Oral immunization with recombinant *Lactococcus lactis* confers protection against respiratory pneumococcal infection

Julio Villena, Marcela Medina, Raúl Raya, and Susana Alvarez

Abstract: In the present work, we evaluated if oral immunization with the pneumococcal protective protein A (PppA), expressed in the cell wall of *Lactococcus lactis* (*L. lactis* PppA+), was able to confer protective immunity against *Streptococcus pneumoniae*. Mice were immunized orally with *L. lactis* PppA+ for 5 consecutive days. Vaccination was performed one (nonboosted group) or 2 times with a 2 week interval between each immunization (boosted group). Oral priming with *L. lactis* PppA+ induced the production of anti-PppA IgM, IgG, and IgA antibodies in serum and in bronchoalveolar (BAL) and intestinal (IF) lavage fluids. Boosting with *L. lactis* PppA+ increased the levels of mucosal and systemic immunoglobulins. Moreover, the avidity and the opsonophagocytic activity of anti-PppA antibodies were significantly improved in the boosted group. The presence of both IgG1 and IgG2a anti-PppA antibodies in serum and BAL and the production of both interferon γ and interleukin-4 by spleen cells from immunized mice indicated that *L. lactis* PppA+ stimulated a mixture of Th1 and Th2 responses. The ability of *L. lactis* PppA+ to confer cross-protective immunity was evaluated using challenge assays with serotypes 3, 6B, 14, and 23F. Lung bacterial cell counts and hemocultures showed that immunization with *L. lactis* PppA+ improved resistance against all the serotypes assessed, including serotype 3, which was highly virulent in our experimental animal model. To our knowledge, this is the first demonstration of protection against respiratory pneumococcal infection induced by oral administration of a recombinant lactococcal vaccine.

Key words: Lactococcus lactis, recombinant PppA, oral vaccine, *Streptococcus pneumoniae*.

Résumé : Dans le travail présenté ici, nous avons déterminé si l'immunisation orale avec la protéine protectrice du pneumocoque A (PppA), exprimée dans la paroi cellulaire de *Lactococcus lactis* (*L. lactis* PppA+), pouvait conférer une immunité protectrice contre *Streptococcus pneumoniae.* Des souris ont été immunisées oralement avec *L. lactis* PppA+ pendant 5 jours consécutifs. La vaccination a été réalisée une ou 2 fois, à 2 semaines d'intervalle entre chaque immunisation le cas échéant (groupes respectivement sans rappel et avec rappel). La sensibilisation active orale avec *L. lactis* PppA+ a induit la production d'anticorps anti-PppA de type IgM, IgG et IgA dans le sérum ainsi que dans les liquides de lavage bronchoalvéolaire (BAL) et intestinal (IF). Le rappel avec *L. lactis* PppA+ a augmenté les niveaux d'immunoglobulines muqueuses et systémiques. Qui plus est, l'avidité et l'activité d'opsonisation-phagocytose des anticorps anti-PppA étaient significativement améliorées chez le groupe avec rappel. La présence d'IgG1 et d'IgG2a anti-PppA dans le sérum et le BAL, et la production d'IFN-γ et d'IL-4 par les cellules de la rate des souris immunisées ont indiqué que *L. lactis* PppA+ a stimulé une réponse mixte Th1 et Th2. La capacité de *L. lactis* PppA+ à conférer une immunité protectrice croisée a été évaluée par des tests de provocation avec les sérotypes 3, 6B, 14 et 23F. Les décomptes des cellules bactériennes pulmonaires et les hémocultures ont montré que l'immunisation avec *L. lactis* PppA+ a amélioré la résistance envers tous les sérotypes évalués, y compris le sérotype 3 hautement virulent dans notre modèle animal expérimental. À notre connaissance, il s'agit de la première démonstration d'une protection contre une infection respiratoire à pneumocoque induite par l'administration orale d'un vaccin lactocoque recombinant.

Mots-clés : Lactococcus lactis, PppA recombinante, vaccin oral, *Streptococcus pneumoniae*.

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Introduction

The oral route is the ideal means of delivering prophylactic and therapeutic vaccines, as it offers significant advantages over systemic delivery. Most notably, oral delivery is associated with simple administration and improved safety. In addition, unlike systemic immunization, oral delivery can induce mucosal immune responses. To facilitate effective immunization with orally administered peptide and protein vaccines, antigens must be protected, vaccine uptake enhanced, and the innate immune response activated (Lavelle and O'Hagan 2006). Thus, great efforts have been made in recent years to combine mucosal delivery with agents that have intrinsic adjuvant activity. In this sense, lactic acid

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bacteria (LAB) represent an interesting alternative for the development of mucosal vaccines, especially because they possess well-documented immunoenhancing properties (Cross 2002; Alvarez et al. 2007).

The majority of research concerning LAB-mediated enhanced immune protection has focused on the gastrointestinal tract, and few studies has been conducted to consider the possibility that LAB might stimulate the respiratory mucosal immune system sufficiently to provide increased protection against pathogens (Alvarez et al. 2001; Villena et al. 2005; Racedo et al. 2006; Villena 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 like the respiratory tract (Kiyono and Fukuyama 2004). This homing pathway of primed lymphoid cells from the inductive sites on the Peyer's patches to distant mucosal sites after antigen stimulation could be exploited to design LAB-derived oral vaccines that could afford protection against respiratory pathogens (Alvarez et al. 2007).

The lactic acid bacterium model *Lactococcus lactis* NZ9000 has been extensively engineered for the production of heterologous proteins using the nisin controlled gene expression (NICE) system (Le Loir et al. 2005). We have demonstrated that this strain, administered by the oral route, is able to improve innate and specific immune responses in the respiratory tract (Villena et al. 2008). In addition, we have used the NICE system as well as the signals of the lactococcal Usp45 secretion peptide and of the cell wall anchoring protein M6 from *Streptococcus pyogenes* to develop a recombinant strain of *L. lactis* able to express the pneumococcal protective protein A (PppA) on its surface after the induction with nisin (Medina et al. 2008). In the present research, the ability of orally administered recombinant *L. lactis* PppA to induce systemic- and mucosal-specific antibodies was evaluated. In addition, the protective efficacy of the oral vaccine was evaluated after respiratory challenges with different serotypes of *Streptococcus pneumoniae*.

Materials and methods

Immunization procedures

Male Swiss albine mice (6 weeks old) 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 5–6 mice for each time point. The Ethical Committee for Animal Care at CERELA approved experimental protocols.

Recombinant *L. lactis* PppA was grown in M17-glu plus erythromycin (5 μg/mL) at 30 °C until cells reached an OD_{590} of 0.6 and then it was induced with 50 ng/mL of nisin for 2 h, as described previously (Medina et al. 2008). *Lactococcus lactis* PppA was harvested by centrifugation at 3000*g* for 10 min, then washed 3 times with sterile 0.01 mol/L PBS, pH 7.2) and finally resuspended in nonfat milk (NFM) to be administered to mice. Mice were immunized orally with recombinant *L. lactis* PppA previously induced with nisin (*L. lactis* PppA+) at a dose of 10^8 cells per mouse per day for 5 consecutive days, which is the optimal dose with adjuvant properties (Villena et al. 2008). Each mouse received 250 μL of the vaccine suspension, which was instilled into the oral cavity. This immunization was performed one (nonboosted group) or 2 times with a 2 week interval between each immunization and the next (boosted group). Mice receiving the recombinant strain without induction (*L. lactis* PppA–) or sterile NFM were used as controls.

ELISA for anti-PppA antibodies

Serum, bronchoalveolar lavage fluid (BAL), and intestinal lavage fluid (IF) antibodies against PppA protein were determined by ELISA modified from Green et al. (2005). Briefly, plates were coated with rPppA (100 μL of a 5 μg/mL stock in sodium carbonate – bicarbonate buffer, pH 9.6, per well). Nonspecific protein binding sites were blocked with PBS containing 5% nonfat milk. Samples were diluted (serum 1:200, BAL 1:20, IF 1:100) with PBS containing 0.05% (*v*/*v*) Tween 20 (PBS-T). Peroxidase-conjugated goat anti-mouse IgM, IgA, IgG, IgG1, or IgG2a (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% H_2O_2), and the reaction was stopped by addition of 1 mol/L H_2SO_4. Readings were carried out at 493 nm (VERSAmax Tunable microplate reader) and samples were considered negative for the presence of specific antibodies when the OD_{493} was <0.1.

Antibody avidity assay

For the measurement of IgM, IgG, and IgA antibody avidity the basic ELISA method was used. After the samples were incubated, plates were washed and incubated for 15 min at room temperature with 0.5 mol/L sodium thiocyanate (NaSCN) to induce the dissociation of the antigen–antibody complexes. Plates were washed and the remaining incubations were performed as described above without modifications. The avidity index for each sample was determined as follows: the antibody concentration in the presence of the chaotropic agent NaSCN was divided by the antibody concentration in the absence of NaSCN and multiplied by 100 (Anttila et al. 1998).

Opsonophagocytosis assay

Opsonophagocytic activity of BAL, IF, and serum antibodies was determined by measuring the killing of live pneumococci by peritoneal macrophages in the presence of antibodies and complement. A modification of the method described by Romero-Steiner et al. (1997) was used. Isolation of peritoneal macrophages was performed as follows: male Swiss albine mice (6 weeks old) were anesthetized and sacrificed by cervical dislocation. The peritoneal cavity was flushed with 5 mL of RPMI 1640 (Sigma). The macrophage suspension was washed twice with the same medium and it was adjusted to a concentration of 10^6 cells/mL. Test samples (serum, BAL, and IF) were also diluted in RPMI. Ten microlitres of pneumococcal solution containing 1000 CFU of *S. pneumoniae* T14 and 20 μL of test sample

was placed in each well of a 96-well microtiter plate. After 30 min of incubation at room temperature, 40 μL of peritoneal macrophages suspension (4×10^5 cells/well) and 10 μL of mouse complement (Sigma) were added to each well. The mixture was incubated for 1 h at 37 °C. Five microlitres of the reaction mixture was plated on Todd–Hewitt agar – yeast extract (Oxoid). The plates were incubated in a candle jar at 37 °C for 18 h, and then surviving bacterial colonies on the plates were counted. The opsonization titer of each sample was defined as the final dilution of a serum, IF, or BAL sample that results in half as many colonies as are seen with the control well containing all the reactants except for the serum, IF, or BAL, respectively.

Spleen cell cytokine production

To determine the cytokine profile induced by *L. lactis* PppA vaccine, spleens were removed 2 weeks after the last immunization and single cell suspensions were prepared. Aliquots of 4×10^5 cells were cultured in RPMI 164 supplemented with 10% fetal calf serum, 2 mmol/L L-glutanine, 50 mmol/L 2-mercaptoethanol, 100 U/mL of penicillin, and 100 μg/mL of streptomycin and in the presence of 10 μg/mL of recombinant PppA (rPppA) from *Escherichia coli* (Medina et al. 2008). Levels of interferon gamma (INF-γ) and interleukin-4 (IL-4) were measured in the supernatant of stimulated cells. 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.

Experimental infection

Freshly grown colonies of capsulated *S. pneumoniae* T14 were suspended in Todd–Hewitt broth (THB) (Oxoid) and incubated 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 3 times with sterile PBS. Challenge with *S. pneumoniae* T14 was performed 14 days after the end of each *L. lactis* PppA+ immunization. Mice were challenged nasally with the pathogen by dripping 25 μL of an inoculum containing 10^6 cells into each nostril. Infection was monitored until day 15 postinfection according to previous studies (Racedo et al. 2006). In addition, cross-protective immunity was evaluated in immunized mice after challenge with different serotypes of *S. pneumoniae*, which were kindly provided by Dr. M. Regueira from the Laboratory of Clinical Bacteriology, National Institute of Infectious Diseases, Argentina. Freshly grown colonies of *S. pneumoniae* strains AV3 (serotype 3), AV6 (serotype 6B), AV14 (serotype 14), and AV23 (serotype 23F) were suspended in THB and incubated at 37 °C until the log phase was reached (Medina et al. 2008). Challenge with the different pneumococcal strains was performed 14 days after the end of each *L. lactis* PppA+ immunization. Mice were challenged nasally with 10^6 pathogen cells as described above. The development of pneumococcal disease was evaluated 48 h after challenge.

Bacterial cell counts in lung and blood

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/g 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.

Statistical analysis

Experiments were performed in triplicate and results were expressed as the mean ± standard deviation (SD). 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 $P < 0.05$.

Results

Mucosal and systemic immune response to *L. lactis* PppA+ vaccination

The ability of *L. lactis* PppA+ to elicit specific anti-PppA mucosal and systemic antibodies after oral immunization was studied. First, we evaluated the levels of the anti-PppA antibodies in mice that received only a priming with *L. lactis* PppA+ (recombinant strain previously induced with nisin). Samples analyzed 14 days after the last day of administration showed that oral immunization with *L. lactis* PppA+ induced production of specific anti-PppA IgM, IgG, and IgA in BAL (Fig. 1A). The production of specific antibodies was also observed in serum (Fig. 1B). No detectable values of anti-PppA-specific antibodies were observed in either serum or BAL collected from controls or animals that received *L. lactis* PppA (recombinant strain without induction). We then assessed if the levels of respiratory- and systemic-specific antibodies were enhanced with a boosting. Specific IgG and IgA anti-PppA antibodies in serum and BAL were significantly improved with the boosting while the levels of specific IgM decreased (Figs. 1A and 1B). Considering that immunizations were performed by the oral route, we determined the levels of specific antibodies in intestine with the aim of evaluating the effectiveness of *L. lactis* PppA+ to stimulate the inductive sites of the gut mucosal immune system. Primary immunization with *L. lactis* PppA+ induced production of anti-PppA antibodies in intestine, mainly IgA, which increased significantly after boosting (Fig. 1C).

Biologic activity of anti-PppA antibodies

Antibody avidity of BAL, IF ,and serum antibodies is shown in Fig. 2. The primary immunization with *L. lactis* PppA+ induced the production of anti-PppA IgM, IgG, and IgA antibodies in BAL with avidity lower than 50% (Fig. 2A). The boosting with *L. lactis* PppA+ increased avidity of BAL IgG and IgA but did not induce changes in IgM antibodies. Mice in the boosted *L. lactis* PppA+ group also showed serum antibodies with higher avidity than that of mice in the nonboosted *L. lactis* PppA+ group (Fig. 2B). In addition, avidity of IF antibodies was studied. Mice in the nonboosted *L. lactis* PppA+ group showed percentages of

Fig. 1. Levels of specific antibodies in bronchoalveolar fluid (A), serum (B), and intestinal fluid (C) samples. Mice were orally immunized, one (nonboosted group) or 2 times (boosted group), with the pneumococcal protective protein A expressed in *Lactococcus lactis* (*L. lactis* PppA+). No detectable values of anti-PppA-specific antibodies were observed in samples collected from animals that received *L. lactis* PppA– (recombinant strain without induction) or nonfat milk (controls). Results are expressed as mean ± SD (n = 5–6). *, Data are significantly different from that of the nonboosted group at the same time point ($P < 0.05$).

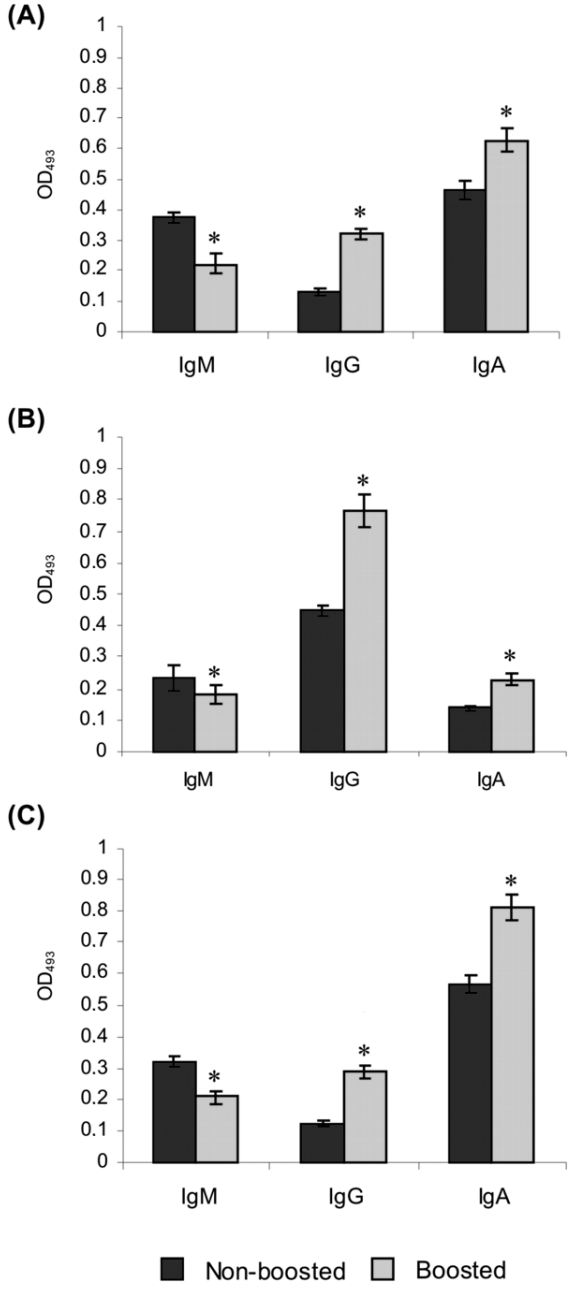


Fig. 2. Avidity of specific antibodies in bronchoalveolar fluid (A), serum (B), and intestinal fluid (C) samples. Mice were orally immunized, one (nonboosted group) or 2 times (boosted group) with the pneumococcal protective protein A expressed in *Lactococcus lactis*. The avidity index for each sample was determined by dividing the OD_{493} in the presence of the chaotropic agent NaSCN by the OD_{493} in the absence of NaSCN and multiplying by 100. Results are expressed as mean ± SD (n = 5–6). *, Data are significantly different from that of the nonboosted group at the same time point ($P < 0.05$).

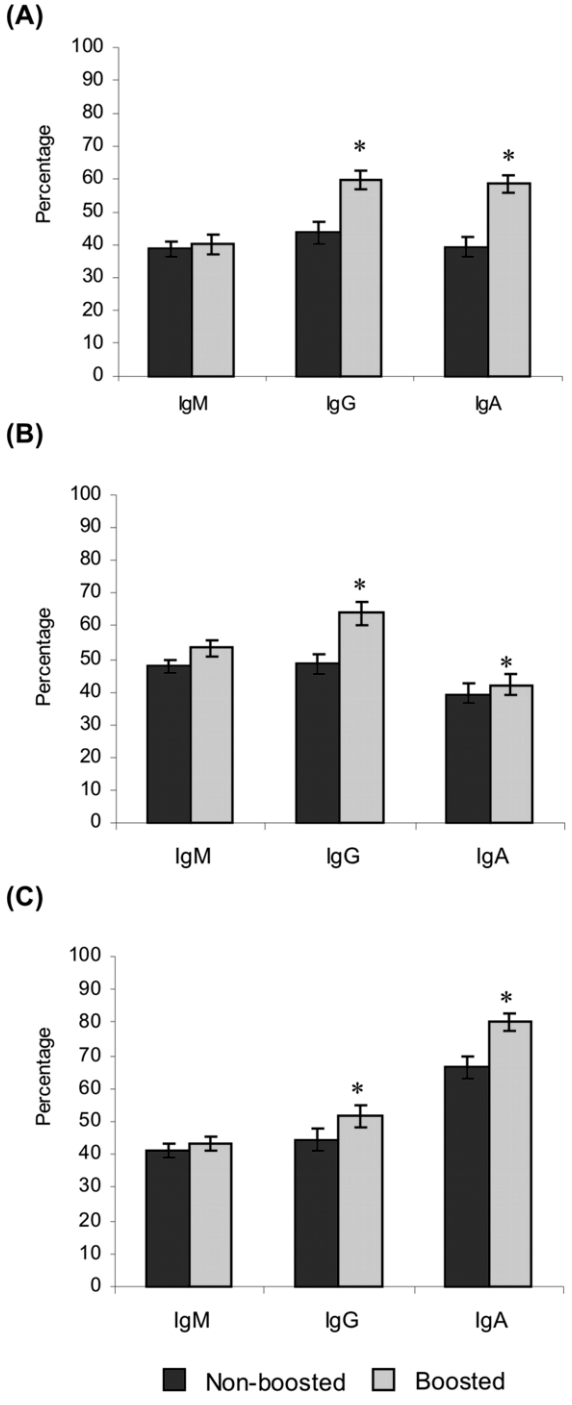


Fig. 3. Opsonophagocytic activity of antibodies in bronchoalveolar fluid, serum, and intestinal fluid samples. Mice were orally immunized, one (nonboosted group) or 2 times (boosted group), with the pneumococcal protective protein A expressed in *Lactococcus lactis*. Results are expressed as mean ± SD (n = 5–6). *, Data are significantly different from that of the nonboosted group at the same time point ($P < 0.05$).

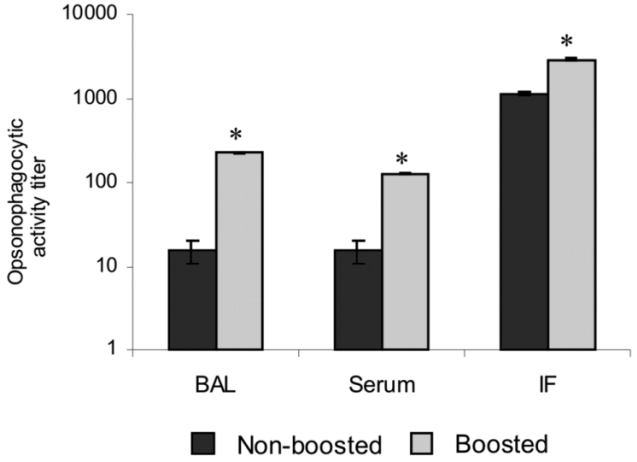


Fig. 4. Levels of specific antibodies in bronchoalveolar fluid (A) and serum (B) samples. Mice were orally immunized, one (nonboosted group) or 2 times (boosted group), with the pneumococcal protective protein A expressed in *Lactococcus lactis*. Results are expressed as mean ± SD (n = 5–6). *, Data are significantly different from that of the nonboosted group at the same time point ($P < 0.05$).

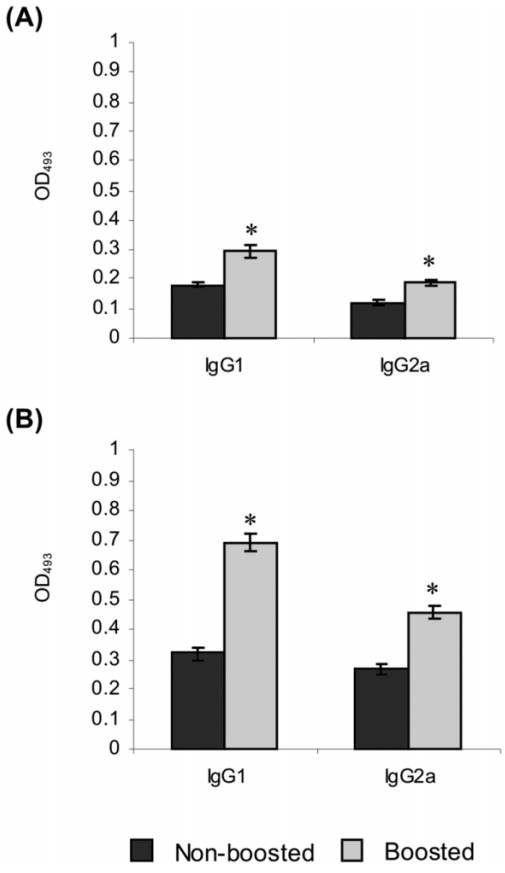
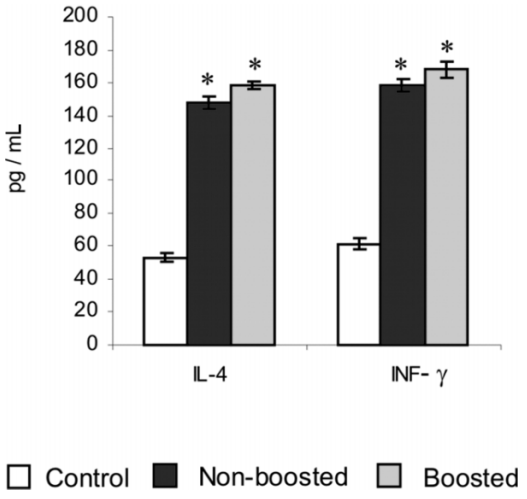


Fig. 5. Spleen cell cytokine production. Levels of interleukin-4 (IL-4) and interferon gamma (INF-γ) were measured in the supernatant of stimulant cells. Mice were orally immunized, one (nonboosted group) or 2 times (boosted group), with the pneumococcal protective protein A expressed in *Lactococcus lactis*. Results are expressed as the mean ± SD (n = 5–6). *, Data are significantly different from that of the nonboosted group at the same time point ($P < 0.05$).

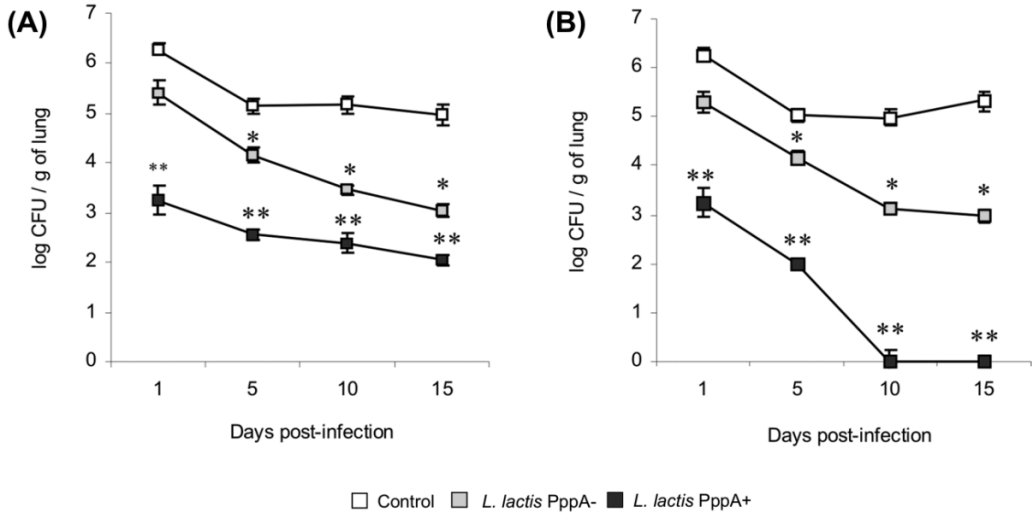


avidity below the 50% for IgM and IgG antibodies, although IgA anti-PppA reached values close to 70% (Fig. 2C). The boosting increased avidity of IgG and IgA in IF, the latter reaching an average of 80%, which was the highest value observed in our study. The study of the opsonophagocytic activity of anti-PppA antibodies showed that mice in the nonboosted *L. lactis* PppA+ group had low titers in serum and BAL (Fig. 3). The animals that received a boosting showed significantly higher titers in serum, BAL, and IF (Fig. 3).

Th immune response induced by *L. lactis* PppA+ vaccination

The subclasses of IgG anti-PppA antibodies were studied as surrogate markers to determine the T-helper (Th) response. Production of both IgG1 (Th2) and IgG2a (Th1) anti-PppA antibodies was detected in serum and BAL of mice immunized with *L. lactis* PppA+ (Fig. 4). Serum and BAL IgG1/IgG2a ratios were 1.51 and 1.46, respectively, thus, a respiratory and systemic Th1- and Th2-type mixed response was observed for the oral immunization with PppA antigen, expressed in *L. lactis*. The levels of anti-PppA IgG1 and IgG2a were enhanced in the boosted group, but the ratio of BAL and serum IgG1/IgG2a, which were 1.55 and 1.51, respectively, was similar to that of the nonboosted group. In addition, the levels of INF-γ and IL-4 were determined in the supernatant of stimulant spleen cells to confirm that the administration of *L. lactis* PppA+ induced a mixed Th1/Th2 response. The mice in the nonboosted and boosted *L. lactis* PppA+ groups showed a significant increase in the levels of IL-4 and INF-γ (Fig. 5), although no differences were observed between the 2 immunization schemes.

Fig. 6. Kinetics of pneumococcal clearance from lungs. Mice were orally immunized, one (nonboosted group, A) or 2 times (boosted group, B), with the pneumococcal protective protein A expressed in *Lactococcus lactis* (*L. lactis* PppA+) and then challenged nasally with 10^6 *Streptococcus pneumoniae* cells. Mice receiving the recombinant strain without induction (*L. lactis* PppA–) or sterile nonfat milk (control) were used as controls. The lower limit of bacterial detection was 1.5 log CFU/g of lung. Results are expressed as mean ± SD (n = 5–6). *, Data are significantly different from that of the control group at the same time point ($P < 0.05$). **, Data are significantly different from that of the control and *L. lactis* PppA– groups at the same time point ($P < 0.05$).



Protective capacity of *L. lactis* PppA+ vaccination

To determine whether the induction of specific antibodies by oral immunization with *L. lactis* PppA+ correlated with an increased resistance to pneumococcal infection, we next determined the ability of orally vaccinated animals to clear *S. pneumoniae* T14 from their lungs and blood. Mice immunized with *L. lactis* PppA+ showed significantly lower lung bacterial cell counts than those in the control group (Fig. 6A). In addition, *L. lactis* PppA+ mice showed negative hemocultures since day 5 postinfection, while the control group revealed pneumococci in blood throughout the assesed period (data not shown). We also observed that mice orally immunized with *L. lactis* PppA– were more resistant to pneumococcal infection than control mice. However, compared with mice in the *L. lactis* PppA+ group, those immunized with *L. lactis* PppA– were less resistant to the infection with pneumococci. On the other hand, mice that received *L. lactis* PppA+ twice (boosted group) showed a higher bacterial clearance than mice immunized only once (nonboosted group). Boosted animals showed negative lung bacterial counts from day 10 postinfection onwards (Fig. 6B), while hemocultures were negative throughout the assessed period (data not shown). In addition, cross-protective immunity was evaluated in immunized mice using challenge–infection experiments with different serotypes of *S. pneumoniae*. The 4 serotypes studied were capable of infecting mice, but the virulence of each strain was different. *Streptococcus pneumoniae* AV3 was the most virulent, followed by strains AV14 and AV6, while strain AV23 was less virulent (Table 1). The mice in the nonboosted *L. lactis* PppA+ group showed significantly lower lung bacterial cell counts than their respective control groups (Table 1). Priming with *L. lactis* PppA+ was effective to avoid the spread of the strains AV14 and AV23 to the bloodstream. On the other hand, boosted mice showed lower pathogen counts in lung than those found in the nonboosted group. Moreover, boosted *L. lactis* PppA+ group was able to prevent the dissemination into blood of strains AV6, AV14, and AV23 (Table 1). None of the immunization schemes studied avoided the bacteremia caused by the strain AV3; however, *L. lactis* PppA+ group was able to eliminate strain AV3 from blood on day 5 postinfection (data not shown). It was further observed that the administration of *L. lactis* PppA– decreased pulmonary infection caused by the 4 serotypes, although the level of protection achieved was lower than the *L. lactis* PppA+ treatment (Table 1).

Discussion

In the present work, we evaluated if oral immunization with a pneumococcal antigen expressed in *L. lactis* was able to confer protective immunity against *S. pneumoniae*. We selected the pneumococcal protective protein A, which is a small surface protein of *S. pneumoniae* that has been described recently (Green et al. 2005). Although the physiological function and role in pathogenesis of PppA in pneumococcal infection are unknown, it has been demonstrated that antibodies against this antigen can reduce *S. pneumoniae* nasal colonization (Green et al. 2005).

The most effective immunity against respiratory infections involves an IgG and IgA response; consequently, the presence and levels of pathogen-specific neutralizing antibody activity in IgA and IgG are considered a vital attribute of any vaccine designed to prevent infection acquired via the respiratory tract (Ogra 2003). Moreover, it has been demonstrated that the opsonophagocytic activity and avidity of these antibodies are more critical determinants of protection than their concentration (Bernatoniene and Finn 2005). The production of serum and BAL anti-PppA IgM, IgG, and IgA antibodies was observed after primary immunization with *L. lactis* PppA+, and the mice in this group showed increased resistance to *S. pneumoniae* T14 infection compared

Table 1. Lung bacterial cell counts and hemocultures after nasal challenge with different pneumococcal serotypes.

	Serotype 3		Serotype 6B		Serotype 14		Serotype 23F	
Group	Lung (CFU/g)	Blood (CFU/mL)	Lung (CFU/g)	Blood (CFU/mL)	Lung (CFU/g)	Blood (CFU/mL)	Lung (CFU/g)	Blood (CFU/mL)
Nonboosted								
L. lactis PppA+	4.8±0.3*	3.9±0.3*	4.3±0.2*	3.1±0.5*	3.9±0.5*	<1.5*	2.3±0.3*	<1.5*
L. lactis PppA–	5.9±0.2*	4.6±0.4	5.2±0.4*	3.3±0.5*	4.8±0.4*	3.2±0.5*	3.5±0.2*	2.7±0.6*
Control	6.9±0.3	4.8±0.2	6.3±0.3	4.0±0.2	6.4±0.5	4.1±0.7*	4.4±0.4	3.8±0.5
Boosted								
L. lactis PppA+	3.5±0.4*,†	3.3±0.7*,†	3.0±0.5*,†	<1.5*,†	2.1±0.5*,†	<1.5*	<1.5*,†	<1.5*
L. lactis PppA–	5.5±0.4*	4.5±0.3	4.9±0.2*	3.5±0.4*	4.3±0.3*	3.1±0.7*	3.3±0.5*	<1.5*,†
Control	6.8±0.6	4.9±0.5	6.2±0.3	4.2±0.5	6.1±0.5	4.3±0.2*	4.3±0.4	3.9±0.3*

Note: Mice were orally immunized, one (nonboosted group) or 2 times (boosted group), with the pneumococcal protective protein A expressed in *Lactococcus lactis* (*L. lactis* PppA+) and then challenged nasally with 10^6 *Streptococcus pneumoniae* cells. Mice receiving the recombinant strain without induction (*L. lactis* PppA–) or sterile nonfat milk were used as controls. The lower limits of bacterial detection were 1.5 log CFU/g of lung and 1.5 log CFU/mL of blood. Samples were taken on day 2 postinfection and results are expressed as mean ± SD (n = 5–6).

*Value is significantly different from that of the respective control groups ($P < 0.05$).

†Value is significantly different from that of the nonboosted group ($P < 0.01$).

with the mice receiving *L. lactis* PppA or controls. However, this immunization protocol did not induce a complete clearance of lung and was unable to avoid dissemination of pneumococci into blood. When mice received a boosting of *L. lactis* PppA+ 2 weeks later, the levels of anti-PppA IgG and IgA antibodies in serum and BAL increased significantly, while the levels of IgM decreased, which would indicate a switch of IgM+ cells to IgA+ and IgG+ cells. Moreover, the avidity and the opsonophagocytic activity of serum and BAL anti-PppA antibodies were significantly improved with the boosting. The *L. lactis* PppA+ boosted group was able to eliminate *S. pneumoniae* T14 from lungs and avoided septicemia. These results shows that when *L. lactis* PppA+ is used as an oral vaccine, it is necessary a boosting to afford full protection against serotype 14.

It is known that Th1 cells secrete IL-2, INF-γ, and TNF-α and are associated with switching to IgG2a, while Th2 cells secrete IL-4, IL-5, and IL-10 and promote switching to IgG1 and IgA (Spellberg and Edwards 2001). Thus, the subclasses of serum and BAL anti-PppA IgG antibodies and the production of IL-4 and INF-γ by spleen cells were determined to study the Th response induced by *L. lactis* PppA+ vaccination. The presence of both IgG1 and IgG2a anti-PppA antibodies and the increase of both INF-γ and IL-4 in immunized mice indicated that *L. lactis* PppA+ stimulated a mixture of Th1 and Th2 response. Probably, this kind of Th response contributed to the protective effect of *L. lactis* PppA+ because,in the development of pneumococcal vaccines the IgG isotype switching to a mixed or IgG1 dominant type along with a mucosal IgA response is preferred to prevent colonization of *S. pneumoniae* in the respiratory tract (McGhee and Kiyono 1993; Bruyn et al. 1992; Wortham et al. 1998).

Since cross-protection is an essential characteristic of a widely successful pneumococcal vaccine, a variety of pneumococcal strains need to be tested to prove the effectiveness of the vaccine construct. Thus, the ability of oral immunization with *L. lactis* PppA+ to confer cross-protective immunity was evaluated with serotypes 3, 6B, 14, and 23F. These serotypes were selected according to epidemiological studies that determined that serotype 14 is the prevailing one in our country (Mollerach et al. 2004) and by also taking into account that serotypes 3, 6B, 9, 14, 18, 19, and 23F are the ones most often associated with invasive disease (Hausdorff et al. 2005). The results showed that immunization with *L. lactis* PppA+ improved resistance against all the serotypes assessed, including serotype 3, which is highly virulent in our experimental animal model. This effect was expected, since Green et al. (2005) showed that the PppA protein is antigenically conserved and exhibits a high level of sequence conservation in serotypes 3, 5, 9, 14, 19, and 23.

Orally administered vaccines have the distinct advantage in that they can potentially reach the highly concentrated gut-associated lymphoid tissue (GALT) and can induce both serum and secretory immune response (McGhee et al. 1992). However, oral immunization has been limited because of inefficient antigen uptake, tolerance induction, and proteolytic degradation of antigens before they reach the immune cells. The efficient generation of a protective immune response after the administration of *L. lactis* PppA+ could be due to the fact that this experimental vaccine overcame the above difficulties. PppA expressed in *L. lactis* may resist digestion by gastric acid and enzymes and may be taken up by M cells and dendritic cells, thus inducing an immune response in the common mucosal immune system. The surface location of PppA in the *L. lactis* vaccine strain assayed may contribute to the augmented immune response by facilitating the adequate exposure of PppA to antigen-presenting cells for processing. In addition, our previous results obtained with nonrecombinant *L. lactis* NZ9000, which showed that the administration of 10^8 cells of *L. lactis* for 5 days was the optimal treatment to improve mucosal immunity (Villena et al. 2008), allowed us to use an *L. lactis* PppA+ dose able to avoid mucosal tolerance. The efficient stimulation of the gut mucosal immune system by *L. lactis* PppA+ was evidenced by the production of specific anti-PppA IgA antibodies in the intestine. The induction of antibodies with high avidity and opsonophagocytic activity in the IF by *L. lactis* PppA+ vaccination, therefore, indicates a potential for our immunization scheme to elicit protective immunity against relevant pathogens in the gut.

This is, to our knowledge, the first demonstration of pro-

tection against respiratory pneumococcal infection induced by oral administration of a recombinant lactococcal vaccine. Other authors have reported the expression of pneumococcal antigens in recombinant LAB that have been used to improve resistance against *S. pneumoniae* (Audouy et al. 2006; Sarno Oliveira et al. 2006; Hanniffy et al. 2007). In these works, nasal immunizations were used to evaluate the efficacy of the experimental vaccines, since it has been demonstrated that the nasal administration of antigens is an efficient route to elicit protective immunity in both the mucosal and the systemic immune compartments (Kiyono and Fukuyama 2004). Further comparison of our and those strategies is difficult because of the differences in (*i*) the antigens used, (*ii*) the number of bacterial cells delivered, (*iii*) the antigen concentration, (*iv*) the animal models, and (*v*) the pneumococcal serotypes used to evaluate protective immunity. Thus, immunization by the nasal route with *L. lactis* PppA+ using a similar scheme has to be evaluated to compare the efficacy of the vaccine administered by different mucosal routes. In addition, whether oral immunization with *L. lactis* PppA+ improves resistance to *S. pneumoniae* infection in animal models that resemble high-risk groups for serious pneumococcal diseases, which includes infants, elderly, and immunocompromised hosts, has to be determined in future studies.

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