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Original article

Lactobacillus casei administration reduces lung injuries in a *Streptococcus pneumoniae* infection in mice

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Abstract

The effect of the oral administration of *Lactobacillus casei* on the prevention of a *Streptococcus pneumoniae* lung infection in a mouse experimental model was studied, analyzing the innate and specific immune response. Adult Swiss albino mice were treated with *L. casei* (10^9 CFU/day) for 2, 5 and 7 d. Mice were infected intranasally with *S. pneumoniae* (10^6 CFU/mouse) after each treatment and the microbiological, histopathological and host responses were determined for 15 d after infection. Feeding *L. casei* for 2 d induced a faster clearance of *S. pneumoniae*, with a lower number of pneumococci in lung and a shorter period of septicemia than in the control group. *L. casei* administration induced activation of phagocytes as evidenced by the strong myeloperoxidase activity and the nitro blue tetrazolium assay in lung. Mice given *L. casei* for 2 d showed higher levels of anti-pneumococcal serum IgG and bronchoalveolar lavage IgA than the control mice. The group fed *L. casei* for 2 d could beneficially regulate the balance between tumor necrosis factor alpha and interleukin 10, allowing a more effective immune response against infection and modulating the inflammatory response, with less damage to the lung.

Keywords: *Lactobacillus casei*; Lung infection; *Streptococcus pneumoniae*

1. Introduction

Streptococcus pneumoniae causes pneumonia, septicemia, otitis media and meningitis. Acute respiratory infection by pneumococci results yearly in more than one million deaths worldwide despite the widespread use of antibiotics [1]. There is growing evidence that certain aspects of the immune response greatly contribute to the high mortality rate: while immunosuppressed patients die as a consequence of poor host response, immunocompetent hosts face overwhelming inflammatory reactions that may lead to tissue injury, shock and, eventually, death [2,3]. Pneumonia is the most common clinical presentation of pneumococcal disease; in fact, 95% of cases appear as pneumonia or meningitis and *S. pneumoniae* continues to cause considerable morbidity and mortality throughout the world [4]. Consequently, it is necessary to develop more effective methods for the prevention of pneumococcal infections, especially in developing countries, where the incidence is high [4].

Abbreviations: BAL, bronchoalveolar lavages; BALT, bronchus-associated lymphoid tissue; FICT, fluorescein isothiocyanate; LAB, lactic acid bacteria; Lc2d (Lc5d, Lc7d), mice fed for 2 (5,7) days with *Lactobacillus casei*; MPO, myeloperoxidase; NBT, nitro blue tetrazolium; NFM, non-fat milk; PMN, polymorphonuclear neutrophils; sIgA, secretory immunoglobulin A.

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The normal host defense response against lung pathogens is performed mainly by the immune system and includes both the innate and the specific immune response. The fact that certain lactic acid bacteria (LAB) activate and modulate the immune system suggests that these microorganisms can be beneficially used as immune modulators [5]. The oral administration of certain LAB induces the activation of peritoneal macrophages, which may be important effector cells in specific and non-specific host defense [6]. Moreover, various strains of probiotic lactobacilli have been well characterized in terms of their ability to induce cytokine production following contact with mononuclear phagocytes or accessory cells [7].

Although most research on probiotic-mediated enhanced immune protection is focused on the gastrointestinal tract, a few recent studies consider the possibility that probiotics might sufficiently stimulate the common mucosal immune system so as to provide increased protection to other mucosal sites [8], including the upper respiratory and urogenital tracts.

We demonstrated previously that *Lactobacillus casei* induces protective immunity in the gut [9] and that the oral administration of different species of *Lactobacillus* enhances bronchial immunity by increasing the number of lymphocytes in the bronchus-associated lymphoid tissue (BALT) [10]. Moreover, we showed that the oral administration of *L. casei* to young mice enhances lung *Pseudomonas aeruginosa* clearance and improves the phagocytic activity of alveolar macrophages with a dose-dependent effect [11]. On the basis of these previous results, the aim of the present research was to study the effect of the oral administration of *L. casei* CRL 431 on the prevention of *S. pneumoniae* lung infection in mice, analyzing the innate and specific immune response.

2. Materials and methods

2.1. Animals and microorganisms

Adult 8-week-old Swiss albino mice (22–28 g) were obtained from CERELA and housed individually during the experiments. Each parameter studied was carried out in 5–6 mice for each time point.

Lactobacillus casei CRL 431 (CERELA culture collection) was cultured for 8 h at 37 °C (final log phase) in Man-Rogosa-Sharpe broth (Oxoid), harvested and washed with sterile 0.01 M phosphate buffer saline (PBS), pH 7.2. Capsulated *S. pneumoniae* serotype 14 (ANLIS, Argentina), one of the ten most frequent serotypes isolated in Argentina [12], was obtained from the respiratory tract of a patient from the Children's Hospital (Tucuman-Argentina).

2.2. Feeding procedures

L. casei was administered to different groups of mice for 2, 5 or 7 consecutive days at a dose of 10^9 CFU/mouse/day (groups Lc2d, Lc5d and Lc7d respectively). *L. casei* was suspended in 5 ml of sterile 10% non-fat milk (NFM) and added to the drinking water (20% v/v). The control group received sterile NFM in the same conditions as the test groups. All mice were fed a conventional balanced diet *ad libitum*.

2.3. Experimental infection

S. pneumoniae was grown on blood agar for 18 h. Colonies were suspended in Todd Hewitt broth (Oxoid), incubated overnight at 37 °C, harvested and washed with sterile PBS. Cell density was adjusted to 4×10^7 CFU/ml. Challenge with pneumococci was performed on the day after the end of each *L. casei* treatment (on the 3rd, 6th or 8th day). 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.

2.4. Bacterial cell counts in lung and blood

Treated and control mice were sacrificed before infection (d 0) and on d 1, 2, 3, 5, 7, 10 and 15 postinfection. Lungs were excised, 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. *S. pneumoniae* was identified by standard techniques [13] and 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.

2.5. Total and differential blood and broncho-alveolar lavage (BAL) leukocyte counts

Blood samples were obtained as described above. BAL samples were obtained according to Bergeron et al. [14] modified as follows: the trachea was exposed and intubated with a catheter and 2 sequential lavages were performed in each mouse with 0.5 ml of sterile PBS. The recovered fluid was centrifuged for 10 min at 900 × *g*; the pellet was used to count BAL leukocytes and the fluid was frozen at −70 °C for antibody analyses. The total number of leukocytes was determined with a hemocytometer. Differential cell counts were performed by counting 200 cells in blood or BAL smears stained with May Grünwald-Giemsa.

2.6. Serum and BAL anti-pneumococcal antibodies

A previously developed ELISA technique was used to determine anti-pneumococcal IgA and IgG in serum and BAL [15]. Briefly, plates were coated with a heat killed *S. pneumoniae*-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 (serum 1:20; BAL 1:2) with PBS containing 0.05% (v/v) Tween 20 (PBS-T). Peroxidase-conjugated goat anti-mouse IgA or IgG (Fc specific, Sigma) was diluted (1:500) in PBS-T. Antibodies were revealed with a substrate solution [3-3′, 5-5′-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).

2.7. Phagocyte activation

2.7.1. Washburn test

Measurement of myeloperoxidase activity of blood neutrophils was carried out using a cytochemical method [15]. Cells were graded as negative or weakly, moderately or strongly positive and used to calculate the score.

2.7.2. Nitro blue tetrazolium (NBT) test

The bactericidal activity of macrophages and neutrophils was measured in the pellet of BAL using the NBT reduction test (Sigma) [15]. A hundred cells were counted and the percentage of NBT positive (+) cells was determined.

2.8. Myeloperoxidase (MPO) assay in lung

Neutrophil infiltration in lung tissue was quantified by measurement of MPO. Lungs were cleared of blood, removed and homogenized in 50 mM acetate buffer, pH 5.4 (MPO-assay buffer). Homogenates were frozen at −70 °C for 15 min, thawed, sonicated for 60 s and centrifuged at 3600 × *g* for 15 min at 4 °C. MPO was evaluated by adding 200 µl of an appropriate dilution of the lysate to 20 mM 3,3′,5,5′-tetramethylbenzidine in dimethylphormamide and 30 µl of 2.7 mM of hydrogen peroxide in MPO-assay buffer. The reaction mixture was incubated for 3 min at 37 °C and stopped with ice-cold 200 mM sodium acetate buffer (pH 3) [16]. Absorbance was read at 655 nm against a standard curve made with commercial MPO (Sigma). The results were expressed as specific activity of MPO (MPO units/mg of total proteins in lung homogenate). Total protein concentration was determined in the cellular lysates by Bradford's method.

2.9. Pro and anti-inflammatory cytokines

Tumor necrosis factor alpha (TNF-α) and interleukin-10 (IL-10) were measured in serum and lung homogenates. Commercially available ELISA kits were used according to the manufacturer's recommendations (R&D systems, TNF-alpha MTA00; IL-10 M1000, Minn., USA). Lungs were homogenized in PBS, centrifuged at 900 × *g* and supernatants were aliquoted and stored at −70 °C.

2.10. Histopathological examination and IgA+ cell identification in lung

To evaluate tissue damage caused by infection, lungs were aseptically removed, fixed in 4% formalin and embedded in histowax (Leica Microsystems). Histopathological assessment was performed on five-micron tissue sections stained with hematoxylin-eosin. At least four tissue sections from various areas of the lung of each mouse in all experimental groups were examined. The number of IgA+ cells in lung was determined by an immunofluorescence technique according to Perdigon et al. [10]. The results were expressed as the number of positive fluorescent cells per 10 fields.

2.11. Statistical analysis

Experiments were performed in triplicate and results were expressed as mean ± standard deviation (S.D.). After verification of a 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. Bacterial cell counts in lung and blood

The pathogen was detected in lung and blood samples of control mice throughout the period assayed. Mice treated preventively with *L. casei* for 2 days (Lc2d) showed lower lung bacterial counts than control mice until d 10 postinfection; from then on, bacterial counts were negative (Fig. 1). In addition, hemocultures from the Lc2d group were negative till the d 2 postinfection (data not shown). Lung bacterial counts were significantly lower in Lc5d mice than in the control group until d 5 postinfection; from then on, there was no difference between the two groups (Fig. 1).

3.2. Total and differential blood leukocyte counts

A significant increase in blood leukocytes was observed in control mice from h 24 till d 5 after challenge followed by

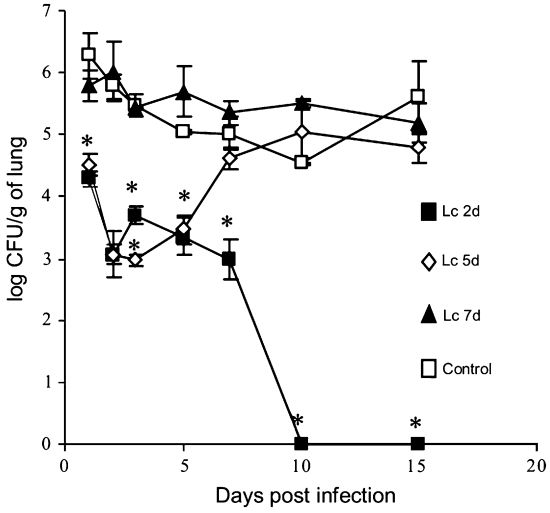

Fig. 1. Kinetics of *S. pneumoniae* clearance from lungs. Mice were challenged intranasally with 10^6 *S. pneumoniae* cells after treatment with *L. casei* for 2, 5 or 7 d (Lc2d, Lc5d, Lc7d, respectively). For evaluation of CFU pathogen counts in individual lung homogenates, mice were sacrificed on d 1, 2, 3, 5, 7, 10 and 15 postinfection. Results are expressed as mean ± S.D. from 6 mice per group at the indicated time point. *Significantly different from the control group at the same time point ($P < 0.005$).

Table 1
Blood leukocyte (BL) and PMN counts (10^9 cells/liter) of mice fed *L. casei*

Time post-challenge (d)	Experimental groups							
	Control		Lc2d		Lc5d		Lc7d	
	BL	PMNs	BL	PMNs	BL	PMNs	BL	PMNs
0	6.9 ± 0.2	0.9 ± 0.05	6.3 ± 0.7	0.9 ± 0.07	5.9 ± 0.3	0.8 ± 0,03	6.2 ± 0.6	0.7 ± 0.06
1	8.9 ± 0.1	1.1 ± 0.07	11.0 ± 0.4*	3.3 ± 0.03*	8.1 ± 0.3	1.9 ± 0.04	8.9 ± 0.3	0.9 ± 0.05
5	9.7 ± 0.9	2.5 ± 0.07	11.5 ± 0.7*	3.2 ± 0.06*	9.4 ± 0.4	2.6 ± 0.03	9.5 ± 0.7	2.5 ± 0.05
10	8.5 ± 0.4	2.3 ± 0.04	10.2 ± 0.5*	2.9 ± 0.06	9.7 ± 0.5	2.7 ± 0.02	8.5 ± 0.5	2.6 ± 0.05
15	7.2 ± 0.7	2.1 ± 0.01	8.9 ± 0.4	2.8 ± 0.08	8.5 ± 0.2	2.6 ± 0.04	7.5 ± 0.5	2.3 ± 0.03

Mice were fed *L. casei* for 2, 5 or 7 d (Lc2d, Lc5d, Lc7d, respectively) before (d 0) and after challenge (d 1, 5, 10, 15) with *S. pneumoniae* (1×10^6 CFU per mouse). Control mice were challenged with the pathogen without previous treatment. Results represent mean ± S.D. for six mice per group at each time point. *Significantly different from the control at the same time point ($P < 0.05$).

a gradual decrease and a return to normal values on d 15 post-infection (Table 1). The changes in the number of leukocytes were mediated principally by the polymorph nuclear neutrophil (PMN) population (Table 1).

Lc2d mice showed a significant increase in the total number of leukocytes and in PMN cells compared to controls, and the numbers remained high until d 10 postinfection.

3.3. Serum and BAL anti-pneumococcal antibodies

Serum and BAL IgA and IgG antibodies increased gradually after challenge in all groups (data not shown) with a peak on d 15 postinfection (Table 2). The Lc2d group showed a significant increase in serum and BAL anti-pneumococcal IgG compared to control mice. Control and treated mice showed similar values of serum IgA. However, the Lc2d and Lc5d groups had significantly higher levels of BAL anti-pneumococcal IgA than the control group. On the basis of the above results, Lc2d was selected as the optimal treatment to improve the immune response against pneumococcal infection. Thus, the following studies were carried out in the Lc2d group.

3.4. Total and differential BAL leukocyte counts

Macrophages were the predominant resident leukocytes in the alveolar spaces of treated and control non-infected mice (data not shown). In the control group, pneumococcal infection induced an early increase in BAL leukocytes, macrophages, lymphocytes (refer to supplementary data, Appendix 1) and PMNs (Fig. 2A). The pneumococcal infection in the Lc2d group caused a recruitment of cells into the alveoli, resulting in a significantly higher number of PMNs (Fig. 2A) than those in the control group on d 2 and 3 postinfection. However, there was a significant decrease in BAL PMNs on d 10 and 15 post-infection compared to control mice.

3.5. Phagocyte activation

3.5.1. Blood peroxidase scores

Normal values of blood peroxidase scores were 112 ± 7, with no significant modifications after Lc2d administration (113 ± 5). After challenge, there was a gradual increase, highest values being reached on d 3 postinfection in both the Lc2d and the control groups (Fig. 2B). However, scores were significantly higher in the Lc2d mice than in the control group. The stronger activation of PMNs observed in the Lc2d group would be involved in the more effective clearance in blood, which would also allow the Lc2d mice to return earlier to basal peroxidase scores.

3.5.2. NBT positive cells

Prior to infection, Lc2d treatment increased BAL NBT+ cells compared to the control group (Fig. 2C). Pneumococcal infection increased the bactericidal function of BAL cells in

Table 2
Anti-pneumococcal IgG and IgA (pg/ml) from mice fed *L. casei*

Time post challenge (d)	Experimental groups	Serum		BAL	
		IgG	IgA	IgG	IgA
0	Control	2.06 ± 0.56	0.28 ± 0.093	0.12 ± 0.09	0.2 ± 0.09
	Lc2d	2.51 ± 0.43	0.32 ± 0.041	0.11 ± 0.09	0.41 ± 0.031
	Lc5d	1.98 ± 0.73	0.3 ± 0.053	0.11 ± 0.07	0.31 ± 0.04
	Lc7d	2.13 ± 1.01	0.25 ± 0.08	0.15 ± 0.06	0.25 ± 0.025
15	Control	28.51 ± 2.14	9.67 ± 1.61	2.01 ± 0.08	2.81 ± 0.05
	Lc2d	32.91 ± 1.54*	12.31 ± 1.43	2.51 ± 0.11**	3.32 ± 0.05*
	Lc5d	29.77 ± 1.39	10.99 ± 1.52	2.06 ± 0.13	3.10 ± 0.08*
	Lc7d	28.03 ± 1.60	9.73 ± 1.12	2.06 ± 0.11	2.32 ± 0.10

Mice were fed *L. casei* for 2, 5 or 7 d (Lc2d, Lc5d, Lc7d, respectively) before (d 0) and after challenge (d 15) challenged with *S. pneumoniae* (1×10^6 CFU per mouse). Control mice were challenged without previous treatment. Results represent mean ± S.D. of each group of animals ($n = 6$). Asterisks represent significant differences from the control group at the same time point (*$P < 0.05$ and **$P < 0.005$).

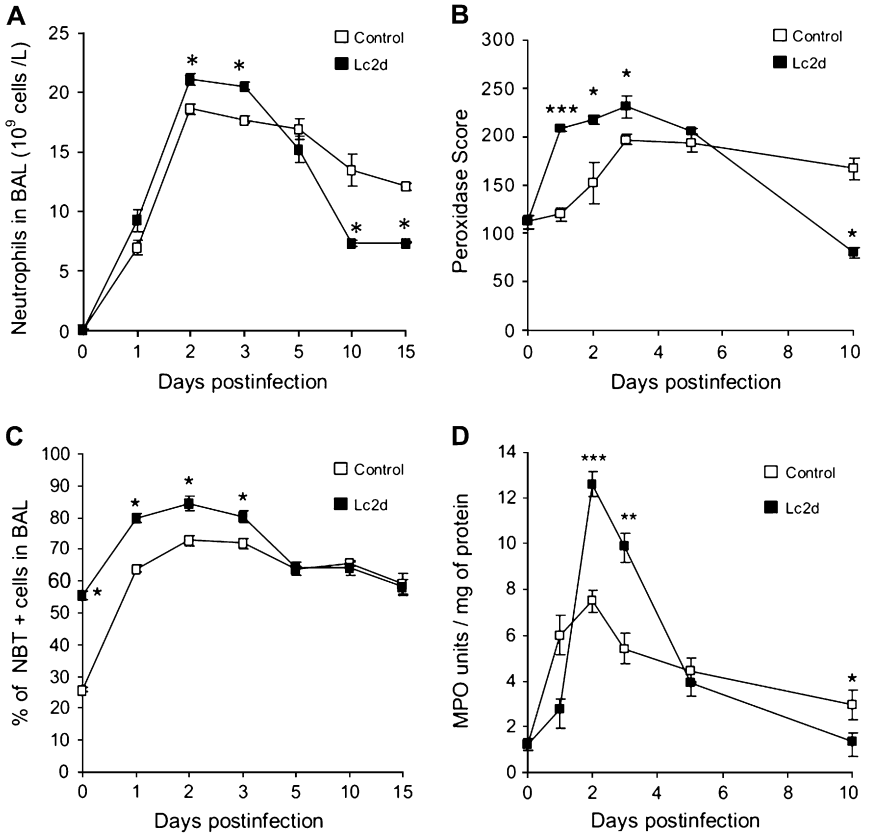

Fig. 2. BAL neutrophil counts (A), blood neutrophil peroxidase score (B), % of BAL NBT+ cells (C), and MPO activity in lung homogenates (D). Mice were fed *L. casei* for 2 d (Lc2d) and challenged with *S. pneumoniae* (1×10^6 CFU per mouse). Control mice were challenged with the pathogen without previous treatment. Results are expressed as mean ± S.D. for 6 mice per group at each time point. Asterisks represent significant differences from the control group at the same time point (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$).

Lc2d and control mice; however, the number of NBT+ cells was higher in the Lc2d group on d 1, 2 and 3 postinfection.

3.5.3. MPO activity in lung

Pneumococcal infection induced an early increase in MPO (Fig. 2D), with a peak on d 2 postinfection in both the Lc2d and control groups that was significantly higher in the former. MPO returned earlier to basal levels in the Lc2d mice, probably because infection was more efficiently controlled than in the control group.

3.6. Histopathological examination

Lung weight in the control mice increased gradually after challenge from 179 ± 10 mg on d 0 to 360 ± 12 mg on d 15 postinfection ($P < 0.01$). Histopathological examination revealed an intense inflammatory response with progressive parenchymal involvement, including widespread cellular infiltration, hemorrhage, increased fibrosis in bronchial walls and vessels and reduction of the alveolar airspaces (Fig. 3B). Lung of Lc2d mice revealed signs of a moderate inflammatory response with no modification of the lung weight throughout the assessed period (198 ± 10 mg). In addition, focal cellular infiltration with no hemorrhage and conserved alveolar airspaces were observed in the Lc2d group (Fig. 3A).

3.7. IgA+ cells in BALT

Treatment with Lc2d enhanced the number of IgA+ cells prior to infection. Challenge with pneumococci induced a progressive increase in IgA+ cells until d 15 postinfection in both control and Lc2d groups. However, Lc2d mice showed significantly higher numbers of IgA+ cells compared to the control group at all assayed periods (refer to supplementary data, Appendix 2).

3.8. Pro- and anti-inflammatory cytokines

TNF-α (associated with the inflammatory response) and IL-10 (associated with the anti-inflammatory response) were evaluated in this study (Fig. 4). In the Lc2d and control groups, lung TNF-α reached high levels at h 8 postinfection compared with pre-infection values, with a peak at h 12 postinfection. However, Lc2d treatment induced a stronger increase in this cytokine, with values higher than those in the control group at h 8 postinfection. In control mice, very low levels of serum TNF-α were detected until h 12 postinfection, when there was a rapid increase that reached a peak at h 72 postinfection. The kinetics of serum TNF-α in the Lc2d mice was similar to that of the control group, but the values were significantly smaller from h 48 postinfection until the end of the experiment.

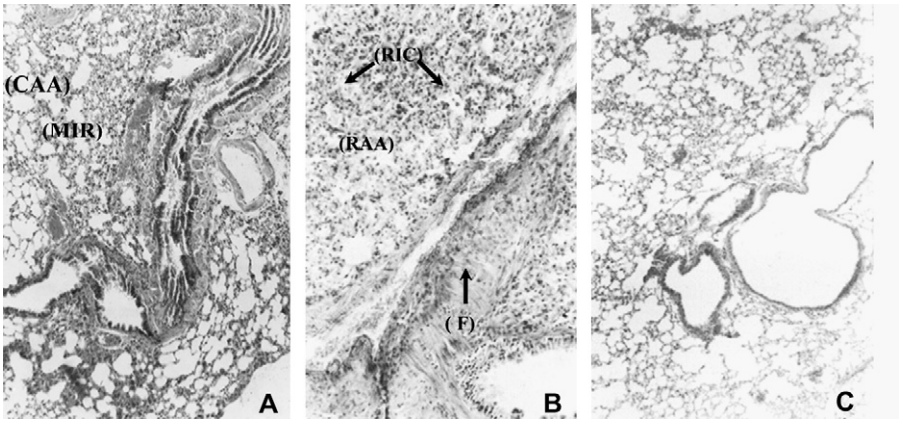

Fig. 3. Representative histological slides of lung tissue from control mice (B) and mice treated preventively with *L. casei* for 2 d (Lc2d) (A) after intranasal challenge with 10^6 CFU *S. pneumoniae* (d 10 postinfection). Lung architecture of normal non-infected mice is shown in (C). Recruited inflammatory cells (RIC), fibrosis (F) and reduction of alveolar airspaces (RAA) are evident in the control group (B). Lc2d showed signs of a moderate inflammatory response (MIR) without hemorrhage and conserved alveolar air spaces (CAA) in comparison with control mice (C). Original magnification ×200.

Lc2d treatment prior to infection significantly increased lung and serum IL-10 compared to the control group. The values of serum IL-10 in the Lc2d group remained higher than in the control group on d 3 and 5 postinfection. After challenge, lung IL-10 in the Lc2d group was lower than in the control mice on d 1 postinfection while it was higher on d 5 and 10 postinfection.

4. Discussion

The present research describes the immune enhancing effect of the oral administration of *L. casei* on an *S. pneumoniae* respiratory infection. Out of the three treatments assayed, *L. casei* administration for two days proved to be the most effective against infection. The protective effect of *L. casei* was evidenced by the stimulation of both the innate and adaptative immune response.

Lc5d and Lc7d treatments were not as effective as Lc2d in improving the immune response against pneumococci, 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 [11,15].

Effective pulmonary host defense against respiratory pathogens is believed to be mainly mediated via phagocytosis by alveolar macrophages and recruited neutrophils [17]. If pneumococci overcome these defenses and gain entry into the bloodstream, systemic protection is afforded by anticapsular antibodies [18]. Such defenses are orchestrated by a rapid inflammatory response in the coordination of which cytokines play an important role [19]. In our infection model, *L. casei* treatment induced a faster clearance of the pathogen, which was reflected in the lower number of pneumococci in the lung and the shorter period of septicemia compared to the control group.

L. casei administration was able to induce activation of the non-specific immune response, inducing a rapid and early increase in PMN cells. In this sense, it is known that the recruited PMNs become functionally activated via stimulation of pro-inflammatory cytokines and other mediators released within the infected compartment, including G-CSF, IL-1, IL-8, MIP-2 and TNF-α [20]. In our research, TNF-α increased earlier in the Lc2d group than in the control group; in the former, the increase in this cytokine was restricted to the lung, since in serum there were lower levels in the Lc2d group than in the control. Evidence is accumulating that the local activity of pro-inflammatory cytokines is required for an adequate antibacterial response at an infection site [19]. It has been reported that TNF-α would inhibit the onset of bacteremia by stimulating antibody production, neutrophil recruitment and granulocyte-macrophage colony-stimulating factor production by endothelial cells [21]. In addition, TNF-α can promote the antimicrobial activity of phagocytic cells by activating the respiratory burst [22]. We found that *L. casei* administration was able to induce the activation of phagocytic cells, which was evidenced by the strong activity of neutrophil MPO in lung homogenate as well as by the increased percentage of BAL NBT+ cells. This effect was presumably mediated by TNF-α. On the other hand, although PMNs are a key component of the host defense response against invading pathogens, they have also been implicated as mediators of tissue injury in a variety of inflammatory disorders [23]. Consequently, regulation of the inflammatory response by anti-inflammatory cytokines prevents damage to the host. Blum et al. have suggested that LAB could participate in tissue protection against the deleterious effect of an ongoing inflammatory process [24]. In our work, Lc2d treatment prior to pneumococcal infection induced a significant increase in IL-10 in lung and serum. This increase could help to reduce the production of pro-inflammatory cytokines and chemokines and to downregulate the expression of adhesion molecules [25]. Consequently, in agreement with other

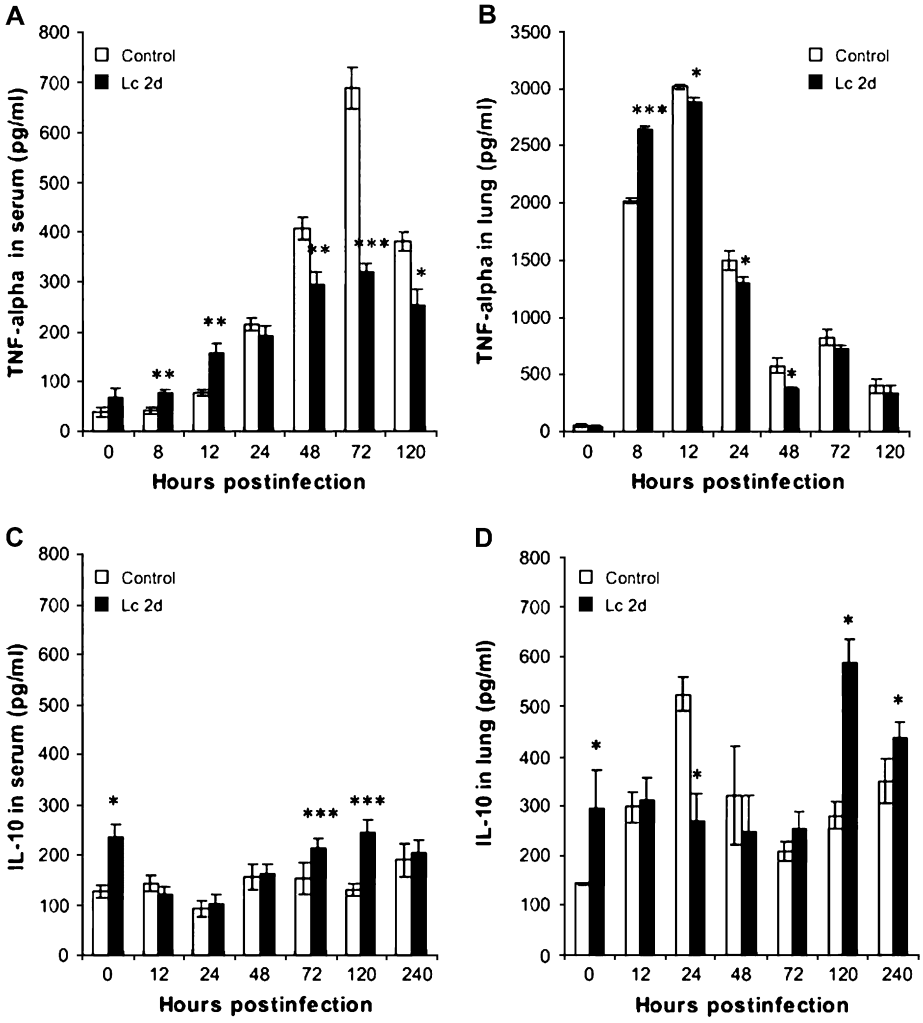

Fig. 4. TNF-α in serum (A) and lung (B), IL-10 in serum (C) and lung (D) of mice fed *L. casei* for 2 d (Lc2d) before (d 0) and after challenge (d 1, 5, 10 and 15) with *S. pneumoniae* (1×10^6 CFU per mouse). Control mice were challenged with the pathogen without previous treatment. Data represent mean ± S.D. for 6 mice per group at each time point. Asterisks represent significant differences from the control at the same time point (*$P < 0.05$, ** $P < 0.01$, ***$P < 0.005$).

reports [20], IL-10 would be valuable for attenuating inflammatory damage and pathophysiological alterations in lung infected with pneumococci. Thus, the histopathological examination of lung showed that both the inflammatory response and tissue damage were significantly lower in mice treated with *L. casei*. According to these results, Lc2d treatment would beneficially regulate the balance between TNF-α and IL-10, allowing a more effective inflammatory response against infection.

On the other hand, IL-10 enhances some B cell functions, which would favor the development of the specific immune response [26]. The mucosal immune system is able to respond to invading pathogens in the respiratory tract by producing pathogen-specific secretory immunoglobulin A (sIgA) antibodies [27]. Mice treated with *L. casei* for 2 d showed higher levels of anti-pneumococcal serum IgG and BAL IgA than the control group. Local sIgA has been known to prevent colonization of mucosal tissues and subsequent spreading into the systemic circulation more efficiently than systemic antibodies [28]. Janoff et al. demonstrated that human pneumococcal capsular polysaccharide-specific IgA initiates killing of *S. pneumoniae* with complement and phagocytes [27]. Activation of these cells requires local inflammation and mediators such as TNF-α and C5a, which can promote phagocyte activation with IgA-mediated uptake and killing of the pathogen [27]. Also, IgG would be involved in opsonization and, to a lesser extent, complement-mediated lysis. Both immunoglobulins may be responsible for the neutralization of toxins produced by bacterial pathogens and the inactivation of other virulence factors [29].

The relevance of the oral administration of *L. casei* lies in its ability to induce an increase in the mucosal and systemic immune response, which is important to diminish pathogen colonization of lungs and prevent spreading into the systemic circulation.

Perdigón et al. [30] demonstrated that *L. casei* CRL 431 orally administered interacts with the M cells of the Peyer's patches and that antigen uptake by M cells results in the initial induction of cell migration. These migrating cells enter mucosal effector sites such as the lamina propria of the upper respiratory tract [30].

On the basis of these previous findings and our own results, we propose a possible mechanism to explain the role of *L. casei* in the improved resistance to pneumococcal infection. *L. casei* would stimulate immune cell migration from the Peyer's patches in the small intestine to BALT. In addition, *L. casei* would affect cytokine expression in a specific or non-specific manner by stimulating distant immune cells. This cell migration as well as the activation of cells in distant sites would allow a faster immune response, both innate and specific, against pneumococcal infection, thus favoring clearance of the pathogen and modulating the inflammatory immune response, with less damage to lung tissue. The results obtained in this study may lead to new insights concerning the use of *L. casei* as an oral adjuvant or as an oral vaccine vector for a wide range of infectious lung diseases, which have a high incidence in Argentina.

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Appendix. Supplementary information

Supplementary information for this manuscript can be downloaded at doi:10.1016/j.micinf.2006.04.022.

References

[1] G.R. Siber, Pneumococcal disease: prospects for a new generation of vaccines, Science 265 (1994) 1385–1387.
[2] R.H. Simon, R. Paine, Participation of pulmonary alveolar epithelial cells in lung inflammation, J. Lab. Clin. Med. 126 (1995) 108–118.
[3] E.I. Tuomanen, R. Austrian, H.R. Masure, Pathogenesis of pneumococcal infection, N. Engl. J. Med. 332 (1995) 1280–1284.
[4] R. Ruvinsky, A. Gentile, M. Regueira, A. Corso, Infecciones invasivas por *Streptococcus pneumoniae*: estudio epidemiológico e importancia del desarrollo de un sistema de vigilancia, Arch. Argent. Pediatr. 100 (2002) 31–43.
[5] K.L. Erickson, N.E. Hubbard, Probiotic immunomodulation in health and disease, J. Nutr. 130 (2000) 403S–409S.
[6] G. Perdigon, M. Nader de Macias, S. Alvarez, G. Oliver, A. Pesce de Ruiz Holgado, Effect of oral administration of lactobacilli on macrophage activation in mice, Infect. Immun. 53 (1986) 404–410.
[7] M.L. Cross, A. Ganner, D. Teilab, M.F. Linley, Patterns of cytokine induction by gram-positive and gram-negative probiotic bacteria, FEMS Immunol. Med. Microbiol. 42 (2004) 173–180.
[8] M.L. Cross, Microbes versus microbes: immune signals generated by probiotic lactobacilli and their role in protection against microbial pathogens, FEMS Immunol. Med. Microbiol. 34 (2002) 245–253.
[9] S. Alvarez, N. Gobbato, E. Bru, A. Ruiz Holgado, G. Perdigón, Specific immunity induction at mucosal level by viable *Lactobacillus casei*. Perspective for oral vaccine development, Food Agric. Immunol. 10 (1998) 79–87.
[10] G. Perdigón, S. Alvarez, M. Medina, E. Vintiñi, E. Roux, Influence of the oral administration of lactic acid bacteria on IgA producing cells associated to bronchus, Int. J. Immunopathol. Pharmacol. 12 (1999) 97–102.
[11] S. Alvarez, C. Herrero, E. Bru, G. Perdigón, Effect of *Lactobacillus casei* and yogurt administration on prevention of *Pseudomonas aeruginosa* infection in young mice, J. Food Protect. 64 (2001) 1768–1774.
[12] H. Zemlickova, M.I. Crisostomo, M.C. Brandileone, T. Camou, E. Castaneda, A. Corso, G. Echaniz-Aviles, M. Pasztor, A. Tomasz, Serotypes and clonal types of penicillin-susceptible *Streptococcus pneumoniae* causing invasive disease in children in five Latin American countries, Microb. Drug Resist. 11 (2005) 195–204.
[13] R. Facklam, J.A. Washington II, Streptococcus and related catalase negative gram positive cocci, in: A. Balows, W.J. Hausler Jr., L.H. Kenneth, H.D. Isenberg, J. Shadomy (Eds.), Manual of Clinical Microbiology, fifth ed. American Society for Microbiology, Washington D.C, 1992, pp. 238–257.
[14] Y. Bergeron, N. Oullet, A. Deslauriers, M. Simard, M. Olivier, M.G. Bergeron, Cytokine kinetics and other host factors in response to pneumococcal pulmonary infection in mice, Infect. Immun. 66 (1998) 912–922.
[15] J. Villena, S. Racedo, G. Agüero, E. Bru, M. Medina, S. Alvarez, *Lactobacillus casei* improves resistance to pneumococcal respiratory infection in malnourished mice, J. Nutr. 135 (2005) 1462–1469.
[16] P.M. Bozeman, D.B. Learn, E.L. Thomas, Assay of the human leukocyte enzymes myeloperoxidase and eosinophil peroxidase, J. Immunol. Methods 126 (1990) 125–133.
[17] S.B. Gordon, G.R. Irving, R.A. Lawson, M.E. Lee, R.C. Read, Intracellular trafficking and killing of *Streptococcus pneumoniae* by human alveolar macrophages are influenced by opsonins, Infect. Immun. 68 (2000) 2286–2293.
[18] M. Anttila, M.J. Voutilainen, V.J. Eskola, H. Kayhty, Contribution of serotype-specific IgG concentration, IgG subclasses and relative antibody avidity to opsonophagocytic activity against *Streptococcus pneumoniae*, Clin. Exp. Immunol. 118 (1999) 402–407.
[19] S. Nelson, W.R. Summer, Innate immunity cytokines and pulmonary host defense, Infect. Dis. Clin. N. Am. 12 (1998) 555–567.
[20] A.R. Kerr, J.J. Irvine, J.J. Search, N.A. Gingles, A. Kadioglu, P.W. Andrew, W.L. McPheat, C.G. Booth, T.J. Mitchell, Role of inflammatory mediators in resistance and susceptibility to pneumococcal infection, Infect. Immun. 70 (2002) 1547–1557.
[21] K. Takashima, K. Tateda, T. Matsumoto, Y. Iizawa, M. Nakao, K. Yamaguchi, Role of tumor necrosis factor alpha in pathogenesis of pneumococcal pneumonia in mice, Infect. Immun. 65 (1997) 257–260.
[22] S. Dusi, V.D. Bianca, M. Donini, K.A. Nadalini, F. Rossi, Mechanisms of stimulation of the respiratory burst by TNF in non-adherent neutropphils: its independence of lipidic transmembrane signaling and dependence on protein tyrosine phosphorylation and cytoskeleton, J. Immunol. 157 (1996) 4615–4623.
[23] P. Zhang, W.R. Summer, G.J. Bagby, S. Nelson, Innate immunity and pulmonary host defense, Immunol. Rev. 173 (2000) 39–51.
[24] S. Blum, Y. Delneste, S. Alvarez, D. Haller, P.F. Pérez, C. Bode, W.P. Hammes, A.M.A. Pfeifer, E.J. Schiffrin, Interactions between commensal bacteria and mucosal immunocompetent cells, Internat. Dairy J. 9 (1999) 63–68.
[25] F. Willems, A. Marchant, J.P. Delville, C. Gerard, A. Delvaux, T. Velu, M. de Boer, M. Goldman, Interleukin-10 inhibits B7 and intercellular adhesion molecule-1 expression on human monocytes, Eur. J. Immunol. 24 (4) (1994) 1007–1009.
[26] A.J. Husband, K.W. Beagley, J.R. McGhee, Mucosal cytokines, in: L.O. Pearay, M.E. Lamm, J. Biewenstock, R. Jerry (Eds.), Mucosal Immunology, second ed. Academic Press, San Diego, CA, 1999, pp. 541–557.
[27] E.N. Janoff, C. Fasching, J.M. Oresnstein, J.B. Rubins, N.L. Osptad, A.P. Dalmasso, Killing of *Streptococcus pneumoniae* by capsular polysaccharide-specific polymeric IgA, complement, and phagocytes, J. Clin. Invest. 104 (1999) 1139–1147.
[28] J.M. Woof, J. Mestecky, Mucosal immunoglobulins, Immunol. Rev. 206 (2005) 64–82.
[29] V.E. García, M.M. Gherardi, M.F. Iglesias, M.C. Cerquetti, D.O. Sordelli, Local and systemic immunity against *Streptococcus pneumoniae*: humoral responses against a non-capsulated temperature-sensitive mutant, FEMS Microbiol. Lett. 108 (1993) 163–168.
[30] G. Perdigón, R. Fuller, M. Medina, The influence of the lactic acid bacteria and other resident microflora on the immune system of the growing animal, in: W. Holzapfel, P. Naughton (Eds.), Microbiological Ecology of Growing Animals, Biology of Growing Animals Series, first ed. Elsevier, UK, 2005, pp. 351–375.