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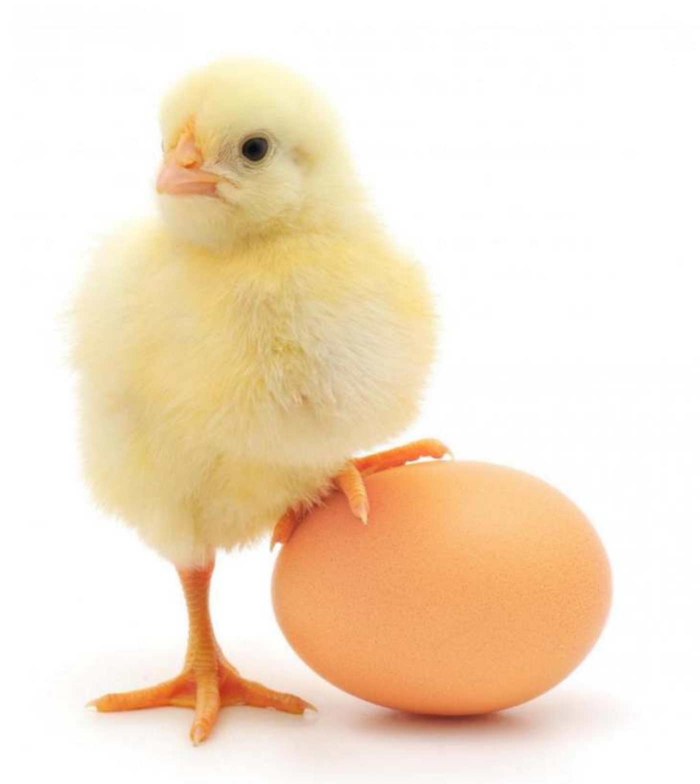

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Effects of *Bacillus subtilis* in the Dynamics of Infiltration of Immunological Cells in the Intestinal Mucosa of Chickens Challenged with *Salmonella* Minnesota

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Abstract: The use of *Bacillus subtilis* (BS) as a probiotic in bird feed was studied through the evaluation of its effect on the infiltration of immune cells in the ileum and cecum mucosa of chickens challenged with *Salmonella* Minnesota (SM). The birds were divided into three treatment groups; Negative control, containing unchallenged birds; Positive control, with SM challenged birds; and Probiotic, with SM challenged birds fed with a diet containing BS (DSM 17299 2.13 x 10^6 cfu/g). The birds fed BS showed increased goblet and CD4+ cell counts in the ileum and cecum before being challenged with SM in comparison to the birds not fed BS. After the SM challenge, the birds fed BS showed a reduction in the *Salmonella* counts at 48 Post Inoculation (PI) in the cloaca and cecum swabs and in litter samples and furthermore a reduction in CD8+ cells in the cecum compared to the challenged birds. Based on the results, it is concluded that feeding BS as a probiotic to broilers reduced the *Salmonella* spp. counts and thus, affected the mobilization of CD4+ and CD8+ cells in the ileum and cecum mucosa.

Key words: Microbiology, probiotic, mucosal immunity, *Salmonella* Minnesota

INTRODUCTION

The intestinal macrobiotic plays an important role in the maintenance of animal health. Beneficial microorganisms, as probiotic bacteria, have been shown to stimulate nonspecific host resistance to microbial pathogens (Perdigon *et al*., 1998). Thereby they aid in immune elimination and to modulate the host's immune responses to potentially harmful antigens with a potential to down-regulate hypersensitivity reactions (Isolauri *et al*., 2001). Components of the bacteria cell wall, e.g. peptidoglycans and glucopolyssacarides are important for the initial activation of the immune system. In addition, the intestinal microbiota is involved in the maintenance of the immunological tolerance because the presence of bacteria is required to keep the hyporresponsivity against antigens in the mucosa (Gaboriau-Routhiau and Moreau, 1996). Probiotics can control the enteric pathogens through a mechanism of competitive exclusion (Nurmi and Rantala, 1973), indirectly helping the inflammatory response modulation and the improvement of the nonspecific intestinal barrier (Maldonado Galdeano *et al*., 2007; Callaway *et al*., 2008).

Bacillus subtilis is a bacteria used as probiotic in poultry production that may improves the performance of birds (Fritts *et al*., 2000; Khaksefidi and Ghoorchi, 2006; Teo and Tan, 2007) and reduced some pathogenic bacteria (Fritts *et al*., 2000). In vitro studies demonstrate that BS cultures are able to inhibit the *Salmonella* Enteritidis invasion of intestinal epithelial cells (Thirabunyanon and Thongwittaya, 2012). It also improves the nonspecific (Teo and Tan, 2007; Lee *et al*., 2011) and the specific immune response of broilers (Khaksefidi and Ghoorchi, 2006).

CD4+ cells are able to produce cytokines in response to subsequent antigen stimulation and to express effector molecules that "help" B lymphocytes and macrophages, whereas CD8+ cells became capable of producing molecules that lyse other cells (Abbas, 2000).

Food toxinfections in humans caused by the consumption of meat and raw or poorly cooked eggs, contaminated with *Salmonella* sp., has significantly increased in the last 5 years (Callaway *et al*., 2008). The Enteritidis and Typhimurium are the most frequently isolated serovars, however, there is increasing concern about the Heidelberg, Senftenberg, Infantis and Minnesota. Voss-Rech *et al*. (2011) identified 20 serovars in a broiler study between 2009 and 2010 and found the Minnesota to be present in 37.93% of the analyzed samples with the highest prevalence of the serovars.

Considering probiotics to have potential beneficial effects in the control of *Salmonella* sp. and its

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interference in the immune response in birds, this study aimed to evaluate the efficiency of *Bacillus subtillis* (BS) probiotic in *Salmonella* Minnesota (SM) control in crop and cecum of broilers challenged with SM and immune cells infiltration of the intestinal mucosa of broilers.

MATERIALS AND METHODS

The current study was approved by the Committee for Ethics on the Use of Animals of the Veterinary Science of the Federal University of Parana (CEUA protocol number 034/2011).

Sixty one-day-old male Cobb® broilers were randomly divided into three treatments, in an entirely randomized experiment, each animal being a replicate. Birds from Negative control didn´t received SM inoculation and birds from Positive control and Probiotic group were inoculated orally with SM solution at 14 days. Birds from Probiotic group received feed with 50g/ton of BS probiotic from 1 to 35 days.

Birds were housed in separate but identical rooms for each treatment, located side by side, under negative pressure. Prior to the beginning of the experiment the rooms were disinfected and the floor covered with wood shavings previously autoclaved at 121°C for 15 min. Sterility tests were performed in the rooms, on the equipment and litter before initiating the experiment. Five additional animals were euthanized and necropsied prior to the experiment; the liver and cecum were collected and evaluated for the presence or absence of *Salmonella*.

The animals were kept at a comfort temperature for their age and received water and feed *ad libitum*. The experimental diets were pelleted and contained nutrient levels equal to or higher than the levels recommended by the NRC (1994).

The probiotic product GalliPro® (Chr. Hansen A/S) consisting of *Bacillus subtilis* (DSM17299) 2.06 x 10^8 cfu/g, was added to the feed at the concentration of 1000 g/t, according to the manufacturer's recommendations. After pelletizing, the microbiological recovery analysis of the feed showed 2.13 x 10^6 cfu/g.

At 14 days of age, the animals from Positive control and Probiotic group were orally inoculated with a SM solution at the concentration of 1x10^8 cfu/mL.

Five swab samples per treatment (*pool* of 3 animals) were collected from cloaca at 48 h Post Inoculation (PI) for analysis of *Salmonella* counts.

At 7 and 35 days of age (5 and 10 animals/treatment respectively), animals were aseptically euthanized and necropsied for the collection of the crop and cecum used in the *Salmonella* analysis. At 21 and 35 days of age, five litter samples were collected in aliquots of 10 g from each of the rooms, housing the animals (5 samples/treatment), for later *Salmonella* counts (Pickler *et al*., 2012).

The *Salmonella* counts were performed in swab samples from cloacae, crops and ceca and in litter samples. All samples were 10^{-1} serially diluted, beginning at 2% peptone water, followed by 0.1% peptone water (RM001, HiMedia Laboratories Pvt. Ltd., Mumbai, IN) until reaching a 10^{-3} dilution. Subsequently, 100 µL of each dilution were plated in duplicate on XLD media (CM469, Oxoid Limited, Hampshire, UK) using a sterile Drigalsky loop. The plates were incubated at 35°C for 24 h and used for the counting of typical colonies.

The initial solution of 2% peptone water was incubated at 35°C for 24 h. After incubation, the samples not showing growth of typical *Salmonella* colonies was added 100 µL of the initial solution to 10 mL of Rappaport-Vassiliadis broth (CM 669, Oxoid Limited, Hampshire, UK) and incubated at 42°C for 24 h for confirmation of negativity/positivity.

The results from the colony counting were expressed according to the Colony Count Procedure Protocol from Normative 62, published in August 26, 2003 (Brazil, 2003). The *Salmonella* colony counts were expressed in Log_{10} for the statistical analysis.

At seven and 35 days samples of five centimeters of ileum (two centimeters above the ileum-cecal junction) and cecum (end part of left cecum) of five birds from each treatment group at seven and 35 days of age. At seven days, the Negative control and Positive control were not inoculated with SM, due to it samples were taken from two birds from Negative control and three birds from Positive control to represent Negative control for histological and immunohistochemistry analysis.

Samples were placed in 10% buffered formalin and processed according to procedure (Smirnov *et al*., 2004) to analyze goblet cells. Briefly the slides were deparaffinized in heated xylene, rehydrated with alcohol and stained with Alcian Blue (stain goblet cells), hematoxylin and eosin.

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

The histological analyses and quantification of CD4+ and CD8+ cells in the intestinal epithelium were performed in light microscopy using an image analyzer system (Motic Image Plus 2.0 - Motic China Group

Co. 2006) coupled to a microscope (Olympus America INC., NY, USA). The quantification of Goblet cells and CD4+ and CD8+ cells was performed by field (100 X magnification) in the ileum and cecum fragments. Ten fields per slide were recorded.

The data was analyzed in the statistical program Statistic for Windows Copyright © 2008. The results were submitted to ANOVA and the PLSD Fisher's test at 5% probability.

RESULTS

The liver and cecum samples collected on the first day and crops and ceca samples collected at day 7 were negative for Salmonella sp. In the cloaca swabs (at 48 h PI) it was found that the isolation of *Salmonella* sp. (84.85%) was significantly reduced ($p \leq 0.05$) in the Probiotic group compared to the Positive control (Table 1).

The microbiological analysis showed significant difference in *Salmonella* counts in crops from 35 days old birds (Table 1) between the Probiotic group and the Positive control. A significant reduction in the *Salmonella* count was also observed in the cecum compared to the Positive control. The use of probiotic reduced *Salmonella* counts by 76.7% in the litter samples from 21 days old birds compared to the Positive control, however, in 35 days old birds the effect was less pronounced (20.9%).

Table 2 presents the results from goblet, CD4+, CD8+ cell counts and CD4+:CD8+ ratio in the ileum and cecum from broilers at 7 days of age. The birds fed the probiotic (Probiotic group) showed a significant increase in the goblet and CD4+ cell counts in the ileum and cecum sections compared to the Negative control. At day 7 no significant difference in the CD8+ cell count was found in the intestinal fragments analyzed. The CD4+:CD8+ ratio in the ileum shows that birds from Probiotic group exhibited higher expression of CD4+ cells in relation to the CD8+ cells than the Negative control; no difference was observed in the ileum.

In the histological assessment performed in the 35 days old birds after SM challenge (Table 2), the goblet, CD4+ and CD8+ cell counts did not show significant difference in the ileum samples between the treatments. However, the CD8+ cell counts were statistically higher in the cecum of birds from the Positive control than in the other groups. Regardless of the CD4+:CD8+ ratio showing higher expression of CD4+ cells than CD8+ cells, the birds from the Positive control showed a decrease in this proportion, related to the increase of CD8+, when compared to the other groups at 35 days of age.

DISCUSSION

In the present study, the significant increase of goblet cells in 7 days old animals fed the probiotic suggests that the presence of BS in the diet can interfere with the innate immune response by expression of goblet cells. The goblet cells are located in the intestinal villi and are responsible for maintaining the mucus layer, that acts as a physical and biological protection against pathogens and is therefore considered a part of the

Table 1: *Salmonella sp.* colony counting (log_{10} cfu/g) in cloaca, litter, crop and cecum samples from broilers in different treatment groups

Groups	Cloaca swabs 48h PI	Litter 21 days	Litter 35 days	Crop 35 days	Cecum 35 days
Negative control	0.00±0.00[b]	0.00±0.00[c]	0.00±0.00[c]	0.00±0.00[b]	0.00±0.00[c]
Positive control	3.95±2.24[a]	4.30±0.07[a]	3.60±0.22[a]	0.87±0.50[a]	4.30±4.28[a]
Probiotic	0.60±0.55[b]	1.00±0.00[b]	2.85±0.08[b]	0.70±0.48[b]	1.63±1.10[b]
p-value	0.002	0.001	0.001	0.025	0.001

[a-c]Different upper-case letters in the same column differ by PLSD Fischer's test with 95% level of confidence ($p<0.05$)

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

	Groups	Goblet cells	CD4+ cells	CD8+ cells	CD4+:CD8+ Ratio
7 days					
Ileum	Negative control	41.40±8.43[a]	4.30±3.71[b]	7.90±2.33	0.63±0.62[a]
	Probiotic	57.25±6.94[b]	8.80±4.60[b]	6.30±3.00	1.62±1.10[b]
	p-value	0.001	0.026	0.195	0.023
Cecum	Negative control	10.30±2.34[a]	8.30±3.30[a]	10.30±4.05	0.87±0.40
	Probiotic	15.70±2.54[b]	13.00±4.52[b]	11.80±3.58	1.22±0.63
	p-value	0.001	0.016	0.392	0.157
35 days					
Ileum	Negative control	64.50±14.95	16.20±6.50	6.90±4.80	3.80±3.10
	Positive control	60.95±12.76	15.50±5.60	11.40±6.35	1.90±1.75
	Probiotic	63.00±11.50	10.80±5.01	6.80±5.01	1.85±1.20
	p-value	0.694	0.091	0.068	0.092
Cecum	Negative control	10.85±6.09	19.60±6.09	11.50±3.56[a]	2.01±1.31[a]
	Positive control	13.70±5.36	23.70±8.35	21.90±5.68[b]	1.11±0.39[b]
	Probiotic	13.70±4.66	25.30±7.91	13.10±4.22[a]	2.04±0.69[a]
	p-value	0.165	0.235	0.001	0.043

[a-c]Different upper-case letters in the same column differ by PLSD Fischer's test with 95% level of confidence ($p<0.05$)

innate immune response and thus, regulated in response to inflammation and infection (Uni *et al.*, 2003).

A significant increase in the CD4+ cell counts of the ileum and cecum fragments was observed in 7 days old probiotic fed birds. According to van Immerseel *et al.* (2002), the encounter between specialized epithelial cells and microorganisms quickly stimulates the release of proinflammatory chemokines that attract innate immune cells e.g. granulocytes and macrophages, which are able to trigger a wide range of new immune responses as the emergence of T helper lymphocytes (CD4+ cells).

At 35 days, no statistically difference in the CD4+ cells counts was observed in the ileum and cecum samples from the different treatments. The CD4+ cells are linked to the initiation of specific immune responses, which are also responsible for the immune modulation but will not be related only by *Salmonella* challenge, other agents also could stimulate these cells even on non-challenged group. The CD4+:CD8+ cells ratio indicates higher presence of CD4+ cells in all groups when compared to the CD8+ cells. However, the Positive control presented the highest number of CD8+ cells in the cecum when compared to the other treatments, which can be the result of the increased presence of SM in the cecum in this group. Berndt and Methner (2001) also observed an increase in the amount of CD8+ cells in the cecum after a *Salmonella* infection.

The significant reduction in the *Salmonella* counts in the cecum of birds from the Probiotic group compared to the Positive control is in agreement with the results reported by Knap *et al.* (2011). The inhibitory effect of probiotics on the population of pathogenic enterobacteria by a competitive exclusion mechanism is well documented (Reid and Friendship, 2002; Callaway *et al.*, 2008). However, the relationship between the presence of probiotics in the diets and the immunological responses in birds was also observed in the present study. These results suggest that the effect of probiotics on the SM reduction can be associated with changes in the dynamics of the infiltration of immune cells in the intestinal mucosa of chickens.

Conclusion: The use a *Bacillus subtilis* based probiotic (DSM17299) was effective in the reduction of *Salmonella* counts in cloaca and cecum swabs from broilers challenged with SM. These results can be associated with changes in the dynamics of the infiltration of immune cells in the ileum and cecum mucosa in response to the SM challenge.

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