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Lactobacillus rhamnosus V5 Prevents *Salmonella enterica* Serovar Typhimurium Invasion in Cell Culture and Mice Infection

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Authors' contributions

This work was carried out in collaboration among all authors. Author CTT performed lactobacilli growth; author PCDS worked with animals in vivo assay; author EKN performed statistical analysis; author RSA identified lactobacilli (16S); author LAP designed the in vivo assays; author AASB performed bacteriocin assay; author SG isolated lactobacilli; authors DOP and EJAA performed histological analysis; author MCG worked with cell culture (adhesion and invasion); author RKTK performed the results analsysis; author GN designed the study. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of this study was to evaluate the protective capacity of the exopolysaccharide-producing *Lactobacillus rhamnosus* V5 against invasion *in vitro* and *in vivo* with *S. typhimurium*.
Methodology: We tested the antimicrobial activity of the compound extracted from the

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lactobacilli against *S. typhimurium* directly, also we tested the interference of this compound in *S. typhimurium* adherence and invasion of HeLa and HEp-2 cells (*in vitro* testings). For *in vivo* experiments, we used 16 BALB/c female mice. Through gavage method we introduced *L. rhamnosus* as probiotic and then infected mice with *S. enterica* serovar typhimurium. After euthanasia, spleen, liver and Peyer's patches removed for microbiological and histopathological analysis.

Results: The results showed that lactobacilli were able to produce antimicrobial compounds against *S. typhimurium*. These lactobacilli inhibited the adhesion and invasion of *S. typhimurium* in HeLa and HEp-2 cells, respectively. The challenge assay in the murine model demonstrated a decrease in pathogen translocation in the spleen and liver from mice treated with probiotic as well as protection of ileal tissue in lactobacilli-treated mice. The histopathological analysis demonstrated the presence of prominent lymphoid nodules in the ileum from the non-treated lactobacilli mice.

Conclusion: Our results suggest that *L. rhamnosus* improved the effectiveness of the intestinal barrier and, thus, could be a potential probiotic to control salmonellosis.

Keywords: Bacteriocins; protection; lactic acid bacteria; adhesion assays; murine model; histopathological analysis.

1. INTRODUCTION

Probiotics are live microorganisms that confer a health benefit for the host through the production of bioactive compounds or equilibrating the gastrointestinal tract microbiota [1] when administered in adequate amounts [2]. Probiotics are usually incorporated into nutritional supplements and pharmaceutical products [3]. The use of probiotics is advantageous by virtue of the property of non-selection of multidrug resistant bacteria, especially for broad-spectrum antimicrobials [4]. Probiotics has not been associated with side effects, they rarely cause a complication in healthy hosts, but they should be used with caution in patients with serious illnesses or in severely immunocompromised people [5]. Probiotics can also act as an alternative growth promoter in animal production [6].

Besides these positive effects, probiotics are useful for prevention and treatment of gastrointestinal diseases, such as irritable bowel syndrome [7], inflammatory bowel disease [8], necrotizing enterocolitis [9], food allergy [10], and infectious diarrhea [11], as well as presenting great effectiveness in the treatment of rotavirus and pouchitis [5].

Lactobacillus and Bifidobacterium are the most common genus of probiotics [12] Lactobacillus belongs to the group of lactic acid bacteria (LAB) that is composed of Gram-positive, non-sporulating, anaerobic or facultative aerobic cocci or rods, which produce lactic acid as one of the main fermentation end products of the catabolism of simple carbohydrates [13]. This lactic acid reduces the pH of the intraluminal environment and inhibits multiplication of pathogenic bacteria. In this sense, it is suggested that organic acids can penetrate the bacterial cell wall and change their normal physiology of species of microorganisms [4]. LAB can provide immune-modulating and immune-stimulating activities [14] or non-immune mechanisms [15]. They can exert direct antimicrobial activity against pathogens by increasing phagocytosis [16], modifying and enhancing the cytokine production [17,18]. The strength in prevention or treatment by LAB probiotics is well demonstrated in investigations concerning *Helicobacter pylori* gastroenteritis, cancer [19,20], lactose intolerance [21] and C. albicans in oral candidiasis [22]. Found in normal intestinal microbiota, *Lactobacillus rhamnosus* is a potential probiotic essential for gut homeostasis and capable of benefit dysbiosis-related diseases [23]. Recent studies have proven that Lactobacillus administration prevents intestinal infection with *Salmonella*, and also act as a probiotic agent capable to attenuate severity of salmonellosis [24,25,26].

According to the Centers for Disease Control and Prevention (CDC) of the United States [27], approximately 1.2 million illnesses are caused by *Salmonella* spp. every year, causing 19,000 hospitalizations and 380 deaths. Children up to four years old are the most likely to contract salmonellosis. The *Salmonella* genus consists of only two species. According to the Kauffman-White classification system, *Salmonella* enterica has more than 2,600 serovars [28]. The serovar

Salmonella typhimurium induces rapid host death, mainly in susceptible hosts [29]. It causes a considerable number of human diseases in developed nations [30] and variants of *S. enterica* serovar typhimurium have been described as causing highly invasive illnesses in Africa [30,31].

S. enterica is one of the most common causal agents of foodborne illnesses associated with the consumption of fresh leafy vegetables [32], tomatoes, alfalfa sprouts, and orange juice [33,34]. This pathogen can be ingested in beef, pork, turkey and principally in chicken, due to the ubiquity of bacteria and its capacity to grow at a wide range of temperatures: from 7 to 45°C [27]. *S. enterica* serovar typhimurium can resist the low pH of gastric secretion, invade and translocate from the intestinal barrier, and survive inside macrophages [35,36]. Robinson [29] proposes that this pathogen induces the production of type I interferon, which drives necroptosis of macrophages and allows them to evade the immune response. In this report, we explored the protective ability of a strain of *Lactobacillus rhamnosus* against the invasion *in vitro* and *in vivo* by *S. enterica* serovar typhimurium.

2. METHODOLOGY

2.1 DNA Extraction and PCR Amplification

The total genomic DNA of Lactobacillus rhamnosus V5 was extracted using the Puregene® Blood core kit B (Qiagen, Hilden, Gemany) according to the manufacturer's instructions. Bacterial ribosomal subunits 16S primers were used in this study (primers set: 16S Fw: 5'-GAGTTTGATCCTGGCTCAG-3' and 16S Rev: 5'-AGAAAGGAGGTGATCCAGCC-3'). The PCR melting temperature was 59°C. A PCR reaction mixture contained: 2 µL of extracted template DNA (50 ng), 2 µL of dNTPs (0,2 mM; Invitrogen, USA), 0.2 µL of Taq High Fidelity (5 U/µL; Invitrogen, USA), 5 µL of buffer (10 x; Invitrogen, USA), 3 µL of MgSO4 (50 mM; Invitrogen, USA) and 37 µL of deionized water, totaling a final volume of 50 µL. The PCR cycles consisted of 94°C of initial denaturation for 5 min, 35 cycles of 94°C for 1 min, 59°C for 1 min and 68°C for 2 min, followed by 10 min of final extension at 68°C. PCR products were analyzed by agarose gel electrophoresis in 1% in TAE (20 mM Tris acetate, pH 8.0; 0.5 mM EDTA) at 80 V and 400 mA for 30 min. After that, the DNA was extracted from the gel and purified using quick gel extraction Kit PureLink TM (Invitrogen, USA).

2.2 Pathogenic Bacteria

The mice were infected with an attenuated pathogen, *S. typhimurium* χ3985 UK1 (ΔcyaΔcrp) strain from the Center for Infectious Diseases and Vaccinology, Biodesign Institute and School of Life Sciences, Arizona State University, Tempe, AZ, United States of America [36]. The use of an attenuated strain allows evaluation of the process of translocation in the mouse, which would not be possible with a virulent strain. *S. typhimurium* χ3985 UK1 (ΔcyaΔcrp) has a deletion in the adenylate cyclase and cyclic AMP receptor protein [37], however, it continues with its immunogenic action, being able to infect and persist in the organs of mice, such as in the Peyer's patches, spleen, and liver. These bacteria were grown in Luria-Bertani (LB) broth (Difco, Franklin Lakes, NJ, USA) at a temperature of 37°C for 18 h.

2.3 Probiotic

The *Lactobacillus rhamnosus* V5 strain was obtained from a mixture of various bacteria from "Viili" given in Department of Food Science and Technology, Agricultural Science Center, State University of Londrina, Londrina, Paraná, Brazil. The *L. rhamnosus* strain was grown in De Man, Rogosa and Sharpe (MRS) broth medium (Difco, Franklin Lakes, NJ, USA), at a temperature of 37°C, in an atmosphere of 5% (v/v) CO2 for 18 h.

2.4 Adhesion and Invasion in Cell Culture

2.4.1 Cultivation of HEp-2 and HeLa cells

Cell cultures were grown in a 24-well plate (BD Falcon, Bedford, MA, USA) in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum which was incubated in a 5% CO2 atmosphere at a temperature of 37°C, for 48 h. The cell monolayer was grown for approximately 24 h at 37°C with 5% CO2 to give at least 80% confluency.

2.4.2 Inhibition of bacterial adhesion

The assay was performed according to the methodology described by Cravioto and collaborators [32]. HeLa cells were cultured in a 24-well plate (BD Falcon microplates, Bedford,

MA, USA) on sterile round coverslips (13 mm in diameter) that were placed before the cells.

First, we added 10^7 cfu of lactobacilli into a well to 1 mL of DMEM, for 2 h in the CO2 oven (5%), at a temperature of 37°C. Next, we added 10^7 cfu of *S. typhimurium*, leaving the well for 3 h under the same conditions. After the period, the monolayers were washed with sterile 0.05 M phosphate buffered saline (PBS, pH 7.4) and incubated for another 3 h. Then, we washed the coverslips five times with PBS, fixed with methanol (Merck, Darmstadt, Germany) for 10 min, and stained with May-Grunwald (Sigma-Aldrich, St. Louis, MO, USA) and Giemsa (Sigma-Aldrich, St. Louis, MO, USA). The slides were examined under a light microscope using an oil immersion lens. Finally, we quantified adhered bacteria for each 100 HeLa cells from different fields of the coverslip.

2.4.3 Inhibition of bacterial invasion

Invasion testing by *S. enterica* serovar Typhimurium strain and inhibition of invasion by *L. rhamnosus*. were performed according to Sansonetti and collaborators [38,39]. First the HEp-2 cells were washed twice with Phosphate Buffered Saline - PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.76 mM KH2PO4, pH 7.4) thereafter they were added to 50µL medium and a suspension containing 108 cfu/mL of *L. rhamnosus* was incubated for 2 h in a CO2 incubator (5%) at 37°C. Next, 100 µL of suspension containing 107 cfu/mL of *S. typhimurium* invading bacteria was added and left to act for 2 h. After this period the plate passed through the washing process as described above. Then 1 mL of gentamicin (Sigma-Aldrich, St. Louis, MO, USA) was added to each well in a concentration of 100 µg /mL and allowed to act for 2 h. The aim of the antibiotic is to kill the bacteria that did not invade the cells.

After the effect of the antibiotic, the plate was passed 3 times through wash steps, after which 500 µL of 1% Triton (Sigma-Aldrich, St. Louis, MO, USA) was added and left to act for 5 min, to lyse the cell function to release the invading bacteria. Next, 100 µL of each well was withdrawn and transferred to a microtube. From this serial dilution (10-1, 10-2,10-3) was carried out and plated in triplicate in MacConkey (MC) agar (Difco, Franklin Lakes, NJ, USA). Analysis was performed after incubating for 24 h at a temperature of 37°C.

2.5 Antibacterial Activity of the Supernatant

2.5.1 Obtaining supernatant from *Lactobacillus rhamnosus*

L. rhamnosus V5 strain was grown in a tube containing 10 mL of MRS broth (Difco, Franklin Lakes, NJ, USA) at a temperature of 37°C, for 18 h. After growth, the culture was centrifuged at 7000 rpm for 10 min. The clear supernatants obtained were used in the experimental trials as follows: (I) Clear supernatants were filter sterilized through membrane filtration, 0.22 µm pore size and 25 mm diameter (Millipore, Billerica, MA, USA), and used in the assay. (II) The pH of clear supernatants was adjusted to pH 6.5–7.0 with 0.1 NaOH and used in the assay after filter sterilization. (III) Clear supernatants with no treatment were subjected to heat treatment at 100°C for 10 and 20 min and then used in the assay after filter sterilization [40].

2.5.2 Antimicrobial susceptibility testing

After obtaining supernatants (I, II, III), susceptibility testing was performed to determine the Minimal Inhibitory Concentration (MIC) using the microdilution method, as standardized by the National Committee for Clinical Laboratory Standards - CLSI [41]. The test was performed in triplicate in a 96-well plate, with a U-bottom shape.

S. enterica serovar Typhimurium was initially cultivated in Nutrient Agar (AN) (Difco, Franklin Lakes, NJ, USA) at 37°C for 18 h and was then standardized to 0.5 McFarland standard (corresponding to ≅ 1.5 x 108 bacteria/mL) and diluted 1:100 in saline (0.9% NaCl) to reach a concentration of approximately 106 cfu/mL. In the positive control, Müller-Hinton (MH) broth (Difco, Franklin Lakes, NJ, USA) medium and the bacteria were added, while in the negative control only MH broth was added. Microdilutions were made with the supernatant final concentrations ranging from 0.62 to 20%. Each well was inoculated with 50 µL of the bacterial suspension prepared above, (bacteria final concentration: ≅ 5x105 cfu/mL) and the 96-well plate was incubated at 37°C for 24 h and the bacterial growth visually assessed.

2.5.3 Time-kill curve

After analyzing the MIC of the supernatant (I) a time-kill curve was performed. *S. typhimurium*

assay was grown in NA (Difco, Franklin Lakes, NJ, USA) medium and incubated at 37°C for 18 h after bacterial growth. The culture was adjusted to a concentration of ≅ 1.5 x 108 bacteria/mL (0.5 of McFarland) and 10 µL placed in three microtubes, with supernatant concentrations of 20%, 10%, and the control, containing the bacterium and MH broth. The microtubes were incubated at 37°C and evaluated at the following moments; 0 h, 2 h, 4 h, 7 h, 10 h, and 24 h. This evaluation consisted in serial dilutions and triplicate plating in MH agar medium were performed in each period. The cfu count was performed after 24 h of incubation at 37ºC.

2.5.4 "Spot-on-the-lawn" antagonism method

The antimicrobial activity of lactobacilli against *S. typhimurium* was determined by the "spot on the lawn" antagonism method, performed according to the methodology described by Lima and collaborators [42]. The lactobacilli were grown in MRS broth, and incubated at a temperature of 37°C, for 24 h under aerobic conditions. Subsequently, aliquots of this culture were punctually added on MRS agar plate. After drying was complete, the plate was incubated under aerobic conditions at a temperature of 37°C, for 8 h.

S. typhimurium was previously seeded in NA at 37°C for 24 h and the culture was adjusted in saline according to the McFarland 0.5 scale. Next, 250 µL of the adjusted culture was transferred to an erlenmeyer flask containing 25 mL of MH semi-solid agar, where it was homogenized and poured onto the *L. rhamnosus* dish. After complete solidification of the upper layer, the plate was incubated for an additional 24 h at 37°C under aerobic conditions. The presence of inhibition halo indicated the *L. rhamnosus* production of substances with antimicrobial activity.

2.6 *In vivo Assay*

2.6.1 Animals

In total, 16 BALB/c female, mice weighting approximately 20 g, 4- to 6-weeks-old, were tested. These animals were maintained in a pathogen-free animal facility of the State University of Londrina (Londrina, PR, Brazil).

2.6.2 *In vivo* challenge using mice

The *in vivo* assay and microbiological analysis were performed according to the protocol of Coconnier et al. [43]. The mice were divided into two groups: the treated group which received orally, through gavage method, three inoculations of an 18 h grown culture of *L. rhamnosus* containing 10^9 cfu in 0.2 mL, on alternate days, and the control group that received 0.2 mL of PBS.

After one day of treatments with *L. rhamnosus*, a 10^8 cfu suspension of *S. enterica* serovar Typhimurium was inoculated. After 10 and 14 days of pathogen inoculation, 4 mice from each group were euthanized by cervical dislocation (treated with lactobacilli and non-treated control) and the spleen, liver, and Peyer's patches removed for microbiological and histopathological analysis.

2.6.3 Microbiological analysis

Microbiological analysis was performed to evaluate the translocation of *S. enterica* serovar Typhimurium. After collection, a small part of the organs was cut and reserved for histology. The other portion of the organs was weighed, crushed with macerators, homogenized, and individually reserved in Falcon type tubes containing 5 mL of PBS. Serial dilutions (10-1, 10-2, 10-3) were made and 10 µL of these bacterial suspensions were plated in triplicate in MC agar at 37°C; after 24 h the cfu was determined by direct counting.

2.6.4 Histopathological analysis

The collected material was processed and analyzed by the Department of Histology, Center for Biological Sciences, State University of Londrina, PR, Brazil. The organs were fixed by immersion in Bouin solution for 24h. All collected organs and the ileum were included in paraffin following the conventional protocol; 7-µm-sections were stained with hematoxylin and eosin (HE). The images were captured using photomicroscopy (Zeiss Axiophot) coupled to a high-resolution camera (Moticam 2300 3.0 MP). Alterations in the histological structure were investigated.

2.7 Statistical Analysis

Differences in the *in vitro* and *in vivo* tests were compared using the Student t test. For statistical analysis *in vivo*, data were normalized by total cfu per milliliter (cfu/mL) for the Peyer's patches, spleen, and liver.

For analysis of the growth and death curve data, analysis of variance (ANOVA) was performed, and the Tukey test to compare the means, considering a factorial design, the factors being the treatments, and the levels the times. The significance level adopted was 5%, and the analyses were performed using software R version 3.4.4 (2018).

3. RESULTS

3.1 Identification of *Lactobacillus rhamnosus* Strain V5

The molecular characterization of the specie was performed through PCR in which the amplified region was the 16S ribosomal RNA gene whose sequence was deposited in GenBank database under accession number MG209517.

3.2 Adhesion and Invasion in Cell Culture

The adhesion assays using HeLa cells showed an inhibition of *Salmonella*-adherence in the presence of *L. rhamnosus*. The addition of probiotic together with *Salmonella* presented the highest inhibition when compared with previous treatment (Table 1).

Table 1. Inhibition of the *Salmonella* adhesion in HeLa cells by *Lactobacillus rhamnosus*

Bacterial treatment	Number of adhered bacteria/ 100 cells
Lactobacillus rhamnosus	0
Salmonella Typhimurium	782 ± 144,25
Lactobacillus + *Salmonella* (0 h)*	40 ± 16,33
Lactobacillus + *Salmonella* (- 3 h)**	128 ± 45,25
Lactobacillus + *Salmonella* (- 5 h)***	370 ± 140,46

**Lactobacillus strain added together Salmonella strain.*
***Lactobacillus strain added 3 h before the addition of Salmonella strain.*
****Lactobacillus strain added 5 h before the addition of Salmonella strain.*

The inhibition of *Salmonella* invasion in HEp-2 cells was also observed in the presence of probiotic (Fig. 1), showing a significant reduction of invasive cells in the presence of *L. rhamnosus* compared to control.

3.3 Antibacterial Activity of the Supernatant

3.3.1 MIC

In the present study, the supernatant (I) demonstrated antimicrobial activity. The minimal inhibitory concentration of the supernatant against *S. typhimurium* was 10%. The supernatant (II) was sensitive to neutralization with 1N NaOH solution, totally losing its inhibitory capacity, demonstrating that the antimicrobial activity verified in the experiment may have been due to the presence of acids, leading to a drop in the pH of the medium. During the growth of lactic acid bacteria, a fall in pH occurs, making the environment quite acid, mainly due to the production of acids such as lactic acid. The supernatant (III) was resistant to thermal treatments. The minimal inhibitory concentration of the supernatant against *S. typhimurium* was 10%, demonstrating that the antimicrobial activity verified in the experiment may have been due to the presence of acids. Bacteriocin produced by LAB has low molecular weight and are easily denatured by thermal treatments [44].

3.3.2 Growth and death curve

After determining the minimum inhibitory concentration of the supernatant not neutralized against *S. typhimurium*, the time-kill curve assay was performed. The results showed statistically significant differences ($p < 0.05$). The 10% supernatant was able to inhibit the growth of the bacterium, but after the period of 10 h the bacteria began to multiply and at the end of 24 h had an increase of one log in relation to the initial inoculum, demonstrating a bacteriostatic effect. However, in the time of 24 h showed a difference of 4 logs (Fig. 2). The supernatant at 20% presented bactericidal action, gradually decreasing the number of viable cells, eliminating 100% of the bacterial population in 24 h (Fig. 2).

3.3.3 Spot-on-the-lawn antagonism method

The "Spot-on-the-lawn" antagonism method showed the antimicrobial activity of lactobacilli against *S. typhimurium*, forming zones of inhibition of 21 mm in diameter.

3.4 *In vivo* Assay

3.4.1 Microbiological analysis

Ten days post-infection with *S. typhimurium*, we observed a significant decrease ($p < 0.05$) in the

number of *Salmonella* colonies in the spleen when treated with probiotic (Fig. 3A). Other organs (liver and Peyer's patches) did not present significant differences between the treated and control animals (non-treated). However, after 14 days the number of *Salmonella* colonies was lower in all organs from mice treated with lactobacilli, mainly the liver ($p < 0.05$), (Fig. 3B). Some non-treated (control) mice died (data not shown) and their organs were not collected for microbiological analysis.

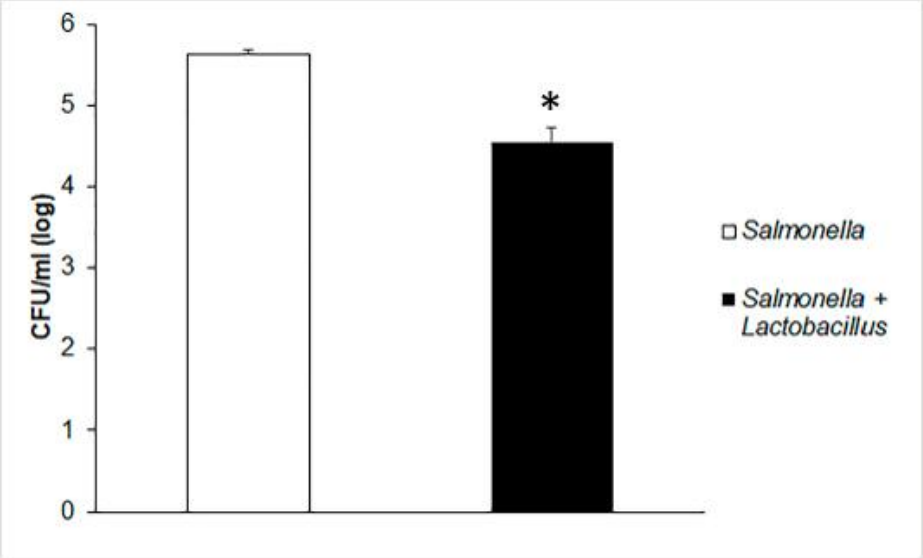

Fig. 1. Count of invasive bacteria (*Salmonella Typhimurium*) in HEp-2 cells treated and not treated with *L. rhamnosus*

(*) Significant decrease in the number of S. typhimurium (P < 0.05)

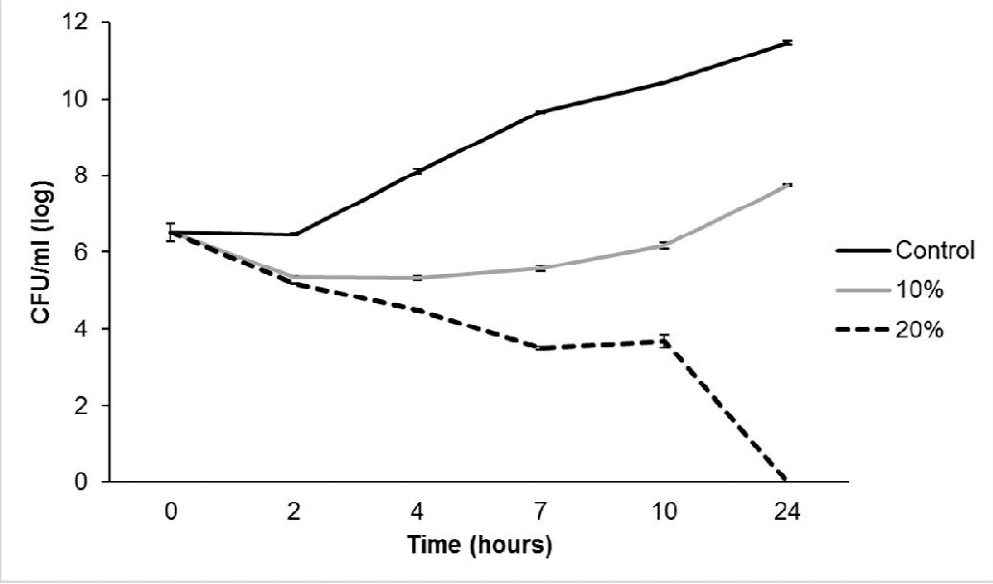

Fig. 2. Growth and death curve of *Salmonella* Typhimurium in the presence of supernatant not neutralized from *L. rhamnosus*

(*) Significant reduced growth of S. typhimurium (P < 0.05)

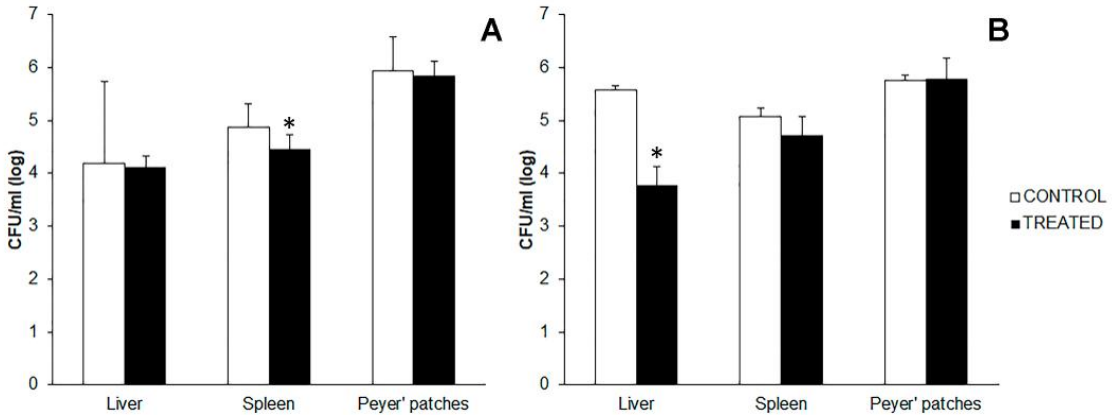

Fig. 3. Counting of colony forming unit from liver, spleen and Peyer' patches after 10 (A) and 14 (B) days post-infection in murine model

(*) Significant decrease in the number of S. typhimurium (P < 0.05)

3.4.2 Histopathological analysis

The ileum samples collected from the control group demonstrated the presence of prominent lymphoid nodules both at 10 days (Fig. 4A) and 14 days (Fig. 4C) post-infection. Mice treated with *L. rhamnosus* did not present alterations in the histological characteristics of the ileum at either moment (Fig. 4B and 4D).

The liver samples of non-treated mice demonstrated the presence of inflammatory foci both at 10 days (Fig. 4E) and at 14 days (Fig. 4G) post-infection. Mice treated with *L. rhamnosus* did not present inflammatory foci at either moment (Fig. 4F and 4H). Intestinal epithelial cells, and the spleen and liver of all groups did not show detectable histopathological alterations under light microscopy.

4. DISCUSSION

Several studies have reported different probiotics to prevent infections against foodborne pathogens [25,26,45]. In this report, we showed that *L. rhamnosus* V5 promoted protection *in vitro* and *in vivo* against *S. enterica* serovar Typhimurium UK1, attenuated ΔcyaΔcrp (χ3985 UK1 [ΔcyaΔcrp]) (strain from Center for Infectious Diseases and Vaccinology, The Biodesign Institute and School of Life Sciences, Arizona State University, Tempe, AZ [36]. This infection model was used to evaluate the translocation process in mice, although *S. typhimurium* mutant decreased its virulence by deletion in the receptor protein of the adenylate cyclase and cyclic AMP, this strain keeps invading host cells [37]. Due to the characteristic of this pathogen strain, this *in vivo* model evaluated the probiotic effect.

Several mechanisms have been proposed to explain the beneficial effects of probiotics. For example, bacteriocins produced by probiotics can inhibit pathogenic bacteria, preventing infection in humans or other animals [46]. However, our results support that the inhibitory activity observed in *in vitro* assays was due to the production of organic acids, which reduced the pH of the medium. This effect was similar to the studies of Ogawa and collaborators [47] and Pereira and Gómez [48].

Probiotics can also inhibit pathogen adherence in host cells by competition in linkage to host receptors [49]. In this sense, we observed that the presence of *L. rhamnosus* decreased the number of *Salmonella*-adhered in HeLa cells, mainly when the probiotic was added together with the pathogen (Table 1). Interestingly, the non-adherence of *L. rhamnosus* in HEp-2 cells suggests that the bacteriocins or presence of acids from probiotics can prevent bacterial adhesion or colonization. Cell invasion ability is an important virulence characteristic of *Salmonella*. *L. rhamnosus* V5 to reduces the number of invasive bacteria in HEp-2 cells, as also demonstrated in previous studies [49,50]. Thus, the results of adhesion in HeLa and invasion assays in HEp-2 cells showed that cell culture is an interesting *in vitro* tool to select an applicant for probiotic.

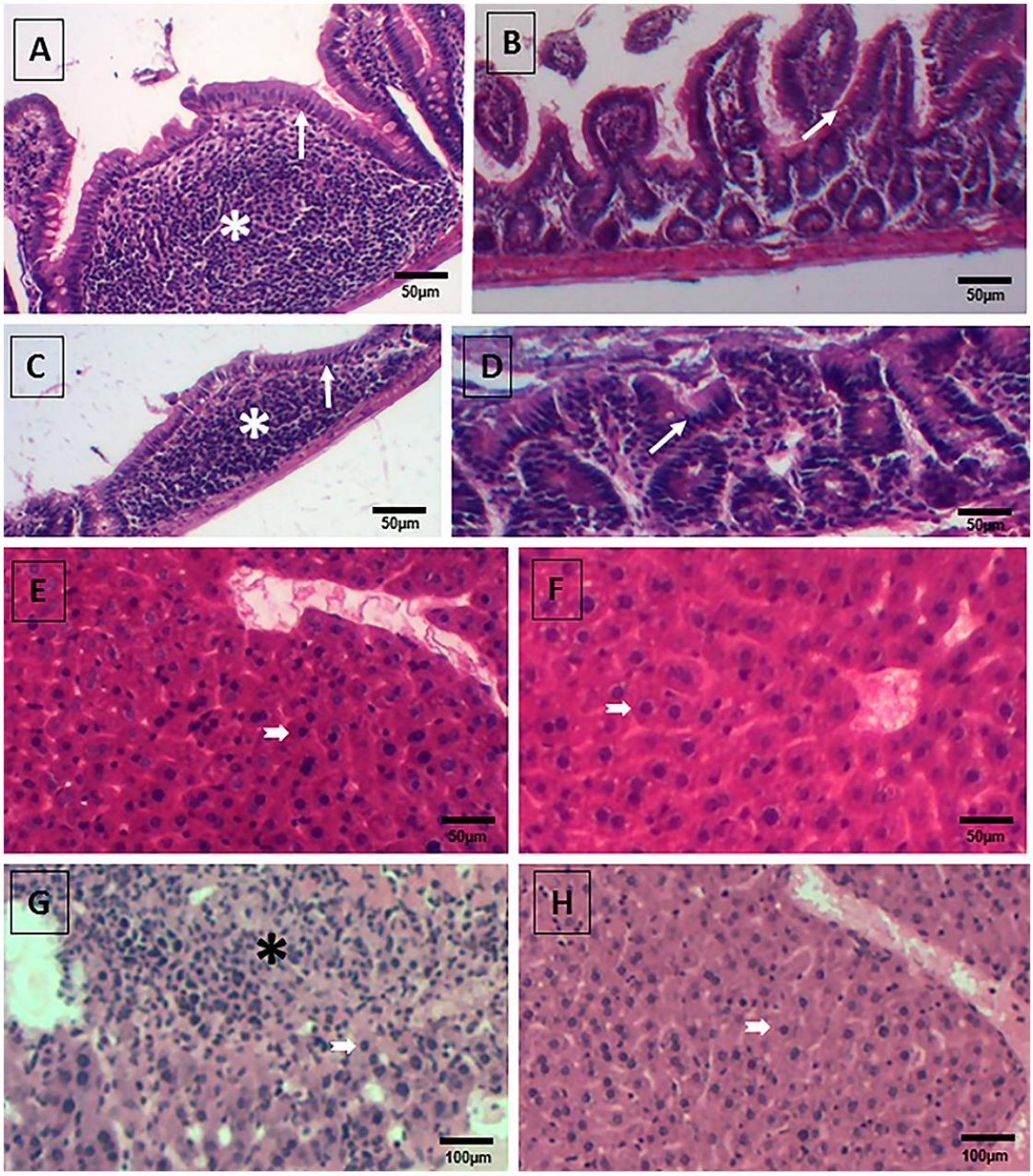

Fig. 4. Photomicrograph of ileus intestinal tissues and liver of mice treated and non-treated with probiotics at 10 and 14 days post-infection with *S. typhimurium*. Photomicrograph of intestine (ileum) and liver of mice infected with *S. typhimurium*. A: Ileum of non-treated mice at 10 days post-infection with *Salmonella*. Note prominent lymphoid nodule (*). B: Group treated with *L. rhamnosus* at 10 days post-infection. C: Non-treated mice at 14 days post-infection with *Salmonella*. Note prominent lymphoid nodule (*). D: Group treated with *L. rhamnosus* at 14 days post-infection. Intestinal epithelium (long arrows). E: Liver of non-treated mice at 10 days post-infection with *Salmonella*. F: Group treated with *L. rhamnosus* at 10 days of infection. G: Non-treated mice at 10 days post-infection with *Salmonella*. Note inflammatory foci (*). H: Group treated with *L. rhamnosus* at 14 days of infection. Hepatocyte (short arrows). Stained with Hematolin-Eosin (HE)

As *L. rhamnosus* V5 demonstrated a protective effect *in vitro* against infection, we evaluated if this positive effect would also be observed in mice infected with *S. enterica* serovar Typhimurium UK1. In this way, it was verified that the number of *Salmonella* bacteria in the organs of the three treated mice was significantly lower than the untreated mice after 14 days of infection. Thus, the microbiological evaluation after 14 days of infection in this model showed effective partial protection.

Acursio et al. [51] tested the protective effect of Lactobacillus plantarum and *L. rhamnosus* inoculating a single dose of fermented milk containing of 8.0 log10 cfu/mL. Afterwards, live *S. typhimurium* was inoculated five days after mono-association with Lactobacillus strains. On day 20 post-challenge with *S. typhimurium*, translocation was found in the liver of mice treated with L. plantarum but not in those treated with *L. rhamnosus*. This result is very important to highlight that not all species of the Lactobacillus genus are able to present a protective effect.

Our *in vivo* results confirmed the decrease in the invasion *in vitro*, showing that cell culture assays have been used previously to assess the effective probiotic potential. Thus, other tests using alternative models would be interesting in an initial screening assessment of the effectiveness of probiotics [52-54].

The presence of prominent inflammatory foci in the intestinal mucosa was observed only in mice non-treated with *L. rhamnosus* It is known that these inflammatory foci are common in the ileal mucosa, but they tend to increase in quantity and size when the intestinal barrier is ruptured. As no change was observed in the larynx of mice treated with probiotics, it is suggested that *L. rhamnosus*. V5 was more effective for the intestinal barrier.

The literature already describes that Lactobacillus protects the integrity of the intestinal epithelial barrier from *Salmonella* infection [55], which corroborates our results. Researchers showed the efficiency of Lactobacillus fructosus and *L. rhamnosus* in maintaining the integrity of Caco-2 culture cells [55,56]. Reduction in inflammatory foci leads to a progressive decrease in intestinal inflammation of the Peyer's patches, spleen, and peritoneum of mice treated with L. casei [22]. The absence of changes in the spleen, liver, and Peyer's patches at 10 days post-infection suggests that bacterial translocation was under control.

It is known that *Salmonella* bacteria attack enterocytes, promoting rupture of occlusive junctions [55,56,57] and M cells, provoking intense inflammatory response [58]. Untreated mice infected with *Salmonella* bacteria presented more inflammatory nodules in the ileum and the presence of bacteria in the liver. On the other hand, mice treated with *L. rhamnosus* V5 and infected with *Salmonella* bacteria presented reduced inflammatory nodules in the ileum and no histological alterations in the liver. Considering this, it is reasonable to consider that *L. rhamnosus* V5 used as a probiotic was able to improve the intestinal barrier. Therefore, the use of *L. rhamnosus* V5 as a probiotic could be a viable alternative for controlling salmonellosis. Further studies using transmission electron microscopy could provide detailed information about the ultrastructure of the intestinal wall of these mice and contribute to understanding the mechanisms involved in the beneficial action of *L. rhamnosus* V5 as a probiotic.

5. CONCLUSION

We conclude that *L. rhamnosus* V5 was able to control *S. typhimurium*, inhibiting the adhesion and invasion of *Salmonella* bacteria *in vitro* and in mice *in vivo*. Because of this, *L. rhamnosus* V5 was able to control pathogen translocation in the spleen and liver. Thus, *L. rhamnosus* V5 could be used as a probiotic to control salmonellosis. Moreover, the histological assay is interesting tool to analyze the probiotic effects in intestinal tissues. This study showed the importance to search new lactobacilli as applicant probiotic for development of a new product.

ETHICAL APPROVAL

The project was approved by the Ethics Committee for Animal Experimentation of the State University of Londrina (CEUA / UEL), protocol no104/2013.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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