Research Note

Enhanced Mucosal Immunity Against *Eimeria acervulina* in Broilers Fed a *Lactobacillus*-Based Probiotic[1]

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ABSTRACT The effect of feeding a *Lactobacillus*-based probiotic on intestinal intraepithelial lymphocyte (IEL) subpopulations and subsequent protection against coccidiosis was investigated in broiler chickens. Day-old male broilers were fed standard rations without control (CONT) or with a commercial probiotic (PROB) Primalac. Differences in IEL subpopulations were assessed by flow cytometry at 21 d postprobiotic treatment. At 25 d of age, a group of randomly selected birds from each diet was inoculated orally with 10,000 (per bird) sporulated oocysts of *Eimeria acervulina* and kept on the same diets. Fecal material, sera, and intestinal washes were collected 10 d postchallenge with *E. acervulina*. Birds on the PROB diet had more IEL expressing the surface markers CD3, CD4, CD8, and $\alpha\beta$TCR than those of the CONT diet. The probiotic-fed chickens produced less oocysts ($P < 0.0001$) compared to the untreated, control group (368×10^6 in CONT vs. 89×10^6 in PROB). The interferon-γ levels in both serum and intestinal secretions were not significantly different between the two groups. However, CONT group showed higher antibody levels against a recombinant coccidial antigen in the intestinal secretions than the PROB group. No significant difference was found in serum antibody levels against the same antigen. These results clearly indicate that the probiotic bacteria impacted the local immune response as characterized by altered IEL subpopulations and increased the birds' resistance to *E. acervulina* as reflected by reduced oocyst shedding.

(*Key words*: *Lactobacillus*, intraepithelial lymphocyte, *Eimeria*, gut immunity, broiler)

2003 Poultry Science 82:62–66

INTRODUCTION

Gut mucosal surfaces play a key role in the exclusion and elimination of potentially harmful dietary antigens and enteric microorganisms and, with the gut microflora, are essential to maintaining healthy flocks and minimizing losses associated with various diseases and stressors. The use of probiotics for poultry is based on the knowledge that the gut flora is involved in resistance to enteric infections including *E. coli* (Watkins et al., 1982; Watkins and Miller, 1983; Chateau et al., 1993; Jin et al., 1996), *Salmonella* (Chateau et al., 1993; Hejlicek et al., 1995; Qin et al., 1995; Jin et al., 1996; Pascual et al., 1999; Stern et al., 2001), and *Campylobacter* (Morishita et al., 1997; Hakkinen and Schneitz, 1999; Stern et al., 2001). Feeding probiotics helps maintain beneficial intestinal microflora and may modulate the mucosal immune system enhancing the host's resistance to enteric pathogens.

Although the protective mechanisms of probiotics are still not fully known, it has been established in laboratory rodents that lactic acid bacteria given orally can significantly affect both the systemic and mucosa-associated immune responses (Perdigon and Alvarez, 1992; Famularo et al., 1997). Despite the fact that several studies have shown disease prevention or immune enhancement resulting from oral administration of probiotics, few studies are available on their specific effects on the gut defense mechanisms in chickens. The present work was conducted to help characterize some of these actions; the specific objectives were to determine the effects of a commercial *Lactobacillus*-based probiotic on the different subpopulations of intestinal intraepithelial lymphocytes (IEL) in broiler chickens, and the potential protection it might provide to the birds against a coccidial challenge.

Received for publication January 15, 2002.

Accepted for publication August 8, 2002.

[1]Part of this research was presented at the 2000 Poultry Science Association meeting, August 18–20, Montreal, Canada (Dalloul et al., 2000).

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Abbreviation Key: Ab = antibody; CONT = control; *EA* = *Eimeria acervulina*; HBSS = Hank's balanced salt solution; IEL = intraepithelial lymphocytes; IFN-γ = interferon-γ; mAb = monoclonal antibody; PBS-T = PBS with Tween 20; PI = postinfection; PROB = probiotic; RT = room temperature.

MATERIALS AND METHODS

Birds and Treatments

One hundred day-old male broiler chicks (Ross 308[3]) were randomly assigned to eight cages (12 to 13 per cage) of an electrically heated battery. Half the birds received a corn-soybean-based broiler diet serving as controls (CONT), and the other half was fed the same diet with the probiotic (PROB) Primalac[4] added at the rate of 1 g/kg feed. The diets were formulated to meet or exceed the nutrient requirements for broilers as recommended by the National Research Council (1994). Weights and feed consumption were recorded weekly. On Day 20, 32 birds were selected on a mean body weight-basis within each treatment and maintained on the same diets for subsequent treatment and sampling.

Lymphocyte Preparation and Flow Cytometric Analysis

Intestinal IEL were prepared from 21-d-old birds (16 total, eight per treatment) and differences in their subpopulations determined as described by Lillehoj and Chai (1988). Briefly, small intestinal sections from the duodenum to mid-jejunum (two birds per cage, pooled) were excised, cut longitudinally, washed in several changes of ice-cold calcium- and magnesium-free Hank's balanced salt solution[5] (HBSS), and cut into small pieces. The pieces were treated with 10 m*M* dithiothreitol,[6] followed by incubation in 10^{-4} *M* EDTA[6] for 20 min, both in calcium- and magnesium-free HBSS supplemented with 5% fetal calf serum[7] with continuous swirling at 37 C. The released IEL were passed through a nylon wool[8] column and resuspended in staining buffer (HBSS without phenol red containing 3% fetal calf serum and 0.01% sodium azide[6]). Cells were then incubated with mouse anti-chicken monoclonal antibodies (mAb) to different T-cell surface markers at 4 C for 40 min and detected with a fluorescein isothiocyanate goat anti-mouse IgG[9] mAb. The cells were then enumerated using a Coulter EPICS XL-MCL flow cytometer,[10] and each population expressed as the percentage of total lymphocytes counted.

Eimeria Infection and Oocyst Production

At 24 d of age, 16 birds (eight per treatment) were randomly selected, transferred to an isolation facility, caged individually, and inoculated esophageally with 10,000 *Eimeria acervulina* (*EA*) sporulated oocysts on the following day. Oocyst production and shedding were assessed as described by Lillehoj and Ruff (1987). Briefly, droppings from each bird were collected for 4 d, starting on day 6 postinfection (PI), water was added to each sample, soaked overnight, and the fecal material ground and homogenized. Two 35 mL samples were taken, diluted, and the oocysts were counted microscopically in duplicate slides using a McMaster counting chamber. The total number of oocysts was calculated using the formula: total oocysts = oocyst count × dilution factor × (fecal sample volume/counting chamber volume).

Collection of Sera and Intestinal Washes

Sera and intestinal washes were collected from both infected (10 d PI) and uninfected birds of the same age. For sera, blood samples were obtained from individual birds (5 cc/bird), allowed to clot overnight at 4 C, and the sera collected. Each bird was then killed by cervical dislocation and the duodenal section of the small intestine, the infection site of *EA*, was quickly removed, cut longitudinally, and incubated in 10 mL of ice-cold PBS[11] containing 0.05 trypsin inhibitory U/mL of aprotinin,[11] 5 m*M* EDTA,[6] 2 m*M* phenylmethylsulfonyl fluoride,[11] and 0.02% sodium azide for 4 h on ice. The tissues were then discarded and the washes collected. All intestinal washes and sera were individually clarified by centrifugation and stored at –20 C until analysis.

Serum Antibodies and Interferon-γ ELISA

Individual samples (each bird) of sera and intestinal washes were tested for interferon-γ (IFN-γ) and antibodies (Ab) to a recombinant coccidial antigen using ELISA as described by Yun et al. (2000a). Interferon-γ was quantified using a direct binding ELISA where flat bottom microtiter plates[12] were coated with 60 μL of sample in 40 μL of sodium carbonate buffer,[11] for 18 h at 4 C, and washed three times with PBS containing 0.05% Tween-20 (PBS-T).[11] Blocking followed using PBS with 2% BSA for 1 h at room temperature (RT), and plates washed three times with PBS-T. To each well, 100 μL of mouse anti-chicken IFN-γ mAb (Yun et al., 2000a) were added, incubated for 1 h at RT, washed as above, and detected with a horseradish peroxidase-conjugated goat anti-mouse IgG (H+L)[11] and its substrate, tetramethylbenzidine.[11] The optical density was read at 450 nm by an automated microtiter plate reader (Bio-Rad).[13] To detect anticoccidial Ab, microtiter plate wells were coated with 2 μg/well of the recombinant coccidial antigen 3-1E (Lillehoj et al., 2000) in 100 μL of 0.1 *M* carbonate buffer, for 18 h at 4 C. The plates were washed, wells blocked with PBS-2% BSA for 1 h at RT, and washed again. Serum and intestinal wash samples (100 μL) were added and incubated for 1 h at RT with continuous gentle shaking. The wells were again washed three times with PBS-T, and bound Ab detected with horseradish peroxidase-conjugated rabbit anti-

[3]Allen's Hatchery, Seaford, DE.
[4]Star-Labs, Inc., St. Joseph, MO.
[5]Life Technologies, Grand Island, NY.
[6]Fisher Scientific, Pittsburgh, PA.
[7]HyClone, Pittsburgh, PA.
[8]Robbins Scientific, Sunnydale, CA.
[9]Southern Biotech, Birmingham, AL.
[10]Coulter Corp., Miami, FL.
[11]Sigma, St. Louis, MO.
[12]Dynex Technologies, Chantilly, VA.
[13]Bio-Rad Clinical Diagnostics, Hercules, CA.

chicken IgG[11] and tetramethylbenzidine as described above. The plates were also read at 450 nm.

Statistical Analysis

Differences among experimental treatments were tested by analysis of variance using the Mixed model procedures of SAS (SAS Institute, 1996) and considered significant at a probability $P \leq 0.05$ unless noted otherwise.

RESULTS AND DISCUSSION

The study was designed to examine the effects of feeding a *Lactobacillus*-based probiotic on the intestinal IEL subpopulations and any subsequent enhancement of intestinal immunity against coccidiosis. Changes in IEL subpopulations within the upper small intestine were analyzed by flow cytometry at 21 d, and the results are presented in Figure 1. Fecal oocyst shedding following *EA* infection was used as an assessment of susceptibility to the pathogen. The ELISA results of IFN-γ and anticoccidial Ab in sera and intestinal secretions 10 d PI are shown in Figure 2.

The data show an increase in most IEL subpopulations in the PROB group in comparison to the CONT group (Figure 1). Birds on the probiotic diet showed an increase in IEL expressing the surface markers CD3, CD4, CD8, and $\alpha\beta$TCR than those of the control diet. The number of IEL expressing the $\gamma\delta$TCR was similar in both groups. The slight but significant increase in the number of IEL in the PROB birds could be the result of a nonspecific stimulation of the local immune system possibly by certain antigens of probiotic bacteria. More significant is the protective role that IEL play during enteric infections, especially that the involvement and importance of T cells in immune responses to coccidiosis has been well documented (Lillehoj and Trout, 1996). The number of duodenal CD4$^+$ and CD8$^+$ IEL in chickens increased following *EA* infection and two-color immunofluorescence analysis of duodenal IEL showed an increased number of $\alpha\beta$TCR$^+$ CD8$^+$ cells following *EA* challenge (Lillehoj, 1994). This stimulation of mucosal immunity by the probiotic bacteria prior to coccidial infection could prove beneficial in protecting against the pathogen.

To determine if such an effect occurred, oocysts shed in feces collected on Days 6 to 9 PI were enumerated (eight birds per treatment). The number of fecal *EA* oocysts

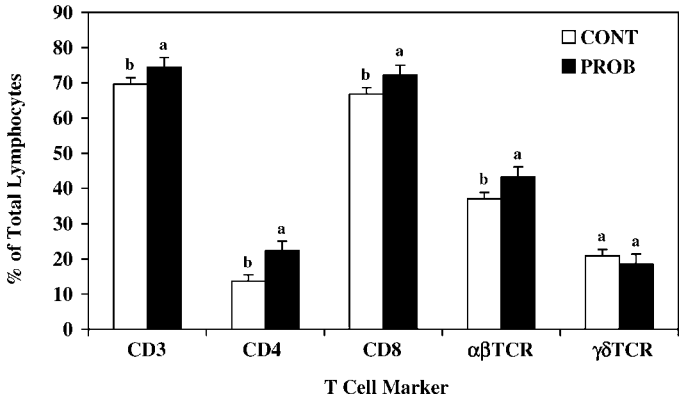

FIGURE 1. Flow cytometric analysis of intestinal intraepithelial lymphocytes (IEL) in the control (CONT) and the probiotic-fed (PROB) broilers. IEL isolated from the upper small intestine (duodenum and jejunum) of 3-wk-old birds were analyzed by flow cytometry for the expression of surface antigens: CD3, CD4, CD8, $\alpha\beta$TCR, and $\gamma\delta$TCR. Data are presented as percentage of total lymphocytes counted (cells stained with a pan lymphocyte monoclonal antibody). Each bar is the mean of 8 birds, and error bars represent SEM. Means lacking common superscripts differ ($P \leq 0.05$).

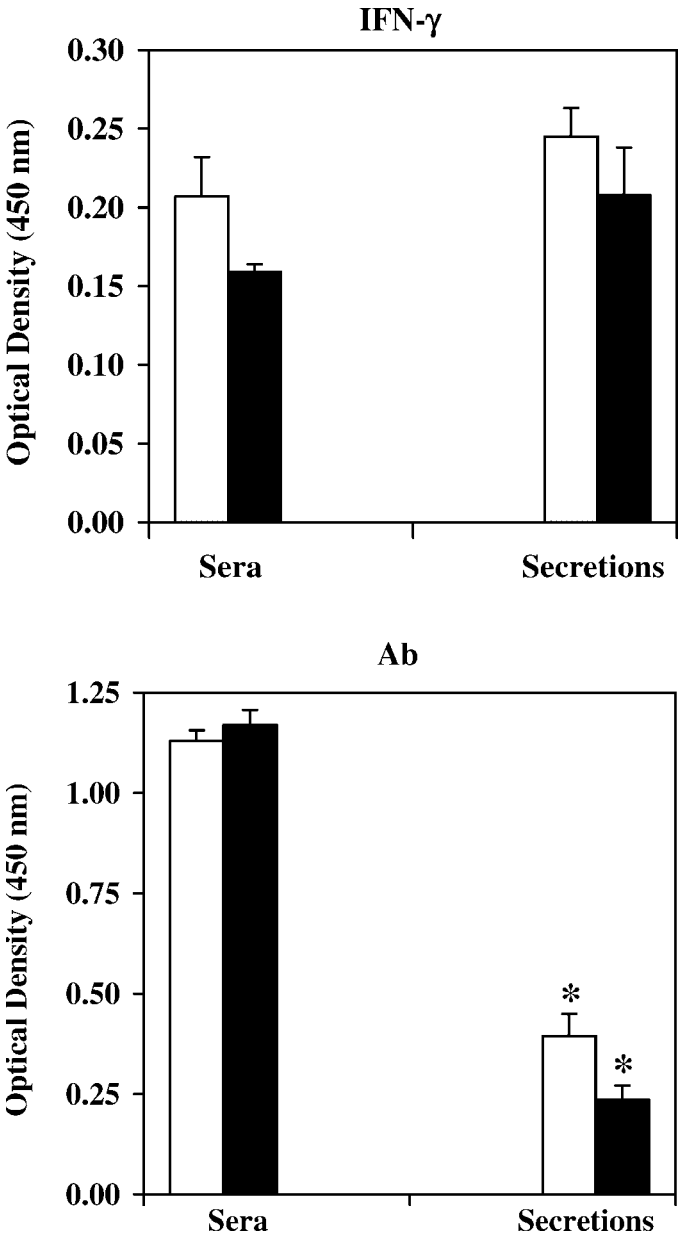

FIGURE 2. Interferon-γ (IFN-γ) and anticoccidial antibody (Ab) responses in sera and intestinal secretions of the control (CONT □) and the probiotic-fed (PROB ■) broilers following *E. acervulina* challenge. Each bar represents an average ELISA reading (optical density) of infected birds (8 per treatment group, tested in duplicate wells) obtained 10 d postchallenge. Each optical density reading (at 450 nm) was calculated by subtracting the background reading (from prechallenge birds) from the reading of infected birds. *Means differ ($P \leq 0.05$).

averaged 368×10^6/bird in the control group, but was reduced ($P < 0.0001$) fourfold (89×10^6/bird) in the probiotic-treated group, a clear indication of improved resistance to *EA* infection. The exact protection mechanism is not clear; it could be partially attributed to the early stimulation of the immune elements within the intestine by probiotic bacteria, namely the IEL, leading to a quicker immune response to *Eimeria*. In mice, depletion of $CD4^+$ cells resulted in increased oocyst production during *Eimeria* primary infection, suggesting a critical role for $CD4^+$ cells or the cytokines they produce in controlling parasite replication (Rose et al., 1992). However, a more recent study showed that depletion of $CD4^+$ cells had no effect on primary *EA* infection in chickens (Trout and Lillehoj, 1996). Trout and Lillehoj (1995) found $CD8^+$ cells to be present in large numbers just 24 h following *EA* infection, and significantly more sporozoites were found in or next to $CD8^+$ cells in *EA*-immune chickens than in naïve chickens, a further indication of T-cell involvement in immune responses to coccidiosis. Therefore, an early stimulation of the immune cells within the epithelium can enhance resistance to *EA* infection in broiler chickens.

However, *Eimeria*, being an intracellular parasite, must invade the host cells in order to replicate. First, it must adhere to epithelial surfaces. Gut-adapted probiotic bacteria may compete for adhesion sites and occupy common receptors on the epithelial cells. This would retard penetration and infiltration by *Eimeria* oocysts and consequently, their replication and shedding. Since this view is consistent with the oocyst data, we sought additional evidence of immune modulation. Serum samples and intestinal secretions taken 10 d PI were tested by ELISA for IFN-γ levels and for antibodies against a recombinant coccidial antigen. Despite the higher frequency of T cells prior to coccidial challenge and the substantial reduction in oocyst production in the probiotic-fed birds, no significant differences in IFN-γ levels occurred between treatments (Figure 2). We hypothesize that IFN-γ would rise, especially since higher levels have been associated with immune responses to coccidial infections (Lillehoj and Trout, 1996; Yun et al., 2000b). Lymphocytes from *Eimeria*-infected chickens produced a higher level of IFN-γ when stimulated with concanavalin A than did lymphocytes from uninfected chickens (Martin et al., 1994; Dimier-Poisson et al., 1999). Therefore, it is unclear why no difference in IFN-γ was detected. However, one explanation is that a temporal relationship exists and that the kinetics of IFN-γ production over time may be the more important variable to measure. For example, cytokine synthesis and release very early postchallenge may have already diminished 10 d later.

Intestinal secretions showed higher antibody level ($P \leq 0.05$) against a recombinant coccidial antigen in CONT birds than in PROB birds, but no difference was found in serum antibody levels against the same antigen (Figure 2). Were permeability across the mucosal barrier increased following the parasite-mediated damage to the epithelial layer, it would be expected that the pathogen would quickly penetrate the submucosa and reach the lamina propria where the majority of Ab-producing B cells reside. Therefore, while higher local Ab levels demonstrate effectiveness of resident B cell and existing T-helper cell populations, it does not necessarily reflect resistance to a primary infection. In fact, it may well document the invasive potential by *EA* seen in CONT birds that was lowered in PROB birds. Hence, there would be less secretory antibody in PROB than in CONT chickens, which was our result.

These results demonstrate an immunoregulatory effect of dietary probiotic on the local immune system in broiler chickens (e.g., IEL), improved resistance to *EA* (e.g., reduced oocyst shedding), and a rationale for further study of the beneficial effects of *Lactobacillus*-based probiotics and to elucidate their protective role.

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