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Review

Lactic acid bacteria in the prevention of pneumococcal respiratory infection: Future opportunities and challenges

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ABSTRACT

Lactic acid bacteria (LAB) are technologically and commercially important and have various beneficial effects on human health. Several studies have demonstrated that certain LAB strains can exert their beneficial effect on the host through their immunomudulatory activity. Although most research concerning LAB-mediated enhanced immune protection is focused on gastrointestinal tract pathogens, recent studies have centered on whether these immunobiotics might sufficiently stimulate the common mucosal immune system to provide protection to other mucosal sites as well. In this sense, LAB have been used for the development of probiotic foods with the ability to stimulate respiratory immunity, which would increase resistance to infections, even in immunocompromised hosts. On the other hand, the advances in the molecular biology of LAB have enabled the development of recombinant strains expressing antigens from respiratory pathogens that have proved effective to induce protective immunity. In this review we examine the current scientific literature concerning the use of LAB strains to prevent respiratory infections. In particular, we have focused on the works that deal with the capacity of probiotic and recombinant LAB to improve the immune response against *Streptococcus pneumoniae*. Research from the last decade demonstrates that LAB represent a promising resource for the development of prevention strategies against respiratory infections that could be effective tools for medical application.

Contents

Abbreviation: α-NBE, α-naphthyl butyrate esterase; β-G, β-glucuronidase, AM: alveolar macrophages; BALF, bronchoalveolar lavage fluid; BALT, bronchus associated lymphoid tissue; BCD, balanced conventional diet; CPS, capsular polysaccharide; DCs, dendritic cells; GALT, gut associated lymphoid tissue; GEM, Gram-positive Enhancer Matrix; HSA, Heat Stable Antigen; IL, interleukine; INF, interferon; LAB, lactic acid bacteria; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MNC, malnourished control; MHC-II, major histocompatibility complex II; MIP, macrophage inflammatory protein; MPO, myeloperoxidase; NALT, nasal associated lymphoid tissue; NBT^+, nitro blue tetrazolium positive; NOD, nucleotide-binding oligomerization domain-like receptor; PCV-7, pneumococcal 7-valent conjugated vaccine; PFD, protein-free diet; PIgR, polymeric immunoglobulin receptor; PRRs, pattern recognition receptors; PpmA, Putative proteinase maturation protein A; PppA, pneumococcal protective protein A; PsaA, pneumococcal Surface Antigen A; PspA, pneumococcal Surface Protein A; PspC, pneumococcal Surface Protein C; SlrA, Streptococcal lipoprotein rotamase A; TLR, Toll-like receptor; TNF, tumor necrosis factor; WNC, well-nourished control.

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1. Introduction

Acute lower respiratory tract infections are a persistent public health problem. They cause a greater burden of disease worldwide than human immunodeficiency virus infection, malaria, cancer, or heart attacks [1]. *Streptococcus pneumoniae* is one of the microorganisms that cause severe respiratory infections. It is a common inhabitant of the upper respiratory tract, existing mainly as a commensal bacterium along with other co-resident microorganisms on the respiratory epithelium. Defects in host defenses can alter this host–pathogen interaction and allow strains to invade the host [2]. Pneumococcus is one of the principal aetiological agents of pneumonia, bacterial meningitis and otitis media and children, the elderly and immunocompromised hosts are particularly at high risk [2]. Despite appropriate therapies, mortality due to the different pneumococcal pathologies remains high: around 1 million children die every year from pneumococcal diseases, mainly in developing countries [3,4]. The rapid emergence of multidrug-resistant *S. pneumoniae* strains throughout the world has led to increased emphasis on the prevention of pneumococcal infections by vaccination. However, available vaccines present disadvantages associated with their low immunogenicity in populations at risk (i.e., the pneumococcal 23-valent polysaccharide vaccine) or with their high cost as a public health strategy in developing countries (i.e., conjugated vaccines) [5,6].

Two of the most important strategies for the prevention of infectious diseases are healthy nutrition and the use of effective vaccines. Historically, deaths from infections have been reduced by improvements in nutrition. Besides, during the last few decades, a large body of literature has established strong links between nutrition, immune function and infectious diseases. Moreover, the development of vaccines and their massive use have enabled the eradication of numerous infectious diseases in various parts of the world. Lactic acid bacteria (LAB) can be used for both strategies.

They have been used for the development of probiotic foods with the ability to stimulate the immune system, which would increase resistance to infections, even in immunocompromised hosts [7,8]. On the other hand, the advances in the molecular biology of LAB have enabled the development of recombinant strains expressing antigens from various pathogens that have proved effective to induce protective immunity. In this review we examine the current scientific literature dealing with the use of LAB strains to prevent respiratory infections. In particular, we have focused on the works that deal with the capacity of probiotic and recombinant LAB to improve the immune response against *S. pneumoniae*.

2. Improvement of respiratory immunity by probiotic lactic acid bacteria

2.1. Effect on immunocompetent hosts

Several studies have demonstrated that certain probiotic LAB strains can exert their beneficial effect on the host through their immunomudulatory activity [7–11]. Although most research concerning LAB-mediated enhanced immune protection is focused on gastrointestinal tract pathogens, a few recent studies have centered on whether immunobiotics might sufficiently stimulate the common mucosal immune system to provide protection to other mucosal sites as well [7,8,11,12]. In this sense, we studied the potential effect of probiotics on the improvement of the immune response against respiratory pathogens using an experimental model of *S. pneumoniae* infection in adult immunocompetent mice. In our experimental model, mice were infected intranasally with *S. pneumoniae* serotype 14. After the challenge, the pathogen was detected in lung and blood samples throughout the period assayed (15 days) [13]. In order to study tissue damage in lungs, biochemical and histological studies were performed in the respiratory tract of the infected mice. Challenge with pneumococci significantly increased bronchoalveolar lavage fluid (BALF), albumin concentration and lactate dehydrogenase (LDH) activity, which indicates that infection increased the permeability of the alveolar-capillarity barrier and cell damage in lungs. Moreover, lung histopathological examination revealed a gradual and intense inflammatory response with progressive parenchymal involvement, including widespread cellular infiltration, passage of blood elements from capillaries to tissues, increased fibrosis in bronchial walls and vessels, hemorrhage and reduction in the alveolar airspaces [13].

The immunostimulating properties of LAB have been proved to be strain- and dose-dependent [7,13–15]. Consequently, the ability of LAB to increase resistance against pneumococcal infection was studied using several *Lactobacillus* and *Lactococcus* strains and different doses and periods of administration [13,16–18]. Only four of the treatments assayed increased the resistance of the mice to challenge with the respiratory pathogen: administration of *Lactobacillus casei* CRL431, a probiotic strain with widely documented immunomodulatory properties [7,13,19], *Lactococcus lactis* NZ9000, a strain used for the expression of heterologous proteins [16,20], *Lactobacillus rhamnosus* CRL1505, a new probiotic strain isolated from goat milk with optimum technological properties [18], and a probiotic yogurt prepared with the immunobiotic strains *Lactobacillus bulgaricus* CRL423 and *Streptococcus thermophilus* CRL412 [17]. Our studies demonstrated that LAB, administered by the oral route at the proper dose, were able to increase *S. pneumoniae* clearance rates in lung and blood, improved survival of infected mice and reduced lung injuries [13,16–18]. In order to elucidate the immunological mechanisms involved in the increased resistance to the pneumococcal infection induced by LAB, we studied both the innate and the specific immunity against the pathogen, and we found that the effects of LAB treatments were related to an up-regulation of both types of immune response in the respiratory tract (Fig. 1).

Innate immunity. Macrophages have a central role in the maintenance of immunological homeostasis and host defense, the key population in the lungs being composed of alveolar macrophages (AM) [21]. Although resting AM are normally maintained in a quiescent state and produce small amounts of pro-inflammatory cytokines, they maintain the capacity to be activated in response to pathogens [1]. The activation of AM results in increased phagocytosis and microbial killing [22]. Moreover, in the event that the invading pathogens are too virulent or represent too large a load to be contained by AM alone, these cells are capable of generating mediators such as TNF-α, IL-1β, MIP-1, MIP-1β, IL-8 and IL-6 that recruit large numbers of neutrophils into the alveolar space. These recruited neutrophils provide auxiliary phagocytic capacities that are critical for the effective eradication of offending pathogens [23,24].

Mice treated orally with LAB had significantly higher amounts of BALF TNF-α than mice in the control group after challenge with *S. pneumoniae* [13]. These increased levels of BALF TNF-α allowed an improved recruitment of neutrophils from the pulmonary vasculature into the alveolar spaces and a higher activation of BALF phagocytes, as shown by the number of BALF neutrophils, lung myeloperoxidase (MPO) and the percentage of BALF nitro blue tetrazolium positive (NBT^+) cells [13,16–18]. In addition, LAB were able to induce activation of the systemic innate immune response, which was evidenced by the increase in the number and microbicidal function of blood neutrophils [13,16–18].

On the other hand, although neutrophils are a key component of the host defense response against invading pathogens, they have also been implicated as mediators of tissue injury in a variety of inflammatory disorders [23]. Consequently, regulation of the inflammatory response by anti-inflammatory cytokines prevents damage to the host. Blum et al. [25] have suggested that LAB could participate in tissue protection against the deleterious effect of an ongoing inflammatory process. In our

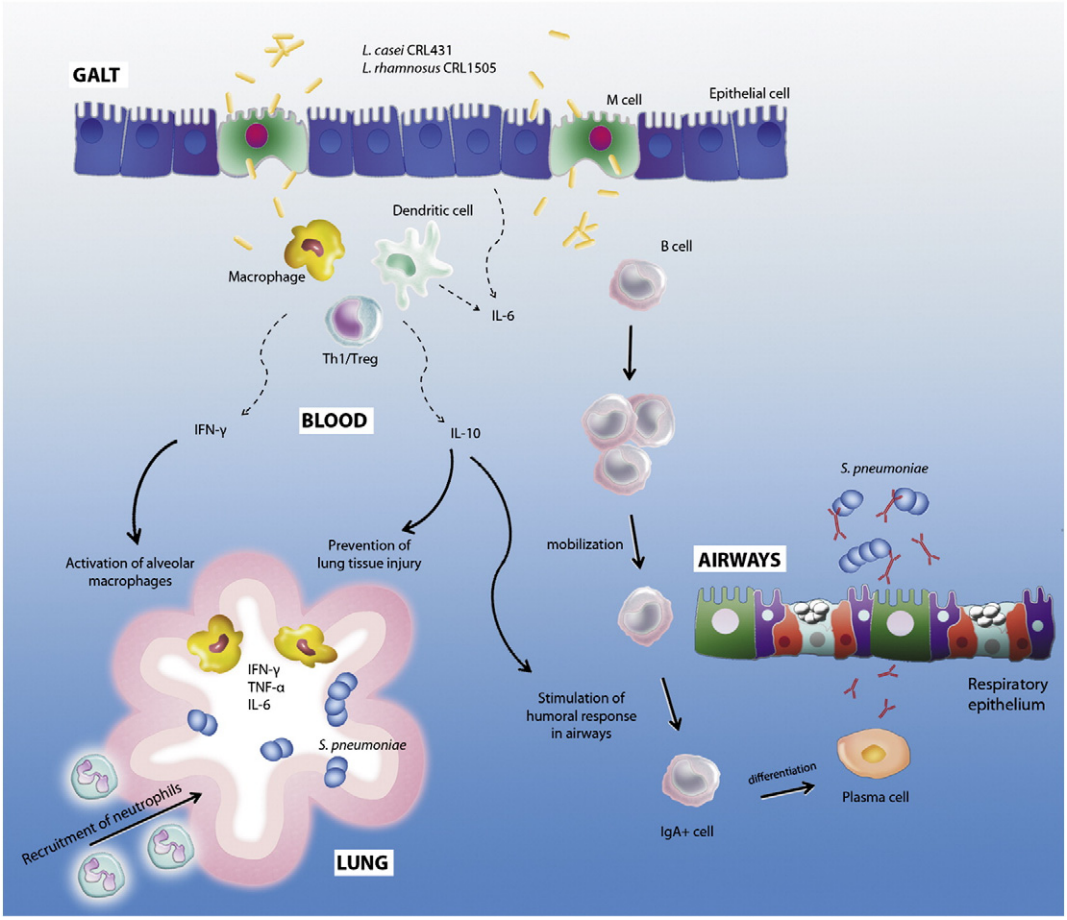

Fig. 1. Improvement of respiratory immune response against *Streptococcus pneumoniae* induced by probiotic lactic acid bacteria (LAB). Oral administration of LAB allows the interaction of lactobacilli with cells in the gut associated lymphoid tissue (GALT). Dendritic cells (DCs) can extend appendices between epithelial cells to take up bacteria. In addition, LAB are transported through microfold epithelial cells (M cells). The contact of lactobacilli with macrophages and DCs induces activation signals that trigger a switch in cytokine and chemokine production and upregulation of costimulatory molecules. Cytokines produced by macrophages and DCs stimulated with lactobacilli can modulate the function of T and B cells. Cytokines orchestrate the conversion of naïve T cells into mature Th1 cells which produce IFN-γ that can be released into blood and stimulate cells in distant mucosal sites from the gut such as alveolar macrophages. In this way, LAB treatments allow a more effective production of pro-inflammatory cytokines, recruitment of neutrophils from the pulmonary vasculature into the alveolar spaces and a higher activation of phagocytes when the pneumococcal infection occurs. Moreover, LAB allow an improved production of IL-10 that would be valuable for attenuating inflammatory damage and pathophysiological alterations in lung. Then, oral administration of LAB is able to beneficially regulate the balance between pro- and anti-inflammatory responses in lung during pneumococcal infection. In addition, the interaction of LAB with intestinal epithelial cells and immune cells can induce the production of IL-6 that allow maturation and proliferation of B cells and stimulate the IgA cycle. The mobilization of IgA^+ cells from the gut to the respiratory tract together with the improved production of specific IgA prevents colonization of mucosal tissues and subsequent spreading into the systemic circulation. Moreover, specific IgA antibodies can bind antigens and minimize their entry with a consequent reduction in inflammatory reactions, which prevents potentially harmful effects on the tissue.

experiments, treatments with immunobiotics prior to pneumococcal infection induced a significant increase in IL-10 in lung and serum [13,16–18]. This increase could help to reduce the production of pro-inflammatory cytokines and chemokines and to downregulate the expression of adhesion molecules [26]. Consequently, in agreement with other reports [27], IL-10 would be valuable to attenuate inflammatory damage and pathophysiological alterations in lung infected with pneumococci. According to these results, LAB treatments would beneficially regulate the balance between TNF-α and IL-10, allowing a more effective inflammatory response against infection (Fig. 1).

Humoral immunity. After exposure to pathogens there is an activation of antibody responses in the respiratory tract. The type and concentration of antibodies produced is dependent on the site of exposure. Upper airway exposure results primarily in an IgA response; however, when organisms reach the deep lung after passing through the upper airway, they induce an increased production of pathogen-specific IgG [28]. The organized lymphoid follicles in the nasal associated lymphoid tissue (NALT) and bronchus associated lymphoid tissue (BALT) are considered important inductive sites for the respiratory mucosal immune response [29]. Following exposure to a pathogen there is an antigen uptake by dendritic cells (DCs) of NALT or BALT, activation of $CD4^+$ T cells and generation of IgA-producing plasma cells that populate airway lamina propria [30].

On the other hand, in the gut immune response induced by commensal bacteria, the antigen presentation from the luminal flora leads to the generation of large quantities of local IgA. Moreover, the increase in the number of IgA producing cells is the most remarkable property induced by probiotic microorganisms [19]. It has been demonstrated that the IgA^+ cells in the lamina propria of the small intestine can be increased by orally administered LAB [14,15]. A common mucosal immune system exists whereby immune cells stimulated in one mucosal tissue spread and relocate to various mucosal sites. This concept implies that oral immune stimulation can induce immunity in distal intestinal mucosal sites. Our lab and others have demonstrated that the oral administration of certain LAB strains is able to induce the IgA cycle and increase the IgA^+ cell population in the respiratory tract [13–17].

When studying the capacity of *L. casei* CRL431, *L. lactis* NZ 9000, *L. rhamnosus* CRL1505 and the probiotic yogurt to stimulate the IgA cycle, we found that these oral treatments were capable of increasing

the number of IgA^+ cells in intestine and bronchus [13,16–18]. We also found that LAB treatments were able to improve the production of anti-pneumococcal IgA in the airways [13,16–18]. Under inflammatory conditions, cytokines in the respiratory tract change dramatically and, when a Th2 response is needed, additional sources of IL-4, IL-5, IL-6 and IL-10 contribute to stimulate B cells to proliferate and mature into polymeric IgA-producing cells and to develop specific antibodies [31,30]. In our studies, IL-4 and IL-10 were significantly increased in the respiratory tract of animals that received LAB, which correlates with the increase in the levels of specific IgA in BALF. The production of specific IgA in the respiratory tract during an infectious process is important because it prevents colonization of mucosal tissues and subsequent spreading into the systemic circulation [28]. Additionally, specific IgA antibodies can bind antigens and minimize their entry with a consequent reduction in inflammatory reactions, which prevents potentially harmful effects on the tissue. Thus, the stimulation of the IgA cycle and the improvement of the levels of pathogen specific IgA induced by the LAB strains could explain at least partly the greater resistance of the treated mice to the challenge with *S. pneumoniae* (Fig. 1).

In the deep lung, when *S. pneumoniae* reaches the alveolar space, there is a differentiation and expansion of antibody-secreting plasma cells that are committed to the production of IgG [32,33]. These antibodies have an important role in the protection against pneumococcal infection since opsonizing IgG antibodies are important for complement fixation and for enhancing the efficiency of macrophage killing. This immune activation also induces the production at the systemic level of antibodies responsible for preventing the passage of *S. pneumoniae* to the blood and their subsequent dissemination [34].

Our experiments demonstrated that LAB treatments improved the production of antipneumococcal IgG in BALF [13,16–18]. This fact could be related to the stimulation of antigen presenting cells in the lung that induces T cell activation and B cell clonal expansion and differentiation into IgG^+ antibody-secreting plasma cells. When studying the activation of the recruited macrophages and the levels of TNF-α in BALF after the challenge with the pathogen, we found that mice treated orally with LAB showed higher BALF TNF-α values and NBT^+ cells percentages than mice in the control group [13,16–18], which would indicate that LAB treatments would be capable of improving macrophage-mediated antigen presentation. In addition, it has been demonstrated *in vitro* that *L. lactis* NZ 9000 cells are able to up regulate the expression of MHC-II and CD86 co-stimulatory molecules in bone marrow derived DCs [35,36]. During the generation of an efficient effector immune response DCs have to overcome suppression by Treg cells. In this sense, production of IL-6 by DCs can release them from the suppression of naturally occurring Tregs [37]. It has also been demonstrated that other cytokines are able to trigger DCs activation/maturation, among them proinflammatory cytokines such as TNF-α [38]. In our studies we observed that preventive treatments with LAB were able to increase the levels of IL-6 and TNF-α in the respiratory tract after challenge with *S. pneumoniae* [13,16,18]. These results would indicate that oral treatment with LAB would be capable of improving antigen presentation mediated by pulmonary DCs. Our laboratory is working actively to demonstrate this effect.

2.2. Effect on immunocompromised hosts

In order to study the effect of probiotics on the respiratory tract defenses of immunocompromised malnourished hosts, we developed a model of pneumococcal infection in protein-malnourished mice [39]. Weaned mice were malnourished after they consumed a protein-free diet (PFD) for 21 days. Malnourished mice were repleted with a balanced conventional diet (BCD) with or without supplemental immunobiotics and challenged with *S. pneumoniae* at the end of the dietary treatments assayed. We studied the effect of probiotics on the recovery of the innate and adaptive immune responses against *S. pneumoniae* respiratory infection [7,39,40]. In this experimental model, pneumococcal colonization of lung and bacteremia were significantly greater in malnourished (MNC) mice compared with the well-nourished control (WNC) group. In addition, we found that MNC mice showed an important increase in LDH and albumin in BALF with respect to the WNC group. These results and the histopathological studies evidenced severe lung injury in the MNC mice [39].

Repletion of malnourished mice with the BCD for 21 days was necessary to obtain levels of infection similar to those in the WNC mice. However, some immunobiotic treatments were able to accelerate the normalization of the immune response to the infection in the malnourished mice [39–41]. Treatment with BCD and supplemental *L. casei* CRL431, *L. rhamnosus* CRL1505 or the probiotic yogurt reduced to seven (lactobacilli) or fourteen (yogurt) days the time needed to normalize the immune response [39,40, Alvarez et al., unpublished results].

2.2.1. Innate immunity

Suppression of neutrophil recruitment or functional activity would predictably result in an increased susceptibility to pulmonary infections. Our experiments showed a decreased number of blood leukocytes and neutrophils in MNC mice suffering from pneumococcal respiratory infection [39,41]. In addition, qualitative alterations of neutrophils have been described in malnourished individuals while a depressed ability to kill ingested bacteria is characteristic of neutrophils in children suffering from malnutrition [42]. These observations agree with our investigations, which showed a decreased bactericidal function of BALF phagocytes in MNC mice. However, administration of immunobiotics during repletion accelerated normalization of this function [39,40, Alvarez et al., unpublished results]. The neutropenia associated with infection in malnutrition is accompanied by a disproportionately large number of band cells. Therefore, the depression reported in the microbicidal activity of neutrophils from infected malnourished individuals may partly reflect the functional limitations of immature cells. In this sense, we found decreased percentages of bone marrow proliferating myeloid cells (myeloblasts, promyelocytes and myelocytes) and reduced peroxidase activity in blood and bone marrow neutrophils in MNC mice during a pneumococcal infection [41]. Repletion with BCD increased the percentage of proliferating cells. However, repletion with *L. casei* CRL431 supplementation caused an even higher proliferation (mitotic pool) than the one found in the WNC mice [41]. In addition, reduced neutrophil migration has been also described in malnutrition [43,44]. When we studied neutrophil and monocyte recruitment in MNC mice, we observed a delay in neutrophil recruitment and significantly decreased lung MPO activity with respect to WNC mice. However, when LAB were administered during repletion, MPO activity was significantly improved [39,40], a fact probably related to the improved levels of TNF-α and IL-1β after infection [45,41].

LAB administration also allowed a more efficient regulation of the inflammatory response against infection [7]. We showed that malnutrition can compromise pulmonary defenses against *S. pneumoniae* and is conducive to excessive inflammation in response to the infection. In our model, neutrophil infiltration in the lungs of malnourished animals did not result in increased bacterial clearance from the lung but was a correlate of unproductive inflammatory response. Malnutrition also prevented production of IL-10 during pneumococcal infection, the critical anti-inflammatory cytokine necessary to control excessive inflammation in the murine lung infected with *S. pneumoniae*. We demonstrated that repletion with immunobiotics was able to increase IL-10 to significantly higher levels than those found in WNC animals [45]. Consequently, the increase in this cytokine observed in the group repleted with immunobiotics could be involved in the lower lung injury reported in our studies [39,40]. According to our results, the use of probiotic bacteria as a supplement in a repletion diet was associated with a pattern of inflammatory and anti-inflammatory cytokines that led to a more

efficient regulation of the inflammatory response, thus limiting the injury caused by the infection.

2.2.2. Humoral immunity

The mucosal secretory IgA antibody response is impaired in malnourished hosts [46,47]. Malnutrition causes a remarkable decrease in the number of IgA^+ cells associated with the lamina propria of the small intestine [47] and has the same effect on the respiratory mucosa, since we found decreased numbers of IgA^+ cells in the BALT of MNC mice [45]. We also observed a significant impairment of the local humoral immune response against *S. penumoniae* infection, which was evidenced by the decreased levels of BALF IgA anti-pneumococcal antibodies [39,40].

The administration of *L. casei* CRL431 was reported to enhance the number of IgA^+ cells in the intestine of malnourished mice [47] and we found that repletion with immunobiotics not only normalized the number of IgA^+ cells in BALT but also induced a significant increase in the number of these cells compared with the WNC group [45]. This improvement in the number of IgA producing cells in the respiratory mucosa was correlated with an enhanced local production of specific antibodies after pneumococcal challenge [39,45]. Moreover, malnourished mice repleted with supplemental *L. casei* CRL431 showed normal levels of serum and BALF IL-6 and higher values of serum IL-4 and BALF and serum IL-10 than those found in the WNC group. Thus, the improvement of the antibody response to pneumococcal infection would be mediated by the different cytokine profile induced by immunobiotics.

In addition, malnutrition produced a remarkable decrease in the levels of specific IgG in BALF and serum [39,40]. It has been suggested that the impairment of the humoral response in malnourished hosts relates to the number and competence of both T and B cells [42]. Lymphoid atrophy, evidenced by the decrease in the size and cellularity of the thymus and of the secondary lymphoid organs, significantly contributes to the alteration in the adaptive immunity in malnourished individuals. In this sense, we observed a significant decrease in blood lymphocytes and bone marrow lymphoid lineage cells in our malnutrition model [41]. Cytochemical assays have been proposed to study maturation of T cells with a scheme that differentiates T cell progress from β-glucuronidase negative (β-G-), α-naphthyl butyrate esterase negative (α-NBE-) to $\beta\text{-G}^+$, α-NBE- and finally to $\beta\text{-G}^+$, $\alpha\text{-NBE}^+$ [49]. Using this cytochemical scheme, we found reduced numbers of blood and bone marrow $\beta\text{-G}^+$ cells and $\alpha\text{-NBE}^+$ cells in MNC mice, which would suggest deficiencies in T cell maturation [41]. Thus, part of the defect in antibody immunity in malnourished mice could be attributed to the profound effect of malnutrition on the maturation of T cells, which results in a reduction in fully functional mature T cells and an excess of poorly functional immature T cells [48]. The administration of immunobiotics to malnourished mice corrected lymphopenia and improved the number of bone marrow lymphoid cells. These treatments, which induced an increase in ß-G^+ cells and $\alpha\text{-NBE}^+$ cells, would improve T cell maturation. Moreover, the normalization of the systemic IgG response was also achieved with the immunobiotic treatments since the levels of serum antipneumococcal IgG were similar to those found in the normal control group [39,40].

We also found a decreased number of bone marrow and spleen B cells ($B220^+$) in malnourished mice. These mice showed a marked decrease in immature B cells ($B220^{low}$ HSA^{high} $IgM^{+/-}$ IgD^-) in bone marrow and mature B cells ($B220^{high}$ HSA^{low} IgM^+ IgD^+) in spleen when compared with the WNC group. In addition, when studying the functionality of B cells, we found that the proliferative capacity in response to LPS and CpG and the production of IgM and IgG were similar in both the WNC and the MNC groups. Thus, malnutrition affects B lymphopoiesis in spleen and bone marrow, decreasing the production and number of B cells without affecting their functionality. The treatment with *L. rhamnosus* CRL1505 induced an improvement in the number of immature B cells in bone marrow and mature B cells in spleen. This effect on B cells could explain the improved specific immune response against *S. pneumoniae* induced by probiotics in malnourished mice (unpublished data from our group).

2.3. Effect of nasal treatments with probiotic lactic acid bacteria

Taking into consideration the fact that the nasal route can induce systemic and respiratory immune responses superior to that to those obtained using oral stimulation [30], more recently we focused on the ability of nasal stimulation with immunobiotics to improve respiratory immune response and analyzed whether the nasal administration of LAB is capable of increasing resistance against *S. pneumoniae* in our challenge-infection mice models [50,51].

The use of non-recombinant LAB nasally administered to prevent respiratory infections has been sparsely investigated. Studies by Cangemi de Gutierrez et al. [52] demonstrated that the intranasal administration of *L. fermentum*, isolated from the pharynx of BALB/c mice, was able to reduce nasal and pharynx colonization by *S. pneumoniae* and to reduce pathogen counts in the lung. Hori et al. [53] studied the effect of the nasal administration of a non-viable lactic acid bacterium on respiratory immunity and observed that nasal treatment of adult BALB/c mice with non-viable *L. casei* Shirota was able to stimulate cellular immunity in the respiratory tract and to significantly increase the resistance of mice to the infection with influenza virus. These reports showed that the intranasal administration of immunobiotics can efficiently improve protection against respiratory infections.

Our laboratory evaluated the effect of nasally administered *L. lactis* NZ9000 and demonstrated that this treatment was able to increase the clearance rate of *S. pneumoniae* by inducing an upregulation of the innate and specific immune responses in both local and systemic compartments [50]. The NALT contains all the immune cells required for the induction and regulation of the mucosal immune response to antigens delivered into the nasal cavity [30]. Hussell and Humphreys [54] suggested that the NALT could fulfill an important role by reducing the pathogen burden to a level that only induces minimal inflammation in the lower lung. In consequence, the intranasal priming of NALT with *L. lactis* before challenge with *S. pneumoniae* was probably able to reduce the number of pathogens in the nasal cavity and of pneumococci that reach the lung. The effect induced by the nasal inoculation of *L. lactis* could be explained by a decreased adherence of *S. pneumoniae* to the respiratory epithelium, as it has been reported that nasally administered LAB could competitively exclude pneumococcal cells [54]. In addition, the increased activation of AM and the increase in the microbicidal function of blood neutrophils observed in mice treated with *L. lactis* would enhance the protective effect [50]. Moreover, levels of BALF IgA and IgG and serum IgG were significantly higher in the *L. lactis* treated group when compared with the control mice [50].

We next evaluated whether the nasal administration of immunobiotics to malnourished immunocompromised mice was capable of increasing respiratory immunity. Our results show for the first time that nasal administration of the probiotic bacterium *L. casei* CRL431 is able to significantly increase the resistance of malnourished mice against a respiratory pathogen [51]. The protective effect of nasal treatment with viable and non-viable *L. casei* against the pneumococcal colonization of lung, bacteremia and lung tissue injury was correlated with the stimulation of the systemic and respiratory immune responses. The protection induced by the nasal stimuli was significantly higher than that achieved with the oral administration of the same probiotic strain [39]. Both the administration of viable and non-viable *L. casei* prevented the dissemination of the pathogen to the blood and induced its lung clearance, whereas the oral treatment, although it prevented the passage of the pathogen to the blood and decreased bacterial counts in the lung, did not induce the complete

clearance of pneumococci from the lung during the period under study [39,50].

The administration of immunobiotics induced a greater production of TNF-α by AM after challenge. Thus, nasal treatments improved the capacity of AM to secrete cytokines in the presence of a pathogen. It has been demonstrated that the interaction of *L. casei* CRL431 with immune cells associated with the gut induced an increase in the expression of TLR-2 and CD-206 receptors in macrophages and DCs [55]. It is possible that nasal administration of *L. casei* CRL431 has a similar effect on the respiratory mucosa. The higher levels of TNF-α allowed the recruitment of phagocytes into the lung, which are of great importance for the control of pneumococcal infection. Moreover, IL-4 and IL-10 were significantly increased in the respiratory tract of animals that received both viable and non-viable *L. casei,* which correlates with the increase in the levels of specific IgA in BALF. In addition, we found that both the IgG1 and the IgG2a anti-pneumococcal antibodies were higher in immunobiotic treated mice [51].

The results from this study suggest that heat-killed LAB are also effective in the immunomodulation of the systemic and respiratory immune system. Therefore, probiotic bacteria in the form of live cells may not be required for this purpose. The effect of non-viable *L. casei* and other LAB on the respiratory immune system should be examined with more detailed studies, as dead bacteria or their cellular fractions could be an interesting alternative as mucosal adjuvants, especially in immunocompromised hosts in which the use of live bacteria might be dangerous. In addition, heatkilled LAB have the advantage of allowing longer product shelf-life as well as easier storage and transportation.

3. Improvement of respiratory immunity by recombinant lactic acid bacteria

3.1. Expression of pneumococcal antigens in lactic acid bacteria

The beneficial effects of LAB against pneumococcal respiratory infections encouraged researchers to develop vaccines that would combine the immunomodulatory properties of such bacteria with antigen delivery [56–58]. During the last decade, the expression of different pneumococcal antigens was achieved in diverse LAB strains, using diverse expression vectors (Table 1).

The first pneumococcal antigen expressed in LAB was type 3 capsular polysaccharide (CPS) [59]. In this work, fragments coding for the pneumococcal type 3 capsule locus were cloned and expressed in *L. lactis* MG1363 for a purpose of studying the role of the genes involved in capsular production. *L. lactis*, a non-capsulated bacterium, was shown to produce significant amounts of extracellular CPS in a similar structure as pneumococcal type 3 CPS. This system was proposed for the study of capsule biosynthesis and the analysis of the biological properties of these polysaccharides in a context where other pneumococcal virulence factors would not be present. Furthermore, a technology for the production of pneumococcal polysaccharides, which are the basis of licensed vaccines, became possible with a non-pathogenic bacterium. As expected, inoculation of purified type 3 CPS in mice elicited T cell-independent responses, characterized by the induction of IgM, low levels of IgG (IgG1 and IgG3 subclasses) and the absence of memory cells. The delivery of type 3 CPS by *L. lactis* did not change the nature of the immune responses to polysaccharides, so a booster effect after a second dose in mice was not observed. In addition, the magnitude of the immune responses elicited by the *L. lactis*-type 3 CPS vaccine was equivalent to the one observed for purified type 3 CPS [59]. Thus, although this strain could be used as a biotechnology tool for the production of type 3 CPS in a safe platform, its application as a recombinant live vaccine against pneumococcal infections remains to be determined.

Recently, the pneumococcal type 14 CPS, which is an example of a complex polysaccharide, was also expressed in *L. lactis*. As in the case of type 3 CPS, the resulting type 14 CPS was expressed in equivalent amounts compared with the expression by *S. pneumoniae* and with an identical structure. Interestingly, type 14 CPS was mainly secreted to the media, a characteristic that could simplify purification steps [60]. Therefore, both works confirm the feasibility of producing pneumococcal polysaccharides in *L. lactis* for purification purposes.

Available pneumococcal vaccines are based on capsular polysaccharide antigens. Conjugated vaccines composed of a mixture of polysaccharides and protein carriers have proved to be more effective for the protection of children and of the elderly than the first generation vaccine composed only of polysaccharides [61]. Still, broad coverage of such vaccines is attained by the inclusion of several polysaccharides in the formulation, a practice that increases production costs. As a result, mass vaccination with pneumococcal conjugated vaccines is beyond the economic reality of developing countries. Moreover, an increase in diseases caused by non-vaccine serotypes was observed in countries where the pneumococcal 7-valent conjugated vaccine (PCV-7) was introduced [62–64], an effect that may potentially occur with the introduction of the newly licensed 10- and 13-valent vaccines. In this context, protein antigens emerge as possible alternatives for effective broadcoverage vaccines at lower costs.

Next, we describe the pneumococcal protein antigens expressed in different lactic acid bacteria for the development of live mucosal vaccines. Data on protective immune responses elicited by the different vaccines in animal models are discussed in the following section.

The first pneumococcal protein antigens expressed in lactic acid bacteria were the Pneumococcal Surface Antigen A (PsaA) and the Pneumococcal Surface Protein A (PspA) [65]. PsaA is a highly conserved 37 kDa lipoprotein expressed by virtually all pneumococcal isolates described to date [66]. The observation that pneumococcal mutants that lack PsaA expression display low capacity to adhere to respiratory epithelial cells and therefore are poor colonizers led to the description of this protein as an adhesin. This conclusion is supported by amino acid sequence similarities with adhesins from other streptococci [67]. Further studies have shown that PsaA is part of an ABC-type transport protein complex and crystallization of recombinant protein demonstrated that it is a Mn^{+2} binding protein responsible for the

Table 1
Recombinant LAB vaccines against pneumococcal infections.

LAB strain	Expression system	Pneumococcal antigen	Protection against colonization	Protection against lethal challenge	Ref.
L. casei CECT5275	lac inducible promoter	PsaA, PspA 1, PspA 3	–	–	[65]
Different LAB strains[a]	P1 constitutive promoter	PsaA	++ to +++[b]	–	[83]
L. lactis MG1363	Nisin-inducible promoter	PspA 3	–	++	[84]
L. casei CECT5275	P1 constitutive promoter	PspA1, PspA5	–	++	[85,86]
L. casei CECT5275	P1 constitutive promoter	PspC	–	–	[86]
L. lactis NZ9000	Nisin-inducible promoter	PppA	+++	+++	[88,103]
L. lactis NZ9000 GEM	–	SLr, IgA1p, PpmA	–	++[c]	[95,97]

[a] *L. lactis* MG1363, *L. casei* CECT5275, *L. plantarum* NCDO1193 and *L. helveticus* ATCC15009.
[b] The best protection was observed for the *L. casei*-PsaA vaccine. No protection was observed for the *L. lactis*-PsaA vaccine.
[c] Increased mean survival time against a fatal pneumonia model.

transportation of Mn^{+2} inside the cells [68]. This raised the question that the deficiency in adhesion observed in PsaA mutants may be a consequence of decreased protein expression triggered by low levels of Mn^{+2}. Despite the controversies on its biological function, several mucosal vaccine formulations composed of PsaA were shown to be protective against pneumococcal nasopharyngeal colonization [69,70].

PspA is a choline binding protein with molecular weights ranging from 67 to 99 kDa that participates in pneumococcal evasion from the immune system by inhibiting complement deposition on bacterial surface [71,72]. Different vaccines composed of PspA were shown to be highly effective against pneumococcal infection in animal models, particularly for the systemic phase of the disease [73,74]. PspA was also shown to bind to the bactericidal apolactoferrin protein that is present on host mucosa [75]. Sequence variability has driven the classification of PspAs into six clades and three families [76], which seem to have a similar distribution in pneumococcal isolates from different parts of the world [77–79]. Thus, approximately 50% of the isolates express PspAs from family 1 (clades 1 and 2) and 50% express PspAs from family 2 (clades 3, 4 and 5). Family 3 PspAs (clade 6) are rarely isolated. Initial cross-reactivity results indicated that a vaccine composed of one PspA from family 1 and one from family 2 would confer broad protection against isolates expressing different PspAs. However, recent studies have indicated that the choice of the PspA molecules for the composition of a broad-coverage vaccine must be carefully analyzed. While some molecules induce antibodies with poor reactivity against PspAs from the same family, others can produce antibodies that react with PspAs from clades 1 to 5 and also elicit cross-protection in mice [80,81].

The first approach for the expression of PsaA and PspA in LAB was conducted using an expression system based on the lactose operon (lacTEGF) from *L. casei* CECT 5275 [82]. Different constructs were obtained for intracellular expression and secretion to the culture media of PsaA and the N-terminal fragments of PspAs from clades 1 and 3 by *L. casei* [65]. In this strategy, expression by the recombinant bacteria was controlled by the addition of lactose to the culture media. As a result, intracellular inducible expression of the three proteins by *L. casei* was successfully achieved, with particularly high levels observed for PsaA that reached about 15% of total protein.

For secretion of the pneumococcal antigens to the culture media, plasmid constructs were developed in which the PsaA and PspA genes were cloned in fusion with the coding region for the *L. casei* cell wall proteinase leader sequence (PrtP). Growth of the recombinant bacteria in the presence of lactose led to the accumulation of both PspA1 and PspA3 in culture supernatants. In contrast, secretion of PsaA to the culture media was not observed. Instead, PsaA appeared to be attached to the *L. casei* cell wall, with only a small fragment exposed on the bacterial surface [65].

Nasal immunizations of mice with recombinant *L. casei* expressing PsaA or PspA, with this inducible system failed to induce systemic or mucosal antibodies against the antigens. In addition, the immunization did not confer protection against established models of pneumococcal colonization and infection (unpublished data from our group). One explanation for these negative data is that the levels of antigens produced in culture upon induction with lactose were not enough to trigger detectable humoral immune responses in mice. Additional expression of the antigens after immunization of mice would not take place because of the absence of an inductor in host mucosa. Further studies would be necessary for the application of these recombinant bacteria for vaccination purposes.

In an extension of this work, the *psaA* gene was cloned under the control of the lactococcal P1 constitutive promoter in the pT1NX vector, in fusion with the first codons of the Usp45 signal peptide [83]. This construct allowed the expression of PsaA attached to the cell wall of four host LAB strains: *L. lactis* MG1363, *L. casei* CECT5275, *L. plantarum* NCDO1193 and *L. helveticus* ATCC15009. All lactobacilli tested expressed similar amounts of PsaA, ranging from 150 to 250 ng per 10^9 cells whereas *L. lactis* expressed approximately 20 ng of the recombinant protein. A comparison of these strains as recombinant vaccines against a model of pneumococcal nasopharyngeal colonization in mice is discussed in the following section.

Three other works described the development of recombinant LAB strains expressing the N-terminal region of PspA under the control of different promoters. The N-terminal region of PspA from clade 3 (amplified from the pneumococcal TIGR4 strain) was expressed in *L. lactis* MG1363 under the control of the nisin inducible promoter [84]. Vaccine formulations, after induction of expression by the addition of nisin to the culture, contained between 250 and 500 ng of PspA per 10^9 *L. lactis* cells. Both live and inactivated recombinant *L. lactis* were tested as nasal vaccines in mice.

Constitutive expression of the N-terminal region of PspA clade 1 (from 435/96 pneumococcal strain) was expressed in the intracellular compartment of *L. casei* CECT5275 [85]. The same vector was used for the expression of PspA from clade 5 (from the 122/02 pneumococcal strain) [86]. In this system, protein levels reached about 100 ng per 10^9 *L. casei* cells for both PspAs. PspA expression did not cause any effect on *L. casei* permanency on mice nasal mucosa, since equivalent CFU numbers could be recovered from animals that received the PspA1 expressing bacteria or the bacteria carrying the empty vector. In both cases, *L. casei* was recovered up to 5 days after inoculation and *in vitro* PspA1 expression was still observed in recovered colonies of *L. casei*-PspA1, thus indicating that heterologous expression could be occurring in mice mucosa.

Recently, Green et al. described a pneumococcal surface-exposed protein that has homology with bactoferritins [87]. This 20 kDa antigen, called Pneumococcal protective protein A (PppA), was found to be highly conserved among pneumococcal isolates, although neither its biological function nor its role in pneumococcal pathogenesis has been determined yet. Nasal immunization of mice with recombinant PppA, in combination with mucosal adjuvants, induced antibodies that reacted with heterologous pneumococcal strains and afforded protection against a model of nasopharyngeal colonization [87].

A construction for the expression of PppA in LAB was developed in which the *pppA* gene was cloned under the control of the nisin inducible promoter. The protein was expressed on the surface of *L. lactis* NZ9000 through the fusion with the Usp45 signal peptide and the anchoring signal peptide CWA-M6, resulting in a final PppA-CWA polypeptide of 34 kDa. Localization of the protein was confirmed by immunoblotting of cellular fractions as well as immunofluorescence of intact bacteria [88]. Different regiments for mucosal vaccination of mice with *L. lactis*-PppA including both live and inactivated bacteria were tested.

Finally, the Pneumococcal surface antigen C was also expressed in the intracellular compartment of *L. casei* CECT5275, under the control of the lactococcal P1 constitutive promoter present in the pT1NX vector [86]. PspC is a polymorphic choline binding protein with molecular and serologic similarity with PspA that has been described to bind to components of the complement system such as C3 and factor H, and the polymeric immunoglobulin receptor (pIgR) [89,90]. All these interactions have been shown to influence bacteria adherence to respiratory epithelial cells and probably facilitate pneumococcal invasion [91,92]. Nasal immunization of mice with recombinant PspC has been shown to protect mice against pneumococcal colonization and sepsis [93,94]. Expression of this antigen in *L. casei* was estimated in 120 ng per 10^9 cells, a concentration similar to the one observed for the expression of PsaA or PspA using the same system. Cloning of pspC using the pT1NXssAnch expression vector which allows a fusion of the gene with the Usp45 signal peptide and the anchoring sequence from *L. casei* peptidase directed PspC to *L. casei* cell wall. However, the concentration of PspC expressed by such a construct was around 10 times lower (unpublished data from our group).

An alternative approach to vaccines based on recombinant LAB as antigen delivery system was called Gram-positive Enhancer Matrix

(GEM). This bacteriuml-shaped particle was produced through the treatment of *L. lactis* NZ9000 with acid, resulting in the degradation of the intracellular content. Expression of target proteins in fusion with a peptidoglycan affinity peptide allows antigen binding and exposure to the surface of GEMs. GEMs have intrinsic adjuvant properties with the advantage of lacking bacterial DNA [95]. In addition, more than one antigen can be combined in a formulation. This system was used to test three pneumococcal antigens: the IgA1 protease, which influences pneumococcal adherence to host cells by cleaving surface-bound IgA1 [96], and two surface associated lipoproteins, the Putative proteinase maturation protein A (PpmA) and the streptococcal lipoprotein rotamase A (SlrA), both with roles in pneumococcal virulence [35,97].

3.2. Protective effect of the nasal immunization with recombinant lactic acid bacteria

The first negative results obtained by nasal immunization of mice with recombinant *L. casei* expressing PsaA through the lactose-inducible system led our group to test the constitutive expression system as an alternative. The advantage of this new approach would be the possibility of studying different bacterial hosts as vaccine vectors. The results obtained in this work showed that both *L. plantarum*-PsaA and *L. helveticus*-PsaA were the best vaccines for the induction of specific anti-PsaA antibodies in mucosa and sera from immunized mice. Nevertheless, although intermediate to low levels of anti-PsaA antibodies were observed in mice immunized with *L. casei*-PsaA, this was the vaccine that induced the best protection against a pneumococcal nasal colonization challenge. Thus, protection induced by the vaccines did not correlate with antibody induction [83]. Effective immunity against pneumococcal colonization in mice was shown to be characterized by the function of $CD4^+$ T cells and the secretion of the pro-inflammatory cytokine IL-17 [98,99]. It remains to be established if the different LAB-PsaA vaccines, in particular *L. casei*-PsaA, can induce such responses. Despite the successful use of *L. lactis* as a delivery vector for antigens against different pathogens [56,58], *L. lactis*-PsaA was shown to be the poorest immunogenic vaccine, no significant reduction in pneumococcal colonization being observed in mice immunized with this vaccine. The apparent discrepancy in these results may be a consequence of the low levels of PsaA expressed by *L. lactis* in this system. Overall, recombinant *L. lactis* vaccines were shown to remain in the nasal mucosa for around 1 day after inoculation while Lactobacilli-derived vaccines usually remained for a few days [83–85].

Nasal immunization of mice with different LAB-PspA vaccines developed in different laboratories conferred increased protection against pneumococcal lethal challenges with very similar survival rates (around 40%). Nasal immunization of CBA/ca, a very susceptible mice strain, with live or inactivated *L. lactis*-PspA3, increased mean survival time after intraperitoneal challenge with the TIGR4 pneumococcal strain (capsule 4, PspA3) and increased survival rates against a respiratory challenge with the same strain. Similarly, nasal immunization of C57Bl/6 mice with *L. casei*-PspA1 significantly increased survival rates after an intraperitoneal challenge with the A66.1 pneumococcal strain (capsule 3, PspA clade 2) [85,30]. Another recombinant vaccine, *L. casei*-PspA5, was also shown to significantly protect mice against a respiratory challenge with the ATCC6303 pneumococcal strain (capsule 3, PspA5) [86]. All these vaccines were shown to induce mucosal and systemic anti-PspA antibodies, with a balanced IgG1:IgG2a ratio. Both protections against intraperitoneal and respiratory challenges correlated with the capacity of the antibodies to induce *in vitro* complement deposition on pneumococcal surface [85,86]. Besides the indications that antibodies are the effectors of the protection observed, mice immunized with *L. casei*-PspA5 also displayed increased recruitment of neutrophils to the respiratory mucosa and increased IFN-γ secretion by lung cells, after the respiratory challenge [86]. Thus, it is clear that the LAB-PspA vaccines induce Th1 responses directed to PspA, which has been described to be a very effective response against pneumococcal infections using different vaccines [100,101]. On the other hand, a rapid control of inflammation in lungs after pneumococcal infection is crucial to improve survival [48,102]. The mucosal immune responses induced by the *L. casei*-PspA5 vaccine seem not to provide this rapid control since high levels of TNF-α are secreted by lung cells collected from immunized mice, 13 h after challenge. This may be the reason for the partial protection elicited by these vaccines (40% survival) [31,86]. Modulation of the immune responses could be achieved by the use of other LAB strains as vaccine vectors, a combination of strains or even co-expression of immunomodulatory molecules.

Neutrophil infiltration in the mice respiratory tract was also induced by the immunization of mice with *L. casei*-PspC 13 h after challenge. Lung and spleen cells also displayed increased secretion of both IFN-γ and IL-17 pro-inflammatory cytokines. However, this vaccine was not able to induce antibodies against PspC and did not protect mice against the lethal pneumococcal respiratory challenge [86]. In fact, Th-17 secreting $CD4^+$ T cells were shown to be critical for native and acquired protection against pneumococcal nasal colonization [98,99], but the presence of antibodies against important virulence factors such as PspA and/or PspC seem to be necessary to limit pneumococcal spread to the bloodstream. Another concern in relation to this data is the low similarity of the PspC fragment used as antigen when compared with the same region in the PspC expressed by the pneumococcal challenge strain ATCC6303 (around 47% of amino acid conservation). This could also have had an influence on the failure of *L. casei*-PspC to afford protection against the invasive respiratory challenge. Further studies using other PspC variants or other challenge strains would be necessary to arrive at a conclusion on this subject.

A very promising approach against pneumococcal infections seems to be the expression of the PppA antigen by LAB strains [88]. Nasal immunization of mice with *L. lactis*-PppA induced systemic and mucosal specific antibodies. Once again, a balanced IgG1:IgG2a ratio was observed. Most importantly, significant protection rates were observed in immunized mice after an intraperitoneal challenge with the pneumococcal T14 strain (capsule 14). Percentages of survival induced by the vaccine reached 60% in adult mice and 70% in young mice. Passive immunization experiments using sera from mice immunized with *L. lactis*-PppA also increased survival of adult and young mice against the challenge with the T14 strain and, moreover, opsonization of bacteria with the immune sera produced similar effects [88]. The antigenic conservation of PppA resulted in protection against respiratory challenges with pneumococcal strains from serotypes 3, 6B, 14 and 2F and therefore this could be proposed as a broad-coverage vaccine formulation. Vaccination also prevented pneumococcal spread to the bloodstream and, together with the results from passive immunization experiments, there is strong evidence that antibodies are the effectors of the protective immune response.

Different immunization protocols were also tested for the *L. lactis*-PppA vaccine [103]. Inactivated *L. lactis*-PppA was also able to induce humoral responses directed to the antigen and to protect mice against a colonization model with type 3 and type 14 pneumococcal strains. However, a great improvement in protection was achieved by a protocol that combines the nasal vaccination with the recombinant *L. lactis*-PppA with oral administration of the probiotic *L. casei* CRL431 strain. Modulation of the immune response profile was accomplished by this strategy, producing both humoral and pro-inflammatory responses, characterized by the secretion of IL-2, IFN-γ and IL-17 cytokines in BALF. Most importantly, the oral administration of *L. casei* also induced the secretion of IL-10 in BALF, which seemed to be responsible for preventing exacerbated inflammatory responses, resulting in effective bacterial clearance with limited tissue damage [103].

In conclusion, promising results against pneumococcal infections were obtained by nasal immunization with different LAB-based vaccines. Such live vectors were proved to be able to induce antibodies against the protective antigens, which correlated with the restriction of the infection in the mucosal tract in models of lethal respiratory challenges or with clearance in models of systemic infections. Th1 as well as Th17 cytokines were also induced by the different vaccines and seemed to account for the protective effect against the colonization models. The control of pro-inflammatory responses, which seems to be of extreme importance to improve protection rates, can be achieved by modulation of the immune responses induced by the vaccines. Fig. 2 summarizes the activation of the immune system and the specific responses elicited by LAB strains expressing pneumococcal antigens.

3.3. Protective effect of the oral immunization with recombinant lactic acid bacteria

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 [30]. 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 LAB-derived oral vaccines that could afford protection against respiratory pathogens [11].

The use of LAB as vectors and adjuvants for the oral delivery of respiratory antigens has been less explored than the use of attenuated pathogens. The use of these microorganisms in the development of vaccines would present several advantages: a) the mucosal administration of LAB is safe and there is no risk of inducing endotoxic shock as they lack lipopolysaccharides (LPS) in their cell wall; b) LAB are resistant to the acids in the gastrointestinal tract and numerous strains can survive passage through the stomach, which makes them

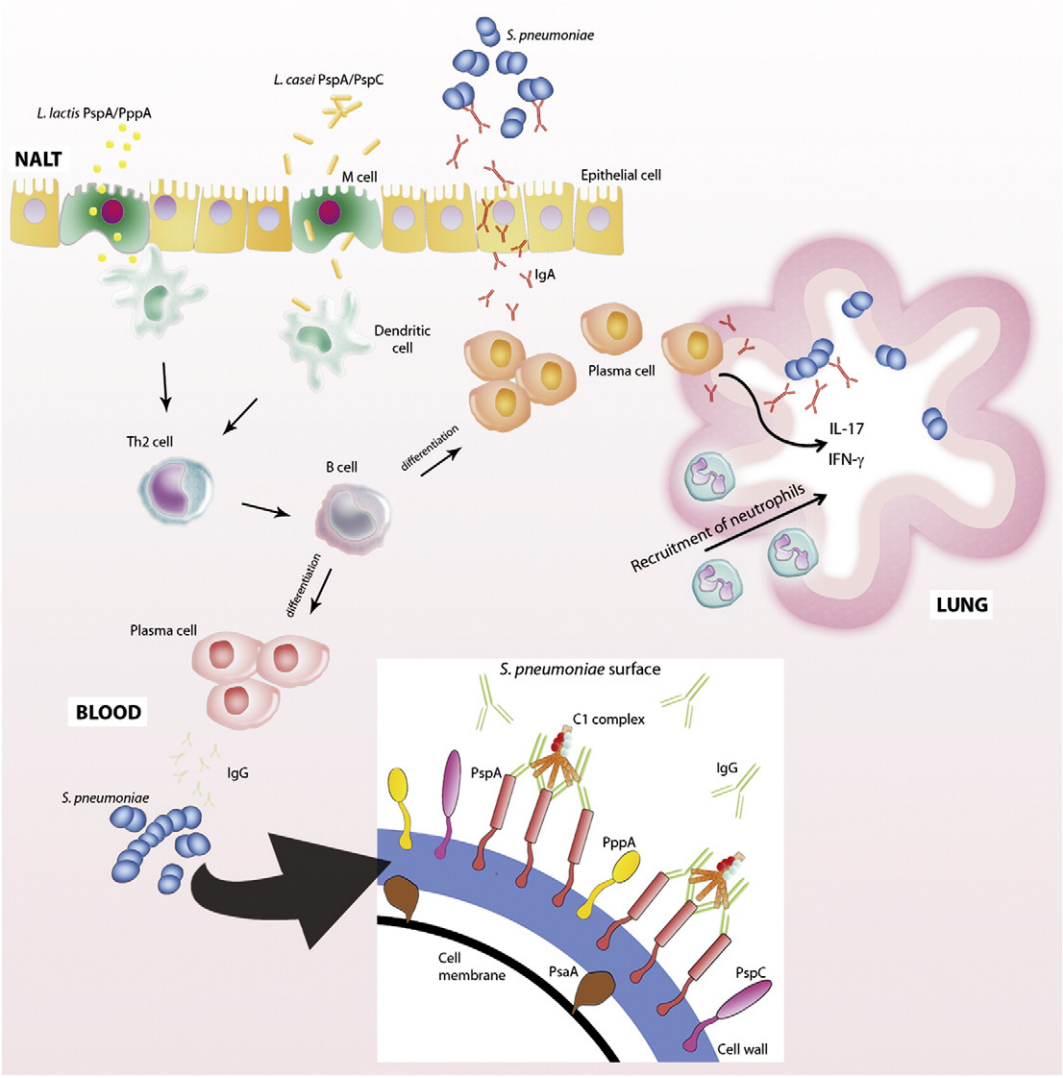

Fig. 2. Immune responses induced by lactic acid bacteria (LAB)-based vaccines expressing different *Streptococcus pneumoniae* antigens. Upon contact with the nasal associated lymphoid tissue (NALT), recombinant LAB carrying heterologous antigens are captured by dendritic cells (DCs) or microfold epithelial cells (M cells). Antigens are then presented to naïve T cells that are differentiated into mature Th2 cells. These cells stimulate B lymphocytes and activate adaptive immune responses inducing the production of specific antibodies: IgG in blood as well as IgA in the respiratory tract. Different recombinant strains are able to induce varying degrees of protection against pneumococcal infection. LAB-based vaccines like *L. lactis*-PppA, *L. casei*-PsaA or *L. casei*-PspC can induce the production of specific antibodies that allow clearance of pneumococcus from nasal mucosa. *L. casei*-PspA is able to stimulate a balanced IgG1/IgG2a response and induce the deposition of complement on pneumococcal surface (C1q complex) resulting in protection against lethal challenges. In addition to the production of specific antibodies, *L. lactis*-PppA can induce the production of cytokines such as IL-17, IFN-γ and IL-2 that improve the recruitment of neutrophils from the pulmonary vasculature into the alveolar spaces and the activation of phagocytes when the pneumococcal infection occurs, conferring protection against lethal challenges.

adequate for immunization through the oral route; c) high level of synthesis of the heterologous antigen in the recombinant LAB would not be necessary, especially if the strains with immunostimulatory capacity are used [16]; d) an additional advantage for the use of LAB is the fact that the food industry has a vast experience in the large scale production and preservation of these microorganisms, which would make vaccine production and distribution easier.

Taking into consideration the fact that bacterial and viral antigens have been efficiently produced in *L. lactis* NZ 9000 as well as the capacity of orally administered *L. lactis* NZ9000 to stimulate the innate and the specific immune responses in the respiratory tract [16], we used the recombinant *L. lactis*-PppA strain to evaluate the capacity of orally administered LAB-based vaccines to induce protective immunity in the respiratory tract. The ability of *L. lactis*-PppA to elicit specific anti-PppA mucosal and systemic antibodies after oral immunization was studied first in adult immunocompetent mice [16]. Oral immunization with *L. lactis*-PppA induced the production of specific anti-PppA IgM, IgG and IgA in BALF and serum [104]. Moreover, we found that the levels, the avidity and the opsonophagocytic activity of serum and BALF anti-PppA antibodies can be significantly improved with appropriate boosting [104]. Considering that the protocols of immunization with boosting were more effective to induce mucosal and systemic specific anti-PppA antibodies, we decided to evaluate the efficacy of the immunization scheme to afford protection against *S. pneumoniae* infection. Experiments of challenge with different pneumococcal serotypes were carried out. Serotypes 3, 6B, 14 and 23F were selected taking into account that serotype 14 is the one with greatest prevalence in our environment and that serotypes 3, 6B, 9, 14, 18, 19 and 23F are the ones most often associated with invasive disease [105]. The four serotypes studied were capable of infecting adult mice, but the virulence of each strain was different. *S. pneumoniae* serotype 3 was the most virulent, followed by serotypes 14 and 6B, while serotype 23F was the least virulent [104]. Adult mice immunized with *L. lactis*-PppA showed significantly lower lung bacterial cell counts than their respective control groups. Moreover, vaccination with *L. lactis*-PppA was able to prevent the dissemination into blood of serotypes 6B, 14 and 23F and allowed the elimination of serotype 3 from blood on day 5 post-infection [104].

Pneumococcal infectious disease is a major cause of human infant mortality. Consequently, one of the major challenges in vaccinology is the development of products that are able to induce protective immunity during 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, our next objective was to find out if the oral immunization protocol with *L. lactis*-PppA, which is effective in inducing protective immunity in adult mice, was able to protect young mice against pneumococcal respiratory infection. Results showed that the oral immunization of young mice with *L. lactis*-PppA was able to induce the production of specific antibodies both in the intestinal tract and at the systemic level [106]. The efficient stimulation of the gut mucosal immune system 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 intestinal fluid. We also observed an efficient stimulation of the systemic immune response after vaccination, which was evidenced by the detection of specific anti-PppA IgG antibodies in the serum [106].

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-γ-producing spleen cells [104]. 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 DCs *in vitro* [36]. In addition, we demonstrated that the oral immunization of young mice with *L. lactis*-PppA is able to induce the production of specific IgA and IgG antibodies in the respiratory tract [106]. Challenge experiments with the different serotypes of the pathogen were also carried out in young mice. Bacterial counts in lung, coupled with haemocultures, allowed us to conclude that young mice are more susceptible than adult mice to respiratory *S. pneumoniae* infection [104,106]. Oral immunization of young mice with *L. lactis*-PppA increased their resistance to infection with the four pneumococcal serotypes, 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 [106]. Our results show that oral immunization with recombinant bacteria represents a promising alternative for improving immunity in young individuals.

4. Probiotic effector molecules

The post-genomics era has strongly stimulated the identification of candidate effector molecules from probiotic microorganisms that are could confer a health benefit to the host via the intestinal immunity, including direct interactions with host epithelial or immune cells. However, there is very limited knowledge on the molecular mechanisms by which probiotics exert their health beneficial effects on the host. To date, only few candidate probiotic effector molecules have been discovered, and while for some there is convincing evidence for their proposed role *in vivo*, others still require validation *in situ* [108,109].

In many studies, whole cells, including live and heat-killed cells, cell wall and cytoplasmic fractions of LAB, have been shown to have various biological functions [8,51,110–114]. The term “immunogenics” has been proposed to include extracellular and intracellular bacterial components with immunoregulatory abilities such as extracellular phosphopolysaccharides, peptidoglycans, lipoteichoic acid and DNA [8,115]. Particularly, the surface cell wall properties of LAB are thought to play an important role in the immunoregulation of the host [116,117]. In LAB the cell wall is typically composed of a thick peptidoglycan layer that serves as a scaffold for the covalent anchoring of other cell-wall polymers, teichoic acid, polysaccharides and surface proteins. These cell wall molecules are key probiotic ligands that can interact with host receptors and induce signaling pathways, resulting in probiotic effects [116,117]. The main cell wall macromolecules have a similar basic architecture among LAB species, but various modifications such as glycosylation can contribute to the strain-specific properties of probiotics. The variation in peptidoglycan structure could be a discriminative feature between probiotics, as the stem peptides can differ substantially. In this sense, research on the bioregulatory function of immunobiotics and immunogenics has shown that structural, chemical and conformational differences in cell surface constituents occur even in genetically related LAB strains, resulting in different immunoregulatory effects [110,114]. In addition to the cell wall components, DNA and oligodeoxynucleotides have been shown to be major immunoregulatory substances in cytoplasm [111–113]. In fact, up to date, several immunostimulatory DNA sequences have been found in genomic sequences of immunobiotic LAB [110].

In recent years enormous efforts have been made to unravel the mechanisms of probiotic actions and various experimental approaches have been developed to characterize the molecular basis of probiotic effects [8,110,117]. It has been shown that cell wall components and DNA motifs from immunobiotic LAB can induce the immunoactivation of GALT. Moreover, it was demonstrated that TLR2, TLR9, nucleotide-binding oligomerization domain-like receptor 1 (NOD1) and NOD2 are able to recognize cell wall components and DNA of dietary LAB, thereby contributing to immunoregulation in the GALT [110,114,118,119].

Although significant progress has been made in the knowledge of the mechanisms of probiotics action in the gut, it is not known how some immunobiotic strains are able to stimulate immunity in distal mucosal sites from the gut when orally administered. Taking into

account all these previous findings, LAB strains or their cell fractions that are capable of activating pattern recognition receptors (PRRs) in intestinal epithelial cells and/or cells of innate immunity are probably the most effective immunobiotics for the stimulation of the respiratory and systemic immune responses. Two recent studies support our hypothesis. Ichinohe et al. [120] showed that commensal microbiota composition critically regulates the generation of virus-specific $CD4^+$ and $CD8^+$ T cells and antibody responses following respiratory influenza virus infection. These authors speculated that a select group of commensal bacteria, mainly neomycin sensitive bacteria (Lactobacilli) could trigger TLRs to stimulate leukocytes either locally or systemically. Then, the factors released by such leukocytes could support steady-state activation of inflammasome-dependent cytokine release by respiratory tract DCs improving their migration to the draining lymph nodes when a viral infection occurs [120]. Moreover, commensal microbiota providing signals for PRRs has been discussed by another recent study showing that peptidoglycan translocated from the gut microbiota to the systemic circulation is sensed by NOD1 receptor, resulting in enhanced systemic innate immunity mediated by neutrophils [121]. Further studies using immunomodulatory LAB strains able to stimulate respiratory immunity as well as their cell components such as non-viable bacterial particles, intact cell walls, cell wall polysaccharide-peptidoglycan complex and chromosomal DNA are necessary to find probiotic effector molecules able to stimulate immunity in distant mucosal sites from the gut. Since the maintenance of the viability of microorganisms such as LAB limits their use, sale and transfer to less accessible or more distant areas, the possibility of developing preventive methods with non-viable microorganisms or their fractions emerges as a good alternative for their use as a medical tool.

5. Conclusions

In this review we describe several research works dealing with the possibility of using LAB for the prevention of *S. pneumoniae* respiratory infection. The results discussed here and the ones described by different groups using LAB for the prevention of other respiratory infections show some important coincidences. In general, LAB strains with immunomodulatory properties are able to stimulate the innate immune system. Then, when an infectious stimulus occurs, adaptive immunity is activated according to signals induced by the pathogen, which would be enhanced by the immunomodulatory effect of LAB. Another important coincidence observed in the different research works is that the ability of LAB to modulate the inflammatory response via the induction of IL-10 production is important to improve protection. In this sense, two important questions should be answered in order to use immunobiotic LAB strains to efficiently prevent respiratory infections: should the selection of LAB with immunomodulatory properties be based on the intensity of mucosal and systemic innate immunity activation rather than on the activation of the adaptive immune response? What is more important in the selection of LAB as adjuvants, their ability to activate or to down regulate the immune response? Studies with the same probiotics strains in different experimental models indicate that the mechanisms of action depend on the nature of the disease being treated [107]. Therefore, we could hypothesize that the effect of LAB would be based primarily on the induction of an early intense innate immune response together with an improved ability to regulate the inflammatory response that prevents tissue damage. These early events during the course of infection would be followed by an appropriate adaptive response activation according to the infectious challenge.

With regard to the prevention of pneumococcal respiratory infection by using LAB, two major lines of research can be said to coexist. The first line studies the possibility of using LAB as adjuvants in order to increase the innate and specific immunity against the respiratory infection. These investigations are particularly important in the protection of immunocompromised hosts. In this sense, it seems clear that the optimal way to improve the respiratory and systemic immunity is the nasal administration. In addition, in order to ensure the safety of the adjuvant, it would be interesting to propose the use of non-viable microorganisms or bacterial components. In this way, it is necessary to advance in the study of the mechanisms involved in the immunomodulatory activity of the bacterial fractions. The results described in this review demonstrate the importance of the use of probiotics in immunocompromised hosts, which not only accelerate the normalization of the immune response against *S. pneumoniae* but can also improve defenses, achieving higher levels of protection than those observed in immunocompetent hosts. Further research in this area opens up important possibilities for future applications of LAB.

The second line of research investigates the possibility of using LAB as vaccines against pneumococcal infections. As reviewed in this work, promising results against pneumococcal infections were obtained by nasal immunization with different LAB-based vaccines. One characteristic of these vaccines is the need of multiple doses (ranging from 3 to 6), which may be a problem for vaccination in different populations due to low degree of commitment of individuals with complex schedules, mainly in developing countries. Combination with probiotics or immunomodulatory molecules or even co-expression of such molecules may be alternatives to overcome this problem. In this sense, there is still a long way to go in order to optimize the conditions to make these vaccines safe and to develop easy and efficient immunization protocols. Progress in clinical studies is also required because, although research conducted in experimental models is needed, it does not guarantee the effectiveness of LAB-based vaccines in humans. On the other hand, GEM-derived vaccines composed of pneumococcal antigens have also been shown to induce humoral and cellular protective immune responses. Bivalent SLr-IgA1p or trivalent SLr-IgA1p-PpmA GEM vaccines elicited increased survival time in a model of fatal pneumococcal pneumonia in mice induced with the D39 strain (capsule 2). Nevertheless, immunization protocols still consist of 3 doses [35,97]. Thus, the induction of robust immune responses using fewer doses is still a challenge for the use of LAB strains as vaccine vectors against pneumococcal diseases that should be pursued by the research groups.

In conclusion, LAB represent a promising resource for the development of prevention strategies against respiratory infections that could be effective tools for medical application.

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