Immunobiology 220 (2015) 109–116

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Safety of a nasal vaccine against *Streptococcus pneumoniae* using heat-killed *Lactobacillus casei* as adjuvant

Guillermina Almada[a], Cecilia Haro[a], Elisa Vintiñi[b], Marcela Medina[c,*]

[a] *Instituto de Bioquímica Aplicada, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán-CONICET, Balcarce 747, CP 4000 San Miguel de Tucumán, Tucumán, Argentina*
[b] *LARIVENOA,Facultad de Agronomía y Zootecnia. Universidad Nacional de Tucumán. Florentino Ameghino S/N. CP: 4105, San Miguel de Tucumán, Tucumán, Argentina*
[c] *Instituto de Microbiología, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán-CONICET, Ayacucho 471, CP 4000 San Miguel de Tucumán, Tucumán, Argentina*

ARTICLE INFO

Article history:
Received 27 May 2014
Accepted 11 August 2014
Available online 10 September 2014

Keywords:
Vaccine safety
Nasal vaccine
Adjuvant
Heat killed-*L. casei*
S. pneumoniae

ABSTRACT

Streptococcus pneumoniae is a highly important respiratory pathogen that causes infections in children, elderly people and immunocompromised people around the world. Pneumococcal vaccines licensed did not reach to eradicate the pneumococcal infection. In a previous study was demonstrated the effectiveness of a nasal experimental vaccine that consisted in a pneumococcal protective protein A (PppA) co-administrated with heat-killed-*Lactobacillus casei* (LcM), in mice model of respiratory pneumococcal challenge. In the present work the safety of the experimental vaccine LcM + PppA and its components were evaluated through hematological, biochemical and immune parameters in a model infection with *S. pneumoniae*. Thus, alanine transaminase activity, creatinine levels, lactate dehydrogenase activity, C reactive protein levels, corticosterone levels in serum, total and differential leukocyte counts in blood and bronchoalveolar lavages (BAL) and IgE in BAL, were evaluated. Experimental vaccine: LcM + PppA nasally administered does not induce harmful effects in our vaccination–infection model. Studied parameters showed LcM + PppA's safety in liver, kidney, pulmonary and systemic levels. Although studies in experimental animals do not guarantee security for the application of the vaccine on humans, they are important evidence for the planning and subsequent clinical trials in humans.

Introduction

Streptococcus pneumoniae is a highly important respiratory pathogen and it is considered one of the primary causes of pneumonia and meningitis in children under 5 years old and adults over 65 years old in both developed and developing countries (Scott, 2008; Mulholland, 2007). This bacterium has kept a high incidence of pathologies worldwide due to a combination of virulence factors, its ability to dodge early components of the host immune response and the emergence of antibiotics resistant strains because of an inappropriate use of antibiotics. Vulnerable groups are also immunocompromised patients and individuals with kidney, heart and lung diseases are considered to be susceptible to pneumococcal infection (Lynch and Zhanel, 2009).

Currently, two types of pneumococcal vaccines are licensed: capsular polysaccharide pneumococcal vaccine (PPV) and protein–polysaccharide conjugate pneumococcal vaccine (PCV). Although the former includes serotypes responsible for most cases of pneumococcal diseases, the humoral immune response produced by this type of vaccine is T independent, which is an

Abbreviations: PPV, capsular polysaccharide pneumococcal vaccine; PCV, protein–polysaccharide conjugate pneumococcal vaccine; *S. pneumoniae*, *Streptococcus pneumoniae*; PppA, pneumococcal protective protein A; LcM, heat-killed-*Lactobacillus casei*; LcM + PppA, heat-killed-*Lactobacillus casei* + pneumococcal protective protein A; *L. casei*, *Lactobacillus casei* CRL431; MRS, Man-Rogosa-Sharpe; PBS, phosphate buffer saline; THB, Todd Hewitt Broth; PppA, pneumococcal protective protein; rPppA, recombinant PppA; LPS, lipopolysaccharide; dpi, days post infection; ALT, alanine transaminase; LDH, lactate dehydrogenase; BAL, bronchoalveolar lavage; CPR, C reactive protein; Ab, antibody; Ag, antigen; RSV, respiratory syncytial virus; NALT, nasopharynx-associated lymphoid tissue.

* Corresponding author at: Instituto de Microbiología, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), Ayacucho 471, CP4000 San Miguel de Tucumán, Tucumán, Argentina. Tel.: +54 381 156583999.

E-mail addresses: mgalmada@ucdavis.edu (G. Almada), anaharo@fbqf.unt.edu.ar (C. Haro), eovintini14@yahoo.com.ar (E. Vintiñi), marcemedina74@yahoo.com.ar (M. Medina).

http://dx.doi.org/10.1016/j.imbio.2014.08.013
0171-2985/

important disadvantage. Moreover, the PPV immunization does not protect children less than 2 years of age and adults over 65. The latter vaccine has a small number of polysaccharides chemically conjugated to proteins acting as carriers, which allows it to produce a T dependent humoral response. At present, there are three types of PCV vaccines developed against the most common pneumococcal serotypes. Even though these vaccines have had a positive impact and have succeeded in reducing the incidence of infections, they have failed to eradicate pneumococcal infections (Moffitt and Malley, 2011; Pletz et al., 2008). Furthermore, the main problem regarding current vaccines is that they are serotype dependent. This implies that protection is only against serotypes whose antigens were incorporated into the formulation of the vaccine. Hence, the development of new strategies continues to be a challenge and nasal vaccines would represent a valuable option to prevent infections produced by respiratory pathogens, whose host colonization occurs along the upper respiratory tract.

An interesting alternative vaccine against pneumococcal would be the use of pneumococcal proteins conserved within most serotypes of *S. pneumoniae*, which would be responsible for developing in the host an immune response at both mucosal and systemic levels. In this sense, previous studies have shown that some pneumococcal antigens expressed in certain strains of *Lactobacillus* succeeded in protecting the host effectively against an infection produced by *S. pneumoniae* in a mouse model (de Lúcia Hernani et al., 2011; Oliveira et al., 2006; Campos et al., 2008). In addition, a prior study demonstrated the effectiveness of a nasal experimental vaccine that consisted in a pneumococcal protective protein A (PppA), conserved within several *S. pneumoniae* serotypes, which was co-administrated with heat-killed-*Lactobacillus casei* (LcM) as an adjuvant, in order to reinforce the mucosal immune response (Vintiñi and Medina, 2011). In addition, a prior study demonstrated the effectiveness of a nasal experimental vaccine that consisted in a pneumococcal protective protein A (PppA), conserved within several S. pneumoniae serotypes, which was co-administrated with heat-killed-*L. casei* (LcM) as an adjuvant, in order to reinforce the mucosal immune response (Vintiñi and Medina, 2011). This vaccine was able to prevent a systemic pneumococcal infection, increasing mice survival and producing high levels of IgA and IgG anti-PppA in both systemic and mucosal compartments. However, the assessment of vaccine safety is a critical aspect that must be considered before their potential use in human health. The aim of the present work was to assess the safety of the experimental vaccine LcM + PppA and its components through hematological, biochemical and immune parameters in a model infection with *S. pneumoniae*.

Materials and methods

Experimental animals

Three-week-old male Swiss albino mice were obtained from the closed colony at CERELA. Each experimental group consisted of 6 mice in each evaluated period. During all the experiments, animals were kept under controlled environmental conditions with light–dark cycles of 12 h and were supplied with balanced rodent food and water *ad libitum*. Experiments were approved by the Animal Care and Ethics committee at CERELA.

Microorganisms and culture conditions

Lactobacillus casei (*L. casei*) CRL 431, obtained from the CERELA culture collection, was cultured for 18 h at 37 °C (final log phase) in Man-Rogosa-Sharpe broth (MRS, Oxoid), then harvested and washed twice with sterile 0.01 M phosphate buffer saline (PBS), pH 7.2. The bacterial suspension was adjusted to the desired concentration (10^9 cell/day/mouse). Finally, bacteria were heated in a water bath at 80 °C for 30 min and the lack of bacterial growth was confirmed by using MRS agar plates (heat-killed *L. casei*: LcM). This lactic acid bacterium was chosen as adjuvant for the vaccine formulation because of its immunomodulatory activity (Maldonado Galdeano and Perdigón, 2006; Racedo et al., 2006).

S. pneumoniae (serotype 14) was kindly provided by the Clinical Bacteriology Laboratory of Hospital del Niño Jesús de Tucumán, Argentina. Pneumococci serotypification was performed by Administración Nacional de Laboratorios e Institutos de Salud-ANLIS "Dr. Malbran", Buenos Aires, Argentina. *S. pneumonia*e was cultured for 18–20 h at 37 °C in microaerophilic atmosphere. Freshly grown colonies of were suspended in THB and incubated at 37 °C. Pathogens were harvested, centrifuged at 5000 rpm for 10 min at 4 °C and washed three times with sterile PBS. Then, the cell concentration of the pathogen was adjusted 10^6 cells/mouse.

Pneumococcal antigen: recombinant PppA (rPppA) was purified by using a His-Bind purification kit (Novagen) and visualized by electrophoresis on 12% SDS polyacrylamide gels. The reagent and PppA solution were below the limit of detection (2 pg/ml) when tested by the E-toxate test for LPS (Sigma). rPppA concentration was determined by Bradford method and 5 μg of this protein was used in immunization protocols (Medina et al., 2008).

Nasal immunization protocol

Young mice were nasally immunized with 50 μL of a mixture of 5 μg of PppA plus heat-killed *L. casei* (LcM) (10^9 cell/day/mouse) as the mucosal adjuvant (LcM + PppA). The immunization was carried out by using a protocol consisting in 3 successive administrations, which included two consecutive days each time with a 14 day interval between each administration (days 0–1, 14–15 and 28–29). Groups that received LcM, PppA and phosphate buffer saline solution (PBS) were used as controls.

Experimental infection

Experimental respiratory infection was induced 14 days after the 3rd immunization. Control and immunized mice were infected intranasally with the pathogen by dripping 25 μL of an inoculum containing 10^6 cells/mouse of *S. pneumoniae* in PBS into each nostril and allowing it to be inhaled. Mice were sacrificed and samples were collected the day before the infection (day 0) and on day 5 and day 10 after the infection had been caused (5 dpi and 10 dpi, respectively). Days 0, 5 and 10 after infection corresponded to days 42, 47 and 52 after the 1st immunization, respectively.

Alanine transaminase (ALT) activity

Enzymatic activity was measured in mouse serum by spectrophotometric kinetic assay (Roche Diagnostics, Mannheim, Germany). Results were expressed as U/L.

Creatinine evaluation

Enzymatic activity was measured in mouse serum by spectrophotometric kinetic assay (Wiener Lab). Results were expressed as mg/dL.

Lactate dehydrogenase (LDH) activity

LDH was measured in bronchoalveolar lavage (BAL) by the formation of a reduced form of nicotinamide adenine dinucleotide using Roche Diagnostic reagents and procedures (Wiener Lab). For BAL collection, trachea was exposed, intubated with a catheter,

and 2 sequential lavages were performed in each mouse by injecting 0.5 mL of sterile PBS. The recovered fluid was centrifuged for 10 min at 3000 rpm (Racedo et al., 2006; Medina et al., 2008). The supernatant fluid was frozen at −70 °C for subsequent biochemical analyses (Bergeron et al., 1998). Results were expressed as U/L of BAL fluid.

C reactive protein detection

This parameter was measured in serum samples by imunoturbidimetric ultrasensitive assay (Roche Diagnostics, Mannheim, Germany). Results were expressed as mg/L.

Corticosterone evaluation

This parameter was measured in serum samples by using a competitive assay based on ultrasensitive electrogenerated chemiluminescence (Roche Diagnostics, Mannheim, Germany). Results were expressed as μg/dL.

Total and differential blood leukocyte counts

Blood samples were obtained by cardiac puncture from sodium pentobarbital anesthetized animals and collected in tubes containing EDTA as an anticoagulant. Total number of leukocytes was determined with a hemocytometer. Differential cell counts were performed by counting 200 cells in blood smears stained with May Grünwald–Giemsa by using a light microscope (100×), and absolute cell numbers were calculated (Dacie and Lewis 1995).

Total and differential BAL leukocyte counts

BAL samples were obtained according to the technique described previously. The recovered fluid was centrifuged for 10 min at 900 × *g*. The supernatant fluid was frozen at −70 °C for subsequent biochemical analyses and the pellet was used to perform the smears. The total number of white blood cells (WBC) was determined with a hemocytometer. Differential cell counts were performed by counting 200 cells in BAL smears stained with May Grünwald–Giemsa using a light microscope (100×) and absolute numbers were calculated (Dacie and Lewis 1995).

IgE

Antibody analyses were performed by using Mouse IgE Ready-Set-Go! ELISA (eBioscience, Vienna, Austria) commercial kit. Manufacturer directions were thoroughly followed. Results were expressed as ng/mL.

Statistical analysis

Experiments were performed in triplicate and results were expressed as mean ± SD. After verification of a normal distribution of data, one- and two-way ANOVA was used. Tukey's test (for pair wise comparisons of the mean values) was used to test for differences between the groups. Differences were considered significant at $P < 0.05$.

Results

Liver, kidney and pulmonary function evaluation

Different parameters were evaluated in this vaccine-infection model in order to analyze whether the protective response induced by experimental nasal vaccine was able to protect the function of vital organs involved in body organism homeostasis such as liver, kidney and lungs. In this sense, the possible adverse manifestations that could appear with the immune response induced by vaccination in infected animals would be made clear.

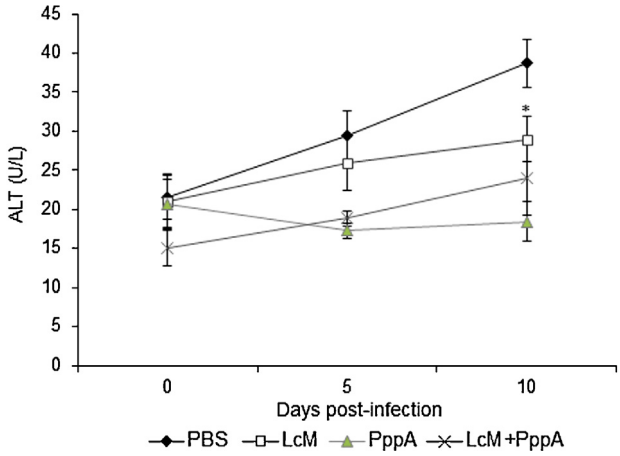

Fig. 1. ALT activity. Animals were divided in 4 groups and were nasally inoculated with PBS (control), LcM (heat-killed *L. casei*), PppA (pneumococcal protein A) and the experimental vaccine LcM + PppA, respectively. Once immunization period was over, all the groups were challenged against *S. pneumoniae*. ALT concentration was determined on the day before the infection with the pathogen (day 0) and on days 5 and 10 after the infection in serum. Results were expressed as means ± SD ($n = 6$). *Significantly different from the PBS group ($P < 0.05$).

Liver function

In order to assess liver normal functionality in the present model, Alanine Transaminase (ALT) activity was employed as injury marker. Results before infection (day 0), showed ALT levels were similar in all groups studied. After the infection with *S. pneumoniae*, PBS control group showed a progressive increase in the levels of this enzyme during all the evaluated period (Fig. 1). A similar behavior was observed among groups which received the probiotic (LcM) and the experimental vaccine (LcM + PppA). However, LcM + PppA and its components (LcM, PppA) showed significantly lower values than control group at day 10 post infection.

Kidney function

Creatinine circulates freely in blood and urine and its values remain relatively constant at all times. This nitrogen compound is excreted from the organism through our kidneys thus systemic high values of this parameter indicate an abnormal kidney function. In order to study kidney function, serum levels of creatinine were analyzed and these values remained constant and similar to basal levels during the evaluated period and in all the experimental groups, before and after pneumococcal infection (Fig. 2).

Pulmonary cytotoxicity

LDH activity measured in acellular BAL fluid was used as a lung cytotoxicity marker. LDH enzyme is regularly present in the cytoplasm of living cells and is released into the extracellular space when the cell membrane is affected by a toxic agent. Previous to pneumococcal infection, LcM, PppA and LcM + PppA did not cause any significant change in LDH concentration in comparison to unimmunized control mice. After pathogen challenge and because of the infection, control group LDH levels suffered an increase, reaching a peak at 5 dpi. Although values decreased by 10 dpi, it did not reach basal levels again. Similar behavior was observed in LcM group. In contrast, LcM + PppA group showed a moderate LDH increase at 5 dpi with significant lower levels ($P < 0.05$) than unimmunized control; these levels remain constant at day 10 post infection (Fig. 3).

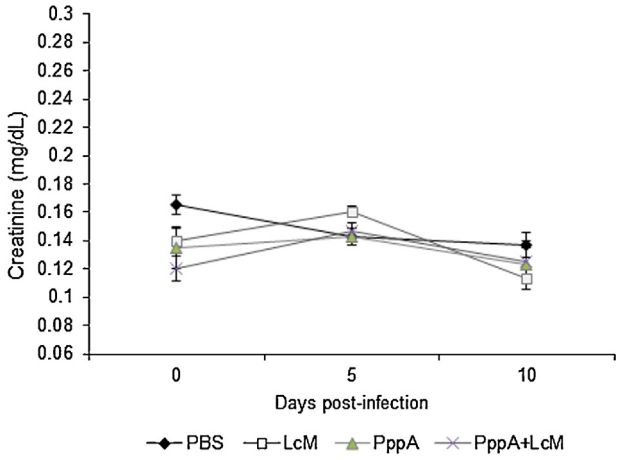

Fig. 2. Creatinine concentration. Animals were divided in 4 groups and were nasally inoculated with PBS (control), LcM (heat-killed *L. casei*), PppA (pneumococcal protein A) and the experimental vaccine LcM + PppA, respectively. Once immunization period was over, all the groups were challenged against *S. pneumoniae*. Creatinine concentration was determined on the day before the infection with the pathogen (day 0) and on days 5 and 10 after the infection in serum. Results were expressed as means ± SD ($n = 6$).

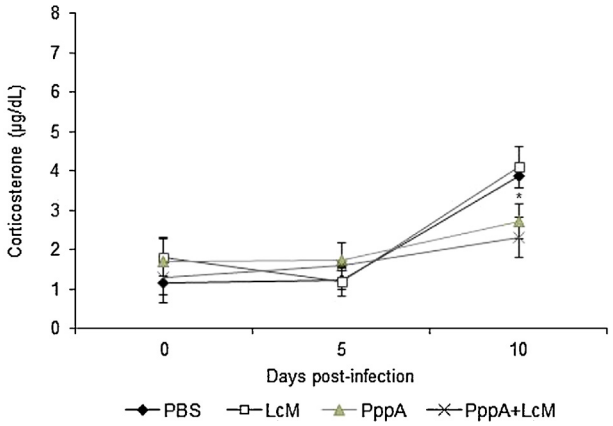

Fig. 4. Corticosterone concentration. Animals were divided in 4 groups and were nasally inoculated with PBS (control), LcM (heat-killed *L. casei*), PppA (pneumococcal protein A) and the experimental vaccine LcM + PppA, respectively. Once immunization period was over, all the groups were challenged against *S. pneumoniae*. Corticosterone concentration was determined on the day before the infection with the pathogen (day 0) and on days 5 and 10 after the infection in serum. Results were expressed as means ± SD ($n = 6$). *Significantly different from the PBS group ($P < 0.05$).

Evaluation of post-vaccine stress and inflammation

Assessment of C reactive protein: acute phase reactant

CPR is a plasmatic protein which participates in the systemic response to swelling; is an important acute phase reactant. For this matter CRP was used in this experimental model as an inflammatory marker. Before infection, the experimental vaccine and its components did not cause an increment in CPR values. After pathogen challenge and at 5 dpi, all groups studied showed a little increase tendency in the protein levels, without presenting significant differences between them. However, control group exhibited higher levels than LcM + PppA group at 5 dpi. This slight increase of CPR levels could have been caused by the pathogen entrance and the inflammatory response triggered by the infection (figure not showed).

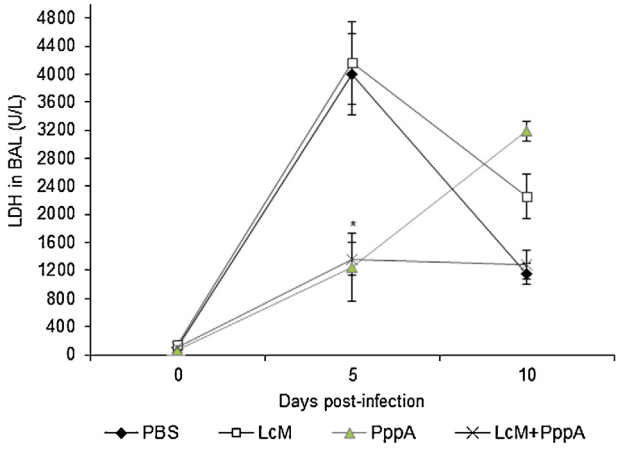

Fig. 3. LDH activity. Animals were divided in 4 groups and were nasally inoculated with PBS (control), LcM (heat-killed *L. casei*), PppA (pneumococcal protein A) and the experimental vaccine LcM + PppA, respectively. Once immunization period was over, all the groups were challenged against *S. pneumoniae*. LDH concentration was determined on the day before the infection with the pathogen (day 0) and on days 5 and 10 after the infection in BAL. Results were expressed as means ± SD ($n = 6$). *Significantly different from the PBS group ($P < 0.05$).

Assessment of corticosterone as a stress post-vaccine parameter

Corticosterone is a glucocorticoid release by mice's adrenal cortex in response to a stressful situation, analogous to human cortisol. The pathogen entrance to the body is a stressful stimulus which causes plasmatic corticosterone levels to increase influencing host immune response against the pathogen. In addition, a vaccine is considered a stressful stimulus because a foreign agent enters the body. A cortisol increase is expected in vaccinated and infected individuals who were immunized with the pathogen. This behavior is part of a normal physiologic response; however, serum cortisol levels should not rise due to its immune suppressor effect. For all the considerations stated above, serum corticosterone levels were also tested. Results exhibited in all the experimental groups showed similar corticosterone levels before infection as well as at 5 dpi. On day 10 dpi, both PppA and LcM + PppA groups, exhibited significant lower levels ($P < 0.05$) than control and LcM groups of this parameter (Fig. 4).

Evaluation of blood and BAL's Leukocyte Population

Leukocytes are part of the innate immune response which is the first line of defense against infections or foreign bodies. In this vaccine-infection model, the pathogen colonizes the host's respiratory tract and it can even reach systemic circulation. In order to evaluate if this experimental vaccine causes a regulated innate immune response against pneumococcus, leukocytes kinetics were assessed in lung and peripheral blood.

Leukocyte evaluation at pulmonary level

Pneumococcal infection led to an increase of total leukocyte count in BAL in all the groups studied. This increase reached a peak at 5 dpi before returning to basal levels at 10 dpi. However, LcM + PppA group showed a statistically significant increase compared to control group before pathogen's challenge and at 5 dpi ($P < 0.05$) (Fig. 5). Thus, before infection, LcM + PppA induced a significant increment of lymphocytes in comparison to control group, whereas neutrophils were the dominant white cells at 5 dpi. Also, at 5 dpi, a significant increase of eosinophils count was observed within the LcM, PppA and LcM + PppA groups. However, those values revealed a decrease toward 10 dpi while the control group showed an increase of this particular leukocyte (Table 1).

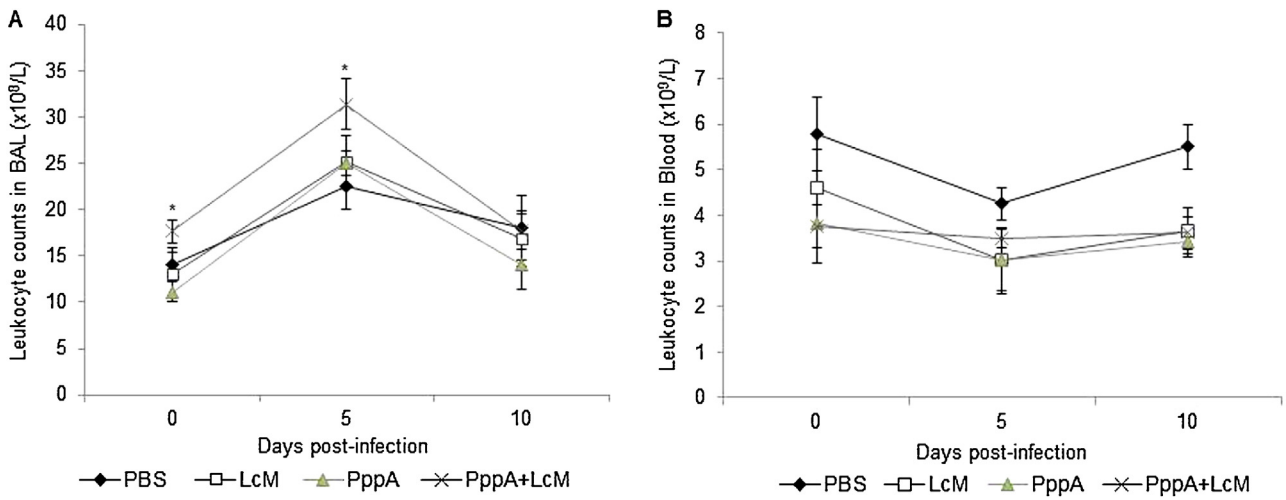

Fig. 5. Total leukocyte counts. Animals were divided in 4 groups and were nasally inoculated with PBS (control), LcM (heat-killed *L. casei*), PppA (pneumococcal protein A) and the experimental vaccine LcM + PppA, respectively. Once immunization period was over, all the groups were challenged against *S. pneumoniae*. Leukocyte counts were determined on the day before the infection with the pathogen (day 0) and on days 5 and 10 after the infection in BAL (A) and in blood (B).

Table 1
Leukocyte differential count in bronchoalveolar lavages (BAL). Animals were divided in 4 groups and were nasally inoculated with PBS (control), LcM (*L. casei* heat killed), PppA (pneumococcal protein A) and the experimental vaccine LcM + PppA, respectively. Once immunization period was over, all the groups were challenged against *S. pneumoniae*. BAL samples were obtain on day 0 (previous infection) and on days 5 and 10 post infection; cells were count in smears stained with May Grünwald–Giemsa. Different letters (a–c, a′–c′, a″–d″, aˆ–dˆ) indicates significant differences between experimental groups.

Experimental group	Time	Macrophages BAL ($\times 10^8$/L)	Neutrophils BAL ($\times 10^8$/L)	Lymphocytes BAL ($\times 10^8$/L)	Eosinophils BAL ($\times 10^8$/L)
PBS	0 dpi	11.44 ± 1.11^{a}	$1.57 \pm 0.41^{a'}$	$0.83 \pm 0.24^{a''}$	$0.27 \pm 0.02^{a\hat{}}$
LcM		10.74 ± 2.13^{a}	$1.68 \pm 0.15^{a'}$	$0.63 \pm 0.03^{a''}$	$0.19 \pm 0.09^{a\hat{}}$
PppA		8.55 ± 1.20^{a}	$1.34 \pm 0.21^{a'}$	$0.41 \pm 0.08^{a''}$	$0.18 \pm 0.07^{a\hat{}}$
LcM + PppA		12.68 ± 1.84^{a}	$2.56 \pm 0.75^{a'}$	$2.28 \pm 0.32^{b''}$	$0.17 \pm 0.01^{a\hat{}}$
PBS	5 dpi	20.38 ± 2.52^{b}	$1.8 \pm 0.18^{a'}$	$0.25 \pm 0.09^{c''}$	$0.22 \pm 0.02^{a\hat{}}$
LcM		19.39 ± 2.81^{b}	$3.18 \pm 0.70^{b'}$	$0.56 \pm 0.06^{a''}$	$1.12 \pm 0.10^{b\hat{}}$
PppA		18.03 ± 2.98^{b}	$3.27 \pm 0.60^{b'}$	$0.60 \pm 0.09^{a''}$	$1.11 \pm 0.13^{b\hat{}}$
LcM + PppA		24.2 ± 2.75^{b}	$4.4 \pm 0.54^{c'}$	$1.25 \pm 0.17^{d''}$	$1.82 \pm 0.51^{b\hat{}}$
PBS	10 dpi	11.79 ± 0.14^{a}	$2.35 \pm 0.55^{b'}$	$0.1 \pm 0.02^{a''}$	$1.86 \pm 0.35^{c\hat{}}$
LcM		10.74 ± 0.23^{a}	$2.43 \pm 0.30^{b'}$	$0.43 \pm 0.1^{a''}$	$0.64 \pm 0.15^{d\hat{}}$
PppA		10.40 ± 0.96^{a}	$2.47 \pm 0.64^{b'}$	$0.22 \pm 0.07^{c''}$	$0.91 \pm 0.10^{b\hat{}}$
LcM + PppA		13.53 ± 1.53^{a}	$2.27 \pm 0.62^{b'}$	$0.90 \pm 0.12^{a''}$	$0.96 \pm 0.15^{b\hat{}}$

Leukocyte evaluation at systemic level

Total blood leukocyte behavior was similar in all the experimental groups throughout the whole experiment; these cells exhibited a decrease at 5 dpi and then returned to basal levels by the 10 dpi (Fig. 5). To assess possible changes in leukocyte population, differential count on peripheral blood was also performed. Results showed that before challenge, only those mice which received experimental vaccine exhibited a significant lower lymphocyte count ($P < 0.05$) compared to control group, whereas no changes were observed in other types of white cells population. At 5 dpi, control group showed a decrease on lymphocyte population but an increase on monocyte population. By day 10 post infection, these

Table 2
Leukocyte differential count in peripheral blood. Animals were divided in 4 groups and were nasally inoculated with PBS (control), LcM (*L. casei* heat killed), PppA (pneumococcal protein A) and the experimental vaccine LcM + PppA, respectively. Once immunization period was over, all the groups were challenged against *S. pneumoniae*. Blood samples were obtain on day 0 (previous infection) and on days 5 and 10 post infection; cells were count in blood smears stained with May Grünwald–Giemsa. Different letters (a–b, a′, a″–d″, aˆ–bˆ) indicates significant differences between experimental groups.

Experimental group	Time	Neutrophils BAL ($\times 10^9$/L)	Eosinophils BAL ($\times 10^9$/L)	Lymphocytes BAL ($\times 10^9$/L)	Monocytes BAL ($\times 10^9$/L)
PBS	0 dpi	0.94 ± 0.18^{a}	$0.1 \pm 0.02^{a'}$	$4.9 \pm 0.48^{a''}$	$0.07 \pm 0.01^{a\hat{}}$
LcM		1.07 ± 0.26^{a}	$0.09 \pm 0.03^{a'}$	$3.35 \pm 0.4^{a''}$	$0.1 \pm 0.04^{a\hat{}}$
PppA		0.61 ± 0.09^{a}	$0.2 \pm 0.08^{a'}$	$2.33 \pm 0.35^{a''}$	$0.07 \pm 0.02^{a\hat{}}$
LcM + PppA		0.81 ± 0.20^{a}	$0.13 \pm 0.05^{a'}$	$2.81 \pm 0.31^{b''}$	$0.05 \pm 0.02^{a\hat{}}$
PBS	5 dpi	0.86 ± 0.14^{a}	$0.13 \pm 0.06^{a'}$	$2.86 \pm 0.30^{b''}$	$0.17 \pm 0.06^{b\hat{}}$
LcM		0.77 ± 0.56^{a}	$0.13 \pm 0.10^{a'}$	$2.05 \pm 0.24^{c''}$	$0.07 \pm 0.03^{a\hat{}}$
PppA		0.78 ± 0.25^{a}	$0.19 \pm 0.09^{a'}$	$1.94 \pm 0.05^{c''}$	$0.09 \pm 0.01^{a\hat{}}$
LcM + PppA		0.64 ± 0.23^{a}	$0.14 \pm 0.03^{a'}$	$2.67 \pm 0.10^{b''}$	$0.07 \pm 0.01^{a\hat{}}$
PBS	10 dpi	1.32 ± 0.10^{b}	$0.17 \pm 0.07^{a'}$	$3.96 \pm 0.40^{d''}$	$0.05 \pm 0.01^{a\hat{}}$
LcM		0.7 ± 0.10^{a}	$0.14 \pm 0.07^{a'}$	$2.67 \pm 0.30^{b''}$	$0.14 \pm 0.06^{a\hat{}}$
PppA		0.66 ± 0.24^{a}	$0.16 \pm 0.08^{a'}$	$2.74 \pm 0.10^{b''}$	$0.05 \pm 0.02^{a\hat{}}$
LcM + PppA		0.69 ± 0.19^{a}	$0.23 \pm 0.10^{a'}$	$2.56 \pm 0.32^{b''}$	$0.13 \pm 0.05^{a\hat{}}$

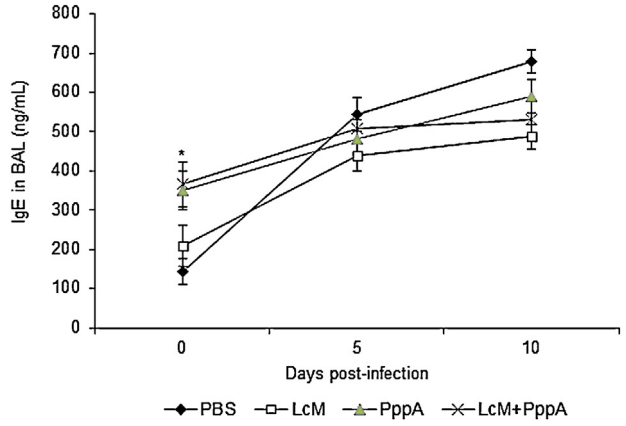

Fig. 6. IgE concentration. Animals were divided in 4 groups and were nasally inoculated with PBS (control), LcM (heat-killed *L. casei*), PppA (pneumococcal protein A) and the experimental vaccine LcM + PppA, respectively. Once immunization period was over, all the groups were challenged against *S. pneumoniae*. IgE concentration was determined the day before the infection with the pathogen (day 0) and on days 5 and 10 after the infection. Results were expressed as means ± SD ($n = 6$). *Significantly different from the PBS group ($P < 0.05$).

values returned to basal levels while neutrophils levels experienced a significant increase in comparison to 0 dpi. Experimental vaccine group experienced an increase in monocyte population at 10 dpi, but no significant changes were detected among the rest of leukocyte populations (Table 2).

IgE as a marker of potential allergy trigger

In order to dismiss potential allergic effects as a vaccination result, total IgE levels were studied in BAL. A high production of this immunoglobulin, together with an eosinophil increase in the respiratory tract, may trigger adverse effects to the vaccinated host's airways.

LcM + PppA immunization caused a significant increase of total IgE levels ($P < 0.05$) compared to control group. A similar behavior was observed in the group which received a nasal dosis of PppA. This component might be the cause of the increase. After infection, control, LcM and PppA groups showed a progressive increment of total IgE values. However, immunized mice with experimental vaccine displayed significant lower levels of total IgE at 10 dpi compared with non-immunized mice (Fig. 6).

Discussion

At present, only a few vaccines are administered through mucosal routes, even when mucous membrane constitutes the main gateway for most pathogens that affect humans. The aim of the present study was to evaluate the safety of a nasal vaccine (LcM + PppA) against *S. pneumoniae* in a vaccination–infection model. This experimental vaccine demonstrated to be efficient in specific antibody production at both systemic and mucosa level (Vintiñi and Medina, 2011). However, a crucial aspect which must be evaluated prior to human studies is to ensure safety in a host that was infected with pneumococcus after vaccine was administered. Nasal vaccination has the capacity to produce a powerful immune response in mucosal and systemic compartments. This response should be effective in the pathogen's eradication but also must be regulated to avoid adverse effects in the host. Thus, in our model, through basic parameters we evaluated liver, liver, kidney and pulmonary function in order to discard possible diseases caused by the experimental vaccine in an infected host. Pneumococcal pneumonia can induce hepatic enzyme alterations such as transaminases alteration (Marrie, 1999). In our infection model, the pneumococcal bacterium reaches the bloodstream in unvaccinated mice (Vintiñi and Medina, 2011), increasing systemic ALT levels. This increase may be associated with an inflammatory process triggered at a systemic level in order to try to control both the infection and the direct effect of the pathogen on the hepatic parenchyma (Marrie, 1999). In contrast, experimental vaccine and its components did not cause serum ALT levels to rise. The absence of unwanted side effects in the liver could be explained by the vaccine's ability to prevent systemic pneumococcal spread in a murine model (Vintiñi and Medina, 2011); hence, the liver damage associated with pneumococcal pneumonia would be prevented. Moreover, renal function assessed by serum creatinine levels was preserved in our model.

Lung is pneumococci's target organ; therefore, the vaccine must not only be effective but also preserve its functionality and promote the clearance of the pathogen. In pneumococcal infection, LDH levels in BAL correlate with an increase of cell lysis in lung. This tissue damage is associated with the pathogen's inherent factors (virulence factors) and the inflammatory response generated by the host to cope with the infection. LDH evaluation levels in BAL proved the vaccine to produce a protective effect on lung parenchyma in relation to what was observed in the infected control group. PppA would exert a similar effect because of the moderate LDH levels released. This result would be associated with the presence of anti-PppA IgA and IgG produced by the nasal administration of PppA (Vintiñi and Medina, 2011). In this sense, specific Ab levels induced by PppA were considerably lower than Ab levels induced by the experimental vaccine (LcM + PppA). This would explain LDH's rise at 10 dpi in the group that was administered with PppA in comparison with the vaccinated group. LcM induced similar LDH levels as the control group at 5 dpi but expressed higher levels at 10 dpi. This fact would be due to innate immune response induced by LcM, more potent compared with LcM + PppA. In this sense, kinetics observed in vaccinated mice for LDH would be the result of an effective specific humoral and cellular immune response against pneumococcal Ag; with an appropriate balance between regulation and activation of the immune system (Vintiñi and Medina, 2011). Both response and balance, in turn, limit lung tissue damage and promotes pathogen's clearance.

A controlled inflammatory response is essential to fight the infectious process without damaging the host tissues. CRP is an unquestionable marker of infection and would be involved in microorganism's clearance during acute phase period (Póvoa, 2002). A similar behavior was observed in all experimental groups, displaying a moderate increase at 5 dpi. Prior studies demonstrated that CRP secretion starts between 4 and 6 h after the body is stimulated and those values duplicate every 8 h and reach a peak between 36 and 50 h. When the stimulus is removed or it disappears, CRP values fall rapidly and return to basal levels, unless stimulation is maintained for long periods of time (Pepys and Baltz, 1983; Vigushin et al., 1993; Hogarth et al., 1997). In our model it is likely that CRP values have been significantly high within the first 24 h of infection since it is one of the earlier acute phase reactants that are raised. However, CRP was measured on day 5 and day 10 post infection because the stated objective was to assess whether the experimental vaccine would induce a prolonged and sustained inflammatory process in mice after infection with *S. pneumoniae* which would be detrimental to the host.

On the other hand, previous studies in infected children with respiratory syncytial virus (RSV) have shown an increase of plasma cortisol during the acute phase of infection which is parallel to a decrease in INF-γ and IL-12 levels, both essential for anti-viral defense (Pinto et al., 2006). These findings suggest a link between the increment in plasma cortisol and the decrease of Th1 type responses. This would produce an imbalance in the ratio of CD4+ Th1/Th2 lymphocyte populations, which brings with it a number of negative effects on the host. Thus, an increment in plasma cortisol

could cause a decrease in Th1 products with an augmentation in Th2 products, such as IL-4 and IL-5, a fact which promotes IgE production and eosinophil influx that might trigger an allergic response in the host. Moreover, studies demonstrated a direct link between levels of plasma cortisol and a decline in the humoral specific immune response after vaccination (Cohen et al., 2001). In this model, an excessive and/or sustained increase of corticosterone would cause negative effects on the host because it would induce a down regulation of the immune response. As a result, Th1 cells and antibody production which are essential for defense against pneumococcus would decrease. In this immunization–vaccination model, the experimental vaccine reduced corticosterone levels accompanying pneumococcal infection due to its ability to efficiently and rapidly control the pneumococcal infection (Vintiñi and Medina, 2011). Further studies are necessary to understand the association between a post-infection hormone increase and the specific immune response induced by the experimental vaccine.

Normal physiologic response against a pathogen's entrance consists in an augmentation of leucocytes involved in inflammation. Inflammatory response represents a key process for the defense against infectious microorganisms but its magnitude must be moderate in order to avoid excessive tissue damage. In the model used in the present work, the pathogen colonizes upper and lower respiratory tract. In this way it invades the bloodstream, and this is the reason why leukocyte's kinetics in lung and peripheral blood was assessed. Our nasal vaccination promoted leukocyte's migration from peripheral blood to the lung in order to protect the body against a possible infection. This behavior was reflected in the increase of leukocytes at 5 dpi observed in LcM + PppA group in comparison with control group ($P < 0.05$). PppA and LcM administration exhibited the same tendency, without expressing significant differences compared to control group. The increment observed within the first half of the tested period corresponds to a leukocyte's migration to the lung, i.e., the infection site. This migration, in turn, would correlate with pneumococci's pulmonary colonization caused by the innate immune response in order to contain infection and prevent the pathogen to spread. The results of the analysis have shown that leukocyte increase, as a result of vaccination, occurred mainly at the expense of neutrophils and lymphocytes augmentation. Vaccine components: LcM and PppA showed a similar kinetic to the vaccinated group; however, the number of neutrophils at 5 dpi was significantly lower than the one induced by the experimental vaccine. Neutrophils' increase would be mainly associated to pathogen's elimination through phagocytosis with controlled pulmonary tissue damage, as it was also shown by LDH levels which were considerably lower in the immunized mice group than in the control group. With regard to lymphocyte population in LBA, the group which received the experimental vaccine exhibited a different kinetics compared to what was observed for neutrophils. Thus, lymphocyte increase induced by LcM + PppA would be associated to its movement from the nasopharynx-associated lymphoid tissue (NALT) (Vintiñi and Medina, 2014) as a result of both direct stimulation and the migration of circulating lymphocytes. Previous studies demonstrated that LcM + PppA induces an activation of T lymphocytes cell population and also an increase of IgA and IgG specific for the antigen (Vintiñi and Medina, 2011, 2014) in nasal lavages, bronchoalveolar lavages and serum. On this basis, the increment of lymphocyte population in LBA would be due to the presence of T and B lymphocytes specific for Ag. In addition, macrophages, cells involved in both innate and adaptive immunity, showed a similar kinetics among all experimental groups evaluated without expressing significant differences between them. Thereby, the experimental vaccine induces an increase in the number of macrophages in LBA similar to the unvaccinated control although the number of activated macrophages within the vaccinated group may be higher. Past works demonstrated that LcM nasal administration increases activation of phagocytes in LBA (Villena et al., 2009). These cells would contribute to the pathogen's depuration by a direct phagocytosis and also promoting the recruitment of neutrophils from the bloodstream to the lung through biological mediators such as TNF-α e IL-1β (Van der Poll and Opal, 2009).

Eosinophils population is primary associated with extracellular parasite's defense and hypersensitivity or allergies reaction. However, these cells are also part of a depuration process since it also phagocyte Gram-positive bacteria and Gram-negative bacteria. Eosinophils are considered multifunctional leukocytes which contribute to a wide variety of physiological and pathological processes, depending on its localization and activation. It has the ability to produce immunoregulatory cytokines that are actively involved in the regulation of Th2 responses. On these occasions, eosinophils are recruited to sites of inflammation where it produces cytokines, lipid mediators and releases proteins from its toxic granulates (Kita, 2011, 2013). Based on eosinophils' potential to participate in processes related to the body defense as well as in pathological processes such as allergies, its evaluation was very important to determine in our vaccination–infection model, especially in the lungs. The experimental vaccine and it compounds induced a significant increment of eosinophils at 5 dpi but a significant decrease was observed at 10 dpi, in comparison to the control for that same day. Eosinophils' increase happened earlier in the vaccinated group than in unimmunized control, which would be associated more with host defense functions rather than allergic processes, attaining in this way a more efficient and earlier control of the pneumococcal infection. Further studies are required in order to affirm eosinophils' implication in the host defense within our model.

Overall, leukocyte increment induced by the experimental vaccine in the pulmonary compartment was mainly mediated by neutrophils and lymphocytes with scarce participation of macrophages and eosinophils. The increase of neutrophils would be an essential component of the necessary inflammatory response in order to reduce pulmonary colonization. In addition, increased lymphocytes and its participation in the innate immune response would be mainly mediated by IgA+ B cells. In this regard, several studies have shown that certain strains of lactic acid bacteria, including *L. casei* used in this work, tend to increment mucosal's IgA+ cells (Vintiñi and Medina, 2014; Maldonado Galdeano et al., 2011; Perdigón et al., 1999), thus raising the levels of secretory IgA. The bind between IgA and *S. pneumoniae* minimizes the entry of the latter into the body by immune exclusion. Furthermore, previous studies have shown that LcM + PppA induces high levels of anti-PppA IgG and IgA in LBA, (Vintiñi and Medina, 2011) for which antigen specific B cells would be responsible for lymphocytes increase in the lungs as part of the adaptive immune response induced by vaccination. These physiologic and specific antibodies (secretory IgA) would diminish the induction of an exacerbated inflammatory response, preventing potentially harmful effects in lung tissue.

On a systemic level, the experimental vaccine causes minor changes in peripheral blood leukocyte populations except for the drop in lymphocyte counts which migrate to lung. LcM + PppA does not induce a sustained inflammatory process on a systemic level, which correlates with CRP, corticosterone, ALT and creatinine values evaluated in serum. In a previous work it was demonstrated that LcM + PppA induces a Th1, Th2 and Th17 lymphocyte population's stimulation. CD4+Th2 lymphocyte population is characterized for producing IL-4 cytokine besides IL5 and IL-13 cytokines. IL-4 cytokine production promotes the humoral immune specific response in our model of nasal administration with the experimental vaccine LcM + PppA. However, IL-4 and IL-13 cytokines stimulate IgE production whereas IL-5 stimulates eosinophils' development and activation. An elevated IgE

production, accompanied with an increase of eosinophils within the respiratory tract, could trigger allergy processes with harmful effects on the host airways. In our model, the experimental vaccine caused a significant increase in the eosinophils population, which was previously discussed. Additionally, on day 14 after the 3rd immunization was performed, total IgE ($P < 0.05$) from BAL samples exhibited a significantly increase compared to the control group. As it was mentioned before, this behavior would be a cause of the increment of IL-4 in BAL within the vaccinated group. A similar effect was observed within the group that received a nasal administration of PppA, a vaccine component which would be the main responsible factor for this effect. Nevertheless, after challenge against the pathogen, LcM + PppA showed IgE levels that were significantly lower than that of the control group at 10 dpi. This result might indicate that experimental vaccine would regulate Th2 response and IL-4 production through the induction of regulatory IL-10 cytokine, as it was proved in a prior study.

Based on the results of evaluated parameters, the experimental vaccine LcM + PppA nasally administered to prevent infections caused by *S. pneumoniae* does not induce harmful effects in our immunization–infection model. Studied parameters showed LcM + PppA's safety in liver, kidney, pulmonary and systemic levels. Although studies in experimental animals do not guarantee security for the application of the vaccine on humans, they are important evidence for the planning and subsequent clinical trials in humans.

Conflict of interest

None declared.

Acknowledgement

This work was supported by grants from PICT2010 No. 828.

References

Bergeron, Y., Ouellet, N., Deslauriers, A.M., Simard, M., Olivier, M., Bergeron, M.G., 1998. Cytokine kinetics and other host factors in response to pneumococcal pulmonary infection in mice. Infect. Immun. 66, 912–922.

Campos, I.B., Darrieux, M., Ferreira, D.M., Miyaji, E.N., Silva, D.A., Areas, A.P., Aires, K.A., Leite, L.C., Ho, P.L., Oliveira, M.L., 2008. Nasal immunization of mice with *Lactobacillus casei* expressing the Pneumococcal Surface Protein A: induction of antibodies, complement deposition and partial protection against *Streptococcus pneumoniae* challenge. Microbes Infect. 10, 481–488.

Cohen, S., Miller, G.E., Rabin, B.S., 2001. Psychological stress and antibody response to immunization: a critical review of the human literature. Psychosom. Med. 63, 7–18.

Dacie, J., Lewis, S., 1995. Basic haematological techniques. In: Dacie, J.V., Lewis, S.M. (Eds.), Practical haematology. , 8th ed. Churchill Livingstone, Philadelphia, Penn, pp. 49–82.

de Lúcia Hernani, M., Ferreira, P.C., Ferreira, D.M., Miyaji, E.N., Ho, P.L., Oliveira, M.L., 2011. Nasal immunization of mice with *Lactobacillus casei* expressing the pneumococcal surface protein C primes the immune system and decreases pneumococcal nasopharyngeal colonization in mice. FEMS Immunol. Med. Microbiol. 62, 263–272.

Hogarth, M.B., Gallimore, R., Savage, P., Palmer, A.J., Starr, J.M., Bulpitt, C.J., Pepys, M.B., 1997. Acute phase proteins, C-reactive protein and serum amyloid A protein, as prognostic markers in the elderly inpatient. Age Ageing 26, 153–158.

Kita, H., 2011. Eosinophils: multifaceted biologic properties and roles in health and disease. Immunol. Rev. 242, 161–177.

Kita, H., 2013. Eosinophils: multifunctional and distinctive properties. Int. Arch. Allergy Immunol. 161, 3–9.

Lynch, J.P., Zhanel, G.G., 2009. *Streptococcus pneumoniae:* Epidemiology. Risk Factors, and Strategies for Prevention. Semin. Respir. Crit. Care Med. 30, 189–209.

Maldonado Galdeano, C., Novotny Núñez, I., de Moreno de LeBlanc, A., Carmuega, E., Weill, R., Perdigón, G., 2011. Impact of a probiotic fermented milk in the gut ecosystem and in the systemic immunity using a non-severe protein-energy-malnutrition model in mice. BMC Gastroenterol. 26 (11), 64.

Maldonado Galdeano, C., Perdigón, G., 2006. The probiotic bacterium *Lactobacillus casei* induces activation of the gut mucosal immune system through innate immunity. Clin. Vaccine Immunol. 13, 219–226.

Marrie, T.J., 1999. Pneumococcal pneumonia: epidemiology and clinical features. Semin. Respir. Infect. 14, 227–236.

Medina, M., Villena, J., Vintiñi, E., Hebert, E., Raya, R.R., Alvarez, S., 2008. Nasal immunization with *Lactococcus lactis* expressing the pneumococcal protective protein A induces protective immunity in mice. Infect. Immun. 76, 2696–2705.

Moffitt, K.L., Malley, R., 2011. Next generation pneumococcal vaccines. Curr. Opin. Immunol. 23, 407–413, http://dx.doi.org/10.1016/j.coi.2011.04.002.

Mulholland, K., 2007. Childhood pneumonia mortality: a permanent global emergency. Lancet 21, 285–289.

Oliveira, M.L., Areas, A.P., Campos, I.B., Monedero, V., Perez-Martinez, G., Miyaji, E.N., Leite, L.C., Aires, K.A., Ho, P.L., 2006. 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. Microbes Infect. 8, 1016–1024.

Pepys, M.B., Baltz, M.L., 1983. Acute phase proteins with special reference to C-reactive protein and related proteins (pentraxins) and serum amyloid A protein. Adv. Immunol. 34, 141–212.

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

Pinto, A.R., Arredondo, M.S., Bono, R.M., Gaggero, A.A., Díaz, V.P., 2006. T helper 1/T helper 2 cytokine imbalance in respiratory syncytial virus infection is associated with increased endogenous plasma cortisol. Pediatrics 117, e878.

Pletz, M.W., Maus, U., Krug, N., Welte, T., Lode, H., 2008. Pneumococcal vaccines: mechanism of action, impact on edpiemiology and adaption of the species. Int. J. Antimicrob. Agents 32, 199–206.

Póvoa, P., 2002. C-reactive protein: a valuable marker of sepsis. Intensive Care Med. 28, 235–243.

Racedo, S., Villena, J., Medina, M., Agüero, G., Rodríguez, V., Alvarez, S., 2006. *Lactobacillus casei* administration reduces lung injuries in a *Streptococcus pneumoniae* infection. Microbes Infect. 8, 2359–2366.

Scott, J.A., 2008. The global edpiemiology of childhood pneumonia 20 years on. Bull. World Health Organ. 86, 494–496.

Van der Poll, T., Opal, S.M., 2009. Pathogenesis, treatment, and prevention of pneumococcal pneumonia. Lancet 374, 1543–1556.

Vigushin, D.M., Pepys, M.B., Hawkins, P.N., 1993. Metabolic and scintigraphic studies of radioiodinated human C-reactive protein in health and disease. J. Clin. Invest. 91, 1351–1357.

Villena, J., Barbieri, N., Salva, S., Herrera, M., Alvarez, S., 2009. Enhanced immune response to pneumococcal infection in malnourished mice nasally treated with heat-killed *Lactobacillus casei*. Microbiol. Immunol. 53, 636–646.

Vintiñi, E., Medina, M., 2014. Immune response in nasopharynx, lung and blood elicited by nasal experimental pneumococcal vaccines containing live and heat-killed lactobacilli as mucosal adjuvants. Can. J. Physiol. Pharmacol. 92, 124–131.

Vintiñi, E.O., Medina, M.S., 2011. Host immunity in the protective response to nasal immunization with a pneumococcal antigen associated to live and heat-killed *Lactobacillus casei*. BMC Immunol. 12 (46), 1471–2172.