Temporal Effects of Lactic Acid Bacteria Probiotic Culture on *Salmonella* in Neonatal Broilers

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ABSTRACT We evaluated the ability of a commercially available lactic acid bacteria-based probiotic culture (LAB) to reduce *Salmonella* Enteritidis or *Salmonella* Typhimurium in day-of-hatch broiler chicks. In these experiments, chicks were challenged with *Salmonella* Enteritidis or *Salmonella* Typhimurium and treated with LAB 1-h postchallenge. Following treatment, cecal tonsils and ceca were aseptically collected for *Salmonella* Enteritidis or *Salmonella* Typhimurium enrichment or *Salmonella* Enteritidis enumeration, respectively. In experiments 1 to 3, LAB significantly reduced the incidence of *Salmonella* Enteritidis (60 to 70% reduction) or *Salmonella* Typhimurium (89 to 95% reduction) recovered from the cecal tonsils of day-old broiler chicks 24 h following treatment as compared to controls (*P* < 0.05). Additionally, administration of LAB caused a >2.9 log₁₀ reduction of total cecal *Salmonella* Enteritidis recovered 24 h following treatment as compared with controls (*P* < 0.05). In experiments 4 to 7, upon sample enrichment LAB significantly reduced the recovery of *Salmonella* Enteritidis from the cecal tonsils at 24 h, but not 6 or 12 h posttreatment (*P* < 0.05). However, in experiments 6 and 7, when total cecal *Salmonella* Enteritidis recovery was enumerated, a significant treatment-associated reduction was observed 12 h posttreatment, although in cecal tonsil samples there was no difference in *Salmonella* Enteritidis incidence at 12 h (*P* < 0.05). In these studies, LAB treatment significantly reduced recovery of *Salmonella* in day-of-hatch broilers.

Key words: *Salmonella*, probiotic, lactic acid bacteria, poultry

INTRODUCTION

In the United States, it is estimated that 1.4 million humans contract salmonellosis, and that the annual cost of this illness, including lost productivity, is $3 billion annually (WHO, 2006). In the year 2004, surveillance data indicated that the greatest number of foodborne illnesses was 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), the poultry industry and governmental agencies are focused on eradicating *Salmonella* in live birds and at the processing plant (Hargis et al., 2001).

The use of probiotics in poultry has been investigated since Rantala and Nurmi (1973) observed that exposure of young chicks to bacteria from the gut of mature birds conferred protection from infection. Selected beneficial bacteria such as lactic acid bacteria (LAB) have been proposed as probiotics for the prevention of various enteric diseases and the improvement of overall health for many years (Tellez et al., 2006). Previous probiotic cultures have successfully reduced enteric salmonellosis in poultry. Specifically, Baba and others (1991) found that using a combination of *Escherichia coli* and *Lactobacillus* spp. was more effective at reducing *Salmonella* Typhimurium colonization in chicks than treating with an individual probiotic isolate. An anaerobic probiotic culture comprised of 29 bacterial strains representing 10 genera also reduced the amount of recoverable *Salmonella* Typhimurium from chicks (Corrier et al., 1995, 1998). Other anaerobic cecal-extracted probiotic cultures have also proved effective at reducing *Salmonella* (Impey et al., 1984) or *Salmonella* and *Campylobacter* (Blankenship et al., 1993; Stern et al., 2001). These findings clearly demonstrate the ability of probiotic cultures to reduce *Salmonella* incidence in poultry.

The commercially available probiotic utilized in the present studies, consisting of 11 LAB isolates, has been shown to improve BW of turkeys experiencing idiopathic diarrhea under commercial conditions (Higgins et al., 2005b) and to increase performance of production turkeys under commercial conditions (Torres-Rodriguez et al., 2007). We evaluated the ability of this product to reduce the amount of recoverable *Salmonella* from the ceca of broiler chicks.

MATERIALS AND METHODS

*Salmonella* Amplification

A primary poultry isolate of *Salmonella* Enteritidis PT 13A or *Salmonella* Typhimurium, each resistant to novobi-
ocin (NO, Catalog No. N-1628, Sigma, St. Louis, MO) and selected for resistance to nalidixic acid (NA, Catalog No. N-4382, Sigma), were used for these experiments. The amplification protocol has been described in detail (Higgins et al., 2005a). Briefly, Salmonella Enteritidis or Salmonella Typhimurium was incubated at 37°C for 24 h and passed every 8 h. Cells were then washed 3 times in sterile saline by centrifugation at 1,864 × g. Concentrations of Salmonella Enteritidis or Salmonella Typhimurium were retrospectively determined by spread plating on xylose lactose differential agar (XLD, Catalog No. 278820, Becton Dickinson, Sparks, MD) plates containing NO (25 μg/mL) and NA (20 μg/mL). Actual determined cfu for each experiment are reported in Table 1.

**Probiotic Culture (LAB)**

Eleven LAB isolates, of poultry gastrointestinal origin, were previously selected and described (Higgins et al., 2005b). This commercial product (FM-B11, Catalog No. 41069, IVS-Wynco LLC, Springdale, AR) was diluted in reconstituted powdered skim milk to an expected concentration of 4 × 10^8 cfu/mL for oral gavage of chicks in these studies. Actual cfu administered per chick from each experiment are reported in Table 1 as determined retrospectively from spread plating on Mann Rogosa sharp agar (Catalog No. R1148, Sigma).

**Experimental Design**

For all experiments, day-of-hatch male broiler chicks were obtained from a local hatchery. Chicks used in all experiments were cared for using procedures approved by the University of Arkansas Institutional Animal Care and Use Committee. Chicks were randomly assigned to treatment groups and then challenged by oral gavage (0.25 mL) with Salmonella Enteritidis or Salmonella Typhimurium at approximately 10^8 cfu/chick (Table 1) and placed into pens (n = 40 per pen). Heated brooder batteries were used for housing and chicks were allowed ad libitum access to unmedicated broiler starter ration and water for the duration of the experiment. One hour post Salmonella challenge, LAB was administered via oral gavage (0.25 mL) with vehicle administered to control groups. At selected times following LAB treatment (24-h experiments 1 to 3; 6, 12, and 24 h experiments 4 to 7), broilers were humanely killed by CO2 inhalation, and cecal tonsils and ceca were collected separately and aseptically (n = 25 per group in experiments 1 to 6; n = 20 per group in experiment 7). Cecal tonsils were enriched in 10 mL of tetrathionate broth overnight at 37°C. Following enrichment, each sample was streaked for isolation 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 examined for the presence or absence of the antibiotic-resistant Salmonella Enteritidis or Salmonella Typhimurium. Cecas were homogenized within sterile sample bags (Catalog No. B00679WA, Nasco, Fort Atkinson, WI) using a rubber mallet. Sterile saline (3 mL) was added to each sample bag and hand stomached with the cecal contents. Dilutions were spread plated 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 cfu of Salmonella Enteritidis per cecal pair were determined.

**Statistical Analysis**

The incidence of Salmonella 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. Ceca cfu data were converted to log10 cfu numbers, then compared using the GLM procedure of SAS (SAS Institute, 2002) with significance reported at P < 0.05.

**RESULTS**

In experiments 1 to 3, LAB significantly reduced the incidence of Salmonella Enteritidis (60 to 70% reduction) or Salmonella Typhimurium (89 to 95% reduction) recovered from the cecal tonsils of day-old broiler chickens 24 h following treatment as compared with controls using this model (Table 2; P < 0.05). Also in experiments 1 to 3, groups involving Salmonella Typhimurium challenge were only subjected to enrichment culture, so no Salmonella Typhimurium cfu data was reported. In the first 3 experiments, administration of LAB caused a >2.9 log10 reduction in Salmonella Enteritidis recovered 24 h following treatment as compared with controls (P < 0.05; Table 2). When Salmonella Enteritidis recovery was evaluated only from samples that were cecal-tonsil-positive following enrichment, significant >1.5 log10 reductions of Salmonella Enteritidis recovery were observed from ceca of LAB-treated chicks (P < 0.05).

In experiments 4 to 7, LAB significantly reduced the recovery of Salmonella Enteritidis from the cecal tonsils at 24 h, but not 6 or 12 h posttreatment (P < 0.05; Table 3). However, in experiments 6 and 7, when total cecal Salmonella Enteritidis recovery was enumerated, a significant treatment-associated reduction in recovered cfu was observed 12 h posttreatment, although there was no difference in Salmonella Enteritidis incidence at 12 h (P < 0.05; Table 3). Similar to the results of experiments
1 to 3 (Table 2), LAB treatment was associated with significant reductions in numbers of Salmonella Enteritidis recovered from ceca even when samples that were cecal-tonsil-positive following enrichment were considered \((P < 0.05); \text{Table } 4\).

**DISCUSSION**

The data from experiments 1 to 3 clearly demonstrate that administration of LAB 1-h post Salmonella Enteritidis or Salmonella Typhimurium challenge significantly reduced the incidence of Salmonella recovery from cecal tonsils of broiler chicks compared with untreated controls 24 h following treatment. Similarly, LAB treatment resulted in significant reductions in the concentrations of Salmonella Enteritidis within the ceca. Interestingly, significant reductions in numbers of Salmonella Enteritidis recovered from ceca derived from LAB-treated chicks were observed when only those samples paired with positive enrichment were considered. This suggests that probiotic-related reductions are not only related to reduced incidence of Salmonella-positive chicks, but infection levels within chicks that were still infected were also reduced.

In experiments 4 to 7 the incidence of Salmonella Enteritidis recovered from cecal tonsils of treated chicks was very similar to the untreated controls 6 and 12 h posttreatment. However, by 24 h a significant reduction associated with LAB treatment was observed. As observed at 24 h in experiments 1 to 3, significant treatment-associated reductions in numbers of Salmonella Enteritidis recovered from ceca were observed as early as 12 h posttreatment, whereas differences in incidence of recovery following enrichment were not observed until 24 h. These data suggest that the mechanism of pathogen reduction had been initiated within the first 12 h following treatment. The 2 most likely mechanisms by which this probiotic reduces the recovery of Salmonella involve bacterial interactions (competitive exclusion) or stimulation of a host innate immune response. Briefly, Mead (2000) proposed 4 methods by which competitive exclusion cultures are able to exclude enteric pathogens: competition for receptor sites, production of volatile fatty acids that are inhibitory of certain enteric pathogens, production of bactericins (antimicrobial peptides produced by 1 bacterium that inhibits another bacterium), or competition with pathogens and native flora for limiting nutrients. The LAB culture could reduce Salmonella recovery through a number of these inhibitory mechanisms. Additionally, because these lactic acid bacteria were isolated from mature poultry, their ability to survive in the gut of poultry is anticipated. Whereas bac-

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**Table 2. Effect of lactic acid bacteria (LAB) on Salmonella Typhimurium or Salmonella Enteritidis recovered from cecal tonsils or ceca of broiler chicks 24 h posttreatment**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>Salmonella Typhimurium cecal tonsil recovery</th>
<th>Salmonella Enteritidis cecal tonsil recovery</th>
<th>Log10 Salmonella Typhimurium Enteritidis</th>
<th>Log10 Salmonella Enteritidis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>20/25 (80)</td>
<td>22/25 (88)</td>
<td>3.81 ± 0.32</td>
<td>4.33 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>LAB</td>
<td>2/25 (8)</td>
<td>8/25 (32)</td>
<td>0.62 ± 0.19*</td>
<td>1.95 ± 0.09*</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>18/25 (72)</td>
<td>25/25 (100)</td>
<td>3.59 ± 0.23</td>
<td>3.59 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>LAB</td>
<td>2/25 (8)*</td>
<td>7/25 (28)*</td>
<td>0.42 ± 0.18*</td>
<td>1.91 ± 0.29*</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>20/25 (80)</td>
<td>25/25 (100)</td>
<td>3.91 ± 0.19</td>
<td>3.91 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>LAB</td>
<td>1/25 (4)*</td>
<td>11/25 (40)*</td>
<td>1.00 ± 0.25*</td>
<td>2.22 ± 0.24*</td>
</tr>
</tbody>
</table>

*A significant \((P \leq 0.05)\) difference was observed between control and treated within a single experiment in each column.

**Table 3. Effect of lactic acid bacteria on Salmonella Enteritidis recovered from cecal tonsils or ceca of broiler chicks 6, 12, and 24 h posttreatment**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>Salmonella Enteritidis cecal tonsil recovery</th>
<th>Log10 Salmonella Enteritidis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>8/26 (31)</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>LAB</td>
<td>6/24 (25)</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>16/25 (64)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>LAB</td>
<td>10/25 (40)</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>Control</td>
<td>6/25 (24)</td>
<td>0.35 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>LAB</td>
<td>5/25 (20)</td>
<td>0.30 ± 0.12</td>
</tr>
<tr>
<td>7</td>
<td>Control</td>
<td>8/20 (40)</td>
<td>0.64 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>LAB</td>
<td>8/20 (40)</td>
<td>0.59 ± 0.17</td>
</tr>
</tbody>
</table>

1ND = not determined.

*A significant \((P < 0.05)\) difference was observed between control and treated within a single experiment in each column.
intestinal interactions are the most accepted mechanism for this reduction of Salmonella, stimulation of an effective innate immune response could be more likely due to the rapidity of this response.

Stimulation of immune responses by probiotic cultures has been described in several animal models. Kim and coworkers (2006) demonstrated that parenteral administration of a probiotic Lactobacillus isolate increased expression of tumor necrosis factor-α, interleukin (IL)-12, IL-18, and interferon-γ in the spleen of mice compared with vehicle-injected mice. Vinderola et al. (2004) also observed that oral administration of probiotics increased number of IgA+, tumour necrosis factor-α-, and IL-10-producing cells in the small intestine, whereas some reduced the number of IL-6 producing cells in the small and large intestine of mice following 5 d of treatment. Galdeano and Perdigon (2006) found that oral administration of Lactobacillus casei to mice did not significantly increase the number of CD3+, CD4+, CD8+, or IgA- cells in the small intestine of mice within the first 5 d of treatment. However, there was a significant increase in the mannose binding CD-206 receptors in the small intestine 48 h following probiotic administration. The CD-206 receptor facilitates phagocytosis of mannosylated antigens and is expressed on antigen presenting cells such as dendritic cells and macrophages. This would indicate that the initial immune response is not T- or B-cell dependent, but rather dendritic cells or macrophages are responsible for the early innate immune response associated with probiotics. In poultry, Dalloul et al. (2003) described significantly higher levels of interferon-γ and IL-2 in the intestine of probiotic-treated chickens 3 d following challenge with Eimeria acervulina. With all of these observations, it has still been difficult to determine the host responses that are most beneficial in the reduction of enteric pathogens. In the present studies, LAB treatment significantly reduced recovery of Salmonella. Further research will be conducted using this probiotic to determine the exact mechanism of pathogen reduction.

REFERENCES


