Induction of immune response in broiler chickens immunized with recombinant FliC and challenged by *Salmonella* Typhimurium[1]

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ABSTRACT.- Baptista A.A.S., Donato T.C., Souza, E.E., Gonçalves G.A.M., Garcia, K.C.O.D., Rodrigues J.C.Z., Sequeira J.L. & Andreatti Filho R.L. 2013. **Induction of immune response in broiler chickens immunized with recombinant FliC and challenged by *Salmonella* Typhimurium.** *Pesquisa Veterinária Brasileira 33(10):1215-1221.* Departamento de Clínica Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista Júlio de Mesquita Filho, Unesp-Botucatu, Distrito de Rubião Júnior s/n, Botucatu, SP 18608-970, Brazil. E-mail: angelita_sampaio@yahoo.com.br

The study examined (1) the immune response in broiler chickens after oral immunization with recombinant flagellin (rFliC) from *Salmonella* Typhimurium conjugated with sodium alginate microparticles, and the immune response enhancement in association with recombinant cholera toxin B subunit protein (rCTB) and *pool* of *Lactobacillus* spp. (PL). The immune responses were evaluated by dosage of IgY serum and IgA from intestinal fluid and immunostaining of CD8+ T lymphocytes in the cecum. The immunized animals were challenged with Salmonella Typhimurium (ST) 21 days after treatment. In all immunized groups, a significant increase ($p < 0.05$) was observed in IgA levels (µg/mL), especially three weeks after immunization. The serum IgY levels (µg/mL) were little affected by the treatments and differed significantly among groups only in the second post-immunization week ($p < 0.05$). After the challenge, the number of CD8+ T cells differed significantly between the treatments and negative control. Retrieval of *Salmonella* Typhimurium was not detected at 48 hours after the challenge in T2 (rFliC+rCTb), T3 (rFliC+PL) and T4 (rFliC+rCTB PL). The rFliC administered orally with or without rCTB and *Lactobacillus* spp. produces significant induction of humoral immune response, and the immunized chickens were more effective in eliminating *Salmonella* after challenge.

INDEX TERMS: *Salmonella* Typhimurium, FliC, flagellin, immune response, broiler chickens.

RESUMO.- [**Indução de resposta imune em frangos de corte imunizados com FliC recombinante e desafiados por *Salmonella* Typhimurium.**] Este estudo investigou a resposta imunitária de frangos de corte após a imunização oral com flagelina recombinante (rFliC) de *Salmonella* Typhimurium conjugada com micropartículas de alginato de sódio, e como intensificador de resposta imune foi associada a proteína subunidade B da toxina colérica (rCTB) e *pool* de *Lactobacillus* spp. (PL). As respostas imunes foram avaliadas por dosagem de IgY sérica e IgA do fluído intestinal e imunomarcação de linfócitos T CD8+ presentes no ceco. Os animais imunizados foram desafiados aos 21 dias após tratamento com *Salmonella* Typhimurium (ST). Foi observado em todos os grupos imunizados um aumento significativo ($p < 0,05$) nos níveis de IgA (µg/mL) principalmente três semanas após as imunizações. Os níveis de IgY sérica (µg/mL) foram pouco influenciados pelos tratamentos, apenas na segunda semana após imunização obser-

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vou-se diferenças significativas (p<0,05) entre os grupos. Observou-se que o número de linfócitos T CD8⁺ apresentou diferença significativa entre os tratamentos e o controle negativo após o desafio. Quanto a recuperação de *Salmonella* Typhimurium, observou-se que 48 horas após o desafio já não havia detecção do agente nos grupos T2 (rFliC+rCTb), T3 (rFliC+PL) e T4 (rFliC+rCTB+PL). Concluí-se que rFliC administrada, via oral, associada ou não a *Lactobacillus* spp e rCTB, demonstrou induzir significativamente a resposta imune humoral e que as aves imunizadas foram mais eficientes na eliminação de *Salmonella* após desafio.

TERMOS DE INDEXAÇÃO: *Salmonella* Typhimurium, FliC, flagelina, resposta imune, frangos de corte.

INTRODUCTION

Salmonellosis is a relevant foodborne disease occurring throughout the world. *Salmonella* Enteritidis and *S.* Typhimurium represent the principle serovars associated with foodborne infection in humans (Berndt & Methner 2001, Calenge et al. 2010).

Salmonella infects humans and other animals by the oral route through contaminated food or water (López et al. 2012). A large variety of food products, especially eggs and broiler meat, represent the main infection sources of *Salmonella* for humans (Orji et al. 2005, Bernadt et al. 2007). Therefore, the ultimate objective of developing measures to prevent and control salmonellas in chickens is to prevent these microorganisms from entering the food chain (Bernadt et al. 2007).

The implementation of biosecurity and the use of prebiotics, probiotics, bacteriophages and vaccines (Chambers & Gong 2011) are among the measures adopted by the aviculture industry to prevent and control salmonellosis.

The protection against colonization by enteric pathogens is highly dependent on secretory IgA and on cellular immune response mediated by cytotoxic T cells (Vandeplas et al. 2010).

Studies show that flagellin (FliC), the main structural component of flagella, is capable of interacting with the innate immune system of the host via *Toll-like* receptor 5 (TLR5) triggering immune responses (Cunningham et al. 2004, Alexan et al. 2009). Recently, this protein has been described as a candidate for a subunit vaccine against *Salmonella* (Toyota-Hanatani et al. 2008, 2009, Okamura et al. 2012).

The recognition of FliC via TLR5, expressed in epithelial cells and monocytes, for example, induces the secretion of pro-inflammatory mediators, such as IL-6, TNF-α, IL-1β, as well as the anti-inflammatory cytokine IL-10 (Bedoui et al. 2012). The oral administration of flagellin induces a type Th2 immune response causes an increase mucosal and systemic IgA levels (Chaung et al. 2012).

In light of the immunostimulatory characteristics of FliC, the current study evaluated the immune response of commercial chickens immunized with recombinant FliC in association with intensifiers of immune response, the B subunit of cholera toxin (rCTB) and strains of *Lactobacillus* spp.

MATERIALS AND METHODS

Bacterial samples and culturing conditions

The strains of *Lactobacillus* spp. used in this study were isolated from chicken and selected according to their adhesion capacity and effect as described by Rocha et al. (2012). The strains were cultured separately in 15 mL of Man Rogosa Sharp (MRS) liquid medium, incubated at 37° C, for 24h in an anaerobic system. On the following day, the cultures were mixed in equal parts and administered to the birds.

The challenge was accomplished through a culture of *Salmonella* Typhimurium, a mutant resistant to nalidixic acid (Nalr) and rifampicin (Rifr). For this, *S.* Typhimurium was cultured in 20 mL of *brain heart infusion* (BHI) liquid medium, containing Nal and Rif (100μg/mL) and incubated at 40°C, for 18-24 h. Subsequently, the culture was diluted 100 times in BHI broth and orally administered to the birds with the aid of a sterile plastic pipette. The number of colony-forming units (CFU) was determined through decimal dilutions in PBS (pH 7.2) and plated in brilliant green agar (BGA).

Recombinant proteins

rCTB. The expression of the recombinant CTB protein (rCTB) was carried out in a strain of *Escherichia coli* BL21(SI), which was transformed with the plasmid pAE/ctb (generously donated by Dr. Paulo Lee Ho). Next, recombinant colonies were selected and incubated at 30°C in LB broth added with ampicillin (100 μg/mL), until reaching the optical density (OD$_{600}$) of 0.6. Thereafter, 300mM of NaCl was added to induce expression, and the product was incubated again at 30°C under shaking (180rpm). The rCTB expression was verified by electrophoresis in 12% polyacrylamide gel (SDS-PAGE). After confirmation of recombinant protein expression, the culturing and large-scale induction were accomplished.

The rCTB protein was expressed as inclusion corpuscles; therefore, the rCTB was purified using the *refolding* protocol as described by Arêas et al. (2002).

To confirm the expression of the rCTB protein in pentameric (native) and monomeric form, the *western blotting* assay was performed using the purified and renatured (*refolding)* protein. For this, the rCTB protein was submitted to SDS-PAGE (12%) and subsequently transferred to a nitrocellulose membrane (Sigma, 0.2um). The result was blocked for one hour in a blocking buffer (PBS+0.1% Tween 20+5% skim powdered milk) and incubated for one more hour with the primary antibody *rabbit anti-Cholera toxin* (Sigma) at a 1:10.000 dilution, which was submitted to five washings (5 min) with PBS solution added with 0.05% Tween 20, and again incubated (1h) with secondary antibody stained with peroxidase (anti-*rabbit*), 1:2.500 dilution. After incubation, five washings of 5 min were performed as previously described and the system was revealed with DAB (DAKO), according to the manufacturer's recommendations.

Flagellin- rFliC. Cloning -The gene fliC from Salmonella Typhimurium was amplified by PCR, using the primers Forw 5'CGCCATATGGCACAAGTCATTAATACAAAC3' and Rev. 5'CCGCTCGAGTTAACGCAGTAAAGAGAGGA 3', which were designed based on the complete sequence of the Flic gene deposited in the geneBank (Nº of Access NC_003197). Restriction sites were added for XhoI and NdeI for subsequent sub-cloning in expression vectors. The PCR reaction was carried out using Platinum Taq DNA Polymerase High Fidelity (Invitrogen), according to the manufacturer's protocol; the reactions were incubated in a thermal cycler (Mastercycler Gradiente, Eppendorf) under the following conditions: 95°C for 2 min; 94°C for 30 sec.; 62°C for 30 sec.; 72°C for 1 min - repeating 30 times and final extension at 72°C for 5 min.

After amplification, the PCR product was visualized in 1% agarose gel and purified with a Qiaquick PCR purification kit (Qiagen), following the manufacturer's protocol. The PCR product was cloned in pGEM T-easy (Promega) and sub-cloned in the vector pET28b (Novagem) using the sites *Xho*I and *Nde*I.

Expression and purification. To induce the expression, *E.coli* BL21(DE) pLySs was transformed with the plasmid pET28b/*fliC*. The recombinant colonies were selected and cultured in LB broth added with kanamycin (30μg/mL) and chloranphenicol (50μg/ml) at 37°C until reaching the optical density (OD_{600}) of 0.6. For induction, 1mM of IPTG was added and the culture was incubated at 37°C under shaking (180 rpm). Aliquots were withdrawn hourly to select the best induction time. The rFliC expression was verified in SDS-PAGE (15%). After the expression of recombinant protein was confirmed, the culture was carried out on a large scale.

The rFliC protein was expressed in a soluble form and purified under native conditions by Ni-NTA immobilized-metal affinity chromatography (QIAGEN - Integrated Solutions for the Life Sciences), according to the manufacturer's protocol.

Protein expression was confirmed by the western blotting assay, similarly to the description for rCTB; however, employing the primary antibody anti-FliC (Flagellin, BioLegend) at 1:5.000 dilution ratio and secondary antibody stained with peroxidase (anti-mouse) at 1:2.500 dilution ratio.

After protein purification, the protocol described by Aida et al. (1990) was adopted to remove the endotoxins.

Microencapsulation of the recombinant proteins

The purified proteins were microencapsulated to avoid possible degradation of the initial portion from the digestive tract. To this end, the protein solution was emulsified in soybean oil containing 5% sorbitan monooleate. Next, the emulsion was re-emulsified in a sodium alginate solution (1%) and subsequently the double emulsion was dripped into a calcium chloride solution (1%), and maintained at -20°C until the moment of use. A pilot assay was performed with the microcapsules to verify integrity along the length of the digestive tract.

Experimental design

The procedures performed with the animals were approved by Ethics Committee on the use of animals from College of Veterinary Medicine and Animal Science, UNESP (nº 71/2009-CEUA).

The one-day-old commercial chickens (210) of Ross lineage were housed in experimental cages in the Ornithopathology Infectorium at College of Veterinary Medicine and Animal Science, Unesp, where they received water and non-medicated ration *ad libitum* and necessary heating according to age. The birds were divided into seven groups composed of 30 animals each. The treatments are described in Table 1.

Table 1. Description of treatments and immunization scheme of chickens with recombinant proteins (FliC, CTB) and *Lactobacillus* spp. Pool (PL)

Treatments	Dose of proteins/ PL Duration [3d]	Age of animals 1st dose/*booster***
T1 - rFliC	50 μg	5-7/21
T2 - rFliC + rCTB	50 μg	5-7/21
T3 - rFliC + *PL**	500 μl/ 50 μg	5-7/21
T4 - rFliC + rCTB + PL	500 μl (10^9)	2-4
T5 - *Salmonella* Typhimurium	-	-
T6 - Microcapsules*	50 μg	5-7/21
T7 - Negative control	-	-

*Microcapsules prepared with PBS. **booster* - single dose of protein (50 μg) administered 15 days after the first immunization. Challenge with *Salmonella* Typhimurium was performed 21 days after the treatments.

On days two, three and four, the animals in treatment T4 received 500μL (10^9CFU/mL) of *pool* of *Lactobacillus* spp. by oral route, with the aid of a gavage needle. Between the fifth and seventh day of life, the animals in treatments T1, T2, T3 and T6 received the encapsulated recombinant proteins orally, with the aid of a plastic spatula. Next, they were supplied 500 μL of carbonated buffer (0.3M $NaHCO_3$; pH 8.0) by means of a gavage needle. One reinforcement dose of protein, according to the group, was administered after 15 days. The animals remained fasting 2h before and 2h after the treatments.

The challenge was applied 21 days after the treatments, with an inoculum of $2x10^5$ CFU/ mL of *Salmonella* Typhimurium (*Nalr and Rifr*).

Serological monitoring

On days zero, seven, 14, 21, 22, 23, 26 after the treatments, blood was collected from birds, by means of venous or jugular puncture using sterile needles and syringes. The blood samples were centrifuged at 8000 x *g* for 5 min. The serum obtained was maintained at -20° C until analysis to determine IgY.

Collection of intestinal fluid

Soon after blood collection, five birds from each group were euthanized by cervical dislocation. The intestines were collected and, with the aid of a sterile disposable syringe, 2mL of washing solution (PBS pH 7.0 added with BSA (1%), thymerosal (0.01%), 5mM of EDTA and 1 mM of phenylmethyl sulfonyl fluoride – PMSF) were injected into the proximal portion of the duodenum to ensure that the entire length of the intestine would be washed. The fluid collected was centrifuged at 1200x*g* for 7 min. The resultant supernatant was retrieved and stored at -20ºC for subsequent quantification of secretory IgA.

Collections of ceca for re-isolation of *Salmonella* Typhimurium

For the bacterial determinations of the cecal content, the ceca were collected from all birds and placed individually in sterile plastic collection bags. Next, they were macerated and diluted in PBS (pH 7.2) at 1:10 ratio. Subsequently, dilutions were performed in series, and plated in BGA added with 100μg/mL of Nal and Rif. The reading of the plates was carried out 18-24 hours after incubation.

All the cecal samples were cultured in tetrathionate broth (100μg/mL of Nal and Rif) at 1:10 ratio, incubated at 40°C for 18-24 hours. Subsequently, they were plated in BGA medium (100μg/ mL of Nal and Rif).

Cecal collections for immunohistochemistry assays

One-centimeter portions were collected from ceca. The fragments were opened with a longitudinal cut, deposited in histological cassettes and fixed in buffered formol (10%) for 24h. Subsequently, the material was washed in distilled water and conditioned in ethanol (70%) until they were blocked in paraffin and the slides were formulated.

Enzyme Linked Immunosorbent Assay - ELISA

Secretory IgA intestinal and serum IgY. The measurement of antibodies was accomplished by Kits Chicken IgA ELISA quantification and Chicken IgY ELISA quantification (Bethyl Laboratories, Montgomery, TX) according to the manufacturer's recommendations. The serum samples were diluted at 1:6.400 and intestinal fluid samples were diluted at 1:3.200 and tested in quadruplicate.

Immunohistochemistry

An immunohistochemistry assay was conducted to determine in influx of CD8^{+} T lymphocytes in the cecum of chickens after immunizations. Slides were assembled with cecal segments of four animals from each group, euthanized on days 21 and 28 after the

treatments. The tissue cuts (3μm) were submitted to deparaffinization at 56°C for 24h. On the following day, the samples were submitted to antigen retrieval, according to the protocol described by Kajiya et al., (2009), with modifications , where the samples were incubated in citrate-phosphate-borate buffer (33mM of $Na_3C_6H_5O_7$; 33mM of NaH_2PO_4, 57mM of $Na_2BO_4O_7.10H_2O$, pH 3.0) and incubated in a water bath at 96° C for 20 min. Subsequently, we adopted the protocol recommended by the manufacturer of the detection kit Envision-HRP (Dako, USA). The primary antibody used was CD8^{+} anti-*chicken* (CT-8; NBP1-28270, Novus Biologicals, USA), diluted 1:50, whereas the revelation was accomplished with 3.3-diaminobenzidine (DAB, Dako, USA). The tissues were photographed randomly in 10 histological fields (229mm[2]), at 40X objective in an optical microscope (Zeiss).

Statistical analysis

The experimental design was completely randomized using a factorial scheme of independent groups. The data obtained were submitted to the analysis of variance, followed by the Tukey test at 5% significance.

The IgA concentrations and the number of CD8^{+} T lymphocytes were transformed into log_{10} and the results are presented as geometric means.

RESULTS

Measurement of IgA and IgY

The results of IgA concentration (μg/mL) are presented in Table 2.

We observed that the IgA concentrations increased in all the groups until 21 days after the treatments (dat). Afterwards, the treated groups presented values higher (p<0.05) than the control groups and the highest concentration of 943 μg/mL was observed for group T1 (rFliC).

On the days (22 and 23) following the challenge with *S*. Typhimurium, we observed a significant drop in IgA concentrations in groups T1, T2 (rFliC+rCTB) and T3 (rFliC+PL), while group T4 (rFliC+ rCTB+ PL) showed a significant decline starting from day 23. However, we found that on day 26 dat, five days after the challenge and the IgA values of all the groups had resumed a significant increase and reached levels similar to those observed prior to the challenge (21 dat).

On day 26 dat, groups T4 and T5 (positive control) presented the highest IgA concentrations, approximately 1511 and 1546 μg/mL, respectively, differing (p<0.05) from negative controls T6 and T7.

Table 3 details the serum IgY concentrations. At 14 dat, we observed a significant difference (p<0.05) between the groups (T1 - T4) as compared to the controls (T6 and T7) and group T1 (rFliC) had the highest concentration, 686μg/mL. Nevertheless, from 21 dat until 26 dat, we did not observe significant differences in the IgY values among the different groups.

Analysis of the IgY means throughout the experiment

Table 2. Concentration of IgA (μg/mL) in intestinal fluid of commercial chickens at zero, seven, 14, 21, 22, 23 and 26 days after treatment with: T1 - rFliC, T2 - rFliC + rCTB, T3 - rFliC + *Lactobacillus* spp., T4 - rFliC + rCTB + *Lactobacillus* spp., T5 - *Salmonella* Typhimurium, T6 - microcapsules or T7 - negative control

Treatments	Days after treatment							Means*
	0	7	14	21	22	23	26	
T1	22.01[a, D]**	55.76[bc, C]	260.27[a, B]	943.45[a, A]	396.67[b, B]	245.17[cd, B]	54.67[ab, A]	223.64[b]
T2	22.01[a, D]	86.76[b, D]	157.23[bc, C]	927.39[a, A]	381.46[b, B]	275.87[bcd, B]	938.12[ab, A]	223.07[b]
T3	22.01[a, E]	89.92[b, D]	181.27[ab, C]	916.64[a, A]	520.96[ab, B]	366.55[abc, B]	592.77[b, AB]	232.95[b]
T4	22.01[a, A]	154.64[a, D]	193.87[ab, D]	832.73[a, B]	701.09[a, BC]	470.94[a, C]	1511.05[a, A]	309.84[a]
T5	22.01[a, E]	73.66[b, D]	172.41[ab, C]	223.44[c, C]	657.38[a, B]	406.11[ab, B]	1546.69[a, A]	221.05[b]
T6	22.01[a, E]	33.47[c, E]	99.69[c, D]	165.00[c, C]	529.21[ab, A]	306.29[abcd, B]	809.60[b, A]	148.47[c]
T7	22.01[a, E]	37.59[cd, E]	173.92[ab, C]	399.80[b, B]	470.16[ab, B]	212.22[d, C]	785.61[b, A]	172.31[c]
Means*	22.01[F]	67.27[E]	171.13[E]	516.78[B]	510.53[B]	315.07[C]	966.74[A]	

* Geometric mean. ** Means followed by the same upper-case letter on the line or lower-case letter in the column do not differ statistically by the test of Tukey (p<0.05).

Table 3. Concentration of IgY (μg/mL) in serum of commercial chickens at zero, seven, 14, 21, 22, 23 and 26 days after treatment with: T1 - rFliC, T2 - rFliC + rCTB, T3 - rFliC + *Lactobacillus* spp., T4 0 rFliC + rCTB + *Lactobacillus* spp., T5 - *Salmonella* Typhimurium, T6 - microcapsules or T7 - negative control

Treatments	Days after treatments							Means
	0	7	14	21	22	23	26	
T1	577.07[a, C]**	576.20[b, C]	686.02[a, AB]	713.24[a, A]	649.87[a, AB]	615.72[a, BC]	713.37[a, A]	647.35[ab]
T2	577.07[a, C]	659.14[a, B]	677.12[ab, AB]	734.66[a, A]	663.12[a, B]	648.98[a, B]	680.78[a, AB]	662.98[a]
T3	577.07[a, C]	617.59[ab, BC]	677.38[ab, AB]	711.39[a, A]	656.86[a, AB]	632.31[a, BC]	684.22[a, AB]	650.97[ab]
T4	577.07[a, D]	605.84[ab, CD]	666.66[ab, ABC]	733.93[a, A]	652.55[a, BC]	641.78[a, BCD]	679.75[a, AB]	651.08[ab]
T5	577.07[a, C]	638.18[ab, BC]	615.25[bc, BC]	708.77[a, A]	650.77[a, AB]	650.34[a, AB]	670.55[a, AB]	644.42[ab]
T6	577.07[a, C]	611.39[ab, BC]	589.52[c, C]	693.35[a, A]	632.59[a, ABC]	620.92[a, BC]	670.03[a, AB]	627.84[b]
T7	577.07[a, C]	625.91[ab, AB]	579.77[c, C]	677.32[a, A]	638.29[a, A]	640.91[a, AB]	671.88[a, A]	630.17[b]
Means	577.07[E]	619.17[D]	641.67[CD]	710.38[A]	649.15[C]	635.85[CD]	681.51[B]	

** Means followed by the same upper-case letter on the line or lower-case letter in the column do not differ statistically by the test of Tukey (p<0.05).

revealed that the values were most pronounced at 21. The general mean on 21 dat was 710μg/mL.

Retrieval of *Salmonella* Typhimurium (*S*T)

As shown in Table 4, all the treated groups had a significant reduction (p<0.05) in *S*T retrieval after the challenge. At 24h after the challenge, group T3 (rFliC+PL) showed a significant difference (p<0.05) in relation to the positive control (T5) and the other groups.

Yet, starting from 48 h post-challenge (23 dat), there was no *S*T retrieval in groups T2, T3 or T4. Furthermore, all the treated groups differed significantly (p<0.05) from the positive control.

The presence of *S*T in the cecum of challenged chickens was not detected 120 h (26 dat) after the challenge in the different groups.

The results from culturing samples of cecal content (data not shown) confirm the findings of the counting of CFU/mL.

Table 4. Quantity of *Salmonella Typhimurium* in cecal content of chickens after oral administration of $2x10^5$ CFU/mL

Hours after infection	Bacterial count ($\log_{10}$ CFU/mL)						Means
	Treatments						
	T1	T2	T3	T4	T5	T6	
24	2.03[a, AB]**	2.22[a, AB]	1.20[a, BC]	1.29[a, ABC]	2.55[a, A]	0.00[a,C]	1.55[a]
48	0.20[b, B]	0.00[b, B]	0.00[b, B]	0.00[b, B]	1.90[a, A]	0.00[a, B]	0.35[b]
120	0.00[b, A]	0.00[b, A]	0.00[b, A]	0.00[b, A]	0.00[b, A]	0.00[a, A]	0.00[b]
Means	0.74[AB]	0.74[AB]	0.40[B]	0.43[B]	1.48[A]	0.00[B]	

** Means followed by the same upper-case letter on the line or lower-case letter in the column do not differ statistically by the test of Tukey (p<0.05).

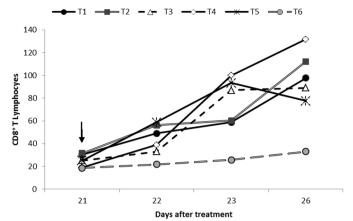## Number of CD8^{+} T lymphocytes

We observed no significant differences (p<0.05) in the number of CD8^{+} T lymphocytes in cecum of chickens at 21 dat among the different groups in relation to the negative control (T6) (Table 5, Fig.1).
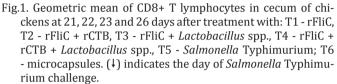
Soon after the challenge (24h), corresponding to 22 dat, we observed a significant increase (p<0.05) in the CD8^{+} T lymphocyte population in the groups T1 (rFliC),

Table 5. Mean of CD8+ T lymphocytes in ceca of commercial chickens at 21, 22, 23 and 26 days after treatment with: T1 - rFliC, T2 - rFliC + rCTB, T3 - rFliC + *Lactobacillus* spp., T4 - rFliC + rCTB + *Lactobacillus* spp., T5 - *Salmonella* Typhimurium or T6 - microcapsules

Treatments	Days after treatment				Means*
	21	22	23	26	
T1	30.20[a, C]**	48.98[a, BC]	58.88[a, AB]	97.72[a, A]	53.95[a]
T2	31.62[a, B]	56.23[a, B]	60.25[a, AB]	112.20[a, A]	58.88[a]
T3	25.12[a, B]	33.11[ab, B]	87.09[a, A]	89.12[a, A]	50.12[a]
T4	19.05[a, C]	38.90[ab, B]	100.00[a, A]	131.82[a, A]	56.23[a]
T5	25.12[a, B]	58.88[a, A]	93.32[a, A]	77.62[a, A]	57.54[a]
T6	18.62[a, A]	21.88[b, A]	25.70[b, A]	33.11[b, A]	24.43[a]
Means*	24.55[C]	40.74[B]	64.56[A]	83.17[A]	

* Geometric means. ** Means followed by the same upper-case letter on the line or lower-case letter in the column do not differ statistically by the test of Tukey (p<0.05).

Fig.1. Geometric mean of CD8+ T lymphocytes in cecum of chickens at 21, 22, 23 and 26 days after treatment with: T1 - rFliC, T2 - rFliC + rCTB, T3 - rFliC + *Lactobacillus* spp., T4 - rFliC + rCTB + *Lactobacillus* spp., T5 - *Salmonella* Typhimurium; T6 - microcapsules. (↓) indicates the day of *Salmonella* Typhimurium challenge.

T2 (rFliC+rCTB) and T5 (positive control) as compared to group T6, which was not submitted to the challenge (Table 5).

On day 23, all groups showed a significant increase (p<0.05) in the number of CD8^{+} T lymphocytes compared to T6. Furthermore, on the same day, we found no difference (p<0.05) between the treated groups and T5. This behavior was also observed on 26 dat (Table 5).

DISCUSSION

The FliC protein, the largest structural component of bacterial flagella, had highly conserved domains that are recognized by TLR5 receptors (Mizel & Bates 2010). Studies show adjuvant activity of FliC when it is associated with other antigens (McSorley et al. 2000, Skountzou et al. 2009, Bedoui et al. 2012) and its capacity to induce humoral and cellular immune response (Bobat et al. 2011)

Alexan et al. (2009) evaluated the immunomodulatory activity of FliC in mice and reported that high titers of antibodies at six weeks after immunization have the capacity to protect against the challenge of virulent strains of *Salmonella* spp. Although recent studies show the immunostimulatory capacity of FliC in chickens (Toyota-Hanatani 2008, 2009, Chaung et al. 2012, Okamura et al. 2012), there is little information in the literature on this subject.

In our study, we have investigated the capacity of the FliC recombinant to induce immune response in commercial chickens, when associated with *Lactobacillus* spp. and CTB and immune response enhancers in the mucosa.

We detected a significant increase of intestinal IgA levels in all the immunized groups, with the most pronounced response observed three weeks after immunization. Strindilius et al. (2004) found similar results and reported high IgA levels in mice immunized with flagellin. Chaung et al. (2012) studied the association de FliC with an inactivated influenza virus (H5N2) and observed that the birds immunized with this combination of antigens showed high IgA levels against H5N2, and concluded that FliC acts as an efficient mucosal adjuvant in birds.

The IgA levels of intestinal fluid observed in our study, at two days after the challenge, showed a significant decrease. Although this behavior is not very clear, we attribute it to a possible local immunological reaction where the presence of opsonized or secretory-IgA-linked microorganisms may have interfered with the method used to detect immunoglobulin.

Nevertheless, on five days after the challenge, IgA levels significantly increased in the challenged groups. These results corroborate Marcq et al. (2011) who demonstrated that after challenge with *Salmonella* Typhimurium, the birds showed higher titers of secretory IgA.

The systemic humoral immune response was not greatly influenced by the treatments regarding mucosal immunity. We observed that two weeks after immunization, the treated groups differed significantly from the control groups; however, this difference disappeared from the third week onwards, when the IgY levels continued highly similar among all groups, and no further significant inter-group differences were observed until the final collection day. These results show that flagellin has little capacity to induce serum IgY and corroborate Sbrogio-Almeida et al. (2001), who verified that oral immunization of mice with flagellin does not provide higher IgG titers. Similar to the results found by Revolledo et al. (2009), in the current study, there were no significant changes in serum IgY levels after the challenge.

The fact that the experimental conditions in our study did not produce significant pre-challenge differences in the population of CD8^{+} T lymphocytes among the different groups allows to infer that rFliC, even when associated with *Lactobacillus* and rCTB, does not cause a significant increase in the number of CD8^{+} T lymphocytes in the cecal content of treated chicken meat. However, we observed a significant increase in lymphocytes after the challenge with *Salmonella* spp.

The effect of the different treatments on intestinal colonization with *Salmonella* Typhimurium showed early elimination of microorganisms by the immunized animals (Table 4). We observed that 48 h after the challenge, *S*T was not detectable in groups T2 (rFliC+rCTb), T3 (rFliC+PL) and T4 (rFliC+rCTB+PL). Although these results did not show a significant difference to group T1 (rFliC), we can infer that the association of rFliC with *Lactobacillus* and/or CTB enabled greater efficacy in the *Salmonella* elimination.

Thus, we can conclude that the different rFliC treatments were capable of inducing mucosal immunity in the animals, conferring high levels of secretory IgA, which may have contributed to the elimination of *Salmonella* Typhimurium soon after the challenge. There was a slight stimulation of the systemic immune response while the population of CD8^{+} T lymphocytes was not influenced by the treatments before the challenge.

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