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# Immunomodulatory activity of *Lactobacillus rhamnosus* strains isolated from goat milk: Impact on intestinal and respiratory infections

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ABSTRACT

The immune stimulation induced by *Lactobacillus rhamnosus* CRL1505 (Lr05) and *L. rhamnosus* CRL1506 (Lr06) on the resistance to infection with an intestinal pathogen (*Salmonella typhimurium*) and a respiratory pathogen (*Streptococcus pneumoniae*) was studied in swiss-albine mice experimental models. The cytokine profiles that induced the innate and specific immune response in both infectious processes were investigated. Both strains were able to improve resistance against the intestinal pathogen. Only Lr05 was able to induce a significant decrease in the number of *S. pneumoniae* in the lung, prevent its dissemination into the blood and induce a significant increase in Th1 (INF-γ) and Th2 (IL-6, IL-4 and IL-10) cytokine levels in the bronchoalveolar lavages (BAL). The changes in the cytokines profiles in BAL were associated with an increase in the number and activity of phagocytic cells and with the increase in specific antibodies in serum and BAL, which would explain the increased resistance to the challenge. The administration of Lr06 did not induce significant effects at the respiratory mucosal level. The results described in the present paper showed that certain LAB strains can share certain functional properties, although some of them can perform a functional role better than others, so that it is important to perform careful studies on specific strains, according to their therapeutic use.



## 1. Introduction

The use of lactic acid bacteria (LAB) in foods is widely distributed due to their beneficial effects on health such as the increase in the innate immune response (Jain et al., 2008), control of intestinal infections (Perdigon et al., 1990), influence on cholesterol levels (Taranto et al., 1998) and anticarcinogenic effect (Commane et al., 2005). At present there is great interest in the development of oral vaccines using recombinant LAB that express heterologous proteins on the surface. These proteins combine the adjuvant properties of the bacterium with the induction of antibodies specific for the pathogen (Villena et al., 2008). Besides, the recombinant bacterium serves as a particulate antigen for the induction of the B cell memory (Corthésy et al., 2005). However, the properties of LAB that are required for specific applications are different, so that the appropriate selection of strains is very important according to their therapeutic use.

Several studies have demonstrated that the oral administration of certain LAB has an influence on intestinal immunity and that they can afford protection against microbial enteropathogens (Perdigon et al., 1991; Medici et al., 2005; Delcenserie et al., 2008). In addition, it has been demonstrated that these bacteria can sufficiently stimulate the common mucosal immune system as to increase protection in distant mucosae such as the respiratory mucosa (Cross 2002; Villena et al., 2005). It is well known that each strain has specific properties and that its effects on the hosts' health cannot be extrapolated to other strains (Boyle et al., 2006; Pineiro and Stanton, 2007).

The selection of probiotic strains is difficult since up to now no clear concepts exist with respect to adequate methodology and criteria. Although until now probiotic products for multiple applications predominated on the market (Gueimonde and Salminen, 2006), the present trend is towards the selection of strains to be made according to specific uses.

There are in the literature comparative studies concerning the immunomodulatory activity between different species of LAB both *in vitro* (Haller et al., 2001) and *in vivo* (Perdigon et al., 1995), but there are very few in which the properties of strains belonging to the same species are compared. Recently *in vitro* studies were carried out to determine the immunomodulatory properties of *Bifidobacterium longum* strains in which the importance of selection criteria according to the therapeutic use is considered (Medina et al., 2007).

The ability to induce an increase in the number of IgA producing cells in the lamina propria of the gut was considered an important characteristic of a probiotic strain (Gauffin Cano et al., 2002; Vinderola et al., 2006). However, recent studies have shown that the induction of cytokines such as INF-γ, TNF-α and IL-6 by probiotic bacteria, are

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more representative than IgA+ cells in the characterization of a probiotic strain (Dogi et al., 2008).

In the last few years it has been demonstrated that the oral administration of different LAB strains has the capacity to induce different cytokine profiles in the gut and possesses differential intrinsic adjuvanticity (Maassen et al., 2000; Maldonado-Galdeano et al., 2007). Consequently, the study of the cytokine profile induced by a certain strain could be an adequate strategy to analyze its potentiality as a probiotic.

In this work we studied comparatively two LAB strains isolated from goat milk (with similar technological properties) and their influence cytokines induction during an infection with an intestinal pathogen (*Salmonella typhimurium*) and against an infection with a respiratory pathogen (*Streptococcus pneumoniae*) for the purpose of elucidating selection criteria of strains for protection against infection in two different mucosae. We also analyzed the relationship between cytokine profiles, the innate immune response, the production of systemic and mucosal antibodies and their probable role in the control of infections.

## 2. Materials and methods

### 2.1. Microorganisms

*Lactobacillus rhamnosus* CRL1505 and CRL1506 were obtained from the CERELA culture collection (Chacabuco 145, San Miguel de Tucumán, Argentina). Both strains were isolated from goat milk from northwestern Argentina. The culture was kept freeze-dried and then rehydrated using the following medium: peptone 15.0 g, tryptone 10.0 g, meat extract 5.0 g, distilled water 1 l, pH 7. It was cultured for 12 h at 37 °C (final log phase) in Man-Rogosa-Sharpe broth (MRS, Oxoid). The bacteria were harvested by centrifugation at 3000*g* for 10 min, washed three times with sterile 0.01 mol/l phosphate buffer saline (PBS), pH 7.2, and resuspended in sterile 10% non-fat milk.

### 2.2. Animals and feeding procedures

Male 6-week-old Swiss albino mice were obtained from the closed colony kept at CERELA. They were housed in plastic cages at room temperature. Mice were housed individually during the experiments and the assays for each parameter studied were performed in 5–6 mice per group for each time point. *L. rhamnosus* CRL1505 or CRL1506 was administered to different groups of mice for 2, 5 and 7 consecutive days at a dose of $10^8$ cells/mouse/day in the drinking water. The treated groups and the untreated control group were fed a conventional balanced diet *ad libitum*.

### 2.3. Phagocytic cell activation

*Nitro blue tetrazolium (NBT) test*. Bronchoalveolar lavage (BAL) samples were obtained as described previously by Villena et al. (2005). The phagocytic bactericidal activity (oxidative burst) of macrophage and neutrophil function in BAL samples were measured using the nitro blue tetrazolium (NBT) reduction test (catalogue no. 840-W; Sigma-Aldrich Co.) in the BAL pellet. NBT was added to all samples with (positive control) or without the addition of bacterial extract, and then they were incubated at 37 °C for 20 min. In the presence of oxidative metabolites, NBT (yellow) is reduced to formazan, which forms a blue precipitate. Smears were then prepared; after staining, these samples were examined under a light microscope for blue precipitates and 100 NBT positive (+) cells were counted.

### 2.4. Cytokine concentrations in serum, BAL and intestinal fluid

Blood samples were obtained through cardiac puncture at the end of each dietary treatment and collected in heparinized tubes. BAL samples were obtained as described previously (Villena et al., 2005). Intestinal fluid (IF) samples were obtained as follows: the small intestine was flushed with 5 ml of PBS and the fluid was centrifuged (10,000*g*, 4 °C 10 min) to separate particulate material. The supernatant was kept frozen until use. Tumour necrosis factor (TNF)-α, interferon (INF)-γ, interleukin (IL)-1β, IL-4, IL-6 and IL-10 concentrations in serum, BAL and IF, were measured with commercially available enzyme-linked immunosorbent assay (ELISA) technique kits following the manufacturer's recommendations (R&D Systems, MN, USA).

### 2.5. S. typhimurium infection

Treated and control mice were challenged with 50 µl of $10^7$ cells/mouse of *S. typhimurium* ($20LD_{50}$) by oral administration. The intestinal pathogen was isolated from infant faeces and provided by the Children's Hospital (Tucuman-Argentina). An aliquot (200 µl) from an overnight culture was placed in 5 ml of sterile BHI broth and incubated for 4 more hours (37 °C, aerobiosis). The concentration of *Salmonella* was adjusted to $1.10^7$ CFU in phosphate buffered saline (PBS). Mice were sacrificed on days 2 and 14 post-infection (Perdigon et al., 1991).

### 2.6. S. pneumoniae infection

The experimental animal model of respiratory infection was used as previously described (Villena et al., 2005). Capsulated *S. pneumoniae* serotype 14 (ANLIS, Argentina) was obtained from the respiratory tract of a patient from the Children's Hospital (Tucuman-Argentina). Treated and control mice were challenged intranasally with the pathogen by dripping 25 µl of an inoculum containing $10^6$ CFU (log phase) in PBS into each nostril and allowing it to be inhaled. Mice were sacrificed on days 2 and 14 post-infection.

### 2.7. Bacterial cell counts in liver, spleen and blood for S. typhimurium infection

The liver and spleen of mice in the different experimental groups were removed on day 2 post-challenge. Organs were homogenized in 0.1% peptone water, diluted appropriately ($10^{-1}$–$10^{-4}$) and plated in MacConkey agar. Plates were incubated at 37 °C for 48 h. Results were expressed as the number (log) of CFU/g of organ. Bacteremia was monitored by blood samples obtained by cardiac puncture which were plated on MacConkey agar. Results were reported as negative or positive hemocultures.

### 2.8. Bacterial cell counts in the lung and blood for S. pneumoniae infection

Lungs were excised on day 2 post-challenge, weighed and homogenized in sterile peptone water. Homogenates were diluted appropriately, plated in duplicate on blood agar and incubated for 18 h at 37 °C. The results were expressed as log of CFU/g of organ. Bacteremia was monitored by blood samples obtained by cardiac puncture which were plated on blood agar. Results were reported as negative or positive hemocultures.

### 2.9. Leukocyte counts in the blood and BAL

Blood and BAL samples were obtained as described above. The total number of leukocytes and differential cell counts were performed as described previously (Villena et al., 2005; Racedo et al., 2006).

*Washburn test*. Measurement of myeloperoxidase activity of blood neutrophils was carried out as described previously by Villena et al. (2005). Cells were graded as negative or weakly, moderately, or strongly positive and were used to calculate the score.

### 2.10. Specific IgA and IgG antibodies (anti-pneumococcal and anti-salmonella antibodies)

Anti-pneumococcal antibodies (IgA and IgG) in serum and BAL were determined on day 14 after challenge using the ELISA technique previously described (Villena et al., 2005). An ELISA test was developed to measure anti-salmonella antibodies (IgA) in IF. Plates were coated with a heat killed *S. typhimurium*–sodium carbonate–bicarbonate buffer (1:100) suspension, pH 9.6. Non-specific protein binding sites were blocked with PBS containing 5% NFM. Samples were diluted (1:10) with PBS containing 0.05% (v/v) Tween 20 (PBS-T). Peroxidase-conjugated goat anti-mouse IgA Fc specific (Sigma) was diluted (1:100) in PBS-T. Antibodies were revealed with a substrate solution [3–30, 5-50-tetramethylbenzidine (Sigma)] in citrate–phosphate buffer (pH 5, containing 0.05% $H_2O_2$) and the reaction was stopped by addition of $H_2SO_4$ 1 M. Readings were carried out at 493 nm. Antibody concentration was expressed as pg/ml determined from a standard curve made with commercial mouse IgA or IgG (Sigma).

### 2.11. Statistical analysis

Experiments were performed in triplicate and results were expressed as mean ± standard deviation (SD). After verification of the normal distribution of data, 2-way ANOVA was used. Tukey's test (for pairwise comparisons of the means) was used to test for differences between the groups. Differences were considered significant at $p<0.05$.

## 3. Results

### 3.1. Phagocytic cell activation

In order to study the effect of the treatments on the phagocytic activity of alveolar and peritoneal macrophages we used the NBT test. The animals were fed *L. rhamnosus* CRL1505 or *L. rhamnosus* CRL1506 for 2, 5 or 7 days and at the end of each treatment the NBT test was performed. The administration of *L. rhamnosus* CRL1505 for 5 or 7 days was able to increase significantly the percentage of peritoneal NBT+ cells, while only the treatment for 5 days with *L. rhamnosus* CRL1506 was able to achieve such effect (Table 1). Only the treatment with *L. rhamnosus* CRL1505 induced a significant increase in the percentage of alveolar NBT+ cells (Table 1).

**Table 1**
Phagocytic cells activation.

| | % Peritoneal NBT+ cells | | % BAL NBT+ cells | |
|---|---|---|---|---|
| Groups | Non-stimulated | Stimulated | Non-stimulated | Stimulated |
| Control | 28.4 ± 0.8 | 33.8 ± 1.1 | 17.5 ± 1.1 | 25.8 ± 0.8 |
| *L. rhamnosus* CRL1505 2d | 30.5 ± 1.0 | 35.6 ± 1.4 | 18.3 ± 0.9 | 27.2 ± 1.1 |
| *L. rhamnosus* CRL1505 5d | 36.5 ± 1.9[a] | 63.5 ± 1.5[a] | 32.9 ± 1.2[a] | 51.9 ± 1.3[a] |
| *L. rhamnosus* CRL1505 7d | 33.4 ± 1.2[a] | 55.2 ± 1.6[a] | 25.2 ± 1.3[a] | 33.5 ± 1.8 |
| *L. rhamnosus* CRL1506 2d | 29.5 ± 0.9 | 35.2 ± 0.9 | 18.9 ± 1.4 | 27.8 ± 1.0 |
| *L. rhamnosus* CRL1506 5d | 37.1 ± 1.3[a] | 68.9 ± 1.3[a] | 20.1 ± 1.1 | 28.5 ± 1.9 |
| *L. rhamnosus* CRL1506 7d | 31.4 ± 1.1 | 37.6 ± 1.4 | 21.3 ± 0.8 | 26.7 ± 0.7 |

Percentage of BAL and peritoneal NBT+ cells of mice that received *L. rhamnosus* CRL1505, or *L. rhamnosus* CRL1506 for 2, 5 and 7 days, compared to control mice. Values are means for $n=6\pm$SD.
[a] Significant differences between test and control groups ($p<0.05$).

### 3.2. Resistance against S. typhimurium infection

The administration of *L. rhamnosus* CRL1505 and *L. rhamnosus* CRL1506 for 5 and 7 days induced a significant decrease in the number of *S. typhimurium* in the liver and spleen with respect to the control (Table 2). Besides, in the animals that received such treatments, the pathogen was not detected in the blood (Table 2).

### 3.3. Resistance against S. pneumoniae infection

Only the group that received *L. rhamnosus* CRL1505 for 5 consecutive days showed *S. pneumoniae* counts in the lung significantly lower than those of the controls. In addition, it was the only treatment that proved effective to limit the dissemination of the pathogen, since hemocultures were negative (Table 3). None of the treatments with *L. rhamnosus* CRL1506 modified the lung clearance of the pneumococcus or prevented its dissemination (Table 3).

On the basis of the above results, we selected as the optimum treatments the administration of *L. rhamnosus* CRL1505 and CRL1506 for 5 days. The time dependent effect of lactic acid bacteria on host immunity has been previously reported. Our studies with the probiotic strain *Lactobacillus casei* CRL431 showed that five and seven days treatments with the probiotic bacteria were not as effective as two days treatment in improving the immune response against respiratory pathogens, probably because the large number of bacterial antigens that enter the gut would induce the appearance of suppressor T cells, which would on the one hand, prevent an undesirable effect on the gut but, on the other, would cause a decrease in the immune stimulation (Villena et al., 2005; Racedo et al., 2006). Thus, the following experiments were performed only with *L. rhamnosus* CRL1505 and CRL1506 5d treatments.

### 3.4. Basal cytokine concentrations

Before assessing the response against pathogens, we studied the effect of the selected treatments on the concentration of cytokines both in the intestinal and respiratory mucosae and at the systemic level.

Animals treated with *L. rhamnosus* CRL1505 and CRL1506 showed significant increases in TNF-α in IF with respect to the control group, without differences between them (Fig. 1a). In contrast, LAB administration failed to induce modifications in TNF-α and IL-1β levels in serum and BAL (Fig. 1b,c). Both treatments with LAB induced significant increases in INF-γ levels in serum and IF with respect to the control. However, only the group treated with *L. rhamnosus* CRL1505 showed an increase in this cytokine in BAL (Fig. 1). The levels of IL-6 and IL-4 in IF increased significantly with respect to the control in

**Table 2**
Resistance against *Salmonella typhimurium* infection.

| *Salmonella typhimurium* counts | | | |
|---|---|---|---|
| Groups | Liver (log CFU/g organ) | Spleen (log CFU/g organ) | Blood |
| Control | 4.46 ± 0.03 | 3.21 ± 0.05 | Positive |
| *L. rhamnosus* CRL1505 2d | 3.97 ± 0.15 | 2.91 ± 0.79 | Positive |
| *L. rhamnosus* CRL1505 5d | 2.65 ± 0.29[a] | 2.23 ± 0.88[a] | Negative[a] |
| *L. rhamnosus* CRL1505 7d | 3.20 ± 0.46[a] | 2.20 ± 0.85[a] | Negative[a] |
| *L. rhamnosus* CRL1506 2d | 3.93 ± 0.50 | 2.25 ± 0.12[a] | Positive |
| *L. rhamnosus* CRL1506 5d | 2.50 ± 0.16[a] | 2.00 ± 0.25[a] | Negative[a] |
| *L. rhamnosus* CRL1506 7d | 3.41 ± 0.44[a] | 2.33 ± 0.11[a] | Negative[a] |

*Salmonella typhimurium* CFU counts in liver and spleen, and hemocultures positive or negative of mice that received during 2, 5 and 7 days *L. rhamnosus* CRL1505 or *L. rhamnosus* CRL1506, compared to control mice after oral challenge with *Salmonella typhimurium* (day 2 post-infection). Values are means for $n=6\pm$SD.
[a] Significant differences between test and control groups ($p<0.05$).

**Table 3**
Resistance against *Streptococcus pneumoniae* infection.

| *Streptococcus pneumoniae* counts | | |
|---|---|---|
| Groups | Lung (log CFU/g organ) | Blood |
| Control | 5.83 ± 0.21 | Positive |
| *L. rhamnosus* CRL1505 2d | 6.32 ± 0.13 | Positive |
| *L. rhamnosus* CRL1505 5d | 3.98 ± 0.17[a] | Negative[a] |
| *L. rhamnosus* CRL1505 7d | 5.56 ± 0.32 | Positive |
| *L. rhamnosus* CRL1506 2d | 5.51 ± 0.22 | Positive |
| *L. rhamnosus* CRL1506 5d | 6.19 ± 0.36 | Positive |
| *L. rhamnosus* CRL1506 7d | 5.67 ± 0.27 | Positive |

*Streptococcus pneumoniae* CFU counts in the lung and hemocultures positive or negative of mice that received *L. rhamnosus* CRL1505 or *L. rhamnosus* CRL1506 for 2, 5 and 7 days, compared to control mice after nasal challenge with *Streptococcus pneumoniae* (day 2 post-infection). Values are means for $n = 6 \pm$ SD.
[a] Significant differences between test and control groups ($p < 0.05$).

animals treated with both LAB (Fig. 1a). Only the group treated with *L. rhamnosus* CRL1505 showed increased levels of IL-4 in serum, and the level of IL-6 was higher than in the group treated with *L. rhamnosus* CRL1506 (Fig. 1b). Moreover, only the *L. rhamnosus* CRL1505 treatment was able to induce significant increases in the level of both cytokines in BAL (Fig. 1c). The treatments with LAB induced significant increases in IL-10 levels in serum, IF and BAL with respect to the control. However, the group that received *L. rhamnosus* CRL1505 showed IL-10 levels significantly higher than the *L. rhamnosus* CRL1506 treated group (Fig. 1).

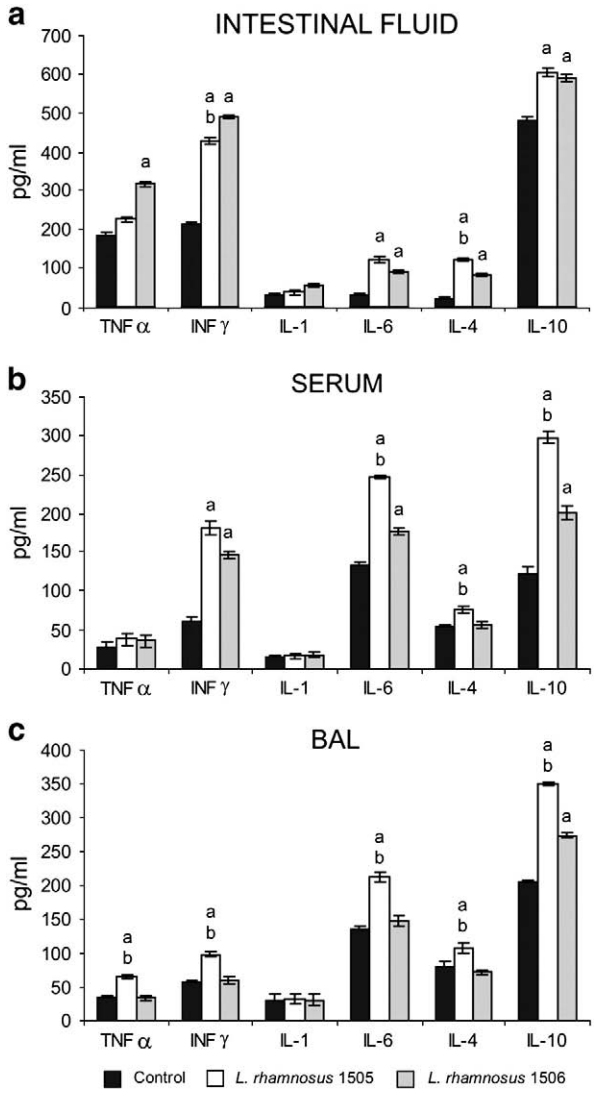


**Fig. 1.** Effect of bacterial administration on the tumour necrosis factor (TNF)-α, interferon (INF-γ), interleukin (IL)-1β, IL-4, IL-6 and IL-10 concentrations, in intestinal fluid (a), serum (b) and BAL (c) of mice that received *L. rhamnosus* CRL1505 or *L. rhamnosus* CRL1506, compared to control mice. Values are means for $n = 6 \pm$ SD. [a] Significant differences between test and control groups ($p < 0.05$) and [b] Significant differences between test and *L. rhamnosus* CRL 1506 groups ($p < 0.05$).

### 3.5. Immune response against S. typhimurium infection

#### 3.5.1. Blood leukocytes

The values of leukocytes, lymphocytes and neutrophils in the blood increased significantly after the challenge with *S. typhimurium* in all the assayed groups. However, these counts were significantly higher in the group treated with *L. rhamnosus* CRL1505 with respect to the groups treated with *L. rhamnosus* CRL1506 and to the untreated control (Table 4). We also determined the score of peroxidase activity as a measure of the activation of the phagocytic cells in the blood. After the challenge, an increase in the peroxidase score was observed in all the experimental groups. However, the animals that received the LAB presented values significantly higher than those in the control group (Table 4).

#### 3.5.2. Specific IgA and IgG antibodies

The values of anti-salmonella IgA in IF and serum anti-salmonella IgG were significantly higher in the groups treated with both LAB compared to the control (Fig. 2). However, the animals treated with *L. rhamnosus* CRL1505 presented values of pathogen-specific IgA significantly higher than the ones found in the group that received *L. rhamnosus* CRL1506 (Fig. 2a).

#### 3.5.3. Intestinal and serum cytokines

The infection significantly increased the levels of TNF-α and IL-1β in serum and IF in all the studied groups, with a greater increase in those that received LAB (Fig. 3). When comparing both strains, we observed that *L. rhamnosus* CRL1506 induced levels of TNF-α and IL-1β in IF significantly higher than the strain CRL1505. In serum, *L. rhamnosus* CRL1505 induced levels of TNF-α significantly higher than the strain CRL1506. At the intestinal level, the group that received *L. rhamnosus* CRL1506 presented levels of INF-γ significantly higher than the one that received *L. rhamnosus* CRL1505 (Fig. 3a). At the serum level, *L. rhamnosus* CRL1505 showed a greater increase in INF-γ compared to the strain CRL1506 (Fig. 3b). After the infection, the levels of IL-6 and IL-4 increased in all experimental groups. However, the treatment with LAB was able to induce a significant increase in IL-4 levels in IF and in the blood with respect to the control. Besides, *L. rhamnosus* CRL1505 presented significantly higher levels compared to the strain CRL1506 (Fig. 3). The treatment with both LAB induced a significant increase in the levels of IL-10 with respect to the control after the infection both in IF and in the blood (Fig. 3). However, the group that received *L. rhamnosus* CRL1505 presented levels of IL-10 significantly higher than those in the group that received *L. rhamnosus* CRL1506.

**Table 4**
Effect of the assayed strains on the blood leukocytes on intestinal infection.

| Groups | Leukocytes | Neutrophils | Peroxidase score |
|---|---|---|---|
| Control | 8.9 ± 0.5 | 1.9 ± 0.07 | 158.9 ± 3.1 |
| *L. rhamnosus* CRL1505 5d | 10.3 ± 0.3[a] | 2.2 ± 0.1[a] | 193.0 ± 2.5[a] |
| *L. rhamnosus* CRL1506 5d | 9.8 ± 0.2 | 1.8 ± 0.2 | 175.0 ± 3.1[a] |

Total and differential count of blood leukocytes ($10^9$cells/l) and peroxidase activity (score) of mice that received *L. rhamnosus* CRL1505 or *L. rhamnosus* CRL1506 for 5 days compared to control mice after oral challenge with *Salmonella typhimurium* (day 2 post-infection). Values are means for $n = 6 \pm$ SD.
[a] Significant differences between test and control groups ($p < 0.05$).

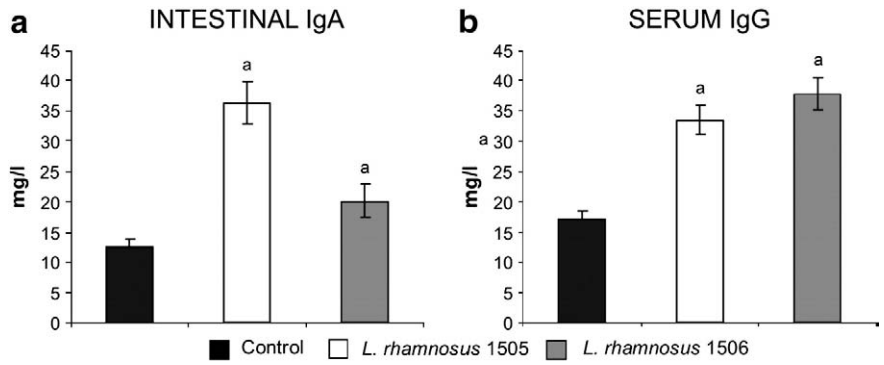


**Fig. 2.** Anti-salmonella antibodies of intestinal fluid (a) and serum (b) after challenge (day 15) with *Salmonella typhimurium* of mice that received *L. rhamnosus* CRL1505 or *L. rhamnosus* CRL1506 for 5 days. Values are means for $n=6\pm$SD. [a] Significant differences between test and control groups ($p<0.05$).

### 3.6. Immune response against S. pneumoniae infection

#### 3.6.1. Blood and BAL leukocytes

The challenge with *S. pneumoniae* induced an increase in the number of leukocytes and neutrophils in the blood. The group treated with *L. rhamnosus* CRL1505 showed values of leukocytes and neutrophils in the blood significantly higher than those in the control group, while the group treated with *L. rhamnosus* CRL1506 showed no significant differences with respect to the controls (Table 5). Besides, the infection induced an increase in leukocytes, macrophages and neutrophils in BAL in all the studied groups (Table 5). The group treated with *L. rhamnosus* CRL1505 presented values of leukocytes and neutrophils in BAL that were significantly higher than those in the control group, as well as a greater activation of the phagocytic cells. The treatment with *L. rhamnosus* CRL1506 induced no modifications in the number of leukocytes in BAL or in the activity of the phagocytic cells with respect to the control (Table 5).

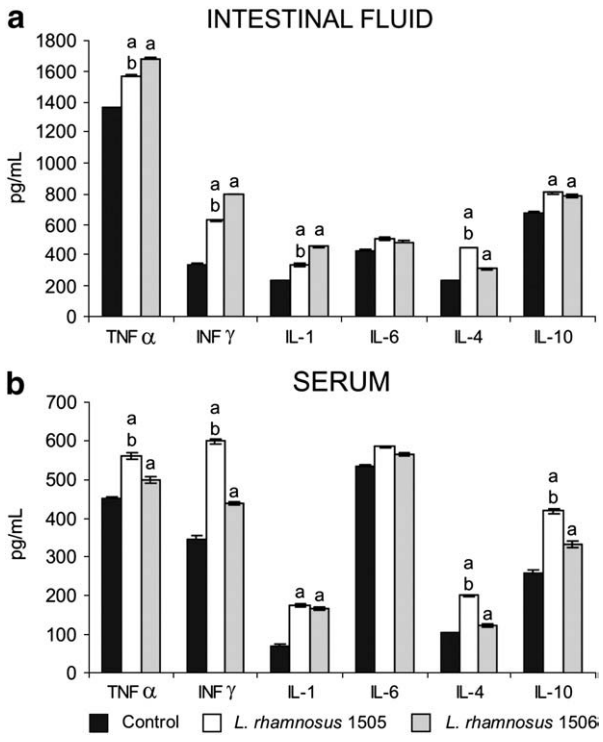


**Fig. 3.** Effect of bacterial administration on the tumour necrosis factor (TNF)-α, interferon (INF-γ), interleukin (IL)-1β, IL-4, IL-6 and IL-10 concentrations, in intestinal fluid (a) and serum (b) after challenge (day 2) with *Salmonella typhimurium* in mice that received *L. rhamnosus* CRL1505 or *L. rhamnosus* CRL1506. Values are means for $n=6\pm$SD. [a] Statistically different between test and control groups ($p<0.05$) and [b] Significant differences between test and *L. rhamnosus* CRL1506 groups ($p<0.05$).

#### 3.6.2. Specific IgA and IgG antibodies

Previous studies carried out in this respiratory infection model showed that, after the challenge with *S. pneumoniae*, the specific mucosal and systemic antibodies progressively increased until they reached a peak after 14 days (Racedo et al., 2006). That is why in this work we studied the levels of anti-pneumococcal IgG and IgA in serum and in BAL on day 14 post-infection. The treatments with *L. rhamnosus* CRL1505 and *L. rhamnosus* CRL1506 induced significant increases in the values of specific IgA in BAL with respect to the control group (Fig. 4a). However, only the group treated with *L. rhamnosus* CRL1505 presented an increase in the levels of anti-pneumococcal IgG in BAL with respect to the control group and the levels of IgA were significantly higher than that of the group that received *L. rhamnosus* CRL1506 (Fig. 4b). No differences were observed among the experimental groups when analyzing the values of serum IgA (Fig. 4c). The values of specific IgG in serum were significantly higher in the group treated with *L. rhamnosus* CRL1505 with respect to the control group (Fig. 4d).

#### 3.6.3. BAL and serum cytokines

The infection with *S. pneumoniae* significantly increased the levels of all the cytokines studied (Fig. 5). However, there were certain differences among the studied groups. Both LAB treatments induced increases in the levels of TNF-α in serum and in BAL, but *L. rhamnosus* CRL1505 showed TNF-α levels higher than those of *L. rhamnosus* CRL1506. Only the *L. rhamnosus* CRL1505 treatment was able to induce a significant increase in serum IL-1β with respect to the control (Fig. 5b). The animals that received *L. rhamnosus* CRL1505 showed INF-γ levels significantly higher than those found in all the other experimental groups in both serum and BAL (Fig. 5). The group treated with *L. rhamnosus* CRL1505 showed values of IL-4 and IL-6 significantly higher than those in the control group in serum samples. The group treated with *L. rhamnosus* CRL1506 did not show significant differences with respect to the control group in the values of these cytokines in either BAL or blood (Fig. 5). The group treated with *L. rhamnosus* CRL1505 presented values of IL-10 in serum and BAL that were higher than those in the other experimental groups.

## 4. Discussion

The oral administration of two *Lactobacillus* strains of the same origin and with similar technological properties was able to induce differential cytokine profiles, especially when their effect was assessed in distant mucosae.

Previous studies have demonstrated that the oral administration of wild type *Lactobacillus* strains can induce differential cytokine profiles

**Table 5**
Effect of the assayed strains on the blood leukocytes on respiratory infection.

| | BAL leukocytes | | | | Blood leukocytes | | |
|---|---|---|---|---|---|---|---|
| Groups | Leukocytes | Macrophages | Neutrophils | NBT+ cells | Leukocytes | Neutrophils | Peroxidase score |
| Control | 25.6 ± 0.98 | 15.3 ± 0.47 | 10.3 ± 0.67 | 71.4 ± 3.1 | 8.9 ± 0.5 | 1.9 ± 0.07 | 161.9 ± 3.3 |
| *L. rhamnosus* CRL1505 5d | 32.0 ± 1.06[a] | 16.1 ± 0.98 | 15.9 ± 0.87[a] | 83.9 ± 2.9[a] | 10.1 ± 0.4[a] | 2.4 ± 0.03[a] | 196.5 ± 2.9[a] |
| *L. rhamnosus* CRL1506 5d | 26.5 ± 1.15 | 15. 6 ± 0.99 | 10.9 ± 1.12 | 74.5 ± 3.0 | 9.0 ± 0.5 | 1.9 ± 0.05 | 173.1 ± 3.4 |

Total and differential count of leukocytes ($10^7$ cells/l) and percentage of NBT+ cells on BAL, and total and differential count of leukocytes ($10^9$ cells/l) and peroxidase activity (score) on blood, of mice that received *L. rhamnosus* CRL1505 or *L. rhamnosus* CRL1506 for 5 days, compared to control mice after nasal challenge with *Streptococcus pneumoniae* (day 2 post-infection). Values are means for $n = 6 \pm$ SD.
[a] Significant differences between test and control groups ($p < 0.05$).

in the gut (Maassen et al., 2000). Besides, the analyses of the cytokines profiles induced by certain lactic acid bacteria revealed that the most remarkable effect was the increase in the levels of TNF-α, IFN-γ and in the regulatory cytokine IL-10 for all the probiotic strains assayed (Maldonado-Galdeano et al., 2007). However, few reports exist concerning the cytokine profiles induced by LAB in distant mucosae such as the respiratory mucosa. In this sense, we demonstrated that a probiotic *L. casei* strain was able to increase the resistance to pneumococcal infection in mice and that this effect was associated with a differential pattern of cytokines induced in the respiratory tract (Racedo et al., 2006). In addition, our previous studies showed that the use of *L. casei* as a supplement in a repletion diet was associated with a pattern of inflammatory and anti-inflammatory cytokines that led to an increased number and functionality of the cells that participate in the immune response against a pneumococcal infection (Agüero et al., 2006; Salva et al., 2008). However, it was reported that different strains may have different functional roles and applications in different pathological conditions (Medina et al., 2007). Consequently, it would be important to have well-defined criteria for the selection of a probiotic microorganism according to the desired application.

Some authors have proposed the study of cytokine profiles induced by probiotic bacteria in the gut (Maldonado-Galdeano et al., 2007) or in the serum (Vinderola et al., 2006) as an important criterion in the study of their probiotic properties. Bearing in mind these facts and the importance of the ability to stimulate distant mucosae, we also included the study of the activation of alveolar macrophages and the cytokine profile induced by LAB in the respiratory mucosa.

In order to compare the efficacy of the strains for the stimulation of the intestinal and respiratory immunity, we used infectious challenges. We used two different models of infection: an intestinal Gram (−) pathogen and a Gram (+) respiratory pathogen, both of great importance from the epidemiological standpoint (Boyle et al., 2007; Rudan et al., 2008). *S. typhimurium* infection in susceptible mouse strains causes an invasive systemic disease that is similar in many respects to typhoid fever. This model is widely accepted as the best experimental system for studying human typhoid fever and has

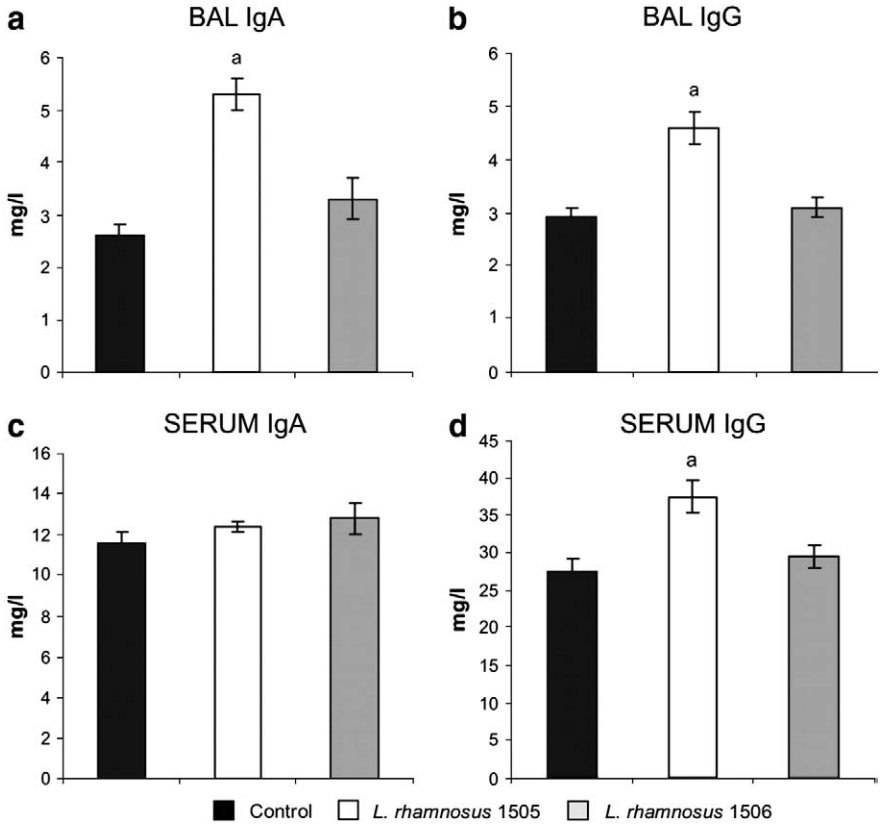


**Fig. 4.** Anti-pneumococcal antibodies of BAL (a, b) and serum (c, d) after challenge (d 15) with *Streptococcus pneumoniae* in mice that received *L. rhamnosus* CRL1505 or *L. rhamnosus* CRL1506 for 5 days. Values are means for $n = 6 \pm$ SD. [a] Statistically different between test and control groups ($p < 0.05$).

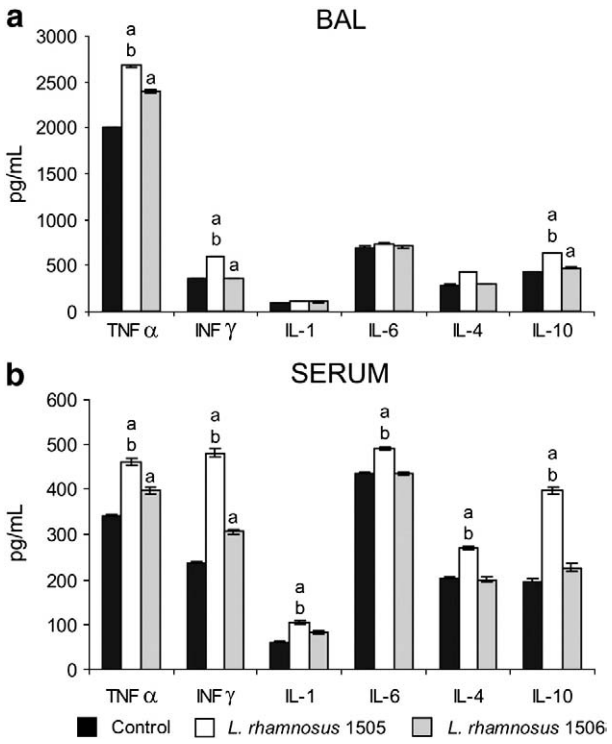


**Fig. 5.** Effect of bacterial administration on the tumour necrosis factor (TNF)-α, interferon (INF-γ), interleukin (IL)-1β, IL-4, IL-6 and IL-10 concentrations, in BAL (a) and serum (b) after challenge (day 2) with *Streptococcus pneumoniae* in mice that received *L. rhamnosus* CRL1505 or *L. rhamnosus* CRL1506. Values are means for $n=6\pm$SD. [a] Significant differences between test and control groups ($p<0.05$) and [b] Significant differences between test and *L. rhamnosus* CRL 1506 groups ($p<0.05$).

proved extremely valuable in uncovering mechanisms of innate and acquired immune resistance to intracellular pathogens (Ravindran and McSorley, 2005). *S. pneumoniae* is one of the most important respiratory pathogens (Ruvinsky et al., 2002). This bacterium can migrate to mucosal areas that are normally sterile and lead to a variety of mucosal infections such as otitis media or pneumonia. In addition, pneumococci can induce invasive cases of septicemia and meningitis. The high morbidity associated with this microorganism remains a leading cause of mortality in the young and the elderly (Foxwell et al., 2003).

In the present paper we analyzed the cytokines induced by two potential probiotic strains of *L. rhamnosus*. In IF we found that both have an influence on the same cytokines, with significant differences only at the levels of two of them (INF-γ and IL-4). The influence of the LAB on the cytokines levels in IF would be related to the improvement of the resistance to infection by *S. typhimurium*. Interferon-γ, produced by activated T cells and natural killer (NK) cells, has been shown to play an important role in host defence against intracellular pathogens such as *S. typhimurium* (Ramarathinam et al., 1991; Benbernou and Nauciel, 1994). In this work we observed a significant increase in IFN-γ in serum and IF of mice fed with both strains of *L. rhamnosus*. *In vitro* studies have shown that, epithelial cells and fibroblasts are resistant to *S. typhimurium* invasion in the presence of IFN-γ (Chen et al., 1989) and that IFN-γ activates mouse peritoneal macrophages, resulting in enhanced *S. typhimurium* killing (Chen et al., 1989). Therefore, the increase in IFN-γ in IF is probably related to the activation of peritoneal macrophages observed in the treated groups. When we analyzed the specific antibodies produced during the *Salmonella* infection, we observed clear differences between strains. While the administration of *L. rhamnosus* CRL1505 induced increases in IgA in IF and serum IgG with respect to the control group, *L. rhamnosus* CRL1506 did not affect these parameters. However, both strains were able to increase resistance to the *Salmonella* infection, probably because even though the production of IFN-γ is essential for the resolution of enteric *Salmonella* infection, the antibodies have little effect on this process (Bao et al., 2000). As to the cytokines profile, we found that the group treated with *L. rhamnosus* CRL1506 showed higher levels of inflammatory cytokines at the local level (IF), while in serum higher cytokines levels were found in the *L. rhamnosus* CRL1505 treated group. In this sense, the significant increases in specific IgA in IF and IgG in serum observed in this group are probably related to the enhancement of IL-4 and IL-10.

When we analyzed the effect of both *L. rhamnosus* strains on the prevention of *S. pneumoniae* infection, we observed that only *L. rhamnosus* CRL1505 was able to induce a significant decrease in the bacterial cell counts in the lung and to prevent pathogen's dissemination. Even more, only *L. rhamnosus* CRL1505 administration induced significant increases in the cytokine levels in BAL, both in the cytokines related to Th1 (INF-γ) and in the Th2 immune response (IL-6, IL-4 and IL-10). This would be associated with the increase in the number and activity of neutrophils and with the increase in specific antibodies in serum and BAL. This effect would be similar to the one found in our previous works performed with *L. casei* CRL431, a strain of proven immunomodulatory activity, which was able to induce an activation of both innate and specific immune response against pneumococcal infection, thus favoring clearance of the pathogen and modulating the inflammatory immune response, with less damage of lung tissue (Racedo et al., 2006). The administration of *L. rhamnosus* CRL1506 did not induce significant effects at the respiratory mucosal level.

It is known that the immune system of the small intestine is anatomically connected to the systemic immune system by the lymph and blood circulation. The immune response induced in the small intestine can spread through the systemic immune system reaching mucosal and non-mucosal sites (Vinderola et al., 2006). Different immune mechanisms are evoked against different infections. *S. typhimurium* is an intestinal pathogen and local activation induced by both LAB is important. However, *S. pneumoniae* is a respiratory pathogen in whose response the activation of distant mucosae is essential. This activation was found only with the administration of de *L. rhamnosus* CRL1505. In the protection against pneumococcal infection both Th1 and Th2 immune responses are important (Yamamoto et al., 2004). *L. rhamnosus* CRL1505 was able to induce increases in IFN-γ (a Th1 cytokine) and IL-4, IL-6 and IL-10 (Th2 cytokines). Then, the cytokine profile induced by *L. rhamnosus* CRL1505 administration would be related to an increase in the resistance to the pneumococcal infection observed in this experimental group. Recently it has been shown that *Lactobacillus* strains can regulate dendritic cells surface molecule expression and cytokine production (Christensen et al., 2002; Zeuthen et al., 2006; Borchers et al., 2009). LAB, including the intestinal inhabitants and those administered orally, are in close proximity to dendritic cells in the gut mucosa. Then, LAB may have immunoregulatory effects through dendritic cells in the gut (Borchers et al., 2009). However, the mechanisms through which various *Lactobacillus* strains induce different dendritic cell responses are still unknown and further studies would be necessary on this subject.

According to our results, the cytokines assayed in the blood serum showed a pattern of production that was quite similar to the one found for the same cytokines in BAL. This fact underlines the importance of blood serum as a tool for the study of the potential immunomodulatory effect of LAB at distant mucosal sites.

In summary, the results described in the present paper showed that: i) certain LAB strains may share certain functional properties. However, some of them can perform a functional role better than others, so that it is important to carry out thorough studies on specific strains, according to their therapeutic use; ii) the basal cytokine profiles induced by LAB administration is a good tool for strain selection both *in vivo* and *in vitro*,

and iii) it is of fundamental importance to demonstrate the effectiveness of the probiotic strain when the host's immune system must respond to an infectious challenge.

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