

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

with controls ($P < 0.05$). Additionally, administration of LAB caused a $>2.9 \log_{10}$ 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

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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 Jenteg, 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-

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Table 1. Challenge level of *Salmonella* Enteritidis, *Salmonella* Typhimurium, or treatment level of lactic acid bacteria (LAB) in colony-forming units per chick

Experiment	Colony-forming units administered per chick		
	<i>Salmonella</i> Enteritidis	<i>Salmonella</i> Typhimurium	LAB
1	4.5×10^3	7.5×10^3	7.5×10^5
2	7.0×10^3	4.0×10^3	2.0×10^6
3	3.5×10^3	2.0×10^3	5.0×10^5
4	6.0×10^3	NA ¹	5.0×10^5
5	5.0×10^3	NA	6.0×10^5
6	3.0×10^3	NA	2.0×10^6
7	5.0×10^3	NA	1.0×10^6

¹NA = not applicable.

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 $1,864 \times g$. 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 $\mu\text{g}/\text{mL}$) and NA (20 $\mu\text{g}/\text{mL}$). 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 4×10^6 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 10^4 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 $\mu\text{g}/\text{mL}$ of NO and 20 $\mu\text{g}/\text{mL}$ 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. Ceca 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 $\mu\text{g}/\text{mL}$ of NO and 20 $\mu\text{g}/\text{mL}$ 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 χ^2 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

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

Experiment	Treatment	<i>Salmonella</i>	<i>Salmonella</i>	Log ₁₀ <i>Salmonella</i>	Log ₁₀ <i>Salmonella</i>
		Typhimurium cecal tonsil positive/total (%)	Enteritidis cecal tonsil positive/total (%)	Enteritidis cecal recovery (all samples)	Enteritidis cecal recovery (positive samples only)
1	Control	20/25 (80)	22/25 (88)	3.81 ± 0.32	4.33 ± 0.17
	LAB	2/25 (8)*	8/25 (32)*	0.62 ± 0.19*	1.95 ± 0.09*
2	Control	18/25 (72)	25/25 (100)	3.59 ± 0.23	3.59 ± 0.23
	LAB	2/25 (8)*	7/25 (28)*	0.42 ± 0.18*	1.91 ± 0.29*
3	Control	20/25 (80)	25/25 (100)	3.91 ± 0.19	3.91 ± 0.19
	LAB	1/25 (4)*	11/25 (40)*	1.00 ± 0.25*	2.22 ± 0.24*

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

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 ($P < 0.05$; 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 post-treatment. 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 bacte-

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

Experiment	Treatment	Time posttreatment (h)					
		6		12		24	
		<i>Salmonella</i> Enteritidis cecal tonsil positive/total (%)	Log ₁₀ <i>Salmonella</i> Enteritidis cecal recovery	<i>Salmonella</i> Enteritidis cecal tonsil positive/total (%)	Log ₁₀ <i>Salmonella</i> Enteritidis cecal recovery	<i>Salmonella</i> Enteritidis cecal tonsil positive/total (%)	Log ₁₀ <i>Salmonella</i> Enteritidis cecal recovery
4	Control	8/26 (31)	ND ¹	20/26 (77)	ND	25/26 (96)	ND
	LAB	6/24 (25)	ND	20/26 (77)	ND	4/26 (15)*	ND
5	Control	16/25 (64)	ND	21/24 (88)	ND	25/25 (100)	ND
	LAB	10/25 (40)	ND	21/25 (84)	ND	11/25 (44)*	ND
6	Control	6/25 (24)	0.35 ± 0.19	15/25 (60)	1.80 ± 0.32	23/25 (92)	2.41 ± 0.24
	LAB	5/25 (20)	0.30 ± 0.12	12/25 (48)	0.93 ± 0.20*	10/25 (40)*	0.90 ± 0.21*
7	Control	8/20 (40)	0.64 ± 0.19	16/20 (80)	2.60 ± 0.37	20/20 (100)	3.39 ± 0.26
	LAB	8/20 (40)	0.59 ± 0.17	14/20 (60)	1.49 ± 0.27*	10/20 (50)*	0.93 ± 0.24*

¹ND = not determined.

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

Table 4. Effect of lactic acid bacteria (LAB) on number of *Salmonella* Enteritidis recovered from ceca of broiler chicks at 6, 12, and 24 h posttreatment

Experiment	Treatment	Time posttreatment (h)		
		6	12	24
(Log ₁₀ <i>Salmonella</i> Enteritidis cecal recovery) ¹				
6	Control	1.48 ± 0.00	2.81 ± 0.25	2.62 ± 0.20
	LAB	1.48 ± 0.00	1.79 ± 0.14*	1.87 ± 0.18*
7	Control	1.61 ± 0.13	3.25 ± 0.28	3.40 ± 0.26
	LAB	1.48 ± 0.00	2.14 ± 0.22*	1.87 ± 0.23*

¹*Salmonella* Enteritidis-positive samples only.

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

rial 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 *Lactobacilli* 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-, tumor 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 mannose-sylated 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

Baba, E., S. Nagaishi, T. Fukata, and A. Arakawa. 1991. The role of intestinal microflora on the prevention of *Salmonella*

- colonization in gnotobiotic chickens. *Poult. Sci.* 70:1902–1907.
- Bean, N. H., and P. M. Griffin. 1990. Food-borne disease outbreaks in the United States, 1973–1987: Pathogens and trends. *J. Food Prot.* 53:804. (Abstr.)
- Blankenship, L. C., J. S. Bailey, N. A. Cox, N. J. Stern, R. Brewer, and O. Williams. 1993. Two-step mucosal competitive exclusion flora treatment to diminish salmonellae in commercial broiler chickens. *Poult. Sci.* 72:1667–1672.
- Corrier, D. E., D. J. Nisbet, J. A. Byrd, B. M. Hargis, N. K. Keith, M. Peterson, and J. R. DeLoach. 1998. Dosage titration of a characterized competitive exclusion culture to inhibit *Salmonella* colonization in broiler chickens during growout. *J. Food Prot.* 61:796–801.
- Corrier, D., D. Nisbet, C. Scanlan, A. Hollister, and J. DeLoach. 1995. Control of *Salmonella typhimurium* in broiler chicks with a continuous-flow characterized mixed culture of cecal bacteria. *Poult. Sci.* 74:916–924.
- Dalloul, R., H. Lillehoj, T. Shellem, and J. Doerr. 2003. Enhanced mucosal immunity against *Eimeria acervulina* in broilers fed a *Lactobacillus*-based probiotic. *Poult. Sci.* 82:62–66.
- FoodNet. 2005. Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food—10 sites, United States, 2004. *Morb. Mortal. Wkly. Rep.* 54:352–356.
- Galdeano, C. M., and G. Perdigon. 2006. The probiotic bacterium *Lactobacillus casei* induces activation of the gut mucosal immune system through innate immunity. *Clin. Vaccine Immunol.* 13:219–226.
- Hargis, B. M., D. J. Caldwell, and J. A. Byrd. 2001. Microbial pathogens of poultry: Live bird considerations. In *Poultry Meat Processing*. A. R. Sams, ed. CRC Press LLC, Boca Raton, FL.
- Higgins, J., S. Higgins, K. Guenther, W. Huff, A. Donoghue, D. Donoghue, and B. Hargis. 2005a. Use of a specific bacteriophage treatment to reduce *Salmonella* in poultry products. *Poult. Sci.* 84:1141–1145.
- Higgins, S., A. Torres-Rodriguez, J. Vicente, C. Sartor, C. Pixley, G. Nava, G. Tellez, J. Barton, and B. Hargis. 2005b. Evaluation of intervention strategies for idiopathic diarrhea in commercial turkey brooding houses. *J. Appl. Poult. Res.* 14:345–348.
- Impey, C., G. Mead, S. George, P. Wyeth, and N. Chettle. 1984. Attempt to control stunting in chickens by competitive exclusion. *Vet. Rec.* 115:36–37.
- Kim, Y., T. Ohta, T. Takahashi, A. Kushiro, K. Nomoto, T. Yokokura, N. Okada, and H. Danbara. 2006. Probiotic *Lactobacillus casei* activates innate immunity via NF- κ B and p38 MAP kinase signaling pathways. *Microbes Infect.* 8:994–1005.
- Mead, G. C. 2000. Prospects for competitive exclusion treatment to control *Salmonellas* and other foodborne pathogens in poultry. *Vet. J.* 159:111–123.

- Persson, U., and S. I. Jendteg. 1992. The economic impact of poultry-borne salmonellosis: How much should be spent on prophylaxis? *Int. J. Food Microbiol.* 15:207.
- Rantala, M., and E. Nurmi. 1973. Prevention of the growth of *Salmonella* in chicks by the flora of the alimentary tract of chickens. *Br. Poult. Sci.* 14:627-630.
- SAS Institute Inc. 2002-2003. Version 9.1. SAS, Cary, NC.
- Stern, N., N. Cox, J. Bailey, M. Berrang, and M. Musgrove. 2001. Comparison of mucosal competitive exclusion and competitive exclusion to reduce *Salmonella* and *Campylobacter* spp. colonization in broiler chickens. *Poult. Sci.* 80:156-160.
- Tellez, G., S. E. Higgins, A. M. Donoghue, and B. M. Hargis. 2006. Digestive physiology and role of microorganisms. *J. Appl. Poult. Res.* 15:136-144.
- Torres-Rodriguez, A., A. M. Donoghue, D. J. Donoghue, J. T. Barton, G. Tellez, and B. M. Hargis. 2007. Performance and condemnation rates analysis of commercial turkey flocks treated with a *Lactobacillus* spp-based probiotic. *Poult. Sci.* 86:444-446.
- Vinderola, C. G., M. Medici, and G. Perdigon. 2004. Relationship between interaction sites in the gut, hydrophobicity, mucosal immunomodulation capacities and cell wall protein profiles in indigenous and exogenous bacteria. *J. Appl. Microbiol.* 96:230-243.
- WHO. 2006. Subject: Drug resistant *Salmonella*. <http://www.who.int/mediacentre/factsheets/fs139/en/> Accessed Nov. 2006.
- Zar, J. 1984. *Biostatistical Analysis*. 2nd ed. Prentice-Hall, Englewood Cliffs, NJ.