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Use of bacteriophages to reduce *Salmonella* in chicken skin in comparison with chemical agents

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ABSTRACT

Salmonella spp. is one of the major causes of foodborne diseases and a problem for the poultry industry. Bacteriophages have emerged as a promising agent to reduce pathogens in foods. Here, we compared bacteriophages and the classically used chemical agents for reducing *S.* enteritidis counts in chicken skin. Five phages were isolated from chicken feces, characterized and selected to be used as biosanitizer. Bacteriophages were assigned to the family *Podoviridae* and revealed identical RFLP profiles, although they showed a different host range and replication dynamics. Bacteriophages were found to be effective at a multiplicity of infection (MOI) of 10 at both 37 °C and 25 °C in liquid medium, with no significant reductions taking place when MOI was less than 10. When samples of chicken skin experimentally contaminated with 1×10^5 CFU/cm^2 *S.* enteritidis were treated with phage cocktail or chemical agents, similar reductions of about 1 log CFU/cm^2 were observed. These data suggest that bacteriophages can be employed as an alternative agent to reduce *S.* enteritidis contamination of poultry carcasses in industrial conditions.

1. Introduction

Salmonella spp. has long been recognized as an important foodborne pathogen and a major public health burden worldwide. According to the Centers for Disease Control and Prevention (CDC), 40,000 cases of salmonellosis are reported annually in the USA (CDC, 2009). Furthermore, non-typhoid salmonellosis is regarded to be the second most common cause of foodborne zoonotic infection in Europe and more than 100,000 cases of human salmonellosis were reported in 2009 (European Food Safety Authority—EFSA [EFSA], 2011).

Although more than 2500 serovars of *Salmonella* enterica have been identified (Grimont & Weill, 2007), the human infections are caused by a limited number of serovars. *S.* enteritidis and *S.* typhimurium belong to the most common serovars isolated during outbreaks of foodborne salmonellosis in the United States and European Union (EFSA, 2011; Finstad, O'Bryan, Marcy, Crandall, & Ricke, 2012). Most infections are associated with food consumption of animal origin, and poultry meat and by-products are considered to be the major vehicles of *Salmonella* spp. to humans (Marin & Lainez, 2009). The contamination may occur throughout the poultry production chain, and processing steps such as head pulling and evisceration are considered potential risk factors that contribute to high incidence of *Salmonella* spp. in chicken carcasses (Muth, Fahimi, & Karns, 2009; Rasschaert et al., 2008; Sams, 2001). Many decontamination technologies based on physical, chemical, and biological approaches have been subjected to scientific trials over the years in order to reduce the microbial meat contamination (Hugas & Tsigarida, 2008). Physical interventions have been used or are being developed to reduce the bacterial contamination on poultry carcasses. They include mainly, water-based and steam treatments, irradiation, ultrasound, high hydrostatic pressure processing and pulsed electric field processing (Buncic & Sofos, 2012; Mukhopadhyay & Ramaswamy, 2012). Amongst these physical methods, water-based and steam treatments have been used frequently for the decontamination of poultry carcasses, and yielded reductions for various bacterial species in the range from 0.9 to 2.1 and 2.3 to 3.8 orders of magnitude, respectively (Loretz, Stephan, & Zweifel, 2010). Changes on the appearance and quality of carcasses, the need for equipment and operator training, and consumer rejection constitute major limitations for application of physical methods. On the other hand, several chemicals such as trisodium phosphate, chlorine-based compounds and organic acids are used in some countries to assist in the reduction of microorganisms in poultry carcasses during slaughter and processing (Del Río, Panizo-Morán, Prieto, Alonso-Calleja, & Capita, 2007; Dinçer & Baysal, 2004). For example, exposure to trisodium phosphate, chlorine-based compounds, and lactic or acetic acid reduced *Salmonella* contamination

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by 0.6–2.3, 0.9–1.1, and 0.8–2.2 log units, respectively (Buncic & Sofos, 2012). These chemical compounds used for the decontamination of poultry carcasses are not selective and act against a wide variety of microorganisms. However, the applicability of some chemicals is limited due to increasing costs, negative effect on sensorial properties, concentrated substances might constitute a health hazard or ecological menace, and some agents show corrosive properties, instability in solution or inactivation by organic matter (Lillard, 1990; Loretz et al., 2010). Moreover, chemical agents are not allowed to be used in the removal bacterial contamination on meat surface in the European Union (OJEC, 2009). The use of chemical agents for decontamination of chicken carcasses has hampered trade relations between the countries. In this context, the search for new decontamination technologies has recently gained the interest of the poultry industry. Bacteriophages are bacterial viruses that have great potential for use as biocontrol agents in foods (Coffey, Mills, Coffey, McAuliffe, & Ross, 2010; Mahony, McAuliffe, Ross, & Sinderen, 2011). They offer a number of desired properties such as specificity for target bacteria, inability to infect human cells, capacity for self-replication and self-limiting, and ubiquitous presence in nature, that makes them excellent tools for food safety purposes (Mahony et al., 2011; Rohwer & Edwards, 2002). However, the major barriers for phage application in bacterial control are the possible emergence of phage-resistant derivatives, phage activity as vectors of virulence and antibiotic-resistance genes, lysogenic conversion, and other adverse factors such as temperature, pH and food compounds which limit the phage action (García, Martínez, Obeso, & Rodríguez, 2008). Therefore, the characterization and selection of bacteriophage are important steps to ensure their safe and effective use as biocontrol agents.

A few publications have demonstrated that bacteriophages can be used to successfully reduce *Salmonella* spp. in foods, especially meat and poultry products (Bielke, Higgins, Donoghue, Donoghue, & Hargis, 2007; Bielke et al., 2007; Bigwood, Hudson, Billington, Carey-Smith, & Heinemann, 2008; Fiorentin, Vieira, & Barioni, 2005; Goode, Allen, & Barrow, 2003; Guenther, Huwyler, Richard, & Loessner, 2009; Hooton, Atterbury, & Connerton, 2011). However, to our knowledge, no previous study has tested the bacteriophage activity in short contact time nor compared its effectiveness with sanitizers, so that it can be applied directly to the poultry processing line.

The major aim of this study was to compare the efficacy of bacteriophages and sanitizers for control of *S.* enteritidis in artificially-contaminated chicken skin, in order to obtain new possibilities for poultry decontamination towards non-chemical methods.

2. Material and methods

2.1. Bacterial species and growth conditions

The bacterial strains (see Table 1) were provided by Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, Brazil, and grown at optimum conditions (37 °C/24 h) in Brain Heart Infusion medium (BHI—Himedia, Mumbai, India). *S.* enterica subsp. enterica serovar enteritidis was used as host for isolation and propagation of bacteriophages and contamination of chicken skin. The other bacterial strains were used to evaluate the host range of isolated bacteriophages. All bacteria were subcultured once on BHI agar plates at 37 °C for 24 h, except *Campylobacter jejuni*, which was incubated at 37 °C for 48 h under microaerophilic atmosphere (5% O_2, 10% CO_2, 85% N_2). After incubation, the colonies were harvested, deposited in BHI broth containing glycerol (20% v/v) and stored frozen at −80 °C until further use.

2.2. Chicken skin samples

Chicken skin was removed aseptically from fresh chicken portions, frozen at −20 °C and cut into approximately 4 cm^2 sections. These chicken skin sections were irradiated at 10 kGy (Nordion, IR214, GB 127—MSD Nordion, Canada) to eliminate the natural microbiota and then stored frozen (−20 °C) in a nylon-polyethylene package until further use. Some chicken skin sections were deposited in BHI broth and incubated at 37 °C for 48 h to confirm the microorganism absence.

Table 1
Lytic spectrum of bacteriophages isolated from chicken feces on salmonellae and other foodborne pathogens.

Microorganisms	Phages (phiSE)				
	7	16	18	36	43
S. typhi ATCC 6539	+	+	+	+	+
S. typhimurium ATCC 14028	+	+	+	+	+
S. enteritidis ATCC 13076	+	+	+	+	+
S. gallinarum ATCC 9184	+	+	+	+	+
S. pullorum ATCC 9120	+	+	+	+	+
S. abony NCTC 6017	+	+	+	+	+
S. choleraesuis ATCC 10708	−	+	+	−	−
S. arizonae ATCC 13314	−	−	−	−	−
Escherichia coli ATCC 11229	−	+	+	−	−
Klebsiella pneumoniae ATCC 10031	−	−	−	−	−
Enterobacter aerogenes ATCC 13048	−	−	−	−	−
Campylobacter jejuni NCTC 12662	−	−	−	−	−
Listeria monocytogenes ATCC 7644	−	−	−	−	−
Enterococcus faecalis ATCC 19433	−	−	−	−	−
Staphylococcus aureus ATCC 6538	−	−	−	−	−

(−) Absence of phage plaques; (+) presence of phage plaques.

2.3. Bacteriophage isolation and purification

The bacteriophages were isolated from free-range chicken feces collected from 16 different poultry farms located in the region of Viçosa-MG. Feces samples from 20 different poultry were mixed to compose the sample units from each poultry farm. A total of 45 feces samples were collected and subjected to bacteriophage isolation according to the method described by Atterbury et al. (2005) with some modifications. Briefly, 1 g of chicken feces was diluted 1:10 in SM buffer (50 mM Tris–HCl, Sigma-Aldrich, Saint Louis, USA [pH 7.5], 0.1 M NaCl—Vetec, Rio de Janeiro, Brazil, 8 mM $MgSO_4 \cdot 7H_2O$—Chemco, São Paulo, Brazil, 0.01% gelatin—Merck New Jersey, USA), resuspended by gentle inversion and then incubated overnight at 17 °C on a gyratory platform shaker (Bio Braun Biotech International, Melsungen, Germany) at 150 rpm to allow phage elution into the buffer. The suspension was afterwards centrifuged (Centrifuge Sigma template 3K30, Osterode am Harz, Germany) for 5 min, 13,000 ×*g*, 4 °C to remove bulk debris and then filtered through a 0.22-μm-pore-size membrane filter (Sigma-Aldrich, Saint Louis, USA) to remove any remaining bacterial cells. Phage detection was done by spotting the phage lysate on *S.* enteritidis lawns as described by Adams (1959). These plates were incubated at 37 °C for 12 h and inspected for plaques. A clear zone in the plate, resulting from the lysis of host bacterial cells, indicated the presence of phage. A unique lysis plaque from each positive sample of chicken feces was purified by serial dilution and plating to soft agar overlays for containing only one single type of phage. Each phage isolate was subjected to a minimum of three successive rounds of serial plaque purification and final lysates were stored in SM buffer at 4 °C for further use.

2.4. Bacteriophage propagation and concentration

Concentrated phage solutions were produced using the plate lysis and elution method as described by Sambrook and Russell (2001) with some modifications. A top agar was prepared containing 1 mL of phage solution and 1 mL of a bacterial overnight culture in 5 mL of soft-agar and applied to the surface of BHI agar plates. After solidification of the top agar layer the plates were incubated at 37 °C overnight. Afterwards, the propagation plates were examined and the

degree of lysis recorded. Five mL of sterile SM buffer was added to the surface of each plate and the phages eluted overnight at 17 °C on a gyratory platform shaker (60 rpm). The phage and cell suspensions were harvested and centrifuged (5 min, 13,000 ×*g*, 4 °C) and the supernatant was then filtered through a 0.22-μm-pore-size membrane filter. Phage stocks were stored in SM buffer at 4 °C until further use.

2.5. Bacteriophage titration

Bacteriophage titer was analyzed as described by Adams (1959). Briefly, phage stocks were serially diluted in SM buffer to achieve a concentration that would provide individual lysis plaques in a bacterial lawn. Aliquots of 100 μL of diluted phage solution, 100 μL of a bacterial overnight culture, and 3 mL of molten agar were mixed and poured onto BHI agar plates. Plates were incubated overnight at 37 °C and the number of plaques was counted on the appropriate dilutions giving between 10 and 100 plaques.

2.6. Electron microscopy

Bacteriophage morphology exam was performed as described by Oliveira et al. (2009) with some modifications. Phage particles were sedimented by centrifugation at 25,000 ×*g* for 60 min. Phages were washed twice in 0.1 M ammonium acetate pH 7.0 (Vetec, Rio de Janeiro, Brazil), filtered through a 0.22-μm-pore-size membrane filter, deposited on copper grids provided with carbon-coated Formvar films (Canemco Inc., Quebec, Canada), stained with 2% uranyl acetate (Sigma-Aldrich, Saint Louis, USA) and examined in a Zeiss EM109 electron microscope (performed by Microscope Center, Federal University of Viçosa, Brazil).

2.7. DNA extraction and restriction enzyme digestion

Purified phage suspensions were previously treated with DNase I (Sigma-Aldrich, Saint Louis, USA) to disrupt bacterial DNA and the phage capsids were disintegrated using proteinase K (Sigma-Aldrich, Saint Louis, USA), ethylenediaminetetraacetic acid—EDTA (Sigma-Aldrich, Saint Louis, USA) and sodium dodecyl sulfate—SDS (Sigma-Aldrich, Saint Louis, USA). Phage DNA were extracted by phenol/chloroform method and precipitated by standard ethanol procedure (Sambrook & Russell, 2001). DNA was digested with *Cfo*I and *Hinf*I (Promega, Madison, USA) following the manufacturer's recommendations. The obtained fragments were visualized after 1% agarose gel (Sigma-Aldrich, Saint Louis, USA) electrophoresis with Blue Green Loading Dye (LGC Biotecnologia, São Paulo, Brazil) and illumination by UV light.

2.8. Phage lytic spectrum

The host range of each phage was determined against 15 bacterial strains representing eight genera and including eight *Salmonella* serovars (Table 1). Susceptibility of various bacterial strains was tested using the drop-on-lawn technique (Adams, 1959). Aliquots of 10 μL of serial dilutions of the different phage suspensions were added to bacterial lawns. The plates were incubated overnight under optimal condition for each strain, and the lytic activity was checked for the formation of clear areas and phage plaque formation on the bacterial lawns.

2.9. Effects of phage concentration and incubation temperature

Overnight culture of *S.* enteritidis was sub-cultured (1% v/v) in fresh BHI broth and incubated at 37 °C or 25 °C under agitation (150 rpm) until cultures reached an OD_{600} of 0.5, approximately 10^8 CFU/mL. Afterwards, bacteriophages were added to final concentrations of 10^3, 10^6 or 10^9 PFU/mL and cultures were re-incubated. *S.* enteritidis cultures without bacteriophage addition were used as control. Bacterial growth was monitored by measuring absorbance at 600 nm (Spectrophotometer Model SP-22, Biospectro, São Paulo, Brazil). This step was used to define phage concentration that should be applied in chicken skin decontamination.

2.10. Salmonella reduction in chicken skin

To determine the effect of bacteriophages and sanitizers on artificially-contaminated chicken skin with *S.* enteritidis, we used an experiment structured by a randomized complete block design with repetition. The treatments were arranged in 10 blocks with three replicates per block. The experimental units of each block were derived from a single poultry. The sections of chicken skin were inoculated with 10^5 CFU/cm^2 and the inoculum was uniformly spread over each side using sterile disposable spreaders. After a drying period of 30 min at 25 °C, the sections of chicken skin were divided into six batches, each containing thirty sections. Samples in threes batches were dipped into 100 mL of decontamination agents, respectively of 200 mg/L sodium dichloroisocyanurate for 10 min; 100 mg/L peracetic acid for 10 min and 2% (v/v) lactic acid for 90 s. These conditions of use are allowed in some countries such as US, and generally applied in decontamination of poultry carcasses during slaughter and processing. The chemical agents were prepared just before use according to the instructions of the manufacturer (Nippon Chemical, São Paulo, Brazil) and their concentrations were determined on each repetition. Samples of another batch were dipped into 100 mL of phage cocktail at 10^9 PFU/mL for 30 min, composed of equal 20 mL volumes of five phages in SM buffer. Samples in the remaining two groups were dipped into 100 mL of sterile distilled water for 30 min (water-dipped control) or not dipped (untreated control). All decontamination agents were previously cooled at 6 °C to simulate industrial conditions. After treatment, sections of chicken skin were deposited in sterile stomacher bags, added to 10 mL volumes of specific solutions to inactivate each decontamination agent, subjected to stomaching and serially diluted. The sections of chicken skin treated with sodium dichloroisocyanurate or peracetic acid were added to buffered peptone water (00.1% w/v) containing sodium thiosulfate (00.25% w/v), while those treated with lactic acid were added of phosphate buffered saline (3.6 mM KH_2PO_4 [Sigma-Aldrich, Saint Louis, USA], 11.4 mM Na_2HPO_4 [Sigma-Aldrich, Saint Louis, USA] and 29 mM NaCl [Vetec, Rio de Janeiro, Brazil]—pH = 7.2 ± 0.2). Skin samples subjected to other treatments were diluted in saline (0.85% w/v).

The droplet method as described by Morton (2001) was applied for quantitative determination of *S.* enteritidis counts. Aliquots of 20 μL of serial dilutions were deposited onto XLT4 agar plates (Difco, Sparks, USA) and incubated at 37 °C for 6–8 h. *S.* enteritidis plate counts were expressed in CFU/cm^2.

2.11. Demonstration of phage activity at low temperature

Additional experiments were carried out to ensure that phage activity occurred on the chicken skins at low temperature and not simply on the surface of the agar plate during counting (Goode et al., 2003). Equal 0.1 mL volumes of *S.* enteritidis overnight culture (10^8 CFU/mL) and phage suspension (10^9 PFU/mL) were mixed with 5 mL of BHI previously cooled and incubated overnight at 6 °C. In parallel, bacterial growth without phage addition was used as negative control. After incubation, the cultures were added to 24 mM EDTA to stop the phage infection process and serially diluted in saline–EDTA solution (24 mM EDTA, 0.85% NaCl). *S.* enteritidis reductions were determined by plating on BHI agar plates. Negative control was made without addition of EDTA to evaluate its effect on *S.* enteritidis cells.

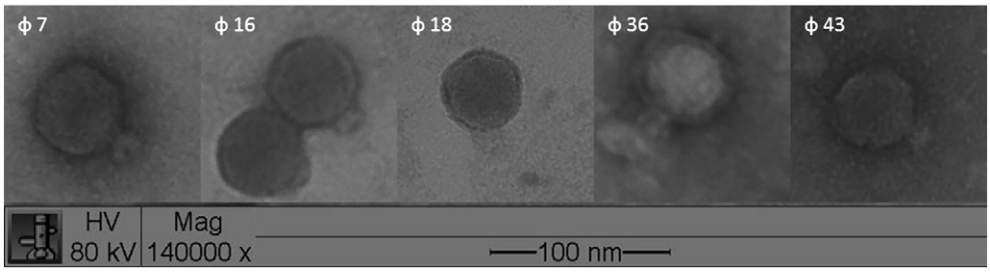

Fig. 1. Electron micrographs of *Salmonella* phages (phiSE). Phage with a short tail and an icosahedral shaped head.

2.12. Phage sensitivity of Salmonella recovered from chicken skin

S. enteritidis colonies were recovered from phage-treated chicken skin to determine the occurrence of resistance against five phages that were used in decontamination treatment. *S.* enteritidis isolates were tested for phage resistance using the drop-on-lawn technique as described above. The plates were incubated at 37 °C for 18 h and checked for the presence of phage plaque.

2.13. Statistical analysis

S. enteritidis counts were all logarithmically transformed prior to statistical analysis and presented as mean values average and standard deviation of the mean. Analysis of variance one-way (ANOVA) followed by Duncan's multiple-range test was used for determining mean significant differences ($p < 0.05$) between decontamination treatments according to the experimental design.

3. Results

3.1. Phage characterization

Bacteriophages were isolated from 24 (53.3%) chicken feces samples obtained from 10 different poultry farms. These phages were isolated initially based on their ability to lyse *S.* enteritidis. The phages were assigned as phiSE (*Salmonella* enteritidis phage) and numbered according to the isolation source. Five phages were selected for use as biosanitization agents against *S.* enteritidis due to their lytic activity (larger plaque size and higher transparency). Analysis of phages using electron microscopy allowed the morphological classification of phage into viral family and order. The five phages isolated were morphologically similar and assigned to the family *Podoviridae* and order *Caudovirales* because of the presence of a short tail and an icosahedral shaped head (Fig. 1). Heads were measured between opposite apices and ranging from 43.4 ± 3 nm to 49.6 ± 5 nm in size. The length tails ranged from 12.8 ± 0.6 nm to 19.5 ± 1 nm. Although these five phages have been isolated from different sources, restriction analysis with *Cfo*I and *Hinf* I endonucleases revealed identical RFLP profiles (Fig. 2). The phage genome sizes may not be calculated as sums of the molecular weights of the restrictional fragments due to the higher DNA fragmentation caused by both enzymes. The phages were assessed for their ability to plaque on a range of bacteria including gram-negative and gram-positive strains. All five phages were capable of lysing six of the eight *Salmonella* serovars that were tested in this study (Table 1). *S.* enterica subsp. arizonae was not susceptible to none of the phages evaluated and *S.* choleraesuis was only infected by phiSE16 and phiSE18 phages. These two phages were also able to infect *E. coli* and can be interesting candidates for use in biosanitization and phage therapy. None of the phages lysed any of the other gram-negative and gram-positive bacteria tested.

3.2. Effects of phage concentration and incubation temperature

Before the five phages were mixed and used as biosanitizer to reduce *S.* enteritidis in chicken skin, the replication dynamics of each phage were characterized in vitro at 37 °C and 25 °C. All five phages were able to reduce significantly the OD of *S.* enteritidis growth at

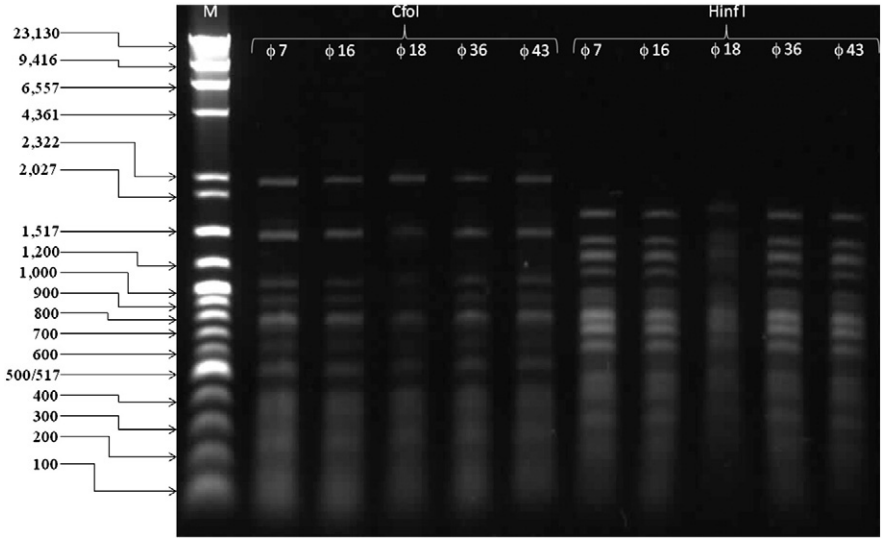

Fig. 2. Digestion of the DNA of *Salmonella* phage (e.g. phiSE 7) with endonucleases *Cfo*I and *Hinf*I. Lane M—lambda *Hind*III molecular weight marker and 100 bp DNA ladder (New England Biolabs, MA, USA).

10^9 PFU/mL (MOI = 10) in both temperatures evaluated (Fig. 3). When *S.* enteritidis was challenged with phages at 10^3 PFU/mL (MOI = 0.00001) and 10^6 PFU/mL (MOI = 0.01) no reduction in bacterial OD were observed between control and test with phages (data not shown). These results demonstrate that bacterial growth inhibition was clearly phage concentration-dependent. The efficiencies of the phages in reducing the OD of *S.* enteritidis varied at both 37 °C and 25 °C. Almost all phages evidently reduced the bacterial OD after 2 h of incubation at 37 °C, except the phage phiSE18 that was slower than others (Fig. 3A). When incubated at 25 °C, a decrease in bacterial OD was evident 2.5 h after infection by phages phiSE7, phiSE18 and phiSE43, while the phiSE16 and phiSE36 phages demonstrated activity after 4.5 h of incubation (Fig. 3B). Subsequent bacterial re-growth was observed from phiSE7 phage at both 37 °C and 25 °C, likely indicating bacterial resistance as previously described by other authors (Kim, Klumpp, & Loessner, 2007).

3.3. Salmonella reduction in chicken skin

The chicken skin sections used in this study exhibited natural contamination of approximately 10^5 CFU/cm^2 of mesophilic aerobic microorganisms. Radiation treatment with a dose of 10 kGy caused a complete elimination of natural microbiota in chicken skin. These chicken skin sections were again contaminated with 10^5 CFU/cm^2 of *S.* enteritidis and used to compare the decontamination effect of bacteriophages and sanitizers. Fig. 4 shows the reductions of *S.* enteritidis on experimentally-contaminated chicken skin sections by decontamination treatments. The bacteriophage cocktail applied to chicken skin experimentally-contaminated achieved significant reductions ($p < 0.05$) in *S.* enteritidis counts. The sample immersion in phage suspensions at 10^9 PFU/mL (MOI = 10,000) for 30 min resulted in a 1.0 log CFU/cm^2 reduction (4.5 ± 0.4 log CFU/cm^2) when compared with untreated controls (5.5 ± 0.4 log CFU/cm^2) (Fig. 4). To evaluate the washing effect caused by immersion in liquid, we compared *S.* enteritidis counts of contaminated chicken skin immersed in distilled water for 30 min with untreated control. There was a 0.2 log CFU/cm^2 reduction (5.3 ± 0.9 log CFU/cm^2) between distilled water treatment and untreated control (5.5 ± 0.4 log CFU/cm^2) that was statistically significant ($p < 0.05$), even being a low value. All treatments with chemical agents routinely used in carcass decontamination also significantly reduced ($p < 0.05$) *S.* enteritidis counts on chicken skin sections when compared with untreated controls. The treatments with sodium dichloroisocyanurate (200 ppm for 10 min), peracetic acid (100 ppm for 10 min) and lactic acid (2% for 90 s) decreased

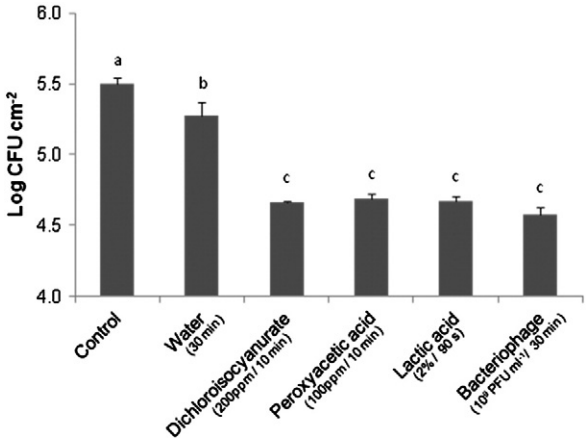

Fig. 4. Reduction of *S.* enteritidis counts in chicken skin treated with bacteriophages and chemical agents. Values followed by same letter do not differ significantly from one another (*Duncan* test). Data reported are the mean ± standard deviation.

about 0.81 ± 0.06 log CFU/cm^2 of *S.* enteritidis counts on chicken skin sections (Fig. 4). These results were not statistically different from the reductions obtained with phage treatment.

3.4. Demonstration of phage activity at low temperature

To demonstrate that phages were able to reduce *S.* enteritidis on the chicken skins at low temperature and not simply on the surface of the agar plate, we used EDTA to stop the infection process before plating. The EDTA concentration (24 mM) used in this study did not affect the *S.* enteritidis viability, but inhibited phage infection on the surface of the agar plate. It was evidenced due to the absence of lysis plaque on the bacterial lawns (data not shown). The *S.* enteritidis count obtained after phage treatment at 6 °C for 16 h was 6.7 ± 0.1 log CFU/mL against 7.7 ± 0.1 log CFU/mL of the control treatment. There was a 1.0 log CFU/mL reduction that demonstrates the phage activity at refrigeration temperature.

3.5. Phage sensitivity of Salmonella recovered from chicken skin

The occurrence of phage-resistant *S.* enteritidis in the chicken skin sections immersed into phage suspension was monitored. Thirty *S.* enteritidis colonies were randomly selected, recovered from the XLT4 agar plates and then tested against five phages that were used in

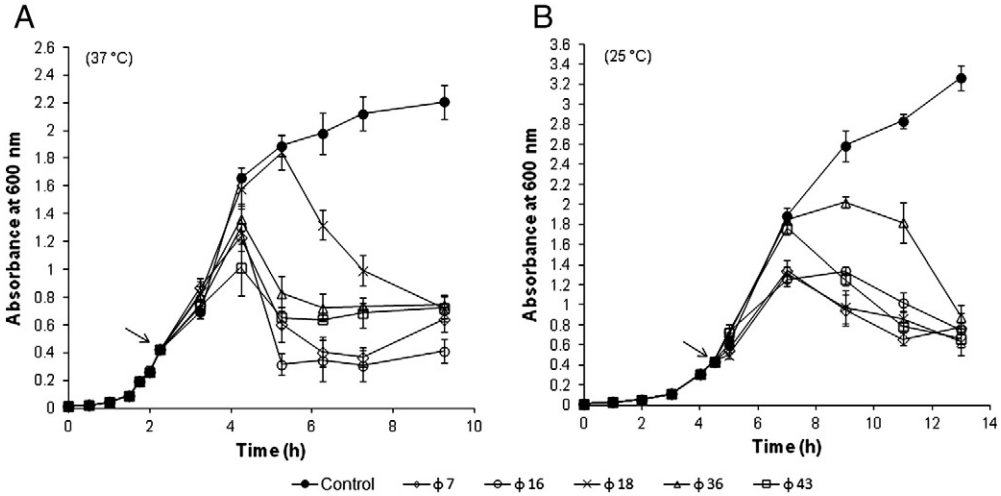

Fig. 3. Inhibition of *S.* enteritidis strain ATCC 13076 with phages in medium liquid (BHI). Cultures were grown at 37 °C (A) and 25 °C (B), and phages were added at a concentration of 10^9 PFU/mL. The arrow indicates the time of phage infection. The data represent the mean ± standard deviation.

decontamination treatment. Testing for phage sensitivity revealed that all *S.* enteritidis isolates were sensitive to all five phages. These results indicate that the contact time was not long enough for the bacteria acquiring phage resistance.

4. Discussion

Physical, chemical and biological methods have been widely studied and applied through the whole food chain to reduce foodborne pathogens, especially in the poultry industry. Recent reviews on the application of decontamination treatments for poultry carcasses reported reductions of microbiological contamination ranging from 0.9 to 3.8 log and 1.0 to 2.2 log by physical and chemical methods, respectively (Hugas & Tsigarida, 2008; Loretz et al., 2010). However, many of these methods might exert an adverse impact on carcass appearance and sensorial properties. Moreover, organic matter often reduces its antimicrobial activity and chemicals are prohibited in the European Union, which makes biological interventions such as the use of bacteriophages, a promising alternative for decontamination of poultry carcasses.

In this study, we have demonstrated that bacteriophages were able to reduce *S.* enteritidis counts on chicken skin at refrigeration temperature and short contact time. In addition, the decrease of *S.* enteritidis count on artificially-contaminated chicken skin after phage treatment corresponded to the reduction achieved by chemical agents commonly used in the poultry industry. Both phage and chemical agents reduced *S.* enteritidis counts by an average of 1 log CFU/cm^2 when compared with untreated control. Our data are consistent with some published studies in which bacteriophages were used to control pathogens in foods (Atterbury, Connerton, Dodd, Rees, & Connerton, 2003; Hooton et al., 2011). In other studies about phage applications, the greatest reductions in host cell numbers were measured when the contaminated samples were incubated with phages for a longer time (Bigwood et al., 2008; Goode et al., 2003). However, one of our goals was to evaluate phage activity in short contact time in order to apply it before the freezing of carcasses. The treatment conditions used in this study were designed according to poultry processing steps, where the residence time of carcasses in chiller varies from 45 to 60 min and temperatures varies from 4 to 6 °C (Sams, 2001). Our results suggest that bacteriophage may be applied to reduce *S.* enteritidis into poultry processing steps such as evisceration and chilling. Although the contamination reduction after phage treatment may be considered little, bacteriophages can remain viable and stable for long time periods at low temperature storage (Fiorentin et al., 2005; Guenther et al., 2009; Hooton et al., 2011). Due to the commercial storage conditions applied to poultry meat and related products, the growth of *Salmonella* spp. is greatly reduced or halted and the phage replication will be inhibited. However, bacteriophages can recover the replication in conditions of temperature abuse, or even exhibit a passive activity that cause reductions without the need for the bacteriophage to replicate and complete their life cycle (Hooton et al., 2011). Our results indicate that the inactivations measured may have been due to lysis from without (Delbrück, 1940) since a high MOI was necessary to obtain *S.* enteritidis count reductions on chicken skin at short contact time. Moreover, we demonstrated in additional experiments with addition of EDTA that the phage activity was not due to infections resulting in lysis upon commencement of cell growth after plating. Lysis from without may occur when a large number of bacteriophages are absorbed per host cell and change the cytoplasmic membrane integrity causing bacterial lysis in the absence of phage replication (Abedon, 2011; Atterbury et al., 2003; Goode et al., 2003).

The ratio of host cells and phages denominated as "multiplicity of infection (MOI)" appears to be of great relevance to the successful application of this technology (Kasman et al., 2002; Whichard, Sriranganathan, & Pierson, 2003). As demonstrated in previous studies (Goode et al., 2003; Hooton et al., 2011; Turki, Ouzari, Mehri, Ammar, & Hassen, 2012), the application of MOI in excess of the bacterial concentration was also important to reach significant reductions of *S.* enteritidis in this study. Our data obtained in experiments evaluating the replication dynamics of phages in liquid medium suggested a necessity of high phage concentrations such as 10^9 PFU/mL for optimum efficacy, since amounts equal or less than 10^6 PFU/mL were not able to reduce *S.* enteritidis growth. Therefore, the chicken skin decontamination was performed with phage cocktail at 10^9 PFU/mL (MOI = 10,000). Although this seems to be relatively high, it is both technically and economically possible.

The phage activity has been found to be sensitive to the physiological state of the host, which has been affected by its growth conditions such as temperature, nutrient availability and oxygen tension (Cohen, 1949; Hadas, Einav, Fishov, & Zaritsky, 1997). There is a similar dependence of phage growth rate on the host growth rate, and when the hosts grow at optimal conditions, the phage infection cycle is faster, corresponding to a shorter latent time, faster progeny production rate, and larger burst size (You, Suthers, & Yin, 2002). In this study, we also investigated the effect of incubation temperature on growth inhibition of *S.* enteritidis in liquid media. We observed that the phage activity varied at different temperatures, and for the most phages evaluated it was slightly faster at 37 °C than at 25 °C. Although these results demonstrate a better phage activity at optimal host growth temperature, this does not hinder the phage application at low temperature, as demonstrated by other authors (Bigwood et al., 2008; Fiorentin et al., 2005; Guenther, Herzig, Fieseler, Klumpp, & Loessner, 2012; Hooton et al., 2011). In this assay, we also observed a bacterial re-growth in *S.* enteritidis cultures inoculated with the phiSE7 phage, indicating probably resistance. However, in the experiment of chicken skin decontamination, we did not find any bacteria isolated from phage-treated samples to be resistant against the five phages used, possibly due to the short contact time and the use of phage cocktail. The development of resistance in the host could reduce efficacy of phage treatment and in order to minimize this problem, some measures should be considered such as the use of virulent phages with a broad host range in mixtures/cocktails and the treatment of products immediately prior to packaging and shipment, thereby preventing re-introduction and recycling of resistant bacteria in the production environment (Guenther et al., 2009; Kunisaki & Tanji, 2010).

Bacteriophages used in this study showed activity against other *Salmonella* serovars, which broadens their spectrum of action against contamination from different sources. In addition, some phages were also able to infect other bacteria assigned to the *Enterobacteriaceae* family. The degree of genetic relatedness between these bacteria and structural similarity between receptors for phages such as pili and LPS may have contributed to these results. Phages that present a broad host range, including other bacteria strains, have also been isolated in other studies, and exhibit great potential to control pathogens in food (Bielke, Higgins, Donoghue, Donoghue, & Hargis, 2007; O'Flynn, Coffey, Fitzgerald, & Ross, 2006). This feature allows simplicity, safety and cost reduction in the production of bacteriophages, as they can be replicated in non-pathogenic bacteria (Bielke, Higgins, Donoghue, Donoghue, Hargis, et al., 2007).

Five phages used in this study presented similar morphology and its restriction digest profiles were indistinguishable from each other. However, there were evident differences between some of these phages, their replication dynamics and host range. Despite that morphological and genetic characterization of these phages suggests that they belong to identical phage-types, the genome sequencing is necessary to complete their identification.

To our knowledge, this is the first study to examine and observe that phages could be used to reduce *S.* enteritidis in a similar industry process as applied in chemical agents. In addition, different phage products would be developed to control other microorganisms in poultry carcasses during processing steps.

5. Conclusion

We believe that bacteriophage-based treatment can be applied for pathogen control in poultry carcasses and has a potential to replace chemical agents in the path toward the production and supply of safer food. However, factors such as phage/host/food interactions, effects of processing on phage viability, and security should be better understood before phages can be used reliably as biosanitizer agents.

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