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Effects of *Enterococcus faecium* on Diet in the Dynamics of CD4+ and CD8+ Cell Infiltration in the Intestinal Mucosa of Broilers Challenged with *Salmonella* Minnesota

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Abstract: The emergence of different *Salmonella* serovars raises concern about human and animal infection and measures of *Salmonella* control should be studied. The use of *Enterococcus faecium* (EF) as probiotic in poultry feed was studied to control *Salmonella* Minnesota (SM) infection in crop and cecum of SM challenged broilers and to assess its effect on immune cell infiltration into ileum and cecum mucosa of broilers. Birds were divided into three treatment groups: Negative control, birds non-inoculated; Positive control, with SM inoculated birds and Probiotic, with SM inoculated birds and treated with EF-containing diet. Before SM challenge, birds of the Probiotic group presented increased goblet cell counts in the ileum and cecum, decreased CD8+ cells in the ileum and increased CD4+ cells in the cecum as compared to birds from the Negative control. After SM challenge, birds from Probiotic group presented decreased *Salmonella* counts in cloacal swabs at 48 hours post-inoculation (p.i.) and also in the cecum and litter at 35 days of age. The CD4+cell in the ileum and CD8+cell counts in the cecum were lower when compared to the Positive control counts. Based on these results, it can be assumed that the use of EF probiotic can reduce *Salmonella* spp. counts and therefore affect CD4+ and CD8+ cells mobilization in the ileum and cecum mucosa of broilers.

Key words: *Enterococcus faecium*, probiotic, CD4+cells, CD8+cells, goblet cells, *Salmonella*

INTRODUCTION

Salmonellosis is a severe public health problem both in developing and developed countries. In broilers, contamination occurs mainly in the crop and cecum (Ramirez *et al*., 1997; Corrier *et al*., 1999). Some mechanisms have been used in poultry production to control this pathogen (Cardoso and Carvalho, 2006). This control is based on biosafety management practices, associated with the use of some additives, one of them being the probiotics. They are defined as a single culture or a mixture of cultures of living microorganisms that, if applied to animals or human beings, favorably affect the host, improving endogenous microbiota properties (Havenaar *et al*., 1992).

The inhibitory effect of probiotics on pathogenic enterobacteria such as *Salmonella* by Competitive Exclusion (CE) mechanism is well documented in the literature (Reid and Friendship, 2002; Hariharan *et al*., 2004; Dahiya *et al*., 2006; Callaway *et al*., 2008). Nurmi and Rantala (1973) were the first to describe the use of CE to control *Salmonella*, linking its mode of action to volatile acids production and a competition between beneficial and pathogenic microorganisms for binding sites in the gut (Soerjadi *et al*., 1981).

Some authors also report that a number of bacteria used as probiotics may act on the immune response (Noujaim *et al*., 2008; Mouni *et al*., 2009; Lee *et al*., 2010). Toll-like receptors provide the interaction between the intestinal cells and probiotic bacteria, stimulating an immune response that is different from the immune response produced by pathogenic bacteria. The intestinal cells are the first line to generate immune signals to the underlying immune cells in the lamina propria (Vinderola *et al*., 2005).

Enterococcus faecium (EF) is a lactic acid bacteria that has inhibitory effects against *Escherichia coli* and *Salmonella* spp. (Lewenstein *et al*., 1979). It was shown to have a probiotic effect improving performance and feed conversion in piglets (Mallo *et al*., 2010) and modulating immunity in rats (Sun *et al*., 2010). The objective of the present work was to evaluate the efficiency of EF-based probiotic in SM control in crop and cecum of broilers challenged with SM and immune cells infiltration of the intestinal mucosa of broilers.

MATERIALS AND METHODS

Animals, facilities and experimental design: The current study was approved by institutional Ethics Committee of the Use of Animals (CEUA protocol number 034/2011).

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Sixty one-day-old male Cobb® broilers were randomly divided into three treatments, in a completely randomized experiment with 20 birds, each animal being a replicate. T1-Negative control, bird did not received SM inoculation, T2 Positive control inoculated with SM and T3 Probiotic, birds inoculated with SM and received feed with probiotic in diet. Each treatment group (n = 20) was housed in a separate room from 1 to 35 days to avoid probiotic and *Salmonella* cross contamination between treatments. The isolated rooms were identical, located side by side, with negative pressure and previously cleaned and disinfected. Wood shavings as litter were autoclaved at 121°C for 15 min. Rooms, equipment and litter were tested for sterility before the experiment started. Upon arrival, five birds were euthanized and necropsied and their liver and cecum collected to test for presence/absence of *Salmonella*.

The birds were kept at an ideal room temperature for comfort consistent with their age and were given ad libitum access to water and feed. The balanced diet was formulated with levels equal to or higher than the NCR (1994) recommendations and pelletized.

Probiotic: The tested probiotic strain was *Enterococcus faecium* (NCIMB 10415. 50 g/ton-Cylactin ME 20® DSM Nutritional Products, Heerlen, Netherlands) from 1 to 35 days of age. The feed containing probiotic presented an average of 1×10^9 CFU/kg of feed.

Salmonella strain: At 14 days of age birds from Positive control (T2) and Probiotic (T3) groups were orally inoculated with 1 mL of a *Salmonella* Minnesota solution at 10^8 CFU/mL concentration. Administration was performed by oral gavage, using a syringe with attached flexible tube.

Collection of material for microbiology testing: At 48 hours post inoculation (PI), five samples of cloacal swabs were taken from each treatment group (3 animals pool) for *Salmonella* counts. Euthanasia was performed on five animals from each treatment group at 7 days of age and on 10 animals from each treatment group at 35 days. Birds were necropsied to aseptically collect the crop and cecum for *Salmonella* counts.

At 21 and 35 days of age, five litter samples (10 g) were collected from the boxes were the animals were housed (five samples/treatment) for *Salmonella* counts.

Samples processing for microbiology tests: Cloacal swabs, crops, cecum and litter were diluted in 2% peptone water (RM001, HiMedia Laboratories Pvt. Ltd., Mumbai, IN) at 1:9. Further dilution was conducted by successively placing 1mL of the solution in a test tube with 9mL of 0.1% peptone water until a 10^{-3} dilution was achieved. Then 100 iL of each dilution were transferred to duplicate plates in Xylose Lysine Desoxycholate (XLD) medium (CM469, Oxoid Limited, Hampshire, UK) and uniformly spread with a sterile Drigalsky loop. The plates were incubated at 35°C for 24 h after which the typical colonies were counted (adapted from Desmidt *et al*., 1998).

The initial 2% peptone water solution was incubated for 24 h. If no typical *Salmonella* colonies had developed after the 24 h incubation, 100 µL of the initial 2% peptone solution were placed in a tube with 10 mL Rappaport-Vassiliadis broth (CM 669, Oxoid Limited, Hampshire, UK) and incubated at 42°C for 24 h to confirm the negative/positive results of samples.

The resulting counts were expressed as accordance to Colony Count Procedures set in the Normative Rules No. 62 published in August 26, 2003 (MAPA, 2003). *Salmonella* colonies counts were Log 10 transformed to conduct statistical analysis.

Sampling for histopathology and immunohistochemistry tests: Samples were taken from the ileum (two centimeter above the ileum-cecal junction) and cecum (final portion of the left cecum) of five birds from each treatment group at seven and 35 days of age. At seven days of age, as the Negative and Positive controls were not inoculated with SM, samples were collected from two birds from the Negative control group and three birds from the Positive control, to serve as Negative control for histological and immunohistochemistry analysis. Samples were placed in 10% buffered formalin and processed according to the procedure (Smirnov *et al*., 2004) to analyze goblet cells. Briefly, the slides were deparaffinized in warm xylene, rehydrated with alcohol and stained with Alcian Blue (to identify the goblet cells), hematoxylin and eosin.

Part of the same samples was frozen in liquid nitrogen to be later analyzed for CD4+ and CD8+ cells as earlier described (Jeurissen *et al*., 2000). Immunohistochemistry slides were placed horizontally in a humid incubation chamber and incubated with 100-500 µL of primary Ab specific for CD4+ or CD8+ (SouthernBiotech, Birmingham, AL, USA), each Ab being placed in a different slide, washed thrice with PBS. The slides were then incubated for 30-60 min with HRP-conjugated Ab specific for the primary Ab (HRP-conjugated rabbit anti-mouse Ig, Dako North America, California, USA), then peroxidase activity was developed using DAB kit for immunocytochemistry (Dako North America Inc., California, USA). Slides were counterstained with hematoxylin solution.

Analysis of histopathology and immunohistochemistry slides: Histologic analyses and quantification of CD4+ and CD8+ cells from the intestinal epithelium were performed under light microscopy with an image

analyzer system (Motic Image Plus 2.0-Motic China Group Co. 2006) coupled to the microscope (Olympus America INC., NY, USA). Quantification of goblet cells and CD4+ and CD8+ lymphocytes on the ileum and cecum was performed in 100X magnification fields, 10 fields per slide.

Statistical analysis: All statistical analyses were conducted with the Statistix for Windows Copyright (C) 2008 statistical program. Results were submitted to ANOVA Fischer's test at 5% significance level.

RESULTS

Liver and cecum samples collected on the first day and crop and cecum samples collected at 7 days of age were all negative for *Salmonella*. Results shown on Table 1 indicate that the probiotic in the diet was able to significantly reduce ($p<0.05$) *Salmonella* excretion in cloacal swabs at 48 hours post inoculation (PI) as compared to the Positive control; at 35 days of age the same result was obtained in the cecum, but not in the crop.

Salmonella litter counts (average±standard deviation) at 21 and 35 days of age (Table 1) showed that treatment with EF-feed did not reduce *Salmonella* in the litter at 21 days but decreased *Salmonella* isolation in the litter at 35 days by 47.20% when compared to the Positive control.

Table 2 shows goblet cells, CD4+ and CD8+ counts. Birds from the Probiotic group showed significant increase in goblet cells at the ileum and cecum at 7 days of age, as compared to the Negative control group. At 7 days of age, there was no statistical difference ($p>0.05$) among treatments as to the CD4+ counts at the ileum and the CD8+ counts at the cecum, although the CD4+ counts in the cecum were higher in the Probiotic group than in the Negative control. The number of CD8+ cells in the ileum is lower in the Probiotic group than in the Negative control. The CD4:CD8 ratio in the cecum is significantly ($p<0.05$) lower for CD4+ than for CD8+ in the Negative control as compared with the Probiotic group, while no significant differences were seen in the ileum.

There was no significant difference among the groups in goblet cell dynamics on ileum and cecum mucosa of birds at 35 days of age. However, the number of CD4+ cells in the ileum was lower in the Probiotic group when compared to the other groups and CD8+ in the cecum in the Probiotic group when compared to the Positive control. The CD4:CD8 ratio in the ileum shows significantly ($p<0.05$) lower CD4+ than CD8+ cells in the Probiotic group as compared to the other groups that received no probiotic in the diet. In the cecum, the Positive control presented higher CD8+ counts than the other two groups.

Table 1: Average and SD of *Salmonella* colony counts (log_{10} cfu/g) in cloacal swabs, crop and litter in different treatment groups

Treatments	Cloacal swabs 48h PI	Crop 35 days	Cecum 35 days	Litter 21 days	Litter 35 days
Negative control	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{c}	0.00±0.00^{b}	0.00±0.00^{c}
Positive control	3.95±2.24^{a}	0.87±0.50^{a}	4.30±4.28^{a}	4.30±0.07^{a}	3.60±0.22^{a}
Probiotic	1.51±1.14^{b}	0.67±0.80^{a}	1.12±1.20^{b}	4.03±0.35^{a}	1.90±0.66^{b}
p-value	0.002	0.025	0.001	0.001	0.001

$^{a-c}$Different upper-case letters in the same column differ by Fischer´s test with 95% level of confiance ($p<0.05$)

Table 2: Goblet cells, CD4+, CD8+ counts and CD4+:CD8+ ratio per field in broilers ileum and cecum at 7 and 35 days of age (100x magnification)

	Treatments	Goblet cells	CD4+cells	CD8+cells	CD4+:CD8+Ratio
7 days					
Ileum	Negative control	41.40±8.43^{b}	4.30±3.71	7.90±2.33^{a}	0.63±0.62
	Probiotic	62.50±5.65^{a}	4.80±2.44	4.90±2.02^{b}	1.21±0.85
	p-value	0.001	0.726	0.007	0.097
Cecum	Negative control	10.30±2.34^{b}	8.30±3.30^{b}	10.30±4.06	0.87±0.40^{b}
	Probiotic	12.80±2.53^{a}	18.40±3.56^{a}	11.70±2.87	1.70±0.70^{a}
	p-value	0.002	0.001	0.385	0.005
35 days					
Ileum	Negative control	64.50±14.95	16.20±6.48^{a}	6.90±4.79	3.77±3.08^{a}
	Positive control	60.95±12.76	15.50±5.58^{a}	11.40±6.36	1.89±1.74^{a}
	Probiotic	62.00±9.56	7.90±3.96^{b}	9.20±4.69	1.07±0.83^{b}
	p-value	0.660	0.003	0.188	0.023
Cecum	Negative control	10.85±6.09	19.60±6.10	11.50±3.57^{b}	2.02±1.31^{b}
	Positive control	13.70±5.36	23.70±8.35	21.90±5.68^{a}	1.12±0.40^{a}
	Probiotic	12.20±4.68	21.80±3.12	14.50±4.65^{b}	1.72±0.89^{ab}
	p-value	0.250	0.353	0.001	0.114

$^{a-c}$Different upper-case letters in the same column differ by Fischer´s test with 95% level of confiance ($p<0.05$)

DISCUSSION

It has been shown that EF produces enterocin A, a bacteriocin capable of reducing *Salmonella* Dusseldorf counts in feces, ileum and cecum of broilers (Lauková *et al*., 2004). In the present study, the use of EF (1×10^9 CFU/g) in broilers feed reduced Salmonella counts by 61.77% in cloacal swabs at 48 h p.i. and by 47.20 and 73.45% respectively in the cecum and litter of birds at 35 days of age as compared to the Positive control. According to Lund *et al*. (2002),EF is able to survive intestinal transit and can be isolated in feces of individuals fed with EF in the diet. Bacteriocins produced by EF are very resistant, not being affected by acids and bile salts (Shin *et al*., 2008). Associated to production of lactic acid (Lewenstein *et al*., 1979) and of bacteriocins against Salmonella (Lauková *et al*., 2004), this feature may in part explain the results found in the present study. The antimicrobial activity of EF can act not only against *Salmonella* sp., but also have an effect on the autochthonous microbiota of the animal, regulating it (Mareková *et al*., 2003; Bhardwaj *et al*., 2010). EF did not affect the SM count in the litter at 21 days, but reduced the SM count at 35 days, suggesting that EF needs perhaps more time to replicate in the litter and reach levels that will inhibit the growth of SM.

There is no significant reduction of SM counts in the crop. However, the observed counts were low if compared to previous studies in our laboratory with serotype Enteritidis (not published), which presented higher counts. This suggests that the Minnesota serovar could have behavior different from that of other *Salmonella* serovars as Enteritidis (Ramirez *et al*., 1997). In addition, there is a large adhesion of *Lactobacillus* spp. just after birds were fed (Fuller, 1997). This adhesion may prevent EF from colonizing the crop. However, the presence of *Lactobacillus* spp. may not reduce *Salmonella* spp. counts in the crop. According to van der Wielen *et al*. (2002), *Lactobacillus crispatus* was not able to inhibit the multiplication of *Salmonella* Enteritidis in broilers crop, which could explain the presence of SM in it.

EF also affected the cell dynamics in the intestinal mucosa of birds at 7 days of age, even before SM inoculation. In the Probiotic group, an increased number of goblet cells was seen in the ileum and cecum of birds compared to Negative control. Goblet cells are responsible for maintaining a mucus layer that acts as physical and biological protection and has a role in the innate immune response (Uni *et al*., 2003). Colonization of different parts of the gastrointestinal tract by specific bacteria may be due to their association to the mucus layer and immunoglobulins in a process described as immune inclusion (Everrett *et al*., 2004), which would act as the first defense barrier against noxious organisms and toxins (Nousiainen *et al*., 2005). This might even explain the decrease in isolated SM in birds fed with this probiotic.

Stimuli from probiotic colonization are essential in the development of a functional and well balanced immune system, including the presence of T and B lymphocytes in the lamina propria and also in the expansion and maturation of IgA and to induce tolerance to antigens present (Borchers *et al*., 2009). Helper T lymphocytes (Th cells), CD4+, orchestrate an acquired immune response by promoting intracellular killing by macrophages, antibody production by B lymphocytes and clonal expansion of cytotoxic T lymphocytes (Fearon and Locksley, 1996), whereas CD8 T lymphocytes are involved in antigen elimination (Zou *et al*., 2006).

After SM challenge, birds from the Probiotic group showed reduced number of CD4+ cells in the ileum as compared to the other groups and a decrease in CD8+ cells in the cecum in relation to the Positive control. According to Van Immerseel *et al*. (2002), when specialized epithelial cells meet microorganisms, pro-inflammatory chemokines are rapidly released and they attract innate immune cells such as granulocytes and macrophages, capable of triggering a variety of new immune reactions such as the appearance of T-helper lymphocytes (CD4+ cells).

Accordingly the reduction of CD8+ cells in the cecum mucosa of birds from the Probiotic group, as compared to the Positive control, may be associated to the reduction of pathogenic bacteria in the intestinal lumen, as other studies (Scharek *et al*., 2005) have shown in relation to reduced E.coli counts. Thus, it is possible to speculate that another mechanism of action of probiotics, besides competitive exclusion (associated with the production of inhibitory compounds against pathogenic bacteria and competition by binding sites) may be related to the improvement of the mucosal immune response.

Conclusion: The use of *Enterococcus faecium* as probiotic efficiently increase goblet cells and specific immune response, showed by increase in CD4+ and CD8+ cells at seven days. It also reduced the count of *Salmonella* in cloaca and cecum swabs from challenged broiler chickens, as well as in the litter. At 35 days there is a reduction in the CD8+ cell, in agreement with the reduction of SM observed in cecum and litter. This can be associated with changes in the dynamics of immune cells infiltration into ileum and cecum mucosa as a response to such a challenge with *Salmonella*.

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