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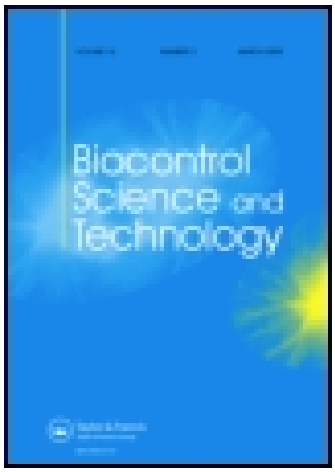

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The use of a bacteriophage cocktail as a biocontrol measure to reduce Salmonella enterica serovar Enteritidis contamination in ground meat and goat cheese

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SHORT COMMUNICATION

The use of a bacteriophage cocktail as a biocontrol measure to reduce S*almonella enterica* serovar Enteritidis contamination in ground meat and goat cheese

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We evaluated the effectiveness of phages on meats and goat cheese contaminated with *Salmonella* Enteritidis (SE). In meats, reductions of SE were observed during the whole experiment, while in goat cheese a reduction was only observed at day 3. We discuss the relevance of phages as a biocontrol in food.

***Keywords*:** Bacteriophage; Bicontrol; *Salmonella* Enteritidis; meat; cheese

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Bacteriophages are viruses that can be found in several biosystems, as well as in foods, and are considered harmless to humans, animals and plants (Hagens & Loessner 2010). There has been an increasing interest in the application of phages as biological agents capable of reducing bacterial contamination in food. Research has been directed towards reducing *Salmonella* spp*., Listeria monocytogenes, Campylobacter jejuni* and *Escherichia coli* O157:H7, which are responsible for most foodborne disease outbreaks worldwide (Anonymous 2013a). First investigations were made on dairy products (Modi, Hirvi, Hill & Griffiths, 2001), melon and apple slices (Leverentz *et al*., 2001) and later, applied on processed and unprocessed meat (Bigwood, Hudson, Billington, Carey-Smith & Heinemann, 2008), and fresh vegetables and fruits (Pao, Randolph, Westbrook & Shen, 2004), among others. These studies demonstrated that the application of phages significantly reduced the population of the target bacteria, ranging from 0.3 to 5.9 $\log_{10}$ colony-forming unit (CFU). As *Salmonella enterica* serotype Enteritidis (SE) is an important cause of foodborne disease (Alerte et al., 2012; Anonymous 2013a; Anonymous 2013b), this study aims to evaluate the effectiveness of a cocktail of phages at reducing SE in foods.

Fresh chicken, turkey, beef meat and goat cheese were obtained from a supermarket. Prior to analysis, each matrix sample was ground and homogenized in a food processor and checked for the absence of contamination by *Salmonella* spp., in accordance with ISO 6579:2002.

A strain of SE phage type 4, with spontaneous resistance to rifampicin and nalidixic acid was used. The inoculum concentration was adjusted to obtain an optical density of 0.5-0.7 OD at 625 nm (approximately 10^8 CFU.ml^{-1}). Serial dilutions were made to obtain concentrations of 10^3 CFU.ml^{-1} for samples incubated at 18°C and 10^5 CFU.ml^{-1} for samples incubated at 5°C, except for goat cheese, which received 10^4 CFU.ml^{-1} to ensure 100% contamination of samples and simultaneously, to recover the challenge strain.

Five lytic phages with activity against the challenge strain were isolated from sewage samples, pickle sauce and ground beef. Isolation was performed according to Santander and Robeson (2002) to obtain concentrations greater than 10^{10} plaque-forming units (PFU) per ml. For food testing, each phage was diluted to obtain 10^7 PFU.mL^{-1} (for samples incubated at 18°C) and 10^9 PFU.mL^{-1} (for samples incubated at 5°C). The lytic activity of each phage stock was assessed against the challenge strain.

Experimental groups of 25 samples each containing 25 g of each matrix were placed in a sterile bag and contaminated with a SE culture in a volume of 2.5 ml. The samples were then mixed and left to stand for 2 h to adapt to the environment and to adhere to the matrix surface. Thereafter, an aliquot of the phage mixture (1:1:1:1:1) was added and mixed with each contaminated sample in a volume of 10% of the sample weight (Day 0). Each experimental group was incubated at 18 and at 5°C for up to 10 days. Each experimental group included a positive control (25 samples contaminated with SE only), and a negative control group (without SE or phages).

SE and phage counts were performed in duplicate in all experimental groups and their positive controls, at 3, 6 and 10 days post incubation. For this, each sample was homogenized by stomacher for two minutes with 225 ml of BPW. Serial dilutions were performed and 100 µl

of each were plated onto XLD agar plates supplemented with antibiotics. The plates were incubated at 36°C ± 1°C for 24 h. Negative count samples were subjected to enrichment in Rappaport-Vassiliadis broth at 42°C to qualitatively determine the presence or absence of the challenge strain. For samples that were positive upon enrichment, it a value of 10^1 CFU.g $^{-1}$ was assumed. Average values of bacterial counts were expressed in logarithm units (log) and subjected to analysis of variance, with a significance level of 5% ($P \leq 0.05$) (InfoStat® v2008). When there were statistically significant differences, Tukey's test was used. For bacteriophage titration, 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 double-plating using the soft-agar overlay technique with the challenge SE strain as an indicator. The plates were incubated at 37°C for 18-24 h prior to counting of plaques.

In the positive control group, SE did not grow at 5°C (Table 1). At 18°C on day 3, average SE counts increased by about 0.77 to 3.23 $\log_{10}$ CFU of the initial dose, while at days 6 and 10, a general decrease ranging of 1.14 to 2.72 $\log_{10}$ CFU was noted (Table 2).

A significant reduction in SE counts ($P \leq 0.05$) on chicken, turkey and beef meat treated with phages at 3, 6 and 10 days of incubation at 5°C (Table 1) was observed. The highest reductions were observed on beef at day 10 (3.54 $\log_{10}$ CFU), followed by turkey (2.84 $\log_{10}$ CFU) and chicken meat (1.67 $\log_{10}$ CFU). Particularly in beef and turkey meat, the phage cocktail reduced the SE counts below the detection level (10^1 CFU.g^{-1}) in a large number of samples. For goat cheese incubated at 5°C, phages significantly reduced ($P \leq 0.05$) the SE counts by 1.42 $\log_{10}$ CFU only on day 3 (Table 1). Similar situation occurred at 18°C (Table 2).

For phage treated groups incubated at 18°C, regardless of the incubation time, bacterial counts significantly decreased between 0.36 to 3.92 $\log_{10}$ CFU, with a highest value on turkey meat at days 3 and 6 (3.92 and 3.88 $\log_{10}$ CFU, respectively). Generally, it was observed that the highest reductions in SE occurred at day 10, except in chicken and turkey meat where this happened at day 3.

Phage titers were stable throughout the experiment at both incubation temperatures, with a maximum variation of 1.5 $\log_{10}$ PFU for 18°C and 1.9 $\log_{10}$ PFU for 5°C (Data not shown). No phages were isolated from the positive control group, and in the negative control group, no phages or challenge strain were isolated.

It was observed that *Salmonella* Enteritidis at 5°C, did not grow in the positive control group samples. A similar situation was observed by Guenther *et al*. (2012) in RTE food contaminated with *Salmonella* Typhimurium, which increased by approximately 2-5 $\log_{10}$ CFU at 15°C, while at 8°C, counts even decreased from 0.5 to 1.4 $\log_{10}$ CFU.

Our results demonstrate that the application of a phage mixture in a high titer on contaminated fresh ground meat and goat cheese, significantly reduces the number of recoverable bacterial cells, between 0.36 to 3.92 $\log_{10}$ CFU, depending of the food matrix, and the time and temperature of incubation. Phages showed a stable lytic activity at 5°C throught

the experiment; this was expected because the five phages were stable in fresh meat and uncontaminated goat cheese, maintained at 4°C (Robeson, Turra, Huber, and Borie 2014).

Ten days after the treatment with phages, the greatest reduction in counts was observed at 5°C (1.67, 2.8, and 3.54 $\log_{10}$ CFU), consistent with the results obtained by Guenther *et al*. (2012), in chocolate milk and mixed seafood, with reductions of approximately 3 $\log_{10}$ CFU at 8°C, until the sixth day. At 15°C, it resulted in a decrease in the counts of *Salmonella* Typhimurium in the first two days, followed by regrowth of the bacteria during the rest of the incubation period (6 days), while at 8°C, the phage reduced the host cell count, even below detection level.

The effectiveness was improved when incubation time was increased, especially at 18°C. In goat cheese, after three days of incubation, phages were unable to achieve a significant reduction, independent of the temperature. With a longer incubation time, most studies showed a greater effect of phages on bacterial inactivation (Bigwood, Hudson, Billington et al., 2008; Guenther, Huwylere, Richard et al., 2009), although a contact time as short as five minutes may be sufficient to reduce viable cell counts.

According to the matrix type, the best results were obtained in fresh meat but not in goat cheese. Throughout the experiment, goat cheese was progressively drying and it could have limited the phage's spread (Bigwood *et al*., 2008). Guenther *et al*. (2009), observed an apparent immobilization of phages in solid food, probably due to limited diffusion, even if they were not physiologically inactive. Experiences on cheese contaminated with *Staphylococcus aureus* shows that an increase of dry matter may restrict the union between phage and their target bacteria, thereby contributing, to bacterial survival (Bueno, García, Martínez, and Rodríguez 2012).

The stability of the phage titer observed throughout the duration of this study has been also reported by others (Leverentz *et al*. 2003; Modi *et al*. 2001; Guenther *et al*. 2012).

In conclusion, the application of lytic phages as biocontrol agents in fresh meat and cheese, contaminated with *Salmonella* Enteritidis is a feasible, safe, simple and specific tool that contributes to food safety.

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Table 1. Means count of SE (log_{10} CFU/g ± S.D) recovered from food experimentally contaminated with *Salmonella* Enteritidis, treated and untreated with phages and incubated at 5° C.

Food	Day 0$^{\Delta}$ log_{10} CFU/g	Day 3* log_{10} CFU/g ± S.D	Day 6* log_{10} CFU/g ± S.D	Day 10* log_{10} CFU/g ± S.D
Chicken breast				
Control non-phage	5.75	5.25^{a} ± 0.19	5.08^{a} ± 0.22	3.35^{a} ± 0.09
Phage-treated	5.75	4.25^{b} ± 0.39	3.78^{b} ± 0.36	1.68^{b} ± 0.43
Turkey breast				
Control non-phage	5.77	5.44^{a} ± 0.26	5.25^{a} ± 0.15	3.53^{a} ± 0.58
Phage-treated	5.77	3.13^{b} ± 0.54	2.92^{b} ± 0.38	0.69^{b} ± 0.47
Beef meat				
Control non-phage	4.71	4.88^{a} ± 0.23	4.97^{a} ± 0.20	4.42^{a} ± 0.17
Phage-treated	4.71	$3,63^{b}$ ± 0,38	4.10^{b} ± 0.43	0.88^{b}± 0.83
Goat cheese				
Control non-phage	4.67	4.01^{a} ± 0.42	3.13^{a} ± 0.35	1.46^{a} ± 0.40
Phage-treated	4.67	2.59^{b} ± 0.68	3.31^{a} ± 0.65	1.36^{a} ± 0.28

Within temperature, food and day, different letters following values indicate statistical differences ($P \leq 0.05$) between the phage-treated and control samples

$^{\Delta}$: Corresponds to the concentration of SE applied to samples at day 0.
*: Mean and Standard Deviation (S.D) of 25 samples for each group on days 3, 6 and 10

Table 2. Means count of SE (log_{10} CFU/g ± S.D) recovered from food experimentally contaminated with *Salmonella* Enteritidis, treated and untreated with phages and incubated at 18° C.

Food	Day 0$^{\Delta}$ log_{10} CFU/g	Day 3* log_{10} CFU/g ± S.D	Day 6* log_{10} CFU/g ± S.D	Day 10* log_{10} CFU/g ± S.D
Chicken breast				
Control non-phage	3.75	6.48^{a} ± 0.28	5.88^{a} ± 0.40	3.76^{a}±0.48
Phage-treated	3.75	5.43^{b} ± 0.49	5.39^{b} ± 1.12	2.88^{b} ± 0.72
Turkey breast				
Control non-phage	3.77	7.00^{a} ± 0.00	6.84^{a} ± 0.29	5.64^{a}±0.27
Phage-treated	3.77	3.08^{b} ± 1.66	2.96^{b} ± 1.73	2.09^{b}± 0.50
Beef meat				
Control non-phage	3.71	5.22^{a} ± 0.26	5.97^{a} ± 0.71	5.29^{a}±0.71
Phage-treated	3.71	4.86^{b} ± 0.49	4.70^{b} ± 1.49	1.64^{b} ± 1.49
Goat cheese				
Control non-phage	3.67	4.44^{a} ± 2.29	2.43^{a} ± 0.58	3.30^{a}±2.06
Phage-treated	3.67	1.95^{b} ± 1.22	2.16^{a} ± 1.19	2.75^{a}± 0.63

Within temperature, food and day, different letters following values indicate statistical differences ($P \leq 0.05$) between the phage-treated and control samples

$^{\Delta}$: Corresponds to the concentration of SE applied to samples at day 0.
*: Mean and Standard Deviation (S.D) of 25 samples for each group on days 3, 6 and 10