Ability of Bacteriophages Isolated from Different Sources to Reduce
Salmonella enterica Serovar Enteritidis In Vitro and In Vivo

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ABSTRACT

Salmonella enterica serovar Enteritidis-lysing bacteriophages isolated from poultry or human sewage sources were used to reduce Salmonella Enteritidis in vitro and in experimentally infected chicks. Cocktails of 4 different bacteriophages obtained from commercial broiler houses (CB4∅) and 45 bacteriophages from a municipal wastewater treatment plant (WT45∅) were evaluated. In experiment 1, an in vitro crop assay was conducted with selected bacteriophage concentrations (10⁵ to 10⁹ pfu/mL) to determine ability to reduce Salmonella Enteritidis in the simulated crop environment. Following 2 h at 37°C, CB4∅ or WT45∅ reduced Salmonella Enteritidis recovery by 1.5 or 5 log, respectively, as compared with control. However, CB4∅ did not affect total SE recovery after 6 h, whereas WT45∅ resulted in up to a 6-log reduction of Salmonella Enteritidis. In experiment 2, day-of-hatch chicks were challenged orally with 3 × 10⁵ cfu/chick Salmonella Enteritidis and treated cloacally with 1 × 10⁹ WT45∅ pfu/chick 1 h postchallenge. One hour later, chicks were treated or not with a commercially available probiotic (Floramax-B11). Both treatments significantly reduced Salmonella Enteritidis recovery from cecal tonsils at 24 h following vent lip application as compared with controls, but no additive effect was observed with the combination of bacteriophages and probiotic. In experiment 3, day-of-hatch chicks were challenged orally with 9 × 10⁵ cfu/chick Salmonella Enteritidis and treated via oral gavage with 1 × 10⁸ CB4∅ pfu/chick, 1.2 × 10⁸ WT45∅ pfu/chick, or a combination of both, 1 h postchallenge. All treatments significantly reduced Salmonella Enteritidis recovered from cecal tonsils at 24 h as compared with untreated controls, but no significant differences were observed at 48 h following treatment. These data suggest that some bacteriophages can be efficacious in reducing SE colonization in poultry during a short period, but with the bacteriophages and methods presently tested, persistent reductions were not observed.

Key words: Salmonella enterica serovar Enteritidis, bacteriophage, crop assay, probiotic, chicken

INTRODUCTION

Food poisoning associated with improper handling of poultry products is a continuing problem (Molbak and Neimann, 2002). Salmonella enterica serovar Enteritidis is the second most common serotype in humans and identified most commonly from clinical and nonclinical chicken sources (Centers for Disease Control and Prevention, 2005). This serovar can cause infection of chickens in the absence of a clinical disease (Gast and Beard, 1990). Several measures to control Salmonella have been used, among them the use of antimicrobial drugs. However, concerns about drug-resistant bacteria and the appearance of drug residues in food animals have stimulated interest in alternative treatments such as the use of probiotics, competitive exclusion products, and bacteriophages (Sulakvelidze et al., 2001; VanImmerseel et al., 2002; Andreatti Filho et al., 2003; Joerger, 2003).

Bacteriophages have received renewed attention in recent years as a possible antibiotic alternative to eliminate or control harmful bacterial infections. Bacteriophages are viruses that infect and replicate in prokaryotic cells rather than eukaryotic cells (Cann, 1993). Lytic bacteriophages infect bacterial cells, multiplying until the bacteria lyses, discharging new bacteriophage particles (Toro et al., 2005). They are ubiquitous and can be isolated from water, sewage, and soil. Often bacteriophages are very host-specific, infecting only 1 serotype within a bacterial species (Ackermann et al., 1978; McLaughlin et al., 2006). Bacteriophages are cultured in host bacteria by traditional microbiological methods and are used to classify bacterial strains, including Salmonella (Ackermann and Nguyen, 1983; Kuhn et al., 2002).

The more recent successes relating to the use of bacteriophages for controlling staphylococcal and Escherichia
coli infections has spurred interest in bacteriophages against additional bacterial species (Slopek et al., 1985; Smith et al., 1987). In chickens, simultaneous bacteriophage administration with E. coli challenge has been shown to significantly reduce mortality as compared with controls (Barrow et al., 1998). Bacteriophages have been demonstrated to be efficacious in treating airsaculitis in chickens caused by E. coli (Huff et al., 2002), reducing E. coli-associated diarrhea (Xie et al., 2005), and strains of Clostridium perfringens (Siragusa et al., 2004). Several experiments have indicated that bacteriophages can reduce systemic Salmonella in chickens (Berchieri et al., 1991; Fiorentini et al., 2005; Toro et al., 2005), broiler carcasses (Higgins et al., 2005), chicken skin (Goode et al., 2003), and poultry products (Whichard et al., 2003).

The objectives of this study were to assess the ability of bacteriophages isolated from commercial broiler houses (CB4∅) and a municipal wastewater treatment plant (WT45∅), administered by different routes, alone or in combination with a probiotic, to reduce Salmonella Enteritidis in vitro and in experimentally infected chicks.

MATERIALS AND METHODS

Salmonella Enteritidis Amplification

A primary poultry isolate of Salmonella Enteritidis, bacteriophage type 13A, was obtained from the USDA National Veterinary Services Laboratory. This isolate was resistant to novobiocin (NO; 25 μg/mL, N-1628, Sigma Chemical Co., St. Louis, MO) and was selected for resistance to nalidixic acid (NA; 20 μg/mL, N-4382, Sigma Chemical Co.) in our laboratory. For these studies Salmonella Enteritidis was grown overnight in tryptic soy broth (TSB) at 37°C. Cells were washed 3 times in sterile saline by centrifugation at 1,864 x g, and the concentration was estimated with a spectrophotometer, using a previously generated standard curve, to approximately 10⁹ cfu/mL and estimated with a spectrophotometer, using a previously generated standard curve, to approximately 10⁹ cfu/mL for 6 h at 37°C. Following incubation, tubes were centrifuged at 1,864 x g for 25 min, and the supernatant was filtered through a 0.8-0.2 μm syringe filter and held at 4°C. A combination of 100 μL of 10⁷ cfu/mL of Salmonella Enteritidis or Salmonella Typhimurium and 1 mL of each broiler house pool was added to 1.5 mL of soft tryptic soy agar (TSA; 211043, Becton Dickinson and Co.). Dilutions were made in sterile saline and poured over a warm TSA plate. Plates were incubated overnight at 37°C. Individual distinct plaques resulting from this plating were then differentiated from plate morphology. Different plaques were sequentially passed on TSA plates at least 3 subsequent times to purify the isolate. From 3 broiler houses, 4 bacteriophage isolates were recovered. Using the same technique, 45 bacteriophages were isolated from 4 wastewater samples (Higgins et al., 2005).

Bacteriophage Amplification

All bacteriophages used in these experiments were amplified in broth using a ratio of 1:3:5 (bacteriophage:turbid Salmonella Enteritidis culture in TSB:fresh TSB) and were incubated at 37°C for 2.5 h. Briefly, an agar plug containing bacteriophage was obtained by pushing a sterile Pasteur pipette into the center of a bacteriophage plaque on a TSA plate where Salmonella Enteritidis was lysed. This bacteriophage plug was then resuspended in 1 mL of sterile saline per plug and then filtered through a 0.8-0.2 μm filter. Turbid Salmonella Enteritidis was obtained from an overnight culture of TSB inoculated with Salmonella Enteritidis and incubated at 37°C. After 2.5 h of incubation, the mixture was filtered, and the bacteriophage was quantified as described above.

In Vitro Crop Assay (Experiment 1)

To evaluate the ability of bacteriophages to reduce Salmonella Enteritidis in vitro, a crop assay system was conducted to simulate the crop environment (Barnhart et al., 1999). An autoclaved, unmedicated corn and soybean meal-based broiler ration, formulated to meet or exceed the levels of critical nutrients for growing broilers, was used (NRC, 1994). The in vitro crop assay consisted of 120 sterile tubes containing 2 g of sterile feed combined with 5 mL of 0.9% sterile saline, 0.5 mL of turbid Salmonella Enteritidis containing 8 x 10⁶ or 8 x 10⁸ cfu/mL, and 1 mL of each filtered bacteriophage pool CB4∅ or WT45∅ with 10-fold dilutions ranging from 10⁶ to 10⁸ pfu/mL. The control tubes received 1 mL of saline. For each treatment, there were 5 replicate tubes.

The test tubes with all contents were vortexed for no more than 5 s and incubated at 37°C. Two or 6 h later, the tubes were vortexed again for no more than 5 s, and 10 μL of each tube was plated for Salmonella Enteritidis enumeration on XLD plates containing 25 μg/mL of NO and 20 μg/mL of NA. The plates were incubated at 37°C for 24 h, and the numbers of colony-forming units of SE were determined.
To determine absence of *Salmonella* Enteritidis, after 6 h of incubation, all the tubes originally inoculated with $8 \times 10^7$ cfu/mL of *Salmonella* Enteritidis were enriched with 5 mL of concentrated (2×) tetrathionate broth (210420, Becton, Dickinson and Co.), vortexed for no more than 5 s and incubated at 37°C overnight, after which the tubes were vortexed again for no more than 5 s, and 1 loop (~20 μL) of each tube was streaked on XLD NO and NA. The plates were then incubated at 37°C for 24 h and examined for presence or absence of *Salmonella* Enteritidis colonies.

### Probiotic Culture

The commercially available probiotic Floramax-B11 (41069, IVS-Wynco LLC, Springdale, AR) was used for this experiment. The product consisted of a defined bacterioprophage containing *Lactobacillus fermentum*, *Lactobacillus helveticus*, *Lactobacillus paracasei*, *Lactobacillus salivaruis*, and *Pediococcus parvulus*. The culture was diluted in reconstituted powdered skim milk to treat chicks cloacally. Actual colony-forming units administered per chick is reported in Table 1 as determined retrospectively. Actual colony-forming units administered per chick is reported in Table 1 as determined retrospectively.

### Bacteriophages in Combination with Probiotic Administered Cloacally (Experiment 2)

One hundred sixty day-of-hatch chicks were obtained from a local hatchery and randomly divided into 4 groups of 25 birds. Each group of birds was placed in cages, provided feed and water ad libitum, and maintained at an age-appropriate temperature for the duration of the experiment. All chicks received a challenge of $3 \times 10^5$ cfu/chick of *Salmonella* Enteritidis by oral gavage (0.25 mL) at the moment of placement. Based on the phenomenon known as cloacal drinking (Sorvari et al., 1977), vent lip application (Corrier et al., 1994) or cloacal drop (Hu et al., 2004), the chicks received 10 μL of WT45∅ or probiotic deposited on the vent lips from a pipette, and such drop was immediately drawn inside the cloaca. Group 1 did not receive vehicle (control group). Group 2 was treated cloacally with $10^8$ pfu/chick of WT45∅ 1 h after challenge. Group 3 was treated cloacally with $10^9$ pfu/chick of WT45∅ 1 h after challenge and then cloacally with $2 \times 10^9$ cfu/chick with the probiotic 2 h after challenge. Group 4 was treated cloacally with $2 \times 10^9$ cfu/chick with the probiotic 2 h after challenge.

All the chicks from each group were euthanized by CO₂ inhalation 24 h posttreatment. Cecal tonsils were aseptically collected from 25 chicks/group, placed individually in tubes containing 10 mL of tetrathionate broth, and incubated overnight at 37°C. After that, 1 loop from each tube was streaked on XLD containing NO and NA, and the plates were incubated overnight at 37°C. Each plate was evaluated for the presence or absence of lactose-negative, NA-resistant *Salmonella* Enteritidis colonies.

### Oral Administration of Bacteriophages (Experiment 3)

One hundred sixty day-of-hatch chicks were obtained from a local hatchery and randomly divided into 4 groups of 40 birds. Each group of birds was placed in cages, provided feed and water ad libitum, and maintained at an age-appropriate temperature for the duration of the experiment. All chicks received a challenge of $9 \times 10^3$ cfu/chick of *Salmonella* Enteritidis by oral gavage (0.25 mL) at the moment of placement. Group 1 did not receive any treatment (control group). Group 2 was treated by oral gavage with $10^9$ pfu/chick of WT45∅ 1 h after challenge. Group 3 was treated by oral gavage with $10^8$ pfu/chick of CB4∅ 1 h after challenge. Group 4 was treated with a mixture of equal ratios of WT45∅ and CB4∅ ($10^9:10^9$ pfu/chick) by oral gavage 1 h after challenge.

Twenty chicks from each group were euthanized by CO₂ inhalation 24 and 48 h after treatments. The cecal tonsils were collected and cultured as above for the presence or absence of *Salmonella* Enteritidis colonies. The chicks used in both in vivo experiments were cared for using procedures approved by the University of Arkansas Institutional Animal Care and Use Committee.

### Statistical Analysis

The incidence of *Salmonella* Enteritidis recovery within experiments was compared using the χ² test of independence (Zar, 1984) to determine significant ($P < 0.05$) differences between control and treated groups. *Salmonella* Enteritidis Enteritidis colony-forming unit data were converted to base-10 logarithm colony-forming unit numbers before analysis using the GLM procedure of SAS (version 9.1, SAS

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Table 1. Recovery of *Salmonella enterica* serovar Enteritidis from cecal tonsils after 24 h of cloacal treatment with bacteriophages isolated from municipal wastewater treatment plant (WT45∅) and probiotic 1 and 2 h, respectively, after *Salmonella* Enteritidis challenge by oral gavage with $3 \times 10^5$ cfu/chick

<table>
<thead>
<tr>
<th>Treatment</th>
<th>24 h</th>
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</thead>
<tbody>
<tr>
<td>Control (only <em>Salmonella</em> Enteritidis)</td>
<td>20/25&lt;sup&gt;a,b&lt;/sup&gt; (80%)</td>
</tr>
<tr>
<td>WT45∅ (10&lt;sup&gt;7&lt;/sup&gt; pfu/chick)</td>
<td>9/25&lt;sup&gt;b&lt;/sup&gt; (36%)</td>
</tr>
<tr>
<td>Probiotic (2 × 10&lt;sup&gt;6&lt;/sup&gt; cfu/chick)</td>
<td>12/25&lt;sup&gt;b&lt;/sup&gt; (48%)</td>
</tr>
<tr>
<td>WT45∅ (10&lt;sup&gt;7&lt;/sup&gt; pfu/chick) and probiotic (2 × 10&lt;sup&gt;6&lt;/sup&gt; cfu/chick)</td>
<td>9/25&lt;sup&gt;b&lt;/sup&gt; (36%)</td>
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<sup>a,b</sup>Values with different superscripts are significantly different ($P < 0.05$).

<sup>1</sup>Number of positive chicks/number of challenged chicks (%).
RESULTS

In experiment 1, using an initial dose of $10^3$ cfu/mL of *Salmonella* Enteritidis, the concentrations from $10^3$ up to $10^9$ pfu/mL of WT45∅ and all the concentrations ($10^3$ to $10^9$ pfu/mL) of CB4∅ significantly reduced *Salmonella* Enteritidis ($P < 0.05$) as compared with the control after 2 and 6 h of incubation (Table 2). However, when the initial dose of $10^6$ cfu/mL of *Salmonella* Enteritidis was used, the CB4∅ only significantly reduced *Salmonella* Enteritidis after 2 h (1.5 log) and not after 6 h of incubation, whereas all the concentrations ($10^3$ to $10^9$ pfu/mL) of the WT45∅ reduced *Salmonella* Enteritidis recovery by 5 to 6 log after 2 and 6 h of incubation (Table 2). The highest concentration ($10^9$ pfu/mL) of WT45∅ reduced *Salmonella* Enteritidis until zero after 2 and 6 h of incubation from an initial dose of $10^3$ cfu/mL of *Salmonella* Enteritidis, confirmed by the enrichment with tetrathionate broth following 24 h of incubation (data not shown).

Data from experiment 2 demonstrate that both treatments by cloaca, $10^9$ pfu/chick of WT45∅, or $2 \times 10^6$ cfu/chick of probiotic significantly reduced *Salmonella* Enteritidis ($P < 0.05$) recovered from cecal tonsils at 24 h following treatment as compared with control (Table 1). Also the mixture of both treatments significantly reduced *Salmonella* Enteritidis, but no additive effect was observed with the combination of bacteriophages and probiotic in this experiment (Table 1).

In experiment 3, the treatments via oral gavage with $10^6$ CB4∅ pfu/chick, $10^8$ WT45∅ pfu/chick, or a combination of both, 1 h postchallenge, significantly reduced *Salmonella* Enteritidis ($P < 0.05$) recovered from cecal tonsils at 24 h as compared with untreated controls (Table 3). However, no significant differences were observed at 48 h following treatment (Table 3).

DISCUSSION

*Salmonella* Enteritidis-lysing bacteriophages isolated from poultry or human sewage sources were used to reduce *Salmonella* Enteritidis in vitro and in experimentally infected chicks. Because the chicken crop has been implicated as an important source of *Salmonella* and other bacteria contamination (Hargis et al., 1995; Smith and Berrang, 2006), an in vitro crop assay was conducted to evaluate the bacteriophage cocktails in the presence of feed, saline, and *Salmonella* Enteritidis, simulating the crop environment. Barnhart et al. (1999) used this model to evaluate several potential disinfectants in the presence of large quantities of organic matter.

This study showed that bacteriophages isolated from a poultry source or wastewater can reduce *Salmonella* Enteritidis. In fact, both CB4∅ and WT45∅ significantly reduced *Salmonella* Enteritidis recovered from cecal tonsils. However, the WT45∅ were more efficacious for reduction of *Salmonella* Enteritidis in vitro than the CB4∅.

The higher number of different bacteriophages in WT45∅ does not explain this improved result in vitro as compared with the 4 bacteriophages used in CB4∅ cocktail treatment, because both treatments were done with the same total bacteriophage concentration. Rather, different lysing ability could explain the better performance of WT45∅. The 4 bacteriophages isolated from broiler houses produced distinct plaques and different killing spectra against several *Salmonella* serovars (data not shown), suggesting that these bacteriophages are not the same. We hypothesized that a cocktail of bacteriophages would decrease the possibility of the development of selection for resistance against all bacteriophages in the cocktail.

### Table 2. In vitro crop assay to determine the ability of bacteriophages isolated from commercial broiler houses (CB4∅) or municipal wastewater treatment plant (WT45∅) to reduce *Salmonella enterica* serovar Enteritidis in the presence of broiler feed

<table>
<thead>
<tr>
<th>Salmonella Enteritidis initial dose (cfu/mL)</th>
<th>Incubation time (h)</th>
<th>CB4∅ concentration (pfu/mL)</th>
<th>WT45∅ concentration (pfu/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$10^5$</td>
<td>$10^6$</td>
</tr>
<tr>
<td>$10^3$</td>
<td>2</td>
<td>4.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.68&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>7.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>$10^6$</td>
<td>2</td>
<td>7.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.49&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>8.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values within columns with different superscripts are significantly different ($P < 0.05$).

### Table 3. Recovery of *Salmonella Enterica* serovar Enteritidis from cecal tonsils of chicks treated by oral gavage with bacteriophages isolated from commercial broiler houses (CB4∅) or municipal wastewater treatment plant (WT45∅) 1 h after *Salmonella* Enteritidis challenge with $9 \times 10^3$ cfu/chick

<table>
<thead>
<tr>
<th>Treatment</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (only <em>Salmonella</em> Enteritidis)</td>
<td>20/20&lt;sup&gt;1&lt;/sup&gt; (100%)</td>
<td>20/20&lt;sup&gt;1&lt;/sup&gt; (100%)</td>
</tr>
<tr>
<td>CB4∅ ($10^6$ pfu/chick)</td>
<td>13/20&lt;sup&gt;1&lt;/sup&gt; (65%)</td>
<td>13/20&lt;sup&gt;1&lt;/sup&gt; (65%)</td>
</tr>
<tr>
<td>WT45∅ ($10^6$ pfu/chick)</td>
<td>14/20&lt;sup&gt;1&lt;/sup&gt; (70%)</td>
<td>14/20&lt;sup&gt;1&lt;/sup&gt; (70%)</td>
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<tr>
<td>CB4∅ ($10^6$ pfu/chick) and WT45∅ ($10^6$ pfu/chick)</td>
<td>9/20&lt;sup&gt;1&lt;/sup&gt; (45%)</td>
<td>9/20&lt;sup&gt;1&lt;/sup&gt; (45%)</td>
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</table>

<sup>1</sup>Values within columns with different superscripts are significantly different ($P < 0.05$).

<sup>1</sup>Number of positive chicks/number of challenged chicks (%).
The data from experiment 2 show use of an alternative route, the cloaca, for bacteriophages, and probiotic treatment reduced Salmonella Enteritidis recovered from cecal tonsils at 24 h following treatment. Though vent lip application may not be practical commercially, this route could be a plausible explanation to what happens in native conditions, because lactobacilli in the cloaca can inhibit growth of Salmonella Enteritidis (Miyamoto et al., 2000). Administration of the WT45∅ by vent lip application caused a significant reduction in Salmonella Enteritidis recovered from the cecal tonsils. This corroborates with the reduction seen by Corrier et al. (1994) following vent application of cecal microflora. There was no additive effect observed with the combination of bacteriophages and the probiotic following vent lip application. Toro et al. (2005) treated chicks orally with bacteriophages and probiotic and likewise observed the absence of a synergistic effect between them for reduction of Salmonella Typhimurium.

The treatment via oral gavage with 10^8 pfu/chick of CB4∅ or WT45∅ or a combination of both reduced Salmonella Enteritidis recovered from cecal tonsils at 24 h as compared with untreated controls, but no differences were observed at 48 h following treatment, suggesting that some bacteriophages can be efficacious in reducing Salmonella Enteritidis colonization in poultry during a short period, possibly without sustained effect due to bacteriophage resistance. Fiorentin et al. (2005) suggested that bacteriophages will be most effective in a short period after administration and only in birds with high colony-forming units per gram of Salmonella Enteritidis. Because the lytic bacteriophage life cycle is usually less than 30 min, it is reasonable to postulate that if there is not sufficient bacteriophage numbers to cause lysis of all Salmonella hosts, some surviving bacteria could become resistant, increasing in number and colonizing the same place during a short period.

Even though the efficacy of these bacteriophages in reducing Salmonella Enteritidis colonization in poultry is short-lived, these data suggest that some bacteriophages can survive some time in the digestive tract. Toro et al. (2005) also demonstrated that some bacteriophages are not inactivated in the digestive tract, reaching their host and replicating successfully.

Our results suggest that the bacteriophages could reduce Salmonella Enteritidis colonization, but there was no sustained Salmonella Enteritidis reduction, probably due to resistance. Alternative treatments of bacteriophage therapy could be plausible; however, further research must focus on improving efficacy for therapeutic use in poultry.

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REFERENCES


