

REVIEW ARTICLE

Immunotherapy of allergic diseases using probiotics or recombinant probiotics

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Summary

Allergic diseases affect up to 30% of the western population, and their prevalence is increasing. Probiotics are able to modulate the mucosal immune response, and clinical trials demonstrated that specific strains, especially lactic acid bacteria (LAB) ones, reduce allergic symptoms. Moreover, the use of recombinant probiotics has been evaluated as possible strategies for the immunotherapy of allergic diseases. The production and delivery of allergens by recombinant LAB in concert with their ability to induce a Th1-type immune response have been shown to be a promising mucosal vaccination strategy in mouse model. The aim of this article is to review the applications of probiotics in allergy immunotherapy with a special focus on recombinant LAB delivering proteins or DNA.

Immunological basis of allergic diseases

Allergic manifestations are ranked as the fourth most important disease in the world, and they are responsible for a substantial healthcare burden on society (Hong *et al.* 2012). The World Health Organization estimates that more than 20% of the world's population suffers from IgE-mediated allergic diseases and that their prevalence over the last 20–30 years is increasing dramatically (WHO/WAO 2002).

Allergies usually occur when the immune system reacts against harmless substances present in the environment.

These substances called allergens can enter in contact with the immune system through various routes such as inhalation, ingestion and skin contact or enter directly into the body through an insect bite. The exposure to environmental allergens can cause a variety of symptoms associated with a range of conditions, including allergic asthma, allergic rhinitis, food allergies, and dermatological problems such as allergic eczema and (Weiner *et al.* 2011).

The allergic immune response is a complex process beginning with the allergen being presented to allergen-specific naïve CD4⁺ T lymphocytes by antigen-presenting

cells. After antigen presentation, these cells become activated and can differentiate into T helper type 2 (Th2) cells. At the second exposure to the same allergen, activated Th2 cells secrete interleukins such as IL-4, IL-5 and IL-13. These interleukins are able to stimulate B-cell activation leading to the secretion of allergen-specific IgE antibodies, which induce cross-linking of the high-affinity IgE receptor (FcεRI) on mast cells and basophiles. Th2 cell cytokines and IgE-activated cells of the innate immune system (eosinophils, mast cells and basophiles) release histamine and other inflammatory mediators into surrounding tissues. The resulting pathophysiological response includes vasodilation, mucous secretion, nerve stimulation and smooth muscle contraction that manifests clinically as itchiness, rhinorrhea, dyspnoea and anaphylaxis characterizing the type I allergic reaction (Gould and Sutton 2008; Bosnjak *et al.* 2011). The role of the Th2-cell-mediated immune response against innocuous environmental allergens is well documented. Furthermore, it is important to mention that the expression of the allergic phenotype also depends on the individual predisposition and the gene–environment interactions (Romagnani 2004).

Overall, allergic manifestations are complex diseases that result in the deregulation of the IgE system homeostasis (Gould and Sutton 2008). The development of type I allergy reflects an imbalance in the T-lymphocyte immunity associated with the dominance of Th2 response. Th2 cells secrete IL-4, IL-5 and IL-13 that trigger IgE production by B lymphocytes, as mentioned above. In contrast, Th1 lymphocytes secrete IFNγ which antagonize IL-4 production and, consequently, Th2-cell development. IFNγ inhibits IgE switching on B lymphocytes and promotes the differentiation of naïve T cells towards Th1 subsets preventing Th2-cell proliferation (Pène *et al.* 1988).

In normal circumstances, allergic reactions against commensal micro-organisms or innocuous antigens contained in food are prevented by the induction of immunological tolerance as the peripheral immune system has evolved many strategies to maintain a state of tolerance to harmless antigens (Akbari *et al.* 2001). In allergic individuals, the unresponsiveness to innocuous antigens is breakdown. One of the major mechanisms of oral tolerance is the induction of regulatory T cells (Tregs cells), favoured by resident dendritic cells (DC) of the mucosal tissue, and the production by mucosal epithelial cells of TGF-β and IL-10 that have been implicated in the down-regulation of immune responses and, therefore, are considered to be key cytokines in tolerance induction via the mucosa (Akbari *et al.* 2001; Weiner *et al.* 2011). By the production of IL-10 and TGF-β, Tregs can suppress IgE production and Th1/Th2 proliferation (Akbari *et al.* 2003).

In the general population, the prevalence of allergies has been estimated to be around 1–3% in adults and 4–6% in children according to the Center for Disease Control (CDC). More than 70 foods have been described to cause food allergies, but several studies indicate that among children, 75% of allergic reactions are due to only a limited number of foods, namely egg, peanut, milk, fish and nut. Fruits, vegetables, nuts and peanuts are responsible for most allergic reactions among adults. Moreover, the prevalence of reported food allergy increased 18% among children under the age 18 from 1997 to 2007. Asthma is estimated by the World Health Organization to affect about 150 million people worldwide and is a major cause of hospitalization for chronic diseases in children in the western population. In more than 50% of adults and in at least 80% of affected children, asthma can be related to an allergic cause. Policies that promote early identification of the disease, to certify adequate treatment and, particularly, improve air quality, are helping to reduce this burden (WHO 2007).

Currently, the only treatment that has been reported to cure allergic diseases is allergen-specific immunotherapy. It involves several injections at increasing doses of the allergen to achieve immunological tolerance over time (Moote and Kim 2011). Even though being considered a potentially disease-modifying therapy, patients' compliance is a problem, as allergen-specific immunotherapy requires the repeated administration of gradually increasing amounts of an allergen for nearly 3 years and carries inherent risks of allergic reactions during the treatment. More recently, a major breakthrough in allergy molecular medicine has been represented by the development of Omalizumab (Xolair), an anti-human IgE monoclonal antibody capable of blocking and preventing IgE binding to FcεRI (Chang 2000). Yet, also this therapeutic intervention presents the discomfort of high-dose injection and is not sufficient to fully protect from allergic manifestations. Recently, the US Food and Drug Administration (FDA) is conducting a safety review of Xolair due to a possible increased risk of heart attack, abnormal heart rhythm, heart failure and stroke in patients treated with Xolair (Nowak-Węgrzyn and Sampson 2011).

Probiotics and allergy treatment

Epidemiological data have demonstrated differences in the intestinal microbial community between allergic and nonallergic children, and for this reason, it was postulated a possible association between allergy and altered microbiota. In fact, it was observed that gut microbiota plays an important role in the early development of the mucosal immune system and as an environmental factor in the development of allergic diseases. Lack of early

microbial stimulation and perturbation of the gastrointestinal microbiota results in aberrant immune responses to innocuous antigens and exerts a negative effect on the development of oral tolerance (Noverr and Huffnagle 2005). Infants that showed higher levels of potentially pathogenic bacteria in their gastrointestinal tract, such as *Clostridium difficile* and *Staphylococcus aureus*, were associated with the increased risk of developing allergy. On the other hand, the intestinal microbial community of nonallergic children was found to be more colonized by lactic acid bacteria (LAB), for example *Lactobacillus*, and other bacteria belonging to *Bifidobacterium* genus, demonstrating that the enhanced presence of these bacteria in the gastrointestinal tract seems to correlate with protection against allergic diseases (Forno *et al.* 2008; Ozdemir 2010).

In this context, the potential use of LAB to develop effective strategies for the immunotherapy of type I allergies is being explored. LAB are food-grade bacteria with a long record of safe oral consumption and are granted as generally regarded as safe (GRAS) by the FDA. Many LAB are able to colonize the mucosa of the oral, urogenital and gastrointestinal tracts, and some species are commensal of mammal microbiota. Specific LAB strains that can provide a health benefit to the host when administered in adequate amount are defined as probiotics (FAO/WHO 2001; Steidler and Rottiers 2006; Wells and Mercenier 2008).

Clinical studies

The first study that illustrates the potential of probiotic bacteria to modulate immune response and prevent allergic diseases was conducted by Kalliomäki *et al.* (2001). The consumption of probiotic *Lactobacillus rhamnosus* GG by either pregnant woman for 4 weeks prior to labour or newborns, both having high-allergy risk factors, decreased significantly the prevalence of atopic eczema. Furthermore, atopic babies that received probiotic strains exhibited reduced symptoms of the disease and higher levels of faecal IgA compared with the control group (Kalliomäki *et al.* 2001). Although their role in allergy is controversial, IgA is resistant to cleavage by secretory proteases and might block allergens penetration within the mucosal tissue, slowing down the allergic reaction (Novak *et al.* 2004). A recent work reports that administration of *Bifidobacterium breve* M16V in cow's milk protein intolerance (CMPI) neonates who underwent small intestine surgery could significantly reduce the incidence of CMPI after surgical procedure (Ezaki *et al.* 2012). Even though *Lact. rhamnosus* GG is the strain that has been most studied, the potential of several probiotic strains for the prevention or treatment of allergic diseases

has been investigated in different clinical trials (for a review see Ozdemir 2010; Kim and Ji 2012). This review will not detail all human clinical trials that are being carried out with probiotics as our intent is to focus on the use of recombinant probiotic as a tool to treat allergic disorders. Nevertheless, many early clinical trials of probiotics against different forms of allergy found in the literature have yielded inconsistent results, probably reflecting the inherent complexity of the allergic syndrome. Lack of alignment of clinical design, for example different target populations, countries, intervention schemes and the formulation of probiotic used in the study, makes difficult to compare results obtained with different probiotic strains (Prescott and Björkstén 2007; Kalliomäki 2010). Moreover, it is important to remark that the variation in the dose, formulation and species of probiotics used in this type of study should be taken into account (Wells 2011a; Kuitunen and Boyle 2012).

Preclinical studies

In attempts to understand the underlying mechanisms of these anti-allergic effects, probiotic bacteria were shown to modulate the balance between the different T helper cells, skewing the immune response to antigens/allergens from a pro-allergic Th2 towards a Th1 one. High levels of type 1 cytokines IFN γ and IL-12 secretion and up-regulation of CD40 and CD86 expression were detected in murine splenocytes co-incubated with *Streptococcus thermophilus* or with *Lactobacillus casei* strains (Ongol *et al.* 2008) as well as in human myeloid dendritic cells (hMDcs) after co-incubation with *Lactobacillus gasseri*, *Lactobacillus johnsonii* or *Lactobacillus reuteri* strains (Mohamadzadeh *et al.* 2005). Niers *et al.* investigated the effect of probiotic-maturated DCs on T-cell polarization. DCs that were co-incubated with *Bifidobacterium bifidum* W23 could drive T cells towards a Th1 response leading to a higher secretion of IFN- γ and IL-10 and lower secretion of IL-4, when compared with the control groups. This strain presented to be a good candidate for primary prevention of allergic diseases (Niers *et al.* 2007). It was also evaluated anti-allergic effects of a Dahi (yogurt) containing probiotic *Lactobacillus acidophilus*, *Lact. casei* and *Lactococcus lactis* biovar diacetylactis (named probiotic Dahi) on ovalbumin (OVA)-induced allergy in mice. Administration of the probiotic Dahi suppressed the production of total and OVA-specific IgE in mice serum. It was also observed higher amounts of INF- γ and IL-12 and lower amounts of Th2-specific cytokines (IL-4 and IL-6) in the cultured splenocytes from mice fed with probiotic Dahi (Jain *et al.* 2010). Wang *et al.* (2012) performed a study using the same OVA-induced allergy mouse model. However, the probiotic chosen for the

study was a mixture named GM080 containing *Lactobacillus paracasei*, *Lactobacillus fermentum* and *Lact. acidophilus*. It was shown that the OVA treatment increased the signalling proteins of inflammation and apoptosis in cardiomyocytes from mice, and administration of GM080 could ameliorate both inflammation and apoptosis in the cells (Wang *et al.* 2012). Using an allergic poly-sensitization model to birch and grass pollen allergens, Schabussova *et al.* (2011) found that the treatment with *Bifidobacterium longum* NCC 3001 and *Lact. paracasei* could prevent inflammation in the lungs, a major target organ of allergic disease. Mice administered with the probiotic strains had increased levels of IgA in their Bronchoalveolar lavage (BAL) fluids, and both strains induced a general immunosuppression of T-cell responses, rather than a shift of the allergen-specific Th2 responses towards the Th1 phenotype. Moreover, IL-10 mRNA expression was elevated in the probiotic-treated group, and the beneficial effects of *Bif. longum* NCC 3001 were maintained for a more prolonged period of time (Schabussova *et al.* 2011). Co-incubation of PBMCs from allergic subjects with a variety of LAB strains inhibited allergen-stimulated Th2-cytokine release and increases the Th1-cytokine response (Ghadimi *et al.* 2008). Recently, it was investigated the prophylactic potential of different probiotic strains using a peanut sensitization model. The prophylactic treatment with both *Lactobacillus salivarius* HMI00 and *Lact. casei* Shirota (LCS) decreased the production of IL-4 and/or IL-5 by mice splenocytes and mast cell responses leading to a reduction in Th2 immune responses. On the contrary, *Lactobacillus plantarum* WCFS1 strain appeared to increase the Th2 phenotype (Meijerink *et al.* 2012). It was also reported that a mixture of eight live probiotic bacteria (VSL#3) when administered orally in mice presensitized with shrimp tropomyosin allergen was capable of suppressing the established Th2 responses and generating regulatory T-cell populations, which were able to control allergic inflammation (Schiavi *et al.* 2011). Thomas *et al.* showed that pigs presensitized with *Ascaris suum* allergen and afterwards fed with *Lact. rhamnosus* HN001 strain had a decreasing severity of allergic skin and lung reactions. This was related to probiotic-induced modulation of Th1 (INF- γ) and regulatory (IL-10) cytokine expression (Thomas *et al.* 2011). Table 1 summarizes some of the *in vitro* and *in vivo* studies performed with probiotics as well as some relevant clinical trials.

Many *in vivo* studies using animal models have shown positive effects of probiotics with respect to the prevention of several allergic diseases. To transpose this results to humans, further works should be carried out regarding the effective probiotic strains, optimal dose, timing

and duration of supplementation as well as the additive/synergistic effects between probiotics and prebiotics. To date, the immunological mechanisms by which a probiotic strain can exert their beneficial effects on humans still remain to be proven (Kim and Ji 2012).

Relevance of preclinical studies

Indeed, Meijerink *et al.* showed that the cytokine profile induced by different probiotic strains was highly variable after co-incubation with peripheral blood mononuclear cells (PBMCs). These data demonstrate that probiotics can have distinct immunomodulatory capacities, which could affect the type of immune responses elicited in the intestine changing their prophylactic/therapeutic properties (Meijerink *et al.* 2012). Even though probiotics may be considered for treatment on the basis of their immunomodulatory properties, it is hard to assure the effect that the probiotic will exert *in vivo* due to the complexity of both allergic diseases and *in vivo* experimentation. For instance, it has been shown that LCS partially protected mice in a peanut sensitization model. Differently, the same probiotic strain did not decrease the food allergic response to peanut extract in Brown Norway rats (De Jonge *et al.* 2008; Meijerink *et al.* 2012). *Bifidobacterium lactis* Bb12, for instance, have yielded a high IL-10/IL-12 ratio after co-incubation with PBMCs being regarded as an anti-inflammatory strain favouring type-2 T-cell responses. Bearing in mind that strains capable of down-regulating Th2 immune responses by favouring Th1 balance might reduce the progression of allergic diseases, a quite surprising effect has been shown after pre- and postnatal administration of *Bif. lactis* Bb12 in a human clinical trial. Besides inducing Th2 immune responses, it was observed a reduced level of allergic sensitization in infants of allergic mothers at 1 year of age. The specific mechanisms by which this strain could exert this effect remain to be discovered (Dotterud *et al.* 2010; Meijerink *et al.* 2012).

Recombinant bacteria and immunotherapy of type I allergy

It was shown that supplementation trials with probiotics can decrease allergic manifestations in children. Some recent studies are also focusing on the use of selected probiotic strains as mucosal antigen delivery vehicles for recombinant allergens, serving as attractive adjuvant systems for improved allergy treatment (Schabussova and Wiedermann 2008). However, the current work on prevention of allergic sensitization using recombinant LAB has been carried out only using murine models (Wells 2011b).

Table 1 Summary of some research studies concerning the use of different probiotic strains as a potential tool to treat/prevent allergic diseases

Strain (s)	Experimental model/disease	Immunological observations	References
<i>In vitro/ex vivo</i> studies with probiotics			
<i>Lactobacillus gasseri</i> (ATCC no. 19992), <i>Lactobacillus johnsonii</i> (ATCC no. 33200) and <i>Lactobacillus reuteri</i> (ATCC no. 23272)	Human myeloid dendritic cells	Induction of bioactive IL-12, IL-18, IFN γ and T-cell proliferation; up-regulation of TLR-2 in the cells	Mohamadzadeh <i>et al.</i> (2005)
<i>Bifidobacterium bifidum</i> W23	Dendritic cells (derived from umbilical cord blood obtained from healthy children); Autologous CD4 $^{+}$ T cells; Chinese hamster ovary (CHO)-cell lines	<i>In vitro</i> cultured neonatal DC were able to drive Th1 responses; high secretion of IFN- γ , IL-10 and lower secretion of IL-4; TLR activation by probiotic bacteria	Niers <i>et al.</i> (2007)
<i>Lactobacillus rhamnosus</i> GG, <i>Lact. gasseri</i> PA, <i>Bif. bifidum</i> MF, <i>Bifidobacterium longum</i> SP, and L.gB.bB.I (a mix of <i>Lact. gasseri</i> , <i>Bif. bifidum</i> , and <i>Bif. longum</i>)	PBMCs from allergic or healthy subjects	Modulation of Th1/Th2 response to allergens	Ghadimi <i>et al.</i> (2008)
Probiotic effects in animal models of allergic disease			
<i>Streptococcus thermophilus</i> 21072 and <i>Lactobacillus casei</i> subsp. <i>casei</i> 027	C57BL/6 and BALB/c mice	Production of IFN- γ from splenocytes, IL-12p70, IL-10 from peritoneal exudate cells and expression of costimulatory molecules in dendritic cells by lactic acid bacteria stimulation	Ongol <i>et al.</i> (2008)
<i>Lactobacillus acidophilus</i> NCDC14, <i>Lact. casei</i> NCDC19 and <i>Lactococcus lactis</i> biovar <i>diacetylactis</i> NCDC-60	Ovalbumin (OVA)-induced allergy in mice	OVA-specific IgE in mice serum; higher amounts of INF- γ and IL-12; lower amounts of IL-4 and IL-6 in the cultured splenocytes	Jain <i>et al.</i> (2010)
<i>Lactobacillus paracasei</i> , <i>Lactobacillus fermentum</i> and <i>Lact. acidophilus</i>	OVA-induced allergy mouse model	Amelioration of both inflammation and apoptosis in cardiomyocytes	Wang <i>et al.</i> (2012)
<i>Lact. paracasei</i> NCC 2461 and <i>Bif. longum</i> NCC 3001	Mouse model of poly-sensitization	Suppression of Th2 responses, up-regulation of IL-10, TLR2 or TLR4 in the draining lymph nodes	Schabussova <i>et al.</i> (2011)
<i>Lactobacillus plantarum</i> WCFS1, <i>Lact. plantarum</i> NCIMB8826, <i>Lactobacillus salivarius</i> HMI001, <i>Lact. casei</i> Shirota, 28 different strains comprising 12 species of probiotics	Human PBMC (hPBMC); mouse peanut extract (PE) sensitization model	Induction of IL-10, IL-12 and IFN- γ in hPBMC; modulation of PE-specific antibody responses by treatment with lactobacilli; <i>ex vivo</i> cytokine response (increased amounts of IL-4, IL-5 and IL-10)	Meijerink <i>et al.</i> (2012)
Probiotic VSL#3 (<i>Lact. acidophilus</i> , <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> , <i>Lact. casei</i> , <i>Lact. plantarum</i> , <i>Bif. longum</i> , <i>Bif. infantis</i> , <i>Bifidobacterium breve</i> , <i>Streptococcus salivarius</i> subsp. <i>thermophilus</i>)	Mouse shrimp tropomyosin sensitization model	Protection of mice against anaphylactic reactions; suppression of established Th2 responses and generation of regulatory T-cell populations	Schiavi <i>et al.</i> (2011)

(Continued)

Table 1 (Continued)

Strain (s)	Experimental model/disease	Immunological observations	References
<i>Lact. rhamnosus</i> HN001	Pig sensitization with <i>Ascaris suum</i> allergen	Induction of INF- γ and IL-10 cytokines in PBMCs from the pigs; diminished allergic skin flare and allergic lung responses	Thomas <i>et al.</i> (2011)
Human clinical studies with probiotics			
<i>Lactobacillus rhamnosus</i> GG	Atopic eczema, allergic rhinitis or asthma	Reduced symptoms of the disease in patients and higher levels of faecal IgA	Kalliomäki <i>et al.</i> (2001)
<i>Bif. breve</i> M16V	Cow's milk protein intolerance (CMPI)	Reduction in CMPI symptoms in neonates after small intestine surgery	Ezaki <i>et al.</i> (2012)
<i>Lact. rhamnosus</i> HN001	Eczema	Protection against eczema, when given for the first 2 years of life only, extended to at least 4 years of age	Wickens <i>et al.</i> (2012)

Charng *et al.* (2006) demonstrated that recombinant LAB are able to inhibit allergen-induced airway allergic inflammation. In this study, mice were intraperitoneally sensitized with Dermatophagoides pteronyssinus group 5 allergen (Der p 5) and orally treated with recombinant LAB containing a plasmid-encoded Der p 5 gene. After sensitization and challenge, it was observed a reduction in the synthesis of Der p 5-specific IgE and hyper-reactivity, thus providing a basis for developing a novel therapeutic method for allergic respiratory diseases (Charng *et al.* 2006). Parental administration of anti-IgE monoclonal antibodies is currently used to treat allergic patients by passive immunization but requires high doses of monoclonal antibodies resulting in high therapy costs. To develop vaccine able to induce natural production of IgE auto-antibodies, *Lact. johnsonii* have been engineered to express either anti-idiotypic scFv or IgE mimotopes fused on its surface. Intranasal administration of the recombinant strains elicits the production of anti-IgE antibodies showing its potential as mucosal vaccine (Scheppeler *et al.* 2005).

Induction of oral tolerance, which can be defined as the process by which the immune system does not respond to unwanted and potentially pathogenic innocuous molecules (Maillard and Snapper 2007), is an attractive therapeutic strategy to treat atopic diseases; indeed, it represents the natural way for the body to prevent allergy development against food antigens. Therefore, inducing a tolerogenic mechanism is suitable to prevent a hyperactive immune system associated with atopic and autoimmune diseases. Recently, it has been shown that the use of genetically modified *L. lactis* to deliver recombinant auto-antigens or allergens can provide a novel therapeutic approach for inducing tolerance (Huibregtse *et al.* 2007).

The potential of *L. lactis* to induce antigen-specific peripheral tolerance has been evaluated by feeding Ovalbumin-sensitized OVA-T-cell receptor transgenic mice with recombinant *L. lactis* expressing OVA. Administration of this strain leads to OVA-specific tolerance by inducing regulatory T cells in a TGF- β dependent manner (Huibregtse *et al.* 2007). This approach can also be used to develop effective therapeutics for systemic and intestinal immune-mediated inflammatory diseases.

To develop mucosal immunotherapy strategies able to modulate the T-cell-mediated response towards a Th1 profile, LAB have been used to produce and deliver allergens at the mucosal surfaces. *Lactobacillus plantarum* has been modified to produce the dust house mite allergen Derp-1. The intranasal administration of the recombinant strain induces Derp-1-T-cell-specific proliferative response with low IFN γ production and reduced IL-5 secretion. Although the inhibitory effect on IL-5 production was shown to be specific to the recombinant strain, the effect on IFN γ was due to *Lact. plantarum* strain. No effect on immunoglobulin production was reported (Kruisselbrink *et al.* 2001). Unlike, Rigaux *et al.* showed that prophylactic intranasal pretreatment of mice with recombinant *Lact. plantarum* strain producing Derp-1 prevents the development of Th2-biased allergic response by a reduction in specific IgE and the induction of allergen-specific IgG2a antibodies. Moreover, both wild-type and recombinant *Lact. plantarum* reduce airway eosinophilia following aerosolized allergen exposure and IL-5 secretion upon allergen restimulation (Rigaux *et al.* 2009).

Recombinant *L. lactis* and *Lact. plantarum* producing the birch pollen allergen Bet v1 have been also reported to modulate the allergic immune response to Bet v1. In prophylaxis protocols, intranasal administration of mice with recombinant strains expressing Bet v1 induces a nonaller-

gic Th1 immune response specific to Bet v1. After sensitization, mice pretreated with the recombinant strains expressing Bet v1 showed a decreased level of specific IgE, decreased level of IL-5 detected in broncho-alveolar fluids (BAL) as well as induction of Bet v1-specific IgA and IFN γ . Intranasal delivery was more efficient in modulating the immune response to Betv1, while oral pretreatment was successful only with recombinant *Lact. plantarum* strain. The differences in the immune responses induced after oral administration by the two recombinant strains might be explained due to the lower amount of expressed antigen by recombinant *L. lactis*; however, differences in immunomodulatory capacities and in gut persistence of the two strains cannot be excluded (Daniel *et al.* 2006, 2007). Schwarzer *et al.* (2011) recently explored the effect of neonatal colonization with a recombinant *Lact. plantarum* NCIMB8826 strain constitutively producing Bet v 1 in a murine model of type I allergy. It has been shown that monocolonization with this strain induced a Th1-biased immune response at the cellular level upon stimulation with Bet v 1 (Schwarzer *et al.* 2011), as demonstrated by Daniel *et al.* (2006, 2007). Furthermore, after sensitization with Bet v 1, immunized mice displayed suppressed IL-4 and IL-5 production in spleen and mesenteric lymph node cell cultures as well as decreased allergen-specific antibody responses (IgG1, IgG2a and IgE) in sera. This suppression was associated with a significant up-regulation of the regulatory marker Foxp3 at the mRNA level in the spleen cells (Schwarzer *et al.* 2011). The capacity of recombinant *Lact. plantarum* expressing another allergen has also been investigated. The strain was engineered to express a major Japanese cedar pollen allergen, Cry j 1, and prophylactic effects *in vivo* were recently evaluated. In attempt to facilitate heterologous expression, the codon usage in the Cry j 1 gene was optimized for *Lact. plantarum* NCL21 strain using the recursive PCR-based exhaustive site-directed mutagenesis. The use of codon-optimized Cry j 1 cDNA and a lactate dehydrogenase gene fusion system led to a successful production of recombinant Cry j 1 in the host strain. Moreover, it was demonstrated that oral administration with *Lact. plantarum* expressing Cry j 1 suppressed allergen-specific IgE response and nasal symptoms in a murine model of cedar pollinosis (Ohkouchi *et al.* 2012).

Marinho *et al.* also evaluate the immunomodulatory effect of *L. lactis* expressing recombinant IL-10 in a cytoplasmic (LL-CYT) or secreted form (LL-SEC) using a mouse model of ovalbumin (OVA)-induced acute airway inflammation. It was observed that mice immunized with LL-CYT and LL-SEC strains had decreased levels of anti-OVA IgE and IgG1 and reduced eosinophils numbers and IL-4 and CCL3 production, when compared to the asthmatic group. Furthermore, LL-CYT strain was more effective to suppress lung inflammation (Marinho *et al.*

2010). Taken together, allergen-secreting LAB could be considered as an alternative strategy to prevent food allergy in the future.

Protein delivery by recombinant lab in cow's milk allergy model

Cow's milk protein allergy (CMPA) is a complex disorder that affects 2–7.5% of children, with the highest prevalence during the first year of age, and is capable of inducing adverse reactions, which may involve skin, gastrointestinal (GI) tract or respiratory system (Caffarelli *et al.* 2010; Du Toit *et al.* 2010). In this allergy, the main allergens are the four proteins of the casein fractions (α s1-, α s2-, β - and κ -casein) as well as well α -lactalbumin and β -lactoglobulin (Wal 2004; Solinas *et al.* 2010). CMPA develops early in infancy within 12–24 months of birth, but 80–90% of affected children recover by acquiring tolerance to cow's milk by the age of 5 years (Exl and Fritsché 2001; Crittenden and Bennett 2005), while 51% of adults develop tolerance. Recent studies associated tolerance to cow's milk with a decreased epitope binding to milk peptides by IgE and a simultaneous increase in corresponding epitope binding by IgG4 (Cerecedo *et al.* 2008; Pecora *et al.* 2009; Savilahti *et al.* 2010; Wang *et al.* 2010).

As described above, LAB are attractive tools to deliver therapeutic molecules at the mucosal level due to their GRAS status and immunomodulatory capacities (Bermúdez-Humarán *et al.* 2011; Pontes *et al.* 2011). Indeed, the model LAB, *L. lactis*, was engineered to express the major cow's milk allergen, BLG. The recombinant allergen was produced predominantly in a soluble, intracellular and mostly denatured form. Mucosal administration of the recombinant strain induced BLG-specific faecal IgA, although allergen-specific IgE, IgA, IgG1 or IgG2a was not detected in mice sera (Chatel *et al.* 2001). Similar immune response was reported after oral administration of recombinant *L. lactis* secreting a T-cell determinant IgE epitope of BLG (Chatel *et al.* 2003).

In a food-hypersensitivity mouse model, Adel-Patient and collaborators showed that oral administration of recombinant strains producing different amounts of recombinant BLG partially prevented mice from sensitization. Oral pretreatment with recombinant strains prevented Th2-type immune response due to a reduction in specific IgE and the induction of allergen-specific IgG2a, immunoglobulins widely recognized as a characteristic of a Th1-type immune response, and faecal IgA antibodies. The intensity of the Th1 immune response induced was correlated with the amount of recombinant BLG produced: the more effective strains were those producing the highest amount of BLG (Adel-Patient *et al.* 2005).

Intranasal delivery of *L. lactis* recombinant strain did not induce the secretion of BLG-specific antibodies but elicited IFN γ production in mice splenocytes after BLG restimulation. Intranasal pretreatment of mice with recombinant *L. lactis* strain producing BLG reduced the airway eosinophilia influx and IL-5 secretion in BAL induced by intranasal allergen challenge. However, unlike after oral administration, no difference in IgE or IgG2a between pretreated and control mice was detected. In the same study, intranasal co-administration of recombinant *L. lactis* strains producing BLG or IL-12 elicited a protective Th1 immune response, inhibiting the allergic response in mice without affecting specific BLG IgE secretion (Cortes-Perez *et al.* 2007). The administration of *L. lactis* expressing cytokines to avoid allergic reactions seems to be a promising approach. After oral challenge with BLG, mice immunized with recombinant *L. lactis* expressing murine IL-10 (LL-mIL10) showed a significant decrease in antigen-specific IgE in their gastrointestinal tract preventing anaphylaxis. Moreover, Th2-type response was completely abrogated, suggesting oral tolerance against BLG. Mice that received LL-mIL10 strain had significantly increased levels of IgA antibodies in their faeces. Considering that T-cell-dependent secretion of IgA antibodies by B cells is largely induced by IL-10 and TGF- β , it was hypothesized that the active secretion of IL-10 by *L. lactis* up-regulated IL-10-secreting cells increasing the levels of plasmatic IL-10. This data suggest a prime role for LL-mIL10 strain in the induction of IL-10 which, consequently, could improve IgA secretion (Frossard *et al.* 2007). The effects of intranasal administration of recombinant *L. lactis* expressing BLG were also tested in a therapeutic protocol. In oral-sensitized mice, intranasal administration of recombinant strain reduced IgG1 production but did not influence specific BLG IgE or IgG2a secretion. After intranasal challenge, mild decrease in IL-4 and IL-5 secreted in BAL was detected (Cortes-Perez *et al.* 2009).

Hazebrouck *et al.* (2006) investigated the effect of *Lact. casei* constitutively producing BLG when established permanently in the gut of gnotobiotic mice. After oral administration, the immune response against BLG as well as BLG production was monitored for 10 weeks; BLG-producing *Lact. casei* strain was successfully able to colonize the gut of mice, and BLG production was detected in the animal faeces. Although immunoglobulins were not detected in sera or faeces, secretion of IFN-gamma and IL-5 was observed in stimulated splenocytes (Hazebrouck *et al.* 2006).

The influence of the administration route on the immune response elicited by a recombinant BLG-producing *Lact. casei* strain was analysed (Hazebrouck *et al.* 2007, 2009). Intranasal pre-administration of the BLG-

producing *Lact. casei* enhances the BLG-specific IgG2a and IgG1 responses in sensitized mice but did not influence BLG-specific IgE production. Unlikely, oral pretreatment led to a significant inhibition of BLG-specific IgE production in sensitized mice, while IgG1 and IgG2a responses were not elicited. The production of IL-17 cytokine by BLG-reactivated splenocytes was similar after both oral and intranasal administrations. However, BLG-reactivated splenocytes from mice intranasally pretreated showed an enhanced secretion of Th1 cytokines (IFN- γ and IL-12) and Th2 cytokines (IL-4 and IL-5) suggesting a mixed Th1/Th2 cell response, whereas only production of Th1 cytokines, but not Th2 cytokines, was enhanced in BLG-reactivated splenocytes from mice orally pretreated. Those results show that the route of administration of recombinant LAB may be critical for their immunomodulatory effects (Hazebrouck *et al.* 2009). Table 2 summarizes the main studies performed with probiotic strains expressing recombinant allergens at different cell locations in several animal models.

DNA delivery by recombinant lab in cow's milk allergy model

Recently, the potential of LAB as mucosal DNA delivery vehicles has been investigated. The advantage of DNA vaccine relies in their ability to induce both cellular and humoral Th1 immune responses, resulting in the specific immune activation of the host against the delivered antigen (Tang *et al.* 1992; Ulmer *et al.* 1993; Liu 2010). DNA immunization has shown great promise in rodent models of allergic disease and, for this reason, had turned out as a promising novel type of immunotherapy against allergy (Li *et al.* 2006).

Genetic immunization with a eukaryotic expression cassette encoding the BLG antigen elicited a Th1 immune response in mice. Preventive immunization reduced BLG-specific IgE production and induced IFN γ -, IL-10- and BLG-specific IgG2a secretion in sensitized mice (Adel-Patient *et al.* 2001). Unlike bacterial delivery of recombinant proteins, bacterial-mediated DNA delivery leads to the expression by the host of post-translationally modified antigens (Fouts *et al.* 2003). Recombinant BLG is expressed mainly in denatured form by *Escherichia coli* or *L. lactis*, whereas its production in eukaryotic cells is in the native conformation (Chatel *et al.* 1999, 2001). *In vitro* delivery of DNA into mammalian cells by LAB was demonstrated employing *L. lactis* containing a BLG eukaryotic expression plasmid. Production of BLG was detected in Caco-2 cells after coculture with *L. lactis* harbouring the expression plasmid. Interestingly, no BLG production was detected in cells incubated with the purified plasmid mixed with *L. lactis*, suggesting that the

Table 2 Main research studies about the use of different recombinant probiotic strains to treat/prevent allergic diseases and major effects on the host immune response

Strain (s)	Recombinant allergen/localization	Experimental model/disease	Immunological observations	References
Recombinant probiotic effects in animal models of allergic disease				
<i>Lactobacillus acidophilus</i> ATCC 4356	<i>Dermatophagoides pteronyssinus</i> group-5 allergen (Der p 5/cytoplasmic expression	BALB/c mice sensitized with Der p 5	Inhibition of Der p 5-specific IgE and airway hyper-reactivity; eosinophilic and neutrophilic cellular infiltration	Chang et al. (2006)
<i>Streptococcus thermophilus</i>	Anti-idiotypic scFv fragments by anti-human IgE antibodies/anchored to the cell wall	BALB/c mice	Induction of an anti-IgE response after intranasal immunization	Scheppler et al. (2005)
<i>Lactobacillus johnsonii</i> NCC2754	<i>Gallus gallus</i> ovalbumin (OVA)/secreted to the extracellular medium	OVA-specific T-cell receptor transgenic mice (DO11.10) on a BALB/c background	Suppression of local and systemic OVA-specific T-cell responses	Huibregtse et al. (2007)
<i>Lactococcus lactis</i> MG1363	Der p 1 of house dust mites/cytoplasmic expression	C57BL/6 J (H-2b) mice	Inhibition of Th1 components of the response and reduced production of IL-5; stimulation of immunoregulatory mechanisms	Kruisselbrink et al. (2001)
<i>Lactobacillus plantarum</i> 256				
<i>Lact. plantarum</i> NCIMB8826, <i>Lact. plantarum</i> EP007	Dust house mite allergen Derp-1/cytoplasmic expression	Der p 1-sensitization murine model	Stimulation of dendritic cells (TLR2-, TLR9- and MyD88-dependent mechanism and via MAPK and NF- κ B activation)	Rigaux et al. (2009)
<i>L. lactis</i> NZ9800 and <i>Lact. plantarum</i> NCIMB8826	Birch pollen allergen Bet v1/cytoplasmic expression	Mouse model of birch pollen allergy	Reduction in allergen-specific IgE; increase in allergen-specific IgA at the mucosae in mice	Daniel et al. (2006)
<i>Lact. plantarum</i> NCIMB8826	Birch pollen allergen Bet v1/cytoplasmic expression	Murine model of type I allergy	Induction of Th1-biased immune response at the cellular level; induction of INF- γ by splenocytes and suppression of IL-4 and IL-5 in spleen and mesenteric lymph node	Schwarzer et al. (2011)

(Continued)

Table 2 (Continued)

Strain (s)	Recombinant allergen/localization	Experimental model/disease	Immunological observations	References
<i>Lact. plantarum</i> NCL21	Japanese cedar pollen allergen Cry j 1/cytoplasmic expression	Murine model of Japanese cedar pollinosis	Suppression of allergen-specific IgE response; amelioration of the cedar pollinosis like clinical symptoms	Ohkouchi et al. (2012)
<i>L. lactis</i> NCDO2118	Full-length <i>Rattus norvegicus</i> IL-10 with the codon usage of <i>L. lactis</i> /cytoplasmic expression and secreted to the extracellular medium	Mouse model of ovalbumin (OVA)-induced acute airway inflammation	Reduction in eosinophil numbers, EPO, IgE anti-OVA levels, IL-4 and CCL3 levels and pulmonary inflammation and mucus hypersecretion	Marinho et al. (2010)
<i>L. lactis</i> NZ9000	Cow's milk allergen, β -lactoglobulin (BLG)/cytoplasmic and extracellular locations	BALB/c mice	Induction of BLG-specific faecal IgA	Chatel et al. (2001)
<i>L. lactis</i> NZ9000	BLG/cytoplasmic and extracellular expression	BLG sensitization murine model	Induction of specific Th1 response down-regulating Th2 balance; reduction in specific IgE responses	Adel-Patient et al. (2005)
<i>L. lactis</i> NZ9000	IL-12 and BLG secreted to the extracellular medium	BLG sensitization murine model	Induction of a specific Th1 immune response regulating systemic and local Th2 and effectors cells	Cortes-Perez et al. (2007)
<i>L. lactis</i> wild type	Murine IL-10 secreted to the extracellular medium	Mouse model of food allergy using BLG in the presence of cholera toxin	Inhibition of anaphylaxis and antigen-specific serum IgE, IgG1 production; increased production of antigen-specific IgA and IL-10 in the gut	Frossard et al. (2007)
<i>Lactobacillus casei</i> BL23	BLG secreted to the extracellular medium	Germ-free C3H/HeN mice	Influence the maturation of the gut immune system; increased levels of INF- γ and IL-5	Hazebrouck et al. (2006)
<i>Lact. casei</i> BL23	BLG	BALB/c mice	Stimulation of systemic IgG1 and IgG2a responses; suppression of BLG-specific IgE production	Hazebrouck et al. (2009)

expression plasmid should be inside the bacteria to achieve efficient delivery (Guimarães *et al.* 2006). The efficiency of DNA delivery to Caco-2 cells was strongly increased by the expression of invasive genes at the surface of *L. lactis*. Recombinant invasive strains expressing either *Listeria monocytogenes* Internalin A or *Staph. aureus* fibronectin-binding protein A genes showed a higher ability to be internalized into mammalian cells compared with the control strain. As a consequence, recombinant invasive strains were more efficient in GFP expression plasmid delivery into Caco-2 cells resulting in higher number of GFP-producing cells (Innocentin *et al.* 2009). *In vivo*, *L. lactis* expressing *L. monocytogenes* InLA was able to invade guinea pig enterocytes after oral administration (Guimarães *et al.* 2005).

Chatel *et al.* demonstrated that *L. lactis* was able to transfer a BLG eukaryotic expression plasmid *in vivo*. After oral administration of BLG delivery strain, BLG cDNA was detected in the epithelial membrane of the small intestine of 40% of mice, and BLG protein was produced in 53% of the mice. Although only mild and transitory secretion of BLG-specific IgG2a was detected in mice sera after oral administration of *L. lactis* harbouring BLG expression plasmid, a significant decrease in BLG-specific IgE in pre-treated sensitized mice sera was reported. Furthermore, BLG-reactivated splenocytes of sensitized mice previously orally fed with the *L. lactis* BLG cDNA delivery strain showed an increased IFN γ production and a decreased IL-5 secretion (Chatel *et al.* 2008).

Recommendation for future research

There is increasing evidence coming from animal models and *in vitro* studies that probiotic strains are able to modulate immune response, promote oral tolerance and inhibit the development of the allergic phenotype. However, there is no definitive proof that probiotics have efficacy in the treatment of any allergic conditions in humans, even though few works have successfully demonstrated its ability to ameliorate allergic symptoms. This fact can be attributed to intrinsic differences in used species, methodologies, probiotic(s) (mixtures) or probiotic strains (Jeurink *et al.* 2012). Therefore, to prevent allergy, we think that patients should follow recommended instructions established by WHO, such as avoidance of cigarette smoke, promotion of breastfeeding or use of hydrolysed formula when this is not possible, besides consuming probiotics (FAO/WHO 2001; Prescott and Nowak-Węgrzyn 2011).

Considering the use of recombinant LAB strains, it is mandatory to remove antibiotic resistance marker from expression vectors used to avoid its escape into the environment by horizontal gene transfer. Another possibility

is to favour antibiotic genes that are not commonly used to treat human infections, for instance kanamycin. Alternative strategies that do not use antibiotic selection are being explored, which may heighten safety of recombinant bacteria, such as the use of selection system based on auxotrophy (Bower and Prather 2009).

Conclusion

Over the past decades, the use of recombinant LAB to develop possible strategies for mucosal immunotherapy of allergic diseases has strongly increased. Growing amount of data regarding the immunomodulatory proprieties of specific LAB strains as well as their persistence in the gut tract will provide rationale choice of LAB strain for specific therapeutic application. Furthermore, the availability of containment systems for genetically modified LAB (Steidler *et al.* 2000) might support progress towards human clinical trials of LAB-based allergen-specific immunotherapy. Allergen-delivering LAB have been implemented mainly in preventive vaccination protocols, but it remains to be seen whether a LAB-based vaccine would be successful in a more realistic scenario, closer to human allergic conditions, and therefore in atopic individuals.

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Author contribution

All authors contributed to conception and design of the review, critically revised the manuscript and approved the final submitted version.

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