

## Effect of Probiotic Treatment in Broiler Chicks on Intestinal Macrophage Numbers and Phagocytosis of *Salmonella* Enteritidis by Abdominal Exudate Cells

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**ABSTRACT** Previous data have indicated that a *Lactobacillus*-based probiotic culture (FM-B11) is efficacious in reducing *Salmonella* Enteritidis colonization within 24 h when administered within 1 h of challenge. We hypothesized that the innate immune system, specifically macrophages, may play a role in the observed reduction of *Salmonella* Enteritidis colonization with probiotic treatment. Day-of-hatch chicks were challenged with *Salmonella* Enteritidis and then treated with the probiotic culture 1 h later. Three other treatment groups were not treated (negative control), challenged only, or treated with probiotic only. In all experiments, probiotic treatment on the day of hatch reduced ( $P < 0.05$ ) cecal *Salmonella* Enteritidis recovery as compared with the control treatment. In experiments 1 and 2, immunohistochemistry was used to evaluate the presence of macrophages (KUL01+) in the ileum and cecum of 7 to 10 chicks per group at 24 h posttreatment. In experiment 1, the number of macrophages observed per 10,000  $\mu\text{m}^2$  in the ileum of *Salmonella* Enteritidis-challenged chicks was higher ( $P < 0.05$ ) than that of nonchallenged chicks ( $4.87 \pm 0.31$  vs.  $3.05 \pm 0.19$ ). In the cecum, there were more ( $P < 0.05$ ) macrophages per 10,000  $\mu\text{m}^2$  in chicks receiving probiotic

treatment without challenge than in negative control chicks ( $5.32 \pm 0.41$  vs.  $3.66 \pm 0.35$ ). However, in experiment 2 we found no differences among treatments in the numbers of macrophages for both the ileum and cecum. Experiments 3 and 4 were performed to evaluate the ability of Sephadex-elicited abdominal exudate cells (AEC) from chicks to phagocytose *Salmonella* Enteritidis in vitro. Abdominal exudate cells were isolated from the abdominal cavity, maintained in tissue culture plates overnight, and then assayed for phagocytic activity by coincubating with *Salmonella* Enteritidis. In experiment 3, more ( $P < 0.05$ ) *Salmonella* Enteritidis was recovered from AEC derived from probiotic-treated chicks than in any other treatment. However, in experiment 4, all treatments resulted in similar levels of elicited AEC, and phagocytosis of *Salmonella* Enteritidis was at low levels in all groups. Although not conclusive, the modest differences detected in experiments 1 and 3, and the fact that those differences were not repeatedly detectable, suggest that these macrophage-related changes were not solely responsible for the reductions of *Salmonella* Enteritidis following probiotic treatment.

**Key words:** *Salmonella*, probiotic, macrophage, chick, phagocytosis

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## INTRODUCTION

*Salmonella* from poultry products has been associated with human foodborne illness (Kimura et al., 2004; Marcus et al., 2007). 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 (World Health Organization, 2006). Competitive exclusion cultures and probiotic cultures consisting of live beneficial bacteria have been used to

reduce levels of *Salmonella* in live poultry, with positive results (Nurmi and Rantala, 1973; Blankenship et al., 1993; Corrier et al., 1995; Waters et al., 2005). Additionally, improved performance has been reported with probiotic cultures (Huang et al., 2004; Higgins et al., 2005; Timmerman et al., 2006). The mechanisms behind these benefits are not well understood, although many studies have observed immunomodulatory effects from probiotic treatments.

Dalloul et al. (2005) described significantly higher levels of both interferon (IFN)- $\gamma$  and interleukin-2 in the intestine of probiotic-fed chickens 3 d following challenge with *Eimeria acervulina*. Another study by Yurong et al. (2005) reported increases in the number of Ig-producing cells (IgM, IgG) detected in Peyer's patches and the cecal tonsils of chicks by d 7 and 10, respectively, following admin-

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istration of a probiotic culture in the drinking water containing *Bacillus subtilis*, *Candida utilis*, and *Lactobacillus acidophilus*. The length of the cecal tonsils was also increased following 3 d of probiotic administration (Yurong et al., 2005). Koenen et al. (2004) found that oral administration of 2 probiotic *Lactobacilli* isolates to broiler chickens for 5 consecutive days caused increased phagocytosis by cells from the cecum and ileum, and also increased serum IgG and IgM.

Probiotics have previously been associated with activation of innate immunity through phagocytic cells. Recently, Farnell and coworkers (2006) reported that specific isolates of probiotic bacteria increased the oxidative burst capacity and degranulation of heterophils isolated from chicks treated 24 h following probiotic treatment, indicating that the innate immune system may also be activated through probiotic treatment. Olivares et al. (2006) reported an increase in both the number of circulating phagocytic cells and their activity in humans following consumption of either 2 lactic acid bacteria or a commercial yogurt. Additionally, Parra et al. (2004) observed that the consumption of fermented milk (containing lactic acid bacteria) by humans increased the oxidative burst capacity of monocytes and increased the tumoricidal capacity of natural killer cells. Sadeyen and coworkers (2004) found that inbred lines of chickens naturally susceptible to *Salmonella* infections had lower expression of IFN- $\gamma$  in the cecal tonsils at 3 wk of age compared with naturally resistant lines of chickens. Because IFN- $\gamma$  is a strong activator of macrophages, the production of this cytokine appears to be important to *Salmonella* resistance. Macrophages are present in most organs and possess effector functions such as phagocytosis, antigen processing and presentation, and cytokine secretion (Qureshi et al., 2000).

Because *Salmonella* spp. have a dynamic relationship with macrophages, we hypothesized that macrophages may play a role in the reduction of *Salmonella* following probiotic treatment. In the following experiments, we evaluated whether a *Lactobacillus*-based probiotic culture would increase the numbers of macrophages in the ileum and cecum. Additionally, we evaluated phagocytic activity in vitro by using Sephadex-elicited abdominal exudate cells (AEC) from chicks receiving the probiotic with or without *Salmonella* Enteritidis challenge.

## MATERIALS AND METHODS

### *Salmonella* Enteritidis

A primary poultry isolate of *Salmonella* Enteritidis, bacteriophage type 13A (*Salmonella* Enteritidis), was obtained from the USDA National Veterinary Services Laboratory. This isolate was resistant to novobiocin (25  $\mu\text{g}/\text{mL}$ , catalog no. N-1628, Sigma, St. Louis, MO) and was selected for resistance to naladixic acid (20  $\mu\text{g}/\text{mL}$ , catalog no. N-1628, Sigma) in our laboratory. For these studies, 100  $\mu\text{L}$  of *Salmonella* Enteritidis from a frozen aliquot was added to 10 mL of tryptic soy broth (catalog no. 211822, Becton Dickinson, Sparks, MD) and incubated at 37 C for 8 h.

Then, 100  $\mu\text{L}$  of culture was passed into 10 mL of fresh medium and incubated again for 8 h. This was repeated one more time for a total of 24 h. Bacterial cells were washed 3 times in sterile 0.9% saline by centrifugation at  $1,864 \times g$ , quantified with a spectrophotometer to a concentration of approximately  $10^9$  cfu/mL in sterile 0.9% saline, and diluted to inoculated concentrations as described below. Concentrations of *Salmonella* Enteritidis were determined retrospectively by spread-plating on xylose-Lys-deoxycholate (XLD) agar and enumeration for each experiment, and actual determined colony-forming units were reported.

### Probiotic Culture

Eleven lactic acid bacterial isolates were previously selected and described (Higgins et al., 2005). These isolates, which were combined to form a probiotic culture that is now commercially available as FM-B11 (catalog no. 41069, IVS-Wynco LLC, Springdale, AR), were used for these experiments. The probiotic culture was diluted in reconstituted powdered skim milk to an expected concentration of  $4 \times 10^6$  cfu/mL for oral gavage of chicks in these studies. Actual colony-forming units administered per chick from each experiment are reported below, which were determined retrospectively from spread-plating on de Man, Rogosa, Sharpe agar (catalog no. R1148, Sigma).

### Experimental Design

These experiments were approved by the Institutional Animal Care and Use Committee of the University of Arkansas. Day-of-hatch broiler chicks were obtained from a local hatchery and were randomly assigned to 1 of 4 treatment groups ( $n = 25/\text{group}$ ). Two groups were challenged by oral gavage with *Salmonella* Enteritidis in 0.25 mL of saline (experiments 1 and 2:  $10^4$  cfu/chick; experiment 3:  $2.5 \times 10^3$  cfu/chick; experiment 4:  $1.4 \times 10^3$  cfu/chick). The 2 remaining nonchallenged groups received the sterile saline vehicle by oral gavage (0.25 mL). All chicks were placed in a battery brooder unit (1 group per level) with unmedicated feed and water and were maintained at an age-appropriate temperature. One hour postchallenge, 1 challenged group and 1 unchallenged group received probiotic treatment by oral gavage (experiments 1 and 2:  $3.0 \times 10^6$  cfu/chick; experiment 3:  $4.1 \times 10^6$  cfu/chick; experiment 4:  $5.7 \times 10^6$  cfu/chick), and the additional 2 groups received the skim milk vehicle by oral gavage.

### *Salmonella* Enteritidis Recovery

In all experiments, *Salmonella* Enteritidis recovery from the cecal tonsils, to determine the efficacy of the probiotic culture, was performed as described below. Chicks were humanely killed by CO<sub>2</sub> asphyxiation 24 h posttreatment. Cecal tonsils were aseptically removed and placed in tetrathionate broth (catalog no. 210420, Becton Dickinson) for *Salmonella* enrichment. The samples were incubated

**Table 1.** *Salmonella* Enteritidis recovered from the cecal tonsils of chicks treated or not treated with a probiotic culture in experiments 1 through 4

Experiment	<i>Salmonella</i> Enteritidis-positive samples/total samples <sup>1</sup> (%)	
	Nontreated chicks	Probiotic-treated chicks
1	24/25 (96)	8/25 (32)*
2	22/25 (88)	9/25 (36)*
3	19/25 (76)	3/25 (12)*
4	22/25 (88)	6/24 (25)*

<sup>1</sup>No *Salmonella* Enteritidis was recovered from groups not receiving the *Salmonella* Enteritidis challenge in these experiments. An asterisk (\*) indicates the values within an experiment are different ( $P < 0.05$ ).

overnight at 37 °C and then streaked for isolation on XLD agar plates (catalog no. 278820, Sigma-Aldrich, St. Louis, MO) containing novobiocin and naladixic acid. Plates were incubated overnight, and the presence or absence of *Salmonella* colonies was recorded.

### Immunohistochemistry (Experiments 1 and 2)

At the same time the cecal tonsils were collected for *Salmonella* Enteritidis recovery, sections of the ileum and cecum were taken from 12 chicks per treatment group. Each sample was approximately 0.5 cm long and was immediately placed into a sterile sample bag and frozen in liquid nitrogen. The ileum sample was obtained from the midpoint between the end of the duodenal loop and Meckel's diverticulum. Cecal tissues were taken from the midpoint of the cecum. Frozen samples were embedded in optimal cutting temperature medium (catalog no. 4583, Sakura Finetek, USA Inc., Torrance, CA) on blocks of dry ice and kept at -80 °C until cut for immunohistochemistry.

Tissue samples were cut 5 µm thick with a cryostat, and sections were mounted on poly-L-Lys-coated slides and briefly fixed in acetone. Immunohistochemistry was performed in humidified chambers at room temperature, and blocking was performed with 10% horse serum in PBS overnight at room temperature. Slides were rinsed, and the KUL01 mouse antichick monocyte-macrophage (catalog no. 8420-01, Southern Biotech, Birmingham, AL) primary antibody, at a dilution of 1:100, was applied to each tissue. Tissues were incubated for 30 min at room temperature. For each sample, an additional

section was prepared and incubated with an antibody of irrelevant specificity to determine nonspecific binding of the primary antibody (isotype control, mouse IgG1, catalog no. M5909, Sigma-Aldrich). After 30 min, all tissues were rinsed with PBS, and horse antimouse IgG was applied at a 1:100 dilution for 30 min. All tissues were rinsed again and then incubated with avidin-enzyme-linked biotin complex (catalog no. PK-4002, Vector Laboratories Inc., Burlingame, CA) for 30 min. Incubation with avidin-enzyme-linked biotin complex was followed by several washes, after which diaminobenzidine substrate activated with 3% H<sub>2</sub>O<sub>2</sub> was added for 5 min. All tissues were placed in methyl green stain for 30 min to counterstain, dehydrated in sequential baths, and mounted with a cover slip.

### Quantification of Macrophages

Macrophages identified by immunohistochemistry were quantified by using a bright field microscope at 400× magnification. Three microscope fields were randomly selected on each sample. For each field, the area was determined and the number of macrophages enumerated. The number of macrophages (KUL01+ cells) per 10,000 µm<sup>2</sup> was determined for each sample. The mean for individual tissues within a group was used to determine the mean for each treatment group.

### Experiments 3 and 4

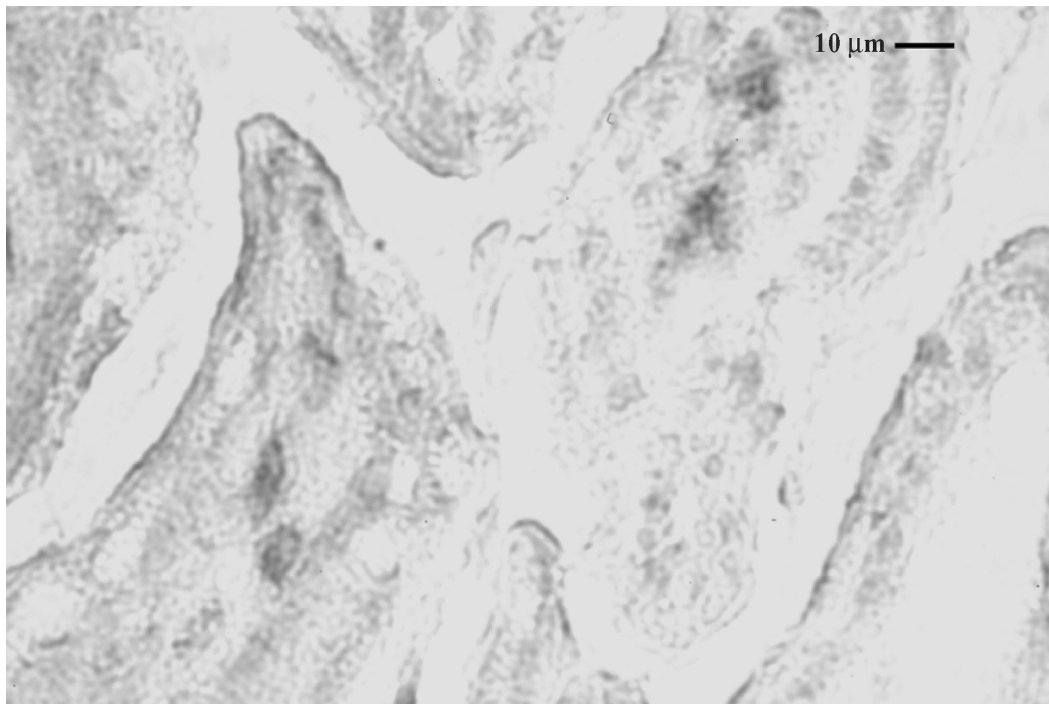
In these experiments, all chicks were injected in the abdominal cavity with 0.5 mL of a 3% Sephadex (catalog no. G5050, Sigma-Aldrich) suspension at the time of probiotic treatment, according to the method described by Qureshi et al. (1986). After 24 h, chicks were humanely killed by CO<sub>2</sub> asphyxiation. Abdominal exudate cells were collected by making an incision in the abdominal cavity and washing the cavity with sterile PBS containing 0.5 U/mL of heparin. The washes were made by using 5-mL pipette tips, which were modified by the addition of 4 holes. The holes were made approximately 0.5 cm above the tip at equal intervals around the pipette tip by perforating the tip with an 18-gauge needle. Collected cells were added to sterile, siliconized glass 15-mL conical tubes and kept on ice for at least 10 min. After collection of AEC, the cecal tonsils were removed and processed as described above for *Salmonella* Enteritidis recovery.

**Table 2.** The enumeration of macrophages in the caeca and upper ileum by immunohistochemistry<sup>1</sup>

Treatment	Experiment 1				Experiment 2			
	n	Macrophages in ileum	n	Macrophages in ceca	n	Macrophages in ileum	n	Macrophages in ceca
Negative control	12	3.05 ± 0.19 <sup>c</sup>	11	3.66 ± 0.35 <sup>b</sup>	8	2.98 ± 0.50	10	4.59 ± 0.50
Probiotic treated	10	4.03 ± 0.51 <sup>ab</sup>	10	5.32 ± 0.41 <sup>a</sup>	10	2.68 ± 0.34	11	4.27 ± 0.35
Challenged	12	4.87 ± 0.31 <sup>a</sup>	11	4.38 ± 0.30 <sup>ab</sup>	10	3.26 ± 0.66	7	6.14 ± 0.85
Challenged and probiotic treated	12	3.73 ± 0.21 <sup>bc</sup>	12	4.54 ± 0.35 <sup>ab</sup>	9	3.12 ± 0.25	9	5.56 ± 0.72

<sup>a-c</sup>Different letters within columns indicate differences between treatment groups ( $P < 0.05$ ).

<sup>1</sup>Number of positive cells/10,000 µm<sup>2</sup> ± SE.



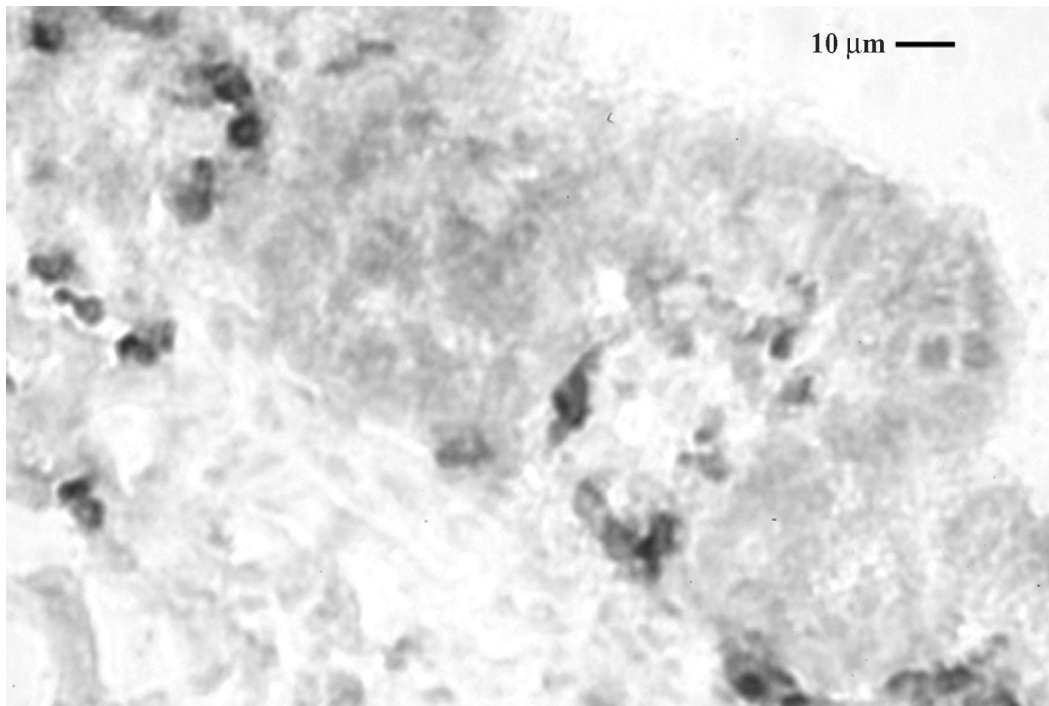
**Figure 1.** Macrophages in the ileum of a probiotic-treated 24-h-old chick. Macrophages in frozen tissue sections (5  $\mu$ m) were identified by using an antichick monocyte-macrophage mouse monoclonal antibody (KUL01) and an indirect immunoperoxidase staining procedure. Immunopositive cells are black.

After collection of the AEC suspension, debris was allowed to settle for 10 min, and the supernatant fluid containing the cells was poured into siliconized sterile 16  $\times$  125 mm borosilicate glass tubes. Supernatants from 3 chicks were pooled into 1 tube, and 8 pools were assayed per treatment group. The AEC containing supernatants were centrifuged at 300  $\times$  g at 8 C for 15 min, and the pellet of cells was resuspended in 5 mL of RPMI medium (catalog no. 11835, Invitrogen, Grand Island, NY) with no phenol red, and 5% fetal bovine serum. The 5 mL of resuspended AEC was added on top of 5 mL of Histo-paque-1077 (catalog no. 10771, Sigma-Aldrich) in sterile siliconized glass tubes. They were centrifuged for 30 min at 500  $\times$  g and then a Pasteur pipette was used to collect the mononuclear AEC at the interface. The cells were washed in PBS by centrifugation at 250  $\times$  g for 8 min, and the pellet was resuspended in 1.1 mL of RPMI medium. From each AEC pool, 10  $\mu$ L of cells was combined with 10  $\mu$ L of 10% Trypan blue for enumeration of mononuclear AEC and determination of cell viability. Each AEC suspension was found to consist of approximately 90 to 95% large viable mononuclear cells. The numbers of cells were recorded for each sample and were found to be between  $10^5$  and  $10^6$  cells/mL. Because of the minimal numbers of cells obtained from each pooled sample, AEC samples were immediately plated into 24-well tissue culture plates (0.5 mL/well, 2 wells per sample, catalog no. 3047, Becton Dickinson Co., Lincoln Park, NJ) and were not adjusted to equal numbers of cells per well. Cells were allowed to adhere for 2 h at 40 C in 5% CO<sub>2</sub>. Following adherence, the wells were carefully washed with sterile

PBS 3 times, and 0.5 mL of RPMI was added. The cells were maintained in tissue culture plates in the incubator overnight.

### **Phagocytosis Assay (Experiments 3 and 4)**

The phagocytosis assay was performed similarly to that described by Desiderio and Campbell (1983) and Kramer et al. (2001) for bovine and avian macrophages, respectively. The next day, AEC cultures were rinsed 1 time with sterile PBS, and 5  $\times$  10<sup>7</sup> cfu of *Salmonella* Enteritidis diluted in 0.5 mL of RPMI medium was added to each well. Samples were incubated in the presence of *Salmonella* Enteritidis for 30 min at 40 C. After *Salmonella* Enteritidis incubation, 0.2 mL of supernatant was taken from each well, serially diluted, and spread-plated on XLD agar plates to determine the numbers of extracellular *Salmonella* Enteritidis. Wells were rinsed with sterile PBS 3 times, and 0.5 mL of RPMI containing 200  $\mu$ g/mL of gentamicin was added to each well and incubated for 45 min at 37 C to kill any extracellular *Salmonella* Enteritidis. Wells were again rinsed 3 times with PBS, and 0.5 mL of PBS containing 1% Triton X was added to the wells for 10 min to lyse the AEC. A sample was taken from each well at this time to determine the numbers of intracellular *Salmonella* Enteritidis recovered. These aliquots were serially diluted and plated on XLD plates for enumeration of viable intracellular *Salmonella* Enteritidis (Desiderio and Campbell, 1983; Kramer et al., 2001). The numbers of cfu of *Salmonella* Enteritidis recovered from each well were then divided by the number of AEC originally counted



**Figure 2.** Macrophages in the cecum of a probiotic-treated 24-h-old chick. Macrophages in frozen tissue sections (5  $\mu\text{m}$ ) were identified by using an antichick monocyte-macrophage mouse monoclonal antibody (KUL01) and an indirect immunoperoxidase staining procedure. Immunopositive cells are black.

in that sample, and data are reported as cfu of *Salmonella* Enteritidis per AEC.

### Statistical Analysis

The incidence of *Salmonella* recovery within experiments was compared by using the chi-squared test of independence (Zar, 1984) to determine significant ( $P < 0.05$ ) differences between control and treated groups. Data from enumerated macrophages in experiments 1 and 2 as well as *Salmonella* Enteritidis cfu in experiments 3 and 4 were analyzed by ANOVA and further separated by Duncan's multiple range test (SAS Institute, 2004).

## RESULTS AND DISCUSSION

To our knowledge, this is the first report of the numbers of macrophages present in intestinal tissue of 1-d-old

chicks. In all experiments, we found significant reductions of *Salmonella* Enteritidis in probiotic-treated chicks compared with nontreated control chicks (Table 1). Using immunohistochemistry (Figure 1), we found higher numbers of ileal macrophages in the lamina propria of chicks in the *Salmonella* Enteritidis-challenged group of experiment 1 compared with the negative control chicks ( $4.87 \pm 0.31$  and  $3.05 \pm 0.19$  macrophages/ $10,000 \mu\text{m}^2$ , respectively,  $P < 0.05$ ; Table 2). Similarly, in the cecum (Figure 2) we observed higher numbers of macrophages in the probiotic-treated chicks ( $5.32 \pm 0.41$  macrophages/ $10,000 \mu\text{m}^2$ ,  $P < 0.05$ ) compared with negative control chicks ( $3.66 \pm 0.35$  macrophages/ $10,000 \mu\text{m}^2$ ). Chicks receiving the *Salmonella* Enteritidis challenge with or without probiotic treatment had intermediate numbers of cecal macrophages ( $4.38 \pm 0.30$  and  $4.54 \pm 0.35$ , respectively). In experiment 2, we observed no treatment differences in macro-

**Table 3.** Phagocytosis of *Salmonella* Enteritidis by abdominal exudate cells (AEC) obtained from chicks treated or not treated with a *Lactobacillus*-based probiotic<sup>1</sup>

Treatment	Experiment 3 ( <i>Salmonella</i> Enteritidis cfu/cell)	Experiment 4 ( <i>Salmonella</i> Enteritidis cfu/cell)
Negative control	$1.94 \pm 0.38^b$	$0.15 \pm 0.10$
Probiotic treated	$2.28 \pm 0.49^b$	$0.76 \pm 0.50$
Challenged	$2.01 \pm 0.32^b$	$0.22 \pm 0.16$
Challenged and probiotic treated	$4.28 \pm 0.97^a$	$0.41 \pm 0.25$

<sup>a,b</sup>Different letters within a column indicate differences between treatments ( $P < 0.05$ ).

<sup>1</sup>In these experiments, chicks from the appropriate groups were challenged with *Salmonella* Enteritidis and then received probiotic treatments and Sephadex injections 1 h postchallenge; 24 h later, AEC were collected from chicks. Samples from 3 chicks were pooled, and 8 pools per treatment group were evaluated in the phagocytosis assay.

phage numbers in either the ileum or cecum (Table 3). However, independent of treatment, the average numbers of macrophages present in the ileum and cecum of 24-h-old chicks were similar in both experiments.

In experiments 3 and 4, we recovered approximately  $10^6$  and  $10^5$  AEC/mL, respectively, in pooled samples from the abdominal cavity. For these assays, the cells were not diluted to maintain constant numbers in each well, but the *Salmonella* Enteritidis recovered was divided by the number of cells plated, so cfu per AEC were reported. In both experiments, there were no differences in the numbers of extracellular *Salmonella* Enteritidis recovered (data not shown). In experiment 3, we recovered significantly higher cfu of intracellular *Salmonella* Enteritidis from AEC collected from chicks receiving the probiotic and then challenged with *Salmonella* Enteritidis (approximately 4 cfu/AEC) compared with all other treatment groups (approximately 2 cfu/AEC; Table 3). The intracellular *Salmonella* Enteritidis recovered from AEC in experiment 4, however, did not differ between treatment groups. Our phagocytosis results (2 to 4 cfu of *Salmonella* Enteritidis per AEC) with AEC derived from 24-h-old chicks corresponded with those of Kramer et al. (2001), who reported that phagocytes isolated from the gut of 7-d-old chicks could phagocytose 2 *Salmonella* Enteritidis or less, but that phagocytes isolated from the spleen of those same chicks could phagocytose 13 to 33 *Salmonella* Enteritidis per cell.

The literature is limited on chicken intestinal macrophages and phagocytosis of *Salmonella* Enteritidis by chicken AEC, especially for very young chicks, and only a few investigators have attempted to enumerate phagocytosed *Salmonella* Enteritidis. The approach used here to enumerate *Salmonella* Enteritidis phagocytosed as cfu per AEC was based on the methods described by Desiderio and Campbell (1983) and Kramer et al. (2001) for bovine and avian macrophages, respectively. The fact that enumeration was based on phagocytosed *Salmonella* Enteritidis that are still alive in the macrophages prior to detection in this phagocytosis assay raises some questions; especially because survival in macrophages is one way that *Salmonella* spp. perpetuate infections in the host. Qureshi et al. (1986) reported that 80% of opsonized *Salmonella typhimurium* are killed within 15 min; hence, the presence of live *Salmonella* Enteritidis in AEC is a relative estimate of ongoing phagocytic activity, independent of downstream killing. Without knowing the downstream impact of surviving phagocytosed *Salmonella* Enteritidis, it is difficult to interpret an increase in cfu per AEC (e.g., experiment 3, *Salmonella* Enteritidis-challenged, probiotic-treated chicks) beyond enhanced phagocytic activity. However, with the same challenge model, recent data from our laboratory (Higgins et al., 2007)—showing that *Salmonella* Enteritidis-challenged, probiotic-treated chicks that did not completely clear *Salmonella* Enteritidis infection consistently had significantly lower counts of *Salmonella* Enteritidis in the cecal contents—support a positive effect of this probiotic treatment on *Salmonella* Enteritidis clearance.

In conclusion, these experiments evaluated the effect of a *Lactobacillus*-based probiotic culture on macrophages in neonatal chicks. This probiotic culture consistently reduced *Salmonella* Enteritidis recovery from the cecal tonsils within 24 h, as compared with nontreated controls. However, differences in the numbers of macrophages in situ were not consistent, and even the observed significant differences were not remarkable. Similarly, observed differences in phagocytosis in experiment 3 were not replicated in experiment 4. These data do not support the hypothesis that the observed reductions in *Salmonella* Enteritidis colonization from day-of-hatch chicks are due to the presence of macrophages or their ability to phagocytose *Salmonella*. However, these data do not preclude the possibility that macrophages are directly or indirectly participating in the consistently observed diminution of *Salmonella* colonization caused by administration of this probiotic.

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