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Anti-infective mechanisms induced by a probiotic *Lactobacillus* strain against *Salmonella enterica* serovar Typhimurium infection

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ABSTRACT

The prevention of pathogen infections is one of the most extensively studied effects of probiotics. *L. casei* CRL 431 is a probiotic bacterium and its effects on the gut immune cells have been extensively studied. The aim of the present study was to determine, using a mouse model, the preventive and therapeutic effect of *L. casei* CRL 431 to achieve protection against *Salmonella enteritidis* serovar Typhimurium infection. In both previous and continuous (previous and post-infection) probiotic administration, the mechanisms induced by this lactic acid bacteria on the first line of intestinal defense (non-specific barrier and the innate immune cells associated to the gut), as a way to understand some of the mechanisms involved in the protection against *Salmonella enteritidis* serovar Typhimurium, were analyzed. The results obtained demonstrated that 7 days *L. casei* CRL 431 administration before infection decreased the severity of the infection with *Salmonella enteritidis* serovar Typhimurium, demonstrating that the continuous administration (even after infection) had the best effect. This continuous administration diminished the counts of the pathogen in the intestine as well as its spread outside this organ. Several mechanisms and cells are involved in this protective effect against *Salmonella enteritidis* serovar Typhimurium. *L. casei* CRL 431 acted on cells of the innate and adaptive immune response. The probiotic administration decreased the neutrophil infiltration with the consequent diminution of intestinal inflammation; activated the macrophage phagocytic activity in different sites such as Peyer's patches, spleen and peritoneum; and increased the number of IgA + cells in the lamina propria of the small intestine which was correlated with increased release of s-IgA specific against the pathogen in the intestinal fluids. The mechanism of the inhibition of cellular apoptosis was not involved.

1. Introduction

Salmonella species are gram-negative, motile, anaerobic facultative, intracellular bacilli. Disease outcome after infection with different serotypes of the species *Salmonella enterica* ranges from self-limiting diarrhoea and localized gastrointestinal inflammation to typhoid fever, a systemic infection with high lethality rates. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is associated exclusively with entero-colitis in humans.

Mice are used by many authors to evaluate possible treatment for this infection. In mice, *S.* Typhimurium spreads systematically via blood circulation and the infection is characterized by severe pathological changes and high bacterial tissue loads in Peyer's patches, mesenteric lymph nodes, the liver and the spleen (Tsolis et al., 1999; Mastroeni and Sheppard, 2004). Mouse septicemia after oral administration of *S.* Typhimurium is generally considered to be a reasonable reflection of typhoid fever observed in *S.* Typhimurium human contaminated patients (Kaufmann et al., 2001).

During typhoid fever, the food-borne pathogen penetrates mucosal barriers and preferentially interacts with M cells of the Peyer's patches, invades and destroys them, and reaches the subepithelial dome, where they come into contact with resident macrophages and dendritic cells (DC) intimately associated with the M cells (Guerrant et al., 1999; Hersh et al., 1999). Macrophages and DC can act as an alternative invasion pathway to M cells and as vehicle in the spread of the pathogen (Vazquez-Torres et al., 1999; Worley et al., 2000).

It is important to considerer the complexity of the intestinal microenvironment where exist a network of interactions among the microorganisms of the resident microbiota, epithelial and immune cells associated with the gut, and nutrients (Hooper and Gordon, 2001; Bauer et al., 2006). The gut associated lymphoid tissue (GALT) plays an important role in the modulation of the immune function, providing an effective barrier against pathogenic bacteria. At this mucosal level, the innate immune response not only provides the first line of defense against pathogenic microorganisms but also provides the biological signals that instruct the adaptive immune system to elicit a response.

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The beneficial effects of the microbiota on the immune system have allowed the proposal to use some non-pathogenic bacteria, named probiotics in improving animal health and protection against infectious agents (Moreau and Gaboriau-Routhiau, 2000; Simon et al., 2003; Galdeano and Perdigon, 2004).

Probiotics are live microorganisms which when are administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2001). Lactic acid bacteria (LAB) are the organisms most commonly used as probiotics, although *E. coli* strain Nissle has also been used as probiotic (Schultz et al., 2004; Sturm et al., 2005; Grabig et al., 2006; Guzy et al., 2008). These microorganisms can influence the composition and activity of the gut microbiota. Some bacterial species (principally belonging to the genera *Lactobacillus* and *Bifidobacterium*) present in fermented foods are regarded as probiotics because of their ability to modulate the GALT through the enhancement of the IgA production (Perdigon et al., 1991, 1993, 2001; Alvarez et al., 1998), the prevention of some intestinal infections (Alvarez et al., 1998 ; Gobbato et al., 2008), the modulation of the inflammatory immune response (de Moreno de LeBlanc et al., 2004) and the reduction of the levels of some tumour promoting factors (de Moreno de LeBlanc et al., 2007).

L. casei CRL 431 is a probiotic bacterium and its effect on the gut immune cells was extensively studied (Perdigon et al., 2002; Galdeano and Perdigon, 2004, 2006; Bibas Bonet et al., 2006; Galdeano et al., 2007). It was reported that this probiotic bacterium interacts with the GALT and makes contact with the immune cells associated to Peyer`s patches and with the lamina propria of the intestinal mucosa (Dogi et al., 2008). Cells from the innate immune response were proposed as the main target of *L. casei* CRL 431 to induce immune stimulation at the gut level (Galdeano et al., 2007).

The prevention of pathogen infections is one of the effects more extensively studied of probiotics. Previously, it was also reported that *L. casei* CRL 431 prevented *Salmonella* and *Escherichia coli* infections in mice and its dissemination to organs, such as the liver and spleen using a malnourished model in mice (Cano and Perdigon, 2003). The inhibition of the cellular apoptosis of macrophage infected with *Salmonella* induced by certain LAB, has been also demonstrated *in vitro* and *in vivo* (Valdez et al., 2001; Gobbato et al., 2008). In this manner, probiotics can be consumed as part of the daily diet to maintain the immune system in an active state and prevent different intestinal disorders.

The aim of the present study was to determine the optimal conditions of administration of *L. casei* CRL431 to achieve the better protection against *S.* Typhimurium infection, and also evaluate the effects of continuous probiotic administration, before and after pathogen challenge, analyzing in both models the influence of this LAB on the first line of the intestinal defense (non-specific barrier and the innate immune cells associated to the gut). The present study is the first step that will allow going deep into the knowledge about how the *lactobacilli* probiotic bacteria works to develop gut anti-infective properties and to establish the scientific basis for the use of the probiotic bacteria as oral adjuvant in the prevention or in the therapy of enteropathogen bacterial infection.

2. Materials and methods

2.1. Animals and bacterial strains

Five to six week-old BALB/c mice weighting 22-26 g were obtained from the closed random bred colony maintained at CERELA (Centro de Referencia para Lactobacilos, San Miguel de Tucumán, Argentina). All the animals were housed in cages kept in a controlled atmosphere (22 ± 2 °C; 55 ± 2% relative humidity) with a 12 h light/dark cycle, and fed *ad libitum* with a conventional balanced diet.

L. casei CRL 431 was obtained from the CERELA culture collection. Overnight cultures were grown at 37 °C in sterile Mann-Rogosa-Sharp (MRS) broth (Britania, Buenos Aires, Argentina). The cells were harvested by centrifugation at 5000 *g* for 10 min, washed three times with fresh PBS and then resuspended in sterile 10% (vol/vol) non-fat milk. *Salmonella enterica* serovar Typhimurium strain was obtained from the Bacteriology Department of the Hospital del Niño Jesus (San Miguel de Tucuman, Argentina). An aliquot (200 µl) from an overnight culture was placed in 5 ml of sterile Brain Heart Infusion (BHI) broth (Britania, Buenos Aires, Argentina) and incubated during 4 hours. The concentration of *Salmonella* was adjusted to 1×10^8 CFU/ml in phosphate buffered saline (PBS).

2.2. Feeding and infection procedures

The assays were performed using 7 experimental groups: For the preventive effect of the probiotic bacterium, three groups (10 mice each one) were performed; mice received *L. casei* CRL 431 for 2, 5, or 7 consecutive days before challenge with the enteropathogen (2d-S, 5d-S, 7d-S). For the effect of the continuous probiotic administration, in the other three groups, mice were administered *L. casei* CRL 431 during 2, 5, or 7 consecutive days, challenged with the pathogen and continued receiving *L. casei* CRL 431 post challenge (2d-S-Lc, 5d-S-Lc and 7d-S-Lc). For the infection control group (S), mice did not receive special feeding and were only challenged with *S.* Typhimurium (See Fig. 1).

L. casei CRL 431 was administered in the drinking water to reach a concentration of 10^8 CFU/ml. All the groups were challenged with 100 µl of 1×10^8 CFU/ml of *S.* Typhimurium (LD_{50}) by gavage. Mice were weighed throughout the experiment and the number of deaths was registered. Animals were sacrificed 7 and 10 days post challenge (7 dpI or 10 dpI) to obtain the samples (Fig. 1). Small and large intestine, liver and spleen tissue was removed and intestinal fluids were collected.

All animal protocols were pre-approved by the Animal Protection Committee of CERELA and all experiments complied with the current laws of Argentina.

2.3. Colonization assays

The large intestine, spleen and liver were aseptically removed, weighed and placed into sterile tubes containing 5 ml of peptone water (0.1%). The samples were immediately homogenized under sterile conditions using a microhomogenizer (MSE, England). Serial dilutions were made and spread onto the surface of MacConkey agar (Britania, Buenos Aires, Argentina) for liver and spleen samples and *Salmonella-Shigella* agar (Britania, Buenos Aires, Argentina) for large intestine samples. The plates were then incubated aerobically at 37 °C for 24 h.

2.4. Determination of total and specific s-IgA in intestinal fluids

Intestinal fluids were collected from the small intestines with 1 ml of 0.85% NaCl and immediately centrifuged at 5000 *g* during 15 min at 4 °C. The supernatant was recovered and stored at −20 °C until IgA determination. All the determinations were performed in triplicate.

ELISA was used to measure the concentration of total s-IgA according to the technique described by LeBlanc et al. (2004). Briefly, affinity-purified monoclonal goat anti-mouse IgA (α-chain specific Sigma, St Louis, MO, USA) was added in 0.05 M carbonate-bicarbonate buffer (pH 9.6) to 96 wells plates and incubated at 37 °C for 1 h. The plates were then washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 0.5% non-fat dry milk in PBS. Plates were washed and incubated for 2 h at 37 °C with either 50 µl of standard kappa IgA (Sigma, St. Louis, USA) or 50 µl of diluted intestinal fluid samples. Plates were washed and incubated in the presence of horseradish peroxidase-conjugated anti-IgA-specific antibodies (Sigma, St. Louis, USA) for 1 h at 37 °C. After that, plates were again washed and trimethylbenzidine (TMB) reagent containing peroxide (BD Biosciences, San Diego, USA) was added. Reactions were stopped with H_2SO_4 (2N). The absorbance was read at 450 nm using a VERSA Max Microplate Reader (Molecular Devices, USA).

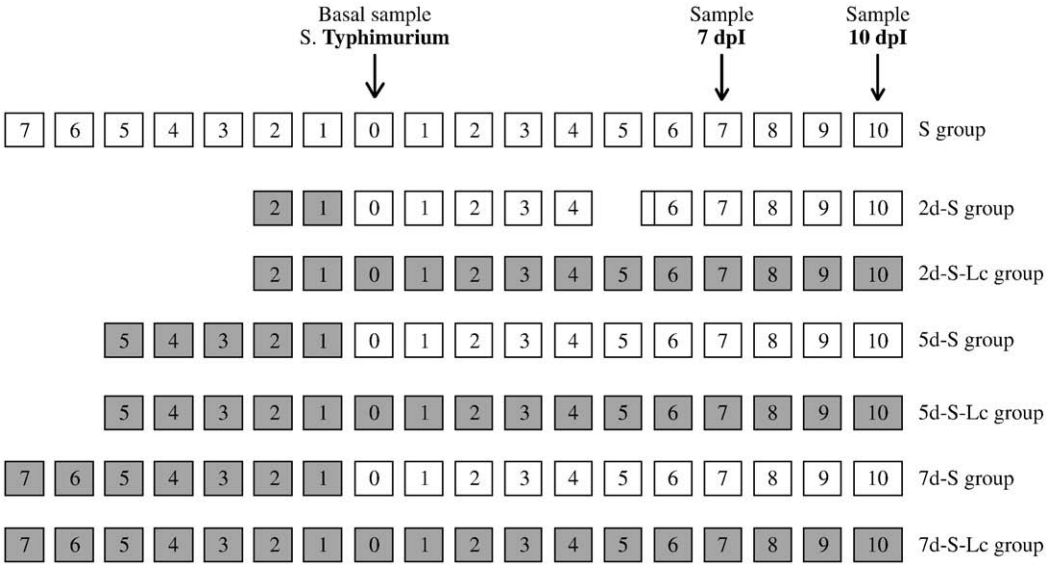


Fig. 1. Feeding and infection protocols. Mice received *L. casei* 431 during 2 (2d-S), 5 (5d-S), or 7 (7d-S) days. After that, animals from each group were sacrificed to obtain the basal sample and the rest of the animals were challenged with *S.* Typhimurium (arrow). Post-infection, each group was divided in two subgroups: one continued receiving the probiotic bacteria (2d-S-Lc; 5d-S-Lc; and 7d-S-Lc) and the other not. To study the effect post-infection (pI), two samples were taken (7 and 10 days pI). Each square represent one different day for each experimental group. Gray squares are used to show the LAB administration to the mice and white squares are used to show the days where the mice did not receive LAB.

For the specific anti-*Salmonella* antibodies determinations, plates were coated with 50 µl of a suspension of concentrated and heat-inactivated *S.* Typhimurium solution (10^{10} CFU/ml) and incubated overnight at 4 °C. Non-specific protein-binding sites were blocked with PBS containing 0.5% non-fat milk. Dilutions in PBS of the test and control samples from the intestinal fluid were then incubated at room temperature for 2 h. After washing with PBS-T, the plates were incubated 1 h with peroxidase-conjugated anti-IgA-specific antibodies. Plates were again washed and the TMB reagent was added. The reaction was stopped with H_2SO_4 (2N). The absorbance was read at 450 nm.

2.5. Histological preparations and immunofluorescence assay for IgA + cells in small intestine tissues

The small intestines were removed, washed with 0.85% NaCl, cut in pieces, and used for histological preparations following the technique described by Sainte-Marie (1962). Serial paraffin sections (4 µm) were stained with hematoxylin-eosin for light microscopy examination.

The numbers of IgA positive cells were determined on histological slides by a direct immunofluorescence assay using an α-chain monospecific antibody conjugated with FITC (Sigma, St Louis, MO, USA). The number of fluorescent cells was counted in 30 fields of view at 1000× of magnification. Results were expressed as the number of positive fluorescent cells per 10 fields of view.

2.6. Determination of myeloperoxidase (MPO) activity

Small intestines were removed and cut into three segments of equal length. Each segment was placed in 1 ml of 0.5% hexadecyltrimethylammonium bromide (HTAB) (Sigma-Aldrich, Inc. St. Louis, USA.) in 50 mM potassium phosphate buffer, pH 6.0 to solubilise myeloperoxidase (MPO) and homogenized using a microhomogenizer. Glass pearls (0.25–0.30 mm Ø, B. Braun, Apparatebau Melsungen) were added and the cells were broken using a cell disrupter (Mini Beadbeater-8; Biospec). The samples were then frozen and thawed 3 times and, centrifuged at 15,000 *g* for 15 min at 4 °C.

The resulting supernatants were assayed for the MPO activity by mixing 10 µl of each sample with 100 µl of TMB reagent containing peroxide (BD Biosciences, San Diego, USA) and measuring the H_2O_2-dependent oxidation expressed as enzymatic units per gram of total protein. One enzymatic unit represents the quantity of enzyme which produces an increase of one absorbance unit per minute. Total protein content of the samples was determined using the Bio-Rad Protein Assay based on the method of Bradford (1976).

2.7. Isolation of mononuclear adherent cells from peritoneum, Peyer's patches and spleen. Determination of phagocytic and microbicidal activities

Peritoneal macrophages were obtained according to Valdez et al. (2001). Macrophages were extracted from peritoneal cavity with 5 ml of sterile PBS, pH 7.4 containing 100 µg/ml of gentamicin (Gm). For the isolation of macrophages from Peyer's patches the protocol described by Galdeano and Perdigon (2006) was used. The small intestine of each mouse was removed, washed and the Peyer's patches were excised in Hank's buffered salt solution (HBSS) containing foetal bovine serum (FBS). The epithelium cells were separated with an HBSS/FBS solution containing EDTA. The sediments were incubated with dispase/DNAse solution and the mononuclear cells were recovered. These cells were collected from the supernatant and washed with RPMI 1640 medium (Sigma, St. Louis, USA). For spleen macrophages isolation, the spleen was collected in 5 ml of HBSS solution containing FBS and aseptically disrupted. The cells were harvested by centrifugation at 800–1000 *g* for 15 min at 4 °C. The resulting pellets were gently mixed with 2 ml of sterile red blood cell lysing buffer (Sigma, St Louise, USA) for 2 min. The haemolysis was stopped with PBS. The samples were again centrifuged and resuspended in RPMI-1640 medium (Sigma, St. Louis, USA) containing FBS.

The adherent cells (macrophages and DC) were separated from the mononuclear population using their adherence property to glass slides.

Phagocytosis was performed using a heat-killed *Candida albicans* suspension at a concentration of 10^7 cell/ml or FITC-labelled *S.* Typhimurium. For the bacteria labelling, an overnight culture was incubated in the dark with 0.1 mg/ml of FITC (Sigma, St. Louise) in sterile PBS for 70 min at 37 °C. Phagocitosis was performed by *ex vivo* assay using equal volumes of opsonised *C. albicans* or FITC- labelled *Salmonella* mixed with 10^6 cells/ml of macrophages. The mixture was incubated for 30 min or 15 min, respectively at 37 °C. Phagocytosis was expressed as the percentage of phagocyting macrophages in 200 cells count using an optical or fluorescence microscope.

Table 1
Changes in the body weight and mortality.

Group	Basal weight	Weight 7 dpI	% of Mortality 7 dpI	Weight 10 dpI	% of Mortality 10 dpI
S	28.4 ± 0.37^a	22.13 ± 0.26^b	30	27.3 ± 0.10^c	70
2d-S	27.94 ± 1.52^a	23.00 ± 1.41^b	20	ND	100
5d-S	27.93 ± 1.70^a	23.27 ± 2.18^b	10	23.90 ± 2.19^b	20
7d-S	26.39 ± 0.37^a	23.29 ± 2.71^b	7	23.03 ± 1.50^b	50
2d-S-Lc	27.79 ± 2.67^a	20.33 ± 1.37^b	10	28.70 ± 5.52^a	50
5d-S-Lc	28.67 ± 2.54^a	27.13 ± 2.27^a	10	26.70 ± 5.51^a	20
7d-S-Lc	27.87 ± 2.82^a	23.53 ± 2.51^a	0	28.16 ± 4.90^a	7

Results express the means ± SD of the body weight of infected control mice and the test groups at 3 time point: the days of the infection (basal data) and 7 and 10 days post-infection (dpI). Means for each value without a common letter differ significantly ($P<0.05$). Mortality is expressed as means ± SD of the mice mortality percentage. The results include three different experimental trials. ND = Not determined.

Microbicidal activity was determined by *ex vivo* assay with the technique described by Gobbato et al. (2008) .Briefly, 2 ml of 10^6 cells/ml extracted from the peritoneum or Peyer's patches were cultured in RPMI-1640 medium (Sigma, St. Louis, USA) and incubated in TC-Plates (6 wells, sterile with LID, Cellstar, Greiner bio-one) for 1 h at 37 °C. Plates were washed with RPMI-1640 medium and the adherent cells were infected with 1 ml of 10^7 CFU of *S.* Typhimurium in a ratio bacteria/ macrophages 10:1. After 30 min of incubation at 37 °C, extracellular bacteria were gently removed by washing and RPMI-1640 medium containing 100 μg/ml of gentamicin was added and incubated 60 min. Cells were lysed with 1 ml of Triton X-100 1% in RPMI-1640. Serial dilutions 10^{-1} to 10^{-3} were plated on MacConkey agar and the number of viable intracellular bacteria was measured after plate's incubation for 24 h at 37 °C.

2.8. Apoptosis determination in the histological cuts of small intestine

Apoptosis was evaluated for the presence of DNA breaks using the Apoptosis Detection System kit (Promega, Madison, WI, USA). The fluorescein-12-dUTP-nick ends labelled DNA were visualised using fluorescence microscopy. Cells were defined as apoptotic if the entire nuclear area of the cell was stained fluorescent. Apoptosis was expressed as the number of apoptotic cells in 10 fields observed at 400× magnification.

2.9. Statistical analysis

Statistical analyses were performed using MINITAB 14 software (Minitab, Inc., State College, PA) by ANOVA GLM followed by a Tukey's posthoc test, and $P<0.05$ was considered significant. All values ($N=15$) were the means of 3 independent trials (no significant differences were observed between individual replicates) ± standard deviation.

3. Results

3.1. Effect of L. casei CRL 431 feeding against S. Typhimurium infection. Analysis of body weight, mortality rates and pathogen colonization. Histological studies

The study of the preventive effect of *L. casei* CRL 431 administration showed that the three groups that received the probiotic had less weight loss and mortality for 7 dpI (Table 1). Pathogen colonization in liver and spleen and their counts in large intestine did not show significant decreases in the test groups compared to the infection control (Table 2).

Mice fed with *L. casei* CRL 431 before or after being challenged with Salmonella both showed a decrease in mortality rates and body weight loss; a weight gain was observed in the group fed during 7 days with this probiotic strain (Table 1). This last group (7d-S-Lc) showed significant decreases ($P<0.05$) of Salmonella concentrations in spleen and large intestine (7 dpI and 10 dpI) and in liver (10 dpI) (Table 2).

The histological studies by hematoxilin-eosin strain on small intestine slides showed no inflammation in the mice which received probiotic during 7 days with continuous probiotic administration after the *Salmonella* challenge. It was only observed an increase in the cellularity without modification of the architecture of the villi (Fig. 2).

3.2. Influence of probiotic administration and Salmonella challenge on s-IgA levels in intestinal fluid and on the IgA + cells associated with the lamina propria of the small intestine

L. casei administration induced a significant increase ($P<0.05$) in the number of IgA + cells, 7 days post-challenge, in the mice of 7d-S-Lc group (136 ± 19, Fig. 3D) compared to the mice of S group (93 ± 24, Fig. 3E). The administration of *L. casei* during 7 days also increased significantly the number of IgA + cells compared with the control (basal sample). No significant differences between any groups were observed 10 days post challenge (Fig. 3A).

Total s-IgA increased in the intestinal fluid in mice fed only previously with the probiotic 10 days post-challenge (55.90 ± 5.48 μg / ml) compared to the infection control (41.75 ± 4.86 μg/ml) (Fig. 3B).

Specific s-IgA against *S.* Typhimurium showed significant increases ($P<0.05$) in both groups (7d-*S* and 7d-*S-Lc*) of mice fed 7 days with *L. casei* CRL 431, 7 and 10 days post challenge, compared to the infected control group. Specific s-IgA levels in the intestinal fluids from the other test groups remained similar to the control in both 7 dpI and 10 dpI samples (Fig. 3C).

3.3. Influence of L. casei CRL 431 administration on PMN myeloperoxidase activity

The ability of *L. casei* CRL 431 to modulate the inflammatory response was determined by measuring the total PMN myeloperoxidase (MPO) in different segments of small intestine (duodenum, jejuna and ileum).

The assay was performed 7 days post infection with only the groups of mice fed *L. casei* 7 days before infection (7d-S and 7d-*S-Lc*) and in the infection control (*S*). The basal sample was obtained before *Salmonella* challenge and was compared the group of probiotic administration during 7 days with the control mice without special feeding.

The results obtained in the ileum were the only ones that showed significant differences between the groups (Fig. 4A).

The basal data showed that probiotic administration did not modify the MPO activity compared to the untreated control. This coincidences with the lack of an inflammatory response induced by a probiotic.

MPO activity increased 7 days post-*Salmonella* challenge in all the experimental groups assayed, nevertheless the infection control showed the highest enhancement (0.048 ± 0.016 UE/g), being significantly lower in both groups of mice given *L. casei* (0.018 ± 0.009 UE/g and 0.007 ± 0.006 UE/g for 7d-S and for 7d-S-Lc, respectively).

Table 2
S. Typhimurim counts in the large intestine and bacterial translocation to spleen and liver.

Organ	Sampling time	Experimental groups						
		S	2d-S	5d-S	7d-S	2d-S-Lc	5d-S-Lc	7d-S-Lc
Spleen	7 dpI	$6.67 \pm 0.83^{a,b,c}$	8.41 ± 0.36^{d}	7.32 ± 0.33^{c}	8.6 ± 0.63^{d}	$7.02 \pm 1.46^{a,b,c}$	6.87 ± 0.07^{b}	3.3 ± 0.37^{e}
	10 dpI	5.7 ± 0.55^{a}	ND	6.73 ± 1.21^{a}	8.56 ± 0.64^{d}	5.21 ± 2.52^{a}	6.62 ± 1.64^{a}	3.4 ± 0.28^{e}
Liver	7 dpI	$7.12 \pm 0.89^{a,b}$	8.96 ± 1.05^{a}	7.22 ± 0.56^{a}	8.6 ± 0.22^{a}	$7.24 \pm 1.42^{a,b}$	$6.62 \pm 1.91^{a,b}$	6.2 ± 0.29^{b}
	10 dpI	$6.93 \pm 2.56^{a,b}$	ND	8.32 ± 1.37^{a}	$7.44 \pm 2.26^{a,b}$	$5.32 \pm 3.18^{a,b}$	$6.5 \pm 0.82^{a,b}$	1.6 ± 0.02^{c}
Large intestine	7 dpI	$5.88 \pm 0.82^{a,c}$	8.41 ± 1^{b}	7.85 ± 1.06^{b}	$6.78 \pm 2.52^{a,b,c}$	$5.83 \pm 2.92^{a,c}$	$7.11 \pm 1.96^{a,b}$	3.8 ± 1.26^{c}
	10 dpI	$4.39 \pm 0.86^{a,c}$	ND	7.74 ± 0.51^{b}	$8.08 \pm 2.02^{a,b}$	$4.36 \pm 1.98^{a,c}$	$5.27 \pm 1.27^{a,c}$	1.1 ± 0.26^{d}

Colony counts are expressed as $\log_{10}$ numbers of bacteria per gram of organ. Each value represents the mean of $N = 15 \pm$ SD. For each organ, comparing all the groups, means without a common letter differ significantly ($P<0.05$). ND = Not determined.

3.4. Determination of the phagocytic and microbicidal capacity of macrophages isolated from peritoneum, Peyer's patches and spleen

Phagocytosis using *Candida albicans* as an antigen was analyzed in the basal samples (after 2, 5 or 7 days of *L. casei* CRL 431 administration). Results showed that 5 and 7 days of probiotic feeding significantly increased ($P<0.05$) the phagocytic activity of magrophages isolated from Peyer's patches (28 ± 2 and 37 ± 1 for 5d and 7d, respectively) compared to the control without special feeding (18 ± 4). Mice fed with *L. casei* CRL 431 during 7 days were the unique ones that showed significant increases ($P<0.05$) of phagocytosis in macrophages isolated from spleen (23 ± 3) or peritoneum (33 ± 1) compared to the control (16 ± 1, and 19 ± 4 for spleen and peritoneal macrophages, respectively, Table 3).

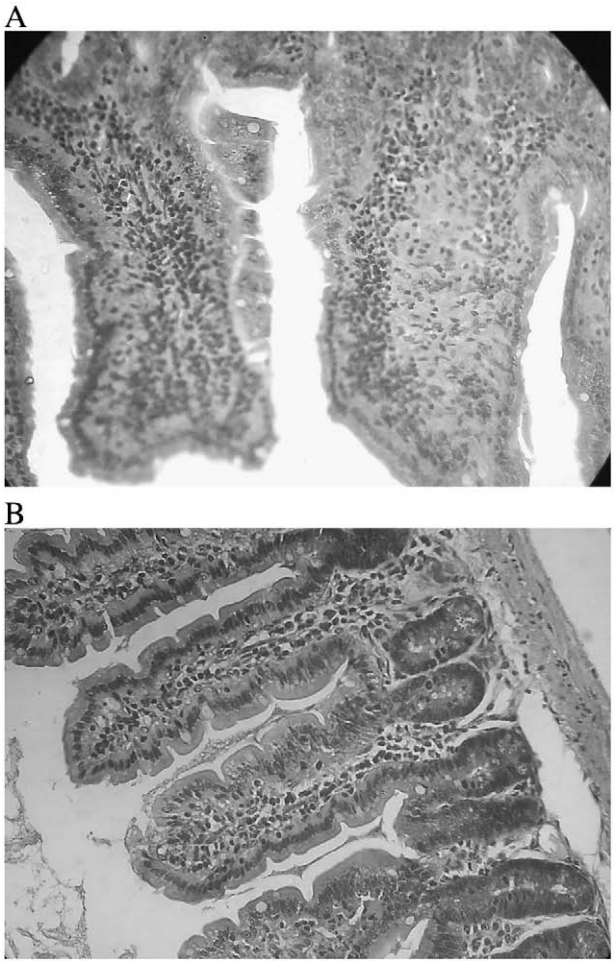


Fig. 2. Histological study of the small intestine. Slices from small intestine of mice were studied after staining with hematoxilin-eosin. The samples were obtained 7 dpI with a magnification of 100×. A) S group: Severe mucosal damages are observed with loss of villi and an influx of inflammatory cells in the mucosa and submucosa. B) 7d-S-Lc group: Tissue maintained the typical structure of the small intestine. It is possible to observe immune cells infiltration in specific areas.

Based on this data, the group of mice fed *L. casei* during 7 days was used to analyze the specific phagocytic activity against *S.* Typhimurium in the basal sample and two days after the challenge with the pathogen. The results obtained showed that 7 days of probiotic administration increased significantly ($P<0.05$) the phagocytic activity of macrophages isolated from peritoneal cavity, Peyer's patches and spleen (64 ± 4, 15 ± 3 and 40 ± 6 respectively) compared to the untreated control group (38 ± 7, 8 ± 2 and 25 ± 8 respectively, Table 3). No significant differences between the groups were found when the macrophages were isolated two days after *Salmonella* infection, even when the mice continued receiving the probiotic bacteria after the challenge with the pathogen (Data not shown).

The microbicidal activity from peritoneal and Peyer's patches macrophages against *S.* Typhimurium was measured by *ex vivo* assay after 2, 5, or 7 days of *L. casei* CRL 431 administration. No significant increases were observed in the microbicidal activity of macrophages isolated from mice that received probiotic compared to the control mice without special feeding (Fig. 4B).

3.5. Analysis of apoptotic cells in small intestine tissues

The assays for apoptosis were carried out only in the groups of mice fed 7 days with *L. casei* CRL 431 (7d Lc, 7d-S and 7d-S-Lc) where the protection of the probiotic against *S.* Typhimurim was observed. No significant differences were observed in the number of apoptotic cells in some of the groups of mice that received the probiotic compared to the infection control at 7 days post infection (Fig. 5).

4. Discussion

The preventive effect of probiotic microorganisms against enterophagenic bacteria has been extensively reported (Callaway et al., 2008; Jain et al., 2008; Szabo et al., 2009). Products containing probiotic microorganisms have been included to the daily diet due to the potential beneficial effects attributed to these microorganisms. The knowledge of the possible mechanisms by which these microorganisms can act against infection is necessary in order to establish the scientific basis by which the consumption of the probiotic microorganisms can prevent specific infections. Furthermore, there is little research performed to evaluate if probiotic administration is required after an intestinal infection to maintain its preventive effect or if this latter administration can add benefits improving the pathogen elimination and recovering the normal intestinal functioning.

On other hand, it is known that each probiotic strain has different properties and that it is not possible to extrapolate the effects found with one probiotic strains to others or its effect against a specific pathogens to other pathogen (Perdigon et al., 2002).

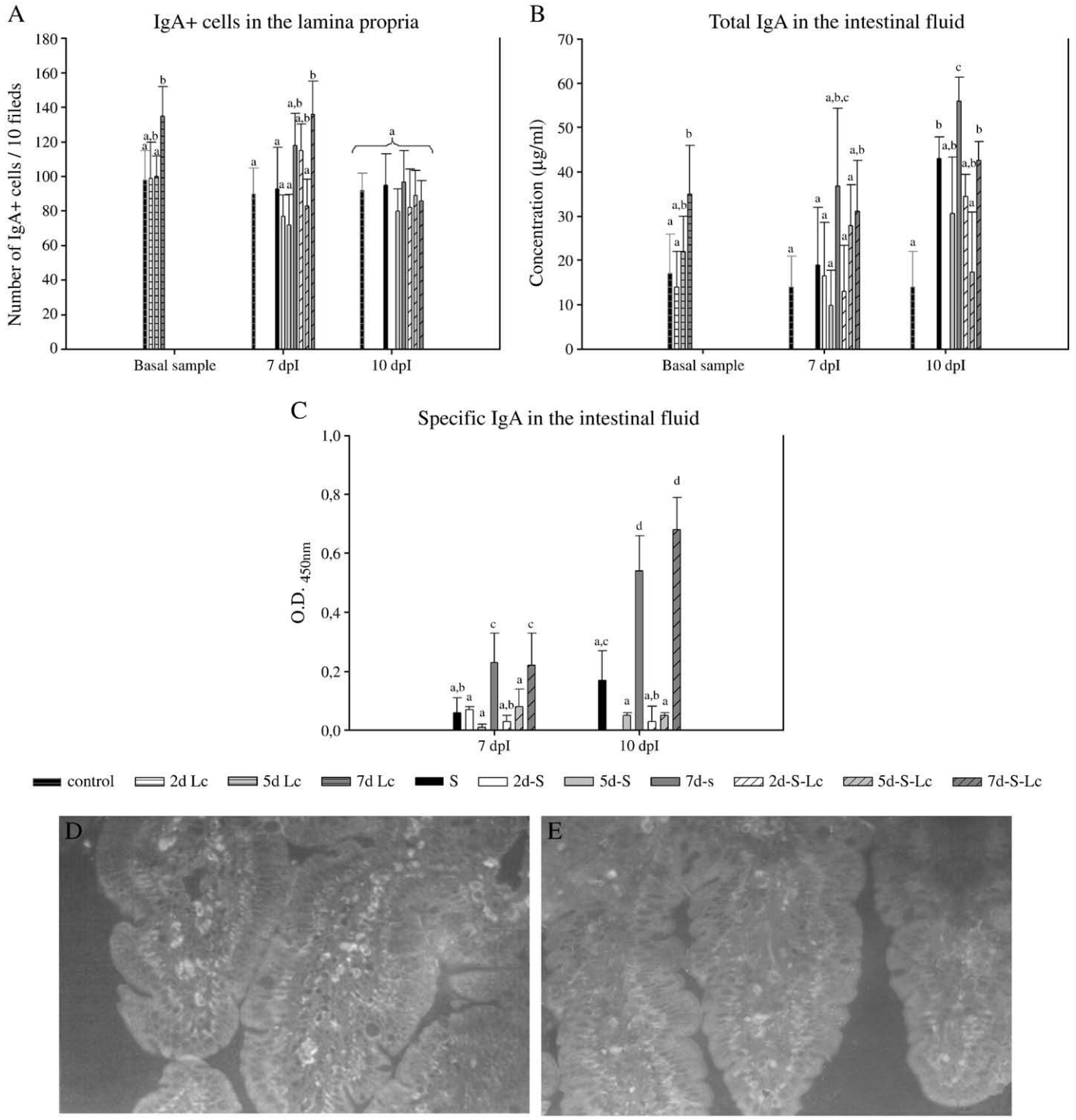


Fig. 3. Effect of *L. casei* 431 administration and *Salmonella* challenge on s-IgA in the small intestine. IgA + cells were counted in histological sections from small intestine obtained the day of the infection (basal sample) and 7 and 10 dpI (A). The microphotographs (1000×) show the increase of IgA + cells (fluorescent cells) in a mouse of 7d-S-Lc group (D) compared to a mouse of S group (E), 7 dpI. ELISA was used to measure the concentration of total s-IgA (B) and specific anti-*S.* Typhimurium s-IgA (C) in the small intestine fluids. The samples were obtained from mice of different groups, the day of the infection (basal sample) for total IgA, and 7 and 10 dpI for both determinations. Data correspond to the means ± SD of results of $N = 15$ animals from three separate experiments. For each figure, means for each value without a common letter differ significantly ($P<0.05$).

In the present work, *L. casei* CRL 431 was evaluated in the prevention of *S.* Typhimurium infection as well as its continuous administration after pathogen challenge. *L. casei* CRL 431 was used in the animal trials because there are many reports demonstrating its capacity to modulate the intestinal immune system, specially the innate immunity (Perdigon et al., 1993; 2002; Galdeano and Perdigon, 2006; Galdeano et al., 2007). The preventive effect of this probiotic strain against *S.* Typhimurium infection was previously demonstrated in a malnourished mice model (Cano and Perdigon, 2003), where the best effect was reported for 7 days of *L. casei* CRL 431 administration.

In the present work, for both models (preventive and continuous administration of probiotic), mice given *L. casei* CRL 431 during 7 days before *S.* Typhimurium were the groups where the best effects were observed, being important the continuous administration of the

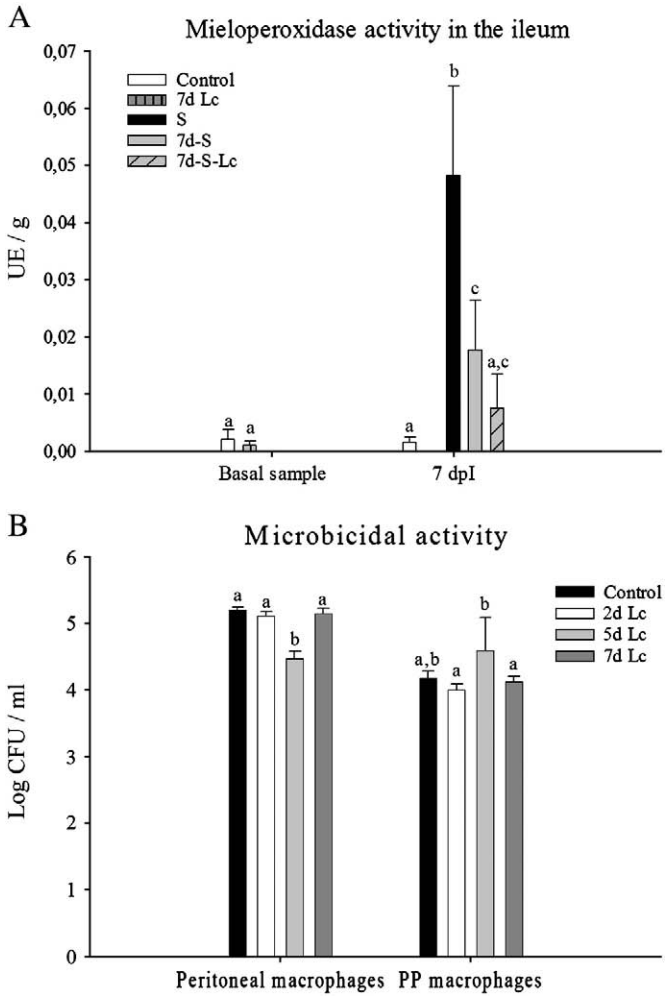


Fig. 4. Mieloperoxidase activity in the ileum fluid and microbicidal capacity of macrophages isolated from peritoneum and Peyer's patches. The activity of the MPO enzyme was measured in the ileum fluids in the basal sample and 7 dpI for control and test groups (A). For this assay the test groups were mice that received *L. casei* CRL 431 during 7 days (7d Lc) and continued or not receiving the probiotic after Salmonella challenge (7d-S and 7d-S-Lc). Microbicidal activity of macropahages isolated from peritoneum and Peyer's patches (PP) was analyzed in the basal sample, after 2, 5 or 7 days of *L. casei* CRL 431 administration (2d Lc, 5d Lc and 7d Lc) compared with the control group without special feeding (B). Data correspond to the means ± SD of results of $N = 15$ animals from three separate experiments. Means for each value without a common letter differ significantly ($P<0.05$). In the figure A the comparisons were performed between different groups and time points. In the figure B, the statistical analysis was performed for each site (peritoneum and PP).

probiotic (before and after challenge) in order to improve the effect of this probiotic bacterium against *S.* Typhimurium infection.

To know the possible mechanisms by which *L. casei* CRL 431 acts in the protection against *S.* Typhimurium infection several parameters were evaluated. Secretory IgA (s-IgA) antibodies are the major effector molecules in the mucosal system and their role as the first defense line against infections has been well demonstrated (Mazanec et al., 1993). One of the functions reported for the intestinal s-IgA is the immune exclusion. In this sense, s-IgA limited the translocation of aerobic bacteria from the intestinal lumen to the mesenteric lymph nodes in adult mice (Macpherson and Uhr, 2004). According to Lamm (1998), IgA can act in the intestinal lumen, into the intraepithelial cells and in the lamina propria; therefore the number of IgA + cells present in the lamina propria was also analyzed in the present work. The study of these cells showed that mice given *L. casei* CRL 431 during 7 days before *S.* Typhimurium infection and that continued with the probiotic administration increased the number of IgA + cells compared to the infection control, in the small intestine, 7 days after infection (Fig. 3A).

IgA + cells can release the antibody in the lumen and the s-IgA levels can increase in the intestinal fluid, this fact was also analyzed. Total s-IgA increased significantly 10 days post-infection only in the group of mice fed *L. casei* CRL 431 during 7 days previous to the infection. We believe that the diminution in the level of total s-IgA after 10 days pathogen challenge in the group with continuous probiotic administration (Fig. 3B), was due to an increase in the level of specific anti-*Salmonella* antibody because this antibody increased more in group 7d-S-Lc than in the group 7d-S where the concentration of the total s-IgA was maintained elevated. However, it was not enough to stop or to diminish *Salmonella* infection. This result showed that even when it is known the important role of the s-IgA to avoid *Salmonella* internalization, other different mechanisms should be implicated in the protection against this infection observed in mice fed continuously with the probiotic bacteria.

Considering the mechanisms implicated in the pathway of internalization of *Salmonella,* it is known that after contact between *Salmonella* and intestinal epithelial cells, and before the epithelium loses its structural integrity, the pathogen induce the release of IL-8 from the epithelial cells favoring the transepithelial migration of neutrophils (PMN) across intestinal epithelia (McCormick et al., 1993; Wall et al., 2007). PMN infiltration into intestine was quantified by measuring MPO enzyme activity (a marker to determine neutrophil infiltration) (Bradley et al., 1982; Colletti et al., 1990; Schmekel et al., 1990). The enzyme activity was evaluated in all the small intestine, being the ileum the intestinal portion where the infection induced the highest MPO activity, compared to those observed in the basal samples previous to the infection. This observation agrees with the prior data where it was reported that *Salmonella enteritidis* attacks the terminal ileum and to a lesser extent the colon (Welton et al., 2008). It is important to note that the probiotic administration did not suppress the PMN infiltration in both groups of mice (previous and continuous administration) fed with *L. casei* where the MPO activity increased significantly after the infection, compared to the basal data. Probiotic administration was only able to diminish the PMN infiltration and consequently, the inflammatory response (Fig 4A). This finding was not an unexpected result, because the neutrophils are the first line of defense that acts when the pathogen comes into contact with the intestine (Wall et al., 2007).

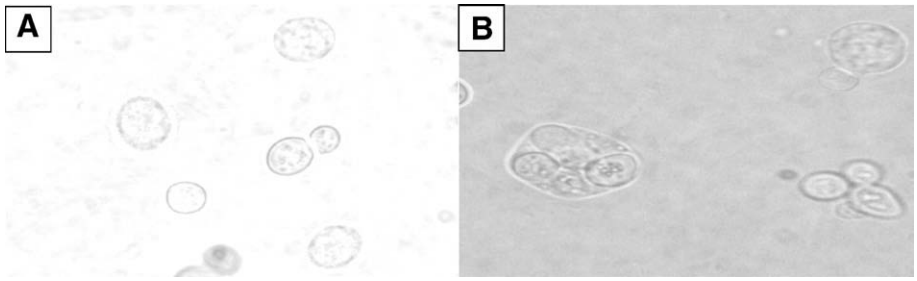
It was reported that *L. casei* CRL 431 stimulates the activity of the immune cells associated to the gut, mainly the cells of innate response (Galdeano et al., 2007). In Peyer's patches, *Salmonella* preferentially interacts with M cells, invades them and then finds macrophages and DC resident in the subepithelial dome (Guerrant et al., 1999). Furthermore, bacteria can migrate to the mesenteric lymph nodes where they can come into contact with other macrophages and spread the infection to deep tissues. For this reason, the phagocytic activity of macrophages against *C. albicans* and *S.* Typhimurium was analyzed in Peyer's patches, spleen and peritoneum.

It was also reported that when the bacterium enters in the phagocytic cells, resides in them resulting in large membrane-bound vacuoles and expressing several gene products that enhance the intracellular survival by neutralising lysosomal killing mechanisms (Uchiya et al., 2004; Hautefort et al., 2008). This pathogen appears to be uncoupled from the main endocytic route, and after a lag period it replicates inside the host cell (Brumell and Grinstein, 2004; Mastroeni and Sheppard, 2004). For this reason, considering that phagocytosis was increased significantly in mice given probiotic during 7 days (Table 3), the microbicidal capacity of these phagocytes was then evaluated by *ex vivo* assay. Significant increases were not found in the test groups compared to the untreated control group (Fig. 4B) suggesting that other mechanisms would also be implicated in the protective effect exerted by this bacterial strain.

Apoptosis was studied due to the fact that after the pathogen replicates inside the host cells, these cells die by apoptosis or lyses, releasing the bacteria to invade other cells (Finlay and Cossart, 1997).

Table 3
Effect of *L. casei* CRL 431 administration on the phagocytic activity of macrophages.

Experimental groups	Percentage of phagocytosis after *L. casei* administration					
	Peritoneal macrophages		Peyer's patches macrophages		Spleen macrophages	
	C. albicans	*S. Typhimurium*	*C. albicans*	*S. Typhimurium*	*C. albicans*	*S. Typhimurium*
Control	$19 \pm 4^{a,b}$	38 ± 7^{a}	18 ± 4^{a}	8 ± 2^{a}	16 ± 1^{a}	25 ± 8^{a}
2d Lc	15 ± 2^{a}	ND	19 ± 1^{a}	ND	$17 \pm 3^{a,b}$	ND
5d Lc	26 ± 4^{b}	ND	28 ± 2^{b}	ND	18 ± 1^{a}	ND
7d Lc	33 ± 1^{c}	64 ± 4^{b}	37 ± 1^{c}	15 ± 3^{b}	23 ± 3^{b}	40 ± 6^{b}



Basal samples (the day of the *Salmonella* infection and before the challenge) were used to isolate the macrophages from peritoneum, Peyer's patches and spleen of mice. The activity of these cells was determined by phagocytosis assay of *Candida albicans* or *S. Typhimurium*. The values are expressed as mean for $N = 15 \pm$ SD of percentage of phagocytosis expressed as the percentage of phagocyting macrophages in 200 cells counted. Means values for each column without a common letter differ significantly ($P<0.05$). ND = Not determined. Figures A and B compare the macrophages from the peritoneum of a mouse of S group (A) with a mouse of 7d Lc group (B). This last photograph shows the yeast taking contact with one macrophage and other yeasts inside of other macrophage (magnification 1000×).

In this manner, apoptosis may be an important step in the pathogenesis of *S.* Typhimurium (Monack et al., 1996; van der Velden et al., 2000) and the possibility that the probiotic can interfere in apoptosis process was considered. Apoptosis studies did not show differences between the infection control group and the groups fed with *L. casei* during 7 days (previous to *Salmonella* or continuously before and after the infection, Fig. 4). The inhibition of apoptosis would not be a mechanism mediated by *L. casei* CRL 431 to avoid *Salmonella* dissemination to the deep tissues.

Our results demonstrated that 7 days of *L. casei* CRL 431 administration decreased the severity of the infection with *S.* Typhimurium in a mouse model, showing the continuous administration the best effect. This continuous administration diminished the counts of the pathogens in the intestine as well as their spreads outside this organ. Several mechanisms and cells are involved in this protective effect against *S.* Typhimurium; *L. casei* acted on cells of the innate and adaptive immune response. The probiotic administration decreased the PMN infiltration with the consequent diminution of intestinal inflammation, activated the macrophage phagocytic activity in different sites such as Peyer's patches, spleen and peritoneum, and the number of IgA + cells in the lamina propria of the small intestine which was correlated with increased release of s-IgA specific against the pathogen in the intestinal fluids. The mechanism of the inhibition of cellular apoptosis was not involved.

Other immune cells and mechanisms that could be also implied in the effect of this probiotic bacterium against *S.* Typhimurium are the differential signals induced in the epithelial cells by the probiotic strain assayed, able to diminish the inflammatory response induced by the pathogen; these studies are currently on way.

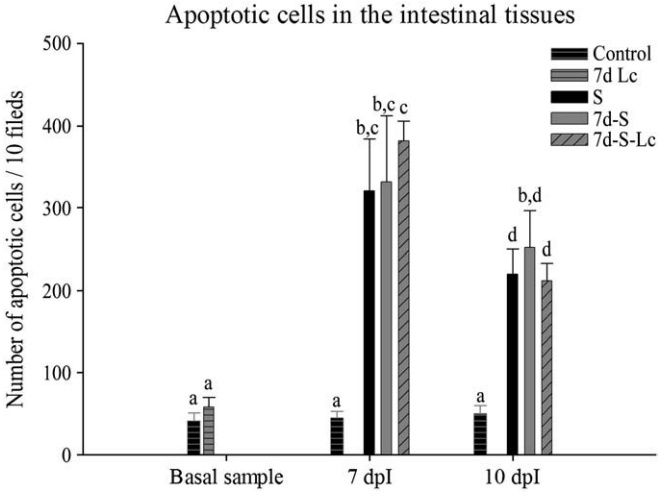


Fig. 5. Determination of apoptotic cells in the small intestine. Apoptotic cells were counted in histological sections from small intestine of mice fed 7d with *L. casei* CRL 431. The samples were taken the day of the infection (basal) and 7 and 10 days post challenge for both 7d-S and 7d-S-Lc groups, compared to the control groups. Means for each value without a common letter differ significantly ($P<0.05$).

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