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Immunobiotic and Recombinant Lactic Acid Bacteria: Soldiers in the Fight Against *Streptococcus pneumoniae*

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ABSTRACT

Respiratory infections are a persistent public health problem and despite the remarkable advances in antibiotic therapies, diagnostic tools, prevention campaigns and intensive care, they are among the main causes of death in the world. *Streptococcus pneumoniae* remain the leading cause of respiratory infections in children younger than 2 years old, elderly and immunocompromised patients. In the last years many efforts have been made to unravel the mechanisms of beneficial bacteria activity and various experimental approaches have been developed to characterize the molecular basis of these effects. Significant progress has been made in the knowledge of the mechanisms of beneficial bacteria action in the gut, and recently great advances have been performed in the knowledge of how orally or nasally administered beneficial bacteria stimulate immunity in the respiratory tract. The purpose of this work is to review the current knowledge on

the effects of probiotics, recombinant lactic acid bacteria and commensal respiratory bacteria on *Streptococcus pneumoniae* infection, and to provide insights on the possible cellular and molecular mechanisms of action, especially those affecting the respiratory immune system.

INTRODUCTION

Respiratory infections are a persistent public health problem and despite the remarkable advances in antibiotic therapies, diagnostic tools, prevention campaigns and intensive care, they are among the main causes of death in the world [1]. *Streptococcus (S.) pneumoniae* remain the leading cause of respiratory infections in immunocompromised patients. Over 44% of total hospitalizations due to bacterial respiratory tract infections in children younger than 2 years old are caused by Pneumococcus. Moreover, studies of etiology of acute pneumonia in severely malnourished children have implicated *S. pneumoniae* as main pathogen causing mortality [2]. Additionally, *S. pneumoniae* is the leading cause of community-acquired pneumonia and of bacteremia and meningitis for those greater than 65 years of age. Worldwide, the mortality-rate associated with pneumococcal pneumonia exceeds 20% but may be as high as 40% for those within nursing homes [3,4].

Pneumococcal pneumonia is characterized by inflammation of lung tissue due to bacterial colonization of the lower respiratory tract, causing fever, malaise, dyspnoea, and productive cough. The infection can progress to an acute respiratory failure, septic shock, and death. Therefore, understanding the interaction of the pathogen with cellular and molecular components of the immune system is highly relevant for the development of preventive and therapeutic tools [5].

Great efforts have been made worldwide to improve resistance against pneumococcal infections and to reduce the associated mortality. Accordingly, the two most important strategies for pneumococcal diseases prevention were healthy nutrition and the use of effective vaccines. Lactic acid bacteria (LAB) can be used for both strategies. During last decade many reports demonstrated that immunobiotics and recombinant LAB are a promising resource for the development of prevention strategies against respiratory infections that could be effective tools for medical application. LAB have been used for the development of probiotic foods with the ability to stimulate the respiratory immune system [6,7]. In addition, the advances in the molecular biology of LAB have enabled the development of recombinant strains expressing antigens from *S. pneumoniae* that have proved to be effective inducing a protective immunity [5]. Therefore, the aim of this review was to analyse the current scientific literature concerning the use of immunobiotic and recombinant LAB strains in the prevention of pneumococcal respiratory infections.

STREPTOCOCCUS PNEUMONIAE VIRULENCE FACTORS AND PATHOGENESIS

Several *S. pneumoniae* molecules, such as proteins and carbohydrates, have been linked to the

capacity of this pathogen to colonize the respiratory tract epithelium and to cause inflammation [8,9] (Figure 1). The capsule is the most important virulence factor. It is composed by a thick layer of polysaccharides that extend from the cell wall. The pneumococcal capsule acts as a hydrated shield protecting the bacterial surface from recognition and interaction with the host immune system. Also, an anti-phagocytic activity has been also shown for the capsule. It was demonstrated that prevented opsonophagocytosis and impaired the recognition of cell wall-deposited C3b/C3bi. In addition, the capsule also promotes bacterial adhesion to respiratory epithelium [9,10].

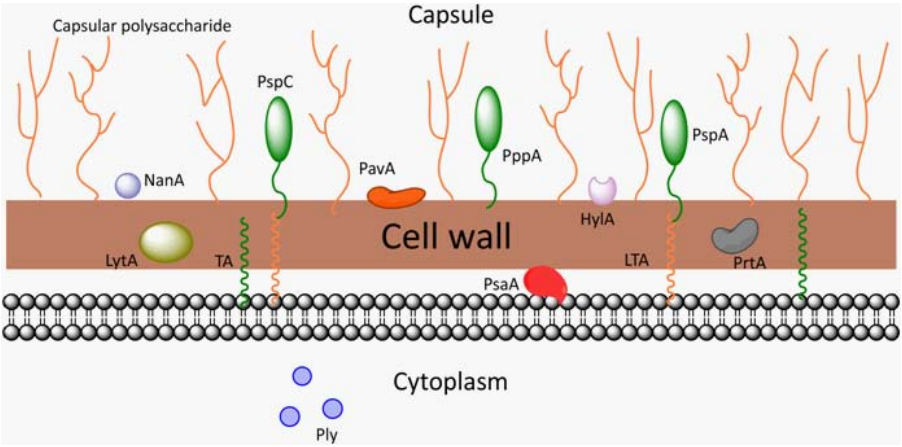

Figure 1: Schematic representation of the major virulence factors of *Streptococcus pneumoniae***.** Different virulence factors from *Streptococcus pneumoniae* are shown with their respective localization in the bacterium (capsule, cell wall, cell membrane and cytoplasm). The capsule is shown as a brown layer, where polysaccharides are represented as branched structures. From left to right: Serin Protease (PrtA), Pneumococcal Surface Adhesin A (PsaA), Pneumococcal Adherence and Virulence factor A (PavA), Pneumococcal Surface Protein A (PspA), HyaluronateLyase (HylA), Pneumococcal Surface Protein C (PspC), Neuraminidase A (NanA), Pneumolysin (Ply), and Autolysin (LytA). The components of the cell wall lipoteichoic acid (LTA) and teichoic acid (TA) are also shown. Disruption of the cell membrane and cell wall mediated by LytA allows the liberation of Ply to the extracellular medium.

Another important virulence factor from *S. pneumoniae* is the pneumolysin, a cholesterol-binding pore-forming toxin. This toxin is released into the alveolus when pneumococci are lysed by an antibiotic or upon autolysis. This virulence factor dramatically impairs alveolar epithelial-capillary barrier function and induces dysfunction of the sodium transporters required for liquid reabsorption in alveolar spaces [11].

S. pneumoniae also possesses an arsenal of surface lytic enzymes, such as hyaluronidases, neuraminidases, and serine proteases, which degrade extracellular matrix components, such as glycolipids and oligosaccharides present in host body fluids and cellular surfaces [9].

Three forms of neuraminidase have been identified in pneumococci, designated as NanA,

NanB, and NanC [12]. Neuraminidase A cleaves terminal sialic acid from cell surface glycans such as mucin, glycolipids, and glycoproteins, with resultant exposure of binding sites on the host cell surface contributing to pneumococcal adhesion and colonization [12]. NanB is involved in the metabolic utilization of sialic acid as a carbon and energy source by the pneumococcus, while NanC has a regulatory role [12,13]. Hyaluronidase is secreted by pneumococci and breaks down the hyaluronic acid component of host connective tissue and extracellular matrix [9]. Increased epithelial permeability caused by hyaluronidase, favours the *S. pneumoniae* spread and colonization, especially when acts together with pneumolysin [14].

Additionally, the adherence and colonization of host tissues by pneumococcus are mediated by surface adhesins. The adhesins, PavA and PavB, promote the invasion of host cells and pneumococcus dissemination. PavA has been shown that bind to fibronectin, while PavB binds both fibronectin and plasminogen [15,16]. It has been suggested that PavA may affect pneumococcal colonization by modulating the expression or function of virulence factors of *S. pneumoniae* [15]. Choline binding proteins (CBPs) binds to terminal choline residues of teichoic or lipoteichoic acids present on the surface of *S. pneumoniae*, anchoring the pathogens to human cell glycoconjugates, favouring the transition from colonization to invasion [8]. The pneumococcal surface proteins A (PspA) and C (PspC) are two other virulence factors belonging to the family of CBPs. PspA is present in virtually all strains of *S. pneumoniae*, although it is highly variable serologically because of the high heterogeneity in the N-terminal region of the *psp*A gene [17]. The variants of PspA have been classified into three families (1, 2 and 3), each composed of different clades. PspA acts by interfering with complement activation and also by binding lactoferrin and apolactoferrin, protecting bacteria against these bactericidal molecules. Among the known pneumococcal antigens, PspA is perhaps the most studied and considered a good candidate for a protein-based vaccine due to its immunogenicity and because it elicits an antibody response that enhances complement deposition and protects against nasal colonization, pneumonia and sepsis in mice [17]. Moreover, the PspA families are able to generate antibodies that show cross-protection among different serotypes of the same family. Same as PspA, PspC (also called CbpA) shows high variability and has been classified into 11 groups. It binds to the complement regulatory protein factor H and to the human secretory IgA [18].

RESPIRATORY IMMUNE RESPONSE AGAINST PNEUMOCOCCAL INFECTION

Invasion of *S. pneumoniae* into the lower respiratory tract represents a serious threat that requires immediate immune responses without damage of lung tissue. Both innate and adaptive immune responses must be regulated to allow the control of infectious agents while avoid damage to the delicate air-exchanging surface. Currently it is well understood how the respiratory immune system acts against *S. pneumoniae* infection. Additionally, great progresses has been made toward understanding how immune response contributes to protection and damage during infection

[19]. Several immune mechanisms are involved in the respiratory defense against *S. pneumoniae*.

Epithelial cells that line the airways and airspaces, alveolar macrophages (AMs) together with the mucociliary elevator of the conducting airways, and the surfactant layer of the alveolar spaces are the first defences against inhaled or aspirated pathogens (Figure 2). The respiratory epithelium forms a complex physicochemical barrier with the mucociliary escalator to provide the first line of defence against inhaled pathogens [20]. Respiratory epithelial cells also produce antimicrobial factors that contribute to mucosal defences. Hundreds of these peptides have been identified in eukaryotes, with highly species-specific variation, but respiratory epithelium primarily expresses members of just two groups: defensins and cathelicidins [21]. Antimicrobial peptides act as important innate immune defence mediators against *S. pneumoniae*. Among a number of antimicrobial peptides, β-defensin 2 has strong antimicrobial activity against this pathogen [22].

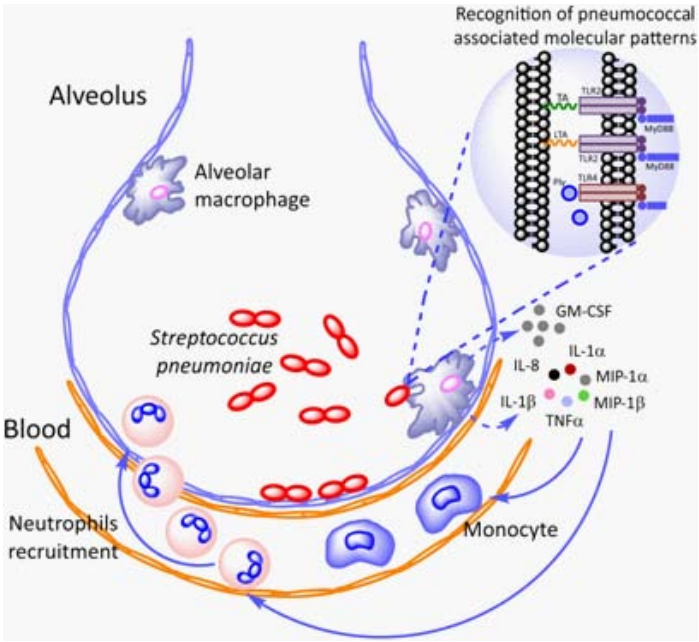

Figure 2: Innate immune response in lung. Alveolar macrophages constitute the first line of phagocytic defence against respiratory pathogens such as *Streptococcus pneumoniae*. Alveolar macrophages play a prominent role in lung immunity by initiating inflammation through the production of chemokines and cytokines and the recruitment of neutrophils from the pulmonary vasculature into the alveolar space. Neutrophils provide auxiliary phagocytic capacities that play a critical role in host defence against *S. pneumoniae.*

In addition to their physicochemical protective role, airway epithelial cells up-regulate several factors and receptors including Toll-like receptors (TLRs), cytokines and chemokines during infections. Many recent studies have focused on the ability of respiratory epithelium to respond

to pathogens through PRRs such as TLRs and NOD-like receptors (NLRs). Most TLRs (TLR1–6, 9) are found on respiratory epithelium and its function in response to several pathogens resulting in lower respiratory infection has been well characterized [23]. For example, signalling through TLR2, which recognizes bacterial peptidoglycans and lipoproteins, appears to be crucial for host response to both extracellular (*S. pneumoniae*, *P. aeruginosa*, *S. aureus*) and intracellular bacteria (*L. pneumophila*, *C. pneumoniae*, *M. pneumoniae*). In response to pneumococcal peptidoglycan, NOD2 signalling stimulates the production of CCL2, recruiting macrophages and neutrophils to the site of infection [24]. Most TLRs and NLRs signalling cascades within airway epithelium result in the release of chemokines (GM-CSF, MIP-2 and KC), thereby allowing the epithelium to modulate the inflammatory response in vast surface area [23]. In addition, it was reported that *S. pneumoniae* induce a prominent production of pro-inflammatory cytokines, IL-1β and TNF-α in human alveolar (A549) and bronchial (BEAS-2B) epithelial cells, and that among the numerous virulence factors encoded by this pathogen, pneumolysin was identified as the major factor involved in the expression of cytokines at the early stage of infection [25].

AMs express typical phenotypic markers ($F4/80^{+}$, $CD11c^{high}$, $CD11b^{int}$) and are normally maintained in a quiescent state. However, they conserve the capacity to be activated in response to pathogens [1]. The activation of AMs results in increased phagocytosis and microbial killing [26]. Moreover, in the case that invading pathogens are too virulent or represent too large a load to be contained by AMs alone, these cells are able to generate 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 [27, 28]. Both, neutrophils and macrophages are critical components of innate defence against pneumococci (Figure 2).

Components of the pneumococcal cell wall, such as lipoteichoic acid (LTA) and lipoproteins, are recognized by TLR2. In addition, TLR9 detects unmethylated CpG motifs of pneumococcal DNA within endosomes and TLR4 appears to be activated by pneumolysin. In addition to inducing cytokines, TLRs activation has also been suggested to enhance pneumococcal phagocytosis and intracellular killing in neutrophils and macrophages [29,30]. It was reported that phagocytosis, lysozyme-dependent digestion of *S. pneumoniae* in macrophages, and subsequent pneumolysin-mediated delivery of pneumococcal peptidoglycan fragments into the host cell cytosol are involved in NOD2 activation [24]. *S. pneumoniae* recognition by NOD2 mediates MCP-1 (CCL2) production that leads to recruitment of additional macrophages into the upper respiratory tract. Accordingly, NOD2 together with TLR2 contribute to clearance of pneumococcal colonization [24]. The inflammasome-forming proteins NLRP3 and AIM2 are other PRRs that contribute to the recognition of *S. pneumoniae*. Previously it was reported that pneumolysin was critical for caspase-1 activation and subsequent IL-1β/IL-18 production in pneumococcal infection [31]. Recent studies demonstrated that the NLRP3 inflammasome is involved in the pneumolysin-stimulated IL-1β production in macrophages [32] and that the AIM2 inflammasome also

mediates IL-1β production in *S. pneumoniae*-infected macrophages [33]. The importance of the inflammasome-IL-1β/IL-18 pathways in macrophages during pneumococcal infection is underlined by studies in mice deficient in IL-1β, the IL-1 receptor or the IL-18 receptor. These mice showed enhanced susceptibility towards *S. pneumoniae* in different disease models [33].

Dendritic cells (DCs) forms an elaborated network in the respiratory tract where constitute an important control point for the induction of immunity or tolerance. Respiratory DCs, originally described as a single population of MHC-II^{+}CD11c^{+} cells, can be subdivided into different subsets in the lung. Conventional DCs express high levels of CD11c compared with CD11c^{dim} plasmacytoid (p) DCs. A highly developed network of CD11c^{+}CD11b^{-}CD103^{+}cDCs is found in the epithelial layer of the conducting airways, and they form long cellular extensions between the basolateral space made up of basal epithelial cells [19]. Immediately below the basement membrane, the lamina propia contains CD11c^{+}CD11b^{hi}CX3CR1^{+} DCs. The conducting airways also contain pDCs. On the other hand, lung parenchymal DCs found in the alveolar septa, can similarly be subdivided into CD11b^{+} and CD11b^{-}DCs, and contain a subfraction of pDCs. In mouse lung, several studies demonstrated the existence of two major DCs subsets identified as MHC-II^{+}CD11c^{+}CD11b^{low}CD103^{+} (CD103^{+} DCs) and MHC-II^{+}CD11c^{+}CD11b^{high}CD103^{-}(CD11b^{high} DCs) cells [34].

DCs perform a sentinel function recognizing inhaled antigens through PRRs. When triggered via PRRs or activated by epithelial cytokines, DCs migrate via afferent lymphatic to the T cell paracortex of the lung-draining mediastinal lymph nodes (MLNs) to select and activate recirculating naive T lymphocytes (Figure 3). The adaptive immune response to lung pathogens depends on the clonal expansion and differentiation of naive T and B cells that occurs primarily in draining lymph nodes. The response is triggered when lung DCs carrying antigen reach MLNs through the afferent lymphatic and present antigen to T cells. They are then processed by resident lymph node DCs and B cells. T lymphoblasts destined to become effector CD4 or CD8 T cells at the site of infection leave the lymph node via the efferent lymphatic, reach the circulation, and enter in the lung through either bronchial circulation, to populate the conducting airways and pleura, or pulmonary arterial circulation, to enter alveolar septa and airspaces [35]. The protective role of DCs against pneumococcal infection is supported by several studies. Virulence factors from *S. pneumoniae* are able to modulate maturation and activation of DCs. It was showed that HSP100/ClpP expression favors the uptake and phagocytosis of pneumococci by human DCs [36]. Furthermore, pneumococcal HSP100/ClpP is required for optimal production of inflammatory cytokines and chemokines, and an efficient activation of adaptive immune response in DCs. In addition, it was reported that PavA is a key factor for pneumococci to induce optimal cytokine productions (IL-1, IL-6, IL-8, IL-12, TNF and IL-10) by DCs and to induce protective adaptive immune responses as well [37]. It was reported that mice lacking the CC chemokine ligand 2 (CCL2) show more severe pneumonia after *S. pneumoniae* infection, as a result of impaired recruitment of lung mononuclear phagocytes, including conventional DCs [38]. In addition, it was demonstrated that respiratory CD103^{+} DCs are critical in the primary activation (IFN-γ and IL-

17 release) of pulmonary iNKT cells that mediate the protective innate immune response to *S. pneumoniae* [39].

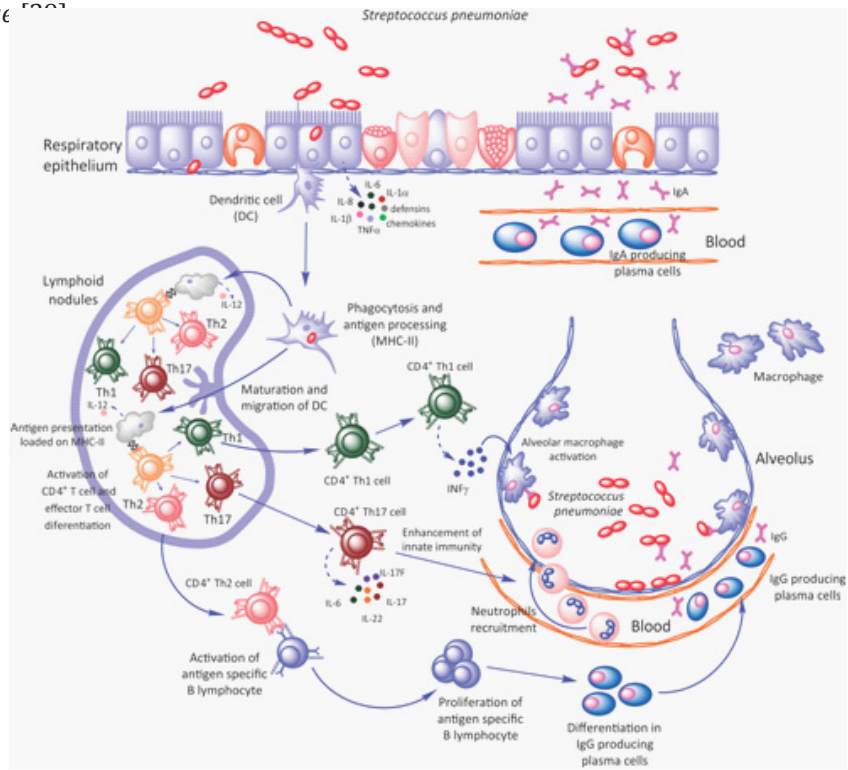

Figure 3: Adaptive immune responses in the airways and lung. Recognition of *Streptococcus pneumoniae* by respiratory antigen presenting cells initiate adaptive immune responses against the pathogen. Th1, Th2 and Th17 responses are involved in the protection against pneumococcal infection. Th1-dependen activation of respiratory macrophages, Th2-dependent production of specific antibodies (IgA and IgG) and, Th17-dependent neutrophilic infiltrate in the lungs are essential components for protection against *S. pneumoniae*.

After exposure to pathogens there is an activation of antibody responses in the respiratory tract. The type and concentration of produced antibodies depend of the site of exposure. Upper airway exposure results primarily in an IgA response (Figure 3). However, when organisms reach the deep lung after passing through the upper airway, they induce an increased production of pathogen-specific IgG [40]. There are two important inductive sites for the respiratory mucosal immune response, the lymphoid follicles in the nasal associated lymphoid tissue (NALT) and the bronchus associated lymphoid tissue (BALT) [41]. Following exposure to a pathogen, the antigen is uptaken by DCs of NALT or BALT, and followed by activation of $CD4^+$ Th2 cells and generation of IgA-producing plasma cells that populate airway lamina propia [42]. IgA reaches the respiratory lumen by interacting with the polymeric Ig receptor on the basolateral surface of epithelial

cells. Secretory IgA binds to bacteria without activating complement or stimulating the release of inflammatory mediators by innate immune cells and has an important role in the protection against respiratory pathogens [43].

In the deep lung, when pathogens reach the alveolar space occurs a differentiation and expansion of antibody-secreting plasma cells that are committed to the production of IgG [44,45] (Figure 3). These antibodies have an important role in the protection against respiratory infections since opsonizing IgG antibodies are important for complement fixation and enhancement of the efficiency of macrophage killing. This immune activation also induces the production at the systemic level of antibodies responsible for preventing the passage of pathogens to the blood and their subsequent dissemination [45,46]. In a recent study, Roche et al. [47] using a murine model of bacterial colonization with *S. pneumoniae* demonstrated the importance of agglutinating IgG antibodies in mucosal defence against the respiratory pathogen. The study showed that systemically delivered anti-pneumococcal IgG accessed the mucosal surface and blocked acquisition of colonization and transmission between littermates.

IMMUNOBIOTICS LACTIC ACID BACTERIA AS ENHANCERS OF RESPIRATORY IMMUNITY

Orally Administered Immunobiotics

Recent studies have centred on whether immunobiotics might sufficiently stimulate the common mucosal immune system to provide protection in other mucosal sites distant from the gut [5-7,48]. The potential protective effect of probiotics on the improvement of resistance against *S. pneumoniae* was studied using an experimental model of infection in adult immunocompetent mice (Table 1). In this model, mice were infected intranasally with *S. pneumoniae* serotype 14. Challenge with pneumococci significantly increased bronchoalveolar lavage fluid (BALF), albumin concentration and lactate dehydrogenase (LDH) activity, indicating that infection increased the permeability of the alveolar-capillarity barrier and induced cell damage in lungs. Additionally, histopathological examination revealed an intense inflammatory response with progressive parenchymal involvement, including cellular infiltration, fibrosis in bronchial walls and vessels, haemorrhage and reduction of alveolar airspaces [49]. Pathogen was detected in lung and blood samples throughout the period assayed (15 days) [49].

The ability of LAB to increase resistance against pneumococcal infection was evaluated [49-52]. Only the administration of *Lactobacillus casei* CRL431, *Lactococcuslactis* NZ9000, *Lactobacillus rhamnosus* CRL1505, and a probiotic yogurt prepared with the immunobiotic strains *Lactobacillus bulgaricus* CRL423 and *Streptococcus thermophilus* CRL412, increased the resistance to the respiratory pathogen, demonstrating that LAB were able to increase *S. pneumoniae* clearance rates in lung and blood, and improve survival of infected mice, up-regulating innate and adaptive immune responses in the respiratory tract [49-53].

Oral treatment with *L. casei* CRL431, *L. rhamnosus* CRL1505 or *L. rhamnosus* CRL1506 on the phagocytic and microbicidal activity of AMs and peritoneal macrophages were studied [54]. It was found that activation of macrophages depended on the LAB strain and on the mucosal and systemic cytokines profiles. LAB increased the microbicidal and phagocytic activity of peritoneal macrophages as well as the cytokines production. In addition, it was found that LAB increased the principal macrophage-activating cytokine (IFN-γ) concentrations in serum that serves critical functions in innate immunity [54]. Only *L. casei* CRL431 and *L. rhamnosus* CRL1505 increased the microbicidal and phagocytic activity of AMs as well as their ability to produce cytokines, with increased concentrations of IFN-γ in BALF [54]. Considering that peritoneal and AMs are activated by cytokines released by immune cells in the gut and not directly by their interaction with lactobacilli, the enhanced phagocytic activity of peritoneal compared to AMs may be due to the fact that the former are located anatomically closer to the place (intestinal environment) where the macrophage stimulating cytokines are produced. However, it is possible that macrophage-stimulating cytokines are produced locally in the respiratory tract; therefore the improved function of AMs after oral treatment with immunobiotics would explain, at least in part, the increased resistance against pneumococcal infection.

Also, mice that received LAB had significantly higher amounts of BALF TNF-α than the control group after challenge with *S. pneumoniae* [49]. The 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. 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 [49-53]. On the other hand, due neutrophils have been implicated in tissue injury in many inflammatory disorders [27], the regulation of inflammatory response by anti-inflammatory cytokines prevented damage to the host. Blum et al. [55] have suggested that LAB could participate in tissue protection against the deleterious effect of an ongoing inflammatory process. Treatments with immunobiotics prior to pneumococcal infection have induced a significant increase in IL-10 in lung and serum [49-53], that could help to reduce production pro-inflammatory cytokines and chemokines production, and to down regulate the expression of adhesion molecules. Consequently, IL-10 attenuated inflammatory damage and pathophysiological alterations in lung infected with pneumococci [56]. In consequence, a more effective inflammatory response against infection could be obtained by LAB treatments that beneficially regulate the balance between TNF-α and IL-10.

On the other hand, it has been demonstrated that oral administration of LAB can increase the IgA^+ cells in the lamina propria of the small intestine [57]. 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. Many authors demonstrated that oral administration of particular LAB strains could induce an increased IgA^+ cell population in the respiratory tract [49-52].

Oral treatments with *L. casei* CRL431, *L. lactis* NZ9000, *L. rhamnosus* CRL1505 and the probiotic yogurt were able to increase the number of IgA^+ cells in intestine and bronchus. Also, it was found that LAB treatments improved the concentration of anti-pneumococcal IgA in the airways [49-53] and increased the levels of IL-4 and IL-10 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 [40]. Additionally, those specific antibodies could bind antigens, minimizing their entry and so reducing the inflammatory reactions, with lower harmful effects on the lung. Thus, the stimulation of the IgA cycle and the improvement of the levels of pathogen specific IgA induced by the LAB strains could be in part responsible of the greater resistance to the challenge with *S. pneumoniae*.

Studies about the effect of LAB on antigen presentation cells, demonstrated that oral administration of LAB resulted in higher BALF TNF-α values and NBT^+ cells percentages, with increased specific anti-pneumococcal IgG. These results indicated that LAB treatments would be able of improving macrophage-mediated antigen presentation in the lung that induced T cell activation and B cell clonal expansion and differentiation into IgG^+ antibody-secreting plasma cells [49-53]. In addition, it was demonstrated *in vitro* that *L. lactis* NZ9000 are able to up regulate the expression of MHC-II and CD86 co-stimulatory molecules in bone marrow derived DCs [58,59]. During the generation of an efficient effector of the immune response, DCs have to overcome suppression by Treg cells. Production of IL-6 by DCs releases them from the suppression by naturally occurring Treg. It has also been demonstrated that other cytokines are able to trigger DCs activation/maturation, among them, proinflammatory cytokines such as TNF-α [60]. Preventive LAB treatments were able to increase IL-6 and TNF-α concentrations in the respiratory tract after challenge with *S. pneumoniae* [49,51-53]. In consequence, oral administration of LAB would be able to improve antigen presentation mediated by pulmonary DCs, as was previously reported [61].

On the other hand, oral administration of non-viable immunobiotics was also able to improve defences against pneumococcal infection. Tanaka et al. [62] reported that oral administration of heat-killed *L. pentosus* b240 did not prolonged the survival time of pneumococcal-infected mice, but significantly reduced body weight loss, as well as bronchitis. Authors suggested that mitogen-activated protein kinases signalling pathways and pro-inflammatory cytokine secretion are involved in the lung damage noted in pneumococcal-infected mice and that administration of b240 strain could suppress the inflammation via JNK pathway and reduce inflammatory tissue damages.

Some clinical studies have been performed in humans in order to evaluate the effect of probiotics in the prevention of respiratory infections, with variable results [63]. The main finding of this review

was that the majority of probiotics, when taken prophylactically by healthy individuals do not reduce the incidence of respiratory infections. However, many authors demonstrated a significant reduction of the severity of symptoms as well as the duration of respiratory tract infections has been associated with probiotic treatments [64-66]. The ability of *L. rhamnosus* CRL1505 for improving respiratory immunity was evaluated in a clinical study, due health benefits of immunobiotics are strain-dependent trait, so the functional effects demonstrated for one probiotic cannot be extrapolated to another ones. Therefore, it was performed a randomized controlled trial in order to evaluate the effect of a probiotic yogurt containing *L. rhamnosus* CRL1505 on both gut and non-gut related illnesses among children [67]. Results demonstrated that *L. rhamnosus* CRL1505 improved mucosal immunity and reduced the incidence and severity of intestinal and respiratory infection in children. When the type of infectious events according to their location and symptoms were studied, the frequency of them was consistent with the prevalence reported in our country. The most common infectious diseases were upper respiratory infections, followed by angina and then lower respiratory infections (acute bronchitis) and diarrhea [68-71]. The 34% of children who consumed the probiotic yogurt showed an infectious event, while in the placebo group this value was reached 66% of children. Also, there was a significant decrease in the presence of fever in children who consumed probiotic yogurt as well as a slight decrease in the need for antibiotic treatment. These results demonstrated a significant reduction in occurrence of infectious events with reduction of associated symptoms, when children received *L. rhamnosus* CRL1505 [67].

Few other studies evaluated whether immunobiotics are capable of preventing respiratory infections and reducing their severity in children. Some studies have examined the role of probiotics in the prevention of respiratory infections in children attending daycare centers [72,73]. Results from a study evaluating *Bifidobacterium lactis* or *Lactobacillus reuteri* versus placebo did not show a beneficial effect on the rate and duration of respiratory illnesses [73]. On the other hand, randomized, double-blind, placebo-controlled studies performed in daycare centers showed that administration of *Lactobacillus GG* resulted in a reduction in the number of children suffering from respiratory tract infections [72,74]. Moreover, treatment with *Lactobacillus GG* significantly reduces the risk for developing nosocomial respiratory tract infections in children who were hospitalized on a pediatric ward [75]. These results suggested that not all the probiotic strains that were able to stimulate intestinal defenses were able to improve respiratory immunity. Therefore some LAB can perform a functional role better than others, so it is important to carry out thorough studies on specific strains, according to their therapeutic use. In this sense, systematic studies in animals on the ability of LAB to improve respiratory immunity, allowed to select one that have beneficial effect in humans [67].

The study of the mechanism responsible for the beneficial role of probiotics on the gut have documented a direct anti-microbial effect and improvement in mucosal barrier function, as a result of the effects of probiotics on both innate and adaptive immunity. However, the mechanisms responsible for the improvement of defences against upper respiratory infections

remain partially unknown. Some progress was made in the knowledge of the immunological mechanisms involved in the protective effect of *L. rhamnosus* CRL1505 against respiratory pathogens [76]. In this regard, it was assumed that the stimulation of the IgA cycle and the improvement of the levels of IgA induced by the *L. rhamnosus* CRL1505 could in part explain the greater resistance of children to respiratory infections [52,67]. On the other hand, it is known that the symptoms associated with common cold are a result of the inflammatory response by the host towards the infection. Therefore, compounds with the capacity to improve immunity and control unproductive inflammation are supposed to be effective antivirals. In this sense, as was previously described *L. rhamnosus* CRL1505 induced a significant increase of IL-10 in lung and serum prior to pneumococcal infection [52] or poly(I:C) challenge [67]. The improved levels of IL-10 would attenuate inflammatory damage and pathophysiological alterations in infected children. This controlled inflammatory response induced by *L. rhamnosus* CRL1505 could explain the beneficial effect in the incidence and severity of common cold [67]. In addition, *L. rhamnosus* CRL1505 is able to augment the number of intestinal $CD3^+CD4^+IFN\text{-}\gamma^+$ T cells and induce a mobilization of these cells into the respiratory tract. Furthermore, the mobilization of $CD3^+CD4^+IFN\text{-}\gamma^+$ T cells from the intestine to the airways induced a higher production of IFN-γ in the respiratory tract. Probably, IFN-γ secreted in response to *L. rhamnosus* CRL1505 stimulation would be capable of functionally modulate the innate immune microenvironment in the lung, inducing the activation of DCs and macrophages, and improving resistance against *S. pneumoniae* [77] (Figure 4).

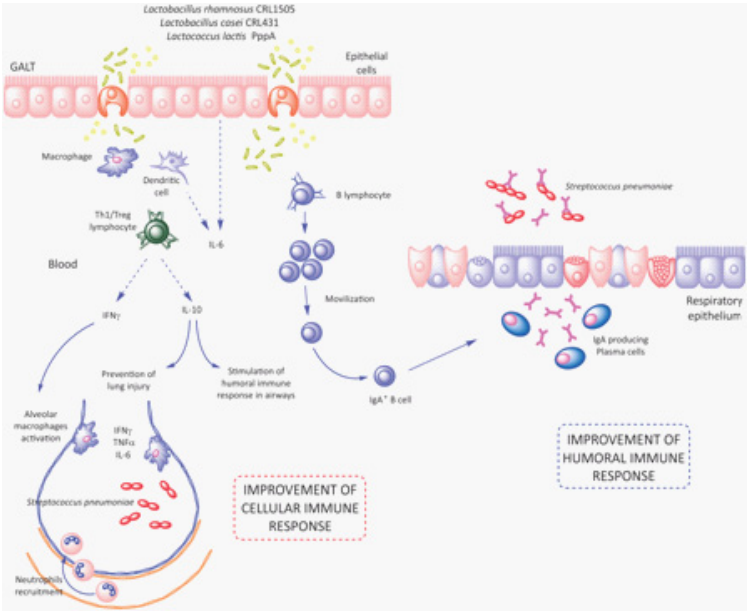

Figure 4: Improvement of respiratory immune response against *Streptococcus pneumoniae* **induced by oral administration of probiotic lactic acid bacteria (LAB).** Orally administered immunobiotic LAB are transported through microfold epithelial cells (M cells), the gut associated lymphoid tissue (GALT), and finally interact with dendritic cells (DCs). Additionally, DCs are able to extend appendices between epithelial cells to take up LAB. The contact of immunobiotic LAB with macrophages and DCs induce activation signals that trigger a switch in cytokine and chemokine production and up-regulation of co-stimulatory molecules. Cytokines produced by macrophages and DCs stimulated with immunobiotic LAB 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, immunobiotic 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, immunobiotic 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 immunobiotic LAB with intestinal epithelial cells and immune cells 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.

Nasally Administered Immunobiotics

Despite the oral way is an attractive route of stimulation because of its acceptability and its simplicity of administration; the nasal mucosa is a practical site for mucosal vaccination because of the absence of acidity, lack of abundant secreted enzymes and small mucosal surface area, that result in a low dose requirement of antigen [41]. Accordingly, the capacity of the nasal administration of immunobiotic LAB to increase the resistance against *S. pneumoniae* has been also evaluated in challenge-infection mice models. Cangemi de Gutierrez et al. [78] 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 reduced pathogen counts in the lung.

In addition, nasal administration of *L. lactis* NZ9000 increased the clearance rate of *S. pneumoniae.* This effect was mediated by an up-regulation of innate and specific immune responses in both local and systemic compartments [79]. As GALT, 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. It was reported that NALT play an important role by reducing the pathogen burden to a level that only induces minimal inflammation in the lower lung [42]. In consequence, nasal administration of *L. lactis* before challenge with *S. pneumoniae* reduced the number of pathogens in the nasal cavity and in lung. This effect could be explained by decreased adherence of *S. pneumoniae* to the respiratory epithelium, by competition between LAB and pneumococci [42]. In addition, the increased activation of AMs with the increased microbicidal function of blood neutrophils together with higher levels of IgA and IgG in BALF and IgG in sserum, would enhance the protective effect [79](Figure 5).

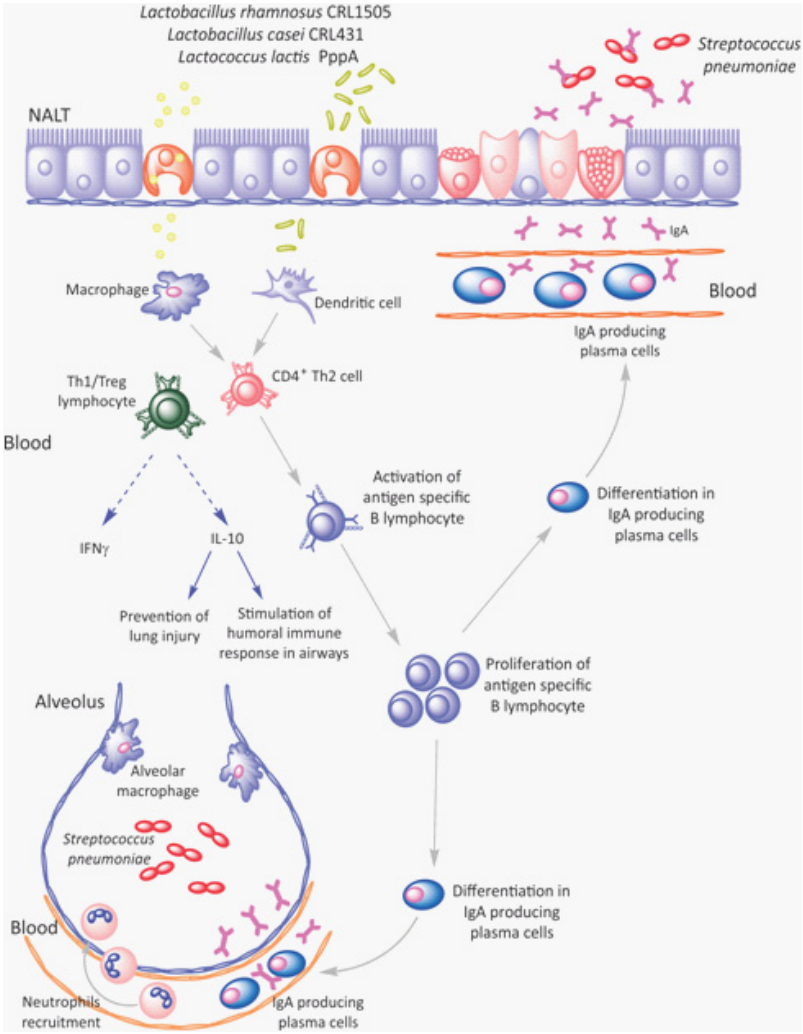

Figure 5: Immune responses induced by nasal administration of probiotic and/or lactic acid bacteria (LAB)-based vaccines expressing different *Streptococcus pneumoniae* **antigens.** Upon contact with the nasal associated lymphoid tissue (NALT), recombinant LAB carrying heterologous antigens are captured 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.

RESPIRATORY COMMENSAL BACTERIA AS POTENTIAL IMMUNOBIOTICS

The mucosal surface that cover the different tracts (gastrointestinal, urogenital, and respiratory) house diverse microbial communities with niche-specific communities. It seems that the presence of a microbiome in mucosal tissues is common fact, considering that mucosal surfaces are a main site of contact with the environmental microorganism. The members of the microbial communities in mucosal tissues interact among them and with the host cells and the multitude of functional characteristics of these communities is involved in the symbiotic interaction between microbiota and host [80-82]. Therefore, commensal microflora significantly affect the human health, in fact, restoration of the commensal microflora composition to the health state has demonstrated to be highly effective for the treatment of several mucosal diseases [83,84].

The respiratory tract is in continuous contact with the environment. This tract shows a great physiological heterogeneity. Beyond differentiated epithelial surfaces, in healthy patients, the respiratory mucosa can express a wide variety of mucins. These glycoproteins protect the host by the capture and clearance of the pathogen and many other substances [85], through a process controlled by the ciliary movement [86]. This is an important point due it was demonstrated that microbes interact with mucins. Recent studies reported the existence of specific species associated with chronic sinus disease (*Corynebacterium tuberculostearicum*) that can induce a significant increase of mucin secretion in the host [87]. The interaction with mucins is an important mechanism because these microbes would have an increased availability of nutrient, protection and increased adherence resulting in a longer residence time. Hence, mucin composition, concentration and homeostasis are important factors related with microbial communities in the respiratory tract.

The first studies in the microbiota associated with the airways demonstrated the presence of distinct resident microbial resident. In healthy patients, the paired nare show a diverse microbiota dominated by Actinobacteria and Firmicutes, while oropharyngeal communities are composed by Firmicutes and Proteobacteria [88]. Many sites of the respiratory tract (eg. lung or sinuses) has been traditionally considered to be sterile, however, currently it is accepted that they harbor a microbiota related with pulmonary health state [87,89,90]. The amount of bacterial communities in the lower airways of healthy persons is significantly lower, representing a transient community

that reminiscent the communities from upper airways and oral cavity [90]. These communities are composed by Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria, Actinobacteria, and Spirochetes [89,91].

There are several studies about microbial communities in lower airways during different diseases, for example, cystic fibrosis, chronic obstructive pulmonary disease [91-93] and asthma [94,95]. Those studies demonstrated the presence of many communities related to the pulmonary health parameters such as pulmonary function [96-99] and bronchial hyperactivity [95]. These results related the airway microbial community with airway health status.

In consequence, a better comprehension of how commensal microbial communities influence the health state of the respiratory tract would allow the development of particular mixtures of specific competing commensal species to get highest efficiency in the treatment and prevention of respiratory diseases.

S. pneumoniae is present in a large percentage of infants and toddlers at a range of 6% to 100% [100], persists for weeks to months escaping the host innate and adaptive immune responses, and constitutes a major risk for auto-infection. *S. pneumoniae* assembles long filamentous pili on their surface through which they adhere to epithelial cells in the upper respiratory tract [101]. One can speculate that some respiratory commensal piliated bacteria might be used to replace pneumococci at the nasal and throat mucous membranes. *Corynebacterium pseudodiphtheriticum*, a piliated [102] Gram-positive normal inhabitant of the nares and throat, could serve as such a probiotic candidate. Bacteriological examinations of the nose and throat in diphtheria carriers showed cases of spontaneous replacement of *C. diphtheriae* by *C. pseudodiphtheriticum* (V. Melnikov, unpublished observation). Kiryukhina et al. (2013) [103] involving volunteers reported the elimination of nasal *Staphylococcus aureus* by implantation of *C. pseudodiphtheriticum* strain isolated from nose of a healthy person. Like *S. pneumoniae* [104], *C. pseudodiphtheriticum* seems to have a potential to produce the wall teichoic acid (WTA), a surface-exposed polymer, essential for nasal colonization [105,106]. This may provide WTA-secured adhesion competitive ability to *C. pseudodiphtheriticum* against pneumococci. Besides, *C. pseudodiphtheriticum* possesses immunomodulatory and adjuvant mycolyl–arabinogalactan–peptidoglycan complex [107] which can also contribute to *S. pneumoniae* elimination.

Colonization of the nasopharyngeal tract is the first and necessary step in the pneumococcal infectious process and often involves the formation of sessile microbial community–biofilm [108]. Biofilm is a stress-tolerant community of microorganisms held together by intercellular junctions and a self-produced extracellular polymeric matrix. It forms on the surface of objects of the environment and the tissues of living organisms. In the view of some authors, the biofilm is a multicellular organism with its inherent development cycle, co-operative behavior of its individuals coordinated by system of quorum sensing [109]. The ability to grow and persist as biofilms is an advantage for many microorganisms, because biofilm grown bacteria show reduced

susceptibility to antimicrobial agents and hinder recognition by the immune system. Biofilms have been associated with up to 80% of all chronic infections [110]. Biofilms are thought to play an important role during colonization of the nasopharynx by *S. pneumoniae*. In comparison to their planktonic counterparts, biofilm pneumococci are hyper adhesive but less invasive and elicit a weaker pro-inflammatory cytokine response. These findings give insight into the requirements for prolonged asymptomatic colonization [111]. Factors influencing pathogen colonization and elimination are not as yet fully understood, but adhesion to mucosal epithelial cells and immune responses are implicated. Potential pathogens *S. pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* are more likely to colonize the nasopharynx of children prone to recurrent otitis media, where impaired local immunity is a main risk factor. Adults with chronic respiratory tract disease also have higher carriage rates [112]. Bearing in mind an implication of probiotics or immunobiotics for balancing the nasopharynx microbiota and struggling *S. pneumoniae* colonization we should not only screen for the beneficial microorganisms commensal for certain biotype? but also search for their occupational, antagonistic and immunomodulating capacities extension.

On the market there is a large number of probiotics, which contain live beneficial microorganisms intended for the restoration of violated intestinal biocenosis. All the current probiotics are planktonic microorganisms obtained by laboratory cultivation on nutrient media. At the same time it has recently become well acknowledged that beneficial bacteria living in nature also grow in a biofilm [113] and appear to be greatly different by the properties from the cultures grown in ''classic'' laboratory conditions [114,115]. So, expecting improvement of the properties of probiotic bacteria it was attempted to obtain a biofilm *in vitro* of the probiotic *Lactobacillus plantarum* 8-RA-3 strain and to evaluate the possibility of its use for the restoration of the disturbed normal intestinal lactoflora by antibiotics in mice. Within the solid substrate cultivation of *L. plantarum* 8-RA-3 strain on the wheat bran saturated with the MRS medium, in 48 – 72 h, the biofilm formation took place. As a result of mice keeping on a diet with the introduction of 0.05% bran fermented by *L. plantarum* 8-RA-3 for 72 h into the fodder, a recovery of normal level of intestinal lactobacilli, inhibited by administration of antibiotic was noted. Five days after the end of the feeding of the test mice, the strains genetically identical to the *L. plantarum* 8-RA-3 were isolated from the faeces of these mice [116]. The results of this study indicate that probiotic bacteria grown in a biofilm could survive in the unfriendly environment, colonize the intestine and balance the impaired microbiota. This might make us think of biofilm grown *C. pseudodiphtheriticum* as nasopharyngeal probiotic prospective candidate, able to eliminate the pathogens like *S. pneumoniae*, by competitive exclusion.

RECOMBINANT LACTIC ACID BACTERIA AS ADJUVANTS AND DELIVERY SYSTEMS TO COMBAT PNEUMOCOCCAL INFECTIONS

Several pneumococcal protein antigens have been expressed in different LAB for the

development of live mucosal vaccines (Table 2). The first pneumococcal protein antigens expressed in LAB were the Pneumococcal Surface Antigen A (PsaA) and the Pneumococcal Surface Protein A (PspA) [117]. 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 [118]. Expression of PsaA and PspA in recombinant bacteria was controlled by the addition of lactose to the culture media. As a result, intracellular inducible expression of the proteins by *L. casei* was successfully achieved. 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 [117]. However, nasally administered *L. casei* PsaA or *L. casei* PspA were not able to induce systemic or mucosal antibodies against the antigens neither conferred protection against pneumococcal colonization and infection in mice [5].

Later, 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 [119]. This construct allowed the expression of PsaA attached to the cell wall of *L. lactis* MG1363, *L. casei* CECT5275, *L. plantarum* NCDO1193 and *L. helveticus* ATCC15009. The results obtained in that 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, authors stated that the protection induced by the vaccines did not correlate with antibody induction [119].

Another work described the development of recombinant *L. lactis* MG1363 expressing the N-terminal region of PspA under the control of the nisin inducible promoter [120]. Both live and inactivated recombinant *L. lactis*-PspA were tested as nasal vaccines in mice. Nasal immunization of CBA/ca with live or inactivated *L. lactis*-PspA increased the mean survival time after intraperitoneal challenge with the pneumococcal strain serotype 4, and increased survival rates against a respiratory challenge with the same strain [14].

Constitutive expression of the N-terminal region of PspA was also expressed in the intracellular compartment of *L. casei* CECT5275 [121,122]. Nasal immunization of C57Bl/6 mice with *L. casei* PspA1 significantly increased survival rates after an intraperitoneal challenge with the A66.1 pneumococcal strain serotype 3 [121]. Another recombinant vaccine, *L. casei*-PspA5, was also shown to significantly protect mice against a respiratory challenge with the ATCC6303 pneumococcal strain serotype 3 [122]. Both experimental 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 [121,122]. Moreover, 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 [122]. Thus, it was clear that this recombinant vaccine induced a Th1 response directed to PspA, which has been described to be a very effective response against pneumococcal infections using different vaccines [123,124]. However, a rapid control of inflammation in lungs during pneumococcal infection is crucial to improve the survival [122], and *L. casei*-PspA5 vaccine was not effective to provide this control. Then, it is probable that the use of other LAB strains as vaccine vectors, could improve the partial protection (40%) elicited by vaccines based in PspA1 or PspA5.

The Pneumococcal surface antigen C (PspC) 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 [122]. 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. Nasal immunization of mice with recombinant PspC has been shown to protect mice against pneumococcal colonization and sepsis. However, *L. casei* PspC was not able to induce antibodies against PspC and did not protect mice against the lethal pneumococcal respiratory challenge [122].

Green et al. described a pneumococcal surface-exposed protein that has homology with bactoferritins [125]. 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 [125]. Taking into account these considerations we expressed PppA antigen in a LAB strain and assessed its efficacy to induce local and systemic immune responses in mice of different ages. In addition, we determined whether the mucosal administration of the recombinant bacteria increased resistance to systemic and mucosal infections caused by the main *S. pneumoniae* serotypes found in our country. For the expression of PppA in LAB, 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 [126]. Different regiments for mucosal vaccination of mice with *L. lactis*-PppA including both live and inactivated bacteria were tested.

Nasal immunization of mice with *L. lactis*-PppA induced systemic and mucosal specific antibodies and significant protection rates were observed in immunized mice after an

intraperitoneal challenge with the pneumococcal T14 strain serotype 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 [126]. 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.

Different immunization protocols were also tested for the *L. lactis*-PppA vaccine [127]. 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 [127].

In addition to nasal immunizations, oral vaccination can be used to induce protective immunity in the respiratory tract. Taking into consideration the capacity of orally administered *L. lactis* NZ9000 to stimulate the innate and the specific immune responses in the respiratory tract [53], 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. Oral immunization of adult immunocompetent mice with *L. lactis*-PppA induced the production of specific anti-PppA IgM, IgG and IgA in BALF and serum [51]. Experiments of challenge with different pneumococcal serotypes were carried out with serotypes 3, 6B, 14 and 23F. 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 [51]. 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 [51].

Also it was evaluated whether the oral immunization with *L. lactis*-PppA 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 [128]. 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. It was 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 [128]. 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 [128]. Results showed that oral immunization with recombinant bacteria represents a promising alternative for improving immunity in young individuals.

CONCLUSIONS

In the last years many efforts have been made to unravel the mechanisms of probiotics' activity and various experimental approaches have been developed to characterize the molecular basis of these effects [129,130]. 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, NOD1 and NOD2 are able to recognize cell wall components and DNA of dietary LAB, thereby contributing to immunoregulation in the GALT [131,132]. Although significant progress has been made in the knowledge of the mechanisms of probiotics action in the gut, it is unknown how some immunobiotics orally administered are able to stimulate immunity in distal mucosal sites from the gut. Recent evidence showed that pattern recognition receptors-mediated sensing of resident commensal microbiota in the steady state regulates the development and function of innate and adaptive immune systems in extra-intestinal sites, and prepares the host against intrusion by pathogenic microorganisms [5,133]. In mice, depletion of gut microbiota by antibiotics can result in reduced surface expressions of TLR2 and TLR4 in peritoneal macrophages, and less inflammation following intraperitoneal LPS injection *in vivo* [134], indicating that intestinal microbiota can constitutively prime peritoneal macrophages in preparation for pathogen invasion. In addition, recognition of peptidoglycan from the microbiota by NOD1 primes systemic innate immunity by enhancing the cytotoxicity of bone marrow-derived neutrophils in response to systemic infection with the bacterial pathogens, *S.pneumoniae* and *Staphylococcus aureus* [135]. Recent studies characterized the cellular and molecular mechanisms by which the gut microbiota regulate respiratory tract immune defense against influenza virus infections [136]. Authors demonstrated 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 specific groups of commensal bacteria, mainly Lactobacilli could induce TLRs to stimulate leukocytes either locally or systemically. Then, the factors released by stimulated leukocytes could support steady-state activation of inflammasome-dependent cytokine release by respiratory tract DCs, improving their migration to the draining lymph nodes in the presence of a viral infection [136]. In general, these studies indicated that gut microbiota can support systemic and respiratory immunity by releasing low

levels of PRR ligands to the circulation. Although, in the case of *L. rhamnosus* CRL1505 and *L. casei* CRL431, it is possible to propose a different mechanism influencing antibacterial and antiviral immune responses in the respiratory tract. Those probiotics could induce a mobilization of cells from intestine ($CD3^+CD4^+IFN\text{-}\gamma^+$ T and IgA^+ cells) and changes in cytokine profile (IFN-γ and IL-10) that would be able to beneficially modulate the respiratory mucosal immunity. In consequence, the activation of respiratory immunity by orally administered probiotics would have a complex mechanism, related to specific strains. Then, a more detailed understanding of the cellular and molecular mechanisms of actions for probiotics is therefore required in order to more effectively target the critical immune pathways that drive respiratory-induced protection. On the other hand, in this review we describe several research works dealing with the possibility of using recombinant LAB as vaccines against *S. pneumoniae* respiratory infection. There are promising results with different LAB-based vaccines. However, there is still a long way to go in order to achieve a robust immune response with a good immunological memory using fewer doses. The development of safe and effective delivery systems is still an ongoing challenge for the use of recombinant LAB and their advantages for mucosal administration. In addition, the detailed study of the mechanisms involved in the capacity of nasal epithelial and immune cells to modulate the inflammatory immune response in the context of antibody generation is still a key challenge.

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