ORIGINAL ARTICLE

CD19 LYMPHOCYTE PROLIFERATION INDUCED BY *Bifidobacterium animalis* subsp. *lactis* IN C57BL/6 MICE EXPERIMENTALLY INFECTED WITH *Toxoplasma gondii*

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SUMMARY

Toxoplasmosis is frequently acquired through the oral route by the ingestion of cysts or oocysts of *Toxoplasma gondii*. Once ingested, the parasites penetrate the intestinal epithelial cells and rapidly disseminate to all organs in the host. During *T. gondii* infection, the intestinal microbiota plays an important role in stimulating a protective immune response against the parasite. In this sense the use of probiotics is worthy of note since they are live microorganisms that have beneficial effects on the host through stimulation of the immune response that can be important in the control of *T. gondii* proliferation and dissemination in the host. In the present study, the action of the probiotic *Bifidobacterium animalis* subsp. *lactis* was investigated in C57BL/6 mice infected with oocysts of ME49 strain of *T. gondii*. The probiotic had an immunomodulatory action, inducing CD19 lymphocyte proliferation and consequently increasing anti-*T. gondii* antibody level. *Bifidobacterium animalis* subsp. *lactis* provided protection in supplemented mice, compared to the control group. In addition, supplemented animals had milder inflammatory process in the small intestine, indicating that the probiotic protects the intestinal mucosa during infection with *T. gondii*. It was concluded that the probiotic *B. animalis* subsp. *lactis* induces humoral immune response capable of providing protection against *T. gondii* infection.

KEYWORDS: *Toxoplasma gondii*; *Bifidobacterium animalis* subsp. *lactis*; Immunomodulation; CD19 lymphocytes; IgG antibodies.

INTRODUCTION

Toxoplasma gondii is the etiologic agent of toxoplasmosis, a protozoonosis of worldwide distribution, which has caused high morbidity and mortality rates, especially for immunosuppressed individuals, constituting a serious public health problem. This disease has two phases: in the acute phase, there is rapid proliferation of tachyzoites, which occasionally causes symptomatology; in the chronic phase, the parasites form cysts that may persist for the whole life of the host in tissues such as the eye, the muscles and the central nervous system[1].

An effective immune response plays an important role in the resistance to the disease. However, the immunological mechanisms of resistance to *T. gondii* infection have not been fully elucidated. The hosts control *T. gondii* infection by inducing a potent immunity mediated by TCD4+ and TCD8+ cells, the secretion of cytokines such as tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) which are essential to control the parasite proliferation and dissemination[2,3]. In addition, the increased humoral immune response will lead to a higher production of anti-*T. gondii* IgG antibodies, and the high IgG levels plays an important role in the protection against *T. gondii* infection, reducing the level of infection by this parasite[3-5].

The intestinal microbiota plays a key role in the development, maturation and modulation of the immune system during infections, and studies in this field have attracted much interest in the last years[6]. In C57BL/6 mice, bacteria of the intestinal microbiota stimulate the development of a protective T helper type 1 (Th1) immune response through toll-like receptors, providing resistance against *T. gondii* infection[7].

For the host's protection against pathogens, the intestinal microbiota can be therapeutically manipulated by means of the administration of several microorganism species, which are called probiotics[8]. The mechanisms of action of probiotics have been recently assessed in infections with protozoa such as *Giardia duodenalis*, *Cryptosporidium parvum* and *Eimeria tenella*[9-11], and in infections with nematodes such as *Toxocara canis* and *Strongyloides venezuelensis*[12,13], yielding promising results.

Considering *T. gondii* infection, only two studies have shown the effects of probiotics. Mice vaccinated with *T. gondii* cytoskeleton

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proteins using *Lactobacillus casei* as adjuvant, had a protective immune response and greater anti-*T. gondii* IgG production[14]. In a second study, immunosuppressed female Wistar rats supplemented with the probiotic *Bifidobacterium animalis* subsp. *lactis* were capable of synthesizing IFN-γ and survived after inoculation of *T. gondii* RH strain, whereas immunosuppressed rats that were not supplemented with the probiotic died five days after the parasite inoculation. These results demonstrate that the immunomodulatory activity of *B. animalis* subsp. *lactis* can be beneficial, especially in individuals infected with *T. gondii*[15].

The aim of this study was to evaluate the immunomodulatory action of *B. animalis* subsp. *lactis* in *T. gondii*-infected mice by quantifying the populations of CD4+, CD8+ and CD19+ lymphocytes in Peyer's patches and by determining the serum antibody level, in order to correlate the immunological profile of animals with protection against *T. gondii* infection.

MATERIAL AND METHODS

Probiotic: *Bifidobacterium animalis* subsp. *lactis* (BB-12®; Christian Hansen) was activated in culture medium De Man Rogosa Sharpe[16] (Oxoid Ltd., Basingstoke, UK), modified with 0.02% sodium carbonate and 0.01% calcium chloride hydrate, and supplemented with 0.05% L-cysteine hydrochloride. Following incubation at 37 °C for 24 hours, *B. animalis* subsp. *lactis* was resuspended in milk at 1.6 x 10^8 CFU/mL.

Animals: This experimental protocol was approved by the Research Ethics Committee of *São Paulo* Institute of Tropical Medicine, University of *São Paulo* (CPE-IMT 2011/125). Male isogenic C57BL/6 mice, weighing approximately 20 g, were purchased from the Animal Facility Center of the School of Medicine, University of *São Paulo*. The animals were kept in the Laboratory of Protozoology of the *São Paulo* Institute of Tropical Medicine, University of *São Paulo*, inside polypropylene boxes containing autoclaved pine shavings; they received commercial animal food Nuvital® (Nuvital Nutrientes S/A, Colombo, PR, Brazil) and water *ad libitum*.

Oocyst production: Cats were infected with cysts of *T. gondii* ME49 strain to allow oocyst formation. Each cat received by gavage 800 tissue cysts of *T. gondii* obtained from previously infected mice[17]. Cats were kept in individual cages and received water and animal food *ad libitum*. Oocyst production was assessed from the 1st day post-infection (PI) to the 20th day PI. Feces were daily collected from each animal for oocyst detection based on the Sheather's method[18]. Every time oocysts were detected, feces were mixed at a proportion of 1:1 with 2.5% sulfuric acid and maintained at room temperature during five days for sporulation. The quantity of oocysts was determined in a Neubauer counting chamber[19]. Oocysts were stored at 4 °C.

Experimental design: For the experiment, nine groups of C57BL/6 mice containing eight animals each were used. Four groups of animals infected with *T. gondii* and four groups of non-infected animals (control groups) were daily supplemented with 0.1 mL of milk containing 1.6 x 10^7 CFU of *B. animalis* subsp. *lactis* or with 0.1 mL milk only. In addition, one group of animals that were neither infected nor supplemented with the probiotic or milk was used as a control. Supplementation of animals started on day 0 and continued until day 45 of the experiment. On day 15 of the experiment, mice were orally infected with 10^2 oocysts of *T. gondii*

ME49 strain. The animals were euthanized in a CO_2 chamber on the 21th day, i.e., seven days PI with *T. gondii* (acute phase of toxoplasmosis) or on the 45th day, i.e., 30 days PI with *T. gondii* (chronic phase of the disease). Blood samples were collected from the animals for the determination of anti-*T. gondii* antibodies. From each mouse, the brain and the intestine were removed for the search of cysts and determination of the lymphocytes populations.

T. gondii antigen preparation: Tachyzoites of *T. gondii* RH strain were harvested from the peritoneal cavity of previously infected mice by PBS washes; suspensions were filtered through a 5 μm polycarbonate filter, and centrifugation was used to recover parasites, which were counted and re-centrifuged. Pellets were suspended in ice-cold water at a parasite density of 10^7 tachyzoites/ mL and subjected to sonication until complete cell lysis[20].

Detection of specific antibodies in the serum of mice: The ELISA technique was used to detect anti-*T. gondii* IgG levels and confirm the infection in the acute and chronic phases. A 96-well polystyrene plate was sensitized with 100 μL of *T. gondii* antigen diluted in 0.1M carbonate-bicarbonate buffer (pH 9.5) and kept overnight in a humid chamber at 4 °C. Then, the plate was washed five times with 0.02% PBS-Tween and blocked with PBSTL solution (PBS containing 0.05% Tween-20 and 0.3% skimmed milk) during 1 hour in an oven at 37 °C. After blockage, 100 μL of the serum from each animal, at 1/100 dilution, were added to each well, and the plate was incubated at 37 °C for 1 hour. Subsequently, the plate was washed five times with PBSTL and received 100 μL/ well of anti-mouse IgG conjugate at 1: 20,000 dilution (Sigma-Aldrich®, St. Louis, MO, USA), followed by incubation for 1 hour at 37 °C. Then, the plate was washed again five times with PBSTL. The reaction was revealed by adding 100 μL of OPD (O-phenylenediamine 1 mg/mL, 0.03% H_2O_2 in 0.2M phosphate-citrate buffer, pH 5.0) for 30 minutes and interrupted by adding 50 μL of 4N HCL. Absorbance of each well was determined in an automatic microplate reader (Multiskan MS® Labsystems Vienna, USA) at 492 nm[21].

Determination of lymphocytes populations in mice: Peyer's plates were removed from mice infected with *T. gondii* and from control animals to determine the population of CD19+ lymphocytes, CD3+CD4+ lymphocytes and CD3+CD8+ lymphocytes by means of flow cytometry. This was obtained by dissociating the intestine of animals under a sterile laminar flow to remove the Peyer's patches on RPMI 1640 culture medium (Sigma®). After centrifugation of isolated cells at 2.800 rpm for 15 minutes (centrifuge Z36HK, Hermle Labortechnik®), supernatant was discarded and cells were re-suspended in 1 mL ISOTON®, counted in a Neubauer chamber and, finally, adjusted to a concentration of 10^6 cells/ mL. Then, the cells underwent surface labeling by using monoclonal antibodies for differentiation of lymphocyte populations: CD3 Pacific blue (BD Biosciences), CD4 Horizon V500 (BD Biosciences), CD8 APC-Cy7 (BD Biosciences) and CD19 PE-Cy7 (BD Biosciences). The cells were then incubated for 30 minutes at 4 °C in the absence of light. After this incubation period, 300 μL of ISOTON® solution were added and the analysis was carried out in a flow cytometer (LSRFortessa BD). A total of 10.000 events were captured by using BD FACSDIVA software and analyzed by Flowjo X software.

Brain cyst research: After 30 days of infection, mice euthanized in the chronic phase of toxoplasmosis had their brain removed and

homogenized in 10 mL of sterile saline. Cysts were counted under a phase contrast microscope[11]. Infection was defined as the presence of cysts and the disease was expressed as the number of cysts/ brain for each challenged animal. Infection protection was defined as the proportion of non-infected challenged animals, while disease protection was considered as the percentage drop of cyst counts in the brains of animals after the challenge[5].

Histological analysis: The intestine of infected animals and control groups were removed, fixed with 10% formaldehyde and embedded in paraffin. Then, 7-μm sections of the material were stained with hematoxylin-eosin and examined for inflammatory changes.

Statistical analysis: The obtained quantitative values such as the level of antibodies, number and percentage of immune cells and number of cysts were statistically assessed by analysis of variance (ANOVA), employing the statistical package GraphPad Prism 6® (GraphPad Prism software, Inc. San Diego, CA, USA). All comparisons were performed at 5% significance level.

RESULTS

Serum humoral immune response in mice: Anti-*T. gondii* IgG levels were determined by the ELISA technique in the serum of mice infected with *T. gondii* oocysts. Serum samples were collected from the animals at seven and 30 days PI. Specific IgG antibody production in the serum of mice is shown in Table 1. There was a greater increase in antibody levels and progression for animals supplemented with *B. animalis* subsp. *lactis*, compared to animals supplemented with milk alone ($p < 0.01$; $p < 0.001$, respectively).

CD4+, CD8+ and CD19+ lymphocyte populations in mice: The influence of *B. animalis* subsp. *lactis* supplementation in C57BL/6 mice challenged with *T. gondii* oocysts was verified by labeling and quantifying immune cell populations by flow cytometry. The proportion of C19+ cells was greater in animals supplemented with *B. animalis* subsp. *lactis* in the chronic phase of toxoplasmosis, compared to control animals ($p < 0.05$). The proportion of CD4+ T cells was smaller in infected mice supplemented with probiotics, compared to infected animals supplemented with milk ($p < 0.001$). Regarding CD8+ T cells, there was a decrease in the population during the infection of animals supplemented with probiotics; however, there was no statistical difference among the evaluated groups (Fig. 1).

Action of probiotics in cystogenesis: To assess whether *B. animalis* subsp. *lactis* supplementation influences *T. gondii* cystogenesis process,
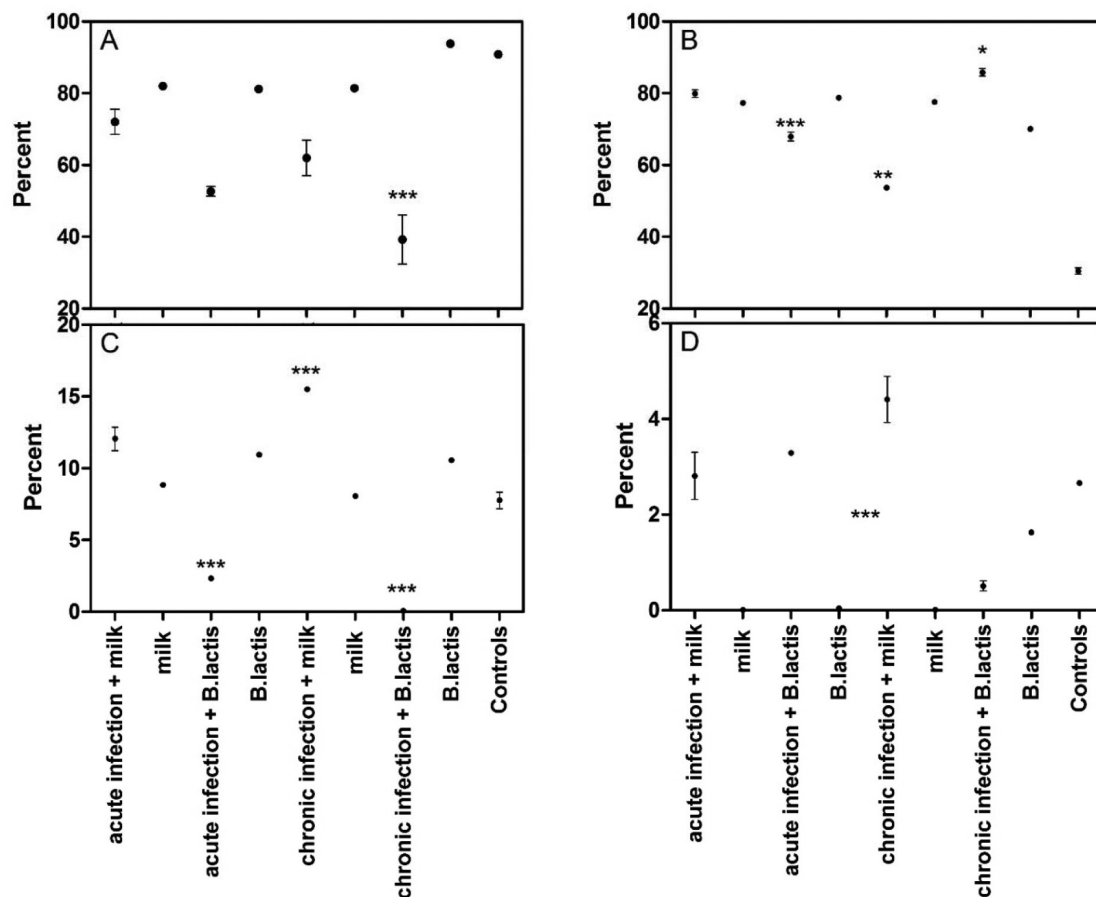
Fig. 1 - Percentage of intestinal free cells during experimental toxoplasmosis in animals receiving *B. animalis* subsp. *lactis* or milk. (A) Lymphocytes total; (B) CD19+ lymphocytes; (C) CD4+ lymphocytes; (D) CD8+ lymphocytes. Bonferroni´s post-test after $p < 0.05$ in ANOVA assays of two individual experiments. (*) represents $p < 0.05$; (**) represents $p < 0.01$; (***) represents $p < 0.001$ in comparison with the adequate control group.

cysts from the brain of animals infected with *T. gondii* strain ME49 and supplemented with probiotics or milk were counted. The number of brain cysts was smaller in the group supplemented with probiotics, compared to the group supplemented with milk ($p < 0.05$). In addition, the number of animals supplemented with probiotics that showed absence of brain cysts was greater when compared to those supplemented with milk (Table 1).

Table 1
Quantitative data on infection and antibody production during experimental toxoplasmosis for animals receiving *B. animalis* subsp. *lactis* or milk

| Infection	Diet	Brain Cysts Mean ± S.E.M.	Anti *T. gondii* IgG (ELISA O.D.)
Acute	Milk	ND	0.423 ± 0.047
	Probiotic	ND	1.218 ± 0.185**
Chronic	Milk	65.00 ± 16.80	1.100 ± 0.093
	Probiotic	30.00 ± 12.54*	3.227 ± 0.052***

T test significance; (*) represents $p < 0.05$; (**) represents $p < 0.01$; (***) represents $p < 0.001$

Probiotic attenuates the inflammatory process in the intestinal mucosa of mice: The epithelium of the small intestine of mice infected with *T. gondii* was intact although, inflammatory infiltrates were present. Nevertheless, the infiltrates were restricted to the intestinal villi of animals supplemented with *B. animalis* subsp. *lactis* (Fig. 2) whereas they were found in the villi and crypts of Lieberkühn in animals supplemented with milk (Fig. 3), indicating a more severe inflammatory process in the latter. Peyer's patches were only found in the intestine of probiotic-supplemented mice, evidencing lymphocyte proliferation (Fig. 2).

DISCUSSION

Lately, much attention has been drawn to probiotics for their potential therapeutic application against several diseases caused by parasites. However, much of the effects of these probiotic microorganisms on parasitic infections has not been fully elucidated. In the present study, the action of the probiotic *B. animalis* subsp. *lactis* on *T. gondii* infection was assessed in C57BL/6 mice. To verify whether the animals were infected after challenge with *T. gondii* oocysts, serum was collected from all the animals to determine anti-*T. gondii* IgG levels. All the animals inoculated with oocysts were seropositive to *T. gondii*. However, anti-*T. gondii* IgG production was greater in animals supplemented with *B. animalis* subsp. *lactis*, and these higher IgG levels were related to the larger numbers of CD19+ lymphocytes found in probiotic-supplemented animals, indicating that *B. animalis* subsp. *lactis* had an immunomodulatory action, and induced humoral immune response in infected mice.

Some probiotics have already been reported to induce protective humoral immune response, increasing specific IgG levels during infection with parasitic protozoa. Broiler chickens infected with *Eimeria acervulina* or *Eimeria tenella* and supplemented with the probiotic microorganisms *Pediococcus acidilactici* and *Saccharomyces boulardii* had higher levels of specific IgG against these protozoa, and a reduced the number of excreted *Eimeria* oocysts was also found[22]. The present study is the first report of increased IgG levels and CD19+ lymphocyte
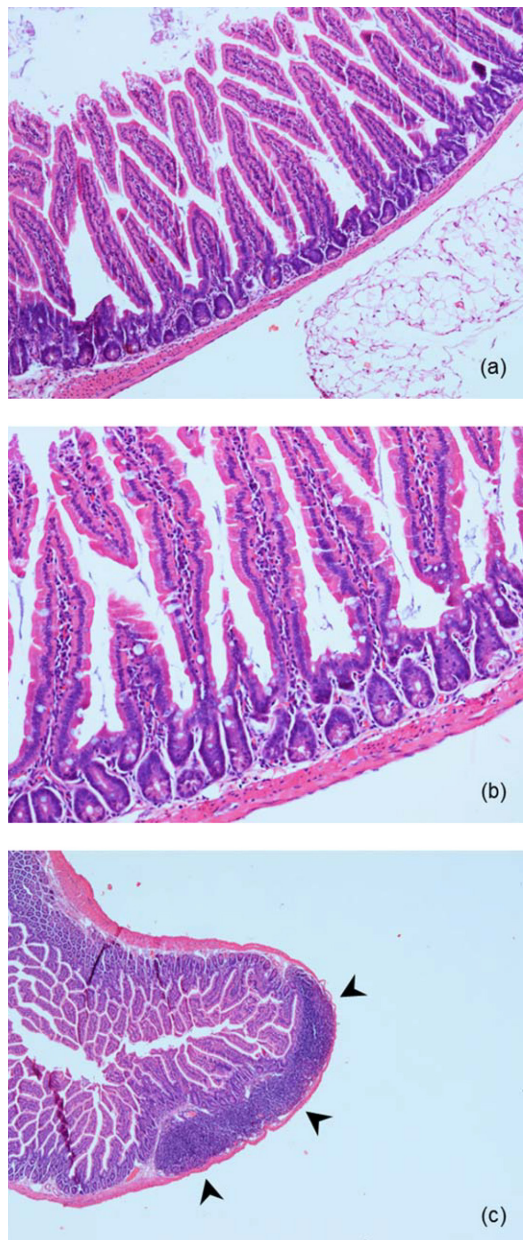
Fig. 2 - Histological images of the small intestine of C57BL/6 mice challenged with oocysts of *T. gondii* ME49 strain and supplemented with probiotic. (a) Intestine of animals in the acute phase of toxoplasmosis. Intact intestinal villi and presence of inflammatory infiltrate in the villi (HE x 100). (b) Intact villosities, inflammatory infiltrate in the villi and discrete edema in the intestinal mucosa of mice in the chronic phase of infection (HE x 200). (c) Peyer's patches in the intestine of a mouse in the chronic phase of toxoplasmosis (arrowhead) (HE x 40).

population induced by the immunomodulatory action of *B. animalis* subsp. *lactis* during *T. gondii* infection.

A humoral immune response with anti-*T. gondii* IgG production has been considered necessary to protect mice against *T. gondii* infection. Azzouz *et al.*[2] noted that immunized BALB/c mice had a great production of anti-*T. gondii* IgG, assuring their survival during infection with *T. gondii* RH strain.
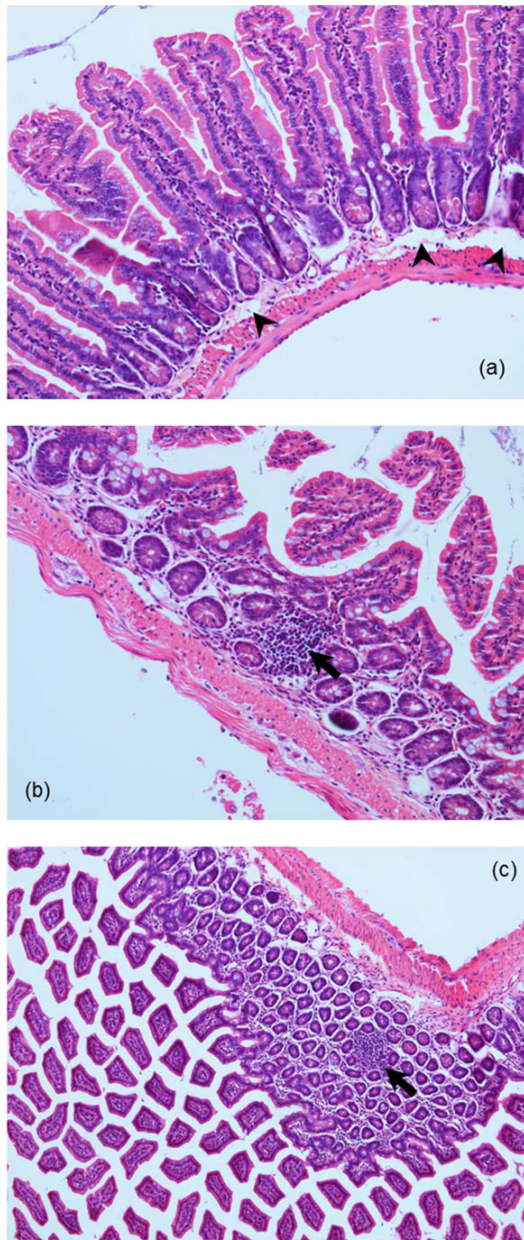
Fig. 3 - Histological changes in the small intestine of mice infected with *T. gondii* ME49 strain and supplemented with milk. (a) Intestine of animals in the acute phase of toxoplasmosis. Intact intestinal villosities, presence of inflammatory infiltrate and pronounced edema in the intestinal mucosa (arrowhead) (HE x 100). (b) Chronic phase of toxoplasmosis. Villi and crypts of Lieberkühn with inflammatory infiltrates (arrow) and presence of a pronounced edema in the intestinal mucosa (HE x 200). (c) Presence of inflammatory infiltrate in the crypts of Lieberkühn in the chronic phase of toxoplasmosis (arrow) (HE x 100).

Protection against *T. gondii* infection can be either qualitative, i.e., can be determined based on the frequency of investigated animals showing absence of cysts in their brain, or quantitative, which can be evidenced by a decrease in the parasite load. In this experiment, quantitative protection was verified by counting the tissue cysts present in the brain of animals infected with *T. gondii* and supplemented with probiotics or milk. C57BL/6 mice challenged with *T. gondii* and

supplemented with *B. animalis* subsp. *lactis* had a smaller number of cysts in the brains, compared to mice that did not receive the probiotic, indicating a less severe infection in comparison with the control animals ($p < 0.05$). Considering the qualitative protection, a larger number of animals supplemented with the probiotic did not have tissue cysts in their brains, compared to control animals. Therefore, quantitative and qualitative protection can be related to the humoral immune response induced by *B. animalis* subsp. *lactis*, since those animals showed higher anti-*T. gondii* IgG levels.

The results of the present study corroborate those described by Zorgi *et al*.[5], who observed that a humoral response with IgG production provided quantitative protection to immunized BALB/c and C57BL/6 mice challenged with *T. gondii* ME49 strain. Other studies have shown similar conclusions since immunized mice challenged with *T. gondii* have produced cell and humoral immune responses with high IgG titers, resulting in reduced formation of brain cysts[3,4].

In addition, *B. animalis* subsp *lactis* provided intestinal mucosa protection since probiotic-supplemented animals showed less severe inflammatory processes, compared to control animals (Fig. 2, Fig. 3). Although the mechanism of action of probiotics in regulating inflammation has not been elucidated, they are believed to interact with immunocompetent cells regulating the production of pro-inflammatory cytokines[23,24]. In *T. gondii* infection, CD4+ lymphocytes play their protective role by synthesizing IFN-γ, which gives the host resistance to infection by this parasite; however, the development of a strong Th1-type response with a large number of CD4+ cells and excessive IFN-γ production is related to several intestinal inflammatory changes associated with the mice mortality[27]. The small number of CD4+ cells verified in this study can be related to a less severe inflammatory process observed in animals supplemented with the probiotic, but future studies must be carried out to elucidate the role of *B. animalis* subsp *lactis* in the regulation of inflammation during *T. gondii* infection.

CD8+ T cells are considered important in the protection against *T. gondii* due to their cytotoxic activity in cells infected with this parasite. There was a reduction in the number of CD8+ T cells in probiotic-supplemented mice, but there was no statistical difference among the assessed groups, indicating that the probiotic did not influence the population of those cells.

In conclusion, *B. animalis* subsp. *lactis* has a immunomodulatory activity since it induced humoral immune response with B lymphocyte proliferation and increased anti-*T. gondii* IgG level in animals infected with *T. gondii*. This immune response provided quantitative and qualitative protection in mice with toxoplasmosis. *B. animalis* subsp. *lactis* has attenuated the inflammatory process, and protected the intestinal mucosa of mice during toxoplasmosis.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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