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

To appear in:
Rev Colomb Cienc Pecu (34, 3)

This unedited manuscript has been accepted by RCCP for future publication and is provisionally published on our website. The manuscript will undergo copyediting, typesetting, and galley review before final publication. Please note that this advance version may differ from the "Ahead of print" and also from the final version.
**SHORT COMMUNICATION**

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

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

*Redução da contaminação por *Salmonella enterica* serovar Enteritidis em alimentos: bacteriófagos líticos em uma matriz caseira semelhante à maionese*

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**To cite this article:**
Borie-Polanco C, Galarce-Gálvez N, Yévenes-Coa K, Yáñez-López JM, Robeson-Camus J, Carbonero-Martínez A. Reducing *Salmonella enterica* serovar Enteritidis contamination in food: lytic bacteriophage cocktail in homemade mayonnaise. Rev Colomb Cienc Pecu 2021; 34(3). Pages pending. DOI: [https://doi.org/10.17533/udea.rccp.v34n3a05](https://doi.org/10.17533/udea.rccp.v34n3a05)

**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, as is the case of eggs. Several control methods have been implemented in egg production process, but they have not been able to effectively reduce the outbreaks. Therefore, the use of bacteriophages for the biocontrol of food-borne pathogens is gaining increasing acceptance. **Objective:** To evaluate the effectiveness of a bacteriophage cocktail to reduce SE counts in mayonnaise-like matrix experimentally contaminated. **Methods:** Homemade mayonnaise was contaminated with SE (10³ CFU/ml) with equal volume to matrix (1:1), treated with a bacteriophage cocktail (five phages, MOI 10⁵), and stored at 21 ºC up to 24 and 72 h.

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Bacterial counts were performed to evaluate the biocontrolling activity of the cocktail, and compared with a contaminated but not treated group. **Results:** Significant (\(p<0.0001\)) reductions (up to 3.75 log\(_{10}\) CFU/ml) were observed in the bacteriophage-treated groups. **Conclusions:** These results demonstrate the effectiveness of using bacteriophages as biocontrol agents for *Salmonella* Enteritidis in a raw-egg-derivative foodstuff, but further studies are needed in order to prove the reduction in an undiluted homemade mayonnaise.

**Keywords:** Biocontrol; eggs; food-borne pathogens; food safety; lytic bacteriophage; mayonnaise; raw food; *Salmonella enterica*.

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**Resumen**

**Antecedentes:** *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 derivados de la avicultura, como es el caso de los huevos. Diferentes métodos de control han sido implementados en el proceso de producción de huevos, pero no han sido capaces de reducir eficazmente los brotes 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 mayonesa experimentalmente contaminada. **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 comparados con un grupo contaminado pero no tratado. **Resultados:** se observaron reducciones significativas (\(p<0.0001\)) (hasta 3,75 log\(_{10}\) CFU/ml) en los grupos tratados con bacteriófagos. **Conclusiones:** estos resultados demuestran la efectividad del uso de bacteriófagos como agentes de biocontrol de *Salmonella* Enteritidis en un alimento crudo derivado del huevo, pero se necesitan mayores estudios para comprobar la reducción en mayonesa casera no diluida.

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

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**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 (p<0,0001) (até 3,75 log10 UFC/ml) foram observadas nos grupos tratados com bacteriófagos. **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; maionese; ovos; patógenos alimentares; *Salmonella enterica*; segurança alimentar.

**Introduction**

Several foodstuffs have been involved in *Salmonella enterica* serovar Enteritidis (SE) outbreaks worldwide (EFSA and ECDC, 2015). However, eggs and egg derivatives are considered as the second food most frequently involved in human salmonellosis. In this sense, 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 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 might be useful as biocontrol agents in foodstuff. Bacteriophages have been isolated from a variety of food, including vegetables, seafood, dairy products, meat and fish (Hudson *et al*., 2005). Bacteriophages are classified into: lysogenic and lytic. Lysogenic bacteriophages are not recommended neither as therapeutic agents nor as biocontrollers in food, because can carry genes encoding virulence factors.
Despite phage-based biocontrol studies in a wide variety of food (i.e. raw meats, ready-to-eat food and fresh products) have been conducted (Bigwood et al., 2008; Guenther et al., 2012), there are few studies in eggs and none in mayonnaise. Thus, Guenther et al. (2012) showed that the application of a bacteriophage reduced by 2.6 log$_{10}$ S. Typhimurium counts in pasteurized egg yolk after 48 hours of incubation at 15 °C. Spricigo et al. (2013) registered a reduction of S. Typhimurium and S. Enteritidis concentrations (0.9 log$_{10}$ CFU/cm$^2$ each) in fresh eggs after being sprayed with 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, there is no information about 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 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 achieve 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, based on their lytic properties against the challenge bacterial strain and nine different SE strains, their stability at 4 and 18 °C for 10 days, host
range against 13 serovars of *Salmonella enterica* (serotypes that usually infect poultry) and pH (4.0 to 9.0) and temperature tolerance between 4 and 45 °C. The bacteriophage insensitive mutants isolation (BIMs) range was $1.3 \times 10^{-6}$ to $2.9 \times 10^{-6}$ 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 MgSO4 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 in order to prepare homemade mayonnaise. Before breaking the eggs, the eggshells were disinfected with 70% v/v alcohol and air-dried. The mayonnaise was prepared inside of a biosafety cabinet, according to the traditional recipe (yolk, albumen, vegetable oil and salt). Only *Salmonella* spp. negative mayonnaise samples by culture (ISO 6579 2002) and by genus-specific PCR analysis targeting invA gene (Malorny *et al.*, 2003) were included in the 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* rif* (10³ 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 the 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 volume of the phage cocktail was added to each contaminated sample. Samples of the control group were contaminated with SE *nal* rif* and added with 2.5 ml of modified SM buffer. Both groups, control and experimental, were incubated at 21 °C, 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 samples of mayonnaise, not inoculated with SE nor 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 ºC 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 the bacteriophage mixture detection from the treated samples, the aqueous phase of the homogenized samples was recovered and then 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 nalr rifr 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 ºC for 18–24 h prior to 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 (log10) and the differences between the means of the phage-treated and untreated samples were evaluated by ANOVA at p ≤ 0.05 significance level. Samples negative to bacterial count but positive to enrichment were assumed as 10³ CFU/ml, corresponding to the minimum detection limit. Data were analyzed using InfoStat® 2008 software.

Results

In the food matrix, the application of the bacteriophage cocktail significantly reduced the 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 obtained.

Table 1. Mean counts and reductions of Salmonella Enteritidis (SE) in homemade mayonnaise-like matrix, with and without phage treatment, incubated for 24 and 72 hours at 21 ºC.
<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Group</th>
<th>SE initial dose (log_{10} CFU/ml)</th>
<th>Mean SE counts (log_{10} CFU/ml) ± SD</th>
<th>SE reduction (log_{10} CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>Experimental</td>
<td>2.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.23&lt;sup&gt;a&lt;/sup&gt; ± 0.3</td>
<td>2.14</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>5.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.49&lt;sup&gt;a&lt;/sup&gt; ± 0.2</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>Experimental</td>
<td>2.98&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.49&lt;sup&gt;a&lt;/sup&gt; ± 0.4</td>
<td>3.75</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>9.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.24&lt;sup&gt;b&lt;/sup&gt; ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

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

The 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<sup>r</sup> rif<sup>r</sup> growth and to the presence of phages.

**Discussion**

In the control samples, SE increased up to 2.75 log<sub>10</sub> CFU/ml at 24 h, and up to 6.26 log CFU/ml at 72 h, showing the effects that cold chain losses can produce in the bacterial load present in foodstuff.

In accordance with our previous results, the application of the lytic bacteriophage cocktail significantly reduced SE counts in 2.14 log<sub>10</sub> CFU/ml at 24 and 3.75 log<sub>10</sub> 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<sup>5</sup> 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 that assess the biocontrolling properties of a bacteriophages cocktail in a matrix similar to homemade mayonnaise, demonstrating its preliminary lytic activity in an egg-derived raw food preparation.

Similarly to our results, Guenther et al. (2012) observed a reduction of 2.6 log<sub>10</sub> CFU/g in pasteurized egg yolk contaminated with S. Typhimurium (10<sup>3</sup> CFU/g) and treated with FO1-E2 phage (3 x 10<sup>8</sup> PFU/g) incubated at 15 °C for 2 days, but towards the fifth day an increase in the bacterial counts, even exceeding the control group, explained by the immobilization of the phage on the 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 present phage cocktail was as low as 2.9 x 10<sup>-6</sup> CFU (Galarce et al., 2016), which hinders the loss of effectiveness due to phage-resistance. In the same context, Spricigo et al. (2013) reported reductions lower than 1 log<sub>10</sub> CFU/cm<sup>2</sup> in fresh eggs contaminated with S. Enteritidis (10<sup>7</sup> CFU/ml), treated with a bacteriophage...
cocktail (10\(^{10}\) PFU/ml) and incubated at room temperature for 2 h. This minor reduction, compared to other matrices analysed was explained by a non-homogeneous *Salmonella* contamination on 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 type strongly influences the phage activity. In liquid matrices, phages can spread almost unrestricted and easily collide with the bacterium, hence, the bacterial reduction should be higher compared to solid matrices (Guenther et al., 2009). As indicated by Guenther et al. (2012), the distribution of the bacteriophages in yolk due to is viscosity is nonhomogeneous and with limited diffusion. For this reason, in the present study, an inoculum volume equivalent to the 50% of the total sample was implemented to improve the bacterial inoculum homogenisation and the distribution of the phages in the mayonnaise. Thus, a higher bacterial reduction was expected in mayonnaise-like matrix than that 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 should be considered that the binding of phages to their ligands on the bacterial surfaces is influenced by intrinsic factors, such as ionic strength, pH, and substances which may interfere with the bactericidal process. Moreover, target bacteria may be embedded within this matrix 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 viscosity of the mayonnaise-like matrix and its relatively complex composition of fat, proteins and carbohydrates, may have interfered with the bactericidal viral activity.

The dilution (50%) of the matrix, decreased the viscosity and changed the characteristics of a 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.

The incubation time can also affect phage activity, with longer incubation times corresponding to higher bacterial reductions (Bigwood et al., 2008). As incubation times (24 and 72 h) did not influence the
bacterial reductions ($p \leq 0.05$), it was excluded from further analysis. Thus, a one-way ANOVA model was adjusted, using treatment (phage vs. no phage) as factor. The coefficient of determination ($R^2$) of the ANOVA performed, ranged between 0.96–0.99, indicating that more than 95% of the total variability of the bacterial count was explained by the adjusted model 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 homemade mayonnaise-like matrix, regardless of the incubation time. Therefore, bacteriophages could constitute an alternative tool for the biocontrol of SE in egg derivatives. However, further studies are necessary to improve the bacterial reduction in undiluted mayonnaise, considering increasing MOI, characterizing the lytic activity of phages in whole egg and in its components by separate; and also, to determine their effectiveness in conjunction with conventional methods.

**Declarations**

**Funding**

This research was supported by The Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT, Proyecto N°1110038) de la Agencia Nacional de Investigación y Desarrollo (ANID), Ministerio de Educación (Chile) and by The laboratorio de Bacteriología Veterinaria, Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile (Chile).

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