

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 10^4 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 10^4 , 10^6 , and 10^8 cfu/chick. In this experiment, doses of 10^6 and 10^8 both significantly reduced *Salmonella* Enteritidis recovery compared with controls (15 vs. 85% *Salmonella* Enteritidis positive), but 10^4 cfu did not significantly reduce *Salmonella* Enteritidis recovery. The second experiment compared the efficacy of oral administra-

tion 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 10^2 to 10^7 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).

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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 genera including *Staphylococcus*, *Enterococcus*, *Streptococcus*, *Listeria*, *Clostridium*, and *Bacillus* (Bogovič-Matijašić 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 naladixic 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 $1,864 \times g$ 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 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 , 10^4 , 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

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

Group	Probiotic treatment ¹ (cfu/chick)	Number <i>Salmonella</i> Enteritidis positive/total samples ² (%)
Control	0	17/20 (85) ^a
Treated	10 ⁴	13/20 (65) ^a
Treated	10 ⁶	3/20 (15) ^b
Treated	10 ⁸	3/20 (15) ^b

^{a,b}Different superscripts indicate significant differences between treatments.

¹Chicks were all challenged with *Salmonella* Enteritidis by oral gavage on the day of hatch (7.5×10^3 cfu/chick). Probiotic treatments were administered by oral gavage 1 h postchallenge.

²Cecal tonsil samples were obtained 24 h posttreatment and were enriched for recovery of *Salmonella* Enteritidis.

and randomly divided into 6 groups of 25 chicks each. All chicks were challenged with 8×10^3 cfu of *Salmonella* Enteritidis by oral gavage in a 0.25-mL volume. One hour later they received one of the following treatments by oral gavage. Chicks were humanely killed and samples taken for *Salmonella* Enteritidis recovery 24 h following treatment.

Negative control chicks were not treated, and instead were orally gavaged with sterile saline. Positive control chicks were treated with 10⁶ cfu of probiotic culture, which was the effective concentration in experiment 1.

Probiotic 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.

Probiotic 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 10⁶ 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- μ 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. 1PEN011, 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 10⁶ 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 10⁶ 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: 1.75×10^4 cfu/chick, experiment 4: 3.7×10^3 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 10⁶ 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

Group	Treatment ¹	Number <i>Salmonella</i> Enteritidis positive/total samples ² (%)
Control	Vehicle	22/25 (88) ^a
Inactivated	Penicillin killed	23/25 (92) ^a
Inactivated	Heat killed	24/25 (96) ^a
Supernatant	Sterile filtered	21/25 (84) ^a
Live probiotic	10 ⁶ cfu/chick	7/24 (29) ^b
Live probiotic	Supernatant removed 10 ⁶ cfu/chick	7/25 (28) ^b

^{a,b}Different superscripts indicate significant differences between treatments.

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

²Cecal tonsil samples were obtained 24 h posttreatment and were enriched for recovery of *Salmonella* Enteritidis.

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

Experiment	Challenge dose (cfu/chick)	Treatment administered (1 h postchallenge)	Route of administration	Number <i>Salmonella</i> Enteritidis positive/total samples (%)
3	1.75×10^4	Control	None	19/19 (100) ^a
		10^6 cfu/mL probiotic	Drinking water	15/20 (75) ^b
		10^2 cfu probiotic	Cloacal	13/20 (65) ^b
		10^4 cfu probiotic	Cloacal	15/20 (75) ^b
4	3.7×10^3	Control	None	20/20 (100) ^a
		4×10^5 cfu/mL probiotic	Drinking water	16/20 (80) ^b
		4×10^3 cfu probiotic	Cloacal	13/20 (65) ^b
		4×10^7 cfu probiotic	Cloacal	11/20 (55) ^b

^{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/10 μ L; experiment 4: 4×10^3 and 4×10^7 cfu/20 μ L). 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).

Lactobacilli 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 *Lactobacilli* exert cytotoxic effects in vivo (Bogovič-Matijašić 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 100 \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.

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