Use of a bacteriophage cocktail to control *Salmonella* in food and the food industry

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# 1. Introduction

Despite all the measures currently implemented in many countries during food production, salmonellosis is the second most commonly reported zoonosis. In the European Union (EU), *Salmonella* is the principal cause of food-borne illness (EFSA, 2012), and in 2010, as in previous years *Salmonella enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium were the serovars most frequently isolated from humans (46.6% and 6.9%, respectively). The principal source of infection is animal-derived foods, mainly eggs and egg products (EFSA, 2012). However, the relevance of vegetables as a source of food-borne outbreaks is increasing and the presence and persistence of *Salmonella* in many vegetables have been reported (Campbell et al., 2001; Greene et al., 2008; Islam et al., 2004; Jain et al., 2009; Little and Gillespie, 2008; Nygård et al., 2008; Weissinger et al., 2000). Many of those studies revealed a possible association between outbreaks of food-borne illness and the contamination of vegetables with fecal pathogens.

Not surprisingly, there is growing concern among producers, regulatory agencies, and consumers about the microbiological safety of food. Disinfection methods such as treatment with organic acids (Dubal et al., 2004; King et al., 2005), water vapor (Gill and Bryant, 1997; Castelo et al., 2001), or both (Pipek et al., 2006) have been used to minimize the microbial contamination of meat but they alter its organoleptic properties. For fruits and vegetables, several different decontamination procedures have been implemented (Warriner et al., 2005), but several factors limit their effectiveness, such as the internalization of food-borne pathogens in fresh produce, biofilm formation by the contaminating bacteria, and the hydrophobicity of plant surfaces (Ollimah and Holley, 2012). The use of bacteriophages has emerged as a promising tool for food preservation and safety (EFSA, 2009; Goodridge and Bisha, 2011; Hagen and Loessner, 2010). The special characteristics of lytic bacteriophages, in particular their target specificity, rapid bacterial killing, and ability to self-replicate, make them especially appropriate for food protection applications. Indeed, the use of bacteriophages in the biological control of different pathogens has been studied in chicken (Atterbury et al., 2003; Fiorentin et al., 2005; Goode et al., 2003; Higgings et al., 2005), pig skin (Hooton et al., 2011) and fresh produce, including sprouts seeds (Pao et al., 2004) and lettuce (Sharma et al., 2009), among others.

The aim of the present work was to determine the effectiveness of a bacteriophage cocktail on several food matrices (pig skin, chicken breast, eggs, and ready to eat lettuce) experimentally contaminated...
with S. Typhimurium and S. Enteritidis. This cocktail was successfully used in a previous study of poultry contaminated with the same bacteria (Bardina et al., 2012).

2. Material and methods

2.1. Bacterial strains and growth conditions

Derivative strains from S. Typhimurium ATCC14028 and S. Enteritidis LK5, were used to experimentally contaminate the foods. The strains were constructed by the insertion of a chloramphenicol (UA1872) and a kanamycin (UA1894) resistance cassette, respectively, in an intergenic sequence of the wild-type strains as described (Bardina et al., 2012). The growth kinetics of these derivative strains did not differ from those of the respective wild-type strains (data not shown).

As needed, overnight cultures of S. Typhimurium UA1872 and S. Enteritidis UA1894 strains were diluted 1:100 in fresh Luria Bertani (LB) broth and incubated at 37 °C until an optical density at 550 nm of 0.3–0.4 was reached. Afterwards, the cultures were appropriately diluted in NaCl 0.9% to achieve the bacterial concentration used to contaminate the different food matrices studied. Viable counts of strains UA1872 and UA1894 in food matrices were routinely determined by plating on xylose lysine deoxycholate (XLD) agar plates (Pronadisa), supplemented with chloramphenicol (3.4 μg/ml) and kanamycin (75 μg/ml), respectively, followed by incubation at 37 °C for 24 h.

2.2. Bacteriophages

The lytic bacteriophages UAB Phi20, UAB Phi78, and UAB Phi87 were chosen from our collection of Salmonella bacteriophages based on their broad host range and the high killing kinetics observed in vitro Salmonella cultures (Bardina et al., 2012). These bacteriophages are protected by a patent application (PCT/EP2012/064797).

The bacteriophage cocktail consisted of a previously described 1:1:1 mixture of the three bacteriophages, each at a titer of 1011 pfu/ml. The cocktail was diluted in MgSO4 10 mM to reach the concentration required for food treatment. The bacteriophage titer was determined by plating adequate dilutions onto lawns of the appropriate bacterial strain.

2.3. Preparation of samples, Salmonella contamination, and bacteriophage treatment

2.3.1. Pig skin

Pig skin from the ventrolateral region of a swine carcass was acquired from a local butcher the day after the slaughter and kept at 4 °C until used in the experiment. The skin was prepared and treated as in the slaughtering process, i.e., hair and dirt were removed followed by cleaning with 70% ethanol, prior to its use in the assays. Sanitized skin pieces were kept, before and after treatment, in polypropylene trays covered with aluminum foil. The trays were previously disinfected with 70% ethanol and UV-irradiated at a dose of 0.7 J/m2 for 30 min. The absence of Salmonella and bacteriophages was confirmed before the skin was contaminated with the two test strains of Salmonella.

The effectiveness of the bacteriophage cocktail was studied in skin pieces 900 cm2 in size that were experimentally contaminated with strains UA1894 or UA1872. A sterile disposable Digralsky spreader was used to homogeneously distribute 1.8 ml of the respective bacterial suspension (1 × 106 cfu/ml) over the skin. Each contaminated skin piece was divided in half, allowed to dry in a biological safety cabinet (Nuaire) at room temperature for 30 min, and then treated with 10 mM MgSO4 (control) or with the bacteriophage cocktail at 1010 pfu/ml. The entire surface of the treated skin was sprayed with 4 ml of the cocktail [multiplicity of infection (MOI) of 4.4 × 106 pfu/cfu]. Both skin halves were dried in a biological safety cabinet at room temperature for 30 min and then incubated at 33 °C, which is the peripheral body temperature of pigs (Huynh et al., 2005). Six samples (25 cm2) were removed from each skin half after 0, 3, and 6 h of treatment and the viable bacterial cell count was determined. Samples were obtained by swabbing the skin surface. The swabs were placed in 2 ml of buffered peptone water (BPW; Merck), homogenized, and the viable counts determined as explained above. Based on these counts, the average bacterial concentration, expressed as cfu/cm2, was calculated for each sampling time. In addition, bacteriophage counts (pfu/cm2) from these swabs were determined by plating adequate bacteriophage dilutions onto lawns of the appropriate bacterial strain.

Bacterial reduction was calculated by subtracting the average bacterial concentration, expressed in log units, on skin pieces treated with the bacteriophage cocktail from that on untreated skin.

2.3.2. Poultry

Fourty-two fresh eggs were acquired at a local supermarket and in the laboratory were aseptically cut into pieces of approximately 30 g. In each experiment, 47 pieces of meat were used, including two that were tested at the beginning of the experiment to confirm the absence of Salmonella. The efficacy of the bacteriophage cocktail was determined by experimentally contaminating the 45 remaining pieces with Salmonella during a 5-min immersion at room temperature in a 106 cfu/ml suspension of strain UA1872 or UA1894 (ratio 1:2 w/v). Excess liquid was removed and the breast pieces were dried in a biological safety cabinet for 15 min at room temperature. Each piece was then transferred to a Whirl-Pak bag (Nasco, Fort Atkinson, WI) and either 100 ml of the bacteriophage cocktail at 1010 pfu/ml (MOI of 1.0 × 103 pfu/cfu) or an equivalent volume of NaCl 0.9% (n = 25) was added. The bags were incubated at room temperature with agitation for 5 min, the excess liquid was removed, and the bags were kept at 4 °C for 7 days. The concentration of Salmonella was determined in five samples not treated with bacteriophages just after Salmonella inoculation and at 1, 2, 5, and 7 days post-contamination and in five bacteriophage-treated samples at 1, 2, 5, and 7 days post-contamination. In addition, in the latter group of samples, bacteriophage concentrations were determined. To quantify Salmonella and bacteriophages, 100 ml of BPW was added to each bag and the contents were homogenized (Bagmixer, Interscience) at maximum speed for 60 s and then diluted and plated as described above. Reduction of Salmonella concentration was calculated as described above.

2.3.3. Fresh eggs

Forty-two fresh eggs were acquired at a local supermarket and their lengths and widths were measured with a caliper (Mitutoyo, Japan). Prior to the assays, the eggs were cleaned with gauze soaked in 70% ethanol and the absence of Salmonella was confirmed in two of them. The efficacy of the bacteriophage cocktail was tested in the remaining 40 eggs by dipping them for 5 min at room temperature into a suspension containing 107 cfu/ml of Salmonella strain UA1894 or UA1872 (200 ml/egg). The eggs were dried for 30 min in a biological safety cabinet at room temperature and then incubated for 2 h 30 min at room temperature. Next, 20 of the contaminated eggs were sprayed with the bacteriophage cocktail at 1 × 1010 pfu/ml (MOI of 1.0 × 103 pfu/cfu) and the remaining 20 eggs with 10 mM MgSO4. All eggs were then incubated for 2 h at room temperature. Bacteria and bacteriophages were subsequently recovered by breaking the egg, using a sterile tweezers and sanitized gloves and then removing their contents but keeping the shells. The latter were placed in bottles with 20 ml of BPW and shaken for 1 min at room temperature. Salmonella cells and the bacteriophages were quantified as described above. Bacterial and bacteriophage concentrations were expressed as cfu/cm2 and pfu/cm2, respectively, according to the formula S = (3.155 – 0.0136 L + 0.0115B) × L × B; where S is the surface of the egg, L its length, and B its width (Narusin, 2005). Reduction of Salmonella concentration was calculated as described above.

2.3.4. Fresh lettuce

Packed fresh-cut romaine lettuce (Lactuca sativa var. longifolia) was purchased from a local supermarket. After the absence of Salmonella
contamination had been confirmed in two samples (25 g each), the remainder were experimentally contaminated by immersion for 5 min at room temperature in a suspension of strain UA1872 or strain UA1894 (1:10 v/w) at 10^5 cfu/ml. Excess liquid was removed and the lettuce was dried in a biological safety cabinet for 15 min at room temperature. Afterwards, 25 Whirl-Pak bags were prepared by adding 25 g of lettuce to each one along with 100 ml of either bacteriophage cocktail at 10^6 pfu/ml (n = 10 bags) (MOI of 1.0 × 10^4 pfu/cfu) or NaCl 0.9% (n = 15 bags). All of the bags were maintained at room temperature for 60 min with 100 rpm of agitation. The Salmonella concentration was determined at 0, 30, and 60 min in the control bags and at 30 and 60 min in the bags containing the bacteriophage-treated lettuce. For both conditions, the number of viable bacterial cells in five bags was determined at each time point by removing the excess liquid and adding 100 ml of BPW, followed by homogenization with a Bagmizer at maximum speed for 60 s. Salmonella and bacteriophages were quantified as described above. Similarly, reduction of Salmonella concentration was calculated as mentioned before.

2.4. Statistical analysis

The logarithm of the bacterial concentrations was determined for the treated and untreated groups in each experiment and the respective values subsequently compared. The database was created using the software: SAS v9.2 (SAS Institute Inc., Cary, NC, USA). The normality within each group was analyzed using the Shapiro-Wilk test. For the analysis of untreated vs. treated groups, a nonparametric (Kruskal-Wallis) test was applied. Each treated sample was compared to its control counterpart and to the t = 0 h sample using a nonparametric bivariate (Wilcoxon) test. The significance level was fixed at 5%.

3. Results

A bacteriophage cocktail composed of a 1:1:1 mixture of the bacteriophages UAB_Phi20, UAB_Phi78, and UAB_Phi87 was evaluated for Salmonella biocontrol in pig skin, two representatives of poultry production (chicken breasts and fresh eggs), and ready to eat product, i.e., packaged lettuce.

In experiments evaluating the cocktail's effectiveness in disinfecting pig skin, a remarkable reduction (p ≤ 0.005) in the concentrations of S. Typhimurium and S. Enteritidis was determined 3 and 6 h post-treatment (Table 1). Both times were chosen because 6 h (Mora M.T., Animal and Food Science Department, Universitat Autònoma de Barcelona, personal communication) and 3 h (Warris et al., 1998) are the duration of the pre-slaughter withdrawal period in Spain and in other countries, respectively. In addition, it should be noted that the bacteriophage concentration remained constant, at about 7 log_{10} pfu/cm², throughout the experiment (Table 1).

Chicken breasts were treated with the bacteriophage cocktail for 5 min and then maintained for 7 days at 4 °C. This experimental design would simulate the method used by the food industry to treat meat with bacteriophages during the pre-packaging washing process and the storage conditions before the meat is consumed. This treatment significantly (p ≤ 0.0001) reduced bacterial cell counts after 7 days at 4 °C. By day 7 of treatment, the S. Typhimurium concentration was 2.2 log_{10} cfu/g lower than the initial concentration (Table 2) whereas the reduction in S. Enteritidis counts was greatest on day 5 post-treatment (1.4 log_{10} cfu/g) and somewhat less on day 7 (0.9 log_{10} cfu/g). Again, the bacteriophage concentration remained invariable throughout the experiment, in the range of 7–7.5 log_{10} pfu/g (Table 2).

The ability of the bacteriophage cocktail to reduce Salmonella contamination in fresh eggs was also demonstrated, with a significant decrease of 0.9 log_{10} cfu/cm² (p ≤ 0.005) in the concentration of both Salmonella serovars (Table 3). The bacteriophage concentration in the eggs after 2 h of treatment was −6 log_{10} cfu/cm². Likewise, there was a reduction of the Salmonella concentration in lettuce (Table 4) treated with bacteriophage and then kept under the same conditions typically used by the consumer to soak vegetables at home before consuming. The cocktail was more effective in S. Typhimurium, with a decrease of 3.4 and 3.9 log_{10} cfu/g (p ≤ 0.005) after 30 and 60 min of immersion, than in S. Enteritidis, in which a significant reduction of 1.9 and 2.2 log_{10} cfu/g, after 30 and 60 min of treatment, respectively (p ≤ 0.005, Table 4) was observed. As in the other tested food products, the bacteriophage concentration in the treated lettuce samples was maintained at high levels, between 8.4 and 9 log_{10} pfu/g.

4. Discussion

Ensuring food safety is a complex process that depends on the implementation of a wide range of coordinated control measures at all levels of the food production chain (based on the farm-to-fork principle). Among the various approaches to food safety currently under exploration, bacteriophages have emerged as a novel tool for the biocontrol of bacterial contamination in foods and are thus of particular interest to the food industry.

In this study, we made use of a bacteriophage cocktail composed of a mixture of three bacteriophages (UAB_Phi20, UAB_Phi78, and UAB_Phi87) that in a previous study was successfully utilized to control bacterial contamination in poultry (Bardina et al., 2012). The effectiveness of this bacteriophage cocktail on the biocontrol of Salmonella was confirmed and expanded in the present work, both in a side product of food production (pig skin) and in several end-products (poultry meat, eggs, and lettuce).

The prevalence of Salmonella in slaughtered pigs in the EU was >30% in 2009 (EFSA, 2011). At the slaughterhouse, the pre-slaughter withdrawal period is a critical determinant of the microbiological quality of the final meat products (Lo Fo Wong et al., 2002), since during this time the animals may be exposed to pathogens such as Salmonella (Berends et al., 1996; Issacson et al., 1999) and skin infections may result in cross-contamination during subsequent processing (Botteldoorn et al., 2004; Farzan et al., 2006). Therefore, eliminating or decreasing the concentration of Salmonella from the animal skin surface is essential and, in terms of food safety, is likely to be most effective if achieved before the animals enter the meat production line.

The treatment of experimentally contaminated pig skin with the bacteriophage cocktail resulted in a very significant reduction in the concentration of both serovars of Salmonella after 3 h. The reduction was maintained or even accentuated 6 h post-treatment (Table 1). To our knowledge, only one other study achieved a significant reduction in Salmonella-contaminated pig skin (Hooton et al., 2011), although the

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<th>Time (h)</th>
<th>Bacteriophage treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Concentration&lt;sup&gt;b&lt;/sup&gt; (log&lt;sub&gt;10&lt;/sub&gt; cfu/cm²)</th>
<th>Reduction&lt;sup&gt;c&lt;/sup&gt; (log&lt;sub&gt;10&lt;/sub&gt; cfu/cm²)</th>
<th>Bacteriophage concentration (log&lt;sub&gt;10&lt;/sub&gt; pfu/cm²)&lt;sup&gt;d&lt;/sup&gt;</th>
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<tr>
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<tr>
<td>0</td>
<td>−</td>
<td>2.6 ± 0.2</td>
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<tr>
<td>3</td>
<td>−</td>
<td>2.9 ± 0.1</td>
<td>2.2 ± 0.1</td>
<td>ND</td>
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<td>6</td>
<td>−</td>
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<td>3.2 ± 0.05</td>
<td>0.03 ± 0.05</td>
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<td>+</td>
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<td>ND</td>
<td>1.2 ± 0.1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.1 ± 0.1&lt;sup&gt;e&lt;/sup&gt;</td>
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<sup>a</sup> Each value is the average of six skin samples ± standard error.
<sup>b</sup> Salmonella colony counting was only possible in three of the six samples plated. In the other three samples, colonies were not observed.
<sup>c</sup> The differences between untreated and treated samples were statistically significant (p ≤ 0.005) in all cases.
<sup>d</sup> ND, not detected.
foodstuffs maintained at 4 °C, due to the slower rate of growth and its influence on bacteriophage replication at that temperature. Other reasons justifying the use of a high-dose cocktail could be the potential inability of the bacteriophages to diffuse through the food matrix and their possible inactivation by components thereof, both of which can hamper the encounter with bacteria, above all when the bacterial load is small.

Eggs and egg products were the most frequently identified food vehicles of salmonellosis outbreaks in humans, accounting for 43.7% of cases (EFSA, 2012). Moreover, according to Regulation (EC) no 589/2008 of the European Commission, fresh eggs cannot be treated or chilled. This would not exclude the use of a bacteriophage-based biocontrol method to prevent contamination with Salmonella given the demonstrated safety of bacteriophages in this setting (Brovko et al., 2012) and the fact that they do not alter the characteristics of food products. However, despite the significant reduction of Salmonella promoted by the bacteriophage cocktail in the eggs tested under the conditions of this study, it was not as high as that obtained in the other matrices (Table 3). As suggested by other authors (Himathongkham et al., 1999), these results may have been due to variations in the Salmonella concentrations of the eggshells, which in turn can be attributed to the characteristics of the eggshell surface. Thus, the effectiveness of the bacteriophage may have been influenced by the translocation of Salmonella from the surface of the eggshell to its outer and inner membranes (Miyamoto et al., 1998). Nonetheless, our results demonstrate the potential of using a bacteriophage cocktail for the biocontrol of Salmonella in fresh eggs although its maximally effective application remains to be determined.

In last few years, vegetables have been implicated as potential vehicles of bacterial pathogens, including Salmonella. These foods can become contaminated via numerous routes, i.e., soil, feces, manure, irrigation water, fungicide and insecticide solutions, dust, insects, wild and domestic animals, and human manipulation (Islam et al., 2004), and cannot be completely disinfected by traditional methods (Legnani and Leoni, 2004). Bacteriophages offer a natural alternative to achieve food safety (Greer, 2005) and their potential as a Salmonella biocontrol agent in minimally processed vegetables, such as packaged romaine lettuce, and other food products is supported by our findings. Specifically, in lettuce immersed in the phage cocktail significant reductions of 3.9 and 2.2 log_{10} cfu/g in the concentrations of S. Typhimurium and S. Enteritidis, respectively, were achieved (Table 4). These results are noteworthy because the use of bacteriophages for the biocontrol of Salmonella-contaminated vegetables has received little attention and previously reported results on their effectiveness in seeds, sprouts, and fruits have been inconsistent (Kochanuchi et al., 2009; Leverentz et al., 2001; Pao et al., 2004; Ye et al., 2010).

In conclusion, this study provides several examples of the effectiveness of a bacteriophage cocktail composed of lytic Salmonella bacteriophages (UBA_Phi20, UAB_Phi78, and UAB_Phi87) as a natural biocontrol agent of this pathogen. The food matrices and treatment

| Table 2 | Effectiveness of a bacteriophage cocktail in reducing the concentrations of S. Typhimurium UA1872 and S. Enteritidis UA1894 in chicken breast. |
| --- | --- | --- | --- | --- |
| Time (day) | Bacteriophage treatment* | Concentration (log_{10} cfu/g)^b | Reduction (log_{10} cfu/g) | Bacteriophage concentration (log_{10} pfu/cm²)^b |
| | | UA1872 | UA1894 | UA1872 | UA1894 | UA1872 | UA1894 |
| 0 | − | 4.6 ± 0.3 | 4.4 ± 0.1 | | | | |
| 1 | − | 4.4 ± 0.2 | 4.4 ± 0.1 | | | | |
| 2 | − | 2.8 ± 0.2 | 3.7 ± 0.2 | 1.6 | 0.7 | 7.6 ± 0.2 | 7.0 ± 0.2 |
| | + | 4.2 ± 0.1 | 4.4 ± 0.2 | | | | |
| 5 | − | 2.6 ± 0.2 | 3.4 ± 0.1 | 1.6 | 1.0 | 7.6 ± 0.2 | 7.0 ± 0.2 |
| | + | 4.0 ± 0.1 | 4.0 ± 0.1 | | | | |
| 7 | − | 2.6 ± 0.2 | 2.6 ± 0.8 | 1.4 | 1.4 | 7.4 ± 0.2 | 6.9 ± 0.2 |
| | + | 4.2 ± 0.1 | 4.0 ± 0.2 | | | | |
| | 2.9 ± 0.2 | 3.1 ± 0.3 | 2.2 | 0.9 | 7.8 ± 0.2 | 7.1 ± 0.2 |

The differences between untreated and treated groups were statistically significant (p ≤ 0.0001) in all cases.

* a, untreated samples; +, bacteriophage-treated samples.

b Each value is the average of twenty eggshell samples ± standard error as determined after 2 h of bacteriophage treatment.

| Table 3 | Effectiveness of a bacteriophage cocktail in reducing the concentrations of S. Typhimurium UA1872 and S. Enteritidis UA1894 in fresh eggs. |
| --- | --- | --- |
| Bacterial strain | Bacteriophage treatment* | Concentration (log_{10} cfu/cm²)^b | Reduction (log_{10} cfu/g) |
| | | UA1872 | UA1894 | UA1872 | UA1894 |
| UA1872 | − | 3.8 ± 1 | | | |
| | + | 2.9 ± 0.5 | | 0.9 | |
| UA1894 | − | 4.5 ± 0.7 | | | |
| | + | 3.6 ± 1 | | 0.9 | |

The differences between untreated and treated groups were statistically significant (p ≤ 0.005) in all cases.

After 2 h of treatment, the bacteriophage concentration was ~6 log_{10} pfu/cm².
conditions used in this study mimicked those commonly encountered in food manufacturing processes. Moreover, our results support the use of bacteriophages as a novel tool for the biocontrol of food-borne pathogens such as *Salmonella*.

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