Use of Lytic Bacteriophages to Reduce Salmonella Enteritidis in Experimentally Contaminated Chicken Cuts

ABSTRACT

Reducing Salmonella contamination in poultry is of major importance to prevent the introduction of this microorganism into the food chain. Salmonellae may spread during storage time (shelf life) whenever pre-harvest control fails or post-harvest contamination occurs. Therefore, preventive measures should also be used in the post-harvest level of poultry production in order to control salmonellae. Chicken skin samples were experimentally contaminated by immersing whole legs (thighs and drumsticks) in a suspension containing 10^6 colony forming units per milliliter (CFU/mL) of Salmonella Enteritidis phage type 4 (SE PT4) at the slaughter day. One day later, samples from one group were immersed in a suspension pool containing 10^9 CFU/mL of each of three wild salmonella-lytic bacteriophages previously isolated from feces of free-range chickens. Salmonella counting was performed at three-day intervals in the chicken legs stored at 5°C and showed a significant reduction (P<0.05) of SE PT4 in bacteriophage-treated cuts on days 3, 6 and 9 post-treatment. These findings suggest that the use of bacteriophages may reduce SE PT4 in chicken skin. Further studies are encouraged and might demonstrate the potential of this approach as an efficient and safe technique to be routinelly used for Salmonella control in chicken products.

INTRODUCTION

Pre-harvest Salmonella elimination might play a significant role in the prevention of pathogen introduction into the food chain and consequently in the reduction of food poisoning in humans (Seo et al., 2000). It is more likely that the pre-harvest control of salmonellae is effective if a multi-factorial program is implemented. Good agricultural practices such as hazard analysis and critical control point (HACCP) (Nayak et al., 2003), vaccination (Zhang-Barber et al., 1999; Yamane et al., 2000), probiotics, prebiotics and symbiotics (Van Immerseel et al., 2002) have been used in poultry production as preventive measures if infection by Salmonella is likely to occur. In spite of that, no measure has been shown to be 100% effective and salmonellae remain as major contaminants to poultry (Persson & Jendteg, 1992; Mead et al., 1999). The potential of phage therapy for Salmonella control has been recently assessed in the pre-harvest level of poultry production (Fiorentin et al., 2005; Sklar et al., 2001).

Decontamination of chicken skin has been of major interest in applied research. Some chemical and physical procedures have been tested, such as carcass washing with aqueous solution of lactic acid (Xiong et al., 1998), use of calcium hypochlorite, hydrogen peroxide and radiation (Nassar et al., 1997), air chilling (Allen et al., 2000), chlorine and sodium triphosphate (Xiong et al., 1998; Whyte et al., 2001), as well as the use...
of natural products such as grapefruit seed extract (Xiong et al., 1998). However, it has not been developed an efficient method that is completely devoid of chemicals and that might not cause food safety concerns.

In a previous study, we isolated and characterized Salmonella-lytic bacteriophages (Fiorentin et al., 2004). Some of these bacteriophages have been administered in vivo to SE PT4-infected broilers and there was a reduction in the colony forming units of SE PT4 per gram of cecal contents by 3.5 orders of magnitude (Log CFU/g) (Fiorentin et al., 2005). Other authors have also successfully reported reductions in Salmonella counts by using bacteriophages in chicken internal organs and feces (Toro et al., 2005), skin (Goode et al., 2003) or poultry products (Whichard et al., 2003). The positive results led to the hypothesis that the bacteriophage isolated previously in our laboratory might also be efficient in reducing SE PT4 in chicken skin. Some advantages of administering phages onto the skin of poultry carcasses are that phages would not recycle in the host and therefore selection of resistant strains would be avoided. Besides, methods of biological control pose fewer risks to the consumer compared to chemical methods.

In the present study, chicken thighs and drumsticks were contaminated with SE PT4 and later treated with a panel of salmonellae-lytic bacteriophages isolated from free-range chickens.

### MATERIAL AND METHODS

**Chicken thighs and drumsticks**

The study evaluated three groups as described in Table 1. The groups were comprised of 25 whole legs (thighs and drumsticks) with mean weight between 300 and 350 grams. The legs were collected during slaughter of a Salmonella-negative flock previously monitored using drag swabs (Waltman et al., 1998). Chicken cuts (or parts) were experimentally contaminated by immersion in a suspension of phosphate buffered saline (PBS pH7.2) containing 10^6 CFU/mL of SE PT4 at slaughter day. The samples of the three groups were transferred to sterile plastic bags, sealed and kept at 5°C. Afterwards, five samples per treatment were randomly taken at each three days for Salmonella and bacteriophage counts.

**Contamination with Salmonella**

SE PT4 isolate P125589 was kindly provided by Dr. Paul Barrow (ARFC Institute for Animal Health, Houghton Laboratory, Cambridge, England). It was originally isolated by Dr B. Rowe (Central Public Health Laboratory, London, UK) from a case of human food poisoning (Barrow & Lovell, 1991).

A fresh colony of SE PT4 was inoculated into 10mL of nutrient broth (NB, 1g/L beef extract, 2g/L yeast extract, 5g/L peptone, 5g/L sodium chloride, pH 7.0) and incubated overnight at 37°C under shaking (200rpm). The culture was frozen at −80°C and one aliquot was used for enumeration of viable cells, by counting colonies grown from tenfold dilutions streaked onto nutrient agar and incubated for 24h at 37°C. After counting, the original culture was diluted with sterile buffered saline (1 L) to produce a solution containing 10^6 CFU/mL that was used to contaminate the chicken cuts.

### Table 1 – Treatments used to assess the effect of bacteriophages on the reduction of Salmonella Enteritidis phage type 4 contamination in broiler skin samples.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cuts</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>Non-contaminated and non-treated</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>Contaminated by immersion on a suspension containing 10^6 CFU/mL of Salmonella Enteritidis phage type 4 at slaughter day</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>Contaminated as in group 2, treated one day later by immersion in a suspension containing 10^9 PFU/mL of a mixture of bacteriophages CNPSA1, CNPSA3 and CNPSA4</td>
</tr>
</tbody>
</table>

**Treatment with bacteriophages**

Bacteriophages CNPSA 1, CNPSA3 and CNPSA4 were isolated from feces of free-range chickens in Brazil and characterized as described elsewhere (Fiorentin et al., 2004). Since resistance to bacteriophages may emerge in growing populations of bacteria, we decided to use a pool of three different viruses. Frozen bacteriophage stocks were amplified on overlay cultures of SE PT4 prepared with Nutrient Broth (NB) containing 0.7% agarose. Enough bacteriophage particles were then used to inoculate one liter of SM buffer (5.8g/L NaCl, 2.0g/L MgSO4-7H2O, 5.0mL/L of a 5% solution of gelatin, 50mL/L of 1M Tris-HCl pH 7.5) to a concentration of 10^9 plaque forming units per milliliter (PFU/mL), which results in a multiplicity of infection of 1,000 (MOI: 1,000), i.e., a thousand PFU of bacteriophages per CFU of SE PT4 was used to contaminate the chicken parts. Bacteriophage titers were determined using tenfold dilutions of the virus preparation mixed to SE PT4 in log-phase growth (10µL : 250µL). The mixtures were
incubated at 37°C for 20 minutes and then mixed with
7mL of melted NB-agarose (45°C), thoroughly
homogenized and overlaid onto 10cm-diameter
Nutrient Agar plates (NB added with 15g/L bacteriologic
agar) (Kudva et al., 1999). Plaques of lysis were counted
after incubation for 24 hours at 37°C. Bacteriophage
titers were obtained by multiplying the number of
plaques in each dilution by the dilution factor. Cuts were
immersed in the bacteriophage suspension, allowed
to dry for a few minutes and replaced into the plastic
bag for storage at 5°C for 15 days.

**Experimental design**

The number of washable viable Salmonella cells and
bacteriophage particles were assessed in five leg
samples taken from each group at three-day intervals.
The cuts were randomly chosen and submitted to
Salmonella and bacteriophage assessment as
described below.

**Salmonella isolation**

Samples from Groups 2 and 3 were individually
rinsed in sterile plastic bags containing 100mL of
buffered peptone water. The number of Salmonella
colonies forming units (CFU) in the rinse solution (rinse
peptone water) was determined by multiplying the number of viable cells in 1mL of the solution multiplied
by 1,000. Rinse peptone water was diluted tenfold in
buffered saline and 100µL of each dilution were spread
onto 10cm-diameter brilliant green agar plates (BGA)
supplemented with novobiocin (40µg/mL) and
incubated at 37°C for 48h. Salmonella CFU per cut
was determined by the number of colonies in plates
with 30 to 300 colonies multiplied by the dilution factor.
The theoretical limit of detection of this method is 30 x
10² CFU per cut, which means that negative results in
Groups 2 and 3 would actually represent <3,000 CFU
per cut. Slight pink-white opaque colonies surrounded
by red medium grown on BGA were confirmed as
Salmonella by slide agglutination test with polyvalent
anti-somatic serum.

Cuts from all groups were also submitted to
qualitative analysis of Salmonella. Rinse peptone water
was incubated at 37°C for 24h for pre-enrichment.
Afterwards, 0.3 mL of each culture was enriched in
2.7 mL of Rappaport-Vassiliadis Soya peptone broth
(RVS) and incubated at 42°C for 24h. A loopful was
then streaked onto BGA plates and incubated at 37°C
for 24h. Salmonella colonies were identified as
described above.

**Bacteriophage titering**

Cuts from Group 3 were individually rinsed in 100mL
of SM buffer and total bacteriophage PFU was titered
as follows. One milliliter of rinse SM buffer was treated
with 5% chloroform to lyse all cells and 10µL were
serially diluted tenfold in sterile SM buffer. From each
dilution, 10µL were then mixed with 250µL of SE PT4
in log-phase growth and incubated for 20 minutes at
37°C to allow infection of Salmonella cells by
bacteriophages. Afterwards, mixtures were added to
7mL of NB containing 0.7% agarose, thoroughly
homogenized and overlaid onto 10cm-diameter
nutrient agar plates, which were then incubated for
24h at 37°C. Plaques of lysis were counted on the
cultures with 30 to 300 plaques. The number of
bacteriophages was determined by multiplying the
number of plaques by 100 and expressed as total PFU
per mL. Total PFU per chicken cut was obtained
multiplying total PFU by 100.

Cuts from Groups 1 and 2 were submitted to
qualitative analysis to confirm their bacteriophage-free
status. One milliliter of rinse SM buffer from each
chicken cut was treated with 5% chloroform, vortexed,
centrifuged (12,000 x g for 4 minutes) and 100µL of
the supernatant was added to 900µL of a SE PT4
culture, which was incubated at 37°C for 24h. After
incubation, this preparation was also treated with 5%
chloroform to lyse all bacteria, centrifuged and 10µL
of the supernatant was used to prepare overlaying SE
PT4 cultures as described before. Positive results were
identified by the presence of lysis plaques with about
1cm of diameter on the Salmonella lawn.

**Statistical analysis**

Analysis of variance followed by t-test was used to
compare means of CFU of SE PT4 per piece according
to the experimental design. Statistical analysis was
performed using a commercial package (SAS, 2001).

**RESULTS AND DISCUSSION**

**Salmonella isolation**

SE PT4 was recovered from all cuts in all five
assessments using the samples of rinse peptone water
from Group 2 and Group 3 submitted to selective
enrichment. Sampling was performed at each three
days after treatment until day 15 (except for day 3).
Therefore, the results indicate a relative long period of
SE PT4 survival considering the shelf life of refrigerated
products. Survival for long periods has been recognized
as an important feature of salmonellae; even in low
temperatures, salmonellae were able to survive in non-
sanitized poultry carcasses at least for 26 days
(Thomson et al., 1979).

The presence of SE PT4 in the bacteriophage-
treated cuts indicates that the conditions used in this
study did not allow bacteriophages to eliminate
Salmonella from poultry skin. However, it is unlikely
that any single technique applied to poultry carcasses
would remove Salmonella completely from the skin,
nor would Salmonella be naturally removed from
contaminated skin only by refrigeration. We have
previously reported that bacteriophages orally
administered to SE PT4-infected broilers reduced the
numbers of Salmonella significantly, but could not
remove completely the bacteria from chicken ceaca
(Fiorentin et al., 2005). The same is probably also true
in other situations, such as when bacteriophages are
applied to poultry skin. In regard to food safety, it is
extremely important to develop techniques that
completely prevent food poisoning in humans. However,
seems hard to achieve this objective using only a
single technique and bacteriophages should probably
be used together with other methods to completely
remove Salmonella from chicken skin. The present
study is our first evidence of Salmonella reduction in
chicken skin by the use of bacteriophages. The findings
encourage us to perform further research in order to
achieve more efficiency regarding bacteriophage
treatment applied on SE PT4-contaminated cuts.

**Bacteriophage isolation**

Rinse peptone water from cuts from Groups 1 and
2 were inoculated with SE PT4 to permit bacteriophage
amplification, in case they were present. This
qualitative analysis confirmed the bacteriophage-free
status of both groups. On the other hand, bacteriophages were isolated from all pieces from
Group 3 in all five samplings according to the qualitative
analysis. These results indicate that bacteriophages were not removed from broiler skin contaminated with Salmonella within a 15 day-period, which might have
resulted from bacteriophage multiplication on the
bacteria or because bacteriophages were not inactivated by natural components of the chicken skin.

A panel of three bacteriophages was used to avoid
selection of strains of SE PT4 resistant to a particular
virus. Although this was a necessary approach, it
prevented us from knowing whether one specific
bacteriophage lasts longer in the cuts or if the three of
them have been recovered from all samples at the same
magnitude. However, we have previously reported
that the three bacteriophages share similar lytic
properties and multiplication rates when cultured in
SE PT4 (Fiorentin et al., 2004) and this might also occur
*in vivo.*

**Salmonella counting**

Enumeration of Salmonella was performed only on
samples from Group 2 and Group 3, because Group 1
was not contaminated and yielded negative results in
the qualitative analysis throughout the experiment.
Total CFU ranged from 0.66 ± 0.05 x 10^8 CFU to 4.840
± 461.09 x 10^6 CFU per cut in the first and last sampling
of Group 2 (SE PT4 contaminated group), respectively.
In the SE PT4-contaminated and bacteriophage-
treated cuts (Group 3), total CFU was 0.29 ± 0.08 x
10^8 CFU and 3,920 ± 738.51 x 10^6 CFU per cut in the
first and the last sampling days, respectively. There was
an increasing trend in total CFU in groups of samples
taken at each three days. These concentrations of CFU
were high, and may be explained because of the high
CFU dose used to contaminate the pieces and also
because the experimental conditions prevented
competition between Salmonella and other organisms
(Coleman et al, 2003). However, lower magnitudes of
total CFU were seen in the bacteriophage-treated
group (Group 3) at all sampling days, except for 12
and 15 days post-treatment (Table 2 and Figure 1).
The means of treated and non-treated parts were
compared by t-test (SAS, 2001), and P values were
highly significant on days 3, 6 and 9, indicating higher
efficiency of bacteriophages in reducing Salmonella
CFU within a 9-day period (Table 2).

Salmonella numbers in samples from Group 2 were
of lower magnitude at 12 days post-inoculation
compared to the samples of Group 3 or even to the
samples of Group 2 in the previous samplings. This
result was probably caused by a technical variation that
occurred during dilution or plating. Therefore, we
suppose that bacteriophages might have actually had
an effect for longer than 9 days. It is also possible,
however, that Salmonella contamination was lower
or bacteriophage effects were greater in the chicken
cuts randomly sampled as one group on day 12. The
curve presented in Figure 1 suggests that the data of
Group 2 on day 12 should logically fall between 4,000
and 8,000 x 10^8 CFU.

Reduction of CFU on treated cuts was about 4.49
times on day 9 post-treatment (Table 2). Such a
reduction is enough to demonstrate a cause-and-effect
relationship, but additional research is required in order
Use of Lytic Bacteriophages to Reduce *Salmonella* Enteritidis in Experimentally Contaminated Chicken Cuts

Table 2 - Mean ± standard deviation of total colony forming units (CFU x 10⁸) recovered from the rinse peptone water of five cuts contaminated with *Salmonella* Enteritidis phage type 4 (Group 2) or contaminated and treated with bacteriophages (Group 3).

<table>
<thead>
<tr>
<th>Days after treatment</th>
<th>Group 2**</th>
<th>Group 3**</th>
<th>P value**</th>
<th>Reduction***</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.65±0.05</td>
<td>0.29±0.08</td>
<td>0.0334</td>
<td>2.27</td>
</tr>
<tr>
<td>6</td>
<td>340.2±124.16</td>
<td>74.6±20.37</td>
<td>0.0026</td>
<td>4.56</td>
</tr>
<tr>
<td>9</td>
<td>7,734±3,875.02</td>
<td>1,720±360.85</td>
<td>0.0195</td>
<td>4.49</td>
</tr>
<tr>
<td>12</td>
<td>772±51.52</td>
<td>1,714±813.74</td>
<td>0.269</td>
<td>-0.45</td>
</tr>
<tr>
<td>15</td>
<td>4,840±461.09</td>
<td>3,920±738.51</td>
<td>0.552</td>
<td>0.97</td>
</tr>
</tbody>
</table>

*Non-contaminated and non-treated pieces (Group 1) were negative throughout the experiment.**t-test.***Difference in mean values between Group 3 (contaminated, treated) and Group 2 (contaminated, non-treated).

Figure 1 - Mean ± standard deviation of total colony forming units (CFU x 10⁸) of SE PT4 recovered from cuts contaminated with *Salmonella* (Group 2) or contaminated and treated with bacteriophage (Group 3). Differences between means were statistically significant on days 3, 6 and 9.

The fact that total CFU increased even in treated cuts leads us to suppose that higher concentrations of bacteriophages might prevent such multiplication of SE PT4 on the skin. However, this is our first report on the use of bacteriophages to reduce *Salmonella* presence in poultry skin. The convincing results presented herein encourage further research in order to achieve greater reductions in contamination of poultry carcasses or cuts by *Salmonella*. The use of higher concentrations of bacteriophages, either alone or coupled with other methods, might determine more efficient reductions of SE PT4 contamination of poultry skin.

**Enumeration of bacteriophages**

Concentration of bacteriophages recovered from the rinse water in Group 3 ranged from 5.8 to 25.4 x 10⁵ PFU per cut (Table 3). The observed variation is probably inherent to the technique used for titering the viruses, once the difference between titers were still smaller than one order of magnitude. This is a low recovery rate compared to the concentration of bacteriophage (10⁹/mL) used to treat the cuts contaminated with SE PT4. A possible explanation is that only a small portion of SM buffer might have actually remained in the cuts after dipping or perhaps bacteriophages adhered poorly to components on the chicken skin; both conditions would have resulted in lower MOI than the expected MOI of 1,000. However, it is noteworthy that bacteriophages were viable and showed similar titers for 15 days at 5°C even in contact with chicken skin.

Table 3 - Means of total plaque forming units (PFU) of bacteriophages recovered from rinse peptone water of contaminated and treated cuts (Group 3).

<table>
<thead>
<tr>
<th>Days after treatment</th>
<th>Total PFU recovered per cut*</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>15 x 10⁵</td>
</tr>
<tr>
<td>6</td>
<td>5.8 x 10⁵</td>
</tr>
<tr>
<td>9</td>
<td>25.4 x 10⁵</td>
</tr>
<tr>
<td>12</td>
<td>8.4 x 10⁵</td>
</tr>
<tr>
<td>15</td>
<td>13 x 10⁵</td>
</tr>
</tbody>
</table>

*Obtained by tittering 10µl of rinse solution multiplied by the dilution factor.

The fact that bacteriophage titers did not increase in Group 3 might have been caused by the reduction observed on CFU of SE PT4. It is also possible that these
bacteriophages did not target any other bacteria present on the chicken skin, otherwise they would have shown higher PFU per cut with longer shelf storage time.

CONCLUSIONS

A panel of bacteriophages reduced SE PT4 countings in experimentally contaminated chicken parts stored at 5°C. We demonstrated that Salmonella CFU was reduced in the bacteriophage-treated cuts on days 3, 6 and 9 post-treatment when compared to their non-treated counterparts.

REFERENCES


Thomson JE, Bailey JS, Cox NA. Phosphate and heat treatments to control Salmonella and reduce spoilage and rancidity on broiler carcasses. Poultry Sciences 1979; 58:139-143.


