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Original article

Induction of systemic and mucosal immune response and decrease in *Streptococcus pneumoniae* colonization by nasal inoculation of mice with recombinant lactic acid bacteria expressing pneumococcal surface antigen A

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Abstract

Mucosal epithelia constitute the first barriers to be overcome by pathogens during infection. The induction of protective IgA in this location is important for the prevention of infection and can be achieved through different mucosal immunization strategies. Lactic acid bacteria have been tested in the last few years as live vectors for the delivery of antigens at mucosal sites, with promising results. In this work, *Streptococcus pneumoniae* PsaA antigen was expressed in different species of lactic acid bacteria, such as *Lactococcus lactis*, *Lactobacillus casei*, *Lactobacillus plantarum*, and *Lactobacillus helveticus*. After nasal inoculation of C57Bl/6 mice, their ability to induce both systemic (IgG in serum) and mucosal (IgA in saliva, nasal and bronchial washes) anti-PsaA antibodies was determined. Immunization with *L. lactis* MG1363 induced very low levels of IgA and IgG, possibly by the low amount of PsaA expressed in this strain and its short persistence in the nasal mucosa. All three lactobacilli persisted in the nasal mucosa for 3 days and produced a similar amount of PsaA protein (150–250 ng per 10^9 CFU). However, *L. plantarum* NCDO1193 and *L. helveticus* ATCC15009 elicited the highest antibody response (IgA and IgG). Vaccination with recombinant lactobacilli but not with recombinant *L. lactis* led to a decrease in *S. pneumoniae* recovery from nasal mucosa upon a colonization challenge. Our results confirm that certain *Lactobacillus* strains have intrinsic properties that make them suitable candidates for mucosal vaccination experiments.

Keywords: Lactic acid bacteria; *Streptococcus pneumoniae*; PsaA

1. Introduction

Streptococcus pneumoniae is the major agent of pneumonia around the world, causing up to one million deaths per year, mainly in developing countries [1]. The high costs of medical care and the appearance of new clinical isolates with multidrug resistance led to the search for efficient new vaccines to prevent pneumococcal infection. Since the pathogen enters the host through the respiratory mucosa, a vaccine inducing the production of protective secretory IgA at this site, as well as systemic IgG antibodies, would be desirable.

Pneumococcal surface antigen A (PsaA) is a membrane-anchored virulence factor, possibly involved in Mn^{2+} and Zn^{2+} transport as predicted by its crystal structure [2]. PsaA deletion

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mutants display low ability to adhere to mucosal cells and therefore are less pathogenic [3]. This characteristic may be due to differences in the modulation of pneumococcal adhesins caused by the absence of Mn^{2+} or Zn^{2+} in the cell [4]. PsaA is conserved among the 90 described *S. pneumoniae* serotypes and is also immunogenic, which makes it a good candidate for vaccine formulations. In fact, antibodies produced against PsaA by nasal immunization, using cholera toxin B subunit as adjuvant, were shown to protect mice against nasopharyngeal colonization by *S. pneumoniae*. This protection can be further increased by the co-administration of PsaA with the pneumococcal surface protein A (PspA), another pneumococcal virulence factor [5,6]. In another approach, oral immunization of mice with PsaA encapsulated in microspheres induced the production of IgG and IgA and resulted in protection against lung colonization and septicemia with five different *S. pneumoniae* strains [7].

Live bacterial vaccine vectors are being extensively studied for mucosal immunization in the prevention of different infectious diseases [8,9]. Among them, lactic acid bacteria (LAB) are especially attractive since they are microorganisms present in the gastrointestinal mucosa of healthy individuals, are widely used in dietary products and possess a GRAS (generally recognized as safe) status. This characteristic is not shared by attenuated pathogen derived live vectors, due to the possibility of reversion of the attenuated phenotype, which could be dangerous mainly for immunocompromised individuals.

Interaction of LAB with the immune system and their potential as antigen carriers are the subjects of a number of recently published studies [10–15]. Different strains and routes of inoculation were evaluated using the fragment C of the tetanus toxin (TTFC), which is so far the best characterized antigen expressed in LAB [9,16–18]. Most of these approaches resulted in protection against tetanus toxin lethal challenge [16,19]. Other antigens like the protective antigen from *Bacillus anthracis* [20], the E7 protein from human papilloma virus 16 [21], the L7/L12 antigen from *Brucella abortus* [22], the Env protein from HIV [23], the M protein from *Streptococcus pyogenes* [24] and the spike glycoprotein from gastroenteritis coronavirus [25] were expressed in LAB and their potential as vaccines against the associated diseases are currently being evaluated. In some cases, such as the immunization with *L. lactis* expressing the HIV Env protein or the M protein from *Streptococcus pyogenes*, protection was observed using adequate animal models [23,24]. In other cases, in vitro neutralizing effects of the antibodies were observed [25].

Using an inducible expression system based on the lactose operon from *Lactobacillus casei* [26] we expressed the PsaA and the PspA antigens from *S. pneumoniae* [27] either intracellularly or secreted to the culture media. These recombinant *L. casei* are being tested as potential anti-pneumococcal vaccines through nasal immunization of mice, but so far we could not detect significant levels of anti-PsaA IgA or IgG (unpublished data). The failure in stimulating the production of antibodies may be a result of the lack of PsaA or PspA expression in the recombinant *L. casei* after nasal immunization, due to the absence of the inducer in the host mucosa. For this reason, we decided to use a system that allows the constitutive expression of PsaA in different LAB strains. In this work, recombinant *Lactococcus lactis*, *Lactobacillus casei*, *Lactobacillus plantarum* and *Lactobacillus helveticus* expressing PsaA were evaluated for their ability to induce systemic and mucosal immune responses in nasally immunized C57Bl/6 mice. Nasal colonization of *Streptococcus pneumoniae* in these mice was also analyzed.

2. Materials and methods

2.1. Bacterial strains and growth conditions

L. casei CECT5275 (formerly ATCC 393 [pLZ15]$^{-}$), *L. plantarum* NCDO1193 (kindly provided by Dr K. Thompsom from the Food and Agricultural Microbiology Research Division, Department of Agriculture, Northern Ireland, UK) and *L. helveticus* ATCC 15009 were routinely grown in MRS medium (Difco), at 37 °C, without shaking. *L. lactis* MG1363 was grown in M17 medium (Difco) containing 0.5% glucose (GM17) at 30 °C without shaking. Plating of bacteria was performed on the respective media with 1.8% agar. For the selection of transformants, 5 μg/ml of erythromycin was used in the media.

2.2. Plasmids and recombinant DNA procedures

The pT1NX vector, kindly provided by Dr Lothar Steidler (Department of Molecular Biology, Flanders Interuniversity Institute for Biotechnology, Ghent University, Belgium) contains the lactococcal P1 constitutive promoter and the usp-45 signal sequence [28]. The 867 bp gene encoding PsaA from *S. pneumoniae* serotype 6B (strain St 472/96 from the Instituto Adolpho Lutz, São Paulo, SP, Brazil) was amplified from pCI-*psaA* plasmid [29] by PCR using the following oligonucleotides:

PsaA-I-forw: 5′ATG CAT CGA TAT CAG CTA GCG GAA AAA AAG AT3′ and
PsaA-I-rev: 5′CCA AGC TTT TAT TTT GCC AAT CCT TCA GC3′

PCR was performed using Taq DNA polymerase (Invitrogen), 200 mM of each deoxynucleoside triphosphate and 20 pmol of each primer. PCR amplification conditions were as follows: 94 °C, 4 min; 30 cycles of 94 °C, 45 s; 45 °C, 45 s; 72 °C 1.5 min; 72 °C, 7 min for final extension. PCR products were cloned into the pGEM-T vector (Promega) for sequence confirmation. One clone was chosen for preparation of the PsaA fragment as follows. The pGEM-T-psaA plasmid was digested with *Hin*dIII and treated with Klenow polymerase (Invitrogen) for generation of a blunt end and was further digested with *Nsi*I. The resulting fragment was cloned into pT1NX previously digested with *Bam*HI and treated with

Klenow DNA polymerase (Invitrogen) for generation of a blunt end and then digested with *Pst*I, providing a protrude end compatible with the *Nsi*I end from the insert. Nucleic acid manipulation and general cloning procedures were performed according to laboratory manuals [30].

For the preparation of competent *L. lactis*, an overnight culture was diluted 1:50 in GM17 containing 0.5% glycine and incubated at 30 °C until OD_{600} reached 0.6. Bacteria were collected by centrifugation at 10,000 × *g*, for 10 min at 4 °C and the pellet was washed two times with 0.5 M saccharose, 10% glycerol (v/v). Bacteria were resuspended in 1:100 of the same solution and electroporated immediately or kept at −80 °C for further use. *L. lactis* was electroporated with ligation mixtures at 2.5 kV, 200 Ω, 25 mF in 0.2 cm cuvettes using a BioRad GenePulser (BioRad, Life Science Research Products, CA, USA). Plasmids isolated from *L. lactis* were used for electroporation of all *Lactobacillus* strains. Electroporation of *L. casei* was carried out as previously described [31]. The same protocol was used for the other lactobacillus strains except that the electroporation buffer was three-fold concentrated. Antibiotic resistant *L. lactis* and *Lactobacillus* clones were screened for the presence of the insert of interest by PCR, using specific primers as described above. Positive clones were frozen in GM17 or MRS containing 15% glycerol, at −80 °C.

2.3. Protein expression and Western blot analysis

Isolated *L. lactis* and *Lactobacillus* clones were grown overnight in GM17 or MRS, respectively. Cultures (10 ml) were collected by centrifugation at 4000 × *g* for 10 min and bacterial pellet was suspended in 1 ml of 100 mM Tris–HCl, pH 8.0. Cell suspensions were transferred to 2 ml tubes containing the same volume of glass beads and lysates were prepared by vigorous shaking in a Bead-Beater (Biospec, Bartlesville, OK, USA) (four cycles of 30 s at maximal speed). Lysates were centrifuged at 15,000 × *g* for 5 min and supernatants were maintained at −20 °C for further analysis. For quantification of PsaA, cultures were grown until OD_{600} reached 2. The CFU values of each culture were determined and extracts corresponding to 10^9 CFU were analyzed by Western blot. A concentration curve was loaded on the same gel using recombinant PsaA expressed and purified from *E. coli* [32]. For the analysis of PsaA in the cell wall, bacteria were incubated with 20 mg/ml of lysozyme in 50 mM glucose, 25 mM Tris–HCl pH 8.0 and 10 mM EDTA, at 37 °C for 30 min. The suspension was centrifuged at 15,000 × *g* for 5 min and the supernatants were collected. Protein extracts or supernatants were fractionated by SDS-PAGE and electrotransferred to nitrocellulose membranes using the Mini Protean II equipment (BioRad, Life Science Research Products, CA, USA). Mouse polyclonal anti-PsaA antiserum was developed against recombinant *S. pneumoniae* PsaA (from strain St 472/96) expressed in *E. coli*. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma Chemical, St. Louis, MO, USA) was used according to the manufacturer's instructions. Detection was performed using the chemiluminescent ECL kit (GE Healthcare).

2.4. Immunization and analysis of immune responses

Six- to eight-week-old female C57Bl/6 mice (five per group) received either the recombinant bacteria expressing PsaA or the respective control bacteria harboring the empty vector. An additional control group received saline. LAB strains were grown until cultures reached an OD_{600} of 2.0; bacteria were collected by centrifugation (4000 × *g*, 20 min at room temperature), washed with saline and then suspended at 10^9 cells in 10 µl. For nasal immunization, mice were anesthetized with a mixture of 0.5% xilazine and 0.2% ketamine and 10 µl of a saline suspension containing 10^9 cells were inoculated into the nostrils with the help of a micropipette on days 0, 1, 14, 15, 28 and 29. Ten days after the last booster mice were bled by the retrorbital plexus. For the collection of saliva, a 0.01% pilocarpine solution was injected intraperitoneally. Nasal and bronchial washes were performed as described elsewhere, using 200 µl and 300 µl of saline, respectively [33]. Anti-PsaA antibodies were detected from sera, saliva, nasal and bronchial washes by enzyme-linked immunoabsorbent assay (ELISA) as previously described [29], using rPsaA purified from *E. coli* as coating and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or anti-mouse IgA (Sigma Chemical, St. Louis, MO, USA). Titers were defined as the last dilution in which absorbances at 492 nm reached 0.1.

The maintenance of the LAB strains harboring the pT1NX or the pT1NX-PsaA plasmids in respiratory mucosa was analyzed up to 7 days after a nasal inoculation of 10^9 bacteria. For this, we performed nasal and bronchial washes in groups of four animals on days 1, 3, 5 and 7 after inoculation (day 0). Dilutions of these washes were plated in MRS or GM17 containing 5 µg/ml of erythromycin. Colonies were counted after 48 h incubation at 37 °C.

2.5. Streptococcus pneumoniae *nasal colonization*

Six- to eight-week-old C57Bl/6 mice (12 per group) were immunized as described above. Individual serum and pooled saliva were collected for analysis of anti-PsaA IgG and IgA, respectively. Fifteen days after the last inoculation, animals were anesthetized and 10 µl of a suspension containing 5×10^6 CFU of *Streptococcus pneumoniae* strain 0603 serotype 6B [34] were inoculated nasally. After 5 days, nasal washes were performed using 200 µl of saline. Serial dilutions of the samples were plated in blood agar containing 8 µg/ml gentamicin. Alpha-hemolytic colonies were counted after incubation of the plates for 24 h at 37 °C, considering the volume recovered. For representation in the graphic and statistical analysis $\log_{10}$ was applied to the values and recovery of 0 CFU was considered 1 CFU.

2.6. Statistical analysis

Differences in antibody titers were analyzed by the Mann–Whitney *U* test. ($P \leq 0.05$ was considered significantly different). The same test was used for the analysis of

S. pneumoniae colonization. ($P \leq 0.02$ was considered significantly different).

3. Results

3.1. Expression of PsaA in different LAB

The *psaA* gene was cloned into the pT1NX vector *Pst*I site, which produced a fusion of a truncated usp45 signal peptide carried by the vector to the PsaA sequence (Fig. 1), under the control of a constitutive promoter. Ligation products were used to transform *L. lactis* and expression of PsaA was analyzed by Western blot of cell lysates. *L. lactis* carrying the constructed vector showed the expression of a protein around 37 kDa reacting against anti-PsaA antibodies, which was not present in extracts from cells carrying the empty vector (Fig. 2A). Recombinant plasmid isolated from *L. lactis* was used to transform different *Lactobacillus* strains. Western blot analysis of protein extracts from erythromycin resistant colonies showed the expression of PsaA in *L. casei*, *L. plantarum* and *L. helveticus*. The PsaA band was not observed in the respective strains carrying the empty vector (Fig. 2A). Using recombinant PsaA as reference, it could be calculated that 10^9 *L. lactis* CFU expressed approximately 20 ng of PsaA, while the amounts synthesized by the different *Lactobacillus* strains were about 150 ng (*L. plantarum*), 200 ng (*L. casei*) and 250 ng (*L. helveticus*) of PsaA (Fig. 2B). PsaA was not detected in 10-fold concentrated culture media from these clones, indicating that secretion of the protein was not occurring (data not shown). However, analysis of the supernatant obtained after incubation of the cells with lysozyme, showed that at least part of the protein is being directed to the cell wall (Fig. 2C). Moreover, when we incubated whole cells with mouse polyclonal anti-PsaA followed by incubation with anti-mouse IgG-peroxidase conjugate and further revealed with OPD and H_2O_2, positive reactions were observed in all LAB expressing PsaA, indicating that at least part of the protein is exposed outside of the cell, in contrast to negative reactions observed for the LAB strains carrying the empty vector (data not shown).

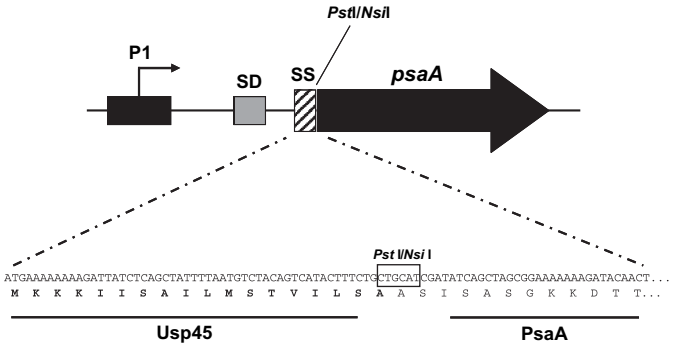

Fig. 1. Schematic representation of the PsaA-expressing plasmid and amino acid sequence resulting from the genetic fusion. P1 represents the constitutive promoter; SD, Shine-Dalgarno sequence; SS, first codons of the Usp45 signal peptide fused to the *psaA* gene.

3.2. Induction of anti-PsaA antibodies by nasal immunization with recombinant LAB

Recombinant LAB were used for nasal immunization of C57Bl/6 mice. As can be observed in Fig. 3A, nasal inoculation with *L. casei*, *L. plantarum* or *L. helveticus* expressing PsaA induced specific anti-PsaA IgA in pooled saliva. No detectable levels of anti-PsaA IgA antibodies were observed in saliva collected from animals that received *L. lactis* expressing PsaA, the LAB strains carrying the pT1NX vector or saline. The animals were then subjected to nasal and bronchial washes for the analysis of the presence of IgA in the respiratory tract. In these samples, the highest IgA titers could be observed in animal groups that received either *L. plantarum* or *L. helveticus* expressing PsaA (Fig. 3A and B) with means significantly different from groups that received saline ($P < 0.001$ and $P < 0.05$, respectively) and the respective controls carrying the empty vector ($P < 0.001$ and $P < 0.05$, respectively). Most of the animals that received *L. casei* expressing PsaA did not display detectable levels of IgA in nasal or bronchial mucosa. In this group, only a single animal showed a very high IgA titer, but the mean of the group was not significantly different from the controls (saline or *L. casei* carrying the empty vector, $P > 0.05$). The group that received *L. lactis* expressing PsaA displayed low levels of anti-PsaA IgA in nasal washes or even no detectable levels in bronchial washes (Fig. 3A and B), with the mean values being not different from the saline group or the *L. lactis* pT1NX group ($P > 0.05$). Analysis of the sera collected from the same animals revealed that the animals that displayed the highest levels of anti-PsaA IgA also displayed the highest levels of anti-PsaA IgG, showing correlation in the production of these two classes of antibodies (data not shown).

For the analysis *S. pneumoniae* colonization, the same experiment was then performed using 12 animals per group. Before challenge, the induction of anti-PsaA IgG was evaluated in the serum collected from each animal and produced similar results as the previous experiment (Fig. 4). Nasal administration of recombinant *L. plantarum* or *L. helveticus* expressing PsaA led to the induction of the highest titers of anti-PsaA IgG in the serum, whereas low levels of anti-PsaA IgG could be observed in the sera of the animals that received recombinant *L. lactis* or *L. casei* (Fig. 4). Statistical analysis showed that the results obtained for the groups that received *L. plantarum* PsaA or *L. helveticus* PsaA were significantly higher than the results obtained for the group that received saline ($P < 0.01$ for *L. plantarum* and $P < 0.001$ for *L. helveticus*) or the respective control strains carrying the pT1NX vector ($P < 0.001$ for both).The levels of anti-PsaA IgG induced by *L. lactis* PsaA or *L. casei* PsaA were not significantly different from the levels found in the saline group ($P > 0.05$). Comparisons with their control strains carrying the empty vector show that no differences were found for *L. lactis* ($P > 0.05$), whereas *L. casei* PsaA induced higher levels of anti-PsaA IgG when compared with *L. casei* pT1NX ($P = 0.01$).

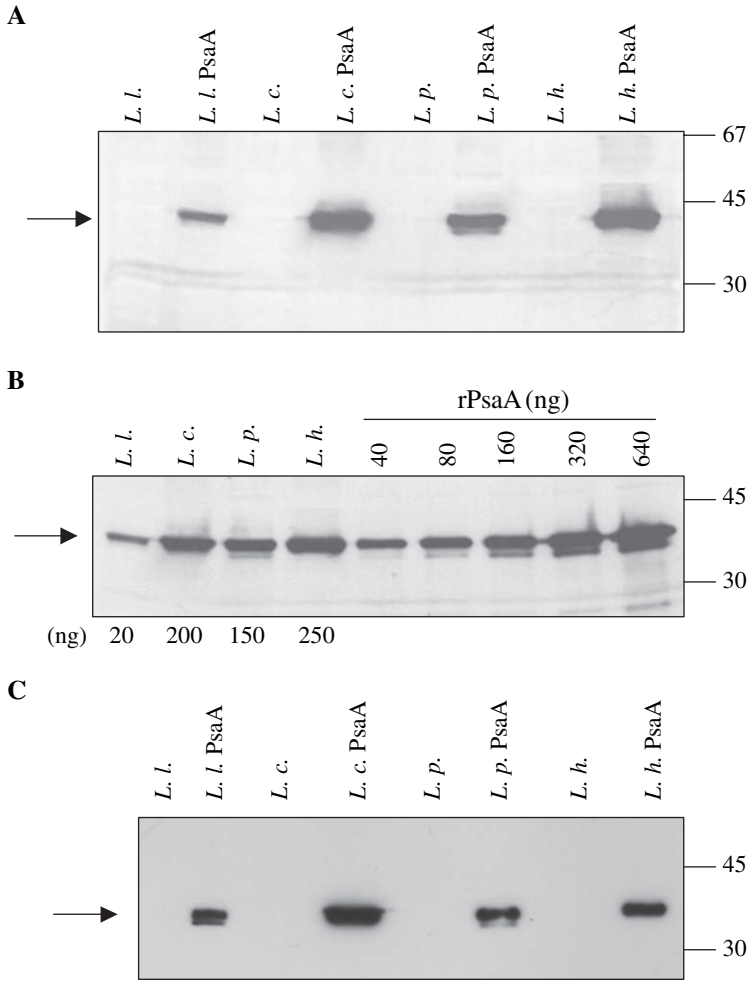

Fig. 2. Expression of PsaA in different LAB strains. (A) Western blot analysis from protein extracts show the constitutive expression of PsaA in *L. lactis* (*L. l.* PsaA), *L. casei*, (*L. c*. PsaA), *L. plantarum* (*L. p*. PsaA) and *L. helveticus* (*L. h*. PsaA). PsaA bands are pointed by an arrow. Lysates from respective control strains carrying the pT1NX empty vector are also shown in the figure. (B) Lysates from 10^9 cells of each recombinant LAB were loaded onto SDS-PAGE and transferred to a nitrocellulose membrane. Concentrations from 40 to 640 ng of rPsaA were used as reference. The estimated amounts of PsaA are shown below the panel (20 ng for *L. lactis*; 200 ng for *L. casei*, 150 ng for *L. plantarum* and 250 ng for *L. helveticus*). (C) Supernatants recovered after treatment of the different strains with lyzosyme. Arrows point to the PsaA band.

3.3. Inhibition of Streptococcus pneumoniae *nasal colonization*

Fifteen days after the last dose, the animals were challenged with *S. pneumoniae* strain 0603. Results from colonies counting (Fig. 5) show significant reduction of *S. pneumoniae* colonization in the *L. plantarum* PsaA and *L. helveticus* PsaA groups, when compared to the animals that received saline ($P = 0.02$ for *L. plantarum* and $P < 0.01$ for *L. helveticus*). When compared with their respective controls, no significant differences were observed for *L. plantarum* ($P > 0.02$), but administration of *L. helveticus* PsaA led to a significant reduction in *S. pneumoniae* colonization in relation to *L. helveticus*-pT1NX ($P = 0.02$). Administration of *L. lactis* PsaA did not induce significant reduction of *S. pneumoniae* colonization either when compared with the saline group or the *L. lactis* pT1NX group ($P > 0.02$). Interestingly, administration of *L. casei* pT1NX led to an inhibition in *S. pneumoniae* colonization when compared with the saline group ($P < 0.01$) and expression of PsaA in this strain produced a much stronger effect ($P < 0.001$). However, the results obtained for the *L. casei* PsaA group were not significantly different from the results obtained for the *L. casei* pT1NX group ($P > 0.02$). No other LAB strain carrying the empty vector induced significant reduction in *S. pneumoniae* colonization when compared with the saline group.

3.4. Recovery of recombinant LAB strains from nasal mucosa

The differences observed in the ability to induce anti-PsaA antibodies as well as the differences in *S. pneumoniae* colonization led us to analyze possible correlations with the permanency of each recombinant strain in mice respiratory mucosa. All LAB strains could be recovered from nasal washes on day 1 in almost the totality of animals analyzed (3 of 4 animals for *L. lactis* PsaA, *L. casei* PsaA and *L. helveticus* PsaA, 4 of 4 animals for *L. plantarum* PsaA). On the other hand,

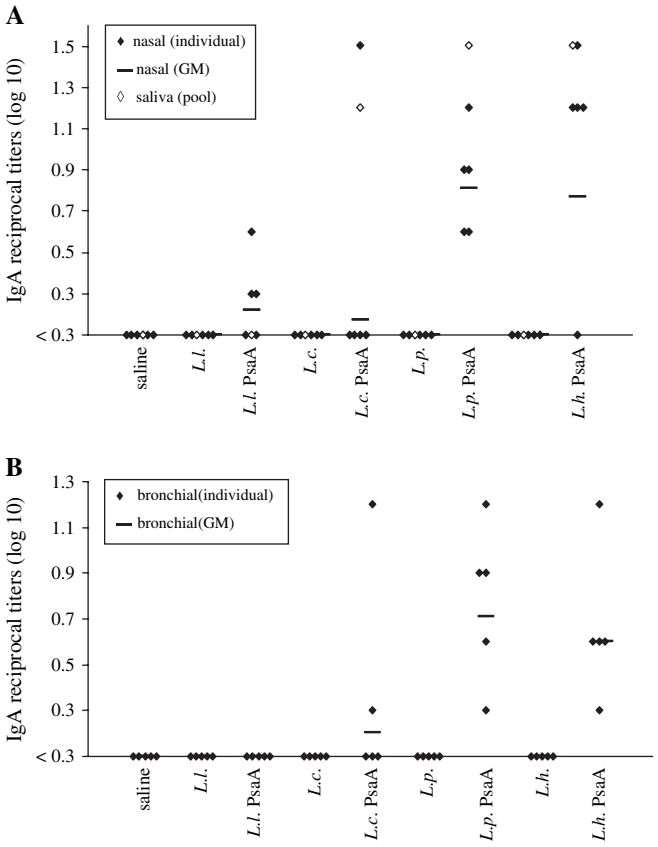

Fig. 3. Analysis of the induction of IgA in saliva, nasal and bronchial washes. Saliva and nasal washes (A) and bronchial washes (B) from individual mice were analyzed by ELISA for anti-PsaA antibodies. Log_{10} of reciprocal antibody titers are shown. Animals that received the respective LAB strains carrying the pT1NX empty vector (*L. l.*; *L. c.*; *L. p.* and *L. h.*) and saline were used as controls. Results are representative of two independent experiments. The individual sera that displayed non-detectable anti-PsaA IgA titers were represented as <0.3.

erythromycin resistant *L. lactis* were already absent on day 3, although all *Lactobacillus* strains were recovered in practically all of the animals analyzed on that day (3 of 4 animals for *L. casei* and *L. plantarum* and 4 of 4 animals for *L. helveticus*). None of the LAB was recovered from nasal washes on days 5 or 7 or from bronchial washes on any of the tested days. Nasal inoculation of the respective strains carrying the empty vector produced similar results, showing that PsaA expression does not exert any effect in bacteria permanency on mice respiratory mucosa (data not shown).

Protein extracts from *L. lactis* recovered on day 1 and *Lactobacillus* strains recovered on day 3 were tested for the expression of PsaA. For this, three colonies of each strain were grown overnight and protein extracts were analyzed by Western blot. All recovered colonies tested were still able to express PsaA in vitro (data not shown).

4. Discussion

Several strategies to induce mucosal immune responses against *S. pneumoniae* antigens are currently being tested [6,32]. One strategy can be the use of LAB as live vectors

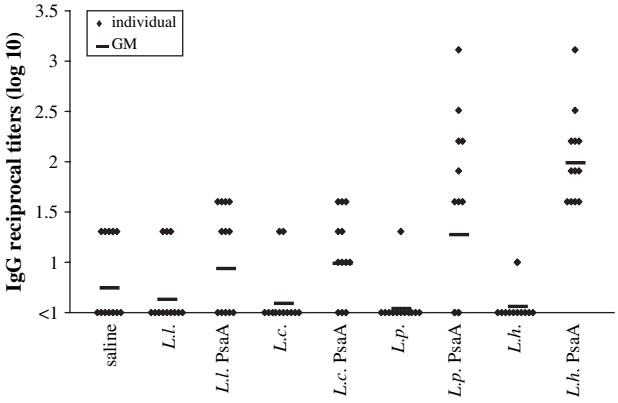

Fig. 4. Induction of IgG by nasal immunization of mice with different LAB strains. Sera from individual mice were analyzed by ELISA for anti-PsaA antibodies. Log_{10} of reciprocal antibody titers are shown. Animals that received the respective LAB strains carrying the pT1NX empty vector (*L. l.*; *L. c.*; *L. p.* and *L. h.*) or saline were used as controls. Results are representative of two independent experiments. The individual sera that displayed non-detectable anti-PsaA IgG titers were represented as <1.

for the delivery of specific antigens to mucosal surfaces due to their adhesion to the epithelium and to their claimed adjuvant properties [17,35,36].

In order to circumvent a possible bacterial host dependent effect that could compromise the success when designing live vectors for mucosal vaccination, we have expressed the PsaA protein from *S. pneumoniae* in *L. casei* CECT 5275, *L. plantarum* NCDO1193 and *L. helveticus* ATCC 15009 as well as in the model LAB *L. lactis* MG1363 strain. After the fusion to the Usp45 signal sequence, the recombinant PsaA protein was directed to the cell wall in the four LAB assayed, with no protein detected in culture supernatants. In a previous report, expression of PsaA in fusion with the leader sequence from the *L. casei* cell wall proteinase PrtP also resulted in the localization of the protein in the cell wall and no secretion to the culture media [27]. Since the sequence which specifies

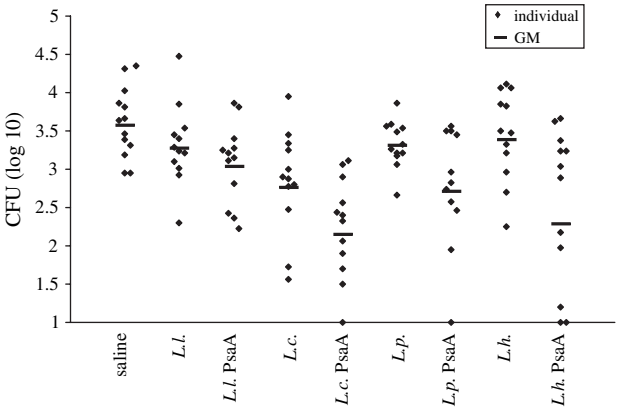

Fig. 5. Nasal colonization by *S. pneumoniae.* Dilutions of individual nasal washes were plated on blood agar and α-hemolytic colonies were counted after 24 h incubation. Log_{10} of total CFU is shown. Animals that received the respective LAB strains carrying the pT1NX empty vector (*L. l.*; *L. c.*; *L. p.* and *L. h.*) or saline were used as controls. Absence of colonies in individual nasal washes is represented as 1.

PsaA covalent anchoring to the cell surface in its natural streptococcal host is absent from our construct, it seems that this protein has the particularity to be retained in the cell wall in different bacteria.

The recombinant LAB inoculated by the nasal route were able to induce different levels of specific anti-PsaA IgA and IgG in C57Bl/6 mice (Figs. 3 and 4). *L. plantarum* and *L. helveticus* turned out to be better in the development of mucosal and systemic anti-PsaA immune response than *L. casei* and *L. lactis*. Pooled saliva from the *L. casei* PsaA group displayed an IgA induction similar to those found in the *L. plantarum* PsaA or the *L. helveticus* PsaA groups (Fig. 3A). However, when nasal or bronchial washes of individual animals in the *L. casei* PsaA group were analyzed, most of them displayed low or even no detectable levels of IgA, with only one animal producing a high titer of anti-PsaA IgA (Fig. 3A and B). The high titer in this individual may be the cause of the anti-PsaA IgA observed in pooled saliva from the *L. casei* PsaA group (Fig. 3A). Since *L. casei* expressed about the same amount of PsaA as *L. plantarum* and *L. helveticus* and was also recovered from mice nasal mucosa in the same period, the differences observed among *L. casei* and the other lactobacilli may reflect differences in their intrinsic adjuvant potential. In another study, Shaw and collaborators have shown that *L. plantarum* NCIMB 8826 expressing TTFC produced better results in oral immunization when compared to *L. casei* 393 [37]. In this case, *L. plantarum* persisted for up to 12 days in mice gastrointestinal tract whereas *L. casei* remained for about 72 h [37]. The inability of *L. lactis* expressing PsaA to significantly induce serum IgG or secreted IgA after nasal inoculation could be explained by the low level of expression in this strain compared to lactobacilli. In addition, it was observed that lactococci carrying the expression vector, remained in mice nasal mucosa only 1 day after the inoculation, while lactobacilli could be detected up to 3 days later.

Nasal inoculation of mice with *L. lactis* PsaA did not exert any effect on *S. pneumoniae* colonization, in comparison with inoculation of saline or *L. lactis* carrying the empty vector (Fig. 5). On the other hand, colonization of *S. pneumoniae* was significantly reduced in mice immunized with *L. helveticus* PsaA, when compared with the animals that received saline or *L. helveticus* pT1NX. A significant decrease in *S. pneumoniae* colonization is also observed in the group that received *L. plantarum* PsaA in relation to the group that received saline, but not in relation to the group that received *L. plantarum* pT1NX. In this case, a possible inhibition effect related to the strain may be taking place. However, it is important to notice that simply inoculation of the *L. plantarum* strain carrying the empty vector is not sufficient to cause a decrease in *S. pneumoniae* colonization. Interestingly the group that received *L. casei* carrying the empty vector displayed a significant reduction in *S. pneumoniae* colonization and this reduction was more accentuated in the group that received *L. casei* PsaA. Since the administration of this strain induced low levels of anti-PsaA antibodies, other mechanisms may be causing this reduction. In addition, although a correlation between antibody induction and inhibition of *S. pneumoniae* colonization was observed when we compared the groups that received *L. lactis* PsaA, *L. plantarum* PsaA and *L. helveticus* PsaA, this correlation is not always maintained when we analyzed the animals individually. Thus, the induction of anti-PsaA antibodies may be only one of the factors contributing to the reduction of *S. pneumoniae* colonization. Nasal immunization with recombinant PsaA in combination with other pneumococcal proteins and CTB as adjuvant [6] as well as oral immunization with microencapsulated PsaA and CTB [38] were able to induce mucosal and systemic antibodies and protect mice against *S. pneumoniae* colonization. These studies suggest a correlation between antibody production and inhibition of colonization. In another study using a colonization model in mice, nasal inoculation of *S. pneumoniae* resulted in mucosal antibody induction that was associated with clearance of the bacteria. However, no correlation was observed between the amount of antibodies detected in the sera or mucosa and the density of colonization of individual animals. In addition, animals with impaired humoral immunity displayed similar densities and durations of *S. pneumoniae* colonization than their normal counterparts. The authors discuss that other components of the adaptative immune response as well as innate immune response may be the main contributors for pneumococcal clearance in their model [39].

The probiotic effects of certain *Lactobacillus* strains have been extensively studied [40]. Among these studies, the *L. casei* strain Shirota has been shown to induce cellular immunity and to reduce influenza virus titers in mice respiratory tract [41]. The administration of *Lactobacillus fermentum* prior to a *S. pneumoniae* challenge led to an increase in anti-*S. pneumoniae* antibodies as well as in activated macrophages in mice lung and a decrease in *S. pneumoniae* colonization [42]. Although the regimen of administration in these type of studies differ from that used in this work, a possible probiotic effect of the *Lactobacillus* strains used here cannot be ruled out and may explain the reduction in *S. pneumoniae* colonization in animals immunized with *L. casei*.

Our results support the use of lactobacilli for vaccination purposes and experimentally proved the importance of two key issues in the design of live vectors for oral vaccination which include the amount of antigen delivered in relation to the expression system and the strain chosen. The relevance of the immunization protocols in these experiments should be stressed and they could still be improved, perhaps to achieve stimulation of the immune system with fewer doses and more efficiently. As an attempt, co-administration with lactobacilli strains expressing other pneumococcal antigens, such as the PspA, is currently being evaluated.

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