INTRODUCTION

Salmonella food poisoning is second most common type of food poisoning in the United States (Foley and Lynne, 2008). The number of worldwide cases has gradually increased beginning in the 1980s, and also in South America since the 1990s (Rodrigue et al., 1990). This increase has been attributed to Salmonella-contaminated eggs and chicken products (Capita et al., 2003).

Salmonella carriage by even a few birds on a farm can become a significant problem when preslaughter and processing conditions spread Salmonella to a larger number of birds and carcasses. One way by which Salmonella loads can increase is during feed withdrawal before transportation and slaughter of the birds. Increased Salmonella loads in crop and ceca can lead to increased opportunities for contamination during processing and a negative effect on food safety (Todd, 1997; Gast, 2008).

Several studies over the last 20 yr have explored the use of bacteriophages to reduce the load of Salmonella in poultry carcasses, skin, and organs. The present study describes the effect of administering bacteriophages on Salmonella Enteritidis loads in crops and ceca of broiler chickens.

MATERIALS AND METHODS

Birds

A total of 150 Cobb lineage broiler chickens aged 45 d were used for this. The birds are housed in metallic cages with water ad libitum. At 40 d of age, 10 birds were tested for the presence of Salmonella. The testing was done on aseptically collected crops, ceca, livers, and spleens by incubating the homogenized tissues in Tetrathionate Brilliant Green medium at 40°C for 24 h and plating on xylose-lysine-deoxycholate agar. The plates were incubated at 40°C for 24 h and inspected for colonies typical of Salmonella. At the age of 45 d, the remaining birds were subjected to a 12-h feed withdrawal, according to guidelines approved by Brazilian legislation (Brazil, 1998).

Preparation and Inoculation of Salmonella Enteritidis

The Salmonella Enteritidis isolate used in the present study was obtained from the collection of the Avian Pa-
The birds were divided into 5 groups as indicated in Table 1. The Salmonella Enteritidis inoculations were carried out 2 h after initiation of the feed withdrawal and the were administered 1 h after the administration of Salmonella Enteritidis. Groups of birds were killed by cervical dislocation (CFMV, 2002) at time 0, and at 30 min 1, 3, 6, and 12 h after phage administration. After removal of the ceca and crops, a medial line incision was made, and the interiors of 5 organ samples were washed with peptone water to collect the contents of the organs. One milliliter of the suspended contents was collected for determination of number of cfu/mL, and an additional 25 mL was put aside for PCR analysis. These studies were done at the Bird Pathology Laboratory of the Department of Clinical Veterinary Medicine of the faculty of Veterinary Medicine and Animal Sciences, São Paulo State University, Botucatu, SP, Brazil. All procedures were done in a closed room with controlled temperature (26 ± 2°C) and humidity (48 ± 5%).

### Experimental Design

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### Isolation, Selection, and Preparation of the Bacteriophage Inoculate

Ten bacteriophages isolated from hospital waste water and 10 bacteriophages from waste water from a poultry house, as well as the bacteriophage P22 (ATCC 19585B1), were used in the study. The select phage exhibited a clean zone of lysis. Phage stocks were obtained by incubating (samples varied between $10^{10}$ and $10^{13}$ pfu/mL) with $10^8$ cfu/mL of Salmonella Enteritidis in 5 mL of tryptic soy broth and incubation at 40°C for 24 h. The lysed culture was centrifuged at 1,096 × g at 5°C and the supernatant was filtered through a 0.22-μm syringe filter. The filtrate was added to 200 mL of tryptic soy broth and 50 mL of the Salmonella Enteritidis culture (overnight culture) and incubated at 40°C for 24 h. The lysed culture sample was subjected to centrifugation at 1,096 × g at 5°C and the supernatant was filtered through a Stericup system (Millipore, Darmstadt, Germany) with a membrane pore size of 0.22 μm. Each bacteriophage isolate was grown individually and aliquots were mixed. The total phage count in the mixture was determined, and the final concentration obtained was $10^{10}$ pfu/mL. Each bird was treated with 10 mL of the solution by oral gavage.

### Monitoring of the Salmonella Enteritidis

A suspension of cecal and crop contents (1:9, wt/vol) was prepared in peptonated water and then decimally diluted in the same medium down to 10. A 100-μL volume of each dilution was spread plated onto brilliant green agar, containing 100 μg/mL of Nal/Rif. The plates were incubated at 40°C for 24 h before typical Salmonella colonies were counted.

Extraction of genetic material for molecular analysis was done using Chelex 100 (BioRad Laboratories, Richmond, VA). Using polypropylene tubes, 1 mL of each sample was homogenized and spun at 1,710 × g for 10 min at 5°C. To each 100 μL of the supernatant thus obtained, 10 μL of chloroform was added, and the solution was homogenized using gentle hand movements for 30 s. The tubes were incubated at 5°C for 10 min, centrifuged at 190 × g for 15 min at 5°C, and 30 μL of the supernatant was added to tubes containing 30 μL of 5% Chelex 100. The mixture was incubated at 99°C for 10 min. The resulting supernatant contained free DNA. The primers used were made by the Invitrogen Corporation (São Paulo, Brazil) and suspended at a concentration of 10 pmol. To detect the presence of Salmonella, PCR was carried out with primers specific for the invA gene (forward 5′-TTGTTACGGCTATTTT-GACCA-3′ and reverse 5′-CTGACTGCTACCTTGCT-...
Table 2. Analysis results of bacterial load of *Salmonella* Enteritidis in poultry fasting before slaughter, treated with bacteriophages from different sources.

<table>
<thead>
<tr>
<th>Time posttreatment</th>
<th>Control</th>
<th>SalE</th>
<th>SalE + Phe</th>
<th>SalE + Pha</th>
<th>SalE + Phe + Pha</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceca</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h</td>
<td>0 (−)ND,c</td>
<td>3 × 10^3 (+)A,a</td>
<td>2 × 10^4 (+)A,b</td>
<td>3 × 10^4 (+)A,b</td>
<td>3 × 10^4 (+)A,b</td>
</tr>
<tr>
<td>30 min</td>
<td>0 (−)ND,c</td>
<td>4 × 10^3 (+)A,a</td>
<td>2 × 10^4 (+)A,b</td>
<td>4 × 10^4 (+)A,b</td>
<td>4 × 10^4 (+)A,b</td>
</tr>
<tr>
<td>1 h</td>
<td>0 (−)ND,c</td>
<td>2 × 10^4 (+)A,a</td>
<td>1 × 10^4 (+)A,b</td>
<td>2 × 10^4 (+)A,b</td>
<td>2 × 10^4 (+)A,b</td>
</tr>
<tr>
<td>3 h</td>
<td>0 (−)ND,c</td>
<td>4 × 10^4 (+)A,a</td>
<td>1 × 10^4 (+)A,b</td>
<td>3 × 10^4 (+)A,b</td>
<td>3 × 10^4 (+)A,b</td>
</tr>
<tr>
<td>6 h</td>
<td>0 (−)ND,c</td>
<td>6 × 10^4 (+)A,a</td>
<td>2 × 10^4 (+)A,b</td>
<td>4 × 10^4 (+)A,b</td>
<td>4 × 10^4 (+)A,b</td>
</tr>
<tr>
<td>12 h</td>
<td>0 (−)ND,c</td>
<td>3 × 10^5 (+)A,a</td>
<td>2 × 10^5 (+)A,b</td>
<td>6 × 10^5 (+)A,b</td>
<td>6 × 10^5 (+)A,b</td>
</tr>
<tr>
<td>Crop</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h</td>
<td>0 (−)ND,b</td>
<td>3 × 10^4 (+)A,a</td>
<td>1 × 10^4 (+)A,a</td>
<td>3 × 10^4 (+)A,a</td>
<td>3 × 10^4 (+)A,a</td>
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<td>4 × 10^4 (+)A,a</td>
<td>2 × 10^4 (+)A,a</td>
<td>4 × 10^4 (+)A,a</td>
<td>4 × 10^4 (+)A,a</td>
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<tr>
<td>1 h</td>
<td>0 (−)ND,b</td>
<td>2 × 10^4 (+)A,a</td>
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<td>2 × 10^4 (+)A,a</td>
<td>2 × 10^4 (+)A,a</td>
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<td>0 (−)D,c</td>
<td>3 × 10^5 (+)A,a</td>
<td>3 × 10^5 (+)A,a</td>
</tr>
</tbody>
</table>

A–D; a–d Capital letters: comparison between means by columns; lowercase letters: comparison of means between rows (P > 0.001). ND = no difference.

The results obtained were analyzed with the ANOVA, and a comparison of means was done using the Tukey test. These analyses were done using SAS statistics software (1999, SAS Institute Inc., Cary, NC).

DISCUSSION

The results obtained in the evaluation of cecal colonization in live birds were similar to those found in previous studies by Berchieri et al. (1991), Sklar and Jongerger (2001), Fiorentin et al. (2005), Toro et al. (2005), Atterbury et al. (2007), Andreatti Filho et al. (2007), and Borie et al. (2008, 2009). In these studies, bacteriophage treatments were done orally or with a spray, in contrast to our study, which employed oral gavage. All of these studies found a reduction in up to 2 log units per gram in the *Salmonella* Enteritidis concentration, as was also observed in our study.

With this form of application of feed withdrawal, we observed a reduction of *Salmonella* Enteritidis load in the ceca as also had been found in the previous studies; moreover, we found no *Salmonella* Enteritidis in the crops. This is a different result to that reported by GATG-3') using the following program: 5 min at 94°C, 35 cycles of amplification (30 s at 94°C, 30 s at 60°C and 30 s at 72°C) with a final polymerization for 4 min at 72°C, as described by Swamy et al. (1996). Detection of the presence of bacteriophage P22 was done by amplifying the gene *sieB* using the primers forward 5′-ATGGTGCAGGTTAATGC-3′ and reverse 5′-CAAAACAAATCCCGAAGCCT-3′ with the following program: 1 min at 94°C, 30 cycles of amplification (30 s at 90°C, 30 s at 58°C, and 60 s at 72°C), with a final polymerization for 8 min at 72°C, according to the method of Mikasová et al. (2005).

Amplifications were done in a final volume of 25 μL: 5 μL of sample to be evaluated: 1 μL of each primer, 12.5 μL of the kit Go Taq Green Master Mix (Promega, Madison, WI), and 5.5 μL of ultradistilled water. The presence of a band on an agarose gel was indicative of the presence of *Salmonella* Enteritidis and bacteriophages in the samples.

Statistical Analysis

Results obtained were analyzed with the ANOVA, and a comparison of means was done using the Tukey test. These analyses were done using SAS statistics software (1999, SAS Institute Inc., Cary, NC).

RESULTS

In general, treatments lowered the number of *Salmonella* Enteritidis cfu in ceca and crops (Table 2). In the ceca, the treatment of birds infected with *Salmonella* Enteritidis with bacteriophages isolated from both residual waste hospital and poultry coop water as well as bacteriophage P22 (SalE + Phe + Pha) gave a reduction in *Salmonella* Enteritidis counts 1 h after the application of the bacteriophage. For the rest of the treatments, the reduction in cfu was found only 3 h posttreatment for the ceca of the birds. In the crop, the treatment of birds infected with *Salmonella* Enteritidis treated with bacteriophages isolated from residual waste hospital water and bacteriophage P22 (SalE + Phe) showed a reduction in cfu 30 min after the application of the bacteriophage; however, the rest of the treatments showed a reduction only after 1 h (posttreatment). At the end of the study, whereas the SalE + Pha (birds infected with *Salmonella* Enteritidis and treated with bacteriophages isolated from residual waste poultry coop water as well as bacteriophage P22) treatment gave a reduction in cfu of SE, the SalE + Phe and SalE + Phe + Pha treatments both decreased the *Salmonella* Enteritidis counts to below the detection limit of 10^2 cfu/g.
Berchieri et al. (1991), who observed a reduction up to tree log units per gram in the *Salmonella* concentration was found in both the ceca and crops with an oral administration of bacteriophage.

Hargis et al. (1995) reported that crop is also responsible for the contamination of *Salmonella* on carcasses; at the time only intestines were considered as potential contaminants on the slaughter line. Ramirez et al. (1997) showed that the withdrawal process before slaughter increases the load of *Salmonella* in contaminated poultry, and Chambers et al. (1998) reported that the presence of *Salmonella* in the crop is common. Years later, Smith et al. (2005), using 3 different biologic agents, showed that the fecal contamination of carcasses is relatively low, and recently Ishola (2010) reported that *Salmonella* remains stable for long periods in the crop.

In the management of chicken production, generally the birds are fasting, with their feed having been removed hours before transport to the abattoir. This is done to decrease the intestinal contents, and thus minimize the possible fecal contamination in the carcass. Consequently, there is an increase in the *Salmonella* spp. population in the crops of infected birds. It seems that the stress induced by fasting may favor colonization by these bacteria (Cardoso and Tessari, 2008).

We postulate that the success in the absence of *Salmonella* Enteritidis from the crop is due to a simple reduction in cfu in the cecum due to chemical, physical, and biological factors that interfere with the concentration of the inoculate of bacteriophages, as the crop receives the full concentration of bacteriophage in the inoculate. In contrast, inoculated bacteriophages arrive in the cecum after a long passage through the intestines, where negative influences (pH differences, diverse digestive chemicals, as well as bacterial defense mechanisms) can influence the viability of the bacteriophages. This can lead to the establishment of an equilibrium situation in the population, as was clearly seen in our results of the treatment of the ceca, 3 h after treatment (Table 2).

An important point that differentiates our study from others is that the other studies used growing birds, which possibly had feed and fecal material in the crops and intestines. By applying the bacteriophage therapy to fasting birds before the slaughterhouse process, we excluded the physical factor that interferes directly in the bacteriophage action on the host agent, and perhaps this was shown by the absence of *Salmonella* during the course of our study.

The ability of bacteriophage therapy to reduce the quantity of *Salmonella* in ceca is well recognized by the scientific community; however, the poultry industry is uncertain of its use, although desiring the elimination of this agent. Given that the intestinal contamination is not the principal site responsible for contamination during the slaughterhouse process and that there is also crop contamination, the absence of contamination in the crops of the birds in our study using bacteriophage therapy by oral gavage makes the use of bacteriophage therapy with this mode of administration more practical to treat a key organ responsible for contamination of poultry products during processing.

Based on our study results, we conclude that the use of lytic bacteriophages as an oral gavage is capable of reducing the cecal bacterial concentration of *Salmonella* Enteritidis and reducing to nondetectable levels in the crop of poultry. We believe that the success in reducing this agent from the crops of broiler chickens was associated with undergoing preslaughter feed withdrawal. More studies are needed to confirm this technique and to improve its practical use.

**REFERENCES**


