Effect of lactic acid bacteria probiotic culture treatment timing on *Salmonella* Enteritidis in neonatal broilers

J. P. Higgins,* S. E. Higgins,† A. D. Wolfenden,‡ S. N. Henderson,‡ A. Torres-Rodriguez,‡ J. L. Vicente,‡ B. M. Hargis,‡ and G. Tellez‡[1]

*Laboratory of Tumor Immunology and Biology, National Cancer Institute/National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892; †Department of Animal and Avian Sciences, University of Maryland, College Park 20904; and ‡Department of Poultry Science, University of Arkansas, Fayetteville 72701

ABSTRACT In the present study, a series of experiments were conducted to evaluate the ability of a combination of 3 ATCC lactobacilli (LAB3) or a commercially available probiotic culture (PROB) to reduce *Salmonella enterica* serovar Enteritidis (*Salmonella* Enteritidis) in broiler chicks. Additionally, we varied the timing of PROB administration in relationship to *Salmonella* challenge and determined the influence on recovery of enteric *Salmonella*. In experiments 1 to 3, chicks were randomly assigned to treatment groups and were then challenged via oral gavage with *Salmonella* Enteritidis. Chicks were treated 1 h after *Salmonella* Enteritidis challenge with LAB3 or PROB. Twenty-four hours posttreatment, cecal tonsils were collected for recovery of enteric *Salmonella*. In experiments 4 to 7, day-of-hatch chicks were randomly assigned to treatment groups and were then treated with PROB via oral gavage and placed into pens. Chicks were challenged with *Salmonella* Enteritidis 24 h after treatment via oral gavage. At 24 h after *Salmonella* Enteritidis challenge, cecal tonsils were collected and recovery of enteric *Salmonella* was determined. In experiments 8 to 10, 1-d-old chicks were randomly assigned to treatment groups and were then challenged via oral gavage with *Salmonella* Enteritidis and placed into pens. Chicks were treated 24 h after challenge with PROB via oral gavage. Twenty-four hours post PROB treatment, cecal tonsils were collected and enriched as described above. It was found that PROB significantly reduced cecal *Salmonella* Enteritidis recovery 24 h after treatment as compared with controls or LAB3-treated chicks in experiments 1 to 3 ($P < 0.05$). Administration of PROB 24 h before *Salmonella* Enteritidis challenge significantly reduced recovery of *Salmonella* Enteritidis in 2 out of 4 experiments and no reduction in cecal *Salmonella* Enteritidis was observed when chicks were challenged with *Salmonella* Enteritidis and treated 24 h later with PROB. These data demonstrate that PROB more effectively reduced *Salmonella* Enteritidis than LAB3, and the timing of PROB treatment affects *Salmonella* Enteritidis-associated reductions.

Key words: *Salmonella*, probiotic, *Lactobacillus*, poultry

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INTRODUCTION

Despite advances in the treatment of infectious diseases, pathogenic microorganisms, including *Salmonella*, are an important threat to health worldwide (Wren, 2000). Recent restrictions on the use of some antimicrobials as growth promoters in animal production have pressured the poultry industry to look for alternatives that can continue to provide performance benefits. Probiotic cultures have been evaluated for this purpose with some success (Cavazzoni et al., 1998; Higgins et al., 2005). However, since Nurmi and Rantala (1973) proposed that competitive exclusion could be used as a method to prevent *Salmonella* infection, numerous researchers have reported the ability of live bacterial cultures (Nisbet et al., 1998; Nisbet, 2000; Bielke et al., 2003) and probiotic organisms (Lu and Walker, 2001; Tellez et al., 2001; Casey et al., 2004) to also reduce colonization of opportunistic microorganisms in the gastrointestinal tract by competition for receptor sites, stimulation of the immune system, and production of some active antimicrobial substances (Resta-Lenert and Barrett, 2003). Probiosis, although not a new concept, has only recently begun to receive an increasing level of scientific interest. January 2006 was the date for the complete ban of antibiotics in animal feed within Europe (Anadon, 2006). A viable alternative to antibiotics

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[1]Corresponding author: gtellez@uark.edu

is, therefore, an important venture. For this reason, the development of new probiotic products that could be licensed for animal use is receiving considerable interest (Kasper, 1998; Rolfe, 2000; Jadamus et al., 2002; Hong et al., 2005; Sleator and Hill, 2008). Currently, there is no universal class of probiotic bacterium, although the most common types available are lactic acid bacteria. These bacteria are found normally in the gastrointestinal tract of humans and animals, and there is the vague notion that the use of indigenous or commensal microorganisms is somehow restoring the natural microflora to the gut. Research conducted in our laboratory has elucidated an effective in vitro screening technique for identification of candidate probiotic organisms (Bielke et al., 2003). Further screening allowed the identification of 11 lactic acid bacteria of the genus or related to *Lactobacillus* in the product FM-B11 (Floramax, IVS-Wynco LLC, Springdale, AR) that were more efficacious in the treatment of *Salmonella*-infected chickens and poults. This probiotic culture has been shown, in both laboratory and field studies, to accelerate development of normal microflora in chicks and turkeys, providing increased resistance to infection by some enteric bacterial pathogens (Higgins et al., 2007; Vicente et al., 2007a,b, 2008). In the present study, we varied the timing of probiotic administration in relationship to *Salmonella* challenge and determined the influence on recovery of enteric *Salmonella*. Additionally, we compared the ability of this probiotic culture with a mixture of 3 ATCC-derived lactobacilli to reduce the incidence of recoverable cecal *Salmonella*.

MATERIALS AND METHODS

Salmonella Amplification

A primary poultry isolate of *Salmonella enterica* serovar Enteritidis phage type 13A (*Salmonella* Enteritidis), resistant to novobiocin (**NO**; Sigma, St. Louis, MO) and selected for resistance to nalidixic acid (**NA**; Sigma), was used for these experiments. Briefly, *Salmonella* Enteritidis 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 were retrospectively determined by spread plating on xylose lactose differential agar (Becton, Dickinson and Company, Sparks, MD) plates containing NO (25 μg/mL) and NA (20 μg/mL). Actual determined colony-forming units for each experiment are reported in Tables 1 and 2.

Lactobacilli Culture

Three *Lactobacillus* isolates were obtained from American Type Culture Collection (Manassas, VA): *Lactobacillus casei* 11578, *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842, and *Lactobacillus fermentum* 14931. These isolates were pooled and incubated in de Man, Rogosa, and Sharpe (**MRS**) broth overnight. The culture was diluted in reconstituted powdered skim milk to an expected concentration of 4×10^6 or 4×10^7 cfu/mL for oral gavage of chicks in these studies. Actual colony-forming units administered per chick from each experiment are reported in Table 1 as determined retrospectively from spread plating on MRS (Sigma) plates.

Probiotic Culture

Eleven lactic acid bacterial isolates, of poultry gastrointestinal origin, were described previously (Higgins et al., 2005; Tellez et al., 2006). This commercial product Floramax (FM-B11, IVS-Wynco LLC) was diluted in reconstituted powdered skim milk to an expected concentration of 4×10^6 cfu/mL for oral gavage to chicks in these studies. Actual colony-forming units administered per chick from each experiment are reported in Tables 1 and 2 as determined retrospectively from spread plating on MRS agar.

Experimental Design

Experiments 1 to 3. Experiments 1 to 3 were conducted as follows. One-day-old 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. Heated brooder batteries were used for

Table 1. Effect of 3 ATCC lactobacilli (LAB3) or a commercially available probiotic culture (PROB) on *Salmonella enterica* serovar Enteritidis (*Salmonella* Enteritidis) from cecal tonsils of broiler chicks 24 h after treatment

Experiment	Treatment	*Salmonella* Enteritidis (cfu per chick)	Treatment (cfu per chick)	*Salmonella* Enteritidis cecal tonsil positive/total (%)
1	Control	4×10^3	0	23/24 (96)[a]
	LAB3	4×10^3	2×10^6	15/22 (68)[b]
	PROB	4×10^3	1×10^6	8/23 (35)[c]
2	Control	9×10^3	0	23/25 (92)[a]
	LAB3	9×10^3	1×10^7	24/25 (96)[a]
	PROB	9×10^3	1×10^6	12/25 (48)[b]
3	Control	4×10^3	0	25/25 (100)[a]
	LAB3	4×10^3	2×10^7	19/25 (76)[b]
	LAB3	4×10^3	2×10^6	19/25 (76)[b]
	PROB	4×10^3	2×10^6	6/25 (24)[c]

[a–c]Values within the same experiment with different superscripts are significantly different ($P < 0.05$).

Table 2. Effect of probiotic (PROB) administration 24 h before or 24 h after *Salmonella enterica* serovar Enteritidis (*Salmonella* Enteritidis) challenge on recovery of *Salmonella* Enteritidis from cecal tonsils of broiler chicks

		1 d of age	2 d of age	3 d of age
Experiment	Treatment	PROB (cfu per chick)	*Salmonella* Enteritidis (cfu per chick)	*Salmonella* Enteritidis cecal tonsil positive/total (%)
4	Control	0	9×10^2	15/25 (60)
	PROB	5×10^5	9×10^2	6/24 (24)*
5	Control	0	7×10^3	14/25 (56)
	PROB	8×10^5	7×10^3	3/25 (12)*
6	Control	0	1×10^4	10/25 (40)
	PROB	1×10^6	1×10^4	11/24 (46)
7	Control	0	1×10^4	13/20 (65)
	PROB	1×10^6	1×10^4	8/20 (40)
		Salmonella Enteritidis (cfu per chick)	PROB (cfu per chick)	*Salmonella* Enteritidis cecal tonsil positive/total (%)
8	Control	6×10^3	0	20/25 (80)
	PROB	6×10^3	3×10^6	23/25 (92)
9	Control	5×10^3	0	17/20 (85)
	PROB	5×10^3	1×10^6	14/19 (74)
10	Control	5×10^3	0	19/20 (95)
	PROB	5×10^3	1×10^6	20/20 (100)

*A significant difference was found between the control and treated values within a single experiment ($P < 0.05$).

housing and chicks were allowed ad libitum access to an unmedicated broiler starter ration, formulated to meet or exceed NRC-recommended levels of critical nutrients (NRC, 1994) and water for the duration of the experiment. Chicks were randomly assigned to treatment groups and were then challenged via oral gavage (0.25 mL) with approximately 10^4 cfu/chick of *Salmonella* Enteritidis (Table 1) and placed into pens (n = 25 per pen). Chicks were treated 1 h after challenge with approximately 10^6 or 10^7 cfu/chick of 3 ATCC lactobacilli (**LAB3**) or commercially available probiotic (**PROB**) cultures via oral gavage (0.25 mL) (Table 1) and PBS as a vehicle was administered to control groups. Twenty-four hours posttreatment, all broilers were humanely killed by CO_2 inhalation and cecal tonsils were collected aseptically. Cecal tonsils were enriched in 10 mL of tetrathionate broth overnight at 37°C. After enrichment, each sample was streaked for isolation on xylose lactose differential agar 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 antibiotic-resistant *Salmonella* Enteritidis.

Experiments 4 to 7. Day-of-hatch male broiler chicks were obtained from a local hatchery and were housed and cared for as described above for experiments 4 to 7. Chicks were randomly assigned to treatment groups and were then treated with approximately 10^6 cfu/chick of PROB culture via oral gavage (0.25 mL) (Table 2) with PBS administered to control groups. Then chicks were placed into pens (n = 25 per pen, experiments 4 to 6; n = 20 per pen, experiment 7). Chicks were challenged 24 h after treatment via oral gavage (0.25 mL) with approximately 10^4 cfu/chick of *Salmonella* Enteritidis (Table 2). At 24 h after *Salmonella* Enteritidis challenge, all broilers were humanely killed and cecal tonsils were collected and enriched as above.

Experiments 8 to 10. Experiments 8 to 10 were conducted as follows. One-day-old male broiler chicks were obtained from a local hatchery. Chicks were randomly assigned to treatment groups and then challenged via oral gavage (0.25 mL) with *Salmonella* Enteritidis at approximately 10^4 cfu/chick (Table 2) and placed into pens (n = 25 per pen, experiment 8; n = 20 per pen, experiments 9 and 10). Chicks were treated 24 h after challenge with approximately 10^6 cfu/chick of PROB via oral gavage (0.25 mL) (Table 2) and PBS was administered to control groups. Chicks were housed and cared for as above. Twenty-four hours post PROB treatment, all broilers were humanely killed and cecal tonsils were collected and enriched as described above.

Statistical Analysis

The incidence of *Salmonella* Enteritidis recovery within experiments was compared, testing all possibilities, using the χ^2 test of independence (Zar, 1984) to determine significant ($P < 0.05$) differences between groups within experiments.

RESULTS AND DISCUSSION

In experiments 1 to 3, chicks were challenged with *Salmonella* Enteritidis and then treated 1 h later with LAB3 or PROB. A significant reduction of *Salmonella* Enteritidis cecal colonization was observed from PROB-treated chicks 24 h after treatment as compared with controls or LAB3 in all 3 experiments (Table 1). Alternatively, LAB3 significantly reduced cecal *Salmonella* Enteritidis recovery 24 h after treatment as compared with controls in experiments 1 and 3, but 10^7 cfu/chick of LAB3 did not reduce cecal *Salmonella* Enteritidis recovery in experiment 2. In experiments 4 to 7, PROB

was administered 24 h before *Salmonella* Enteritidis challenge. It was found that PROB significantly reduced recovery of *Salmonella* Enteritidis as compared with the control treatment in experiments 4 and 5, but not experiments 6 and 7 (Table 2). In experiments 8 to 10, chicks were first challenged with *Salmonella* Enteritidis then treated 24 h later with PROB, and no reduction in cecal *Salmonella* Enteritidis was observed 24 h after treatment (Table 2). These results suggest that the timing of the PROB treatment in relation to the *Salmonella* Enteritidis challenge alters the ability of the probiotic to significantly reduce cecal *Salmonella* Enteritidis incidence in broiler chicks because only marginal protection was observed as compared with previously described competitive exclusion or probiotic cultures (Nisbet, 2000; La Ragione and Woodward, 2003). Additionally, PROB administration 24 h after *Salmonella* Enteritidis challenge provided no protection from enteric *Salmonella* Enteritidis colonization. Together these data suggest that the mode of action for PROB might be different than traditional competitive exclusion cultures (Mead, 2000). Although bacterial interactions are the most accepted mechanism for this reduction of *Salmonella*, stimulation of an effective innate immune response is also possible. With all of these observations, we are just starting to understand that host responses, in addition to or instead of bacterial competition, may actually constitute effector mechanisms for reduced enteric pathogens. In spite of considerable published data regarding the efficacy of probiotics in reducing intestinal colonization by enteric pathogens, the mechanisms of action of probiotics are not fully understood. Several mechanisms have been proposed for probiotic functions, among which modulation of the immune system has recently received attention (Dalloul et al., 2003; Vinderola et al., 2004; Galdeano and Perdigon, 2006). Probiotic bacteria can exert immunomodulatory activities through their interactions with the host immune system. These interactions may lead to enhancement of natural and antigen-specific antibodies (Haghighi et al., 2005), activation or suppression of T cells (Castellazzi et al., 2007), and changes in cytokine expression profiles (Kim et al., 2006; Haghighi et al., 2008). Moreover, probiotics are able to induce the expression of antimicrobial peptides by host cells (Wehkamp et al., 2004; Akbari et al., 2008; Schlee et al., 2008). Collectively, the above-mentioned mechanisms contribute to the immunomodulatory activities of probiotics. In experiments 1 to 3, we observed that the PROB culture was significantly more effective at reducing cecal *Salmonella* Enteritidis than the LAB3 culture. This demonstrates that all lactic acid bacteria are not equally effective at reducing enteric salmonellosis in poultry, as has been described previously (Yokokura, 1997; Kim et al., 2006). Slight differences in surface protein expression between lactobacilli could help describe just one of the possible differences between lactic acid bacteria that effectively prevent enteric infections and those bacteria that confer no protection. Our laboratory is currently studying the source of antimicrobial peptides in the ileum and cecal tonsils of *Salmonella*-infected chicks as well as the mechanisms of action of probiotics in downregulating antimicrobial peptide genes in infected chickens. Overall, more research must be conducted to elucidate the conditions necessary for probiotic bacteria to elicit a beneficial immune response from the host that prevents or treats enteric infections.

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