Microbiology and Immunology

Microbiol Immunol 2012; **56**: 771–781
doi:10.1111/j.1348-0421.2012.00495.x

ORIGINAL ARTICLE

Stimulation of macrophages by immunobiotic *Lactobacillus* strains: influence beyond the intestinal tract

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ABSTRACT

Lactobacillus rhamnosus CRL1505 (Lr1505), _L. rhamnosus_ CRL1506 (Lr1506) and _L. casei_ CRL431 (Lc431) are able to stimulate intestinal immunity, but only Lr1505 and Lc431 are able to stimulate immunity in the respiratory tract. With the aim of advancing the understanding of the immunological mechanisms involved in stimulation of distant mucosal sites, this study evaluated the effects of orally administered probiotics on the functions of alveolar and peritoneal macrophages. Compared to a control group, these three lactobacilli were able to significantly increase phagocytic and microbicidal activities of peritoneal macrophages. After intraperitoneal challenge with pathogenic _Candida albicans_, mice treated with immunobiotics had significantly lower pathogen counts in infected organs. Moreover, lactobacilli-treated mice had a stronger immune response against _C. albicans_. On the other hand, only Lc1505 and Lc431 were able to improve activity of and cytokine production by alveolar macrophages. Only in these two groups was there better resistance to respiratory challenge with _C. albicans_, which correlated with improved respiratory immune response. The results of this study suggest that consumption of some probiotic strains could be useful for improving resistance to infections in sites distant from the gut by increasing the activity of macrophages at those sites.

Key words *Lactobacillus casei* CRL431, *Lactobacillus rhamnosus* CRL1505, macrophages, respiratory immunity.

Lactobacillus species are members of the commensal microflora in the oral cavity, gastrointestinal and genitourinary systems in humans and animals. There are also lactobacilli in various food products such as milk, yogurt and cheese. Some strains of certain species of *Lactobacillus* are able to beneficially influence host health. There are many reports showing that the immunomodulatory capacity of certain probiotic strains may, at least in part, mediate such beneficial effects (1). The immunomodulatory and immunoadjuvant properties of probiotic lactobacilli cannot be attributed to all genera, since in most cases these properties are restricted to certain strains and depend on the administered dose (1–3). Their capacity for increasing the number of IgA^+ cells in the intestinal mucosa and stimulating macrophages and dendritic cells are among the beneficial effects of lactobacilli on the immune system (4). In fact, some probiotic strains are able to decrease the severity of intestinal infections, this effect being related to improved activation of macrophages' phagocytic activity in PPs (5).

Recent research in probiotics has moved beyond enhanced protection in the gut and progressed to

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Received 13 June 2012; revised 9 July 2012; accepted 13 July 2012.

List of Abbreviations: BAL, broncho-alveolar; *C. albicans*, *Candida albicans;* CD, cluster of differentiation; CERELA, Reference Center for *Lactobacilli;* CFU, colony forming unit; IFN, interferon; IgA, immunoglobulin A; IL, interleukin; LAB, lactic acid bacteria; Lc431, *Lactobacillus casei* CRL431; Lr1505, *Lactobacillus rhamnosus* CRL1505; Lr1506, *Lactobacillus rhamnosus* CRL1506; NBT, nitro blue tetrazolium; NFM, non-fat milk; PPs, Peyer's patches; SD, Standard deviation; TLR, Toll like receptor; TNF, tumor necrosis factor.

investigation of improvement of protective responses in distal mucosal sites (2, 3). In particular, it is now apparent that probiotic feeding can influence immune responses in the respiratory tract and improve protection against bacterial and viral pathogens (6–11). In this regard, we previously showed that the immunomodulatory probiotic strain Lc431 is able to improve immunity in the respiratory tract in both immunocompetent and immunocompromised hosts (7, 8). In these studies, we observed that mice orally treated with the optimal dose with adjuvant effect of Lc431 had a higher resistance to challenge with the respiratory pathogen *Streptococcus pneumoniae* (7, 8). In addition, our laboratory has isolated different lactobacilli strains from goat milk and studied their ability to stimulate host defenses. We selected two of the strains evaluated, Lr1505 and Lr1506, because of their capacity to improve intestinal immunity and increase resistance against *Salmonella typhimurium* (12). In addition, our studies have demonstrated that oral administration of Lr1505 is also able to improve resistance against pneumococcal infection (12).

In order to improve understanding of the mechanisms through which certain probiotic strains exert their immunomodulatory effect at sites distant from the gut, in this study we evaluated the influence of oral treatment with Lc431, Lr1505 or Lr1506 on the activity of macrophages at sites distant from the gastrointestinal tract. In particular, we studied the effect of these treatments on the phagocytic and microbicidal activity of alveolar and peritoneal macrophages.

MATERIALS AND METHODS

Animals and microorganisms

Male 6-week-old Swiss albino mice were obtained from the closed colony at CERELA. They were housed in plastic cages and their environmental conditions kept constant, in agreement with the standards for animal housing. The Ethical Committee for Animal Care at CERELA approved the experimental protocols. Lc431, Lr1505 and Lr1506 were obtained from the CERELA culture collection. Bacteria were cultured for 8 hr at 37°C (final log phase) in Man-Rogosa-Sharpe broth (Oxoid, Cambridge, UK), then harvested by centrifugation at 3000 *g* for 10 min, washed three times with sterile 0.01M PBS, pH 7.2, and finally resuspended in NFM at appropriate concentrations for administration to the mice. Lc431 was administered by the oral route for 2 consecutive days at dose of 10^9 cells/mouse/day, which is the optimal dose able to achieve stimulation of respiratory immunity (8, 9). Lr1505 and Lr1506 were administered by the oral route for 5 consecutive days at doses of 10^8 cells/mouse/day (12). Lactic acid bacteria were suspended in 5 mL sterile 10% NFM and added to the drinking water (20% v/v). The control group received sterile NFM under the same conditions. All mice were fed a conventional balanced diet *ad libitum*.

In vivo cytokine production

Cytokine concentrations were measured in serum and intestinal and BAL fluids. Blood from treated or control mice was obtained from the hearts of anesthetized animals, allowed to stand for 1 hr at 37°C and centrifuged for 10 min at 6000 *g*. The small intestines of treated and control mice were flushed with 5 mL of PBS and this fluid centrifuged for 10 min at 10,000 *g* to separate particulate material. BAL samples were obtained according to technique described previously (8, 11). Briefly, the tracheas were exposed and intubated with catheters, then two sequential BALs were performed in each mouse by injecting 0.5 mL of sterile PBS; the recovered fluid being centrifuged for 10 min at 900 *g*. The samples were frozen at −70°C for subsequent cytokine analyses. IFN-γ and TNF-α were determined using the corresponding mouse ELISA kits (R & D Systems, Minneapolis, MN, USA).

Nitro blue tetrazolium test

The bactericidal activity (oxidative burst) of alveolar and peritoneal macrophages was measured in the pellets of peritoneal and BAL fluids using the NBT reduction test (Sigma-Aldrich, St Louis, MO, USA) (10, 11). NBT was added to each sample with (positive control) or without addition of the bacterial extract; then samples 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 prepared and, after staining, the samples were examined under a light microscope for blue precipitates. A hundred cells were counted and the percentage of NBT positive (+) cells determined.

Killing of *Candida albicans*

The candidacidal activity of alveolar and peritoneal macrophages was determined using a technique modified from Vonk *et al*. (13) and Molero *et al*. (14). Two *C. albicans* strains were used: *C. albicans* AV3, a non-pathogenic strain isolated from contaminated food and *C. albicans* AV4, a pathogenic strain isolated from the blood of an infected, immunosuppressed patient (15). Alveolar and peritoneal macrophages were dispersed into the wells of a 96-well flat bottom plate (Nunc, Roskilde, Denmark), 5×10^5 cells in 100 uL of RPMI-1640 and incubated for 2 hr at 37°C in 5% CO_2. The wells were washed gently to remove non-adherent cells. Parallel control wells (without macrophages) were used. For determination of

anti-*C. albicans* activity, macrophages were infected with 100 uL containing 10^5 cells of *C. albicans* AV3 or AV4. After 3 hr of incubation at 37°C in 5% CO_2, 200 uL of distilled water was added to each well to achieve lysis of phagocytes. This procedure was repeated three times and the pooled washes adjusted to a final volume of 1 mL with distilled water. Microscopic examination of the culture plates showed complete removal of phagocytes. Serial dilutions were made up in distilled water and plated (triplicate samples) on Sabouraud agar plates. Results were expressed as percentages of *C. albicans* survival.

Ex vivo phagocytosis assay

Alveolar and peritoneal macrophages were collected aseptically from mice. The macrophages were washed twice with PBS containing BSA and adjusted to a concentration of 10^6 cells/mL. Phagocytosis was performed using a heat-killed *C. albicans* AV3 suspension (100°C, 15 min) at a concentration of 10^7 cells/mL. Mixtures of opsonized *Candida* in mouse autologous serum (10%) were added to 0.2 mL of macrophage suspension. The mixture was incubated for 30 min at 37°C. The percentage of phagocytosis was expressed as the percentage of phagocytosing macrophages in 200 cells counted using an optical microscope (15).

In vitro cytokine production

Alveolar and peritoneal macrophages monolayers were prepared as described above. In order to determinate the influence of lactobacilli on the capacity of macrophages to produce cytokines, alveolar and peritoneal macrophages were challenged *in vitro* with heat-killed *C. albicans* AV4 at a concentration of 10^7 cells/mL. After incubation at 37°C in 5% CO_2, the supernatant was recovered and kept frozen until cytokine analyses. IL-1β and TNF-α were determined using the corresponding mouse ELISA kits (R & D Systems).

Candida albicans infection

In order to evaluate the influence of lactobacilli treatments on the immune response against *C. albicans in vivo*, challenges with pathogenic *C. albicans* AV4 were performed. Yeast cells were grown in Sabouraud broth at 37°C until the log phase was reached. The pathogens were harvested by centrifugation at 3600 *g* for 10 min at 4°C and washed three times with sterile PBS. Intraperitoneal challenge with *C. albicans* AV4 was performed on the day after the end of each *Lactobacillus* treatment (third or sixth days). The mice were challenged with injections of 200 μL of an inoculum containing 10^8 cells. For yeast cell counts in blood, liver and spleen, mice were killed on day 2 post-infection. The livers and spleens were excised, weighed and homogenized in 5 mL of sterile peptone water. The homogenates were diluted appropriately, plated in duplicate on Sabouraud agar and incubated at 37°C. *C. albicans* colonies were counted and the results expressed as $\log_{10}$ CFU/g of organ or mL of blood. Intranasal challenge with *C. albicans* AV4 was performed on the day after the end of each *Lactobacillus* treatment (third or sixth days). The mice were challenged nasally with the pathogen by dripping 25 μL of an inoculum containing 10^7 cells into each nostril. To facilitate migration of the inoculum to the alveoli, the mice were held in a head-up vertical position for 2 min. For yeast cell counts in lung and blood, mice were killed on day 2 post-infection. The lungs were excised, weighed and homogenized in 5 mL of sterile peptone water. The homogenates were diluted appropriately, plated in duplicate on Sabouraud agar and incubated at 37°C. The *C. albicans* colonies were counted and the results expressed as $\log_{10}$ CFU/g of organ or ml of blood. In order to evaluate innate immune responses after challenges, the concentrations of TNF-α and IFN-γ and the number of leukocytes and neutrophils were determined in BAL and peritoneal fluid according to techniques described in a previous report (15). Briefly, a portion of peritoneal or BAL fluid was used to determine the total number of leukocytes using a hemocytometer, and the remaining sample of fluid was centrifuged for 10 min at 900 $\times$ *g*, the pellets used to make smears and differential cell counts performed by counting 200 cells stained with May Grünwald-Giemsa (15).

Statistical analysis

Experiments were performed in triplicate and results expressed as the means $\pm$ SD. Data were evaluated by one-way or two-way ANOVA tests. Tukey's test (for pairwise comparisons of the mean values of the different groups) was used to test for differences between the groups. Significant difference was defined as $P < 0.05$.

RESULTS

Cytokine concentrations in intestine, serum and respiratory tract

The *in vivo* immunomodulating activities of LAB and fermented dairy products containing LAB are in part attributable to altered production of cytokines that play pivotal roles in coordinating immune function. Thus, we first analyzed the concentrations of cytokines in intestinal fluid, serum and BAL, to determine the local and systemic effects induced by stimulation with the *Lactobacillus* strains assayed. We focused our study especially on TNF-α and IFN-γ, whose main biological roles are activation of

innate immunity. Oral administration of Lc431, Lr1505 or Lr1506 significantly increased the concentrations of IFN-γ in intestinal fluid, although the concentrations were higher in Lc431 mice than in Lr1505 or Lr1506 mice (Fig. 1a). Moreover, concentrations of INF-γ were increased in serum of Lc431, Lr1505 or Lr1506 mice (Fig. 1b). In addition, all treatments increased concentrations of TNF-α in intestinal fluid, however, only Lc431 and Lr1505 groups showed higher concentrations of serum TNF-α than did controls (Fig. 1a). There were no changes in TNF-α concentrations in BAL with any of the treatments (Fig. 1c) or in values for BAL INF-γ in mice treated with Lr1506. However, animals in Lc431 and Lr1505 groups had concentrations of BAL IFN-γ that were significantly higher than in the control group (Fig. 1c).

Peritoneal and alveolar nitro blue tetrazolium positive cells

In order to study the activation of the respiratory burst in macrophages, we used the NBT method. All treatments increased the percentage of NBT+ cells in the peritoneal cavity; we observed no significant differences between groups (Fig. 2a). The BAL of mice treated with Lr1505 or Lc431 had significantly greater concentrations of NBT+ cells did that of control mice (Fig. 2b). Moreover, the percentage of NBT+ cells in BAL of the Lc431-treated group was greater than in that of Lr1505-treated mice. Administration of Lr1506 did not induce changes in the percentage of NBT+ cells in BAL (Fig. 2b).

Phagocytic and microbicidal activities of peritoneal and alveolar macrophages

Administration of the three lactobacilli significantly increased the phagocytic activity of peritoneal macrophages against both pathogenic and non-pathogenic *C. albicans* strains (Table 1). We observed no differences between the three treatments. In addition, we observed a significant increase in the microbicidal activity of peritoneal macrophages in mice treated with Lc431, Lr1505 or Lr1506, as evidenced by lower survival rates of *C. albicans* when compared with the control group (Table 1). Lr1505 and Lc431 treatments significantly increased the phagocytic activity of alveolar macrophages, whereas administration of Lr1506 did not induce any change in this variable compared to the control group (Table 1). We also observed an increase in the microbicidal activity of alveolar macrophages of Lr1505- and Lc431-treated mice; this activity was significantly greater in the latter group (Table 1). Furthermore, the microbicidal activity of alveolar macrophages from the Lr1506-treated group was similar to that of the control mice.

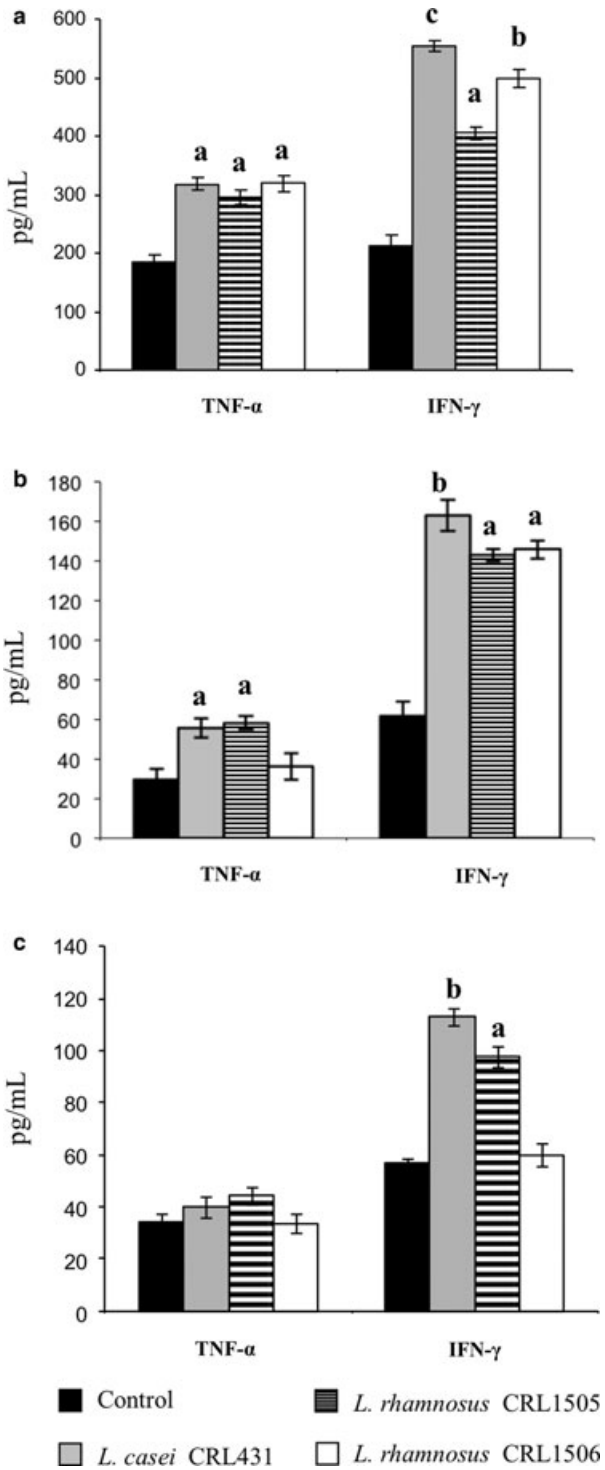

Fig. 1. Modulation of serum and mucosal cytokines by lactobacilli. Effect of bacterial administration on (a) TNF-α and IFN-γ concentrations in intestinal fluid, (b) serum and (c) BAL of mice that had received *Lactobacillus casei* CRL431, *L. rhamnosus* CRL1505 or *L. rhamnosus* CRL1506. The results represent data from three independent experiments. Values with different superscript letters are significantly different ($P<0.05$).

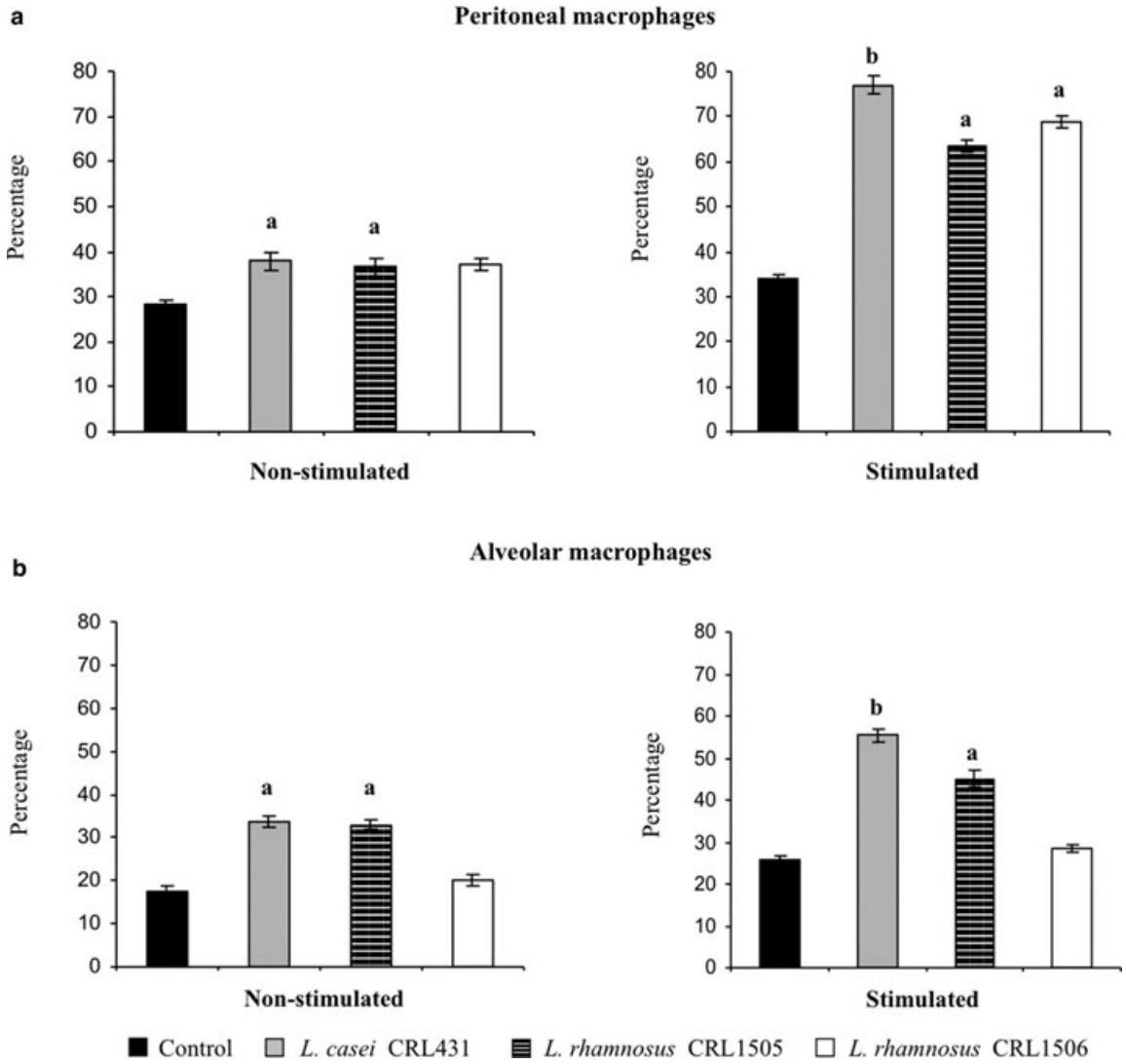

Fig. 2. Bactericidal activity of alveolar and peritoneal macrophages. The bactericidal activity (oxidative burst) of macrophages was measured in pellets of peritoneal and BAL fluids using the NBT reduction test, with or without previous stimulation. Effects of bacterial administration on percentage of NBT+ cells in (a) peritoneal fluid and (b) BAL of mice that had received *Lactobacillus casei* CRL431, *L. rhamnosus* CRL1505 or *L. rhamnosus* CRL1506. The results represent data from three independent experiments. Values with different superscript letters are significantly different ($P<0.05$).

Table 1. Phagocytic and microbicidal activities of peritoneal and alveolar macrophages

	Non pathogenic *Candida albicans*				Pathogenic *Candida albicans*			
	Peritoneal macrophages		Alveolar macrophages		Peritoneal macrophages		Alveolar macrophages	
Group	Phagocytosis (%)	Survival (%)	Phagocytosis (%)	Survival (%)	Phagocytosis (%)	Survival (%)	Phagocytosis (%)	Survival (%)
L. casei CRL431	53.9 ± 1.2^a	38.1 ± 3.3^a	36.1 ± 0.8^b	56.5 ± 4.2^b	46.9 ± 0.8^a	51.3 ± 4.5^a	25.5 ± 0.8^a	71.3 ± 2.7^b
L. rhamnosus CRL1505	49.8 ± 1.1^a	42.5 ± 4.3^a	30.7 ± 1.1^a	64.1 ± 5.6^a	45.1 ± 1.1^a	58.1 ± 3.9^a	26.1 ± 0.9^a	78.4 ± 5.5^a
L. rhamnosus CRL1506	47.9 ± 0.9^a	41.1 ± 3.8^a	19.1 ± 0.9	84.3 ± 4.6	46.0 ± 1.1^a	52.4 ± 5.3^a	12.5 ± 1.1	91.0 ± 3.8
Control	28.9 ± 0.9	74.5 ± 1.9	15.8 ± 0.8	88.9 ± 2.8	22.8 ± 0.8	79.8 ± 3.4	11.9 ± 0.8	90.9 ± 3.7

Cytokine production by *in vitro* stimulated macrophages

We next evaluated cytokine production by macrophages challenged *in vitro* with the pathogenic strain *C. albicans* AV4. All treatments increased production of TNF-α and IL-1β in peritoneal macrophages; we observed no significant differences between treatments (Fig. 3a). Administration of Lr1505 and Lc431 increased the capacity of alveolar macrophages to produce TNF-α and IL-1β in response to *C. albicans* challenge, whereas administration of Lr1506 did not induce changes in the concentrations of these cytokines (Fig. 3b).

Resistance against pathogenic *Candida albicans*

To evaluate the effect of lactobacilli treatments on peritoneal macrophages *in vivo*, we challenged the various groups of mice intraperitoneally with 10^8 cells of pathogenic *C. albicans* AV4 and took samples from liver, spleen and blood 48 hours later to analyze the presence of yeasts. Untreated control animals had positive counts of the pathogen in all the studied tissues (Table 2). Lc431, Lr1505 and Lr1506 treatments significantly reduced *C. albicans* counts in the liver during the studied period. In addition, animals treated with the

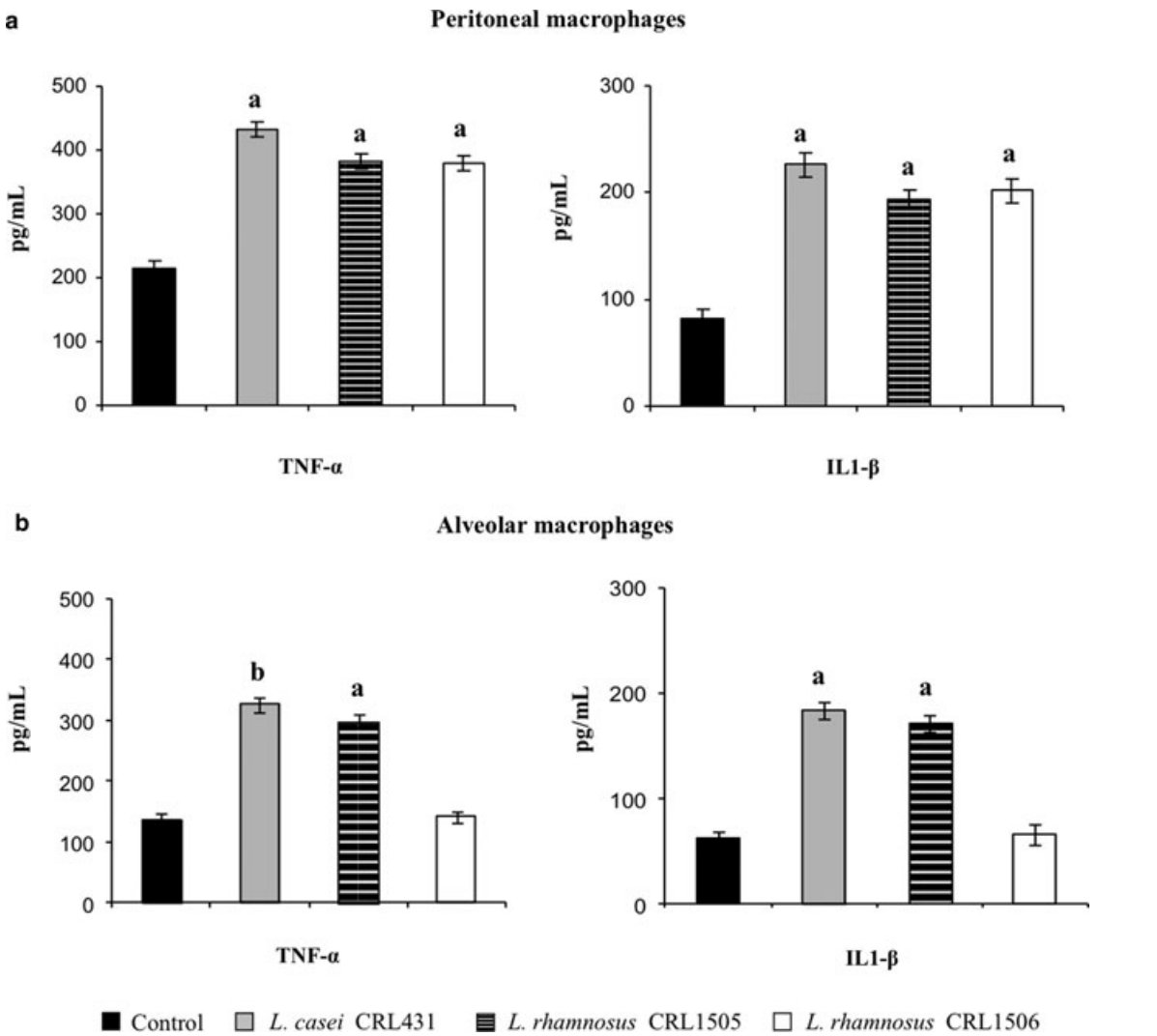

Fig. 3. Modulation of macrophage cytokine production by lactobacilli. Effects of bacterial treatment on TNF-α and IL-1β concentrations in the supernatant of *in vitro* cultured (a) peritoneal and (b) alveolar macrophages. Cell cultures were treated with *Lactobacillus casei* CRL431, *L. rhamnosus* CRL1505 or *L. rhamnosus* CRL1506. The results represent data from three independent experiments. Values with different superscript letters are significantly different ($P < 0.05$).

Table 2. *Candida albicans* cell counts in infected tissues

	Intraperitoneal challenge			Intranasal challenge	
	Liver (log CFU/g)	Spleen (log CFU/g)	Blood (log CFU/mL)	Lung (log CFU/g)	Blood (log CFU/mL)
L. casei CRL431	2,4 ± 0,7[a]	<1.5[a]	<1.5[a]	2,5 ± 0,9[a]	<1.5[a]
L. rhamnosus CRL1505	2,3 ± 1,0[a]	<1.5[a]	<1.5[a]	2,7 ± 0,4[a]	<1.5[a]
L. rhamnosus CRL1506	2,6 ± 0,9[a]	<1.5[a]	<1.5[a]	4,5 ± 1,3	<1.5[a]
Control	4,3 ± 0,5	4,9 ± 0,8	3,7 ± 0,9	4,9 ± 0,6	3,9 ± 0,8

different lactobacilli strains were able to eliminate the pathogenic yeast from blood and spleen (Table 2). In addition, in order to evaluate the influence of Lc431, Lr1505 and Lr1506 on the activity of alveolar macrophages *in vivo*, we challenged the various groups of mice intranasally with 10^7 cells of pathogenic *C. albicans* AV4 and 48 hours later, took samples of lung and blood to determine the presence of yeast. The control animals had positive pathogen counts in both lung and blood (Table 2). Mice treated with Lc431 or Lr1505 had significantly lower *C. albicans* counts in the lungs than did the control group; Lr1506 did not induce changes in this variable. Moreover, all treatments were able to induce clearance of the pathogenic yeast from blood (Table 2).

Immune response in peritoneal cavity

We next studied the immune response in the peritoneal cavity after challenge with *C. albicans* AV4. The number of leukocytes, macrophages and neutrophils in the peritoneal cavity increased in all experimental groups after challenge with the pathogen (Fig. 4a, b). However, mice treated prophylactically with Lc431, Lr1505 or Lr1506 had significantly greater macrophage and neutrophil counts than did those in the control group (Fig. 4a, b). We also observed increased concentrations of TNF-α and IFN-γ in peritoneal fluid after challenge with the pathogen in all experimental groups (Fig. 4c, d). However, groups receiving lactobacilli had greater cytokine concentrations than did controls.

Immune response in the respiratory tract

Nasal challenge with pathogenic *C. albicans* AV4 increased the number of leukocytes, macrophages and neutrophils in BAL in all experimental groups (Fig. 5a, b). Mice treated with Lr1505 or Lc431 had significantly higher macrophage and neutrophil counts than did the control group (Fig. 5a, b). We also observed increased concentrations of TNF-α and IFN-γ in the respiratory tract after challenge with pathogenic yeast in all experimental groups (Fig. 5a, b). However, in the groups receiving Lc431 or Lr1505 the concentrations of both cytokines were significantly higher than in the control group (Fig. 5c ,d).

DISCUSSION

Several studies have reported beneficial effects of probiotic bacteria and products containing these microorganisms on intestinal health. In the present study, we observed that oral administration of Lc431, Lr1505 and Lr1506 stimulates production of TNF-α and IFN-γ in the intestine. This is in line with other studies showing that, of the cytokines induced by immunomodulatory LAB, the most remarkable effect is the increase in TNF-α, IFN-γ, and the regulatory cytokine IL-10 in all probiotic strains assayed (16). In addition, that TNF-α and IFN-γ are both reportedly produced by antigen presenting cells (17). Therefore, our results indicate that the three lactobacilli strains evaluated in this study are able to stimulate macrophages and dendritic cells in the gut. In addition, we observed a strain-dependent difference in the concentrations of TNF-α and IFN-γ after Lc431, Lr1505 or Lr1506 treatments. This effect has been also observed by other authors who have reported strain-dependent differences in the number of gut TNF-α+ and IFN-γ+ cells after oral administration of *Lactobacillus* strains (18). Local activation of the gut immune system induced by Lc431, Lr1505 and Lr1506 would explain the improved resistance of treated mice to oral challenge with the intestinal pathogen *Salmonella typhimurium* (12, 15).

We were particularly interested in the effect of lactobacilli strains beyond the intestinal tract. It is known that the gut immune system is anatomically connected to the systemic immune system by the lymphatic and blood circulation. Therefore, immune responses induced in the small intestine can spread through the systemic immune system and reach mucosal and non-mucosal sites (19). Thus, in the present study, we simultaneously studied the effects of oral administration of *Lactobacillus* strains on sites distant from the gastrointestinal tract by assessing macrophage activity in the peritoneal and alveolar compartments. We found that activation of macrophages at

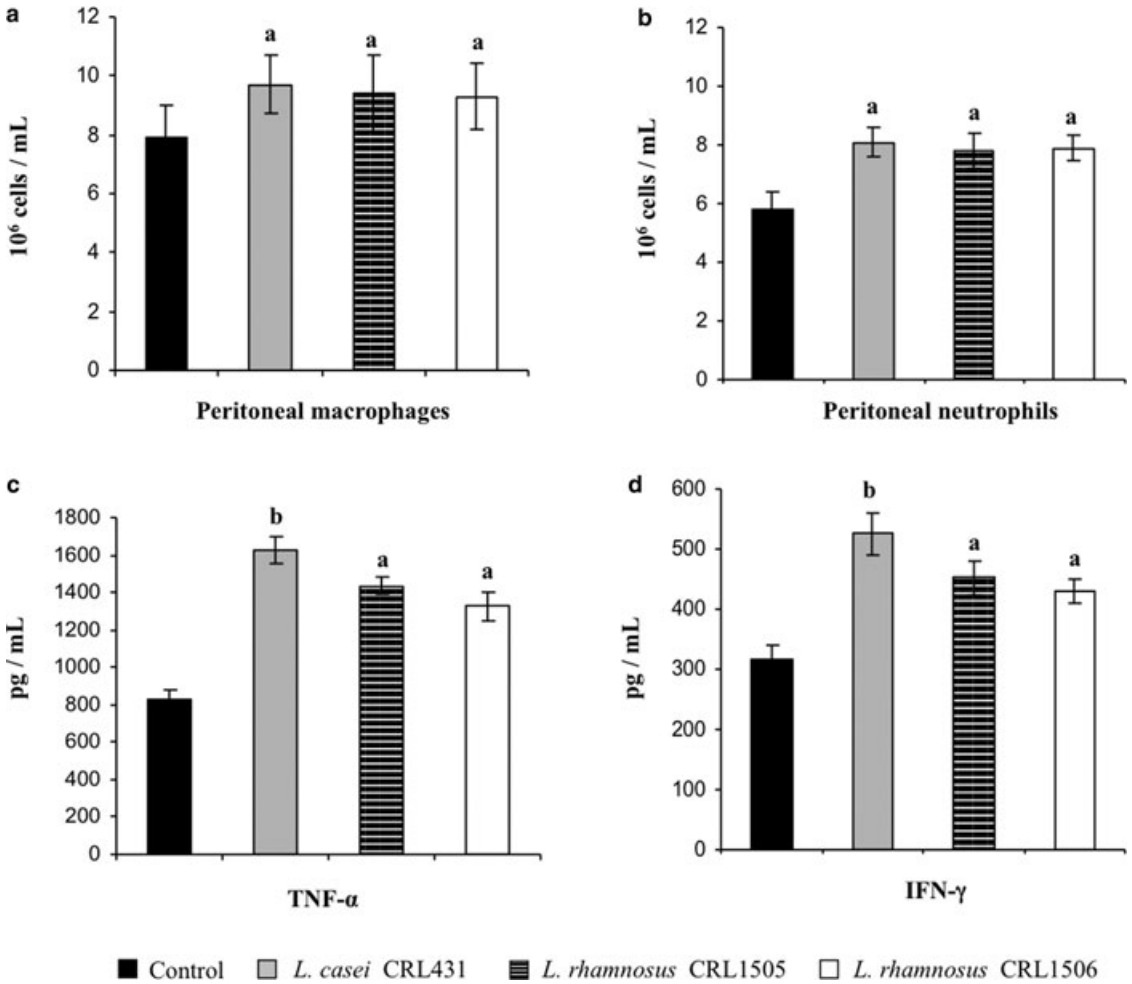

Fig. 4. Immune responses in the peritoneal cavity after challenge with *Candida albicans*. Mice were orally administered *Lactobacillus casei* CRL431, *L. rhamnosus* CRL1505 or *L. rhamnosus* CRL1506 and then challenged by intraperitoneal injection of 10^8 cells of pathogenic *C. albicans*. (a) Number of macrophages, (b) neutrophil leukocytes, and (c) TNF-α and (d) IFN-γ concentrations in the peritoneal cavity on day 2 post-challenge. The results represent data from three independent experiments. Values with different superscript letters are significantly different ($P<0.05$).

sites distant to the gastrointestinal tract is dependent on the strain of LAB employed. We also demonstrated that the stimulatory effects of the LAB are related to the ability of each strain to influence profiles of mucosal and systemic cytokines.

Interaction of macrophages with microorganisms often results in phagocytosis. The phagocytic process comprises several fundamental steps, including attachment to the macrophage surface, internalization, and endosomal processing. Killing accompanies phagocytosis; otherwise, macrophages could serve as a vehicle for dissemination of infection. In addition, cytokine and chemokine synthesis by macrophages likely occurs during each of these steps (20). Our *ex vivo* studies showed that administration of the three strains Lc431, Lr1505 or Lr1506 significantly increases the microbicidal and phagocytic activity of peritoneal macrophages as well as their ability to produce cytokines. Therefore, all functions of peritoneal macrophages are increased by lactobacilli.

Reportedly, cytokines produced in the small intestine after probiotic stimulation can be released into the circulation (21). When studying the concentrations of IFN-γ in serum, we found that LAB treatments induced significant increases in the concentrations of this cytokine. Considering that IFN-γ is the principal macrophage-activating cytokine and serves critical functions in innate immunity, improved production of this cytokine would mediate the stimulation of peritoneal macrophages by the lactobacilli strains. Researchers evaluating the effect of continuous administration of fermented milk containing the

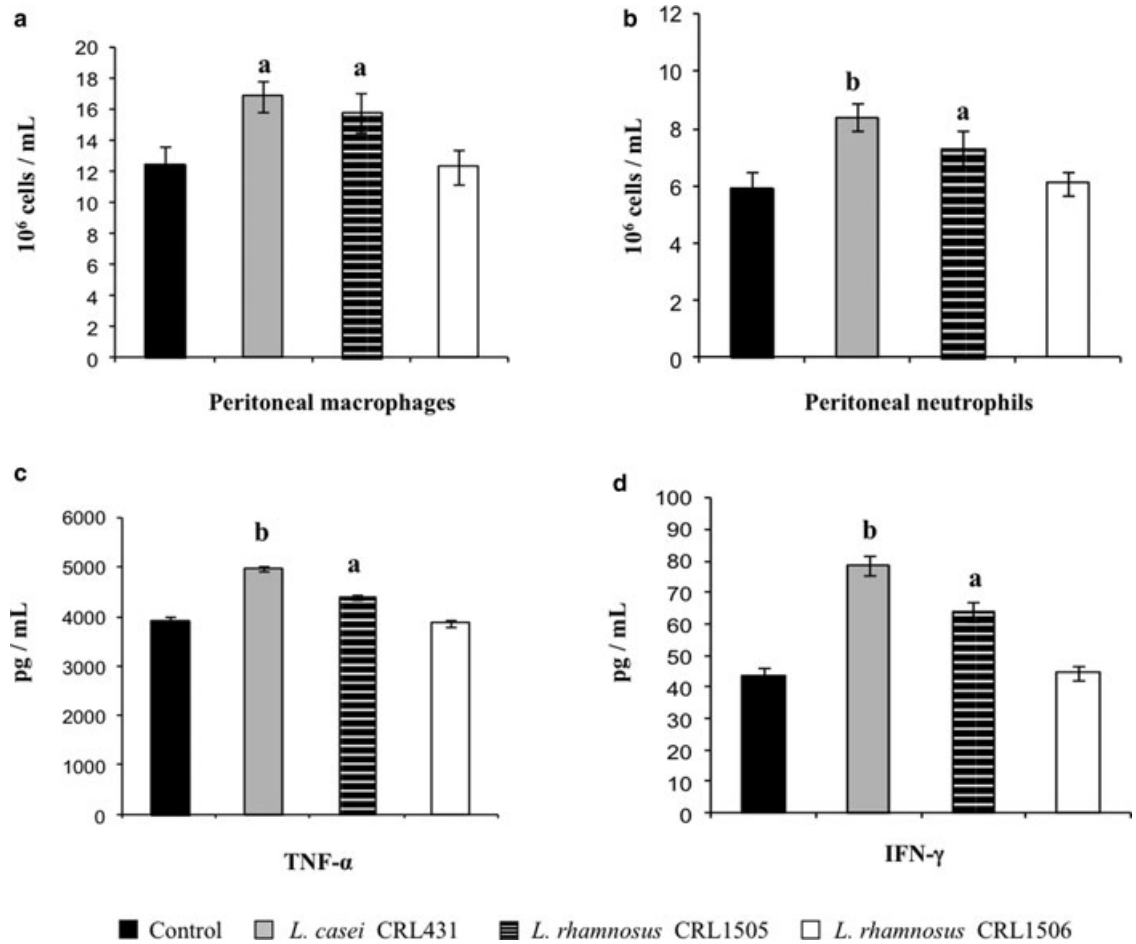

Fig. 5. Immune responses in the respiratory tract after challenge with *Candida albicans*. Mice were orally treated *Lactobacillus casei* CRL431, *L. rhamnosus* CRL1505 or *L. rhamnosus* CRL1506 and then challenged by intranasal administration of 10^7 cells of pathogenic *C. albicans*. (a) Number of macrophages, (b) neutrophil leukocytes, and (c) TNF-α and (d) IFN-γ concentrations in BAL fluids on day 2 post-challenge. The results represent data from three independent experiments. Values with different superscript letters are significantly different ($P<0.05$).

probiotic bacterium *L. casei* DN-114001 have previously described a correlation between improved production of IFN-γ and activity of peritoneal macrophages (22). Considering that several studies have demonstrated the importance of activated macrophages in controlling systemic and mucosal *C. albicans* infections, we decided to confirm our *ex vivo* results with *in vivo* studies using infection-challenge experiments in mice. We observed that mice treated with Lc431, Lr1505 or Lr1506 were able to control the infection induced by intraperitoneal challenge with pathogenic *C. albicans*. This protective effect correlated with increased production of pro-inflammatory cytokines and increased recruitment of phagocytic cells in the peritoneal cavity compared to control mice. Thus, the present study extends our and others previous observations by demonstrating that activation of peritoneal macrophages by orally administered probiotic bacteria improves resistance to pathogens. Administration of probiotic lactobacilli stimulates macrophages and dendritic cells in the gut, inducing production of IFN-γ in the intestine and consequently increasing blood concentrations of IFN-γ. IFN-γ activates peritoneal macrophages that, in the presence of a pathogen such as *C. albicans*, have an increased capacity for phagocytosis and killing of yeasts and induction of recruitment and activation of additional phagocytic cells that contribute to further control of the infection. Furthermore, the extent of peritoneal macrophage activation depends on the amounts of IFN-γ induced by each probiotic strain; we observed increased activation of these cells in animals treated with Lc431, the strain that induced the greatest concentrations of IFN-γ in both the gut and serum.

When we studied the effects of lactobacilli treatments on alveolar macrophage activity, we found two important

differences regarding their effects on peritoneal phagocytes: (i) activation of alveolar macrophages was milder than that of peritoneal macrophages; and (ii) not all strains were able to stimulate the phagocytes in the respiratory tract, despite all the strains stimulated intestinal immunity and activity of peritoneal macrophages. Considering that peritoneal and alveolar macrophages are activated by cytokines released by immune cells in the gut and not directly by their interaction with lactobacilli, the enhanced phagocytic activity of peritoneal compared to alveolar macrophages may be due to the fact that the former are located anatomically closer to the place (intestinal environment) where the macrophage stimulating cytokines are produced. However, it is possible that macrophage-stimulating cytokines are produced locally in the respiratory tract. When we studied cytokines in BAL, we found that, although there were increased concentrations of this cytokine in serum in all lactobacilli-treated groups, only in mice receiving Lr1505 or Lc431 concentrations of IFN-γ were significantly greater than in controls.

Recent evidence has shown that pattern recognition receptor-mediated sensing of resident commensal microbiota in the steady state regulates the development and function of innate and adaptive immune systems in extra-intestinal sites. In mice, depletion of gut microbiota by antibiotics reduces surface expression of TLR2 and TLR4 in peritoneal macrophages and decreases inflammation caused by intraperitoneal lipopolysaccharide injection *in vivo* (23). In addition, recent studies have shown that neomycin-sensitive bacteria in the gastrointestinal tract are required for supporting immune responses to respiratory influenza infection (24). These studies indicate that the gut microbiota support respiratory immunity by releasing small amounts of pattern recognition receptors ligands into the circulation. Although our present study does not disprove this mechanism for Lc431 or Lr1505, we suggest the following alternative mechanism for influencing immune response in the respiratory tract: some immunobiotic strains are able to stimulate the Th1 response in the gut and induce mobilization of Th1 cells from inductive sites in the gut to effector sites in the respiratory tract. These activated Th1 cells would produce cytokines (IFN-γ) that can stimulate the activity of local respiratory immune cells such as alveolar macrophages. Because these macrophages have already been activated, they would be able to efficiently phagocytose pathogens that reached the alveolar space, induce specific immune responses and increase resistance to respiratory infections (6, 7, 11, 24). There is some evidence that supports our hypothesis. Myeloid dendritic cells in PPs express TLR2 and TLR4 and are able to stimulate naïve T cells to differentiate into Th1 cells that secrete a large amount of IFN-γ (22). It has been demonstrated that Lc431 increases expression of both TLR2 and CD-206 in dendritic cells and macrophages in PPs and the lamina propria of the small intestine (15, 23, 25). Thus, the increase in numbers of TLR2+ and IFN-γ+ cells induced by Lc431 could indicate activation of myeloid dendritic cells in PPs and activation of the Th1 response. In addition, considering the concept of a common mucosal immune system, it is possible that some Th1 cells, when moving from inductor to effectors sites in the gut, are directed to and located in the respiratory tract. In fact, preliminary results from our laboratory demonstrate increased numbers of $CD3^+CD4^+$IFN-γ^+ T cells in the lungs of Lc431 and Lr1505 treated mice and not in the lungs of mice receiving Lr1506 (Villena *et al*., unpublished results, 2012).

In conclusion, we have demonstrated an immunomodulatory effect of three probiotic lactobacilli on immune cells distant from the gut: peritoneal and alveolar macrophages. We accordingly suggest that consumption of some probiotic strains could be useful as an adjuvant for the respiratory immune system. More studies are necessary to prove this mucosal adjuvant effect against different respiratory pathogens and to confirm the possibility that the improved function of alveolar macrophages after oral treatment with probiotics is related to the mobilization of $CD3^+CD4^+$IFN-γ^+ T cells from the gut to the lungs.

ACKNOWLEDGMENTS

This work was supported by grants from Proyectos de Investigación Plurianuales (PIP 632/2009), Consejo de Investigaciones de la Universidad Nacional de Tucuman (CIUNT 26 D/403) and Proyectos de Investigación Científica y Tecnológica (PICT 1381/2010).

DISCLOSURE

G. Marranzino, J. Villena, S. Salva and S. Alvarez all have no conflicts of interest to disclose.

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