Prevention of *Salmonella* Typhimurium colonization and organ invasion by combination treatment in broiler chicks

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ABSTRACT The effects in broiler chicks of treatment with a competitive exclusion (CE) product, an experimental dietary probiotic, and the abiotic β-glucan on cecal colonization, organ invasion, and serum and intestinal IgG and IgA levels to *Salmonella* challenge was evaluated. Four groups of 1-d-old chicks were treated by oral gavage on d 1 with an appropriate dose of a commercial CE product. Three groups received daily doses of probiotic, β-glucan, or both, for 6 d. Three other groups were fed daily from d 1 onwards with probiotic, β-glucan, or both. Subgroups of 30 chicks from each group were challenged on d 1, 9, 16, or 23 with 10^7 cfu/mL of *Salmonella* Typhimurium (1769NR) and killed 7 d later. Control groups were maintained untreated and remained unchallenged (negative control), or were challenged with *Salmonella* Typhimurium (1769NR; positive control), as described above. Cecum, liver, and spleen samples were examined for the presence of *Salmonella*, whereas serum and intestinal fluid samples were assayed for total antibody (IgG and IgA) concentrations. Data were analyzed by 1-way ANOVA, and means were compared using Duncan's multiple range test. In comparison with other treatments, those involving CE product and β-glucan, with or without probiotic during the first week, resulted in a superior inhibition of cecal colonization and organ invasion by *Salmonella* and also offered a higher level of protection ($P < 0.05$). During the second week, treatments containing experimental dietary probiotic and β-glucan, with or without CE product, resulted in an inhibition of liver invasion ($P < 0.05$). The IgA levels were significantly higher ($P < 0.05$) in intestinal fluid compared with serum, whereas IgG had low levels. The results in the first and third week indicate that combination treatments involving CE product, probiotic, and β-glucan are a more effective control of *Salmonella* colonization than the corresponding individual preparations.

Key words: *Salmonella* Typhimurium, immune response, competitive exclusion, dietary probiotic, β-glucan

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INTRODUCTION

Salmonella, a genus of zoonotic bacteria, represents one of the primary causes of food poisoning throughout the world (Fantasia and Filetici, 1994) and is thus of considerable public health and economic importance (Uzzau et al., 2000). More than 2,500 serotypes of the bacteria have been identified, and most of those isolated from humans or animals can cause acute gastroenteritis that is characterized by a short incubation period and a predominance of intestinal over systemic symptoms.

Foodborne *Salmonella* continues to be a major cause of salmonellosis. Outbreaks and sporadic cases have indicated that food vehicles such as poultry and poultry by-products are among the most common sources of *Salmonella* infections (Fantasia and Filetici, 1994; Sadeyen et al., 2004; Dunkley et al., 2008). Some *Salmonella* serotypes are more efficient to colonize or invade the gastrointestinal tract and to localize in organs than others (Smith and Tucker, 1980; Barrow et al., 1988; Aabo et al., 2002). Foodborne *Salmonella* in poultry possesses the innate ability to disseminate extraintestinally and to invade numerous avian tissues including the ovaries (Gast and Beard, 1990), whereby it may be deposited inside the egg, infect the embryo, and be transmitted vertically to the progeny. The protection afforded by the microbiota of healthy adult chickens against colonization of *Salmonella* in the digestive tract has been widely studied (Nurmi and Rantala, 1973; Snoeyenbos et al., 1978; Bailey, 1988). The bacterium is not a native member of the microbiota of poultry, and cross-infection and transfer of *Salmonella* to carcasses by fecal contamination during processing represents a common route of transmission (Waters et al., 2005).

Various prophylactic measures have been employed to control *Salmonella* infection in poultry production,

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including the use of antibiotics, competitive exclusion (**CE**) products and probiotics, genetic selection of chicken lines for improved immune responses, and the development of *Salmonella* vaccines (Lillehoj et al., 2000).

The term CE is used to describe the protective effect of natural or native intestinal bacterial flora in limiting the colonization of bacterial pathogens. Numerous trials around the world have demonstrated conclusively that CE is an effective approach in preventing the colonization of *Salmonella* in poultry. To obtain the maximum protective effects, however, CE cultures should be administered to chicks as soon as possible after hatching (Bolder et al., 1992).

Probiotics are live, naturally occurring microorganisms that are employed as feed supplements. Consumption of probiotics benefits the host animal by improving intestinal microbial balance (Fuller, 1993) and by altering the immune system to reduce colonization by pathogens under certain conditions (Patterson and Burkholder, 2003). It is well known that oats, mushrooms, and yeast are considered to be particularly good for the health of humans and animals because they stimulate intestinal movement (White et al., 1981; Englyst and Cummings, 1985; Knudsen et al., 1993; Dongowski et al., 2002; Jamroz et al., 2002). The major component of oats, mushrooms, and yeast is β-glucan, a sugar that cannot be digested by humans, which may be an important potentiator of mucosal immunity in the digestive tract (Tsukada et al., 2003). Moreover, when applied as an abiotic feed additive, β-glucan provided significant protection against organ invasion by *Salmonella* Enteritidis in young chicks (Lowry et al., 2005).

The objectives of the present study were to assess the effects on 1-d-old Ross chicks of an experimental dietary probiotic (**LEB**), the abiotic β-glucan (**G**), and the CE product Aviguard (Bayer, São Paulo, Brazil), administered either separately or in association, by evaluating their influence on IgA and IgG response in serum and intestinal fluid and on cecal colonization and organ invasion after challenge by a nalidixic acid-resistant strain of *Salmonella* Typhimurium.

MATERIALS AND METHODS

Details of the project were submitted to and approved by the Ethical Committee on Animal Research of the Faculty of Veterinary Medicine at the University of São Paulo. All procedures were carried out in compliance with current international regulations relating to the use of experimental animals (NRC, 2002).

Experimental Animals

A total of 420 Ross chicks, each 1 d old and of the same breed line and hatch, were supplied by a commercial hatchery. The birds were housed in floor pen facilities with pine-shaving litter and were provided ad libitum with water and a balanced diet of nonmedicated corn and soybean meal. Transport boxes and house environment were tested according to ISO 6579:2002/Amd 1:2007 (ISO, 2007). Additionally, samples were preenriched in Difco tetrathionate broth (Sparks, MD) and cultured on Difco xylose-lysine-deoxycholate agar, xylose-lysine-tergitol-4 (**XLT4**), and brilliant green agar. No evidence of *Salmonella* infection was detected in the transport boxes or in the animal house environment.

Study Products

CE Product. The CE product Aviguard (Bayer) comprised freeze-dried, viable, partially characterized intestinal bacteria derived from healthy specific-pathogen-free chickens and was administered by crop gavage at the rate of 0.1 mL per bird, as recommended by the manufacturer.

Experimental LEB. The defined LEB preparation contained 12 strains of *Lactobacillus* [*Lactobacillus plantarum*, *Lactobacillus murinus*, *Lactobacillus delbrueckii* ssp. *lactis* (2 strains), *L. delbrueckii* ssp. *delbrueckii*, *Lactobacillus reuteri*, *Lactobacillus salivarum*, *Lactobacillus casei pseudoplantarum*, *Lactobacillus amylophilus* (2 strains), *Lactobacillus agilis*, and *Lactobacillus viridians*], 5 strains of *Enterococcus* (*Enterococcus faecium*, *Enterococcus mundtii*, *Enterococcus faecalis*, *Enterococcus casseliflavus*, and *Enterococcus gallinarum*), and 1 strain of bifidobacteria (*Bifidobacterium bifidus*).

All strains were isolated from poultry sources in the Avian Pathology Laboratory at the Faculty of Veterinary Medicine, University of São Paulo. The *Lactobacillus* strains were cultured individually in de Man, Rogosa, and Sharpe broth and de Man, Rogosa, and Sharpe agar (Atlas, 1997) and then were combined to form a bacterial pool. The *Enterococcus* strains were cultured individually in tryptose-soy broth and were subsequently mixed to form a bacterial pool. *Bifidobacterium bifidus* was cultured in selective broth (Atlas, 1997). The probiotic was prepared by mixing the *Lactobacillus* pool (1 part) together with the *Enterococcus* pool (3 parts) and *B. bifidus* (9 parts) in *B. bifidus* selective broth supplemented with 5% of 199 medium (Cultilab, Campinas, São Paulo, Brazil). One portion of the mix was incubated under aerobic conditions for 24 h, whereas a second portion was incubated under anaerobic conditions for 36 h. After incubation, the cultures were centrifuged (9,000 × *g*; 10 min; 4°C), and the pellets were collected and mixed in equal proportions. The resulting LEB product was added to the feed at a final concentration of approximately 1.6×10^{11} cfu/g.

β-Glucan. The abiotic G was supplied by Biorigin (Lençois Paulista, São Paulo, Brazil) and contained at least 30% of β-1,3-linkages, 11% of β-1,6-linkages, and 23% of β-1,4-linkages. Product G was added to the feed to give a final concentration of 1 mg/kg.

Challenge Organism

The nalidixic acid-resistant challenge strain *Salmonella* Typhimurium (1769NR) (obtained from J. S. Bailey, USDA, Athens, GA) was maintained in Difco brain heart infusion medium. A stock solution of challenge strain inoculum containing 1×10^7 cfu/mL was prepared from 3-h brain heart infusion broth cultures maintained at 37°C. Bacteria were counted in duplicate on Difco plate count agar.

Experimental Design

One-day-old chicks were divided into 9 experimental treatments. Treatments 1 to 7 included 46 birds, whereas treatments 8 and 9 included 49 birds per group. Chicks (3 replicates in each treatment) were distributed randomly. All treatments were housed separately, including 49 birds from negative control (treatment 8) that were kept uninfected in a separate room. Experimental design is shown in Table 1. On d 2, 9, 16, and 23, subgroups of 10 chicks from each treatment (except those of group 8) were challenged with 10^7 cfu/mL of *Salmonella* Typhimurium (1769NR) and killed 7 d later to evaluate antibody responses, cecal colonization, and organ invasion. Birds were moved to another house before challenge. Three experimental trials using this experimental design were conducted on 3 different dates.

Sample Collection and Antibody Detection

Collection of Serum Samples. To assess systemic antibody responses, a blood sample from each bird was collected 1 wk postchallenge and allowed to clot at room temperature for 2 h. Serum was separated, clarified by centrifugation ($580 \times g$; 10 min), and stored at −20°C until required for analysis. Concentrations of serum IgG and IgA were not determined in the first week of treatment because of maternal antibodies. Serum antibodies were measured on d 16, 23, and 30.

Extraction of Intestinal Fluid. For the evaluation of the mucosal antibody response, chickens were killed 1 wk postchallenge by cervical dislocation; the duodenum, jejunum, and ileum were collected; and all connective tissue and fat were removed. Intestinal fluid was collected in 5 mL of a protease inhibitor cocktail containing EDTA, 0.3 mg/mL; phenylmethylsulphonyl fluoride, 75 μg/mL; pepstatin, 0.7 μg/mL; and leupeptin, 0.5 μg/mL (Sigma, St. Louis, MO) in ice-cold PBS. After centrifugation ($10{,}000 \times g$; 10 min; 4°C), the supernatant was separated and stored at −20°C until required for analysis. Concentrations of IgG and IgA in the intestinal fluid were measured after challenge on d 9, 16, 23, and 30.

Antibody Detection. The levels of IgA and IgG in serum and intestinal fluid were quantified using commercial ELISA assay kits (Bethyl Inc., Montgomery, TX). Sera and intestinal antibody levels were determined in 60 birds at 1 d of age in subgroups of 20 chicks (3 replications of 20 chicks each), to evaluate serum and intestinal fluid antibodies at the beginning of the experiment.

Cecal Colonization and Organ Invasion by Salmonella

To investigate challenge strain of *Salmonella* Typhimurium in ceca, liver, and spleen, samples were taken for culture from all birds on d 9, 16, 23, and 30 after challenge. Cecal colonization is the mean *Salmonella* counts calculated for each treatment. Percentage of cecal colonization was calculated to show the proportion of birds colonized by the challenge strain.

To determine cecal colonization, the whole cecum was removed aseptically and was placed in a sterile bag, weighed, and diluted 1:10 with a sterile solution of 0.1% peptone in water. One hundred microliters was spread on XLT4 agar (Difco) and incubated at 37°C for 24 h. Enumeration of *Salmonella* Typhimurium in cecal con-

Table 1. Experimental design

Treatments	Treatment (d 1)	Treatment (d 1 onwards)
1 CE[1]	CE	WT
2 LEB[2]	WT[3]	Dietary probiotic
3 G[4]	WT	β-glucan
4 CE + LEB	CE	Dietary probiotic
5 CE + G	CE	β-glucan
6 CE + LEB + G	CE	Dietary probiotic + β-glucan
7 LEB + G	WT	Dietary probiotic + β-glucan
8 NC[5]	WTN[6]	WTN[7]
9 PC[7]	WTC[8]	WTC[8]

[1]CE = competitive exclusion.
[2]LEB = experimental probiotic.
[3]WT = without treatment.
[4]G = β-glucan.
[5]NC = negative control.
[6]WTN = without treatment, unchallenged.
[7]PC = positive control.
[8]WTC = without treatment, challenged.

Table 2. Sera and intestinal fluid IgA levels in groups of chicks treated on d 1 with CE product and/or fed with either experimental probiotic or β-glucan, or both, from d 1 onwards

Treatments	IgA sera (ng/mL)			IgA intestinal fluid (ng/mL)			
	Second week	Third week	Fourth week	First week	Second week	Third week	Fourth week
1 CE[1]	$502.94^{a} \pm 26.07$	$440.35^{b} \pm 16.99$	$351.77^{b} \pm 11.10$	$16.07^{e} \pm 6.12$	$239.08^{c} \pm 11.25$	$414.46^{b} \pm 12.67$	$330.29^{c} \pm 12.99$
2 LEB[2]	$481.94^{a} \pm 17.07$	$321.01^{c} \pm 19.26$	$355.33^{b} \pm 18.05$	$29.14^{d} \pm 6.46$	$211.83^{c} \pm 10.98$	$398.97^{c} \pm 14.36$	$260.83^{e} \pm 15.77$
3 G[3]	$439.70^{b} \pm 24.18$	$340.36^{c} \pm 7.24$	$258.97^{d} \pm 27.88$	$17.04^{e} \pm 7.31$	$297.68^{b} \pm 10.76$	$330.77^{d} \pm 27.19$	$240.45^{e} \pm 11.25$
4 CE + LEB	$418.13^{b} \pm 7.07$	$325.62^{c} \pm 11.23$	$317.82^{c} \pm 13.41$	$29.39^{d} \pm 8.34$	$196.34^{d} \pm 16.34$	$291.41^{e} \pm 14.79$	$164.05^{g} \pm 18.61$
5 CE + G	$453.02^{a} \pm 10.44$	$335.53^{c} \pm 9.57$	$329.35^{c} \pm 15.98$	$34.99^{d} \pm 10.27$	$150.90^{e} \pm 11.76$	$271.42^{e} \pm 15.29$	$346.05^{b} \pm 14.93$
6 CE + LEB + G	$429.39^{b} \pm 10.22$	$247.42^{e} \pm 11.06$	$305.83^{d} \pm 32.04$	$133.44^{a} \pm 15.10$	$365.71^{a} \pm 9.57$	$399.55^{c} \pm 10.12$	$373.18^{a} \pm 9.24$
7 LEB + G	$415.68^{b} \pm 25.69$	$424.34^{b} \pm 16.05$	$439.04^{a} \pm 28.66$	$69.45^{b} \pm 12.65$	$383.04^{a} \pm 8.52$	$430.66^{a} \pm 14.81$	$287.29^{d} \pm 8.76$
8 NC[4]	$359.68^{c} \pm 31.34$	$268.73^{d} \pm 15.26$	$395.49^{b} \pm 22.35$	$13.46^{e} \pm 7.41$	$156.89^{e} \pm 12.98$	$330.77^{d} \pm 26.23$	$208.77^{f} \pm 16.34$
9 PC[5]	$493.02^{a} \pm 12.25$	$460.54^{a} \pm 15.43$	$330.29^{h} \pm 17.02$	$41.39^{c} \pm 9.34$	$193.47^{d} \pm 10.77$	$398.97^{c} \pm 17.04$	$350.76^{b} \pm 19.87$

[a–h]Different superscript letters associated with mean values in the same column indicate significant differences between treatments ($P \leq 0.05$).

[1]CE = competitive exclusion.

[2]LEB = experimental probiotic.

[3]G = β-glucan.

[4]NC = negative control.

[5]PC = positive control.

tent was performed on XLT4 agar (Difco) supplemented with nalidixic acid (Sigma) to facilitate selection of the antibiotic-resistant challenge organism. In cases in which growth was detected, a series of biochemical and serological confirmatory tests were performed.

To determine organ invasion after challenge, samples of liver and spleen were taken from birds of each experimental treatment. Samples were appropriately removed, and after recording liver and spleen weights, organs were homogenized separately. The homogenates of each organ were diluted 1:10 with a sterile solution of 0.1% peptone water and 100 μL was spread on XLT4 agar as described above. Organ invasion was evaluated by enumeration of *Salmonella* Typhimurium in liver and spleen samples using the method as described above.

Negative samples were placed in tetrathionate broth (1:10), incubated at 37°C for 24 h and streaked on XLT4 agar supplemented with nalidixic acid.

Efficacy of Treatment

In birds treated at the first week with CE product, or combinations thereof, the efficacy of each treatment, except treatment 3, was assessed from the values of the infection factor (**IF**) and the protection factor (**PF**) as described by Mead et al. (1989). The IF is the geometric mean of the number of salmonellae per gram of cecal content for all chicks in a particular group and PF is obtained by dividing the IF value for the control birds by that for the treated group (Mead et al., 1989).

Statistical Analysis

The data from each experimental group were pooled for statistical analysis. Statistical differences between treatments groups were tested by 1-way ANOVA and were considered significant for values of $P \leq 0.05$. Post hoc analysis was carried out using Duncan's multiple range test.

RESULTS

Antibody Levels

Concentrations of serum and intestinal fluid IgG and IgA levels in chicks at 1 d of age were as follows: serum IgG, 156.46 ng/mL; IgG intestinal fluid, 115.12 ng/mL; serum IgA, 21.11 ng/mL; and intestinal fluid IgA, 386.91 ng/mL.

Table 2 shows the effects of these treatments on IgA levels measured in sera and intestinal fluid. After the first week of treatments, intestinal fluid IgA was significantly ($P \leq 0.05$) higher in the birds treated with CE + LEB + G (treatment 6) than any other treatment. The intestinal fluid IgA levels in groups treated with CE (treatment 1) and G (treatment 3) had no differences compared with the negative control (treatment 8). Immunoglobulin A was significantly ($P \leq 0.05$) higher in the positive control (treatment 9) compared with the negative control (treatment 8) and the other treatments (treatment 1 to 5) except for treatments 6 and 7. Levels of intestinal fluid IgA decreased after 1 wk of treatment in all groups in comparison with the value obtained at 1 d of age (386.91 ng/mL).

During the second week after treatments, intestinal fluid IgA was significantly higher ($P \leq 0.05$) in the birds treated with CE + LEB + G (treatment 6) and LEB + G (treatment 7) than any other treatment. Intestinal fluid IgA had no difference in the positive control (treatment 9) compared with the CE + LEB group (treatment 4) but the value was significantly ($P \leq 0.05$) lower than any of the other treatments except groups CE + G and positive control (treatment 9).

After the third week of treatments, IgA was significantly ($P \leq 0.05$) higher in the positive control (treatment 9) than the other treatments except birds treated with CE (treatment 1) and LEB + G (treatment 7). Birds treated with CE + LEB (treatment 4) and CE + G (treatment 5) had significantly ($P \leq 0.05$) lower values than the negative control group (treatment 8).

Table 3. Intestinal fluid and serum IgG levels in groups of chicks treated on d 1 with CE product and/or fed with either experimental probiotic or β-glucan, or both, from d 1 onwards

Treatments	IgG sera (ng/mL)			IgG intestinal fluid (ng/mL)			
	Second week	Third week	Fourth week	First week	Second week	Third week	Fourth week
1 CE[1]	115.44[b] ± 12.12	77.47[a] ± 8.34	49.39[b] ± 7.34	18.59[c] ± 5.89	21.36[a] ± 9.34	18.94[b] ± 7.19	15.79[a] ± 6.05
2 LEB[2]	145.17[a] ± 16.21	32.47[d] ± 9.53	46.05[b] ± 7.85	16.23[c] ± 6.34	18.19[b] ± 8.66	15.84[b] ± 6.14	16.89[a] ± 8.01
3 G[3]	106.78[b] ± 13.06	33.46[d] ± 8.25	36.57[d] ± 6.75	15.27[c] ± 8.12	15.78[b] ± 5.23	18.08[b] ± 7.49	15.41[a] ± 7.98
4 CE + LEB	78.92[c] ± 12.78	48.21[c] ± 9.61	47.38[b] ± 9.02	23.18[b] ± 6.45	20.76[a] ± 7.99	16.56[b] ± 8.45	16.17[a] ± 7.62
5 CE + G	100.99[b] ± 10.04	48.97[c] ± 7.85	55.34[a] ± 8.07	21.84[b] ± 4.33	17.23[b] ± 9.21	17.12[b] ± 10.65	13.96[b] ± 6.76
6 CE + LEB + G	116.91[b] ± 9.25	52.24[c] ± 5.08	38.73[c] ± 4.29	21.42[b] ± 7.89	16.01[b] ± 7.33	14.92[b] ± 8.10	18.02[a] ± 5.32
7 LEB + G	98.89[b] ± 9.78	51.72[c] ± 5.68	52.41[b] ± 5.76	15.17[c] ± 8.21	16.28[b] ± 6.67	21.72[a] ± 5.56	15.89[a] ± 6.93
8 NC[4]	109.15[b] ± 10.45	56.25[b] ± 7.29	44.76[c] ± 6.87	24.05[b] ± 6.77	22.32[a] ± 7.78	20.82[a] ± 9.11	17.18[a] ± 8.34
9 PC[5]	106.37[b] ± 10.56	73.58[a] ± 6.05	40.41[c] ± 7.22	28.17[a] ± 5.98	17.56[b] ± 6.23	17.45[b] ± 7.32	15.29[a] ± 4.86

[a–d]Different superscript letters associated with mean values in the same column indicate significant differences between treatments ($P \leq 0.05$).

[1]CE = competitive exclusion.

[2]LEB = experimental probiotic.

[3]G = β-glucan.

[4]NC = negative control.

[5]PC = positive control.

During the fourth week after treatments, intestinal fluid IgA was significantly ($P \leq 0.05$) higher in the positive control (treatment 9) than any other treatment except birds treated with CE + LEB + G (treatment 6). The IgA values were significantly ($P \leq 0.05$) lower in the group treated with CE + LEB (treatment 4) compared with the negative control (treatment 8).

The second week of treatment exhibited that serum IgA value in the positive control (treatment 9) was significantly ($P \leq 0.05$) higher than treatments G (treatment 3), CE + LEB (treatment 4), CE + LEB + G (treatment 6), and LEB + G (treatment 7). Serum IgA levels had no differences between the positive control (treatment 9) when compared with treatments CE (treatment 1), LEB (treatment 2), and CE + G (treatment 5). The lower level of serum IgA was exhibited by the negative control (treatment 8). During the third week of treatment, the highest level of serum IgA was observed in the positive control (treatment 9) compared with any other treatment. During the fourth week, the high serum response attained by birds treated with LEB + G (treatment 7) was significantly ($P \leq 0.05$) higher than the positive control (treatment 9), and the negative control (treatment 8) experienced significantly ($P \leq 0.05$) higher levels than the positive control (treatment 9). All treatments showed high levels of sera IgA compared with IgA value measured in birds at 1 d of age (21.11 ng/mL).

The intestinal fluid IgA titers were also increased significantly ($P \leq 0.05$) in the challenged control group at wk 2, 3, and 4 in comparison with the value of 41.39 ng/mL obtained at the first week of treatment (Table 2). In the second week, all groups treated had intestinal fluid IgA values significantly ($P \leq 0.05$) higher than the positive control (treatment 9) except birds treated with CE + LEB (treatment 4) and CE + G (treatment 5). During the third week, intestinal fluid IgA levels in birds treated with CE + LEB (treatment 4) and CE + G (treatment 5) had significantly ($P \leq 0.05$) lower values than the negative control (treatment 8). In the fourth week, the high intestinal fluid IgA levels in birds challenged (treatment 9) was significantly ($P \leq 0.05$) higher than any other treatment except birds treated with CE + G (treatment 5).

As shown in Table 3, serum and intestinal IgG showed significant ($P \leq 0.05$) differences between treatments throughout the study period. Antibody levels, however, were low (Table 3), including the challenged control group.

Cecal Colonization and Organ Invasion by Salmonella

Table 4 shows that administration of CE + G (treatment 5), CE + LEB + G (treatment 6), and LEB + G (treatment 7) for 1 wk reduced the percentage of cecal colonization (20, 10, and 10%, respectively) by the challenge strain (Table 4). Cecal colonization was significantly ($P \leq 0.05$) lower from the positive control in all groups except the group treated with G (treatment 3). The lowest IF (0.4, 0.2, and 0.4, respectively) were established for treatments mentioned above, and the group treated with CE + LEB + G (treatment 6) was significantly ($P \leq 0.05$) lower than any other treatment except the negative control (treatment 8). Organ invasion (liver and spleen) was significantly ($P \leq 0.05$) inhibited in all animals treated with CE + LEB (treatment 4), CE + G (treatment 5), CE + LEB + G (treatment 6), and LEB + G (treatment 7) compared with the challenged control group (Table 4). Spleen invasion in groups treated with CE (treatment 1) and G (treatment 3) was significantly ($P \leq 0.05$) higher than the positive control (treatment 9).

During the second week after treatments (Table 5), percentage of cecal colonization was not inhibited in any treatment compared with the positive control (treatment 9). The IF values in all groups treated were similar, indicating that all groups, except treatment 8 and

Table 4. Infection and protection factors, colonization, and organ invasion in groups of chicks treated on d 1 with CE product and/or fed with either experimental probiotic or β-glucan, or both, d 1 to 6[1]

Treatments		IF[2]	PF[3]	Mean *Salmonella* count ($\log_{10}$ cfu/g)			Cecal colonization (%)
				Cecal colonization	Liver invasion	Spleen invasion	
1	CE[4]	1.6[b]	1.75[e]	3.23[b]	2.43[a]	4.15[a]	60
2	LEB[5]	0.8[c]	3.5[d]	3.11[b]	1.19[b]	0.45[d]	60
3	G[6]	1.6[b]	1.75[e]	3.98[a]	2.67[a]	2.37[b]	70
4	CE + LEB	0.9[c]	3.11[d]	2.46[d]	0[c]	0[e]	40
5	CE + G	0.4[d]	7.0[c]	2.42[d]	0[c]	0[e]	20
6	CE + LEB + G	0.2[d]	14.0[b]	2.79[c]	0[c]	0[e]	10
7	LEB + G	0.4[d]	7.0[c]	2.71[c]	0[c]	0[e]	10
8	NC[7]	0[e]	>28[a]	0[e]	0[c]	0[e]	0
9	PC[8]	2.8[a]	1.0[e]	3.99[a]	2.41[a]	1.29[c]	88.8

[a–e]Different superscript letters associated with mean values in the same column indicate significant differences between treatments ($P \leq 0.05$).

[1]Ten chicks from each group were challenged with *Salmonella* Typhimurium on d 2 and killed 7 d later.

[2]IF = infection factor.

[3]PF = protection factor.

[4]CE = competitive exclusion.

[5]LEB = experimental probiotic.

[6]G = β-glucan.

[7]NC = negative control.

[8]PC = positive control.

9, had high proportions of *Salmonella*-infected birds. However, the IF values in all groups treated were significantly ($P \leq 0.05$) reduced compared with positive control (treatment 9). The IF value in groups treated with CE + G (treatment 5) and CE + LEB + G (treatment 6) was significantly ($P \leq 0.05$) lower than the positive control group (treatment 9) but the percentage of cecal colonization was at least 90% in all groups except birds treated with LEB (treatment 2). The PF values showed that any treatment was efficacious for reducing *Salmonella* colonization. Liver invasion was inhibited in chickens fed with LEB + G (with or without CE product), a result that was significantly ($P \leq 0.05$) different from those observed in the other treatment groups (Table 5). However, treatments with CE (treatment 1), G (treatment 3), and CE + LEB (treatment 4) did not prevent spleen invasion when compared with the positive control (treatment 9).

As shown in Table 6, during the third week after treatments, the incidence of cecal colonization (%) was reduced in all groups except CE + LEB (treatment 4) and LEB + G (treatment 7) compared with the positive control (treatment 9). Cecal colonization was also reduced in the positive control group (treatment 9) with a percentage of positive birds of 60%. The lowest IF was exhibited by birds treated with CE + LEB + G (treatment 6) and LEB + G (treatment 7) and these groups showed the highest PF (7.0). Birds treated

Table 5. Infection and protection factors, colonization, and organ invasion in groups of chicks treated on d 1 with CE product and/or fed with either experimental probiotic or β-glucan, or both, from d 1 onwards[1]

Treatments		IF[2]	PF[3]	Mean *Salmonella* count ($\log_{10}$ cfu/g)			Cecal colonization (%)
				Cecal colonization	Liver invasion	Spleen invasion	
1	CE[4]	1.7[c]	2.23[c]	2.92[d]	0.34[c]	2.54[b]	90
2	LEB[5]	1.7[c]	2.23[c]	2.96[d]	0.19[d]	0[d]	60
3	G[6]	2.0[b]	1.90[d]	3.31[b]	1.00[b]	1.91[c]	100
4	CE + LEB	2.3[b]	1.65[d]	3.16[c]	1.24[b]	2.97[a]	100
5	CE + G	2.0[b]	1.90[d]	2.23[e]	0.45[c]	0[d]	90
6	CE + LEB + G	1.6[c]	2.38[c]	2.49[e]	0[e]	0[d]	100
7	LEB + G	1.2[d]	3.17[b]	3.43[b]	0[e]	0[d]	90
8	NC[7]	0[e]	>38[a]	0[f]	0[e]	0[d]	0
9	PC[8]	3.8[a]	1.0[e]	3.63[a]	1.48[a]	0[d]	90

[a–f]Different superscript letters associated with mean values in the same column indicate significant differences between treatments ($P \leq 0.05$).

[1]Ten chicks from each group were challenged with *Salmonella* Typhimurium on d 9 and killed 7 d later.

[2]IF = infection factor.

[3]PF = protection factor.

[4]CE = competitive exclusion.

[5]LEB = experimental probiotic.

[6]G = β-glucan.

[7]NC = negative control.

[8]PC = positive control.

Table 6. Infection and protection factors, colonization, and organ invasion in groups of chicks treated on d 1 with CE product and/or fed with either experimental probiotic or β-glucan, or both, from d 1 onwards[1]

Treatments		IF[2]	PF[3]	Mean *Salmonella* count ($\log_{10}$ cfu/g)			Cecal colonization (%)
				Cecal colonization	Liver invasion	Spleen invasion	
1	CE[4]	0.8[b]	1.75[d]	0.28[d]	0[b]	0[a]	30
2	LEB[5]	0.4[c]	3.5[c]	0.26[d]	0[b]	0[a]	10
3	G[6]	0.4[c]	3.5[c]	0.39[c]	0.34[a]	0[a]	20
4	CE + LEB	0.4[c]	3.5[c]	1.22[a]	0[b]	0[a]	30
5	CE + G	0.4[c]	3.5[c]	0.22[d]	0[b]	0[a]	10
6	CE + LEB + G	0.2[c]	7.0[b]	0.23[d]	0[b]	0[a]	10
7	LEB + G	0.2[c]	7.0[b]	0.96[b]	0[b]	0[a]	20
8	NC[7]	0[d]	>14[a]	0[e]	0[b]	0[a]	0
9	PC[8]	1.4[a]	1.0[e]	0.34[c]	0[b]	0[a]	60

[a–e]Different superscript letters associated with mean values in the same column indicate significant differences between treatments ($P \leq 0.05$).

[1]Ten chicks from each group were challenged with *Salmonella* Typhimurium on d 16 and killed 7 d later.

[2]IF = infection factor.

[3]PF = protection factor.

[4]CE = competitive exclusion.

[5]LEB = experimental probiotic.

[6]G = β-glucan.

[7]NC = negative control.

[8]PC = positive control.

with G (treatment 3) showed liver invasion significantly ($P \leq 0.05$) higher compared with the positive control group (treatment 9). Spleen invasion was inhibited in all groups including untreated and challenged birds (treatment 9).

The fourth week after treatment (Table 7), just 1 chicken in the group treated with CE product (treatment 1), and only 30% of the birds in the positive control group (treatment 9), tested positive for cecal colonization. The IF values in all groups treated, except birds treated with CE (treatment 1), were 0, indicating absence of *Salmonella*-infected birds. However, the IF value in the group treated with CE (treatment 1) was significantly ($P \leq 0.05$) reduced compared with the positive control (treatment 9). The PF values showed that all treatments were efficacious for reducing *Salmonella* colonization except birds treated with CE (treatment 1). Organ invasion was not observed in all treatments including the untreated and challenged group (treatment 9).

DISCUSSION

Mucosal immunity provides the first line of defense after oral exposure to pathogens, and secretory IgA (**SIgA**) provides protection against bacterial, parasitic, and viral pathogens (Fagarasan, 2006) and other foreign proteins from penetrating the intestinal surface

Table 7. Infection and protection factors, colonization, and organ invasion in groups of chicks treated on d 1 with CE product and/or fed with either experimental probiotic or β-glucan, or both, from d 1 onwards[1]

Treatments		IF[2]	PF[3]	Mean *Salmonella* count ($\log_{10}$ cfu/g)			Cecal colonization (%)
				Cecal colonization	Liver invasion	Spleen invasion	
1	CE[4]	0.4[b]	2.25[c]	0.08[b]	0[a]	0[a]	10
2	LEB[5]	0[c]	>9[a]	0[c]	0[a]	0[a]	0
3	G[6]	0[c]	>9[a]	0[c]	0[a]	0[a]	0
4	CE + LEB	0[c]	>9[a]	0[c]	0[a]	0[a]	0
5	CE + G	0[c]	>9[a]	0[c]	0[a]	0[a]	0
6	CE + LEB + G	0[c]	>9[a]	0[c]	0[a]	0[a]	0
7	LEB + G	0[c]	>9[a]	0[c]	0[a]	0[a]	0
8	NC[7]	0[c]	>9[a]	0[c]	0[a]	0[a]	0
9	PC[8]	0.9[a]	1.0[b]	0.28[a]	0[a]	0[a]	30

[a–c]Different superscript letters associated with mean values in the same column indicate significant differences between treatments ($P \leq 0.05$).

[1]Ten chicks from each group were challenged with *Salmonella* Typhimurium on d 23 and killed 7 d later.

[2]IF = infection factor.

[3]PF = protection factor.

[4]CE = competitive exclusion.

[5]LEB = experimental probiotic.

[6]G = β-glucan.

[7]NC = negative control.

[8]PC = positive control.

and can neutralize toxins and infectious microorganisms (Mestecky et al., 1999). The present study showed that colonization by *Salmonella* Typhimurium was reduced and invasion inhibited in treatment group 6 chicks that had been treated with CE product, LEB, and G (Table 4), and this group also exhibited high levels of total IgA in the intestinal fluid. The timing of clearance of *Salmonella* has been correlated with the peak antibody and T-cell responses and, considering the luminal location of the *Salmonella*, the specific IgA response is a prime candidate effector mechanism (Smith and Beal, 2008). In this context, chemical bursectomy treatment resulted in higher cecal carriage and fecal excretion of *Salmonella* (Arnold and Holt, 1995; Desmidt et al., 1998) in the absence of detectable *Salmonella*-specific antibodies (Corrier et al., 1991). In contrast, reduced shedding has been reported in bursectomized chickens compared with normal birds after infection with *Salmonella* Typhimurium (Brownell et al., 1970). This result is justified in the fact that birds were bursectomized at 8 or 9 d posthatch and this procedure should not result in complete B-cell deficiency. Nevertheless, Beal et al. (2006) demonstrated that infection by *Salmonella* Typhimurium induces a high level of specific antibodies, but B cells do not play an essential role in clearance of primary infection or in the enhanced clearance after secondary challenge.

In the present study, the efficacy of CE cultures against *Salmonella* could be demonstrated in chicks that were only a few days old, confirming that the age of the bird is crucial in the prophylactic use of CE preparations (Methner et al., 1999; Schneitz, 2005). The effect of CE treatment in combination with other products was to prevent *Salmonella* colonization and multiplication in the cecum. This can be explained by the fact that the entry of a microorganism into a given environment can be prevented if the space is already occupied by a competitor microorganism that is better suited to establishing and maintaining itself in the environment or is excreting substances that inhibit the competitor (Bailey, 1987).

Regarding the colonization by *Salmonella* Typhimurium of all treated groups, it is likely that as birds aged they acquired resistance to cecal colonization and organ invasion independent of the treatment applied. Such a possibility is supported by the results presented by the positive control treatment 9 (Tables 4 to 7). These findings corroborate previous reports showing that young birds are more susceptible to colonization (Bailey, 1987) and that susceptibility to oral infection diminishes rapidly with increasing age (Milner and Shaffer, 1952). In contrast, Linton et al. (1985) observed increased infection rates from the third week followed by a decrease.

The mechanism of the protective effect of CE treatment recorded in this study is unknown, but it seems unlikely that any single mechanism would be wholly responsible for the observed effects of the combination of products tested. Some factors believed to be involved in the efficacy of CE treatment are as follows: (a) creation of a restrictive physiological environment caused by microbial formation of volatile fatty acids and low oxidation-reduction potentials; (b) competition among different microbes for receptor sites; (c) elaboration of antibiotic-like substances, such as bacteriocins, by the competing microorganism; and (d) microbial competition for essential nutrients (Mead, 2000; Schneitz and Mead, 2000).

The results shown in Tables 4 and 6 suggest that a combined treatment with CE product and G, with or without LEB, provided more consistent protection to chicks against *Salmonella* challenge compared with other treatments, regarding organ invasion. The results shown in Table 4 were that treatments 6 and 7 had 10% of cecal colonization when compared with other groups including positive control. The results obtained at the second week (Table 5) indicated that group 3 treated with experimental LEB had 60% cecal colonization. No differences were observed in the other treatment groups. During the third week (Table 6), cecal colonization was reduced in treated chicks in groups 2, 5, and 6, whereas the positive control group had 60%. Chicks receiving all 3 components of the treatment showed the highest levels of total IgA or IgA antibodies, a result that was significantly ($P \leq 0.05$) correlated with cecal colonization. In addition to the CE effect, the experimental probiotic bacteria must have been in contact with immune cells stimulating SIgA, the most important component in the protection of mucosal surfaces against pathogens (Galdeano and Perdigon, 2004). Probiotic bacteria have the ability to bind to the intestinal mucus, a factor that is key to their protective effect (Ouwenhand et al., 1999a; Collado et al., 2007). However, other protective mechanisms include production of inhibitory substances (Corthier et al., 1985), immunomodulation (Hatcher and Lambrecht, 1993), and modulation of cytokine patterns (Ouwenhand et al., 1999b). It has been shown that administration of CE products and probiotic bacteria, or their products, generate immunostimulatory effects and may enhance the antibody response (Isolauri et al., 2001; De Vrese et al., 2005; Revolledo et al., 2006) including systemic and mucosal immunity (Perdigon et al., 1999). The addition of the abiotic G to the feed has also been shown to decrease the incidence of organ invasion by *Salmonella* Enteritidis (Lowry et al., 2005).

As noted in other studies (Mast and Goddeeris, 1999; Bar-Shira et al., 2003; Bar-Shira and Friedman, 2006), the number of IgA-secreting cells and the level of SIgA in the intestine increases rather slowly over time, suggesting that mucosal immunity is not fully developed in newly hatched chicks and requires a period to mature. The present study showed, however, that at 3 wk of age, levels of IgA were higher in challenged chicks compared with their unchallenged counterparts (Table 2), suggesting that the mucosal response was better developed and able to respond efficiently to enteric bacteria. This hypothesis is supported by the absence of organ invasion by *Salmonella* and by reduced levels of

cecal colonization in the positive control group (Table 7). It is suggested that oral inoculation of *Salmonella* Typhimurium increased the concentration of IgA in the intestinal fluid and may reflect the activation of the intestinal immune system in protecting chicks against pathogen colonization. The significant increase ($P \leq 0.05$) in IgA levels (serum and intestinal fluid) during the experimental period that was observed in all challenged groups (including the positive control) suggests that the defense mechanisms mediated by IgA may contribute to the reduction or inhibition of colonization by *Salmonella* Typhimurium. The response of the positive control group was similar to that described by Lessard et al. (1995), who demonstrated that the immune responses, both cellular and humoral, are activated after infection by *Salmonella* Typhimurium.

The results shown in Table 3 indicate that the levels of serum and intestinal IgG were low in all challenged groups (including the positive control) throughout the experimental period. This demonstrates that experimental infection with *Salmonella* Typhimurium, as well as the treatments supplied in the feed, did not stimulate the production of IgG in the serum and intestinal mucosa. Fukutome et al. (2001) observed a small IgG response after oral immunization of birds, although other authors have reported significant increases in serum and intestinal IgG levels after inoculation of birds with *Salmonella* Typhimurium (Brito et al., 1993).

As far as we are aware, this is the first report of the effect of a CE product used in combination with LEB or G, or both. The results obtained indicate that combined treatments including a CE product are more effective than individual preparations in reducing *Salmonella* colonization and organ invasion in the first week of age (Table 4) and liver invasion at the second week of age (Table 5). Additionally, the reported findings underline the high value of such treatments in stimulating the systemic and mucosal immune response mediated by IgA in broiler chickens. However, further work is required to determine the mechanisms by which the interaction of the combined products regulates avian intestinal immune responses. Moreover, it is necessary to elucidate the overall role of humoral and cellular innate and acquired immunity in the protection afforded by CE products, LEB, and G, together with appropriate means of enhancement of the immune response in a directed and predetermined way. In general, strategies for controlling enteric pathogens need to be further improved to reduce *Salmonella* infections, contamination of poultry products, and, consequently, the incidence of paratyphoid salmonellosis.

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