Stimulation of respiratory immunity by oral administration of *Lactococcus lactis*

Julio Villena, Marcela Medina, Elisa Vintiñi, and Susana Alvarez

Abstract: This work demostrates that nonrecombinant *Lactococcus lactis* NZ, administered by the oral route at the proper dose, is able to improve resistance against pneumococcal infection. *Lactococcus lactis* NZ oral administration was able to improve pathogen lung clearance, increased survival of infected mice, and reduced lung injuries. This effect was related to an upregulation of the respiratory innate and specific immune responses. Administration of *L. lactis* NZ improved production of bronchoalveolar lavage (BAL) fluid TNF-α, enhanced recruitment of neutrophils into the alveolar spaces, and induced a higher activation of BAL phagocytes compared with the control group. *Lactococcus lactis* NZ administered orally stimulated the IgA cycle, increased IgA+ cells in intestine and bronchus, and improved production of BAL IL-4 and IL-10 during infection. Moreover, mice treated with *L. lactis* NZ showed higher levels of BAL anti-pneumococcal IgA and IgG. Taking into consideration that orally administered *L. lactis* NZ stimulates both the innate and the specific immune responses in the respiratory tract and that bacterial and viral antigens have been efficiently produced in this strain, *L. lactis* NZ is an excellent candidate for the development of an effective pneumococcal oral vaccine.

Key words: Lactococcus lactis, oral adjuvant, *Streptococcus pneumoniae*, respiratory immunity.

Résumé : Ce travail démontre que *Lactococcus lactis* NZ non recombinante, administrée par voie orale à une dose appropriée, peut améliorer la résistance aux infections à pneumocoques. L'administration par voie orale de *L. lactis* NZ peut améliorer l'élimination du pathogène des poumons, augmenter la survie des souris infectées et réduire les dommages pulmonaires. Cet effet est relié à l'augmentation des réponses immunes respiratoires innées et spécifiques. L'administration de *L. lactis* NZ a amélioré la production de TNF-α recueilli du liquide de lavage broncho-alvéolaire (LBA), a augmenté le recrutement des neutrophiles dans les espaces alvéolaires, et a induit une plus forte activation des phagocytes du LBA comparativement au groupe contrôle. *Lactococcus lactis* NZ administrée oralement a stimulé la production d'IgA, a augmenté le nombre de cellules IgA+ dans l'intestin et les bronches et a amélioré la production d'IL-4 et d'IL-10 du LBA lors de l'infection. De plus, les souris traitées avec *L. lactis* NZ ont présenté de plus hauts niveaux d'IgA et d'IgG anti-pneumocoque dans le LBA. Considérant que *L. lactis* NZ administrée par voie orale stimule aussi bien la réponse immune innée que spécifique des voies respiratoires et que des antigènes bactériens et viraux ont été produits efficacement chez cette souche, *L. lactis* NZ est une excellente candidate pour développer un vaccin anti-pneumocoque oral efficace.

Mots-clés : Lactococcus lactis, adjuvant oral, *Streptococcus pneumoniae*, immunité respiratoire.

[Traduit par la Rédaction]

Introduction

Numerous studies have demonstrated that lactic acid bacteria (LAB) are able to stimulate the gut immune system and to prevent intestinal infections (for a review, see Cross 2002). Moreover, it has been reported that the oral administration of certain LAB strains can not only increase the local immune response in the intestine but also elicit systemic as well as distal mucosal immune responses (Cross 2002; Alvarez et al. 2007). In these sense, our laboratory has reported that the oral administration of probiotic lactobacilli can improve the immune response against *Pseudomonas aeruginosa* respiratory infection in young mice (Alvarez et al. 2001). Moreover, our studies using models of pneumococcal infection in immunocompetent and immunodeficient mice demonstrated that the oral administration of a probiotic *Lactobacillus casei* strain can improve the respiratory and systemic immune responses against *Streptococcus pneumoniae* (Villena et al. 2005; Racedo et al. 2006).

The protective effect in distant mucosal sites mediated by LAB is possible because of the existence of a common mucosal immune system. When the mucosal immune response is induced, primed T and B cells migrate through the lymphatic system and then enter the peripheral blood circulation via the thoracic duct. Extravasation of the immune cells occurs not only in the gut lamina propia but also in other mucosal sites such as the respiratory tract (Kiyono and Fukuyama 2004; Alvarez et al. 2007). This homing pathway of primed lymphoid cells from the inductive sites on the Peyer's patches to distant mucosal sites after antigen stimu-

Received 12 February 2008. Revision received 14 May 2008. Accepted 16 May 2008. Published on the NRC Research Press Web site at cjm.nrc.ca on 28 July 2008.

J. Villena, M. Medina, and S. Alvarez.[1] Laboratorio de Bioquímica y Clínica Experimental, Centro de Referencia para Lactobacilos (CERELA-CONICET), Chacabuco 145, Tucumán 4000, Argentina; Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Balcarce 747, CP 4000, San Miguel de Tucumán, Tucumán 4000, Argentina.
E. Vintiñi. Laboratorio de Bioquímica y Clínica Experimental, Centro de Referencia para Lactobacilos (CERELA-CONICET), Chacabuco 145, Tucumán 4000, Argentina.

[1]Corresponding author (e-mail: salvarez@cerela.org.ar).

 doi:10.1139/W08-052

lation could be exploited to design LAB-derived oral vaccines that could afford protection against respiratory pathogens (Perdigón et al. 1999; Racedo et al. 2006).

The use of lactobacilli as vaccine vehicles is complex and limited progress has been made with this genus, since some species are refractory to transformation (Perdigón et al. 2001). In contrast, highly efficient expression systems have been developed that successfully express and secrete heterologous antigens in *Lactococcus lactis* (Corthier and Renault 1999). However, no studies exist on the capacity of orally administered lactococci to stimulate respiratory immunity. Establishing the effective immunomodulatory activities of lactococci could lead to the development of oral vaccines that efficiently protect mucosal airways. Thus, the purpose of the present study was to investigate whether the oral administration of nonrecombinant *L. lactis* NZ9000 (NZ), an LAB extensively engineered for the production of heterologous proteins, activates the respiratory immune system and whether it reduces the susceptibility to *S. pneumoniae* infection.

Materials and methods

Animals and microorganisms

Male 6-week-old Swiss albino mice were obtained from the closed colony at CERELA. Animals were housed in plastic cages and environmental conditions were kept constant, in agreement with the standards for animal housing. Each parameter studied was carried out in five or six mice for each time point. The Ethical Committee for Animal Care at CERELA approved experimental protocols. *Lactococcus lactis* NZ was grown until the final log phase in M17 supplemented with 1% glucose (M17-glu) broth (Difco Laboratories) at 30 °C. Bacteria were harvested by centrifugation at 3000*g* for 10 min, washed three times with sterile 0.01 mol/L phosphate-buffered saline (PBS), pH 7.2, and finally resuspended in nonfat milk (NFM) at the proper concentrations to be administered to the mice. *Lactococcus lactis* NZ was administered to different groups of mice by the oral route for 2 or 5 consecutive days at doses of 10^7 or 10^8 cells per mouse per day. *Lactococcus lactis* NZ was suspended in sterile NFM and 150 μL of an inoculum containing the appropriate dose was administered to each mouse. The control group received sterile NFM under the same conditions.

Capsulated *S. pneumoniae* serotype 14 was isolated from the blood of a patient at the Niño Jesús Children's Hospital in Tucumán, Argentina, and was termed *S. pneumoniae* T14. Pneumococci were grown in Todd–Hewitt broth (Oxoid) 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; cell density was adjusted to 4×10^7 cells/mL. Challenge with *S. pneumoniae* T14 was performed on the day after the end of each *L. lactis* NZ treatment (third and sixth days). Mice were challenged nasally with the pathogen by dripping 25 μL of an inoculum containing 10^6 cells into each nostril. To facilitate migration of the inoculum to the alveoli, mice were held in a head-up vertical position for 2 min.

Bacterial cell counts in lung and blood

For bacterial cell counts in lung, mice were sacrificed on days 1, 5, 10, and 15 post-infection and their lungs were excised, weighed, and homogenized in 5 mL of sterile peptone water. Homogenates were diluted appropriately, plated in duplicate on blood agar, and incubated for 18 h at 37 °C. *Streptococcus pneumoniae* colonies were counted and the results were expressed as $\log_{10}$ CFU per gram of organ. Progression of bacterial growth to the bloodstream was monitored by blood samples obtained by cardiac puncture with a heparinized syringe. Samples were plated on blood agar and bacteremia was reported as negative or positive hemocultures after incubation for 18 h at 37 °C.

Leukocyte counts in blood and bronchoalveolar lavages (BAL)

BAL samples were obtained according to the technique previously described (Villena et al. 2005). Briefly, the trachea was exposed and intubated with a catheter and two sequential BALs were performed in each mouse by injecting 0.5 mL of sterile PBS. The recovered fluid was centrifuged for 10 min at 900*g*; the pellet was used to determine total and differential counts of BAL leukocytes and the fluid was frozen at –70 °C for subsequent antibody analyses. Blood and BAL samples were obtained as described above. The total number of leukocytes in blood and BAL was determined with a hemocytometer. Differential cell counts were performed by counting 200 cells in blood or BAL smears stained with May Grünwald-Giemsa.

Peroxidase activity of blood and lung neutrophils

Measurement of myeloperoxidase (MPO) activity of blood neutrophils was carried out using a cytochemical method (Washburn test) with benzidine as a peroxidase chromogen. Cells were graded as negative or weakly, moderately, or strongly positive and were used to calculate the score (Villena et al. 2005). Neutrophil infiltration in lung tissue was quantified by measurement of MPO as previously described (Racedo et al. 2006). Briefly, lungs were cleared of blood, removed, and homogenized in 50 mmol/L 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 mL of an appropriate dilution of the lysate to 20 mmol/L 3,3′,5,5′-tetramethylbenzidine in dimethylphormamide and 30 mL of 2.7 mmol/L hydrogen peroxide in MPO assay buffer. The reaction mixture was incubated for 3 min at 37 °C and stopped with ice-cold 200 mmol/L sodium acetate buffer, pH 3. 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 per milligram of total proteins in lung homogenate). Total protein concentration was determined in the cellular lysates by Bradford's method.

Alveolar phagocytes activation

The bactericidal activity (oxidative burst) of macrophages and neutrophils was measured in the pellet of BAL fluid using the nitroblue tetrazolium (NBT) reduction test (Sigma-Aldrich) (Villena et al. 2006). 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, samples were examined under a light microscope for blue precipitates. A hundred cells were counted and the percentage of NBT-positive (+) cells was determined.

ELISA for anti-pneumococcal antibodies

Specific anti-pneumococcal antibodies (IgA and IgG) in serum and BAL were measured using a previously developed ELISA technique (Villena et al. 2005). Briefly, plates were coated with a heat-killed *S. pneumoniae* – sodium carbonate – bicarbonate buffer (1:100) suspension, pH 9.6. Nonspecific protein binding sites were blocked with PBS containing 5% NFM (PBS–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 Chemical) were diluted (1:500) in PBS–T. Antibodies were revealed with a substrate solution (*o*-phenylenediamine (Sigma Chemical)) in citrate–phosphate buffer, pH 5, containing 0.05% hydrogen peroxide and the reaction was stopped by addition of 1 mol/L sulfuric acid. Readings were carried out at 493 nm (VERSAmax Tunable microplate reader). Antibody concentration of each unknown sample was expressed as micrograms per millilitre determined from a standard curve made with commercial mouse IgA or IgG (Sigma).

Immunofluorescence test for IgA+ cells in lung and intestine

To determine the number of IgA+ cells in lung and intestine, immunofluorescence assays were performed on histological sections. Mice were sacrificed on day 0 and on days 1, 5, 10, and 15 post-infection and organs were aseptically removed and processed following Sainte-Marie's technique (Vintiñi et al. 2000). The slices were incubated with α-chain monospecific anti-mouse antibody conjugated with fluorescein isothiocyanate (Sigma). Cells were counted with a fluorescent microscope using 100× magnification. The results were expressed as the number of positive fluorescent cells per 10 fields. They represent the mean of six histological slices for each animal (five mice per group for each time point).

Cytokines concentrations in BAL fluid

Tumor necrosis factor alpha (TNF-α) and interleukin-10 (IL-10) and IL-4 levels were measured in BAL fluid. Commercially available ELISA kits were used according to the manufacturer's recommendations (R&D Systems, USA). OD readings of samples were converted to picograms per millilitre using standard curves generated with varying concentrations of recombinant cytokine supplied with the kit. The limit of detection was 20 pg/mL for each assay.

Biochemical assay of BAL fluid

Albumin content, a measure to quantitate increased permeability of the bronchoalveolar capillarity barrier, and lactate dehydrogenase (LDH) activity, an indicator of general cytotoxicity, were determined in the acellular BAL fluid. Albumin content was determined colorimetrically based on albumin binding to bromcresol green using an albumin diagnostic kit (Wiener Lab). LDH activity, expressed

Fig. 1. Kinetics of pneumococcal clearance from lungs. *Lactococcus lactis* NZ was orally administered at a dose of (A) 10^8 or (B) 10^7 cells per mouse day day for 2 or 5 consecutive days and then mice were challenged nasally with 10^6 *Streptococcus pneumoniae* cells. Results are expressed as means ± SD (n = 5 or 6). *Significantly different from the control group at the same time point ($p < 0.05$).

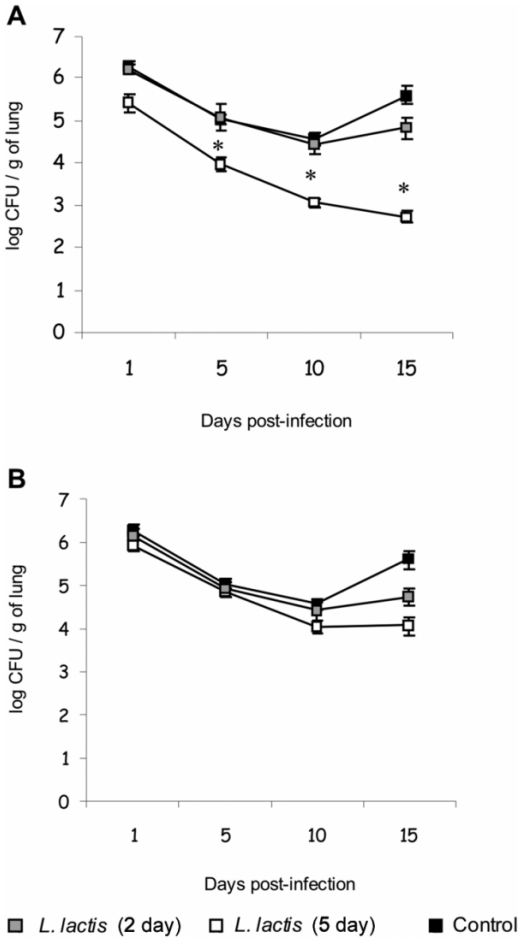


Fig. 2. Survival of infected mice. *Lactococcus lactis* NZ was orally administered at a dose of 10^8 cells for 5 days and then mice were challenged nasally with 10^6 *Streptococcus pneumoniae* cells.

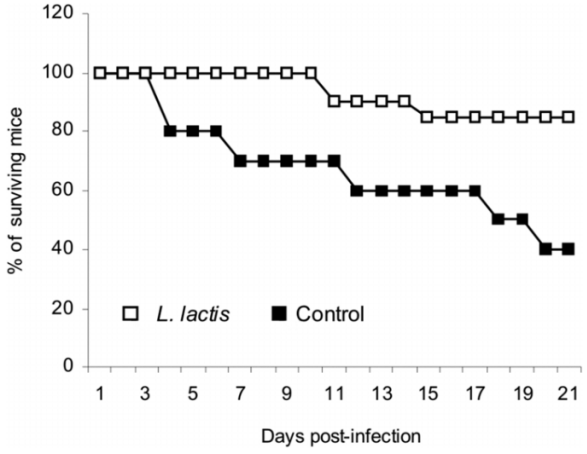
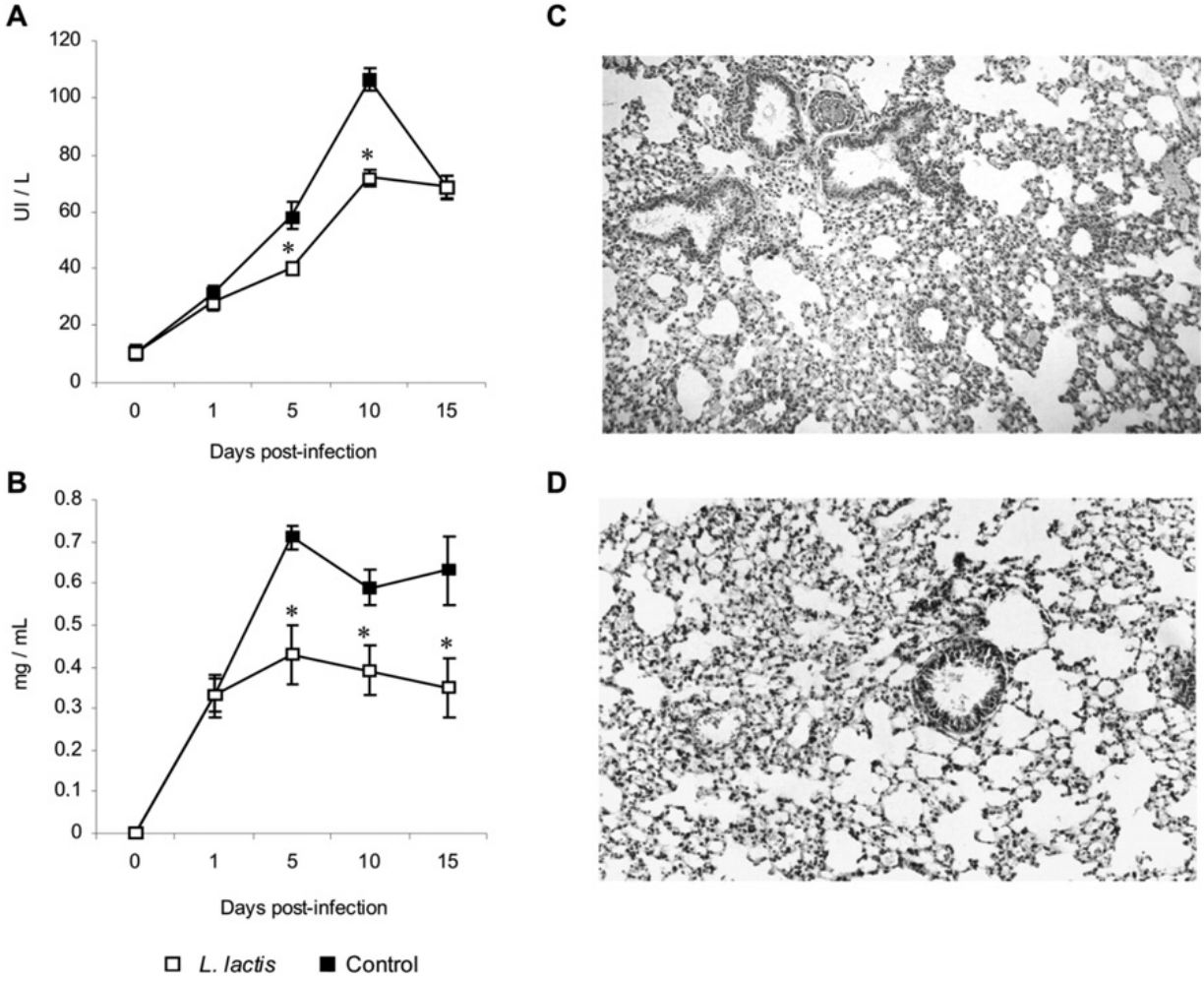


Fig. 3. Lung tissue injuries. *Lactococcus lactis* NZ was orally administered at a dose of 10^8 cells for 5 days and then mice were challenged nasally with 10^6 *Streptococcus pneumoniae* cells. (A) BAL albumin concentration, (B) BAL LDH activity, and histological changes in lungs were studied. (C) Control mice; (D) *L. lactis* treated mice. Original magnification 200×. Results are expressed as means ± SD (n = 5 or 6). *Significantly different from the control group at the same time point ($p < 0.05$).



as units per litre of BAL fluid, was determined by measuring the formation of the reduced form of NAD using Wiener reagents and procedures (Wiener Lab).

Histopathological examination

Tissue damage was evaluated on days 1, 10, and 15 post-infection. Lungs were aseptically removed, fixed in 4% formalin, and embedded in histowax (Leica). Histopathological assessment was performed on 5 μm tissue sections stained with hematoxylin-eosin for light microscopy.

Statistical analysis

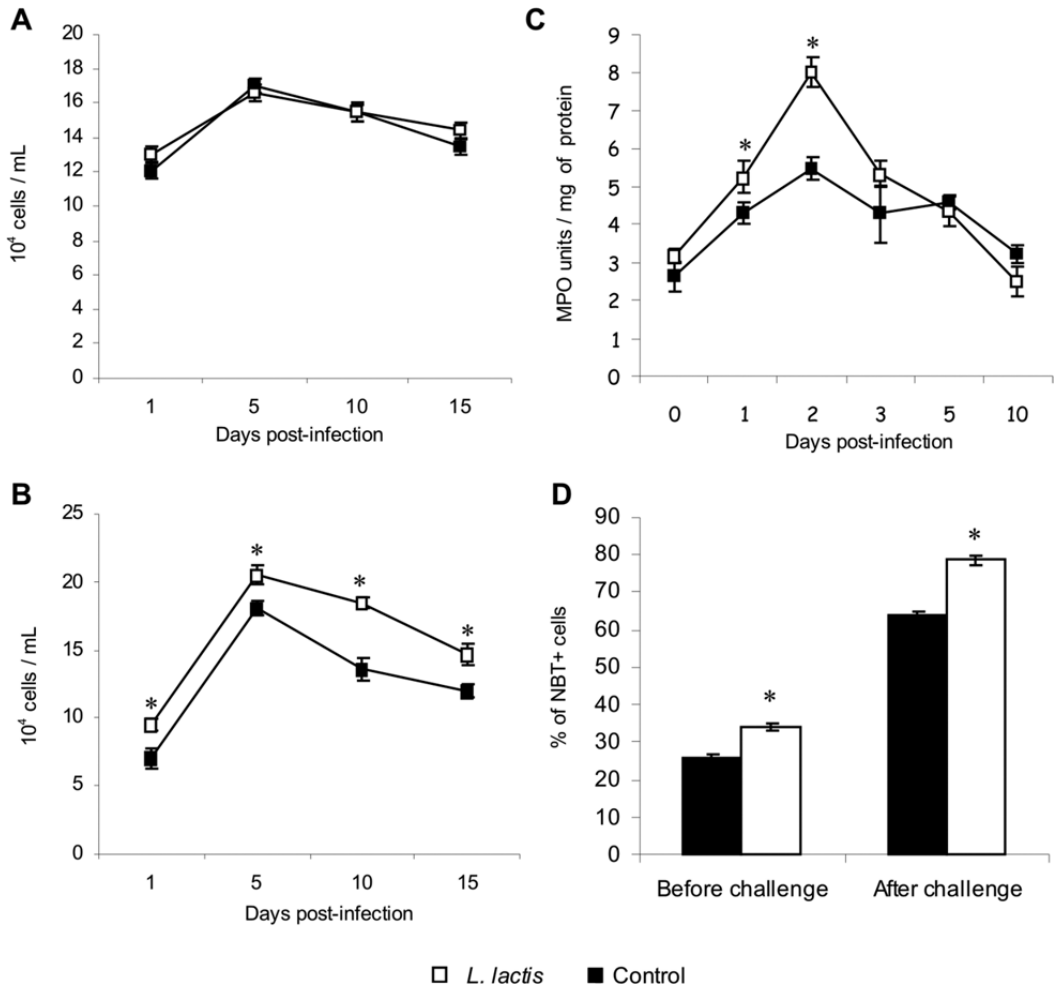
Experiments were performed in triplicate and results were expressed as means ± SD. After verification of a normal distribution of data, two-way ANOVA was used. Tukey's test (for pairwise comparisons of the means) was used to test for differences between the groups. Differences were considered significant at $p < 0.05$. For survival analysis, Pearson's χ^2 test and MV-G-square test were used. Differences were considered significant at $p < 0.05$.

Results

Resistance against pneumococcal infection

The immunostimulating properties of LAB have been proved to be dose dependent (Alvarez et al. 2001; Racedo et al. 2006); consequently, the ability of *L. lactis* NZ to increase resistance against *S. pneumoniae* infection was studied using two doses (10^7 and 10^8 cells per mouse per day) and different periods of administration (2 and 5 days). The oral administration of *L. lactis* at a concentration of 10^8 cells per mouse per day for 5 days was the optimal dose able to reduce pneumococcal colonization in lung (Fig. 1A) and blood, showing negative hemocultures since day 10 post-infection (data not shown). In contrast, the other treatments were unable to reduce the number of pneumococci in lung and blood (Fig. 1). Survival of infected mice was monitored after challenge with the pathogen (Fig. 2). Only 40% of the mice in the control group survived until day 21 post-infection, while 85% of the mice treated with *L. lactis* at a dose of 10^8 cells per mouse per day for 5 days were

Fig. 4. Leukocytes in the respiratory tract. *Lactococcus lactis* NZ was orally administered at a dose of 10^8 cells for 5 days and then mice were challenged nasally with 10^6 *Streptococcus pneumoniae* cells. (A) BAL macrophages, (B) BAL neutrophils, (C) lung MPO activity, and BAL NBT+ cells were studied. Post-challenge values of NBT+ cells correspond to the first day post-infection. Results are expressed as means ± SD (n = 5 or 6). *Significantly different from the control group at the same time point ($p < 0.05$).



alive at this time point. The other *L. lactis* treatments did not modify survival rates, which were similar to those in the control group (data not shown). On the basis of these results, administration of 10^8 cells of *L. lactis* NZ for 5 days was selected as the optimal treatment to improve resistance against pneumococcal infection; consequently, the following studies were carried out with this experimental group.

Lung tissue damage

To establish whether the reduction in pneumococcal cell counts was correlated with a lower tissue damage in lungs, biochemical and histological studies were performed in the respiratory tract of infected mice. Challenge with *S. pneumoniae* significantly increased BAL albumin concentration and LDH activity in treated and control groups (Figs. 3A and 3B), which indicates that infection increased permeability of the bronchoalveolar capillarity barrier and cell damage in lungs. However, *L. lactis* treatment was able to reduce significantly both LDH and albumin compared with the control group. Lung histopathological examination of control mice revealed a gradual and intense inflammatory response with progressive parenchymal involvement, including widespread cellular infiltration, passage of blood elements from capillaries to tissues, increased fibrosis in bronchial walls and vessels, hemorrhage, and reduction of the alveolar airspaces (Fig. 3C). Furthermore, the lung parenchyma was observed to have a distorted appearance with loss of alveolar architecture. Lung examination of mice that received *L. lactis* revealed signs of moderate inflammation with focal cellular infiltration, without hemorrhage and with conserved alveolar airspaces (Fig. 3D).

Innate immune response

To elucidate the mechanism through which the treatment with *L. lactis* exerts its protective effect, we studied the number and functionality of phagocytes at the systemic level and in the respiratory tract. After challenge with *S. pneumoniae*, an increase in the total number of BAL leukocytes was observed in treated and control mice. There were no significant differences between the two groups concerning the number of BAL macrophages (Fig. 4A). In contrast, BAL neutrophil values were significantly higher in treated mice than in the control group (Fig. 4B). To corroborate that *L. lactis* had induced a larger recruitment of neutrophils toward

the lungs, we determined MPO activity in lung homogenates. Lung MPO activity increased after challenge in both groups, but treated mice showed significantly larger values during the earlier stages of infection (Fig. 4C). When activation of alveolar phagocytes was assessed before challenge with the pathogen, the percentage of BAL NBT+ cells was found to have increased levels in treated mice compared with the control group (Fig. 4D). Challenge with *S. pneumoniae* increased the bactericidal activity of BAL phagocytes in both groups. However, percentages of NBT+ cells were significantly higher in mice treated with *L. lactis* than in controls (Fig. 4D).

Pneumoccocal infection increased the number of blood leukocytes in both experimental groups, but treated mice showed higher values than the control group (Fig. 5A). Differential analysis of blood leukocytes showed that these changes were mediated by neutrophils, which were significantly increased in the *L. lactis* group (Fig. 5B). To determine whether *L. lactis* administration was able to activate blood phagocytes, peroxidase activity was assessed. There were no differences between treated and control groups in blood peroxidase scores before challenge (Fig. 5C). After infection, the score values increased in both groups but treated mice showed significantly higher values than the control group (Fig. 5C).

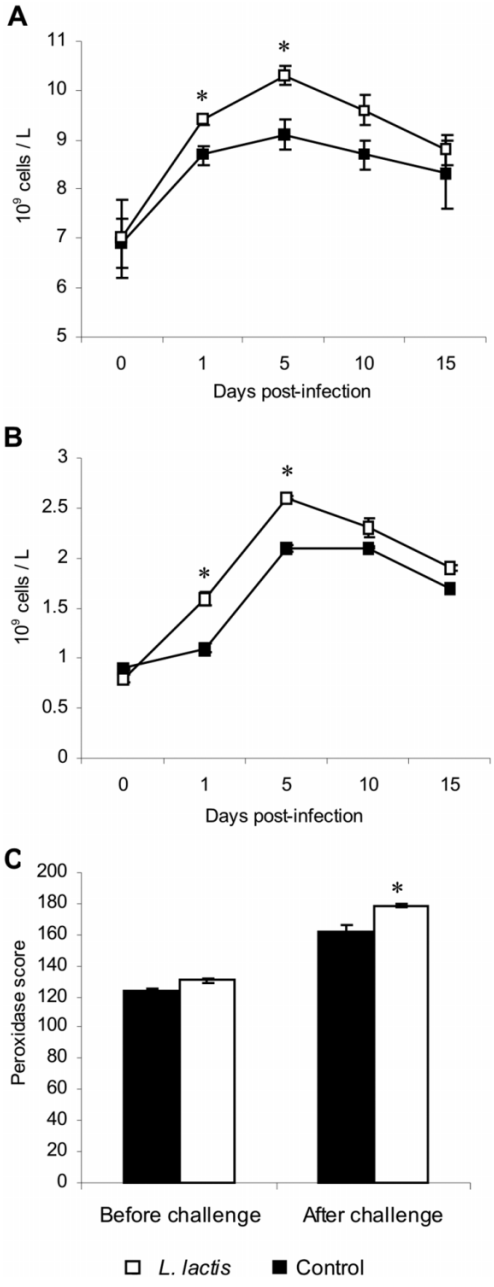
Specific immune response

Respiratory and systemic antibody production was examined to study specific immune response; thus, serum and BAL anti-pneumococcal IgG and IgA antibodies were determined until day 15 post-infection (Fig. 6). Levels of serum anti-pneumococcal IgG and IgA in mice that received *L. lactis* were similar to those in the control group (Figs. 6A and 6B). In contrast, *L. lactis* treatment induced a significant increase in specific pneumococcal BAL IgA and IgG antibodies compared with controls (Figs. 6C and 6D). The higher amount of BAL IgA would indicate that *L. lactis* administration is able to stimulate the IgA cell cycle. Thus, we next determined the number of IgA+ cells in the small intestine and bronchus. Figure 7A shows that treatment with *L. lactis* enhanced the number of intestinal IgA+ cells prior to infection. In addition, IgA+ cells in bronchus tissue were higher in mice that received the LAB (Fig. 7B). Challenge with *S. pneumoniae* did not modify the number of intestinal IgA+ cells but increased respiratory IgA+ cells in both treated and control groups. However, mice in the *L. lactis* group presented higher values than those in the control animals (Fig. 7B).

Production of cytokines in the respiratory tract

Previous studies demonstrated that TNF-α in BAL increased after challenge with pneumococci with a peak at 12 h post-infection (Agüero et al. 2006). Thus, in the present study, the levels of BAL TNF-α were determined at this time point. Mice treated orally with *L. lactis* showed higher values of BAL TNF-α than mice in the control group (Fig. 8A). In addition, the production of BAL IL-4 and IL-10 was determined at 120 h post-infection and results showed that these two cytokines were significantly increased in *L. lactis* treated mice compared with those in the control group (Figs. 8B and 8C).

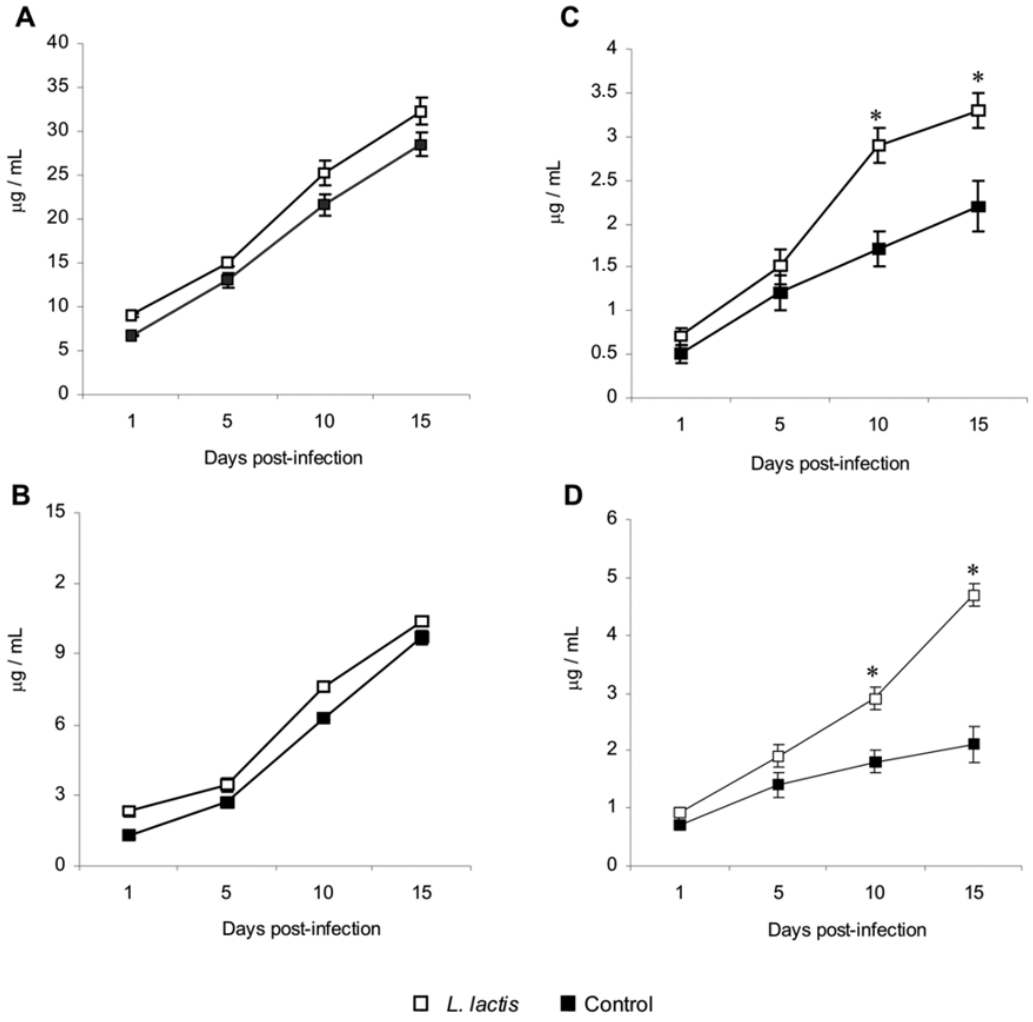
Fig. 5. Leukocytes in blood. *Lactococcus lactis* NZ was orally administered at a dose of 10^8 cells for 5 days and then mice were challenged nasally with 10^6 *Streptococcus pneumoniae* cells. (A) Blood leukocytes, (B) blood neutrophils, and (C) blood peroxidase score were studied. Post-challenge values of blood peroxidase score correspond to the first day post-infection. Results are expressed as means ± SD (n = 5 or 6). *Significantly different from the control group at the same time point ($p < 0.05$).



Discussion

Most research concerning LAB-mediated enhanced immune protection has been focused on the gastrointestinal tract. In contrast, only a few studies deal with the possibility that LAB might sufficiently stimulate the common mucosal immune system as to provide increased protection for the respiratory tract (Cross 2002; Alvarez et al. 2007). There are also few studies concerning the ability of lactococci to

Fig. 6. Anti-pneumococcal antibodies. *Lactococcus lactis* NZ was orally administered at a dose of 10^8 cells for 5 days and then mice were challenged nasally with 10^6 *Streptococcus pneumoniae* cells. (A) Serum IgG, (B) serum IgA, (C) BAL IgG, and (D) BAL IgA anti-pneumococcal antibodies were studied. Results are expressed as means ± SD (n = 5 or 6). *Significantly different from the control group at the same time point ($p < 0.05$).

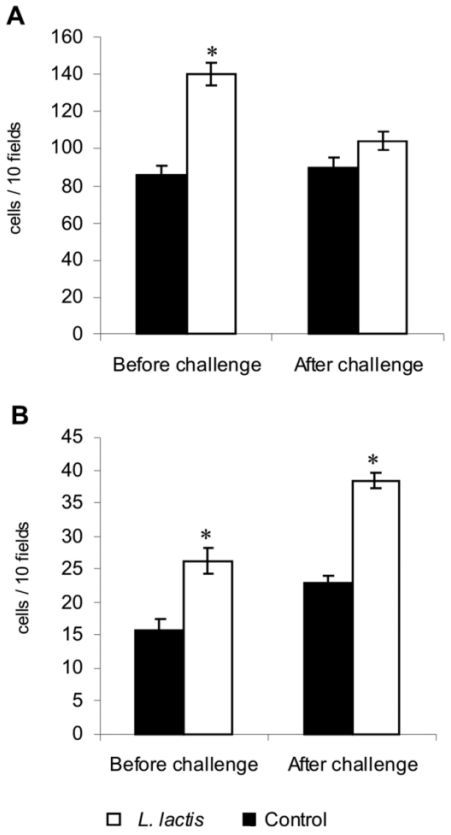


modulate the host's immune response (Kimoto et al. 2004). In the present work, we studied the immunoenhancing properties of nonrecombinant *L. lactis* NZ and demonstrated that this strain, administered by the oral route at the proper dose, is able to improve defense mechanisms against pneumococcal infection. Results showed that the administration of 10^8 cells of *L. lactis* for 5 days was the optimal treatment to improve resistance against pneumococci. In previous studies in our laboratory, we demonstrated that the oral administration of a probiotic *L. casei* strain was able to reduce susceptibility to *S. pneumoniae* infection, with only 2 days of treatment with *L. casei* being required to achieve the protective effect (Racedo et al. 2006). It seems possible that the need for 5 days of treatment with *L. lactis* to obtain the same effect would be related to the fact that lactococci are not able to colonize the intestine (Kimoto et al. 2003). Thus, a longer period of administration would be necessary to stimulate the inductive sites of the gut mucosal immune system.

The oral administration of *L. lactis* was able to increase *S. pneumoniae* clearance rates in lung and blood, improved survival of infected mice, and reduced lung injuries. The beneficial effect of *L. lactis* treatment was related to an up-regulation of the innate and specific immune responses in the respiratory tract.

The protection of the respiratory tract against pathogens relies on both innate and specific defense mechanisms located in the airways and in the alveolar space (Boyton and Openshaw 2002). Alveolar macrophages play a prominent role in lung immunity by initiating inflammatory and immune responses. In the event that the invading pathogens are too virulent or represent too large a load to be contained by alveolar macrophages alone, these cells are capable of generating mediators that recruit large numbers of neutrophils into the alveolar space. These recruited neutrophils provide auxiliary phagocytic capacities that are critical for the effective eradication of offending pathogens (Zhang et al. 2000). The recruitment and activation of phagocytes in the respiratory tract is induced by chemokines and pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, which are expressed rapidly after infection (Kyd et al. 2001). Mice treated orally with *L. lactis* had significantly in-

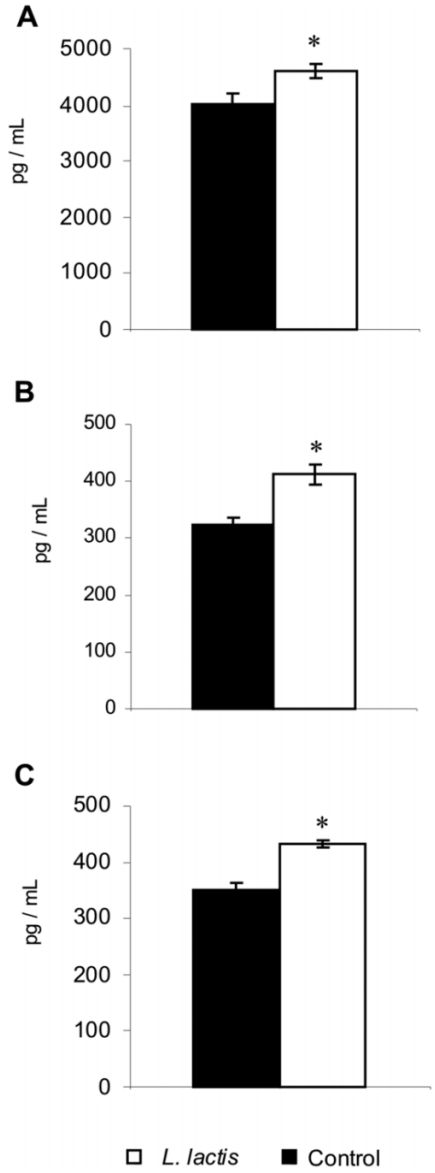
Fig. 7. IgA+ cells in mucosal tissues. *Lactococcus lactis* NZ was orally administered at a dose of 10^8 cells for 5 days and then mice were challenged nasally with 10^6 *Streptococcus pneumoniae* cells. The number of IgA+ cells in (A) intestine and (B) lungs was studied. Results are expressed as means ± SD (n = 5 or 6). *Significantly different from the control group at the same time point ($p < 0.05$).



creased amounts of BAL TNF-α than mice in the control group after challenge with *S. pneumoniae*. These higher levels of BAL TNF-α allowed an improved recruitment of neutrophils from the pulmonary vasculature into the alveolar spaces and a higher activation of BAL phagocytes, as shown by the number of BAL neutrophils, lung MPO, and the percentage of BAL NBT+ cells.

It is known that together with innate immune cells, the production of IgG and IgA antigen-specific antibodies is important to reduce colonization of the respiratory epithelium and to avoid dissemination into blood (Twigg 2005). Mice treated with *L. lactis* showed an improved respiratory humoral immune response, which was evidenced by the higher levels of BAL anti-pneumococcal IgA and IgG. The type and concentration of antibodies produced in the respiratory tract are dependent on the site of exposure. Upper airway exposure results primarily in an IgA response; however, when organisms reach the deep lung after passing through the upper airway, they induce an increased production of pathogen-specific IgG (Twigg 2005). *Lactococcus lactis* treatment improved the production of anti-pneumococcal IgG in BAL. This fact could be related to a stimulation of antigen-presenting cells in the lung that induce T cell activation and B cell clonal expansion and differentiation into IgG+ antibody-secreting plasma cells. This agrees with the

Fig. 8. Cytokines in the respiratory tract. *Lactococcus lactis* NZ was orally administered at a dose of 10^8 cells for 5 days and then mice were challenged nasally with 10^6 *Streptococcus pneumoniae* cells. The levels of (A) BAL TNF-α, (B) BAL IL-4, and (C) BAL IL-10 were studied. Post-challenge values of BAL TNF-α correspond to 12 h post-infection and values of BAL IL-4 and IL-10 correspond to 120 h post-infection. Results are expressed as means ± SD (n = 5 or 6). *Significantly different from the control group at the same time point ($p < 0.05$).



enhanced activation of alveolar macrophages in *L. lactis* treated mice and with recent findings that demonstrated in vitro that *L. lactis* NZ cells are able to stimulate the production of TNF-α by murine peritoneal macrophages and to upregulate the expression of costimulatory molecules in bone marrow derived dendritic cells (Audouy et al. 2006). In addition, *L. lactis* treatment improved the production of BAL anti-pneumococcal IgA. The airways environment, in the absence of bacterial stimuli, is characterized by a weak Th2 response to inhaled harmless antigens (Lambrecht et al. 2001). Under inflammatory conditions, cytokines in the respiratory tract change dramatically. When a Th2 response is

needed, additional sources of IL-4, IL-5, IL-6, and IL-10 contribute to stimulate B cells to proliferate and mature into polymeric IgA-producing cells and to develop specific antibodies (Corthésy 2002; Kiyono and Fukuyama 2004). In this study, IL-4 and IL-10 were significantly increased in the respiratory tract of animals that received *L .lactis*, which is in accordance with the increase in the levels of specific IgA in BAL. Moreover, *L. lactis* administered orally was able to stimulate the IgA cycle, increasing IgA+ cells in intestine and bronchus. The production of specific IgA in the respiratory tract during an infectious process is important because it prevents colonization of mucosal tissues and subsequent spreading into the systemic circulation (Twigg 2005). Additionally, specific IgA antibodies can bind antigens and minimize their entry with a consequent reduction in inflammatory reactions, which prevents potentially harmful effects on the tissue.

It has been established that one of the most important features in the design of a mucosal vaccine formulation is the ability to stimulate the innate immune system so that the appropriate adaptive immune response is triggered (Ryan et al. 2001). Taking into consideration that orally administered *L. lactis* NZ stimulates the innate and the specific immune responses in the respiratory tract and that bacterial and viral antigens have been efficiently produced in this strain (Le Loir et al. 2005), *L. lactis* NZ is an excellent candidate for the development of an effective pneumococcal oral vaccine. Studies of respiratory and systemic immune responses to *S. pneumoniae* infection after oral immunization with recombinant *L. lactis* NZ expressing pneumococcal antigens are currently underway in our laboratory.

Acknowledgements

Supported by grants from FONCyT PICT 99 No. 5-7206, CIUNT 26 D/202, CIUNT D/305, and CONICET Res. 1257/4.

References

Agüero, G., Villena, J., Racedo, S., Haro, C., and Alvarez, S. 2006. Beneficial immunomodulatory activity of *Lactobacillus casei* in malnourished mice pneumonia: effect on inflammation and coagulation. Nutrition, **22**: 810–819. doi:10.1016/j.nut.2006.03.013. PMID:16815495.

Alvarez, S., Herrero, C., Bru, E., and Perdigón, G. 2001. Effect of *Lactobacillus casei* and yogurt administration on prevention of *Pseudomonas aeruginosa* infection in young mice. J. Food Prot. **64**: 1768–1774. PMID:11726157.

Alvarez, S., Villena, J., Racedo, S., Salva, S., and Agüero, G. 2007. Malnutrition, probiotics and respiratory infections. *In* Research advances in nutrition. *Edited by* Global Research Network, India. pp. 9–23.

Audouy, S.A.L., van Roosmalen, M.L., Neef, J., Kanninga, R., Post, E., van Deemter, M., et al. 2006. *Lactococcus lactis* GEM particles displaying pneumococcal antigens induce local and systemic immune responses following intranasal immunization. Vaccine, **24**: 5434–5441. doi:10.1016/j.vaccine.2006.03.054. PMID:16757068.

Boyton, R.J., and Openshaw, P.J. 2002. Pulmonary defences to acute respiratory infection. Br. Med. Bull. **61**: 1–12. doi:10.1093/bmb/61.1.1. PMID:11997295.

Corthésy, B. 2002. Recombinant immunoglobulin A: powerful tools for fundamental and applied research. Trends Biotechnol. **20**: 65–71. doi:10.1016/S0167-7799(01)01874-1. PMID:11814596.

Corthier, G., and Renault, P. 1999. Future directions for research on biotherapeutic agents: contributions of genetic approaches on lactic acid bacteria. *In* Biotherapeutic agents and infectious diseases. *Edited by* G.W. Elmer. Humana Press Inc., Clifton, N.J. pp. 269–304.

Cross, M.L. 2002. Microbes versus microbes: immune signals generated by probiotic lactobacilli and their role in protection against microbial pathogens. FEMS Immunol. Med. Microbiol. **34**: 245–253. doi:10.1111/j.1574-695X.2002.tb00632.x. PMID:12443824.

Kimoto, H., Nomura, M., Kobayashi, M., Mizumachi, K., and Okamoto, T. 2003. Survival of lactococci during passage through mouse digestive tract. Can. J. Microbiol. **49**: 707–711. doi:10.1139/w03-092. PMID:14735220.

Kimoto, H., Mizumachi, K., Takashi, O., and Kurisaki, J. 2004. New *Lactococcus* strains with immunomodulatory activity: enhancement of Th1-type immune response. Microbiol. Immunol. **48**: 75–82. PMID:14978331.

Kiyono, H., and Fukuyama, S. 2004. NALT-versus Peyer's patch mediated mucosal immunity. Nat. Rev. Immunol. **4**: 699–710. doi:10.1038/nri1439. PMID:15343369.

Kyd, J.M., Foxwell, A.R., and Cripps, A.W. 2001. Mucosal immunity in the lung and upper airway. Vaccine, **19**: 2527–2533. doi:10.1016/S0264-410X(00)00484-9.

Lambrecht, B.N., Prins, J.B., and Hoogsteden, H.C. 2001. Lung dendritic cells and host immunity to infection. Eur. Respir. J. **18**: 692–704. PMID:11716176.

Le Loir, Y., Azevedo, V., Oliveira, S.C., Freitas, D.A., Miyoshi, A., Bermúdez-Humarán, L.G., et al. 2005. Protein secretion in *Lactococcus lactis*: an efficient way to increase the overall heterologous protein production. Microb. Cell Fact. **4**: 2–12. doi:10.1186/1475-2859-4-2. PMID:15631634.

Perdigón, G., Alvarez, S., Medina, M., Vintiñi, E., and Roux, E. 1999. Influence of the oral administration of lactic acid bacteria on IgA producing cells associated to bronchus. Int. J. Immunopathol. Pharmacol. **12**: 97–102. PMID:12783652.

Perdigón, G., Fuller, R., and Raya, R. 2001. Lactic acid bacteria and their effect on the immune system. Curr. Issues Intest. Microbiol. **2**: 27–42. PMID:11709854.

Racedo, S., Villena, J., Medina, M., Agüero, G., Rodríguez, V., and Alvarez, S. 2006. *Lactobacillus casei* administration reduces lung injuries in a *Streptococcus pneumoniae* infection. Microbes Infect. **8**: 2359–2366. doi:10.1016/j.micinf.2006.04.022. PMID:16904925.

Ryan, E.J., Daly, L.M., and Mills, K.H.G. 2001. Immunomodulators and delivery systems for vaccination by mucosal routes. Trends Biotechnol. **19**: 293–304. doi:10.1016/S0167-7799(01)01670-5. PMID:11451471.

Twigg, H.L. 2005. Humoral immune defense (antibodies): recent advances. Proc. Am. Thorac. Soc. **2**: 417–421. doi:10.1513/pats.200508-089JS. PMID:16322592.

Villena, J., Racedo, S., Agüero, G., Bru, E., Medina, M., and Alvarez, S. 2005. *Lactobacillus casei* improves resistance to pneumococcal respiratory infection in malnourished mice. J. Nutr. **135**: 1462–1469. PMID:15930453.

Villena, J., Racedo, S., Agüero, G., and Alvarez, S. 2006. Yogurt accelerates the recovery of defence mechanisms against *Streptococcus pneumoniae* in protein malnourished mice. Br. J. Nutr. **95**: 591–602. doi:10.1079/BJN20051663. PMID:16512946.

Vintiñi, E., Alvarez, S., Medina, M., de Budeguer, M.V., and Perdigón, G. 2000. Gut mucosal immunostimulation by lactic acid bacteria. Biocell, **21**: 223–232.

Zhang, P., Summer, W.R., Bagby, G.J., and Nelson, S. 2000. Innate immunity and pulmonary host defense. Immunol. Rev. **173**: 39–51. doi:10.1034/j.1600-065X.2000.917306.x. PMID:10719666.