Evaluation of a *Lactobacillus*-Based Probiotic Culture for the Reduction of *Salmonella* Enteritidis in Neonatal Broiler Chicks

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**ABSTRACT** We evaluated the effect of a *Lactobacillus*-based probiotic culture (FM-B11) for reduction of *Salmonella* Enteritidis in neonatal broiler chicks. In all experiments, chicks were challenged with approximately 10⁴ cfu of *Salmonella* Enteritidis upon arrival at our laboratory, and the treatments were administered 1 h postchallenge. Cecal tonsil samples were obtained 24 h posttreatment and enriched for *Salmonella* Enteritidis recovery. The first experiment compared the effects of oral administration of doses of 10⁴, 10⁶, and 10⁸ cfu/chick. In this experiment, doses of 10⁶ and 10⁸ both significantly reduced *Salmonella* Enteritidis recovery compared with controls (15 vs. 85% *Salmonella* Enteritidis positive), but 10⁴ cfu did not significantly reduce *Salmonella* Enteritidis recovery. The second experiment compared the efficacy of oral administration of the live probiotic culture, with or without supernatant removed, to inactivated cultures or supernatant alone. Live probiotic organisms, with or without supernatant, significantly reduced *Salmonella* Enteritidis in this experiment, but inactivated or cell-free treatments did not reduce *Salmonella* Enteritidis. In the final 2 experiments, differing doses of probiotic culture were administered on the vent lips, where the treatment was taken into the lower gastrointestinal tract via cloacal drinking. Concentrations of probiotic culture from 10² to 10⁷ cfu/chick significantly reduced *Salmonella* Enteritidis, and there was no difference in *Salmonella* Enteritidis recovery between treatment concentrations. These data suggest that this *Lactobacillus*-based probiotic culture may be efficacious for reduction of *Salmonella* Enteritidis in neonatal chicks.

**Key words:** probiotic, *Salmonella*, *Lactobacillus*, cloacal drinking, chick

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**INTRODUCTION**

In the United States, it is estimated that 1.4 million humans contract salmonellosis annually and that the annual cost of this illness, including lost productivity, is $3 billion (WHO, 2006). In the year 2004, surveillance data indicated that the greatest number of foodborne illnesses were caused by *Salmonella*, comprising 42% of all laboratory diagnoses (FoodNet, 2005). Because poultry and poultry products often serve as the vehicle for human *Salmonellosis* (Bean and Griffin, 1990; Persson and Jendteg, 1992, Kimura et al., 2004, Marcus et al., 2007), the poultry industry and governmental agencies are focused on eradicating *Salmonella* in live birds and at the processing plant (Hargis et al., 2001). Additionally, public pressure to reduce usage of antimicrobials has influenced development of alternative methods for reduction of pathogens, including probiotics.

Probiotics are beneficial bacteria that influence the host by improving intestinal health (Isolauri et al., 2001). Bacterial cultures have previously been utilized for reduction of *Salmonella* in chicks with some success (Blankenship et al., 1993, Corrier et al., 1995). Although many probiotic cultures consist of live organisms, some researchers have reported benefits from administration of inactivated or killed organisms. Huang et al. (2004) administered killed, cobalt-enriched *Lactobacillus casei* and *Lactobacillus acidophilus* in the feed of broiler chickens and observed increased BW at 6 wk of age. Application of various formalin-killed probiotic cultures in the feed of rainbow trout fry challenged with *Aeromonas salmonicida* significantly reduced mortality compared with controls (Irianto and Austin, 2003). Other reports indicate that killed cultures are capable of initiating changes in the immune system parameters. Sashihara et al. (2006) applied heat-killed *Lactobacillus plantarum* and *Lactobacillus gasseri* to cultures of splenocytes and mesenteric lymph node cells, and observed an increase in production of IL-12. Administration of live or dead *Lactobacillus* GG to cultures of Caco-2 cells resulted in a decrease of tumor necrosis factor-α induced interleukin-8 production (Zhang et al., 2005).
Some lactic acid bacteria have been reported to produce soluble antimicrobial peptides, called bacteriocins, which are postulated to contribute to their ability to improve intestinal health. An isolate of L. acidophilus has been reported to produce 2 bacteriocins, which inhibited growth of 2 nonpathogens: Lactococcus and Pediococcus. These bacteriocins also inhibited growth of several pathogenic organisms in vitro, from genuses including Staphylococcus, Enterococcus, Streptococcus, Listeria, Clostridium, and Bacillus (Bogović-Matijasović et al., 1998). Ocaña et al. (1999) reported isolation of a bacteriocin from a Lactobacillus salivarius strain that inhibited Enterococcus and Staphylococcus. Other isolates have also been reported to produce bacteriocins, including Lactobacillus delbruekii whose bacteriocin only inhibited other strains of Lactobacillus, which may confer an advantage during colonization. However, there is a dearth of information regarding the effects of bacteriocins in vivo, likely due to the difficulty of measuring these effects in vivo.

We recently used intensive screening of bacteria, which allowed the identification of 11 lactic acid bacteria that were efficacious in the treatment of Salmonella-infected chicks and poults (Tellez et al., 2006). The present studies evaluated the optimal dose necessary for reduction of Salmonella in neonatal chicks, and to evaluate whether inactivated cultures or cell-free supernatant are efficacious in the absence of live bacteria for reduction of Salmonella Enteritidis. Additionally, we tested whether the probiotic organisms are capable of inhibiting Salmonella colonization following cloacal administration.

**MATERIALS AND METHODS**

**Chickens**

Broiler chicks were obtained from a local hatchery on the day of hatch. In all experiments they were housed in battery brooder units at age-appropriate temperatures and were provided feed and water at all times. Use of birds in these experiments was approved by the Institutional Animal Care and Use Committee at the University of Arkansas.

**Salmonella**

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 (catalog No. N-1628, Sigma, St. Louis, MO; 25 μg/mL) and was selected for resistance to nalidixic acid (catalog No. N-1628, Sigma; NA, 20 μg/mL) in our laboratory. For these studies Salmonella Enteritidis was grown overnight in tryptic soy broth (catalog No. 211822, Becton Dickinson, Sparks, MD) at 37°C. Cells were washed 3 times in sterile saline by centrifugation at 1,864 × g and the concentration was estimated with a spectrophotometer to approximately 10^9 cfu/mL in sterile saline and then diluted to inoculated concentrations as described below. Concentrations of *Salmonella* Enteritidis were retrospectively determined by spread plating on xylose lysine deoxycholate agar (catalog no. 278820, Becton Dickinson, Sparks, MD) plates containing novobiocin (25 μg/mL) and NA (20 μg/mL), and enumeration for each experiment. Actual determined colony-forming units for each experiment are reported.

**Probiotic Culture**

Eleven lactic acid bacterial isolates were previously selected and have been previously described (Higgins et al., 2005). This mixture (FM-B11; Ivesco, LLC, Springdale, AR) was used for these experiments. The probiotic culture was diluted in sterile saline to reported concentrations for each experiment. Actual colony-forming units administered per chick from each experiment are reported, which are determined retrospectively from spread plating on Mann Rogosa sharp agar (catalog No. R1148, Sigma).

**Salmonella Recovery**

For recovery of *Salmonella* Enteritidis, chicks were humanely killed by CO2 asphyxiation. The cecal tonsils were aseptically removed and placed in sterile tubes containing 10 mL of tetrathionate broth (catalog No. 210420, Becton Dickinson, Sparks, MD). These samples were incubated 18 h at 37°C and then streaked for isolation on xylose lysine deoxycholate agar plates. Plates were incubated for 18 h at 37°C and then observed for the presence or absence of characteristic *Salmonella* colonies, which are black on this selective media. The recovery of *Salmonella* Enteritidis is reported as the number of positive samples/number of total samples. The incidence of *Salmonella* recovery within experiments was compared using the χ^2_2_ test of independence (Zar, 1984) to determine significant (P < 0.05) differences between control and treated groups.

**Experiment 1**

We performed this experiment to evaluate the optimal numbers of probiotic organisms necessary to reduce *Salmonella* Enteritidis in this model. Eighty chicks were obtained on the day-of-hatch from a local hatchery and were each challenged with 7.5 × 10^3 cfu of *Salmonella* Enteritidis by oral gavage in a 0.25-mL volume. They were then randomly divided into 4 groups and placed on individual levels within the battery brooder, with access to feed and water for 1 h. After 1 h, chicks received the appropriate dose of probiotic culture (10^2, 10^3, or 10^6 cfu/chick) or sterile saline (control group) by oral gavage. Chicks were humanely killed and samples taken for *Salmonella* Enteritidis recovery 24 h following treatment.

**Experiment 2**

This experiment compared the administration of live probiotic organisms with or without supernatant included, supernatant alone, or inactivated cultures. One hundred fifty chicks were obtained on the day-of-hatch...
and randomly divided into 6 groups of 25 chicks each. All chicks were challenged with $8 \times 10^3$ cfu of *Salmonella Enteritidis* by oral gavage on the day of hatch (7.5 $\times$ 10$^3$ cfu/chick). Probiotic treatments were administered by oral gavage 1 h postchallenge.

### Probiorotic Culture with Supernatant
Commercial probiotic culture was amplified in MRS broth for 16 h at 37°C. This culture contained eleven lactic acid bacteria isolates: 3 *Lactobacillus bulgaricus*, 3 *Lactobacillus fermentum*, 2 *Lactobacillus casei*, 2 *Lactobacillus cellobiosus*, and 1 *Lactobacillus helveticus*. Dilutions were made in sterile saline to reach the desired concentration.

### Probiorotic without Supernatant
The probiotic culture was prepared and then washed in sterile saline following centrifugation 3 times. Briefly, the culture was centrifuged for 15 min at 1,864 $\times$ g, then the supernatant was removed and the cells were resuspended in sterile saline. This was repeated 2 more times, then the culture was diluted to a concentration of $10^8$ cfu in 0.25 mL for administration by oral gavage.

### Supernatant
Probiotic culture was centrifuged for 15 min at 1,864 $\times$ g, and the supernatant was transferred to a sterile tube. The supernatant was then filtered using a syringe filter (catalog No. 4187, Pall Corporation, Ann Arbor, MI) with 0.2-$\mu$m pore size. A sample was streaked on both MRS and TSA agar plates to confirm that no bacteria were present.

### Penicillin Inactivated
Probiotic culture killed by penicillin was prepared by combining equal parts live bacteria with sterile saline containing 100,000 units/mL of Penicillin G Potassium (catalog No. 1PENO11, Bimeda Inc., Riverside, MO) and incubated overnight at 37°C. The culture was then centrifuged for 15 min at 1,864 $\times$ g, then the supernatant was removed and the cells were resuspended in sterile saline. This was repeated 2 more times; then the culture was diluted to an approximate concentration of $10^6$ dead organisms in 0.25 mL for administration by oral gavage, based on colony-forming units determined prior to killing. A sample was streaked on MRS and TSA agar plates to confirm inactivation of the culture.

### Heat Inactivated
The probiotic culture was incubated at 60°C in a waterbath for 3 h. The culture was then washed 3 times with saline as described above and diluted to an approximate concentration of $10^6$ dead organisms in 0.25 mL for administration by oral gavage, based on colony-forming units determined prior to killing. A sample was streaked on MRS and TSA agar plates to confirm the inactivation of the culture.

### Experiments 3 and 4
In these experiments we evaluated the ability of the probiotic culture to reduce *Salmonella Enteritidis* when applied in the drinking water or directly to the vent lips. Eighty chicks were obtained on the day of hatch for each experiment, and upon arrival at the laboratory all chicks were challenged orally with *Salmonella Enteritidis* (experiment 3: 1.75 $\times$ 10$^4$ cfu/chick, experiment 4: 3.7 $\times$ 10$^5$ cfu/chick). They were then randomly divided into 4 groups and placed on individual levels in a battery brooder with access to feed and water for 1 h. One group remained untreated, and one group received $10^6$ cfu/mL of probiotic treatment in the drinking water, with 1% skim milk.

### Table 2. Evaluation of efficacy of the supernatant from a probiotic culture with inactivated or live organisms following challenge with *Salmonella Enteritidis*

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Number <em>Salmonella Enteritidis</em> positive/total samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Vehicle</td>
<td>22/25 (88)$^a$</td>
</tr>
<tr>
<td>Inactivated</td>
<td>Penicillin killed</td>
<td>25/25 (92)$^a$</td>
</tr>
<tr>
<td>Inactivated</td>
<td>Heat killed</td>
<td>24/25 (96)$^a$</td>
</tr>
<tr>
<td>Supernatant</td>
<td>Sterile filtered</td>
<td>21/25 (84)$^a$</td>
</tr>
<tr>
<td>Live probiotic</td>
<td>$10^6$ cfu/chick</td>
<td>7/24 (29)$^b$</td>
</tr>
<tr>
<td>Live probiotic</td>
<td>Supernatant removed $10^6$ cfu/chick</td>
<td>7/25 (28)$^b$</td>
</tr>
</tbody>
</table>

$a,b$Different superscripts indicate significant differences between treatments.

$^a$Chicks were all challenged with *Salmonella Enteritidis* by oral gavage on the day of hatch ($8 \times 10^3$ cfu/chick). All treatments were administered by oral gavage 1 h postchallenge.

$^b$Cecal tonsil samples were obtained 24 h posttreatment and were enriched for recovery of *Salmonella Enteritidis*.

### Table 1. Evaluation of different concentrations of a probiotic culture for reduction of *Salmonella Enteritidis* in neonatal chicks 24 h posttreatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Probiotic treatment (cfu/chick)</th>
<th>Number <em>Salmonella Enteritidis</em> positive/total samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>17/20 (85)$^a$</td>
</tr>
<tr>
<td>Treated</td>
<td>$10^6$</td>
<td>13/20 (65)$^a$</td>
</tr>
<tr>
<td>Treated</td>
<td>$10^4$</td>
<td>3/20 (15)$^b$</td>
</tr>
</tbody>
</table>

$^a,b$Different superscripts indicate significant differences between treatments.
added as a stabilizer. Two groups were treated by vent lip application of the culture. The chicks were gently inverted, and probiotic treatment was applied to the vent lips using a pipette (experiment 3: $10^2$ and $10^4$ cfu/mL; experiment 4: $4 \times 10^3$ and $4 \times 10^7$ cfu/mL). The chicks continued to be held inverted until the treatment was taken into the cloaca by cloacal drinking (Corrier et al., 1994); then they were placed again into the brooder battery. Chicks were humanely killed and samples taken for Salmonella Enteritidis recovery 24 h following treatment.

**RESULTS AND DISCUSSION**

In the first experiment, we observed a distinct effect due to the concentration of probiotic treatments administered (Table 1). The lowest concentration examined ($10^4$ cfu/chick) did not result in a significant reduction of Salmonella Enteritidis. However, both $10^6$ and $10^8$ cfu/chick did result in a significant reduction of Salmonella Enteritidis, with only 15% of chicks remaining positive in the cecal tonsils. Remarkably, there was absolutely no improvement of effect following administration of $10^8$ cfu/chick, even though this is a 2-log increase in administered bacteria (Table 1). These data suggest that the effects of this culture are limited, in that an increase in the number of administered bacteria will not further reduce Salmonella Enteritidis colonization.

In the second experiment, reduction in Salmonella Enteritidis colonization only occurred in the groups receiving live probiotic organisms, with or without removal of the supernatant. In other studies with probiotic cultures, improvements in health have been reported due to direct feeding of dead or inactivated cultures (Irianto and Austin, 2003; Huang et al., 2004). Although a marked reduction in Salmonella Enteritidis colonization was observed due to administration of the live culture, inactivation by penicillin or heat negated this effect (Table 2).

Lactobacillii have also been widely reported to produce antibacterial compounds called bacteriocins, and the effect of bacteriocins have been hypothesized to be the mechanism by which Lactobacillii exert cytotoxic effects in vivo (Bogović-Matišić et al., 1998; Ocaña et al., 1999). We hypothesized that a soluble peptide could mediate the reduction of Salmonella Enteritidis we have observed using this probiotic culture. Although administration of the live culture, washed by centrifugation prior to administration, markedly reduced Salmonella Enteritidis colonization in experiment 2, no effect of administration of the supernatant alone was observed (Table 2). These data suggest that the effect of this probiotic is not due to the presence of preformed antimicrobial compounds. However, this does not rule out the possibility of local production of such molecules within the enteric microenvironment.

We further investigated the effect of this probiotic culture when administered by the cloaca in experiment 3. Cloacal drinking has been hypothesized to be a mechanism of sampling the environment and priming the immune system (Sorvari et al., 1975). In these experiments, application of probiotic bacteria by vent application resulted in significant reductions of Salmonella Enteritidis infection, similar to that achieved by drinking water application in experiments 3 and 4 (Table 3). Remarkably, there was no observed difference in Salmonella Enteritidis infection following application of cloacal treatments over a wide range of concentrations ($10^2$ to $10^7$ cfu/chick). Previous studies have indicated that enteric infection with Salmonella was accomplished with lower challenge numbers when applied via the cloaca as compared with oral gavage, presumably due to more direct access to the lower small intestine and ceca and bypassing the more hostile actions of low gastric pH and upper small intestine enzymatic and bile actions (Cox et al., 1990). It is possible that the lower effective dosage observed for Salmonella Enteritidis colonization reduction in the present experiment through vent application is due to a similar mechanism(s).

In conclusion, oral administration of $10^6$ or $10^8$ cfu of this Lactobacillus-based probiotic culture, within 1 h of challenge, significantly reduced Salmonella Enteritidis recovery from neonatal chicks, whereas a $10^6 \times$ lower dosage had no significant effect. Administration of cell-free supernatant or inactivated cultures did not reduce Salmonella Enteritidis infection, indicating that these effects of this probiotic are mediated by live bacteria. Further evaluation revealed that administration of a broad range of probiotic concentrations by vent application resulted in significant reductions of Salmonella Enteritidis 1 h following oral Salmonella Enteritidis challenge.

**Table 3. Comparison of oral or cloacal administration of a probiotic culture for reduction of Salmonella Enteritidis**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Challenge dose (cfu/chick)</th>
<th>Treatment administered (1 h postchallenge)</th>
<th>Route of administration</th>
<th>Number of Salmonella Enteritidis positive/total samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>$1.75 \times 10^4$</td>
<td>Control</td>
<td>None</td>
<td>19/19 (100)$^a$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^6$ cfu/mL probiotic</td>
<td>Drinking water</td>
<td>15/20 (75)$^b$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^7$ cfu probiotic</td>
<td>Cloacal</td>
<td>13/20 (65)$^b$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^3$ cfu probiotic</td>
<td>Cloacal</td>
<td>15/20 (75)$^b$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^6$ cfu/mL probiotic</td>
<td>Cloacal</td>
<td>11/20 (55)$^b$</td>
</tr>
<tr>
<td>4</td>
<td>$3.7 \times 10^3$</td>
<td>Control</td>
<td>None</td>
<td>20/20 (100)$^a$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$4 \times 10^5$ cfu/mL probiotic</td>
<td>Drinking water</td>
<td>16/20 (80)$^b$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$4 \times 10^6$ cfu probiotic</td>
<td>Cloacal</td>
<td>13/20 (65)$^b$</td>
</tr>
</tbody>
</table>

$^a,b$Different superscripts indicate significant differences between treatments.
REFERENCES


