimentos crus derivados de ovos, mas são necessários mais estudos para verificar a redução da maionese caseira não diluída.

Palavras-chave: *bacteriófagos líticos; biocontrole; comida crua; doenças transmitidas por alimentos; maionese; ovos; patógenos alimentares; produtos alimentícios; Salmonella enterica; segurança alimentar.*

Introduction

Several food products have been associated with *Salmonella enterica* serovar Enteritidis (SE) outbreaks worldwide (EFSA and ECDC, 2015). Eggs and egg derivatives are the second foodstuff most frequently involved in human salmonellosis. A total of 54 and 112 outbreaks linked to consumption of egg or egg derivatives, particularly homemade mayonnaise, have been reported between 2002 and 2014 in the UK and USA, respectively (Chousalkar *et al.,* 2017). Thus, several control methods have been implemented in the egg production process, but they have not been able to reduce outbreaks. A promising alternative tool to reduce *Salmonella* contamination in food production and processing is the use of lytic bacteriophages.

Bacteriophages (or phages) are viruses that infect and lyse prokaryotic cells, are target-specific, self-replicating, with rapid bactericidal activity, and do not alter the original properties of food (Jorquera *et al.,* 2015a). Therefore, they could be useful as biocontrol agents in foodstuffs. Bacteriophages have been isolated from various foods, including vegetables, seafood, dairy products, meat, and fish (Hudson *et al.,* 2005). Bacteriophages are classified into lysogenic and lytic. Lysogenic bacteriophages are recommended neither as therapeutic agents nor as bio-controllers in food because they can carry genes encoding virulence factors.

Although phage-based biocontrol studies in a wide variety of foods (*i.e.*, raw meats, ready-to-eat food, and fresh products) have been conducted (Bigwood *et al.,* 2008; Guenther *et al.,* 2012), there are limited studies in eggs and none in mayonnaise. Guenther *et al.* (2012) showed that application of a bacteriophage reduced by 2.6 $\log_{10}$ *S.* Typhimurium counts in pasteurized egg yolk after 48 hours of incubation at 15 ºC. Spricigo *et al.* (2013) registered a reduction of *S.* Typhimurium and *S.* Enteritidis concentrations (0.9 $\log_{10}$ CFU/cm² each) in fresh eggs after being sprayed bacteriophages and incubated at 25 °C for 2 hours.

Our previous experiments demonstrated the SE-reducing effectiveness of a cocktail of five lytic bacteriophages in chicken breast, goat cheese (Jorquera *et al.,* 2015b), salmon (Galarce *et al.,* 2014), and sausages (Galarce *et al.,* 2016). However, to the best of our knowledge, there is no information on the use of bacteriophages as biocontrol agents in raw-egg derivative products. Therefore, the present study aimed to preliminary determine the effectiveness of a phage cocktail to reduce SE counts in a homemade mayonnaise-like matrix maintained at 21 °C, simulating a cold chain loss.

Materials and methods

Bacterial strain

A spontaneous mutant SE phage type 4 (PT 4) strain resistant to nalidixic acid and rifampicin (SE *nal^r rif^r*), originally isolated from a laying hen in Chile, was used to inoculate the mayonnaise samples. This strain was grown in Luria-Bertani broth (Difco®, Franklin Lakes, NJ, USA) and incubated at 37 °C for 18 h. The SE *nal^r rif^r* culture was then adjusted to 0.6−0.8 OD_{625} (Spectroquant Pharo 300, Merck, Darmstadt, Germany) to achieve 10^8 CFU/ml. This suspension was serially diluted in buffered peptone water (BPW, Difco®, Franklin Lakes, NJ, USA) to obtain the bacterial concentrations used to contaminate the samples (10^3 CFU/mL). These concentrations were confirmed by viable counts on xylose-lysine-deoxycholate (XLD, Difco®, Franklin Lakes, NJ, USA) agar plates, supplemented with rifampicin and nalidixic acid (50 µg/ml each, Sigma®, Franklin Lakes, NJ, USA), and incubated at 37 °C for 24 h.

Bacteriophages

Five *Salmonella* specific phages were selected from our collection. The selection was based on their lytic properties against the challenge bacterial strain and nine different SE strains, their stability during the required conditions (4 to 18 °C during 10 days), the host range against 13 serovars of *Salmonella enterica* (serotypes that usually infect

poultry), and tolerance to pH (4.0 to 9.0) and temperature (4 to 45 °C). The bacteriophage-insensitive mutants (BIMs) isolation range was 1.3×10^{-6} to 2.9×10^{-6} CFU (Robeson *et al.,* 2014; Galarce *et al.,* 2016). Due to its morphological characteristics, all phages belonged to the *Siphoviridae* family and were originally isolated from sewage samples (fSE7, fSE8, and fSE12) and foodstuff (fSE1C and fSE4S) (Robeson *et al.* 2014). High titer lysates were individually prepared using a spontaneous mutant SE PT4 strain (VAL 222) resistant to both nalidixic acid and rifampicin as the host bacterial strain. The original wild type SE PT4 strain was provided by Dr. Roy Curtiss III (The Biodesign Institute, Arizona State University). Phages were suspended equitably in modified SM buffer (50 mM Tris-HCl, 8 mM $MgSO_4$ heptahydrated, pH 7.5) to reach the required concentration (10^8 PFU/mL each). A multiplicity of infection (MOI) of 10^5 was used. The phage titer was determined by plating adequate dilutions onto lawns of the target strain (Robeson *et al.,* 2008).

Food matrix

Fresh eggs were acquired from a local supermarket to prepare homemade mayonnaise. Before breaking the eggs, the eggshells were disinfected with 70% v/v alcohol and air-dried. According to the traditional recipe (yolk, albumen, vegetable oil, and salt), mayonnaise was prepared inside of a biosafety cabinet. Only *Salmonella* spp. negative mayonnaise samples by culture (ISO 6579 2002) and by genus-specific PCR analysis targeting *inv*A gene (Malorny *et al.,* 2003) were included in this study.

Trial

Two groups (control and experimental) of 48 mayonnaise samples at 25 ml per sample were individualized in sterile Whirl-Pack bags (VWR, Lutterworth, United Kingdom). Samples were experimentally contaminated with SE *nal^r rif^r* (10^3 CFU/ml) in a biological safety cabinet (Heal Force safe 1200®, Shanghai,

China) by homogenization with an inoculum volume corresponding to 50% of the sample to improve homogenization of bacteria and phages in the samples. The contaminated samples were kept at room temperature for 2 h to allow bacterial adaptation. In the experimental group, 2.5 ml of the phage cocktail was added to each contaminated sample. Samples of the control group were contaminated with SE *nal^r rif^r* and added with 2.5 ml of modified SM buffer. Both groups, control and experimental, were incubated at 21 ºC, and SE enumeration was performed at 24 h of incubation in 24 samples, and then at 72 h in the remaining 24 samples. Experimental and control groups were kept and processed separately, and the bacteria enumerated on the same days as the experimental samples.

Five mayonnaise samples not inoculated with SE or phages were kept in the laboratory throughout the experiment to confirm the absence of intra-laboratory contamination.

After incubation (24 and 72 h), and for bacterial count, 225 ml of BPW were added to each bag and homogenized (Stomacher 400 circulator, Seward LTD®, Worthing, UK) for 1 min. Dilutions with BPW were made and 100 µl of each dilution was plated on XLD agar supplemented with rifampicin and nalidixic acid and incubated at 37 ± 1 °C for 24−48 h. Bacterial counts were performed in duplicate. Samples without evident bacterial growth were subjected to enrichment following ISO 6579:2002, and results recorded as positive or negative.

For bacteriophage mixture detection from treated samples, the aqueous phase of homogenized samples was recovered and treated with chloroform to inactivate bacterial cells, which were discarded by centrifugation. Serial dilutions were carried out in SM buffer, followed by duplicate plating using the soft-agar overlay technique, with the challenge SE *nal^r rif^r* strain as an indicator. Thus, 100 µL of each dilution and 100 µL of the challenge strain were mixed in a tube with 4 mL of molten soft LB agar (Difco®, Franklin Lakes, NJ, USA). The suspension was poured onto solid LB agar plates and incubated

at 37 °C for 18–24 h before plaque detection (Galarce *et al.,* 2016). Detected bacteriophages were not individually identified nor quantified.

Statistical analysis

Mean bacterial counts (CFU/ml) were expressed in logarithmic units (log[10]). ANOVA was used to compare mean differences between phage-treated and untreated samples at $p \leq 0.05$ significance level. Samples negative to bacterial count but positive to enrichment were assumed as 10^3 CFU/ml, corresponding to the minimum detection limit. Data were analyzed using InfoStat® 2008 software.

Results

The application of bacteriophage cocktail in the food matrix significantly reduced bacterial counts, ranging from 2.14 to 3.75 log10 CFU/ml, at 24 and 72 h, respectively. Table 1 shows the bacterial reductions and mean SE counts observed.

Lytic activity was stable throughout the incubation time and phages were detected in all treated samples (data not shown). No phages were isolated from any of the control samples. Mayonnaise samples that were not inoculated with SE and were not treated with phages also showed negative results to SE *nal[r] rif[r]* growth and the presence of phages.

Table 1. Mean counts and reductions of *Salmonella* Enteritidis (SE) in a homemade mayonnaise-like matrix with and without phage treatment, incubated for 24 and 72 hours at 21 ºC.

Incubation time (h)	Group	SE initial dose (log[10] CFU/ml)	Mean SE counts (log[10] CFU/ml) ± SD	SE reduction (log[10] CFU/ml)
24	Experimental	2.62[a]	3.23[a] ± 0.3	2.14
	Control		5.37[b] ± 0.2	
72	Experimental	2.98[a]	5.49[a] ± 0.4	3.75
	Control		9.24[b] ± 0.2	

Different superscript letters (a, b) within columns indicate significant differences ($p \leq 0.0001$).

Discussion

In the control samples, SE increased up to 2.75 log[10] CFU/ml at 24 h, and up to 6.26 log CFU/ml at 72 h, showing the effects that cold losses can produce in the bacterial load present in foods. According to our previous results, the lytic bacteriophage cocktail application significantly reduced SE counts in 2.14 log[10] CFU/ml at 24 and 3.75 log[10] CFU/ml 72 h at the incubation temperature. These reductions can significantly enhance food safety, considering that the infective dose for humans for various serovars, including *S.* Enteritidis, is 10^5 CFU, and that high concentration doses are associated with higher rates of illness and shorter incubation periods (Kothary and Babu, 2001). This is the first study assessing the bio-controlling properties of a bacteriophage cocktail in a matrix similar to homemade mayonnaise, demonstrating its

preliminary lytic activity in an egg-derived raw food preparation.

Similar to our results, Guenther *et al.* (2012) observed a reduction of 2.6 log[10] CFU/g in pasteurized egg yolk contaminated with *S.* Typhimurium (10^3 CFU/g) and treated with FO1-E2 phage (3×10^8 PFU/g) incubated at 15 °C for 2 days. Towards the fifth day, they observed increased bacterial counts, even exceeding the control group, explained by phage immobilization on food surfaces and matrix, possibly in combination with the appearance of phage-insensitive bacteria. Our previous findings suggest that the frequency of emergence of bacteriophage insensitive mutants to the phage cocktail was as low as 2.9×10^{-6} CFU (Galarce *et al.,* 2016), hindering the loss of effectiveness due to phage-resistance. In the same context, Spricigo *et al.* (2013) reported

reductions lower than 1 $\log_{10}$ CFU/cm^2 in fresh eggs contaminated with S. Enteritidis (10^7 CFU/ml) treated with a bacteriophage cocktail (10^{10} PFU/ml) and incubated at room temperature for 2 h. Compared to other matrices, this minor reduction was explained by a non-homogeneous *Salmonella* contamination of the eggshells or by the translocation of microorganisms from the shell surface to its external and internal membranes, which could have affected the phage effectiveness.

Mayonnaise is an edible oil emulsion with additives, forming a semi-liquid matrix. It is well-established that the matrix strongly influences the phage activity. In liquid matrices, phages can spread almost unrestricted and easily collide with the bacterium. Hence, bacterial reduction should be higher compared to solid matrices (Guenther *et al.,* 2009). Guenther *et al.* (2012) indicated that, due to its viscosity, bacteriophages distribution in yolk is non-homogeneous and has limited diffusion. For this reason, in the present study an inoculum volume equivalent to 50% of the total sample was used to improve bacterial inoculum homogenization and phage distribution in the mayonnaise. Thus, higher bacterial reduction was expected in the mayonnaise-like matrix than observed in our previous studies in solid matrices, with reductions of 3.19 log in salmon fillets (Galarce *et al.,* 2014) and 3.92 log in turkey breasts (Jorquera *et al.,* 2015b). It is known that phage binding to ligand on the bacterial surface is influenced by intrinsic factors, such as ionic strength, pH, and substances that may interfere with the bactericidal process. Moreover, target bacteria may be embedded within this matrix and could be partially shielded from diffusing liquid, and therefore are also protected from the phage (Gunther *et al.,* 2009; Jorquera *et al.,* 2015a). Our results suggest that the mayonnaise-like matrix viscosity and its relatively complex composition of fat, proteins and carbohydrates may have interfered with bactericidal viral activity.

Matrix dilution (50%) decreased the viscosity and changed the characteristics of homemade mayonnaise, which prevents inferring the behavior of these phages; however, it suggests that it could be used in sauces containing mayonnaise. More studies are needed to determine the reducing effect of these phages on mayonnaise.

The lytic activity showed similar levels of bacterial reduction at 24 and 72 h of incubation. Although we have demonstrated the stability of these bacteriophages in 10 different food matrices, where they remain stable over 10 days of incubation at 18 and 4 °C (Robeson *et al.,* 2014), their stability in mayonnaise or other egg-derived raw food has not yet been determined; thus, the influence of this factor on the current results should be considered in future studies.

Incubation time can also affect phage activity, with longer incubation time corresponding to higher bacterial reductions (Bigwood *et al.,* 2008). As incubation time (24 and 72 h) did not influence bacterial reduction ($p \leq 0.05$), it was excluded from further analysis. Thus, a one-way ANOVA model was adjusted, using treatment (phage *vs.* no phage) as a factor. The coefficient of determination (R^2) of the ANOVA ranged between 0.96−0.99, indicating that the adjusted model explained more than 95% of the bacterial count's total variability in each case and, consequently, by the treatment with bacteriophages.

In conclusion, our results suggest that under experimental conditions, this bacteriophage cocktail significantly reduces SE counts in a homemade mayonnaise-like matrix, regardless of incubation time. Therefore, bacteriophages could be used to control SE in egg derivatives. Further studies are needed to improve bacterial control in undiluted mayonnaise focusing on increased MOI, characterization of lytic activity of phages in whole egg and its components by separate, and determining their effectiveness in conjunction with conventional methods.

Declaration

Conflict of interest

The authors declare they have no conflicts of interest with regard to the work presented in this report.

Author contributions

Consuelo Borie-Polanco: responsible for study design; administered the project; critical reading and editing of the paper; final approval of the version to be published. Nicolás Galarce-Gálvez: collected bacteria data; writing, critical reading and editing of the paper. Karina Yévenes-Coa: collected bacteria data; data analyses; writing and critical reading of the paper. José M. Yáñez-López: statistical analyses and data interpretation; critical reading and editing of the paper. James Robeson-Camus: responsible for the design of the study; collected bacteriophages data; writing, critical reading and editing of the paper. Alfonso Carbonero-Martínez: responsible for the design of the study; writing, critical reading and editing of the paper.

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