



## Original Article

## Resistance of Young Mice to Pneumococcal Infection can be Improved by Oral Vaccination with Recombinant *Lactococcus lactis*

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**BACKGROUND/PURPOSE:** Oral immunization with *Lactococcus lactis* PppA (LPA+), a recombinant strain that is able to express the pneumococcal protective protein A, can improve the resistance to respiratory challenge with *Streptococcus pneumoniae* in adult mice. In this study, we investigated whether oral immunization protocols using LPA+ are able to protect young mice against pneumococcal respiratory infection.

**METHODS:** Young mice (aged, 3 weeks) were immunized orally with LPA+ for 5 consecutive days. Vaccination was performed once (non-boosted group), or twice with a 2-week interval between each immunization (boosted group). At the end of treatment, the specific immune responses and the resistance to pneumococcal infection were studied.

**RESULTS:** We found that the oral immunization with LPA+ was able to induce the production of specific antibodies in the respiratory and intestinal tracts as well as systemically. Analysis of IgG subtypes showed that LPA+ immunization stimulated a mixed Th1 and Th2 response. To assess whether the production of mucosal and systemic antibodies was able to afford protection against respiratory pneumococcal infection, challenge experiments with the pathogenic serotypes 3, 6B, 14, and 23F were carried out. Vaccination with LPA+ was able to increase resistance to infection with the four serotypes of *S. pneumoniae*, although the protective capacity of the experimental vaccine was different for each of them. Immunization decreased colonization in the lung, prevented bacteremia of serotypes 6B, 14, and 23F, and decreased colony counts of serotype 3.

**CONCLUSION:** We have shown that the oral immunization of young mice with LPA+ effectively induces the production of specific antibodies against the antigen PppA, both in mucosae and at the systemic level.

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The antibodies produced may play an important role in the protection against pneumococcal disease, since the young mice immunized with the experimental vaccine showed an increased resistance to infection with different serotypes of the pathogen.

**KEYWORDS:** *Lactococcus lactis*, oral vaccine, recombinant PppA, *Streptococcus pneumoniae*, young mice

## Introduction

*Streptococcus pneumoniae* is a major cause of morbidity and mortality worldwide. It asymptomatically colonizes the upper respiratory tract in approximately 50% of young children under the age of 2 years.<sup>1,2</sup> In addition, this pathogen is a leading cause of bacterial meningitis, sepsis, pneumonia, and otitis media in young children.<sup>3,4</sup> This problem is exacerbated by the increasing emergence of multiple-antibiotic-resistant strains, an important worldwide issue since infants are colonized very early by resistant strains, especially in developing countries.<sup>2</sup> This fact has stimulated renewed interest in the prevention of pneumococcal infections using vaccines. Three avenues of pneumococcal vaccination have been explored: (1) polysaccharide vaccines; (2) protein-polysaccharide conjugate vaccines; and (3) recently, new vaccine strategies focused on the use of pneumococcal surface-associated proteins. This third strategy is the most desirable because immunizations with these antigens would elicit protection in high risk groups like children; and this vaccine would be serotype independent and cheap to produce.<sup>1</sup> However, mucosal immunization has been limited because of inefficient antigen uptake, tolerance induction, and proteolytic degradation of antigens before they reach the immune cells. 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 bacteria represent an interesting alternative for the development of mucosal vaccines, especially because they possess well documented immuno-enhancing properties.<sup>5-9</sup>

The lactic acid bacterium model, *Lactococcus lactis* NZ9000, has been extensively engineered for the production of heterologous proteins using the nisin-controlled gene expression system.<sup>10</sup> We demonstrated that this strain, administered by the oral route, is able to improve the innate and specific immune responses in the respiratory tract.<sup>11</sup> In addition, we used the nisin-controlled gene

expression 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 induction with nisin.<sup>12</sup> We showed that oral immunization with the recombinant *L. lactis* PppA strain was able to induce the production of both systemic and mucosal (intestinal and respiratory) antibodies in adult immunocompetent mice.<sup>13</sup> Moreover, oral vaccination with *L. lactis* PppA strain improved the resistance of adult mice to respiratory challenge with different pneumococcal serotypes.<sup>13</sup>

Infectious disease is a major cause of human infant mortality. There are over 2.2 million annual deaths in children between the ages of 1 and 6 months. The infectious agents responsible for these deaths may produce acute respiratory infections (e.g. respiratory syncytial virus, *Bordetella pertussis*, *Haemophilus influenzae* B, *S. pneumoniae*) or diarrheal disease (e.g. rotaviruses, *Salmonella* spp., *Shigella* spp.).<sup>14,15</sup> Consequently, one of the major challenges in vaccinology is the development of products that are able to induce protective immunity in the early life period. Most experimental vaccines designed to prevent pneumococcal infections have been studied in infection models with adult immunocompetent mice with a view to their future application to high risk populations (children, the elderly and immunocompromised individuals). Thus the objective of this work was to find out if the oral immunization protocols with *L. lactis* PppA that are effective in inducing protective immunity in adult mice are able to protect young mice against pneumococcal respiratory infection.

## Methods

### Immunization procedures

Male Swiss albino mice (aged, 3 weeks) were obtained from the closed colony at Centro de Referencia para

Lactobacilos. 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 Centro de Referencia para Lactobacilos approved all experimental procedures.

Recombinant *L. lactis* PppA was grown in M17-glu plus erythromycin (5 µg/mL) at 30°C until cells reached an OD<sub>590</sub> of 0.6, and then induced with 50 ng/mL of nisin for 2 hours as previously described.<sup>12</sup> *L. lactis* PppA was harvested by centrifugation at 3,000g for 10 minutes, then washed three times with sterile 0.01 M PBS, pH 7.2, and finally resuspended in non-fat milk to be administered to the mice. Mice were immunized orally with recombinant *L. lactis* PppA previously induced with nisin (*L. lactis* PppA+) at a dose of 10<sup>8</sup> cells/mouse/day, for 5 consecutive days, which is the optimal dose with adjuvant properties.<sup>13</sup> Each mouse received 250 µL of the vaccine suspension orally. This immunization was performed once (non-boosted group), or twice with a 2-week interval between each immunization (boosted group). Mice receiving either the recombinant strain without induction (*L. lactis* PppA–), or sterile non-fat milk, were used as controls.

#### ***Immunofluorescence test for IgA+ cells in lung and intestine***

To determine the number of IgA+ cells in the lung and intestine, immunofluorescence staining were performed on histological sections. Mice were sacrificed on Day 0 and at the end of each immunization treatment, and organs were aseptically removed and processed following Sainte-Marie's technique.<sup>11,16</sup> The slices were incubated with α-chain anti-mouse monoclonal antibody conjugated with fluorescein isothiocyanate (Sigma, St. Louis, MO, USA). Cells were counted with a fluorescent microscope using at a magnification of 100×. The results were expressed as the number of positive fluorescent cells per 10 fields, representing the mean of six histological slices for each animal (five mice per group for each time point).

#### ***Enzyme-linked immunosorbent assay for anti-PppA antibodies***

Serum, bronchoalveolar lavage fluid (BAL), and intestinal lavage fluid (IF) antibodies against PppA protein were

determined by an enzyme-linked immunosorbent assay method modified from Green et al<sup>17</sup> as previously described.<sup>12</sup> Briefly, plates were coated with recombinant PppA protein (100 µL/well of a 5 µg/mL stock in sodium carbonate-bicarbonate buffer, pH 9.6). Non-specific binding were blocked with PBS containing 5% non-fat milk. Samples were diluted (serum 1:200; BAL 1:20; IF 1:100) with PBS-Tween 20 [PBS-T; 0.05% (v/v)]. Peroxidase-conjugated goat anti-mouse IgM, IgA, IgG, IgG1 or IgG2a (Fc specific; Sigma) were diluted (1:500) in PBS-T. Antibodies were revealed with a substrate solution (o-phenylenediamine; Sigma) in citrate-phosphate buffer, pH 5.0 (containing 0.05% hydrogen peroxide) and the reaction was stopped by addition of 1M sulfuric acid. Readings were measured at 493 nm (VERSAmax™ Tunable microplate reader; Molecular Devices, Sunnyvale, CA, USA) and samples were considered negative for the presence of specific antibodies when OD<sub>493</sub> < 0.1.

#### ***Antibody avidity assay***

For the measurement of IgM, IgA, and IgG antibody avidity, the Enzyme-linked immunosorbent assay method was used. After samples were incubated, plates were washed and incubated for 15 minutes at room temperature with 0.5 M 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 modification. 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.<sup>18</sup>

#### ***Opsonophagocytosis assay***

Opsonophagocytic activity of BAL, IF, and serum antibodies were determined by measuring the killing of live pneumococci by peritoneal macrophages in the presence of antibodies and complement.<sup>13</sup> Isolation of peritoneal macrophages was performed as follows: mice (aged 6 weeks) were anesthetized and sacrificed by cervical dislocation. The peritoneal cavity was flushed with 5 mL of RPMI 1640 medium (Sigma). The macrophage suspension was washed twice and adjusted to a concentration of 10<sup>6</sup> cells/mL. Test samples (serum, BAL, and IF) were also diluted in RPMI 1640 medium. Pneumococcal solution

(10 µL) containing 1,000 CFU of *S. pneumoniae* T14, and 20 µL of test sample were placed in each well of a 96-well microtiter plate. After incubating for 30 minutes at room temperature, 40 µL of the peritoneal macrophage suspension ( $4 \times 10^5$  cells/well) and 10 µL of mouse complement (Sigma) were added to each well. The mixture was incubated for 1 hour at 37°C. A total of 5 µL of the reaction mixture was plated onto Todd-Hewitt agar-yeast extract (Oxoid, Basingstoke, United Kingdom). The plates were incubated in a candle jar at 37°C for 18 hours, and then the surviving bacterial colonies on the plates were counted. The opsonization titer of each sample was defined as the final dilution of the serum, IF, or BAL sample that resulted in 50% of the colonies seen with the control well containing all the reactants except for the serum, IF or BAL respectively.

### Experimental infection

Cross protective immunity was evaluated in immunized mice after challenge with different serotypes of *S. pneumoniae*, which were kindly provided by Dr M. Regueira of 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 Todd-Hewitt broth and incubated at 37°C until the log phase of growth.<sup>12,16,19</sup> Challenge with different pneumococcal strains 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. The development of pneumococcal disease was evaluated 48 hours 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 hours at 37°C. *S. pneumoniae* colonies were counted and the results were expressed as log<sub>10</sub> CFU/g of organ. Progression of bacterial growth to the bloodstream was monitored using blood samples obtained by cardiac puncture with a heparinized syringe. Samples were plated on blood agar and bacteremia was reported as either

negative or positive hemocultures after incubation for 18 hours at 37°C.<sup>16,19</sup>

### Statistical analysis

Experiments were performed in triplicate and results were expressed as mean ± standard deviation. After verification of a normal distribution of data, 2-way analysis of variance was used. For pairwise comparisons of the means, Tukey's test was used. Differences were considered significant at  $p < 0.05$ .

## Results

### *L. lactis* PppA+ vaccination induced specific antibodies in serum and intestinal fluid

To evaluate the ability of *L. lactis* PppA+ to stimulate the immune system associated with the intestinal mucosa, the levels of IgA-type anti-PppA antibodies were studied first. The two oral immunization protocols with *L. lactis* PppA+ induced the production of specific antibodies in intestinal fluid, although the immunization protocol designed with two stimulations, 14 days apart from each other, more efficiently induced the production of IgA-type anti-PppA antibodies in the gut (Table 1). No antibodies were found in the groups treated with the recombinant bacterium without boosting (*L. lactis* PppA-) or with non-fat milk. Oral immunization with *L. lactis* PppA+ also induced the production of specific antibodies in serum, while no serum antibodies were found in the control groups (Table 1). In agreement with observations at the level of the intestinal mucosa, oral immunization with boosting induced the production of IgG-type anti-PppA antibodies in serum more efficiently. We also studied the IgG1 and IgG2a levels induced by immunization with *L. lactis* PppA+, to assess the type of immunological response induced (Th1 or Th2), taking into account that Th1 cells secrete Interferon (IFN)-γ, and are associated with class switching to IgG2a, while Th2 cells secrete IL-4, IL-5 and IL-10 and promote class switching to IgG1. We observed that the oral administration of *L. lactis* PppA+ induced a mixed Th1/Th2 response in the young mice, since the IgG1/IgG2a ratios were  $1.5 \pm 0.1$  and  $1.8 \pm 0.3$  for the boosted and non-boosted groups, respectively (Table 1).

**Table 1.** Levels of specific antibodies in serum and intestinal fluid samples<sup>a</sup> ( $n=5-6$ )

Group	Boost	Intestinal fluid		Serum	
		IgA	IgM	IgG	IgG1/IgG2
<i>L. lactis</i> PppA+	No	0.577±0.41	0.245±0.22	0.443±0.33	1.5±0.1
<i>L. lactis</i> PppA-	No	<0.1	<0.1	<0.1	ND
Control	No	<0.1	<0.1	<0.1	ND
<i>L. lactis</i> PppA+	Yes	0.845±0.43*	0.188±0.31*	0.719±0.28*	1.8±0.3
<i>L. lactis</i> PppA-	Yes	<0.1	<0.1	<0.1	ND
Control	Yes	<0.1	<0.1	<0.1	ND

<sup>a</sup>Data presented as mean ± standard deviation; \*significantly different from the non-boosted group at the same time point ( $p<0.05$ ). ND = not determined.

### Oral vaccination with *L. lactis* PppA+ induced specific antibodies in the respiratory tract

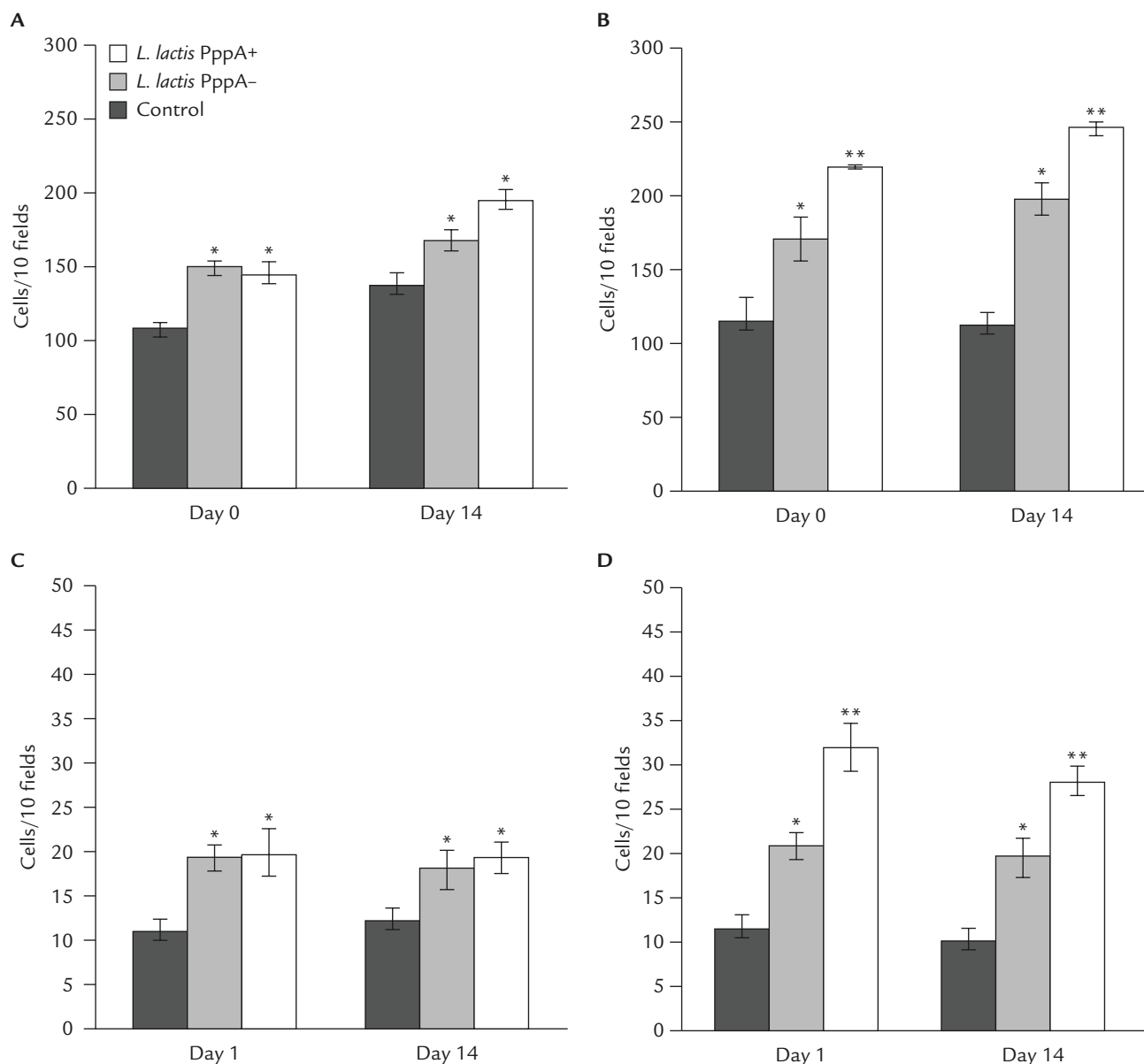
To evaluate whether oral immunization with *L. lactis* PppA+ was able to stimulate the mobilization of IgA-producing lymphocytes from the intestine toward the respiratory tract, the number of IgA+ cells in these mucosae was determined. The number of IgA+ cells in the gastrointestinal tract increased significantly after the administration of *L. lactis* PppA+ (Figures 1A and 1B), immunization with boosting being the most efficient in stimulating this increase. The oral administration of *L. lactis* PppA+ also increased the number of IgA+ cells in lung (Figures 1C and 1D) and, in this case, the protocol with boosting was also the most efficient. The oral administration of *L. lactis* PppA- also increased the number of IgA+ cells in both intestine and lung, which confirms our previous results showing that the non-recombinant strain is able to stimulate the IgA cycle.<sup>11</sup> Since the administration of *L. lactis* PppA+ induces the mobilization of IgA+B lymphocytes from the intestine to the lungs, this treatment should be capable of increasing the levels of anti-PppA IgA in the respiratory tract. To verify this, we determined the levels of these antibodies in BAL. Bearing in mind that other immunoglobulins can diffuse from the serum toward the pulmonary alveoli, and that they have an important role in respiratory defenses, we also determined the levels of IgM and IgG anti-PppA in BAL. After oral immunization with *L. lactis* PppA+, we detected specific IgA, IgM and IgG antibodies in BAL (Table 2). The vaccine plus boosting protocol was the most efficient in increasing the levels

of anti-PppA antibodies in the respiratory mucosae. This effect was evidenced by the IgG and IgA concentrations in BAL, which were significantly higher in this group than in the animals without boosting (Table 2). The IgG1/IgG2 ratios in the respiratory tract also indicated the stimulation of a mixed Th1/Th2 response (Table 2).

### Avidity and opsonophagocytic activity of anti-PppA antibodies

The avidities of the BAL, IF, and serum antibodies are shown in Figure 2. The avidity of the IF antibodies in the non-boosted *L. lactis* PppA+ group were below 40% for both IgM and IgG antibodies, although IgA anti-PppA reached values close to 60% (Figure 2A). Boosting increased the avidity of the IgG and IgA antibodies in IF, the latter reaching an average of 80%, which was the highest value observed in our study. Primary immunization with *L. lactis* PppA+ induced the production of anti-PppA IgM, IgG, and IgA antibodies in BAL with avidities lower than 50% (Figure 2C). Boosting with *L. lactis* PppA+ increased the avidity of BAL IgG and IgA, but did not induce changes in IgM antibody avidity. Mice in the boosted *L. lactis* PppA+ group also showed serum antibodies with higher avidity than mice in the non-boosted *L. lactis* PppA+ group (Figure 2B). Study of the opsonophagocytic activity of the anti-PppA antibodies showed that mice in the non-boosted *L. lactis* PppA+ group had low titers in serum and BAL (Figure 3). Boosted animals showed significantly higher titers in serum, BAL, and IF (Figure 3).





**Figure 1.** Number of IgA+ cells in (A) intestinal tissues of non-boosted group; (B) intestinal tissues in boosted group; (C) lung tissues in non-boosted group; (D) lung tissues in boosted group. \*Significantly different from the control group at the same time point ( $p < 0.05$ ); \*\*significantly different from the control and the *L. lactis* PppA- groups at the same time point ( $p < 0.05$ ).

### Safety of *L. lactis* PppA+ immunization

To assess the safety of *L. lactis* PppA+ immunization in infant mice, weight gain and the number of deaths were monitored daily. No deaths occurred, and weight gain was observed in infant mice immunized with the experimental vaccine compared with the unimmunized mice (data not shown).

### Protective capacity of *L. lactis* PppA+ vaccination

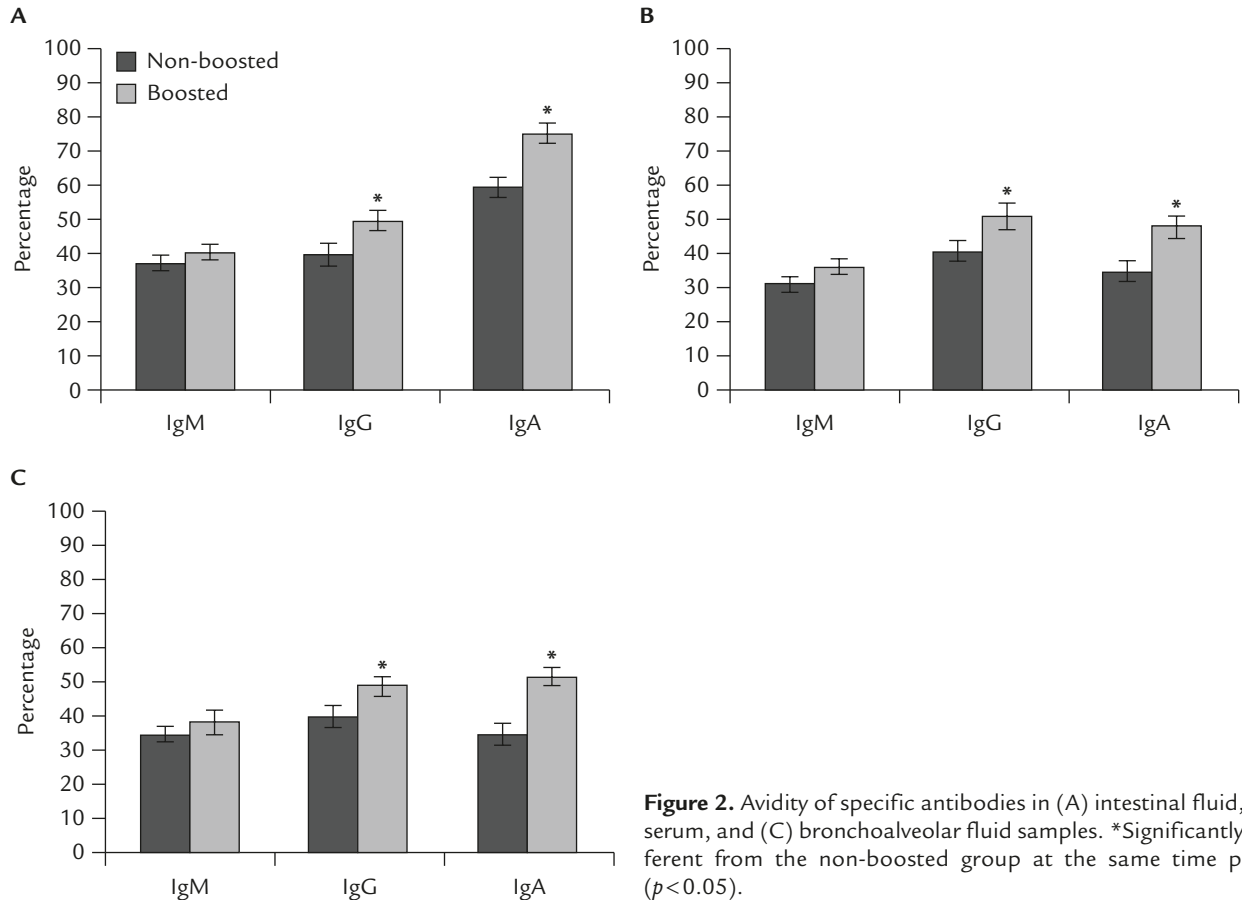
Considering that the immunization protocol with boosting proved to be more effective in inducing systemic and

mucosal specific anti-PppA antigen antibodies, and in mobilizing the IgA+ cells from the inductor sites of the immune system in the intestinal mucosae toward the lungs, we decided to assess the effectiveness of this immunization scheme in providing protective immunity. 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

**Table 2.** Levels of specific antibodies in bronchoalveolar fluid samples<sup>a</sup> ( $n = 5-6$ )

Group	Boost	Bronchoalveolar fluid			
		IgA	IgM	IgG	IgG1/Ig2
<i>L. lactis</i> PppA+	No	0.425±0.31	0.389±0.23	0.216±0.21	1.6±0.1
<i>L. lactis</i> PppA-	No	<0.1	<0.1	<0.1	ND
Control	No	<0.1	<0.1	<0.1	ND
<i>L. lactis</i> PppA+	Yes	0.613±0.25*	0.245±0.15*	0.379±0.15*	1.7±0.2
<i>L. lactis</i> PppA-	Yes	<0.1	<0.1	<0.1	ND
Control	Yes	<0.1	<0.1	<0.1	ND

<sup>a</sup>Data presented as mean ± standard deviation; \*significantly different from the non-boosted group at the same time point ( $p < 0.05$ ). ND = not determined.



**Figure 2.** Avidity of specific antibodies in (A) intestinal fluid, (B) serum, and (C) bronchoalveolar fluid samples. \*Significantly different from the non-boosted group at the same time point ( $p < 0.05$ ).

immunity to the young mice 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,<sup>20</sup> and also taking into account that serotypes 3, 6B, 9, 14, 18, 19, and 23F are the ones most often associated with

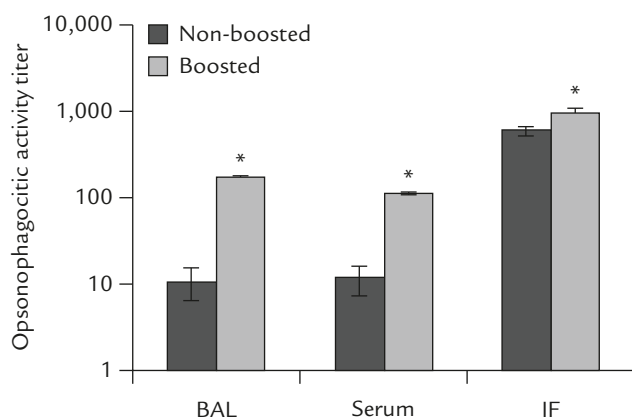
invasive disease.<sup>21</sup> Young mice were challenged intranasally with virulent pneumococcal serotypes 2 weeks after the last vaccine dose, and the development of pneumococcal disease was evaluated 48 hours after infection. In the control animals without treatment, all the *S. pneumoniae* serotypes used were detected in the lung cultures and

hemocultures 2 days after challenge (Table 3). These results indicate that the four serotypes of the pathogen were able to colonize the respiratory tract and spread to the blood stream. However, the bacterial counts for serotype 3 in the lung were higher by one order of magnitude compared with serotype 6B, and by two orders of magnitude compared with serotypes 14 and 23F. The mice immunized with *L. lactis* PppA+ by the oral route showed bacterial counts in the lung that were significantly lower than their respective controls (Table 3). Oral immunization with the recombinant oral bacteria was effective in limiting the dissemination of serotypes 6B, 14, and 23F, since the hemocultures in these groups were negative. However, the oral administration of *L. lactis* PppA+ to infant mice did not prevent bacteremia caused by serotype 3, which would be related to the greater virulence of this strain in our experimental model. Oral treatment with

*L. lactis* PppA- improved resistance to pneumococci, but its protective effect was significantly lower than that observed in the *L. lactis* PppA+ groups (Table 3). This fact is probably related to our previous observation showing that the strain *L. lactis* NZ9000 has the capacity to activate both the innate and specific immune response in the respiratory tract when administered by the oral route at the proper dose.<sup>11</sup>

## Discussion

Orally administered vaccines have the distinct advantage of being able to potentially reach the highly concentrated gut associated lymphoid tissue, and induce both serum and secretory immune responses.<sup>22</sup> Thus, unlike systemic immunization; oral delivery can induce mucosal and systemic immune responses. Vaccination in early life may be important in protecting the individual later on. For example, it is difficult to effectively protect elderly people (one of the most vulnerable groups in the population) due to age-related changes in the immune system (immunosenescence). Vaccination, or exposure to a given disease, early in life has been shown to confer a certain level of immunological memory in later life.<sup>23</sup> In this study, we showed that the oral immunization of young mice with the recombinant lactic acid bacteria, *L. lactis* PppA+, was able to induce the production of specific antibodies both in the intestinal tract and at the systemic level. The efficient stimulation of the gut mucosal immune system by *L. lactis* PppA+ was evidenced by the increase in the number of IgA+ cells in the intestine, and by the production of specific anti-PppA IgA antibodies in the IF. We also observed an efficient stimulation of the systemic



**Figure 3.** Opsonophagocytic activity of antibodies in bronchoalveolar fluid, serum and intestinal fluid samples. \*Significantly different from the non-boosted group at the same time point ( $p < 0.05$ ).

**Table 3.** Lung bacterial cell counts and hemocultures after nasal challenge with different pneumococcal serotypes<sup>a</sup> ( $n = 5-6$ )

Group	Bacterial cell counts <sup>a</sup>							
	Serotype 3		Serotype 6B		Serotype 14		Serotype 23F	
	Lung	Blood	Lung	Blood	Lung	Blood	Lung	Blood
<i>L. lactis</i> PppA+	4.3±0.3*	3.5±0.5*	3.3±0.2*	<1.5*	2.3±0.2*	<1.5*	2.5±0.3*	<1.5*
<i>L. lactis</i> PppA-	6.7±0.5*	4.7±0.3	4.5±0.3*	3.7±0.5*	4.8±0.3*	<1.5*	4.9±0.3*	<1.5*
Control	8.4±0.2	5.1±0.4	7.5±0.5	4.8±0.3	6.3±0.4	4.7±0.6	6.1±0.4	4.1±0.3

<sup>a</sup>The lower limits of bacterial detection were 1.5 log CFU/g of lung and blood. Samples were taken on day 2 post-infection and data presented as mean±standard deviation; \*significantly different from the control group ( $p < 0.05$ ).



immune response after vaccination with *L. lactis* PppA+, which was evidenced by the detection of specific anti-PppA IgG antibodies in the serum. Recent *in vitro* studies showed that *L. lactis* NZ9000 has intrinsic adjuvant properties, since it is able to stimulate the maturation of dendritic cells derived from bone marrow, and to induce the production of cytokines with adjuvant properties such as IL-1 $\beta$ .<sup>24</sup> Moreover, previous *in vivo* studies in our lab demonstrated that this lactic acid bacterium is able to stimulate both intestinal and systemic immunity when administered at the appropriate dose.<sup>11</sup> These properties of *L. lactis* NZ9000 would explain the efficacy of oral immunization with *L. lactis* PppA+ in stimulating specific immune responses against the *S. pneumoniae* antigen in young mice.

The analysis of IgG subtypes showed that *L. lactis* PppA+ immunization stimulated a mixture of Th1 and Th2 responses, which is consistent with our previous studies in adult mice in which we observed that oral immunization with *L. lactis* PppA+ was able to induce the production of IL-4- and IFN- $\gamma$ -producing spleen cells.<sup>13</sup> These findings were confirmed recently by other authors, who demonstrated that *L. lactis* NZ9000 is able to stimulate the production of both IL-12 and IL-10 by bone marrow derived dendritic cells *in vitro*.<sup>24</sup>

It has been demonstrated that oral vaccination can be used to induce protective immunity in distant mucosal sites. The protective effect in those sites mediated by oral vaccines 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 enter the peripheral blood circulation via the thoracic duct. Extravasation of immune cells occurs not only in the gut lamina propria, but also in other mucosal sites such as the respiratory tract.<sup>25</sup> Thus 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 recombinant *L. lactis*-derived oral vaccines that could afford protection against respiratory pathogens.<sup>6</sup> In this study, we demonstrated that the oral immunization of young mice with *L. lactis* PppA+ induced the production of specific IgA and IgG antibodies in the respiratory tract. The relative proportion of immunoglobulins in the upper respiratory tract differs

substantially from that in the lower respiratory tract; IgA prevailing in the former and IgG in the latter.<sup>26</sup> That is why the presence of both immunoglobulin types with specificity for antigens of *S. pneumoniae* is necessary to provide better protection of the airways. Anti-pneumococcal IgA would reduce the colonization of the respiratory tract because of its capacity to limit the attachment of the pathogen to the epithelial cells, while anti-pneumococcal IgG would have a protective role in the alveoli by promoting phagocytosis, and by preventing local dissemination of the pathogen and its passage to the blood stream.<sup>26</sup>

To assess whether the production of mucosal and systemic antibodies induced by oral immunization with *L. lactis* PppA+ was able to afford protection against respiratory infection with *S. pneumoniae*, challenge experiments with the different serotypes of the pathogen were carried out. Bacterial counts in lung, coupled with hemocultures, allowed us to conclude that young mice are more susceptible than adult mice to respiratory *S. pneumoniae* infection, since pathogen counts in blood and lung were significantly higher than those previously found in adult mice.<sup>13</sup> Oral immunization of young mice with *L. lactis* PppA+ increased their resistance to infection with the four serotypes of *S. pneumoniae*, although the protective capacity of the experimental vaccine was different for each of them. Immunization decreased colonization in lung and prevented bacteremia of serotypes 6B, 14, and 23F, and decreased serotype 3 counts.

In conclusion, we have shown that the oral immunization of young mice with the recombinant lactic acid bacteria *L. lactis* PppA+ effectively induces the production of specific antibodies against the PppA antigen of *S. pneumoniae*, both in mucosae and at the systemic level. The antibodies produced play an important role in protection against pneumococcal disease, since young mice immunized with the experimental vaccine showed increased resistance to infection with different serotypes of the pathogen. The identification of effective and safe strategies for the induction of protective immunity in the early life period requires further research, but the use of selected efficacious vaccines could make a tremendous difference in terms of infant survival worldwide. The results of the present paper show that oral immunization with recombinant bacteria represents a promising alternative for improving immunity in young individuals.

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