Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola
ISSN 1516-635X Jan - Mar 2017 / v.19 / n.1 / 019-026

http://dx.doi.org/10.1590/1806-9061-2016-0409

Evaluation of a Probiotic and a Competitive Exclusion Product Inoculated In Ovo on Broiler Chickens Challenged with Salmonella Heidelberg

■Author(s)

Silva IGO[I]
Vellano IHB[I]
Moraes AC[II]
Lee IM[II]
Alvarenga B[II]
Milbradt EL[I]
Hataka A[III]
Okamoto AS[I]
Andreatti Filho RL[I]

[I] Avian Pathology Laboratory, College of Veterinary Medicine and Animal Science (FMVZ), Department of Clinical Veterinary Medicine, Botucatu, SP, Brazil
[II] BioCamp Laboratories, Campinas, SP, Brazil
[III] Veterinary Pathology Laboratory, College of Veterinary Medicine and Animal Science (FMVZ), Department of Clinical Veterinary Medicine, Botucatu, SP, Brazil

■Mail Address

Corresponding author e-mail address
Isabella Goulart Oliveira da Silva
Rua Nove de Julho nº 45, Marapé, Santos, SP, Brazil
11070-150
Tel: (55 14) 998421401
Email: isabella.goliveira@hotmail.com

■Keywords

Chickens, Competitive exclusion, In-ovo inoculation, Probiotic, Salmonella Heidelberg.

Submitted: October/2016
Approved: November/2016

ABSTRACT

The present study evaluated a probiotic and a competitive exclusion product injected *in ovo* on day 18 of incubation together with Marek's disease vaccine in eggs of 56-week-old broiler breeders. Three experiments were carried out. The first trial evaluated the effect of treatments on hatchability, cecal colonization of *Salmonella* Heidelberg (SH), and intestinal mucosa immunity (immunoglobulin A levels in the intestinal fluid). The second trial evaluated the viability of the microorganisms in the products inoculated in a solution containing diluent and Marek's disease vaccine. The third trial evaluated the colonization of the cecal microbiota in non-challenged chickens during first four days of life by culturing cecal samples under aerobic and anaerobic conditions. Hatchability was not affected by the treatments. SH cecal counts were reduced in three-day-old broilers inoculated *in ovo* with the competitive exclusion product. Liver and spleen pool SH counts were not different among treatments. Broilers inoculated *in ovo* presented higher intestinal IgA titers 24 hours after SH challenge compared with the controls. When birds were not challenged, lower cecal microbial counts in aerobic culture were determined in the control group than in the probiotic group on day 3, and in the competitive exclusion group on day 2 when cultured in anaerobiosis. The products inoculated and diluted in the vaccine solution were viable at all analyzed periods when cultured in anaerobiosis. The results of this study suggest *in-ovo* inoculation is an effective route for the administration of the evaluated products, which effectively enhanced the broilers' immune response to a SH challenge, as shown by the increase in IgA titers, and the reduction in cecal *Salmonella* Heidelberg colonization with the *in-ovo* inoculation with the competitive exclusion product.

INTRODUCTION

Paratyphoid serovars of *Salmonella* spp. are known worldwide as the main agents responsible for foodborne diseases caused by the consumption of contaminated poultry meat products. Foley *et al*. (2011) suggests that vaccination against *Salmonella* Enteritidis and its suppression favor the emergence of other, more invasive, paratyphoid serovars. In Brazil, *Salmonella* Heidelberg has been identified as one of the most frequent serotypes involved in food poisoning outbreaks due to the consumption of egg and meat products (Kottwitz *et al*., 2010) contaminated during slaughter and processing, as identified by swab samples collected from the cloaca, scalding water, and the chiller (Colla *et al*., 2012). This serovar was the third main serovar isolated from swabs collected on commercial chicken farms in recent years (Voss-Rech *et al*., 2015) and it is considered an emergent serovar.

The ban on the use of antimicrobials at subtherapeutic doses as growth promotors by the European Union in 2006 motivated the search

for alternative treatments to improve the productivity and to reduce the incidence of enteropathogens in animal production. Examples are probiotics and competitive exclusion products, which have been applied to protect poultry against health challenges. Such products compete with pathogenic bacteria for nutrients and attachment sites on the intestinal mucosa, and produce antimicrobial compounds, such as bacteriocins and volatile fatty acids (Castillo *et al*., 2012), consequently favoring the establishment of beneficial intestinal bacteria. Moreover, this beneficial microbiota has an immuno modulating effect on the gut mucosa by activating macrophages, inducing cytokine production by the intraepithelial lymphocytes, and stimulating the intestinal production of immunoglobulin A (IgA), when birds are exposed to pathogens. IgA then attaches to the binding sites on the intestinal epithelium, establishing an immune barrier (Edens, 2003).

The in-feed treatment of poultry with probiotics and competitive exclusion products has been widely applied; however, the contamination of newly-hatched chicks by *Salmonella* spp. in the hatchery soon after hatching or during the transport to the farms indicates the need for the establishment of favorable bacteria in the gastrointestinal tract as early as possible.

Incubation represents a significant period of the lifespan of modern broilers. During embryonic development, intestinal villus length and crypt depth increase, and on day 17 of incubation, the gastrointestinal tract is physiologically complete, with fully active intestinal enzymes. The ingestion of amniotic fluid on day 18 of incubation is considered the first digestive experience of a broiler chick (Maiorka *et al*., 2002). At the end of the incubation period, the embryo requires energy for hatching, which is provided by glucose and protein breakdown, including of IgA (Rocha & Maiorka, 2013).

Newly-hatched chicks still do not have an established intestinal microbiota, making them highly susceptible to contamination by enteropathogens. Feeding probiotic bacteria to newly-hatched chicks has demonstrated several benefits, including reduced infection by *Salmonella* Enteritidis (Ávila *et al*., 2006). Furthermore, the earlier such treatment is administered, the greater its chances of being efficient.

The technique of *in-ovo* inoculation started to applied in the 1980s with the vaccination against Marek's disease. The first *in-ovo* inoculation study was published by Cox *et al*. (1992), who administered a competitive exclusion culture in broiler eggs on day 18 of incubation, and obtained protection against *Salmonella* Typhimurium in chickens challenged on hatching day. Later, Edens *et al*. (1997) evaluated the effect of *Lactobacillus reuteri* inoculated *in ovo* and the competitive exclusion mechanism of the bacteria. The *in-ovo* inoculation of *Lactobacillus casei*, *Lactobacillus plantarum*, and *Enterococcus faecium* reduced *Salmonella* Enteritidis was demonstrated to be more effective than the inclusion of the probiotic product in the feed to protect broilers challenged with *Salmonella* Thyphimurium (Leandro *et al*., 2010).

Based on that context, this study aimed at evaluating the effects of the *in-ovo* inoculation of a probiotic and a competitive exclusion product on day 18 of incubation, together with a Marek's disease vaccine, on the cecal microbiota of broilers challenged with *Salmonella* Heidelberg.

MATERIALS AND METHODS

Three experiments were performed using 480 eggs acquired from a commercial hatchery.

Experiment I evaluated the efficacy of products in birds challenged with *Salmonella* Heidelberg. In experiment II, the bacterial viability of the probiotic and the competitive exclusion products in a solution containing Marek's disease vaccine was determined. In experiment III, the cecal microbiota of chickens inoculated *in ovo* and not challenged was evaluated.

The experimental procedures were approved by the Committee of Ethics in Animal Experimentation of FMVZ/UNESP, under protocol n. 93/2015.

Experiment I

On day 18 of incubation, 480 eggs from a flock of 56-week-old COBB® broiler breeders were submitted to egg candling to determine the presence of live embryos. Eggs were then set in incubation trays, with a capacity of 96 eggs each, and were injected in the amniotic fluid, using Embrex® *in-ovo* vaccination equipment, according to the following treatments:

Group 1 - Chicken embryos were inoculated with a 0.05-mL solution, containing a vaccine against Marek's disease and a commercial probiotic product. The probiotic included 21 bacterial strains with the following guaranteed levels: one strain of *Bacillus subtilis* ($1.0x10^8$ CFU/g), six strains of *Enterococcus faecium* ($6.0x10^8$ CFU/g), one strain of *Lactobacillus acidophilus* ($1.0x10^8$ CFU/g), one strain of *Lactobacillus delbrueckii* ($1.0x10^8$ CFU/g), three strains of *Lactobacillus plantarum* ($3.0x10^8$ CFU/g), five strains

of *Lactobacillus reuteri* ($5.0x10^8$ CFU/g), one strain of *Lactobacillus salivarius* ($1.0x10^8$ CFU/g), and three strains of *Pediococcus acidilactici* ($3.0x10^8$ CFU/g).

Group 2 - Chicken embryos were inoculated with a 0.05-mL solution containing a vaccine against Marek's disease and a commercial competitive exclusion product, which included, according to the manufacturer, 10^6 CFU/g of total anaerobic bacteria and 10^6 CFU/g of lactic acid-producing bacteria.

Group 3 - Control treatment: Chicken embryos were inoculated with a solution containing only the vaccine against Marek's disease.

At hatch, 240 birds were transported to the Avian Pathology Laboratory of the School of Veterinary Medicine and Animal Science, UNESP, Botucatu campus, SP. Birds were randomly divided into three treatment groups (80 birds each) and housed in experimental cages, at a density of 10 birds per cage. Birds were offered water and feed *ad libitum* for 35 days. The feed did not contain any antimicrobials or growth promoters.

Hatchability

At hatch, the effect of treatments on the hatching rate was evaluated as the number of hatched live chick relative to the number of eggs set.

Bacterial quantification of the commercial products

The probiotic and the competitive exclusion products were diluted according to the manufacturer's recommendations in a solution containing the vaccine against Marek's disease and its diluent. A 10-mL aliquot of each product solution was stored in sterile collection tubes and maintained between 2 to 8ºC.

The inoculum of each product was quantified in successive serial decimal dilutions in phosphate-buffered saline solution (PBS), plated in Man Rogosa Sharpe (MRS) agar, and cultivated under aerobiosis and anaerobiosis. The plates were incubated at 41ºC for 24 hours to quantify lactic acid bacteria. The results are expressed in CFU/mL.

Preparation of the *Salmonella* Heidelberg inoculum and bird challenge

The utilized *Salmonella* Heidelberg strain was resistant to 100µg of nalidixic acid (Nal) and 100µg of rifampicin (Rif). The sample was cultured in 250 mL brain-heart infusion (BHI) broth and incubated at 37ºC for 24 hours. Colony-forming units (CFU) were counted after decimal dilution of the sample in phosphate-buffered saline solution (PBS) and plating on brilliant green agar (BGA) containing nalidixic acid (100µg/mL of medium) and rifampicin (100µg/mL of medium).

On day 2, chicks in all groups received 0.5 mL of the *Salmonella* Heidelberg inoculum, containing $6.8x10^5$ CFU/mL, by gavage using a sterile needle.

Quantification of *Salmonella* Heidelberg in the ceca and liver and spleen pool

At 3, 7, 14, 21 and 35 days of age, ten birds per treatment were euthanized by cervical dislocation and aseptically necropsied for collection of the ceca, liver, and spleen. Individual cecal samples and pooled liver and spleen samples were placed in sterile plastic collection bags. Samples were then macerated and diluted in PBS at the proportion of 1:10. Serial decimal dilutions were plated on BGA containing nalidixic acid (100µg/mL of medium) and rifampicin (100µg/mL of medium), incubated at 37ºC for 24 hours, and characteristic *Salmonella* Heidelberg colonies were counted. Results are expressed in CFU/mL.

Intestinal fluid collection and IgA titering

At 1, 3, 7, 14, 21 and 35 days of age, five chickens per treatment were euthanized and their intestines aseptically collected. Intestines were rinsed with a PBS solution containing 0.01% thimerosal, 1% bovine serum albumin, 1 mM phenylmethyl sulfonyl fluoride, and 5 mM ethylene-diamine tetra-acetic acid (EDTA), according to the description of Donato (2013), with the aid of a 2-mL syringe positioned in the duodenum to allow rinsing the entire gastrointestinal tract. The samples containing intestinal fluid were centrifuged at 1200 xg for 7 minutes, and the supernatant was collected and stored at -20ºC.

Immunoglobulin A (IgA) titers of the intestinal content were determined by enzyme-linked immunosorbent assay (ELISA) using commercial kits (Chicken IgA ELISA Quantitation and ELISA Starter Accessory Kit; Bethyl Laboratories, Montgomery, TX, USA), according to the manufacturer's instructions. Polystyrene plates with 96 flat-bottomed wells were sensitized by the addition of 100µL of affinity purified goat anti-chicken IgA coating antibody diluted in a solution of 0.005M bicarbonate-carbonate buffer at 1:100 to each well. After incubation for one hour at 25ºC, wells were washed five times with ELISA Wash Solution (50mM TRIS, 0.14M NaCl, 0.05% Tween 20, pH 8.0), after which 200µL/well of ELISA Blocking Solution was added (50mM TRIS, 0.14M NaCl, 1% BSA, pH 8.0) Plates were then incubated at 25°C for 30 minutes

and washed three times. Intestinal fluid samples were added at 100µL/well, followed by incubation at 25°C for 60 minutes. Plates were again washed five times in ELISA Wash Solution, and 100µL of diluted HRP detection antibody were added at 1:75.000 to each well. Plates were incubated at 25°C for 60 min and then washed five times. After this period, 100µL of TMB Substrate Solution were added to each well, and the plates were incubated in darkness at 25°C for 15 minutes. The reaction was stopped with 100µL/well of Stop Solution composed by H_2SO_4 (2M).

Titers were read using a spectrophotometer microplate reader at 450 ηm wavelength.

Salmonella Heidelberg selective enrichment

Cecal and liver and spleen pool samples that did not present any *Salmonella* Heidelberg growth when incubated on BGA were submitted to selective enrichment in Rappaport-Vassiliadis and Tetrathionate broths, and incubated at 41°C for 24 hours. After incubation, samples were plated on BGA and Xylose Lysine Deoxycholate agar (XLD) and incubated at 37°C for 24 hours, after which characteristic *Salmonella* Heidelberg colonies were counted. Results are expressed in CFU/mL.

Experiment II

The commercial probiotic and competitive exclusion products were individually diluted according to the manufacturer's recommendations in a diluent solution containing the vaccine against Marek's disease. The solutions were then submitted to successive serial decimal dilutions in PBS, plated in MRS and incubated at 41°C for 24 hours under anaerobiosis and aerobiosis immediately after preparation, and one, three and six hours later. After incubation, the viability of the bacteria present in the products at different hours was determined by bacterial counts, expressed in CFU/mL.

Experiment III

Eggs were submitted to the same treatments and injected according to the same methodology applied in experiment I. However, birds were not challenged with *Salmonella* Heidelberg. At hatch, 60 birds per treatment were selected, and housed for four days in experimental cages at the Avian Pathology Laboratory, School of Veterinary Medicine and Animal Science of UNESP, Botucatu, SP. Water and feed, with no addition of antimicrobials or growth promoters, were offered *ad libitum*.

On days 1, 2, 3, and 4, 10 birds per treatment were euthanized by cervical dislocation and aseptically necropsied for removal of the ceca. Samples were conditioned in sterile plastic collection bags, macerated, and diluted in PBS at 1:10. Colony-forming units (CFU) present in the cecal microbiota were quantified from serial decimal dilutions in PBS and plated on MRS agar. The plates were incubated under aerobic and anaerobic conditions at 41°C for 24 hours.

Statistical analysis

Hatchability results of experiment were statistically evaluated by Goodman Association Test for contrasts within binomial populations. *Salmonella* Heidelberg counts and ELISA results were subjected to analysis of variance, and means were compared by Tukey's test at a 5% significance level. The results of experiment III were submitted to analysis of variance according to a randomized experimental with two factors (collection times and treatments), and means were compared by Tukey's test at a 5% significance level.

RESULTS

Experiment I

The competitive exclusion inoculum presented $3.2x10^3$ CFU/embryo in the aerobic culture and $1.17x10^5$ CFU/embryo in the anaerobic culture. The probiotic inoculum presented $1.59x10^5$ CFU/embryo and $1.11x10^4$ CFU/embryo in the aerobic and anaerobic cultures, respectively.

Hatchability results are shown in Table 1. Despite the lack of statistical difference among treatments, the hatchability of the eggs inoculated with the competitive exclusion product (87.92%) and with the probiotic (86.25%) tended to be higher than that of the control group (85.20%).

Table 1 – Hatchability of eggs inoculated *in ovo* with a probiotic, a competitive exclusion product, or only with the vaccine.

Treatments	N. hatched eggs	N. unhatched eggs	Hatchability*	Total
Probiotic	414	66	86.25%	480
Competitive Exclusion	421	59	87.92%	480
Control	408	72	85.20%	480

*Number of incubated eggs /Number of chicks hatched.

Salmonella Heidelberg (SH) counts in the cecal contents are presented in Table 2. On day 3 post-hatch, 24 hours after the challenge, chicks from eggs inoculated with the competitive exclusion product

presented lower cecal SH counts (3 log_{10}CFU) than those inoculated with the probiotic and control eggs (3.7 and 4.7log_{10}CFU, respectively). SH cecal counts were not statistically different among treatments on days 7, 14, 21, and 35 days post-hatch. SH counts in the liver and spleen pool were not statistically different among the evaluated periods, and this bacterium was detected only on day 7, and in values below 2 log_{10}CFU in the three treatments.

Table 2 – Mean *Salmonella* Heidelberg (log_{10}CFU) counts in the cecal content.

Treatments	Age (days)				
	3	7	14	21	35
Control	4.7 b*	5.1	3.0	≤ 2	0
Competitive Exclusion	3.3 a	4.8	3.2	≤ 2	0
Probiotic	3.7 b	5.3	2.9	≤ 2	0

*Means followed by different lowercase letters in the same row statistically differ by Tukey's test (p<0.05).

The IgA titers obtained in the intestinal fluid, as measured by ELISA, are described in Table 3. On day 1 post-hatch, the control group presented higher IgA titers (428ŋg/mL) than the probiotic (264.1ŋg/mL) and the competitive exclusion (262.3ŋg/mL) groups. On day 3, 24 hours after the challenge with *Salmonella* Heidelberg, there was a considerable drop in intestinal IgA titers in the control group (45.5ŋg/mL), but not in the probiotic and competitive exclusion groups. IgA titers were not statistically different among treatments on days 7, 14, 21 or 35.

Table 3 – IgA titers in the intestinal fluid (ŋg/mL) of chickens before and after challenge with *Salmonella* Heidelberg.

Age (Days)	Treatments		
	Probiotic	Competitive Exclusion	Control
1	264.1	262.3	428
3	272.8 b*	230.6 b	45.5 a
7	194.2	148.9	199.4
14	1141.9	1513.2	1708.6
21	1480	1635.4	1110.8
35	993.5	892.3	1179.9

*Means followed by different lowercase letters in the same row statistically differ by Tukey's test (p<0.05).

The selective enrichment of the cecal samples revealed the presence of *Salmonella* Heidelberg in all groups, except on day 35, when all treatments were negative. Birds of the competitive exclusion group tended to show lower liver and spleen contamination from day 7 post-hatch (Table 4), whereas this effect was detected in the group inoculated with the probiotic *in ovo* later, from day 14.

Table 4 – Liver and spleen pool samples positive for *Salmonella* Heidelberg after selective enrichment.

Age	Probiotic		Competitive Exclusion		Control	
	Positive/ Total	%	Positive/ Total	%	Positive/ Total	%
3	7/9	77.7	4/9	44.4	8/8	100
7	3/3	100	2/4	50	*	*
14	1/6	16.6	1/6	16.6	2/7	28.5
21	0/10	0	2/10	20	3/10	30
35	0/10	0	0/10	0	0/10	0

*Samples that presented SH growth in the SH quantification test were not submitted to selective enrichment.

Experiment II

The bacterial viability results of the probiotic and competitive exclusion product solutions tested under aerobiosis and anaerobiosis are shown in Table 5. Under aerobiosis, bacterial growth was not detected in the solution containing the competitive exclusion product collected one to six hours after its preparation, whereas viable bacteria were present in the probiotic solution at all collection times. Bacterial viability remained constant in the probiotic and competitive exclusion solutions under anaerobiosis at all collection times.

Table 5 – Bacterial viability (log_{10} CFU) in the probiotic and competitive exclusion solutions collected between 0 and six hours after preparation and cultured under aerobiosis and anaerobiosis.

Treatments	Aerobiosis				Anaerobiosis			
	0h	1h	3h	6h	0h	1h	3h	6h
Probiotic	5.09	5.05	5.64	4.95	5.71	5.65	5.79	5.61
Competitive Exclusion	4.83	-	-	-	5.25	5.25	5.05	5.41

Experiment III

On day 3 post-hatch, bacterial counts were statistically different among treatments when cecal samples were cultured under aerobiosis (Table 6), with higher counts in the chicks injected *in ovo* with the control solution compared with those injected with the probiotic solution, whereas those injected with competitive exclusion solution presented intermediate values. No statistical differences among treatments were detected on days 1,2, and 4 post-hatch.

Table 6 – Mean bacterial counts (log_{10} CFU) in the cecal samples cultured in aerobic conditions.

Treatments	Broiler age (days)			
	1	2	3	4
Control	7.31 A*	8.32 B	8.27 b,B	7.42A
Competitive Exclusion	7.45 A	8.45 B	7.94 ab,AB	7.39 A
Probiotic	7.65 AB	8.18 B	7.49 a,A	7.22 A

* Means followed by the same uppercase letters in the same row or lowercase letters in the same column are not statistically different by Tukey's test (p>0.05).

When cultured under anaerobiosis (Table 7), lower cecal bacterial counts ($p<0.05$) were detected on day 2 post-hatch in the chicks injected *in ovo* with the competitive exclusion solution compared with those injected with the probiotic solution, whereas the control birds presented intermediate counts. No statistical differences were detected on days 1, 3, and 4 post-hatch.

Table 7 – Mean bacterial counts (log_{10} CFU) in the cecal samples cultured in anaerobic conditions.

Treatments	Broiler age (days)			
	1	2	3	4
Control	6.73	7.24 ab*	7.15	6.74
Competitive Exclusion	7.22	6.72 a	7.10	6.67
Probiotic	6.94 AB	7.29 b,B	7.22 B	6.41 A

*Means followed by the same uppercase letters in the same row or lowercase letters in the same column are not statistically different by Tukey's test ($p>0.05$).

DISCUSSION

The treatment of poultry with probiotics as an alternative to antimicrobials has been widely used for the establishment of a healthy intestinal microbiota and to prevent *Salmonella enterica* infections. The first report on a competitive exclusion product administrated *in ovo* was published by Cox *et al.* (1992), who demonstrated that a bacterial culture diluted in a Marek's disease vaccine was able to reduce the colonization of the ceca by *Salmonella* Typhimurim. Edens *et al.* (1997) studied the principles of competitive exclusion by injecting *Lactobacillus reuteri* into the air cell or amniotic fluid of broiler embryos. Those authors did not detect any effects on hatchability; however, the cecal colonization of *Salmonella* Typhimurium was reduced.

The present study assessed the potential applicability of the *in-ovo* inoculation of a competitive exclusion product and of a probiotic on day 18 of incubation of broiler embryos, their protective effects against *Salmonella* Heidelberg post-hatch, product viability as a function of time, and their influence on the modulation of the cecal microbiota during the first days of life.

The results of experiment I showed that, although hatchability was not statistically affected by the treatments ($p>0.05$), the numbers of chicks alive at hatch tended to be higher in the competitive exclusion and the probiotic groups than in the control group. The hatchability obtained in competitive exclusion group (87.92%) in the present study was higher than that reported by Meijerhof & Hulet (1997) with the *in-ovo* inoculation of a competitive exclusion product (84.9%). The 86.25% hatchability obtained in the eggs inoculated with the probiotic product is consistent with the value of 83% observed by Yamawaki *et al*. (2013), who inoculated probiotic strains in the air chamber of broiler chicks on day 18 of incubation. On the other hand, Andreatti Filho *et al*. (2006) inoculated *Lactobacillus salivarius* or an undefined cecal microbiota in an air chamber of broiler chicks, but observed only 60% and 55% hatchability, respectively.

In the present study, cecal *Salmonella* Heidelberg counts were reduced in 3-d-old chicks inoculated *in ovo* with the competitive exclusion product (Table 2). This result is in contrast with those of Andreatti Filho *et al*. (2006), who did not find any reduction in the cecal colonization with *Salmonella* Enteritidis in broilers inoculated during incubation with cecal microbiota. These differences may be explained by the fact that the microbiota composition of the competitive exclusion product may allow greater interaction with the host and more extensive association among the different bacteria of the microbiota (Mead, 2000). On the other hand, the results obtained here with the probiotic inoculation are consistent with the findings of Yamawaki *et al*. (2013), who observed that the *in-ovo* inoculation of broiler embryos with *Lactobacillus acidophilus*, *Lactobacillus fermentum*, and *Lactobacillus salivarius* did not reduce cecal *Salmonella* Enteritidis counts five days post-hatch. Martins (2015) inoculated broiler embryos on day 18 of incubation with the same commercial probiotic product employed in the present study and challenged the chicks at hatch with *Salmonella* Enteritidis. Those authors did not detect any reduction cecal *Salmonella* Enteritidis counts in the challenged birds, as also observed with the challenge with the serovar Heidelberg in the current experiment. Leandro *et al*. (2010) demonstrated that the *in-ovo* administration of probiotic strains is more efficient in reducing *Salmonella* Enteritidis cecal colonization than their in-feed administration after challenge, which effects are detected later, at 21 days of age. There was no effect of treatments on *Salmonella* Heidelberg counts in the liver and spleen pool. In contrast, Andreatti Filho *et al*. (2006), inoculating broiler embryos with cecal microbiota or a probiotic (*Lactobacillus salivarius*), did not observe any *Salmonella* Enteritidis growth in the liver of the group inoculated with the probiotic five days after the challenge.

The immuno modulatory effects of the treatments were evaluated as a function of intestinal IgA titers.

Higher IgA titers were measured in the chicks inoculated *in ovo* with the probiotic and the competitive exclusion solutions compared with the control group 24 hours post-challenge, suggesting these products enhanced the immune status of these birds. We did not find any literature reports on the stimulation of secretory IgA by *in-ovo* inoculation of broilers. Gut-associated lymphoid tissue (GALT) matures when broilers are about two weeks old; however, Haguighi *et al* (2006) reported that the administration of probiotics to broiler chickens during the first days of life may promote earlier GALT maturity as a result of the release of IgA.

Salmonella Heidelberg counts in the liver and spleen pool obtained after selective enrichment suggests that the *in-ovo* inoculation with the competitive exclusion product reduced the systemic infection earlier compared with the probiotic product. In addition, the selective enrichment method demonstrated to be effective to detect small quantities of that bacterium.

There are few studies on the viability of the commercial of probiotic and competitive exclusion products, as evaluated by bacterial counts at different times, highlighting the need of further research on this subject. Menconi *et al*. (2014), evaluating two samples of lactic acid bacteria present in a commercial probiotic and incubated for two and four hours, respectively, at temperatures of 15ºC and 45ºC, detected growth under microaerophilic conditions. In the present study, the bacteria in the commercial probiotic product maintained at 25ºC grew under both aerobiosis and anaerobiosis up to six hours of incubation. The absence of growth of microorganisms in the competitive exclusion product under anaerobiosis from one hour of incubation may be explained by the presence of strictly-anaerobic bacteria and the weak interaction with oxygen of facultative anaerobic strains present in that product.

The cecal microbiota of broilers inoculated *in ovo* with the evaluated products and the vaccine and not challenged (Experiment III) was not significantly influenced by the treatments during the first four days of life. However, intestinal microbiota colonization starts to stabilize at a later age (Stanley *et al*., 2014), which was not evaluated in the present study. Although bacterial counts were not different among treatments, molecular techniques were not applied in this study to allow comparing the effects of treatments on cecal bacterial profile. The similar microbiota bacterial counts among treatments, including the control group, determined in the present study are consistent the results of Borges (2015), who identified the *in-ovo* inoculated *Bacillus subtillis* strain in the cecal content of all groups studied. Comparing different routes of administration, Gérard *et al*. (2008) observed that the supply of *Lactobacillus* spp. via drinking water did not influence the cecal bacterial counts of 4-d-old broilers. Mountzouris *et al*. (2010) found that the addition of probiotic strains to broiler diets influenced cecal microbiota counts only at the end of the rearing period.

There are few studies on the use of probiotics and their *in-ovo* inoculation in broilers, which warrants further research on several aspects, such as protection against *Salmonella* infection and time required for the solutions to colonize cecal microbiota.

CONCLUSION

The *in-ovo* inoculation of broiler embryos with the evaluated competitive exclusion product reduced the presence of *Salmonella* Heidelberg in the cecal content of broilers post-hatch. The immuno modulatory effect of the tested products was demonstrated by the increase in IgA titers in the intestinal fluid of the birds challenged with *Salmonella* Heidelberg. The growth of the bacteria several hours after the inoculation of the solutions of the evaluated commercial products were prepared demonstrated that the products remained viable. The results of this study show that the microorganisms present in the evaluated commercial probiotic and competitive exclusion products inoculated *in ovo* on day 18 of incubation are capable of colonizing the ceca of broilers until four days post-hatch.

ACKNOWLEDGMENTS

The authors thank the company BioCamp Laboratories for supplying the probiotic and competitive exclusion products.

REFERENCES

Andreatti Filho RL, Okamoto AS, Lima ET, Gratão PR, Delbem SR. Effect of cecal microflora and *Lactobacillus salivarius in ovo* administration used on chicken previously challenged with *Salmonella enterica* serovar Enteritidis. Arquivo Brasileiro de MedicinaVeterinária e Zootecnia 2006;58(4):467-471.

Ávila LAF, Nascimento VP, Salle CTP, Moraes HLS. Effects of probiotics and maternal vaccination on *Salmonella* Enteritidis infection in broiler chicks. Avian Diseases 2006;50(4):608-612.

Borges, LL. *Bacillus subtilis* na qualidade dos pintinhos ao nascer, desempenho e características instestinais dos frangos [thesis]. Jaboticabal (SP): Universidade Estadual Paulista; 2015.

Castillo NA, Le Blanc AM, Galdeano CM, Perdigón G. Probiotics: an alternative strategy for combating Salmonellosis. Immune mechanisms involved. Food Research International 2012;45(2):831-841.

Colla FL, Rodrigues LB, Dickel EL, Nascimento VP, Santos LR. *Salmonella* Heidelberg isolated at different points of the broiler slaughter house. Arquivos do Instituto Biológico 2012;79(4):603-606.

Cox NA, Bailey JS, Blankenship LC, Gildersleeve RP. Research note: in ovo administration of a competitive exclusion culture treatment to broiler embryos. Poultry Science 1992;71(10):1781-1784.

Donato TC. Ação da serotonina associada à *Lactobacillus* spp. Na resposta imune e morfometria intestinal de frangos de corte (*Gallus gallus domesticus*– Linnaeus 1758) desafiados com *Salmonella* Enteritids [dissertation]. Botucatu (SP): Universidade Estadua Paulista; 2013.

Edens FW, Parkhurst CR, Casas A, Dobrogosz WL. Principles of Ex Ovo Competitive Exclusion and In Ovo Administration of *Lactobacillus reuteri*. Poultry Science 1997;76(1):179-196.

Edens FW. An alternative for antibiotic use in poultry:Probiotics. Revista Brasileira de Ciência Avícola 2003;5(2):75-97.

Foley SL, Nayak R, Hanning IB, Johnson TJ, Han J, Ricke SC. Population dynamics of *Salmonella enteric* serotypes in commercial egg and poultry production. Applied and Environmental Microbiology 2011;77(13):4273-4279.

Gérard P, Brézillon C, Quére F, Salmon A, Rabot S. Characterization of cecal microbiota and response to an orally administered *Lactobacillus* probiotic strain in the broiler chicken. Journal of Molecular Microbiology and Biotechnology 2008;14(1-3):115-122.

Haghighi HR, Gong J, Gyles CL, Hayes MA, Zhou H, Sanei B, et al. Probiotics stimulate production of natural antibodies in chickens. Clinical and Vaccine Immunology 2006;13(9):975-980.

Kottwitz LBM, Oliveira TCRM, Alcocer I, Farah SMSS, Abrahão WSM, Rodrigues, DPR. Epidemiology of Salmonellosis outbreaks occured in the period of 1999-2008 in the state of Paraná, Brazil. Acta Scientiarum Health Sciences 2010;32(1):9-15.

Leandro NSM, Oliveira ASC, Gonzales E, Café MB, Stringhini JH, Andrade MS. Probiotic in diet or inoculated in fertilized eggs. Performance of broiler chicks challenged with *Salmonella* Enteritidis. Revista Brasileira de Zootecnia 2010;39(7):2509-1516.

Maiorka A, Boleli Ic, Macari M. Desenvolvimento e reparo da mucosa intestinal. In: Macari M, Furlan RL, Gonzalez E, editors. Fisiologia aviária aplicada a frangos de corte. 2nd ed. Jaboticabal: FUNEP/UNESP; 2002. p.113-126.

MARTINS BB. Efeito da inoculação de probiótico *in ovo* sobre a morfometria intestinal e controle de *Salmonella* Enteritidis [dissertation]. Botucatu (SP): Universidade Estadual Paulista; 2015.

MEAD GC. Prospects for "competitive exclusion" treatment to control salmonellas and other foodborne pathogens in poultry. The Veterinary Journal 2000;159(2):111-123.

Meijerhof R, Hulet RM. In ovo injection of competitive exclusion culture in broiler hatching eggs. The Journal of Applied Poultry Research 1997;6(3):260-266.

Menconi A, Kallapura G, Latorre JD, Morgan MJ, Pumford NR, et al. Identification and characterization of latic acid bacteria in a commercial probiotic culture. Bioscience of Microbiota Food and Health 2014;33(1):25-30.

Mountzouris KC, Tsitrsikos P, Palamidi I, Arvaniti A, Mohnl M, Schatzmayr G, et al. Effects of probiotic inclusion levels in broiler nutrition on growth performance, nutrient digestibility, plasma immunoglobulins, and cecal microflora composition. Poultry Science 2010;89(1):58-67.

Rocha C, Maiorka A. Nutrição *in ovo*. In: Macari M, Gonzalez E, Patrício IS, Naas IA, Martins PC. Manejo da incubação. 3rd ed. Campinas: FACTA; 2013. p.223-239.

Stanley D, Hughes RJ, Moore RJ. Microbiota of the chicken gastrointestinal tract, productivity and disease. Applied Microbiology and Biotechnology 2014;98(10):4301-4310.

Voss-Rech D, Vaz CSL, Alves L, Coldebella A, Leão JA, Rodrigues DD, Back A. A temporal study of *Salmonella enterica* serotypes from broiler farms in Brazil. Poultry Science 2015;94(3):433-441.

Yamawaki RA, Milbradt EL, Coppola MP, Rodrigues JCZ, Andreatti Filho RL, Padovani CR, et al. Effect of immersion and inoculation in ovo of *Lactobacillus* spp. in embryonated chicken eggs in the prevention of *Salmonella* Enteritidis after hatch. Poultry Science 2013;92(6):1560-1563.