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BRIEF REPORT

Bacteriophage cocktail reduces *Salmonella enterica* serovar Enteritidis counts in raw and smoked salmon tissues

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Abstract
The use of bacteriophages for the biocontrol of food-borne pathogens is increasingly gaining acceptance. In this study, the effectiveness of bacteriophages to reduce *Salmonella* Enteritidis counts was evaluated in raw and smoked salmon tissues. Groups of 25 samples each were contaminated with S. Enteritidis, treated with a phage mix and then incubated for ten days at 18 °C and 4 °C. A significant bacterial reduction was obtained on days 3, 6 and 10 in raw salmon samples incubated at 18 °C (from 0.75 to 3.19 $\log_{10}$ CFU/g) and at 4 °C (from 2.82 to 3.12 $\log_{10}$ CFU/g), whereas in smoked salmon lower reductions were achieved (from 1.02 to 1.96 $\log_{10}$ CFU/g at 18°C and from 0.50 to 1.16 $\log_{10}$ CFU/g at 4 °C). These results show the potential effectiveness of this bacteriophage cocktail as a biocontrol agent against S. Enteritidis in raw and smoked salmon tissues.

PALABRAS CLAVE
Salmonella;
Bacteriófago;
Biocontrol;
Tejidos de salmón

Una mezcla de bacteriófagos reduce los recuentos de *Salmonella enterica* serovar Enteritidis en tejidos de salmón fresco y ahumado

Resumen
El uso de bacteriófagos en el biocontrol de patógenos está adquiriendo cada vez más aceptación. En este estudio se evaluó la efectividad de bacteriófagos en la reducción de los recuentos de *Salmonella* Enteritidis en salmón fresco y ahumado. Para ello, 25 muestras por grupo fueron contaminadas con S. Enteritidis, tratadas con una mezcla de bacteriófagos e incubadas durante 10 días a 18 °C o a 4 °C. A los días 3, 6 y 10 se obtuvo una reducción significativa de los recuentos de S. Enteritidis en las muestras de salmón fresco

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incubadas a ambas temperaturas: la reducción fue de entre 0,75 y 3,19 $\log_{10}$ UFC/g a 18 °C y de entre 2,82 y 3,12 $\log_{10}$ UFC/g a 4 °C. En salmón ahumado las reducciones fueron menores (entre 1,02 y 1,96 $\log_{10}$ UFC/g a 18 °C y entre 0,50 y 1,16 $\log_{10}$ UFC/g a 4 °C). Los resultados indican que estos bacteriófagos constituyen una potencial herramienta de biocontrol de S. Enteritidis en tejidos de salmón fresco y ahumado.

Salmonellosis is one of the most commonly reported zoonotic diseases in many countries. In Chile, since 2005 to 2010, *Salmonella enterica* serovar Enteritidis was the principal pathogen involved in food-borne disease outbreaks, where seafood and fish were the two major food products implicated[2]. These foods have been involved in salmonellosis outbreaks in other countries, such as the United States, due to tuna sushi consumption[5], and in the Netherlands, due to smoked salmon consumption[6].

To prevent these outbreaks as well as other food-borne diseases, it would be necessary to develop new tools to control and reduce their incidence. The use of lytic bacteriophages (or phages) to reduce food-borne pathogens has emerged as a promising tool for food safety. These viruses are target-specific, self-replicating, rapid bactericidal and, do not alter normal food properties. In this regard, Guenther *et al.*[10] used a phage mix to reduce *Listeria monocytogenes* in several ready-to-eat foods, including smoked salmon. They observed a decrease of 2.2 $\log_{10}$ colony-forming units per gram (CFU/g). Additionally, Soni and Nannapaneni[14] observed a 2.3 $\log_{10}$ CFU/g reduction of *L. monocytogenes* in raw salmon by using a commercial phage mix (LISTEX™ P100). Guenther *et al.*[9], assessed the effect of the addition of a lytic phage to reduce *Salmonella* Typhimurium on ready-to-eat food, including a seafood mix, obtaining a reduction of 1.9 $\log_{10}$ CFU/g in the pathogen count. Given these results, they conclude that phages can be highly effective for the biological control of food-borne pathogens in these types of food.

The aim of the present work was to determine the effectiveness of a phage cocktail to reduce S. Enteritidis counts in raw and smoked salmon tissues. In a previous study, this cocktail demonstrated to be effective on chicken and goat cheese[7,12].

A spontaneous mutant *Salmonella* Enteritidis strain, resistant to both nalidixic acid and rifampicin, was used to inoculate the salmon samples (S. Enteritidis *nal^r rif^r*). This strain was grown in fresh Luria Bertani broth (LB, Difco) and incubated at 37°C for 18 h. Then, the OD_{625} of the S. Enteritidis culture was adjusted to 0.6-0.8 (Spectroquant Pharo 300, Merck), a range in which the bacterial suspension reaches 10^8 CFU/ml. Afterwards, serial dilutions were prepared in Buffered Peptone Water (BPW, Difco) to achieve the bacterial concentrations used to contaminate the food matrices studied. Concentrations were confirmed by viable counts on Xylose Lysine Deoxycholate (XLD, Difco) agar, supplemented with rifampicin (50 μg/ml, Sigma) and nalidixic acid (50 μg/ml, Sigma), and incubated at 37 °C for 24 h.

Five phages specific for *Salmonella* were chosen from our collection, based on their lytic properties against the bacterial strain, their stability over time, pH and temperature tolerance, and their host range[13]. Phages were suspended equitably in modified SM buffer (50 ml 1 M Tris-HCl, 2 g $MgSO_4 7H_2O$, pH 7.5). A multiplicity of infection (MOI) of 10^4 was used. The phage titer was determined by plating adequate dilutions onto lawns of the target strain[12].

The raw salmon (RS) fillets and smoked salmon (SS) slices were acquired in sealed packages and transported in refrigerated boxes to the laboratory. Only negative samples to *Salmonella* spp. by traditional culture (ISO 6579:2002) and genus-specific PCR[12] were included in the study.

Meat samples were washed with sterile distilled water, milled in a disinfected food processor (Moulinex®), excess liquid was eliminated when necessary (especially in RS) and subsequently contaminated with S. Enteritidis *nal^{r}rif^r*. For the RS fillets, the inoculum was 10^3 and 10^5 CFU/ml, for 18 °C and 4 °C respectively; and for the SS slices 10^3 and 10^4 CFU/ml for 18 °C and 4 °C, respectively. Groups of 50 samples of 25 g each were used, which were individualized in Whirl- Park bags.

Contamination was carried out in a Heal Force Biological Safety cabinet (HF safe 1200), with an inoculum volume corresponding to 10% of the sample (2.5 ml) and gently homogenized. Then, the samples were kept at room temperature for two hours to propitiate bacterial adaptation.

A 2.5 ml volume of the phage cocktail was added to each contaminated sample and incubated for 10 days: 25 samples at 18 ± 1 °C and 25 samples at cooling temperature (4 ± 1 °C). Afterwards, the samples were analyzed to determine S. Enteritidis counts on days 3, 6 and 10 of incubation. Furthermore, for each experimental group (for both temperatures), a control group of 25 samples was established. These groups were contaminated with S. Enteritidis *nal^r rif^r*, sterile modified SM buffer was added, then kept and processed separately from the groups that received the phage cocktail and subjected to bacterial counts on days 3, 6 and 10 of incubation.

On sampling days, 225 ml of BPW was added to each bag and homogenized (Stomacher 400 circulator) for 1 min. Bacterial counts of the pathogen were performed in XLD agar with the addition of rifampicin (50 μg/ml) and nalidixic acid (50 μg/ml). Sodium pyruvate 1% w/v (Merck®) was added to the plates from samples kept at cooling temperature to improve the recovery and diminish the effect of temperature stress on the bacterial strain[15]. The plates were then incubated at 37 °C for 24-48 h. Negative samples (without evident bacterial growth) were subjected to qualitative bacteriology following ISO 6579:2002. Bacterial counts were carried out in duplicate.

Mean values of bacterial counts (CFU/g) were expressed in logarithmic units ($\log_{10}$) and subjected to analysis of

variance (ANOVA), with a significance level of 5% ($p \leq 0.05$). When there were statistically significant differences, the Tukey's test was used. Tests were performed within each sampling time using the type of fish as a factor with two categories: in the presence and in the absence of the phage cocktail.

In accordance with our results, the application of phages significantly reduced the bacterial counts at both incubation temperatures (Table 1), in comparison with control groups. In the RS group incubated at 18 °C, reductions in bacterial counts were of 0.75, 2.57 and 3.19 $\log_{10}$CFU/g on days 3, 6 and 10, respectively. On the other hand, under cooling temperature, significant reductions of 3.12, 2.83 and 2.82 $\log_{10}$ UFC/g were observed (Table 1). In addition, it should be noted that the phage cocktail concentration remained invariable throughout the experiment (Table 1).

The ability of the bacteriophage cocktail to reduce *Salmonella* Enteritidis counts in SS was also demonstrated, with statistically significant reductions (Table 2). Reductions in bacterial counts were of 1.69, 1.02 and 1.96 $\log_{10}$ CFU/g (p <0.0001) in samples stored at 18 °C. Similarly, at 4 °C, reductions were significant (p <0.0001), corresponding to 0.50, 0.35 and 1.16 $\log_{10}$ CFU/g at days 3, 6 and 10 respectively (Table 2).

No phages were isolated from any of the control samples, corroborating the absence of cross-contamination with their respective experimental group.

In this study, it was demonstrated that the five phage cocktail significantly reduced *Salmonella* Enteritidis counts in SS slices and RS fillets, stored for 10 days at both temperatures. It should be noted that the largest reductions were achieved in RS (3.19 $\log_{10}$CFU/g at 18 °C and 2.82 $\log_{10}$

Table 1 Effectiveness of the bacteriophage cocktail in reducing the concentration of *Salmonella* Enteritidis in raw salmon fillets

Incubation (days)	Phage cocktail[a] addition	*S.* Enteritidis ($\log_{10}$ CFU/g)[b]		Reduction ($\log_{10}$ CFU/g)		Phage cocktail ($\log_{10}$ PFU/g)	
		18 °C	4 °C	18 °C	4 °C	18 °C	4 °C
3	–	7.51 ± 0.16	4.76 ± 0.20				
	+	6.76 ± 1.20	1.64 ± 0.36	0.75	3.12	6.57± 0.24	9.32 ± 0.23
6	–	6.70 ± 0.60	5.07 ± 0.17				
	+	4.13 ± 0.95	2.24 ± 0.45	2.57	2.83	7.32 ± 0.27	9.04 ± 1.82
10	–	5.90 ± 0.49	3.12 ± 0.45				
	+	2.71 ± 0.98	0.30 ± 0.43	3.19	2.82	7.80 ± 0.40	9.68 ± 0.39

The differences between untreated and treated samples were statistically significant ($p \leq 0.0033$) in all cases.
Day 0: contamination day. The bacterial inoculum was 2.9 and 4.9 $\log_{10}$ CFU/g, for 18 °C and 4 °C, respectively; and the phage titer was 7 and 9 $\log_{10}$ PFU/g, for 18 °C and 4 °C, respectively.
[a] –, control samples (without phage), +, samples with phage cocktail addition.
[b] Each value is the average of 25 samples ± standard error.

Table 2 Effectiveness of the bacteriophage cocktail in reducing the concentration of *Salmonella* Enteritidis in smoked salmon slices

Incubation (days)	Phage cocktail[a] addition	S. Enteritidis ($\log_{10}$ CFU/g)[b]		Reduction ($\log_{10}$ CFU/g)		Phage cocktail ($\log_{10}$ PFU/g)	
		18 °C	4 °C	18 °C	4 °C	18 °C	4 °C
3	–	8.23 ± 0.13	3.84 ± 0.08				
	+	6.54 ± 0.28	3.34 ± 0.18	1.69	0.50	7.30 ± 0.37	8.32 ± 0.23
6	–	8.34 ± 0.15	3.73 ± 0.26				
	+	7.32 ± 0.37	3.38 ± 0.19	1.02	0.35	6.61 ± 0.36	8.80 ± 0.07
10	–	6.96 ± 0.42	2.28 ± 0.24				
	+	5.0 ± 0.48	1.12 ± 0.32	1.96	1.16	6.27 ± 0.19	8.66 ± 0.33

The differences between untreated and treated samples were statistically significant ($p \leq 0.0001$) in all cases.
Day 0: contamination day. The bacterial inoculum was 3.2 and 4.2 $\log_{10}$ CFU/g, for 18 °C and 4 °C, respectively; and the phage titer was 7 and 8 $\log_{10}$ PFU/g, for 18 °C and 4 °C, respectively.
[a] –, control samples (without phage), +, samples with phage cocktail addition.
[b] Each value is the average of 25 samples ± standard error.

CFU/g at 4 °C), compared to the SS (1.96 $\log_{10}$ CFU/g at 18 °C and 1.16 $\log_{10}$ CFU/g at 4 °C), regardless of the storage temperature. This difference could be explained by the different water content in both matrices. Moreover, despite being acquired in frozen form, RS had a larger amount of ice due to its industrial processing. This characteristic may have favored phage mobilization in achieving greater reductions in RS, in accordance with the results made by Guenther *et al.*[10]. Due to the lower water activity SS has a dry texture, and added to the long incubation period, phage diffusion could be restricted. This was observed by Bigwood *et al.*[3] that studied the application of a phage cocktail in raw and cooked beef samples contaminated with *Salmonella* Typhimurium. The minor pathogen inactivation was achieved in cooked meat, being this attributed to the dry consistency, which would prevent the phage mobilization. Additionally, the smoking and drying process as a method for food preservation, can limit the subsequent growth of pathogens. Therefore, it is conceivable that this process may have affected the growth of the challenge strain in SS, partially explaining the low effectiveness of the phage cocktail. However, bacterial counts in the control group showed a similar growth rate than that of RS (4.0 and 4.7 $\log_{10}$ CFU/g for RS and for SS, respectively). Therefore, one of the factors that could explain these results is the food matrix's dryness rather than the smoking process itself.

The results obtained are consistent with those from other studies, which indicate that the phage reduction effectiveness depends strongly on the food type. This is associated with intrinsic factors, such as ionic strength, pH and its own components, which can interfere in the phage-binding process to receptors on the bacterial surface[3,10].

Interestingly, the highest reductions were achieved at 18 °C in both matrices, which can be attributed to the target cell. In order to synthesize their components and produce bacterial lysis ("lysis from within"), phages require the enzymatic machinery of the host cell, which at 18 °C is in active growth, compared with the metabolic and structural status at cooling temperatures[15]. By contrast, in the case of the samples stored at cooling temperature, it may occur by "lysis from without", in which case cell death occurs when a large number of phages bind to the bacterial surface receptors, resulting in damage to the cell wall, physiological stress and eventually cell lysis. The latter phenomena is the most likely in the biocontrol of some bacterial enteric pathogens in foods stored under cooling temperatures, where the temperature prevents the full viral lytic cycle, coupled with the low bacterial growth rate at this temperature[1]. To avoid this type of lysis, in the present study the bacterial concentration of all the samples incubated at cooling temperature was higher than the inoculum applied to the samples incubated at 18 °C.

The application of the bacteriophage cocktail to reduce *Salmonella* counts showed that its lytic activity was stable at both temperatures during the whole experience. This was expected since the five phages were not affected by temperatures between –20 °C and 25 °C (data not shown). The greatest reduction in counts at cooling temperature was observed in the first three days after application of the phage cocktail in RS fillets, similarly to that was observed by Guenther *et al.*[9] in chocolate milk and seafood mix with approximate to 3 $\log_{10}$ reductions at 8 °C until the sixth day of the trial. After day 3, pathogen growth rate might also affect the effectiveness of phage infection, even at a high MOI[8]. Low bacterial growth at 4 °C could be responsible for low reductions revealed on days 3 and 6 in SS slices. Furthermore, bacterial reduction in samples incubated at 18 °C increased during incubation, except on day 6 in SS slices. The bacterial growth rate in the samples incubated at 18 °C was normal and suitable for a proper bactericidal phage activity.

Even though reductions in *S.* Enteritidis counts achieved in this study using a multiplicity of infection (MOI) of 10^4 in matrices and storage conditions are satisfactory and consistent with other publications, such values could be improved by increasing the MOI. Some authors emphasize that they had achieved higher bacterial reductions at higher phage levels. Guenther *et al.*[10] studied the effect of a phage against *L. monocytogenes* at different concentrations in ready-to-eat foods, stored for six days at 6 °C. They observed reductions of 2.2 and 2.7 $\log_{10}$ CFU/g in sausages by using a MOI of 10^3 and 10^4, respectively. Moreover, a MOI of 10^5 was enough to completely control the pathogen. Similarly, Hudson *et al.*[11] evaluated the activity of a phage at several concentrations of *E. coli* O157:H7 in samples of raw beef stored at 37 °C for 1 h. This study showed that the greatest bacterial inactivation was achieved (>2.6 $\log_{10}$/food piece) by using a MOI of 10^4, while no inactivation was obtained using a MOI of 10^1. Therefore, it is important to emphasize that the phage concentration must be high enough to ensure its contact with the bacterial host, considering physical limitations of the food matrix for proper dissemination.

The incubation time also affected the effectiveness of the phage cocktail. In general terms, phage effectiveness was higher at longer incubation periods, especially in RS fillets stored at 18 °C. In most studies, the reducing effect of phages is dependent on the incubation time, thus a longer incubation would provide a higher bacterial reduction[3,10], although a contact time as short as 5 minutes may be sufficient for some phages to significantly reduce the contamination levels of their bacterial target[4].

The results obtained in this work reveal the effectiveness of a phage cocktail in reducing *Salmonella* Enteritidis counts in raw salmon fillets and smoked salmon, incubated at 18 °C or at 4 °C for 10 days. Therefore, this cocktail could be an alternative for the biocontrol of *S.* Enteritidis in salmon matrices, although further studies are needed to improve it, such as the isolation of new phages having a stronger lytic activity against *S.* Enteritidis or by increasing the MOI.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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