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Detection of CD4+ and CD8+ Lymphocytes in the Intestine of Broiler Chicks Treated with *Lactobacillus* spp. and Challenged with *Salmonella enterica* serovar *Enteritidis*

■ Author(s)

Noujaim JC
Andreatti Filho RL
Lima ET de
Okamoto AS

Department of Clinical Veterinary Medicine
College of Veterinary Medicine and Animal Science - FMVZ
São Paulo State University - UNESP
Botucatu, SP, Brazil.

■ Mail Address

Raphael Lucio Andreatti Filho
Department of Clinical Veterinary Medicine
College of Veterinary Medicine and Animal Science. São Paulo State University
Distrito Rubião Júnior S/N
18.618-000, Botucatu, SP, Brazil
Telephone/fax: +55 (14) 3811 6293

E-mail: andreatti@fmvz.unesp.br

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ABSTRACT

The expression of immune response as a leukocytic infiltrate by CD4+ and CD8+ cells in the epithelium and in the intestinal lamina propria of chicks fed *Lactobacillus* spp or cecal microflora (CM) and experimentally challenged or not with *Salmonella enterica* serovar *Enteritidis* (SE) was studied using immunohistochemistry. Three hundred and twenty day-of-hatch broiler chicks were divided into four groups of 80 birds each and orally received *L. reuteri*, *L. salivarius*, *L. acidophilus*, or CM. Each group was subdivided into four subgroups of 20 birds each, classified as follows: a subgroup did not receive any oral treatment (negative control), subgroup treated with *L.* spp or CM, subgroup treated with *L.* spp or CM and challenged with SE, and subgroup only challenged with SE (positive control). The results show that the oral treatment with *L. reuteri*, *L. salivarius*, *L. acidophilus*, or CM and challenge or not with SE stimulated bird immune response as determined by the leukocytic infiltrate by CD8+ lymphocytes followed by CD4+ in the epithelium and in the lamina propria of the duodenum, jejunum, and cecum of chicks up to 12 days of age. CD8+ lymphocyte number was significantly higher in the intestine of chicks receiving CM and challenged with SE. The duodenum, followed by the jejunum, were the segments in which the immune response, as shown by T, CD4+ and CD8+ cells, was stimulated with the greatest intensity.

INTRODUCTION

Major risk factors for food poisoning caused by *Salmonella* are associated with chickens, such as eating raw or undercooked eggs (Molbak & Neiman, 2002). *Salmonella enterica* serovar *Enteritidis* (SE) is the second most common serotype in humans and most commonly identified from clinical and non-clinical chicken sources (Centers for Disease Control and Prevention, 2005). This serovar can infect chickens in the absence of clinical disease (Gast & Beard, 1990). Several measures to control *Salmonella* have been used, such as antimicrobial drugs. However, concerns with drug-resistant bacteria and the detection of drug residues in food of animal origin have stimulated the interest in alternative treatments, such as the use of probiotics, competitive exclusion products, and bacteriophages (Sulakvelidze *et al*., 2001; VanImmerseel *et al*., 2002; Andreatti Filho *et al*., 2003; Joerger, 2003).

Lilly & Stillwell (1965) were the first to utilize the term probiotic and the work of Nurmi & Rantala (1973) was the initial milestone of studies involving the concept of competitive exclusion. The principle of competitive exclusion is particularly interesting because it provides some protection of birds against their contamination by pathogens in the first days after hatch, although at this age, neither the intestinal microflora or the immune system of birds is fully developed (Day, 1992).

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The intestinal microflora is composed of various bacterial species, including the prominent genus *Lactobacillus*, which most common species, such as *L. reuteri*, *L. salivarius*, *L. animalis*, and *L. acidophillus*, have shown to be important for the intestinal health of chicks (Ramesh *et al*., 2000).

Oral treatments with *Lactobacillus* spp. have demonstrated immunostimulant effects on the intestinal mucosa (Simon *et al*., 2001). In chickens, the presence of *Lactobacillus* spp. in the intestine stimulates synthesis of IgA by the immune system through the release of short chain peptides, increasing the resistance to diseases (Pulverer *et al*., 1990). Furthermore, lactobacilli are also associated to beneficial effects in the therapy against tumors in humans, exerting antimutagenic effects and modulating immune response, including T-cells, probably due to the influence of cytokines on CD4+ cells (Schifrin *et al*., 1997; Pelto *et al*., 1998). Therefore, the presence of *Lactobacillus* is essential for regulating the composition of intestinal microflora, developing intestinal immunity, and promoting chicken health (Muir *et al*., 2000).

The objective of this study was to evaluate immune system stimulation, in the form of CD4+ and CD8+ lymphocyte leukocytic infiltrate in the intestinal epithelium and lamina propria, of broiler chicks orally treated with *Lactobacillus reuteri*, *L. salivarius*, *L. acidophilus*, or cecal microflora (CM), isolated from breeders, and challenged or not with *Salmonella enterica* serovar *Enteritidis* (SE).

MATERIAL AND METHODS

Birds

Three hundred and twenty day-of-hatch broiler chicks were obtained from a local hatchery. Chicks were housed in metal cages, maintained under heating for 12 days, and were offered water and a commercial feed *ad libitum*, with no addition of antibiotics. Only *Salmonella*-free chicks were utilized, as certified by the results of Salmonella isolation and identification tests of a flock sample, according to the methodology described by Nagajara *et al*. (1991).

Experimental design

The 320 birds utilized were divided into four groups containing 80 chicks each and submitted to oral treatments with *L. reuteri*, *L. salivarius*, *L. acidophilus*, and CM. Each group was subdivided into four subgroups of 20 birds each, which were divided as follows: a subgroup which was not orally treated or challenged (negative control), subgroup orally treated with **L**. spp or CM, subgroup orally treated with **L**. spp or CM and challenged with SE, and a subgroup only challenged with SE (positive control). At two, four, eight, and 12 days of age, five birds from each subgroup were euthanized by cervical dislocation, and fragments of duodenum, jejunum and ceca were aseptically removed and fixed in 10% Bouin solution for 24 hours.

Preparation of cecal microflora and *Lactobacillus* cultures

CM was obtained from three 35-week-old breeders, euthanized by cervical dislocation, and aseptically necropsied for the removal of the ceca, which contents were cultivated in 10mL thioglycolate broth in an anaerobic jar containing the Anaerobac® system for 24 hours at 40°C. Concomitantly, this material also was tested to verify the presence of *Salmonella* spp. (Nagajara *et al*., 1991). Samples positive for *Salmonella* spp. were discarded. After incubation, the number of colony-forming units (CFU) of intestinal bacteria was determined, after which decimal dilution series were performed in pH 7.2 phosphate buffer saline solution (PBS). From those decimal dilutions, 0.1mL was inoculated in Petri dishes containing thioglycolate agar and cultivated under anaerobiosis for 24 hours at 40°C. Day-of-hatch chicks received 107 CFU/chick (0.5mL) of CM, *Lactobacillus acidophilus*, *L. reuteri*, or *L. salivarius* by oral gavage utilizing graduated pipette. All the lactobacilli were isolated from the ceca of 35-week-old breeders, biochemically identified, stored in nutrient broth containing 10% glycerol, and frozen in liquid nitrogen.

Biochemical identification was performed by carbohydrate fermentation test. Lactobacilli samples were propagated in 3mL DeMan-Rugosa-Sharpe broth (MRS) free from meat extract and glucose, with 0.002% bromocresol purple as indicator, and 0.01% of the following carbohydrates were individually added: arabinose, fructose, galactose, glucose, mannitol, mannose, maltose, sucrose, salicyl, and sorbitol. Tubes were incubated at 37°C for 48 hours (Kandler & Weiss, 1986).

Each cryotube containing 1.5mL of culture of *Lactobacillus* spp. was thawed, resuspended in MRS broth, and incubated under anaerobiosis for 24 hours at 40°C before determination of inoculum CFU, which was carried out by decimal dilution series of MRS broth in PBS and plating 0.1mL of these dilutions on Petri dishes containing MRS agar. Cultured were incubated under anaerobiosis for 48 hours at 40°C.

Preparation of *Salmonella* Enteritidis inoculum

A *SE* strain resistant to nalidixic acid and to rifampicin, and isolated from the liver of chickens was utilized for the challenge. This resistance was developed through successive culture in brilliant green agar (BGA), containing 100µg/mL nalidixic acid and rifampicin (Weinack *et al*., 1982). The inoculum was obtained by culture in brain-heart infusion broth (BHI) incubated at 40°C for 12 hours. The challenge inoculum was quantified by CFU number per chick, as previously described. In order to determine CFU number, 100µg/mL of these dilutions were plated in BGA increased from antibiotics. After a period of incubation of 24 hours at 40°C, reading was performed for CFU determination. On the third day of life, each bird was challenged with 106 CFU/chick of SE by oral gavage (0.5mL).

Immunohistochemistry

Immunohistochemical reactions were performed according to Hsu *et al*. (1981) for tissue cuts embedded in paraffin.

All collected intestinal fragments were fixed in 10% Bouin solution for 24 hours and embedded in paraffin. Sections were cut to a thickness of 3mm, cleared in xylene, rehydrated in graded ethanol series, and rinsed in distilled water. Endogenous peroxidases were neutralized with 3% hydrogen peroxide for 15 minutes, followed by rinsing for 5 minutes in distilled water. Antigen was retrieved by incubating slides in EDTA solution (ethylenediaminetetraacetic acid disodium salt), pH 8.0, in a steamer at 96°C for 30 minutes.

Nonspecific immunoglobulin was blocked by incubating the slides for 60 minutes with 5% bovine serum albumin (BSA) before application of the primary antibody. Slides were incubated for 18 hours at 4oC with a monoclonal anti-chicken CD4+ T-lymphocytes (UNLB, Southern Biotechnology Associates, Inc.) at 1:25 dilution and monoclonal anti-chicken CD8+/144B T-lymphocytes (Dakocytomation CD8, T-cell BGM 7103) at 1:100 dilution. A streptavidin-immunoperoxidase staining procedure (LSAB) was used for immunolabeling CD8+ positive cells. CSA procedure was used for immunolabeling CD4+ positive cells. The immunoreactions were observed with 3' 3-diaminobenzidine substrate (DAB). Sections were counterstained with Mayer's hematoxylin.

The negative controls were tested with mouse immunoglobulins (Ig) (for the monoclonal antibody); with one slide with only secondary antibody and one with only streptoavidine and peroxidase. All tested slides were negative.

After the immunohistochemical reactions, marked cells were counted under an optical microscope (JENAMED 2 - Carlzeiss Jena) with 40x magnification. CD4+ and CD8+ lymphocytes were counted in ten random fields in each slide of the epithelium and of the lamina propria of each intestinal segment (duodenum, jejunum, and cecum).

Statistical analysis

Data were submitted to analysis of variance using a completely randomized experimental design (Zar, 1996) in a factorial arrangement. Treatments consisted of combinations of the following factors: 1) oral treatment - CM, L. acidophillus, L reuteri, or L. salivarius; 2) challenge - treated and challenged birds, treated birds, challenged birds (positive control), and birds neither treated nor challenged (negative control); 3) bird age - birds of two, four, eight, and 12 days of age. There were five replicates per treatment, which were compared as to their effects on CD4+ and CD8+ lymphocyte counts at a 5% level of significance.

RESULTS

Positive reactions for CD4+ lymphocytes in the lamina propria of the ceca of two-day-old chicks treated with *L. reuteri* are illustrated in Figure 1. Figure 2 depicts the positive reaction for CD8+ lymphocytes in the duodenum of eight-day-old birds treated with *L. salivarius*.

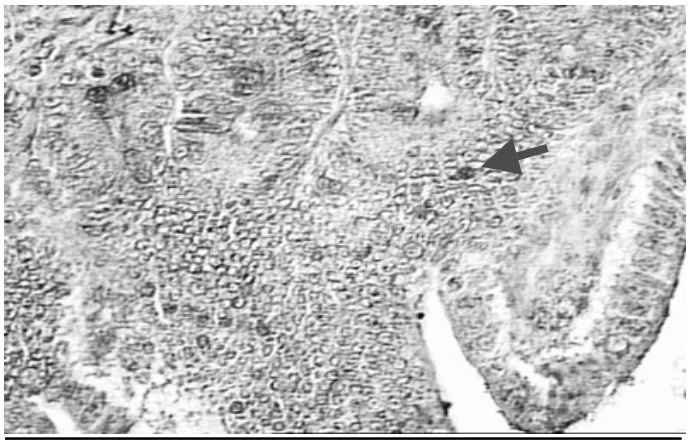

Figure 1 - Positive reaction of CD4+ lymphocytes (arrow) in the lamina propria of the cecum of 2-day-old chicks treated with Lactobacillus reuteri (kit CSA, DAB for 30 seconds, Mayer's hematoxylin counterstaining, 40x).

Table 1 presents the results of mean CD4+ lymphocyte counts obtained with treatment with

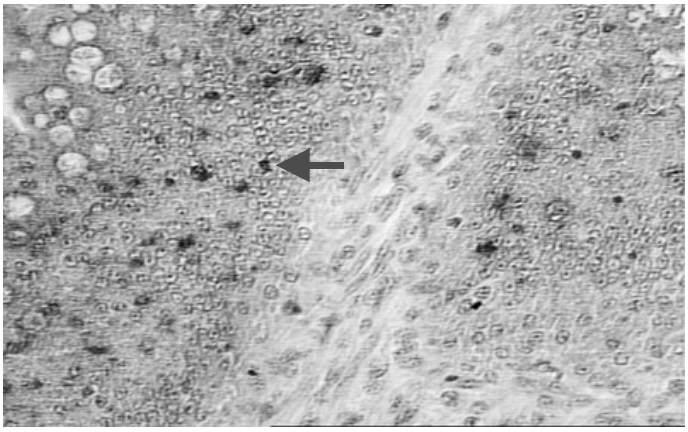

Figure 2 - Positive reaction of CD8+ lymphocytes (arrow) in the lamina propria of the duodenum of 8-day-old chicks treated with *Lactobacillus salivarius* (kit LSAB, DAB for five minutes, Mayer's hematoxylin counterstaining, 40x).

Lactobacillus spp. or CM, and challenge or not with *SE* according to bird age. The data show that at the first three ages (two, four, and eight days of age), no differences were observed (p<0.05) between the negative control and the group treated with L. salivarius and challenged with *SE*. Furthermore, it is observed that CD4+ lymphocyte mean counts of negative controls and birds treated with *L. salivarius* and *L. acidophillus* and challenged with *SE* were lower than those obtained in the other groups. However, at the fourth age (12 days of age), there was a difference (p<0.05) in CD4+ lymphocyte counts between the negative control and the groups treated with *L. reuteri*, *L salivarius*, *L. acidophilus*, or cecal microbiota and challenged with SE. There was no effect of bird age (p<0.05) on CD4+ T cell counts.

Table 1 - Mean quantities of CD4+ lymphocytes marked with specific antibody by immunohistochemistry and counted in ten random fields in each tissue section of the duodenum, jejunum, and ceca of chicks orally treated with *Lactobacillus* spp. or cecal microflora (CM) and challenged or not with *Salmonella enterica* serovar *Enteritidis* (SE) according to bird age (C.V.= 29.2%).

Treatment	Age (days)			
	2	4	8	12
Negative control	3.4BCa	3.1Ba	2.4Aa	3.9Ca
SE	8.0Bc	6.9ABb	6.2Ab	6.6Ab
L. reuteri	8.1Bc	7.8Bc	6.4Ab	6.8Ab
L. salivarius	6.7ABb	7.4Bc	6.0Ab	7.0ABb
L. acidophilus	6.7ABb	7.4Bc	5.9Ab	6.9ABb
CM	8.1Bc	7.8Bc	6.4Ab	6.8Ab
L. reuteri + SE	8.9Bc	6.5Ab	6.1Ab	6.1Ab
L. salivarius + SE	3.6Aa	3.3Aa	3.4Aa	6.1Bb
L. acidophillus + SE	3.6Aa	3.3Aa	3.4Aa	3.1Aa
CM + *SE*	8.9Bc	6.5Ab	6.1Ab	6.1Ab

Upper-case letters compare mean ages for each treatment (Row). Lower-case letters compare means of treatment for each age (Column). Means followed by at least one same letter are not significantly different (p<0.05).

Average CD4+ lymphocyte counts obtained after birds were orally treated *Lactobacillus* spp. or CM and challenged or not with *SE* in relation to the different intestinal segments of chicks are shown in Table 2. There was a difference (p<0.05) between average CD4+ lymphocyte counts between the negative controls and birds treated with Lactobacillus spp or CM, challenged or not with *SE*, in the duodenum and jejunum of chicks. In addition to the significant difference between the average CD4+ lymphocyte counts, their values are higher in the treated groups as compared to the negative control. Moreover, in relation to the ceca, there was no difference (p<0.05) between the negative control and the treatments with *L. salivarius* and *L. acidophillus* and challenged with *SE*.

Table 2 - Mean quantities counts of CD4+ lymphocytes marked with specific antibody by immunohistochemistry and counted from ten random fields in each tissue section of chicks orally treated with *Lactobacillus* spp. or cecal microflora (CM) and challenged or not with *Salmonella enterica* serovar *Enteritidis* (SE) according to intestinal segment (C.V.=29.2%).

Treatment	Segment		
	Duodenum	Jejunum	Ceca
Negative control	3.5Aa	3.1Aa	3.0Aa
SE	7.7Bc	6.8Ade	6.3Abc
L. reuteri	7.4ABc	7.9Bf	6.5Ac
L. salivarius	7.9Bc	6.6Acd	5.8Ab
L. acidophilus	7.8Bc	6.1Ac	5.8Ab
CM	7.4ABc	7.9Bf	6.5Ac
L. reuteri + SE	7.0Ac	7.1Ae	6.5Ac
L. salivarius + SE	4.5Bb	4.6Bb	3.3Aa
L. acidophillus + SE	4.5Bb	4.6Bb	3.3Aa
CM + *SE*	7.0Ac	7.1Ae	6.5Ac

Upper-case letters compare mean ages for each treatment (Row). Lower-case letters compare means of treatment for each age (Column). Means followed by at least one same letter are not significantly different (p<0.05).

Average CD8+ lymphocyte counts in the intestine of chicks, obtained after treatments with *Lactobacillus* spp. or CM and challenge or not with *SE* related to different chick ages are shown in Table 3. Analyzing CD8+ lymphocyte counts, a difference was found (p<0.05) between the negative control and the other groups, independent of oral treatment and/or challenge. The chicks treated with CM and challenged with *SE* presented the highest CD8+ lymphocyte counts in eight- and 12-day-old chicks.

Table 4 shows average CD8+ lymphocyte counts in the duodenum, jejunum and ceca of chicks treated with *Lactobacillus* spp. or CM and challenged or not with *SE*. The negative control was significantly different

(p<0.05) from the other groups, again with higher lymphocyte counts in the treated groups as compared to the negative control. Comparing mean cell counts among the different intestinal segments of the same treatment, no difference was observed (p<0.05) between the negative control and the treatments with *L. salivarius* and *L. acidophillus* challenged with *SE*. The chicks treated with CM and challenged with *SE* and only challenged with SE presented the highest CD8+ lymphocyte counts in the duodenum, jejunum, and ceca.

Table 3 - Mean quantities of CD8+ lymphocytes marked with specific antibody by immunohistochemistry and counted in ten random fields in each tissue section of the duodenum, jejunum, and ceca of chicks orally treated with Lactobacillus spp. or cecal microflora (CM) and challenged or not with *Salmonella enterica* serovar *Enteritidis* (SE) according to bird age (C.V.=26.7%).

Treatment	Age (days)			
	2	4	8	12
Negative control	9.5Ba	7.0Aa	6.8Aa	10.6Ba
SE	25.2Bg	25.4Be	24.0Bf	18.0Acd
L. reuteri	17.1Ade	13.9Ab	15.3Ab	22.0Be
L. salivarius	16.8Ad	20.4Bd	23.8Cf	15.6Ab
L. acidophilus	15.8Acd	18.0ABc	19.3Bcd	19.0Bd
CM	21.7Bf	19.8ABd	22.1Be	17.4Ac
L. reuteri + SE	18.7ABe	16.9Ac	21.0Bde	21.4Be
L. salivarius + SE	14.6Abc	14.9ABbc	17.9Bc	16.2Bbc
L. acidophilus + SE	12.4Ab	14.1ABbc	15.9Bb	15.1Bb
CM + *SE*	23.1Afg	24.9ABe	27.3Cg	25.5BCf

Upper-case letters compare mean ages for each treatment (Row). Lower-case letters compare means of treatment for each age (Column). Means followed by at least one same letter are not significantly different (p<0.05).

Table 4 - Mean quantities of CD8+ lymphocytes marked with specific antibody by immunohistochemistry and counted in ten random fields in each tissue section of chicks orally treated with Lactobacillus spp. or cecal microflora (CM) and challenged or not with *Salmonella enterica* serovar *Enteritidis* (SE) according to intestinal segment (C.V.=26.7%).

Treatment	Segment		
	Duodenum	Jejunum	Ceca
Negative control	8.8Aa	8.4Aa	8.2Aa
SE	25.3Cf	23.7Bf	20.3Ade
L. reuteri	18.5Bcd	17.7Bc	14.9Abc
L. salivarius	20.3Ade	17.7Ac	19.5Ad
L. acidophilus	20.6Bde	18.9Bd	14.3Ab
CM	20.6ABde	22.0Be	18.1Ad
L. reuteri + SE	21.4Be	20.7Bde	16.3Ac
L. salivarius + SE	16.6Abc	15.1Ab	16.0Ac
L. acidophillus + SE	14.9Ab	14.1Ab	14.1Ab
CM + *SE*	27.5Bf	25.8Bf	22.4Ae

Upper-case letters compare mean ages for each treatment (Row). Lower-case letters compare means of treatment for each age (Column). Means followed by at least one same letter are not significantly different (p<0.05).

DISCUSSION

In the present study, samples of *Lactobacillus reuteri*, *L. salivarius*, *L. acidophilus* and CM isolated from breeders supplied by oral gavage to chicks challenged or not with *SE* demonstrated capacity to stimulate the immune system, in the form of leukocytic infiltrate of CD4+ and CD8+ lymphocytes in the intestinal epithelium and lamina propria of chicks as detected by immunohistochemistry. Thus, the results obtained in the present study agree with Lillehoj & Chung (1992), who asserted that the development and composition of intestinal T-cells are influenced by the exposure to environmental antigens, suggesting that the presence of such lymphocytes in the intestinal epithelium is due to intraluminal antigenic stimulus.

A similar result was obtained by Vervelde *et al*. (1998), who identified, also by immunohistochemistry, a high quantity of leukocytic infiltrate of CD3+ lymphocytes, and particularly of CD4+ and CD8+ cells, in the epithelium and in the lamina propria of chick's intestine seven days after the treatment with a mixture of recombinant antigen of *Eimeria* and choleric toxin. Our results are also consistent with the assertion of Elwood *et al*. (1997) that in humans, swine, and rodents, T-cells are deposited as much in the intestinal epithelium as in the intestinal lamina propria. In addition to identifying these same structures, another marked similarity between the study by Vervelde *et al*. (1998) and the present study was that, in both, CD8+ lymphocyte counts were higher than those of CD4+.

McSorley *et al*. (2000) demonstrated that a significant fraction of *Salmonella*-specific CD4+ T-cells respond to the flagellar filament protein, FliC, and that this antigen is capable of protecting against lethal Salmonella infection. Choi *et al*. (1999) described the alterations in T-cell subpopulations, including CD4+, CD8+, TCR1, and TCR2 lymphocytes, as well as the transcription of IFN-γ, and TGF-ß4 mRNA in the intestine of chicks after oral inoculation with *E. acervulina*. According to Songserm *et al*. (2002), the influx of T-cells into the intestinal epithelium is related to the presence of bacteria in the intestinal lumen. Those authors suggest that cell-mediated intestinal immunity is directly related with the elimination of enteropathogens, such as *Pasteurella multocida* and Eimeira, from the intestine of chicks. Therefore, the results obtained by those researchers are consistent with the present experiment, where an increase in CD4+ and CD8+ lymphocytes population was shown four days after the beginning of treatments.

Yet, according to Songserm *et al*. (2002), in addition to bacterial stimulation, the increase in chick age is responsible for the influx of lymphocytes into the intestinal epithelium of chicks. A study performed by Lillehoj & Chung (1992) with broiler chickens verified that the percentage of CD3+ lymphocytes increased 24.4% by one week of age, 42% by the second week, and 62.6% by the fourth week. In relation to the variation in the number of CD4+ lymphocytes with chick age, the percentages obtained in the present study also agree with the findings of Lillehoj & Chung (1992), who observed a decrease in CD4+ lymphocyte counts as chicks aged. Still in relation to CD8+ lymphocyte counts, the numbers obtained by Lillehoj & Chung (1992) are different from those of present study as they showed a gradual and discrete increase in the number of these cells as the birds aged.

Thus, it can be concluded that the oral treatment with *L. reuteri*, *L. salivarius*, *L. acidophilus*, or CM of chicks challenged or not with SE elicit an immune response in the form of leukocytic infiltrate by CD8+ lymphocytes, followed by CD4+, in the intestine in the period from two to 12 days of age. CD8+ lymphocyte counts were significantly higher in the intestine of chicks treated with CM and challenged with SE. The duodenum, followed by the jejunum, were the segments in which the immune response by T, CD4+, and CD8+ cells was stimulated with the strongest intensity.

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