INTRODUCTION

Contact has been established between enteropathogens and the host in intestinal mucosa (Muir et al., 2000). Thus, the immunity system in the mucosa acts as a first line of defense against bacterial and viral infections (Liu et al., 2010).

In the gastrointestinal tract of birds, Lactobacillus spp. keep close contact with the lymphoid tissue associated with the intestine (gut-associated lymphoid tissue; Brisbin et al., 2008). These interactions between the host cells and microorganisms can stimulate the production of secretory IgA and increase the expression of cytokines, providing protection against infections (Ogra et al., 2001; Haghighi et al., 2006; Brisbin et al., 2010).

Some antigens, when administered via mucosa without adjuvants, are weakly immunogenic; therefore, they present a challenge for vaccine development. This highlights the need to develop safe and efficacious mucosal adjuvants. Currently, the most promising adjuvants include the protein flagellin (FliC) and the subunit B of cholera toxin (CTB), which have shown capacity to augment immune responses with high levels of secretory IgA and of cytokines (Zhang et al., 2005; Sun et al., 2010; Chaung et al., 2012).

Flagellin is the main structural protein of flagellae of Salmonella spp. (Salazar-Gonzalez and McSorley, 2005) and has a molecular weight of approximately 51 kDa, presenting 4 structural domains, with domains 0 and 1 highly conserved among the different bacterial species. However, domains 2 and 3 show greater variability and are situated in a hypervariable region (Eaves-Pyles et al., 2001; Haghighi et al., 2006; Brisbin et al., 2010).

The expression of FliC differs among the serotypes of Salmonella enterica, therefore, biphasic systems exist in which the genes fljC (phase 1) and fljB (phase 2) are responsible for the expression, although they do not...
Mucosal immunization, mainly by the oral route, is of great interest because of its practical and logistic advantage in the vaccination process and for favoring the establishment of local immunity (Holmgren et al., 2005). The current study evaluated the immune response of broiler chickens treated with the proteins CTB and recombinant FliC in association with strains of Lactobacillus spp. aimed to develop strategies to induce immune response of mucosa in broiler chickens.

MATERIALS AND METHODS

Bacterial Samples

The strains of Lactobacillus spp. (Lactobacillus reuteri, Lactobacillus acidophilus, Lactobacillus plantarum) used in the present study were isolated from broiler chickens and selected according to adhesion capacity and immunomodulatory effect as described by Rocha et al. (2012). Strains of Escherichia coli BL21 (SI) and E. coli BL21 (DE3) pLysS were used to assess expression of recombinant proteins.

Recombinant Proteins

Recombinant CTB. The expression of the recombinant protein CTB (rCTB) was evaluated in the E. coli strain BL21 (SI), which was transformed using the plasmid pAE/ctb (donated by Paulo Lee Ho, Instituto Butantan, Sao Paulo, Brazil]). The rCTB protein was expressed as corpuscles inclusion was purified following the refolding protocol described by Aida et al. (2002).

To confirm the expression of the rCTB protein in pentameric (native) and monomeric forms, a Western blotting assay was carried out using purified and renatured protein (refolding). For those procedures, the rCTB protein was run in an SDS-PAGE (12%) and subsequently transferred to a nitrocellulose membrane (0.2 μm; Sigma, St. Louis, MO). The result was blocked for 1 h in a blocking buffer (PBS + 0.1% Tween 20 + 5% powdered milk) and incubated for an addition hour with the primary antibody rabbit anti-Cholera toxin (Sigma) in a dilution of 1:10,000. The membrane was then submitted to washings (5 min) with a PBS solution supplemented with 0.05% Tween 20. Incubation was carried out once more with the peroxidase labeled antirabbit secondary antibody diluted at 1:2,500. After incubation, washings were performed as previously described and the reaction was revealed with DAB (DAKO, Glostrup, Denmark) according to manufacturer’s protocol.

Recombinant FliC. For cloning, the gene fliC from Salmonella Typhimurium was amplified by PCR, using the primers 5’CGCCATATGCGCAGTAT-TACAAAAC3’ (forward) and 5’CCGCTCGAGTATACGCGATGAGCGAGGA 3’ (reverse), which were designed based on the complete sequence of the fliC gene deposited in the GenBank (NC_003197). Restriction sites were added to Xho I and Nde I for subsequent subcloning in expression vectors. The PCR reaction was carried out using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Life Technologies, Carlsbad, CA), according to the manufacturer’s protocol. The reactions were incubated in a thermal cycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany) at 95°C for 2 min, 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min, with 30 repetitions and a final extension at 72°C for 5 min.

After amplification, the PCR product was visualized in 1% agarose gel and purified with Qiaquick PCR purification kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Subsequently, they were cloned in pGEM T-easy (Promega, Madison, WI) and subcloned in the vector pET28b (Novagen, Merck Chemicals B.V., Amsterdam, the Netherlands) using the cleavage (restriction) sites mentioned previously.

To induce expression, E. coli BL21 (DE3) pLysS was transformed with the plasmid pET28b/fliC. The recombinant colonies were selected and cultivated in Lactobacillus broth containing kanamycin (30 μg/mL) and chloramphenicol (50 μg/mL) at 37°C until the optical density of 0.6 was reached. For induction, 1 mM isopropyl β-D-1-thiogalactopyranoside was added and the culture was incubated at 37°C under shaking at 180 rpm. The expression of recombinant FliC (rFliC) was verified in SDS-PAGE (15%).

The rFliC protein was expressed in a soluble form and purified under native conditions by immobilized metal-ion affinity chromatography (Ni-NTA, Qiagen) according to the manufacturer’s protocol. Protein expression was confirmed in the Western blotting assay, similar to the rCTB description; however, we used the primary antibody anti-FliC (Flagellin, BioLegend, San Diego, CA) at 1:5,000 dilution ratio and secondary antibody stained with peroxidase (antimouse) 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 solution containing the proteins was emulsified in soy oil containing 5% of sorbitan monooleate. Next, the
solution was re-emulsified in sodium alginate (1%) and, subsequently, the double emulsion was dripped into a calcium chloride solution (1%) and kept at −20°C until the moment of use.

**Experimental Design**

One-day-old broiler chickens (n = 210) of the Ross lineage were allocated into experimental cages. They received water and a nonmedicated ration ad libitum, as well as necessary warming according to age. The broiler chickens were divided into 7 groups composed of 30 animals each. The treatments are specified in Table 1.

On d 2, 3, and 4 of life, 500 μL from a pool of *Lactobacillus* spp. (*L. reuteri*, *L. acidophilus*, *L. plantarum*) was administered orally with the aid of a gavage needle. Then, between d 5 and 7, birds received the encapsulated recombinant proteins (50 μg/d) administered orally with the aid of a plastic spatula followed by 500 μL of carbonate buffer (0.3 M NaHCO₃; pH 8.0) on gavage. A booster (a single protein dose of 50 μg), was administered 15 d after the first immunization. The animals were restricted from eating or drinking for 2 h before and after treatment. Phosphate-buffered saline microcapsules were used as a control material for the capsules preparation.

For serological monitoring, blood was collected from broiler chickens (n = 6 birds/group) at d 0, 7, 14, 21, and 28 posttreatment by puncture of an ulnar or jugular vein. The blood samples were centrifuged at 8,000 × g for 5 min at 5°C. The serum was kept at −20°C until analysis to determine IgY, as described by Rocha et al. (2012).

For collection of intestinal fluid, 6 birds per group were euthanized by cervical dislocation soon after blood collection. The intestines were collected and the intestinal fluid was collected to quantify IgA, as described by Rocha et al. (2012).

To complete cecal collections for immunohistochemical assays, 1-cm portions of cecum were collected and fixed in 10% formalin for 24 h. Subsequently, the material was washed in distilled water and conditioned in 70% ethanol until histological preparation.

For ELISA on intestinal secretory IgA and serum IgY, antibodies were measured using commercial kits Chicken IgA ELISA quantification and Chicken IgY ELISA quantification (Bethyl Laboratories, Montgomery, TX) following the manufacturer’s instructions. The samples of serum and intestinal fluid were diluted at 1:6,400 and 1:3,200, respectively, and tested in 4 repetitions.

The immunohistochemistry assay was carried out to determine the influx of CD8⁺ T lymphocytes into the cecum of the broiler chickens after the immunizations. Slides were prepared with cecum segments from 4 animals euthanized at d 21 and 28 posttreatment. The tissue cuts (3 μm) were submitted to deparaffinization at 56°C for 24 h and antigen retrieval was completed the next day, as described by Kajiya et al., (2009). The slides were immersed in citrate-phosphate-borate buffer (33 mM Na₃C₆H₅O₇, 33 mM NaH₂PO₄, 57 mM Na₂BO₄·10H₂O; pH 3.0) and incubated in a water bath at 96°C for 20 min, following the manufacturer’s protocol using the Envision-HRP detection kit (Dako). The primary antibody used was CD8⁺ antichicken (Novus Biologicals, Littleton, Co) at a dilution ratio of 1:50 and the revelation was carried out with 3,3′-diaminobenzidine (DAB, Dako). Ten histological fields (229 mm²) were randomly photographed (40× objective) in an optical microscope (Zeiss, Jena, Germany).

**Statistical Analysis**

The experimental design was completely randomized using a factorial scheme of independent groups. The data obtained were submitted to the ANOVA, followed by the Tukey test at 5% significance. The IgA concentrations and the number of CD8⁺ T lymphocytes were transformed into log₁₀ and the results are presented as geometric means.

**RESULTS**

**Expression of Recombinant Proteins**

The expressed and purified rFliC proteins and rCTB pentameric showed molecular masses of approximately

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Duration (d)</th>
<th>Age of bird at first dose (d)</th>
<th>Age of bird at booster (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>50 μg</td>
<td>3</td>
<td>5-7</td>
<td>21</td>
</tr>
<tr>
<td>T2</td>
<td>50 μg</td>
<td>3</td>
<td>5-7</td>
<td>21</td>
</tr>
<tr>
<td>T3</td>
<td>50 μg</td>
<td>3</td>
<td>5-7</td>
<td>21</td>
</tr>
<tr>
<td>T4</td>
<td>500 μL (10⁹)</td>
<td>3</td>
<td>2-4</td>
<td>21</td>
</tr>
<tr>
<td>T5</td>
<td>50 μg/500 μL (10⁹)</td>
<td>3</td>
<td>2-7</td>
<td>21</td>
</tr>
<tr>
<td>T6</td>
<td>50 μg</td>
<td>—</td>
<td>5-7</td>
<td>21</td>
</tr>
<tr>
<td>T7</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

1T1 = recombinant flagellin (rFliC); T2 = recombinant subunit B of cholera toxin (rCTB); T3 = rFliC + rCTB; T4 = pool of *Lactobacillus* spp.; T5 = rFliC + rCTB + pool of *Lactobacillus* spp.; T6 = capsules prepared with PBS; and T7 = negative control.
50 and 51 kDa, respectively. The protein reactivity, visualized in Western blotting assays, confirmed the expression (Figures 1 and 2).

Concentration of Intestinal IgA

The results of IgA concentrations in intestinal fluid are shown in Table 2. The IgA concentration was influenced by the treatments and the number of days posttreatment, with a significant interaction between these factors.

The IgA concentrations resulting from all the treatments showed a significant increase ($P < 0.05$) until d 21 posttreatment, when treatments 2 (rCTB) and 3 (rFliC + rCTB) produced the highest values of IgA in the assay, 1,332 and 882 μg/mL, respectively, being statistically equivalent. But only treatment 2 presented a significant difference ($P < 0.05$) if compared with the other treatments.

As expected, the mean of treatment 6 (capsule-placebo) did not differ significantly from treatment 7, demonstrating that the microcapsules formulation is not antigenic. Therefore, all the immune responses observed in the groups that received encapsulated rFliC and rCTB were caused by the protein effect.

Table 2. Concentration of IgA in the intestinal fluid of broiler chickens at d 0, 7, 14, 21, and 28 after treatment

<table>
<thead>
<tr>
<th>Treatment $^1$ (μg/mL)</th>
<th>d 0</th>
<th>d 7</th>
<th>d 14</th>
<th>d 21</th>
<th>d 28</th>
<th>Geometric mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>22.25</td>
<td>59.59</td>
<td>200.59</td>
<td>769.59</td>
<td>615.08</td>
<td>165.97</td>
</tr>
<tr>
<td>T2</td>
<td>22.25</td>
<td>34.57</td>
<td>359.31</td>
<td>1331.92</td>
<td>699.16</td>
<td>186.28</td>
</tr>
<tr>
<td>T3</td>
<td>22.25</td>
<td>86.97</td>
<td>244.55</td>
<td>882.52</td>
<td>639.67</td>
<td>192.91</td>
</tr>
<tr>
<td>T4</td>
<td>22.25</td>
<td>32.57</td>
<td>178.73</td>
<td>588.09</td>
<td>661.69</td>
<td>138.20</td>
</tr>
<tr>
<td>T5</td>
<td>22.25</td>
<td>54.79</td>
<td>212.85</td>
<td>620.88</td>
<td>618.26</td>
<td>158.38</td>
</tr>
<tr>
<td>T6</td>
<td>22.25</td>
<td>65.46</td>
<td>76.19</td>
<td>484.12</td>
<td>485.16</td>
<td>121.12</td>
</tr>
<tr>
<td>T7</td>
<td>22.25</td>
<td>67.13</td>
<td>63.53</td>
<td>446.22</td>
<td>433.50</td>
<td>112.92</td>
</tr>
<tr>
<td>Geometric mean</td>
<td>22.26</td>
<td>54.37</td>
<td>164.40</td>
<td>685.81</td>
<td>574.36</td>
<td></td>
</tr>
</tbody>
</table>

$^a$–$^d$ Means followed by the same lowercase letter in a column do not differ statistically in the Tukey test ($P < 0.05$).

$^A$–$E$ Means followed by the same capital letter in a row do not differ statistically in the Tukey test ($P < 0.05$).

$^1$ T1 = recombinant flagellin (rFliC); T2 = recombinant subunit B of cholera toxin (rCTB); T3 = rFliC + rCTB; T4 = Lactobacillus spp.; T5 = rFliC + rCTB + Lactobacillus spp.; T6 = capsules prepared with PBS; and T7 = negative control.
**Serum Concentration of IgY**

The observed IgY concentrations were dependent on the day and on the treatment, but no interaction ($P < 0.05$) was observed between these 2 factors (Table 3). The mean IgY concentration in all treatments increased until d 14 posttreatment, when it reached 635.73 μg/mL, showing a significant difference ($P < 0.05$) in relation to the other sampling days. At d 21 posttreatment, these values decreased but remained stable until d 28 posttreatment.

Regarding the influence of IgY treatments on production, treatments 2 and 3 produced the highest concentrations, approximately 530 and 540 μg/mL, respectively, differing ($P < 0.05$) from the controls. Conversely, the other treatments (1, 4, and 5) showed high, but statistically equivalent values in relation to the controls.

**CD8+ T Lymphocytes**

The results of the recruitment of CD8+ T lymphocytes, visualized by immunohistochemistry in cecum of broiler chickens at d 21 and 28 posttreatment, are presented in Table 4. The average numbers of CD8+ T lymphocytes found at d 21 posttreatment were significantly higher ($P < 0.05$) than at d 28 posttreatment. At d 21 posttreatment, all the treatments differed ($P < 0.05$) from the negative control (treatment 7), whereas at d 28 posttreatment only treatments 2 (rCTB), 4 (Lactobacillus spp.), and 5 (rFliC+rCTB+Lactobacillus spp.) showed a significant difference in relation to treatment 7.

**DISCUSSION**

The immunomodulatory and adjuvant effects of rCTB and rFliC have been widely studied, especially in mammals, and have been explored in different immunization routes (Sbrogio-Almeida and Ferreira, 2001; Strindelius et al., 2004; Nempont et al., 2008). However, few studies evaluate the activities of these antigens in broiler chickens. In the current study, we evaluated the immune response of broiler chickens after oral treatment with strains of Lactobacillus spp. and recombinant proteins (FliC and CTB). We observed that IgA levels increased in all the treatments, showing a significant stimulation of the humoral immune response.

Our results corroborate previous studies that also demonstrated the immunostimulatory effect of Lactobacillus spp. in broiler chickens at d 21 posttreatment.

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**Table 3. Concentration of IgY in serum of broiler chickens at d 0, 7, 14, 21, and 28 after treatment**

<table>
<thead>
<tr>
<th>Treatment 1</th>
<th>d 0</th>
<th>d 7</th>
<th>d 14</th>
<th>d 21</th>
<th>d 28</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>309.07</td>
<td>512.87</td>
<td>659.67</td>
<td>577.80</td>
<td>548.04</td>
<td>521.49</td>
</tr>
<tr>
<td>T2</td>
<td>309.07</td>
<td>524.55</td>
<td>675.47</td>
<td>585.77</td>
<td>573.25</td>
<td>533.62</td>
</tr>
<tr>
<td>T3</td>
<td>309.07</td>
<td>534.12</td>
<td>666.85</td>
<td>604.56</td>
<td>583.98</td>
<td>539.72</td>
</tr>
<tr>
<td>T4</td>
<td>309.07</td>
<td>503.85</td>
<td>665.69</td>
<td>562.98</td>
<td>559.95</td>
<td>520.31</td>
</tr>
<tr>
<td>T5</td>
<td>309.07</td>
<td>566.29</td>
<td>622.93</td>
<td>571.50</td>
<td>559.95</td>
<td>519.95</td>
</tr>
<tr>
<td>T6</td>
<td>309.07</td>
<td>505.42</td>
<td>603.18</td>
<td>549.33</td>
<td>530.43</td>
<td>499.49</td>
</tr>
<tr>
<td>T7</td>
<td>309.07</td>
<td>503.03</td>
<td>556.35</td>
<td>543.90</td>
<td>534.79</td>
<td>489.43</td>
</tr>
<tr>
<td>Mean</td>
<td>309.07</td>
<td>512.87</td>
<td>635.73</td>
<td>570.83</td>
<td>555.77</td>
<td>555.77</td>
</tr>
</tbody>
</table>

a-c Means followed by the same lowercase letter in a column do not differ statistically in the Tukey test ($P < 0.05$).

A-D Means followed by the same capital letter in a row do not differ statistically in the Tukey test ($P < 0.05$).

1T1 = recombinant flagellin (rFliC); T2 = recombinant subunit B of cholera toxin (rCTB); T3 = rFliC + rCTB; T4 = Lactobacillus spp.; T5 = rFliC + rCTB + Lactobacillus spp.; T6 = capsules prepared with PBS; and T7 = negative control.

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**Table 4. Number of CD8+ T lymphocytes in cecal fragments of broiler chickens at d 21 and 28 after treatment**

<table>
<thead>
<tr>
<th>Treatment (no.)</th>
<th>d 21</th>
<th>d 28</th>
<th>Geometric mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>125.68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>93.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>96.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>78.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T5</td>
<td>76.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T7</td>
<td>21.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Geometric mean</td>
<td>72.44</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a,b,c Means followed by the same lowercase letter in a column do not differ statistically in the Tukey test ($P < 0.05$).

A,B Means followed by the same capital letter in a row do not differ statistically in the Tukey test ($P < 0.05$).

1T1 = recombinant flagellin (rFliC); T2 = recombinant subunit B of cholera toxin (rCTB); T3 = rFliC + rCTB; T4 = Lactobacillus spp.; T5 = rFliC + rCTB + Lactobacillus spp.; T6 = capsules prepared with PBS; and T7 = negative control.
(Rocha et al., 2012). Similar effects were observed in mice treated with *Lactobacillus casei* (Galdeano and Perdigón, 2006).

An immunomodulatory capacity was observed starting in the first week of treatment, when the IgA secretory levels of groups treated remained higher than in the control groups. At d 21 posttreatment, we observed the highest IgA concentrations, especially in treatments 2 (rCTB) and 3 (rFliC+rCTB), showing evidence of the pronounced immunostimulatory CTB capacity.

Previous studies showed the induction of an immune response in mice treated with CTB associated with other antigens (Strindelius et al., 2004; Zhang et al., 2005), providing evidence of a strong adjuvant capacity of the protein (Girard et al., 1999) and that CTB association with the recombinant protein 1EP1, from *Eimeria tenella*, is capable of stimulating production of specific IgA and IgY in broiler chickens. These results are similar to our results where the association of rCTB with the protein rFliC generated high IgA levels. We also observed that administration of unassociated rCTB conferred high IgA levels.

Mucosal surfaces comprise the largest and most important interface between the body and the external environment; therefore, the vast majority of pathogens establish contact with the host via mucosa (Lawson et al., 2011; Kweon, 2011). The mediation of mucosal immune defense is initiated by the activation of lymphocytes and local secretion of IgA. This immunoglobulin represents the principal element of humoral immune response, which provides protection against pathogens on the mucosal surface (Muir et al., 2000; Snoeck et al., 2006). Overall, the treatments also induced the production of IgY serum, but with more stable values than for IgA.

Similar to the results observed for the immune response of mucosa, treatments 2 and 3 showed the greatest responses. These observations allow us to conclude that rCTB was effective to induce the systemic and local immune response, corroborating Broos et al. (2008), who reported an induction of a Th2 response in mice, with high IgG1 levels after rCTB treatment.

The significant immunogenic CTB capacity modulates the differentiation of helper T cell, allowing the stimulation of a Th2 response (Broos et al., 2008), and therefore increasing the IgA and IgG levels (Cox et al., 2006; Sanchez and Holmgren, 2008). The CTB properties induce immune responses by binding to GM1 ganglioside receptors in several cell types, including cells from the immune system (Strindelius et al., 2004; Sanchez and Holmgren, 2008).

The association between rCTB and rFliC induced high IgA levels, corroborating Strindelius et al. (2004), who found high IgA levels after immunizing mice with the same proteins by intranasal and subcutaneous routes. We recognized that the treatments carried out with rFliC and rCTB, associated or not, induced IgA levels significantly higher than in the control groups until d 21 posttreatment. This behavior was not observed when the proteins were associated with *Lactobacillus spp.*

The treatments using pools of *Lactobacillus spp.* induced a significant increase of the IgA concentration until d 14 posttreatment, when these levels decreased, even in association with recombinant proteins. Therefore, it seems that the presence of *Lactobacillus* influenced only the performance of recombinant proteins. Given that the mechanisms that favor this behavior are still not clear, further studies need to be carried out to investigate them.

Antibodies are most effective in eliminating extracellular antigens, as their interaction with the antigen promotes the removal or destruction of the antigen. Nevertheless, when the antigens are located in the cell interior, the immune system activates responses mediated by cytotoxic T lymphocytes (CD8+), which lead to antigenic elimination (Erf, 2004).

We investigated the induction of cellular immune response by recombinant proteins and *Lactobacillus spp.* for the presence of CD8+ T lymphocytes stained by immunohistochemistry in cecal fragments of treated broiler chickens. We observed that, at d 21 posttreatment, all groups showed levels of T CD8+ lymphocytes significantly higher than the control. At d 28 posttreatment, the groups that received *Lactobacillus spp.*, associated or not with proteins, were able to maintain a significant difference in the number of CD8+ T cells compared with the values found in the control. These observations allow us to infer that the presence of *Lactobacillus* was a determinant to achieve a more prolonged cellular immune response. These data corroborate Noujaim et al. (2009), who observed an increase in the number of CD8+ T lymphocytes in the cecum of broiler chickens after immunization with strains of *Lactobacillus* spp. However, this type of response was not observed in mice immunized with *Lactobacillus casei* (Galdeano and Perdigón, 2006). The different results may be correlated with the strain of *Lactobacillus* spp. and the animal species studied.

Our study aimed to aggregate more information regarding the immune response of chickens submitted to treatments with rCTB, rFliC, and *Lactobacillus* spp. We conclude that the recombinant microencapsulated proteins, administered orally, can stimulate humoral and cellular immune response and the association of these antigens with *Lactobacillus* spp. confers a lasting population of CD8+ lymphocytes in the cecum of broiler chickens.

**ACKNOWLEDGMENTS**

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