



Distinctive anti-allergy properties of two probiotic bacterial strains in a mouse model of allergic poly-sensitization

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ABSTRACT

We compared the immunomodulatory properties of *Bifidobacterium longum* NCC 3001 and *Lactobacillus paracasei* NCC 2461 in a mouse model of poly-sensitization to birch and grass pollen allergens. Mucosal application of both strains at the time of sensitization and challenge led to significant suppression of airway inflammation and down-regulated allergen-specific immune responses. In contrast, in the mice treated with probiotics prior to sensitization and challenge, only *B. longum* displayed protective effects. Our findings stress that the choice of probiotic strain and the timing of the application are crucial for tolerance induction. Furthermore, this is the first demonstration of anti-allergic effects of probiotic bacteria in poly-sensitized mice.

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1. Introduction

Commensal bacteria in different mucosal compartments of the body represent an important interface between the external environment and the host immune system. Colonization starts immediately after birth and is considered to be crucial for stimulating and directing the development of the host immune system [1,2]. Reduced microbial contact or presence of altered intestinal microbiota early in life due to improved sanitation, changes in nutrition, or pervasive antibiotic use in industrialized countries have been associated with aberrant immune responses to normally innocuous antigens, such as allergens, later in life [3–7]. These observations deliver a rationale for the increasing interest in probiotic bacteria to prevent allergy development.

Probiotic bacteria are non-invasive and non-pathogenic Gram-positive bacteria, which, when consumed in adequate numbers, confer a health benefit on the host [8–11]. Most intervention studies in animals and humans were performed with probiotic *Lactobacillus* and *Bifidobacterium* species, which were administered by different mucosal routes. For instance, perinatal oral administration of *Lactobacillus rhamnosus* GG significantly reduced the incidence of atopic dermatitis in at-risk children [12,13] for up to 7 years of age [14]. Similarly, application of *L. rhamnosus* to pregnant mothers or offspring suppressed allergic sensitization and airway inflammation

in a mouse model of ovalbumin induced allergy [15,16]. In contrast, application of the same bacteria during adulthood had no beneficial effects [17]. Likewise, Wheeler et al. did not find any improvement in birch pollen allergic patients treated orally with *Lactobacillus acidophilus* [18]. Also, the recent study by Roso et al. failed to show evidence for protective effects of *L. rhamnosus* GG on atopic dermatitis or airway symptoms in young children [19]. These studies indicated that the beneficial effects of probiotics are dependent on the use of specific strains and on the timing of treatment. Along these lines, a recent meta-analysis has shown that the evidence for efficacy of probiotics is more convincing in prevention than in treatment [20].

The precise mechanisms responsible for the anti-allergic effects of probiotics are still poorly understood. Studies by Viljanen et al. [21] and Marschan et al. [22] proposed that induction of low-grade inflammation might help to prevent atopic eczema in infants. *In vitro* studies showed that *Lactobacillus*-exposed human dendritic cells triggered a T cell polarization towards a Th1 response [23,24]. Using a mouse model of birch pollen allergy, we previously demonstrated that mucosal application of *Lactobacillus plantarum* and *Lactococcus lactis*, strains with Th1-inducing capacity *in vitro*, administered together with the allergen reduced allergen-induced basophil degranulation [25]. However, as excessive Th1 responses have been also associated with enhanced airway inflammation [26,27], as well as with increased prevalence of Th1-type mediated disease such as rheumatic arthritis [28,29] or type 1 diabetes [30], a risk of side effects of excessive probiotics-mediated Th1 immunity has often been a matter of debate. Thus, the profound

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induction of Th1 responses by certain probiotic strains might not be the ultimate goal of allergy prevention/therapy. More recently, allergic diseases have been linked with a deficiency in regulatory T cell (Treg) numbers and/or function [31–33] and successful aeroallergen immunotherapy has been associated with the induction of Treg that secrete the suppressive cytokines IL-10 and TGF- β [34]. Thus, probiotic bacteria inducing regulatory immune responses might have beneficial effect in the prophylaxis/treatment of allergic disease. In the present study, we compared the potential of *Lactobacillus paracasei* NCC 2461 and *Bifidobacterium longum* NCC 3001, strains with the capacity to induce IL-10 *in vitro*, to prevent or modulate allergic immune responses when given at different time points. In a mouse model of poly-sensitization to tree and grass pollen allergens, representing the complex allergic parameters of human pollinosis, live bacteria were given intranasally (i) during or (ii) before sensitization and allergen challenge. The results suggest that both strains possess different immunomodulatory properties *in vivo*, most likely involving distinct immunological mechanisms including signalling pathways.

2. Material and methods

2.1. Animals

Female BALB/c mice were purchased from Charles River (Sulzfeld, Germany) at 6–10 weeks of age. Female TLR2-deficient and wild type mice with C57BL/6 genetic background were obtained from R. Maizels (Edinburgh, UK). The animals were maintained under conventional housing conditions and provided with food and water *ad libitum*. All experiments were approved by the Animal Experimentation Committee of the University of Vienna and the Federal Ministry of Science and Research.

2.2. Stimulation of HEK293 cells stably transfected with TLRs

HEK293 cells stably transfected with plasmid carrying human TLR2/CD14 gene were generously provided by M. Yazdanbakhsh (Leiden, Netherlands). HEK293 cells transfected with human TLR4/MD2/CD14 were a gift of B. Bohle (Vienna, Austria). Cells were stimulated with LPS (100 ng/ml, Sigma), Pam3CSK4 (100 ng/ml, Invivogen) or bacteria (10^8 and 10^7 CFU/ml) in 96-well plates. Supernatants were harvested after 20 h of incubation with the indicated stimuli. Concentrations of human IL-8 in cell culture supernatants were analysed by ELISA (Endogene Thermo Scientific, IL, USA) according to the manufacturer's instructions.

2.3. Bacterial strains

Two strains of probiotic bacteria, *L. paracasei* NCC 2461 (CNCM I-2116 deposited by Nestlé), and *B. longum* NCC 3001 (ATCC BAA-999 deposited by Morinaga Milk Japan, Brand BB536) were used for *in vivo* and *in vitro* studies. For intranasal application in the murine model of poly-sensitization spray-dried bacterial preparations were used, for *in vitro* experiments fresh bacterial biomass harvested, washed and frozen 3–4 h after reaching stationary growth phase was utilized.

2.4. Recombinant allergens and natural extracts

Recombinant (r) allergens Bet v 1, Phl p 1 and Phl p 5 were obtained from Biomay GmbH (Vienna, Austria). Pollen from birch (*Betula verrucosa*) and timothy grass (*Phleum pratense*) were purchased from Allergon (Välinge, Sweden) and used for preparation of an aqueous extract as previously described [35].

2.5. Sensitization protocols

Female BALB/c mice were poly-sensitized three times intraperitoneally (i.p.) with a mixture of rBet v 1, rPhl p 1 and rPhl p 5 (5 μ g each) emulsified in aluminium hydroxide (Alum, Serva, Heidelberg, Germany) on days 0, 14 and 28 (experimental design 1; Fig. 1A) or on days 20, 32 and 44 (experimental design 2; Fig. 1B). To induce airway inflammation, mice were challenged 1 week after the last i.p. sensitization by exposure to aerosolized 1% w/v birch pollen (BP) and timothy pollen (TP) extracts on two consecutive days as previously described [36]. Mice were bled 2 days after final airway challenge; sera were collected and stored at -20°C until used. Mice were killed 72 h after final airway challenge.

2.6. Intranasal pre-treatment with probiotics

In the first experimental design, *L. paracasei* NCC 2461 or *B. longum* NCC 3001 were applied intranasally (i.n.) 4 h before poly-sensitization and 4 h before each challenge. Altogether, five doses of 5×10^8 CFU of either bacterial strain in 0.9% NaCl were applied to sedated mice in total volume of 30 μ l per one application. For the second experimental design, 10^9 CFU of bacteria were intranasally applied four times on consecutive days (days 0–3), followed by 4 doses of 5×10^8 CFU (days 7–10). Control mice were sham treated with 0.9% NaCl during or before sensitization (experiments 1 and 2, respectively).

2.7. Bronchoalveolar lavage (BAL) fluids

To evaluate the allergic airway inflammation, mice were terminally anesthetized, the tracheas were cannulated, and internal airspaces were lavaged as previously described [37]. BAL fluids were centrifuged and cell-free supernatants were stored at -20°C for further analysis. Cell pellets were recovered for cellular analysis. After counting, cytopins were prepared by spinning cells onto microscope slides (Shandon Cytospin®, Shandon Southern Instruments, USA) and staining with H & E (Hemacolor®, Merck, Darmstadt, Germany). Cytospin preparations were differentiated according to standard morphologic criteria by counting 200 cells via light microscopy.

2.8. Detection of allergen-specific antibody levels

Allergen-specific antibody levels in mouse sera (IgG1 and IgG2a) or in bronchial lavages (IgA) were determined by ELISA as previously described [35]. Briefly, microtitre plates (Nunc, Roskilde, Denmark) were coated with each of the recombinant allergens Bet v 1, Phl p 1 or Phl p 5 (5 μ g/ml) and incubated with mouse sera in dilutions of 1/1000 for IgG1 or 1/500 for IgG2a detection. Lavage fluids were applied undiluted for detection of IgA. Antibody detection was performed using rat anti-mouse IgG1, IgG2a or IgA (1:500; Pharmingen, San Diego, CA, USA) followed by peroxidase-conjugated mouse anti-rat IgG (1:1000, Jackson Immuno Lab, West Grove, PA, USA). Results show the optical density (OD) values after subtraction of baseline levels from pre-immune sera.

2.9. Rat basophil leukaemia (RBL) cell degranulation

The determination of the allergenic antibody serum activity was performed as previously described [38]. Briefly, RBL-2H3 cells were passively sensitized by incubation with serum samples of the respective experimental groups and their degranulation was induced by addition of recombinant allergens diluted in Thyrode's buffer. Supernatants were analysed for β -hexosaminidase activity. Results are reported as percentages of total β -hexosaminidase

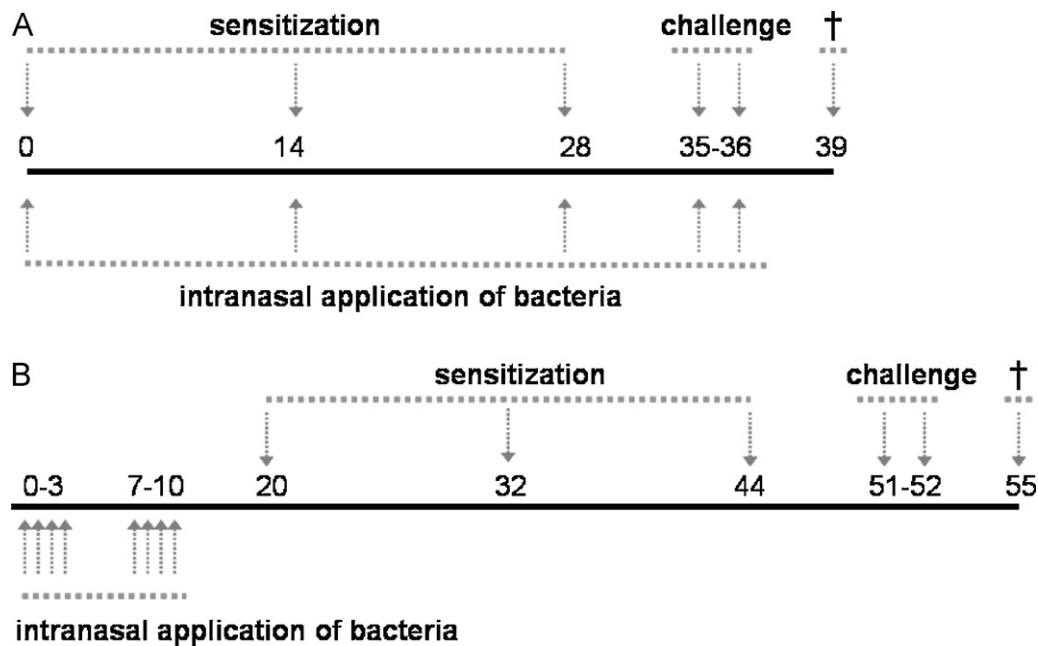


Fig. 1. (A) Application of probiotic bacteria at the time of sensitization and challenge: *Experimental design 1*: Female BALB/c mice ($n=8$) were sensitized three times intraperitoneally with a mixture of rBet v 1, rPhl p 1 and rPhl p 5 on days 0, 14 and 28 (sensitization). One week after the last immunization mice were challenged by exposure to aerosolized 1% birch pollen and timothy pollen extracts on two consecutive days. *L. paracasei* NCC 2461 or *B. longum* NCC 3001 (each 5×10^8 CFU in 30 μ l in 0.9% NaCl) were applied intranasally 4 h before each poly-sensitization and 4 h before each challenge. Control animals were sham treated with 0.9% NaCl. Mice were sacrificed 72 h after the last airway challenge and blood, BAL fluid, spleens and lungs were collected for further analysis. (B) Application of probiotic bacteria prior to allergic sensitization and challenge: *Experimental design 2*: Female BALB/c mice ($n=8$) were intranasally treated with *L. paracasei* NCC 2461 or *B. longum* NCC 3001 (each 10^9 CFU in 30 μ l in 0.9% NaCl) four times on consecutive days (days 0–3), followed by 4 doses of 5×10^8 CFU (days 7–10). Control animals were sham treated with 0.9% NaCl. 10 days after the last intranasal treatment mice were poly-sensitized (days 20, 32 and 44) and challenged (days 51 and 52). 72 h after the last airway challenge and blood, BAL fluid, spleens and lungs were collected for further analysis.

release after adding 1% Triton X-100 and are shown after subtraction of baseline release levels obtained with pre-immune sera.

2.10. In vitro stimulation

Spleen cell cultures and pooled lung cell cultures (10^7 cells/ml) were incubated for 48 h with BP (50 μ g/ml) or phleum extract (50 μ g/ml) as described [39]. Levels of IL-5, IL-10 and IFN- γ in culture supernatants were measured with mouse ELISA kits (sensitivity <5 pg/ml; Endogen, Cambridge, MA, USA). All cytokine levels are shown in pg/ml after subtraction of baseline levels of unstimulated cultures. Moreover, splenocytes obtained from naïve BALB/c mice were cultured with *L. paracasei* NCC 2461 and *B. longum* NCC 3001 at a final concentration of 5×10^7 , 5×10^6 or 5×10^5 CFU/ml for 48 h. Similarly, splenocytes from TLR2-deficient and control mice were cultured with *L. paracasei* NCC 2461 and *B. longum* NCC 3001 at a final concentration of 10^7 , 10^6 or 10^5 CFU/ml for 48 h. Cytokine concentrations in the supernatants were analysed using an ELISA specific for murine IL-10, IFN- γ , IL-12p70 and TNF- α (eBioscience, San Diego, CA, USA), according to the manufacturer's instructions.

2.11. Quantification of mRNA expression by RT-PCR

Total RNA was extracted from pooled mediastinal and submandibular lymph node cell suspensions of mice treated with probiotic bacteria at the time of sensitization and challenge and control mice (experimental design 1) at the end of the experiment and reverse-transcribed into cDNA. Expression of TLR2, TLR4 and IL-10 mRNA was measured by RT-PCR as described previously [40]. Amplification of the housekeeping gene 5-aminolevulinate synthase (ALAS1) (Universal Probe Library probe #64; Roche) was performed in order to standardize the amount of sample cDNA. Data are presented as the ratio of the target genes to ALAS1.

2.12. Statistics

Data are expressed as means $\pm$ SEM. Differences between groups were analysed using non-parametric Mann–Whitney *U*-test; *p*-values <0.05 were considered significant.

3. Results

3.1. Intranasal application of *L. paracasei* or *B. longum* at the time of sensitization and challenge suppressed Th2 responses

We compared the ability of *L. paracasei* NCC 2461 or *B. longum* NCC 3001 to modulate allergic reactions in an established mouse model of allergic poly-sensitization to the major birch and grass pollen allergens (Fig. 1A; experimental design 1). The treatment with both bacterial strains reduced the IgE-dependent basophil degranulation to all three allergens, however, significant down-regulation was observed only against Bet v 1 in *L. paracasei* NCC 2461 treated mice ($p=0.0082$) and against Phl p 5 in *B. longum* NCC 3001 treated mice ($p=0.035$) (Table 1). The application of probiotics did not influence the development of allergen-specific IgG antibody responses in serum, as neither the Th2-related IgG1 nor Th1-related IgG2a serum antibody levels differed significantly between treated or control mice (Table 1).

To evaluate whether intranasal intervention with probiotic bacteria would influence systemic cellular responses, we examined allergen-specific recall responses in spleen cells of treated and control mice. Treatment with both strains reduced the generation of allergen-induced Th2 responses, as seen by significant lower levels of IL-5 in spleen cells of treated mice compared with controls (Fig. 2A). Interestingly, the production of IL-10, which is known to induce regulatory responses and is reported to exert beneficial effects in immune modulation by probiotic bacteria [41,42], was also down-regulated (Fig. 2B). Similarly, application of *L. paracasei*

Table 1
Levels of allergen-specific antibodies in serum and BAL fluid after intranasal administration of *L. paracasei* NCC 2461 and *B. longum* NCC 3001 during poly-sensitization and challenge.

Isotype	Units	Levels of allergen-specific immunoglobulin								
		Anti Bet v 1			Anti Phl p 1			Anti Phi p 5		
		Polysens	+ <i>L. para</i>	+ <i>B. long</i>	Polysens	+ <i>L. para</i>	+ <i>B. long</i>	Polysens	+ <i>L. para</i>	+ <i>B. long</i>
IgE ^a	% release ^c	19.98 ± 4.81	3.48 ± 2.88**	7.81 ± 3.62	3669 ± 7.78	14.56 ± 8.94	13.22 ± 6.16	19.06 ± 3.27	12.15 ± 6.40	7.12 ± 3.76*
IgG1 ^a	OD	1.28 ± 0.13	1.16 ± 0.16	1.17 ± 0.24	1.24 ± 0.03	1.13 ± 0.06	1.22 ± 0.09	1.62 ± 0.05	1.47 ± 0.13	1.42 ± 0.1
IgG2a ^a	OD	0.16 ± 0.04	0.16 ± 0.04	0.2 ± 0.07	0.38 ± 0.16	0.31 ± 0.07	0.96 ± 0.45	2.56 ± 0.24	1.72 ± 0.45	1.97 ± 0.0
IgA ^b	OD	0.16 ± 0.2	0.3 ± 0.03*	0.46 ± 0.15*	0.13 ± 0.03	0.21 ± 0.04	0.39 ± 0.22	0.27 ± 0.04	0.43 ± 0.10	0.71 ± 0.5

Statistics from poly-sensitized mice as compared with *L. paracasei* treated or *B. longum* treated mice are shown as * $p < 0.05$, ** $p < 0.01$.

^a Antibody levels in serum.

^b Antibody levels in BAL fluid.

^c % release of β -hexosaminidase by RBL-2H3 cells sensitized with mouse sera and challenged with allergen.

NCC 2461 and *B. longum* NCC 3001 led to reduction in Bet v 1- and Phl p 1-specific IFN- γ production, however, the level of this cytokine was not influenced in Phl p 5 re-stimulated splenocytes of treated mice (Fig. 2C). These results strongly suggest that generalized suppression of both Th1 and Th2 responses, rather than a shift towards the Th1 immune responses was responsible for the suppression of the allergic phenotype in *L. paracasei* NCC 2461 and *B. longum* NCC 3001 treated animals.

Both bacterial strains were able to markedly suppress the local allergic inflammatory reaction as demonstrated by a significant decrease in the number of eosinophils in the BAL fluid in treated mice compared with control animals. In contrast, the numbers of BAL neutrophils were not, and those of macrophages were only slightly influenced (Fig. 3A). Application of both probiotics significantly reduced the levels of IL-5 in BAL fluids compared to controls (Fig. 3B). Moreover, application of *L. paracasei* NCC 2461 or *B. longum* NCC 3001 increased the production of Bet v 1-specific IgA in BAL fluids (Table 1), but had no effect on total IgA levels

(data not shown). In addition, data from allergen stimulated lung cells, obtained from sensitized and challenged mice, revealed a trend to reduction in IL-5 levels in animals that had received either *L. paracasei* NCC 2461 or *B. longum* NCC 3001 through the intranasal route (Fig. 3C). The reduction of IL-5, which is the key agent in the mobilisation of eosinophils, provides mechanistic explanation for the dramatically reduced airway eosinophilia in probiotic pre-treated mice. Moreover, similarly to our data obtained in allergen re-stimulated spleen cells, both treatments led to reduction of the regulatory cytokine IL-10 also in allergen re-stimulated lung cells (Fig. 3D).

3.2. Intranasal application of *B. longum* but not *L. paracasei* prior to sensitization and challenge induced suppression of Th2 responses

We next tested whether application of the two probiotics before starting sensitization would be equally protective. Mice

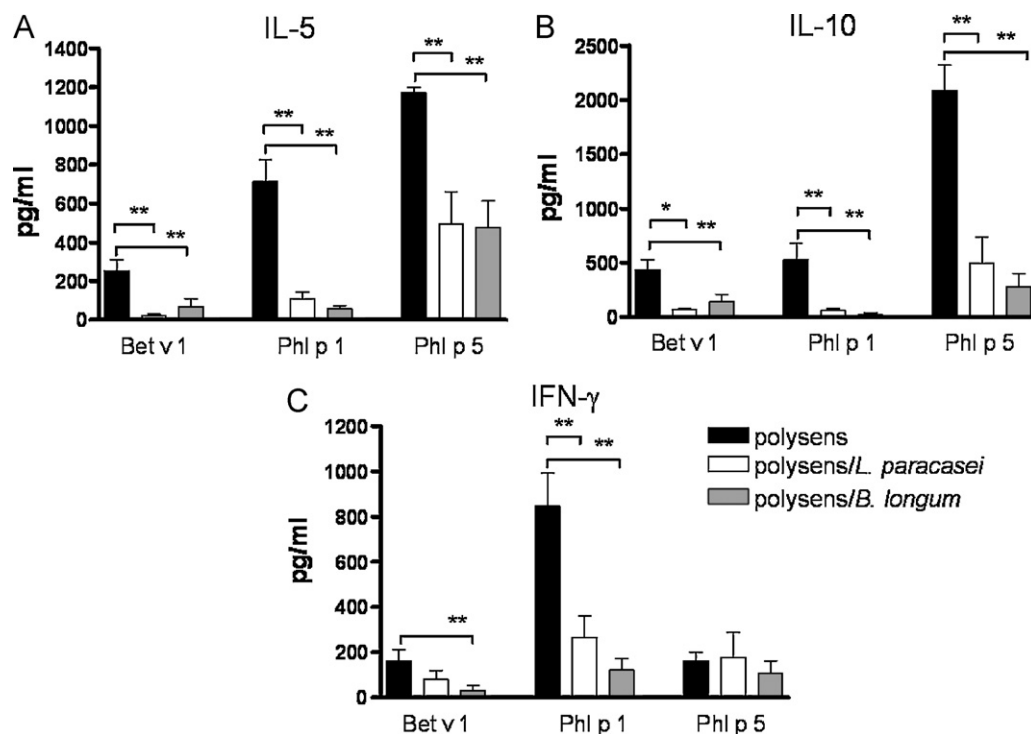


Fig. 2. Intranasal administration of *L. paracasei* NCC 2461 (white bars) and *B. longum* NCC 3001 (grey bars) during poly-sensitization and challenge reduced allergen-specific IL-5 (A), IL-10 (B) and IFN- γ (C) levels in allergen re-stimulated spleen cell cultures compared to levels from poly-sensitized control mice (black bars). Data represent means $\pm$ SEM from groups of 6–8 mice assayed individually. One representative out of two independent experiments is shown. Statistical analysis was performed by Mann–Whitney *U*-test. Statistics from poly-sensitized mice as compared with *L. paracasei* NCC 2461 treated or *B. longum* NCC 3001 treated mice are shown as * $p < 0.05$ and ** $p < 0.01$.

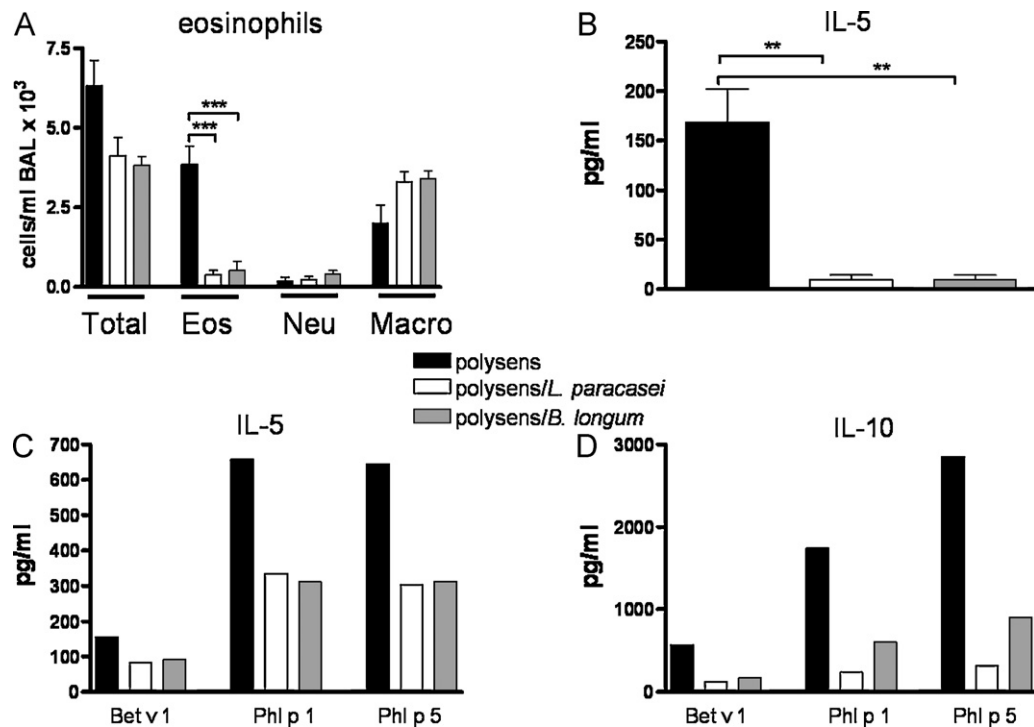


Fig. 3. Intranasal administration of *L. paracasei* NCC 2461 (white bars) and *B. longum* NCC 3001 (grey bars) during poly-sensitization and challenge significantly reduced the number of eosinophils (A) and IL-5 levels (B) in BAL fluid as well as allergen-specific IL-5 (C) and IL-10 levels (D) in allergen re-stimulated pooled lung cell cultures compared to poly-sensitized control mice (black bars). BAL data represent means $\pm$ SEM from groups of 6–8 mice assayed individually. One representative out of two independent experiments is shown. Lung cell cultures were pooled. Statistical analysis was performed by Mann–Whitney *U*-test. Statistics from poly-sensitized mice as compared with *L. paracasei* NCC 2461 treated or *B. longum* NCC 3001 treated mice are shown as **p* < 0.05 and ***p* < 0.01.

were treated intranasally with *L. paracasei* NCC 2461 or *B. longum* NCC 3001 8 times, starting 20 days and ending 10 days prior to the first poly-sensitization (Fig. 1B; experimental design 2). Application of *B. longum* NCC 3001 reduced eosinophil infiltration into the airways (Fig. 4B) and nearly abolished the production of IL-5 in BAL fluids (Fig. 4D). These effects were not observed in the mice treated with *L. paracasei* NCC 2461 (Fig. 4A; C). Likewise, only application of *B. longum* NCC 3001, but not of *L. paracasei* NCC 2461, significantly reduced Th2 responses in allergen re-stimulated spleen cells, as shown by decreased IL-5 production (Fig. 4F). At humoral level, probiotic treatment did not prevent development of allergen-specific antibody responses in serum: neither IgG1 nor IgG2a antibody levels differed between treated and control mice (data not shown). Similarly, there was no difference in the allergen-specific IgE-dependent basophil degranulation in treated mice compared to control (data not shown). However, both *B. longum* NCC 3001 and *L. paracasei* NCC 2461 applications induced allergen-specific IgA production to all 3 allergens in BAL (Figs. 4G and H).

3.3. *L. paracasei* and *B. longum* induced equal production of IL-10 but differed in the production of pro-inflammatory cytokines

To address the difference between these two probiotic strains observed on long term protection *in vivo*, we compared their immunomodulatory properties *in vitro*. Splenocytes of naïve wild type BALB/c mice were stimulated with increasing concentrations of *L. paracasei* NCC 2461 and *B. longum* NCC 3001. Although both bacterial strains were able to induce the production of regulatory cytokine IL-10 at comparable levels (Fig. 5A), *L. paracasei* NCC 2461 was a distinctly more potent inducer of Th1 cytokines IL-12p70, TNF- α and IFN- γ (Fig. 5B–D).

3.4. Role of TLR4 and TLR2 for *in vitro* cytokine production induced by *L. paracasei* and *B. longum*

To assess the role of TLRs in mediating signals from *L. paracasei* NCC 2461 or *B. longum* NCC 3001, we stimulated HEK293 cells stably transfected with TLR2/CD14 or TLR4/MD-2/CD14 with both probiotic strains. Pam3Cys and LPS were used as positive controls for TLR2 and TLR4, respectively. After 20 h of incubation, supernatants were harvested and analysed for IL-8 production. At lower concentration (5×10^7 CFU/ml), both *L. paracasei* NCC 2461 and *B. longum* NCC 3001 activated TLR2 in an analogous manner, as observed by similar induction of IL-8 (Fig. 6A). However, at higher concentration (5×10^8 CFU/ml), *B. longum* NCC 3001 induced significantly more IL-8 in comparison to *L. paracasei* NCC 2461 or the positive control, suggesting that *B. longum* NCC 3001 may preferentially activate the innate immune system through ligation of TLR2. On the other hand, *L. paracasei* NCC 2461 induced significant and dose-dependent expression of IL-8 in TLR4/MD-2/CD14 transfected cells which was not the case with *B. longum* NCC 3001 (Fig. 6B). These results suggest that *L. paracasei* NCC 2461 and *B. longum* NCC 3001 have distinct patterns of usage of TLR2 and TLR4. Stimulation of spleen cells derived from TLR-deficient mice further confirmed that the effect of *B. longum* NCC 3001 on IL-10 and TNF- α production is mediated by TLR2 (Fig. 6C and D) while induction of these cytokines by *L. paracasei* NCC 2461 is TLR2-independent (Fig. 6C and D). This suggests that spleen cells cytokine responses to *L. paracasei* NCC 2461 or *B. longum* NCC 3001 depend on different signal transduction pathways.

3.5. *L. paracasei* or *B. longum* induce up-regulation of IL-10, TLR2 or TLR4 in the draining lymph nodes of their application sites

The stimulation of naïve spleen mononuclear cells with *L. paracasei* NCC 2461 or *B. longum* NCC 3001 resulted in high production

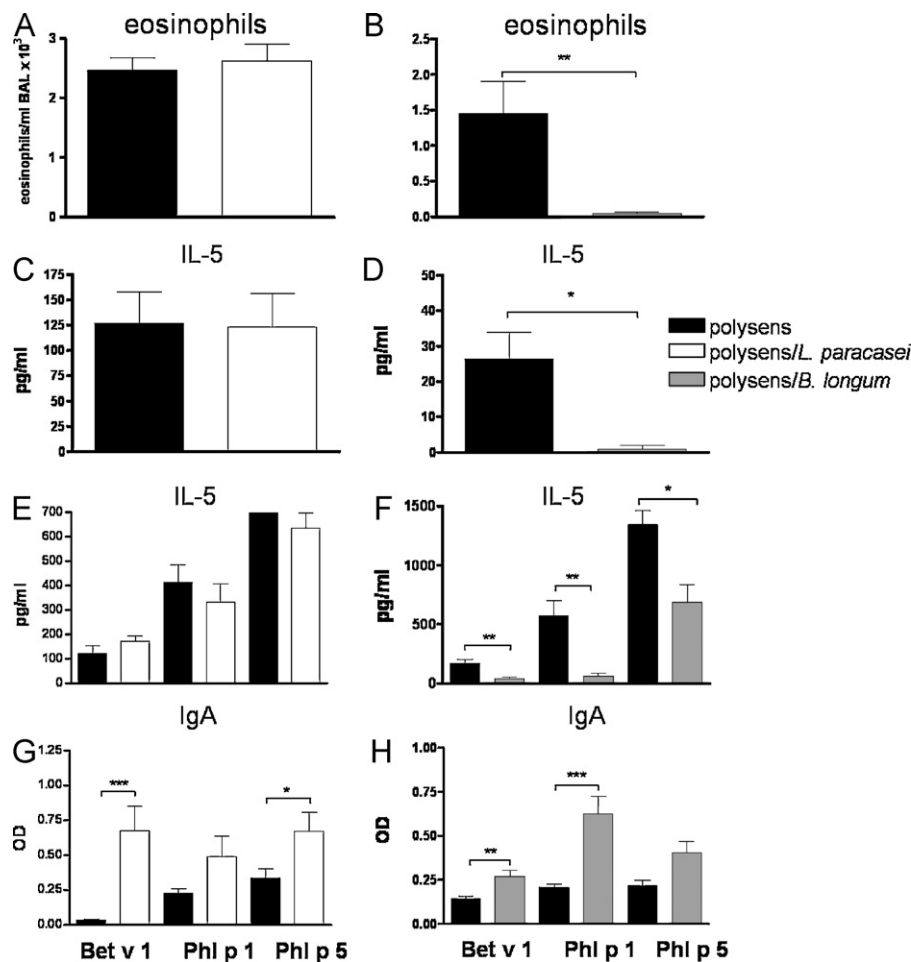


Fig. 4. Intranasal administration of *B. longum* NCC 3001 (grey bars) but not of *L. paracasei* NCC 2461 (white bars) before poly-sensitization significantly reduced the number of eosinophils (A and B) and IL-5 levels (C and D) in BAL fluid compared to poly-sensitized control mice (black bars). Allergen-specific IL-5 levels in spleen cell cultures were significantly reduced after intranasal application of *B. longum* NCC 3001 (grey bars; F) but not after intranasal application of *L. paracasei* NCC 2461 (white bars; E) compared to poly-sensitized controls (black bars). Allergen-specific IgA production in BAL fluid was increased in groups intranasally treated with *L. paracasei* (white bars; G) and *B. longum* (grey bars; H). Data represent means $\pm$ SEM from groups of 6–8 mice assayed individually. One representative out of two independent experiments is shown. Statistical analysis was performed by Mann–Whitney *U*-test. Statistics from poly-sensitized mice as compared with *L. paracasei* treated or *B. longum* treated mice are shown as **p* < 0.05, ***p* < 0.01 and ****p* < 0.001.

of regulatory cytokine IL-10 (Fig. 5A). However, in *ex vivo* experiments, allergen-specific recall stimulations of splenocytes and lung cells from probiotic treated mice resulted in lower IL-10 production compared to untreated controls (Figs. 2B and 3D). Therefore we investigated the production of IL-10 on the mRNA level in the draining lymph nodes of the site of probiotic application, i.e. submandibular and bronchial lymph nodes. The results show that the IL-10 mRNA was up-regulated in lymph nodes of *L. paracasei* NCC 2461 as well as *B. longum* NCC 3001 treated mice in comparison to untreated sensitized controls (Fig. 7A and D).

As both bacterial strains interacted with TLR2 or TLR4 in a HEK293 cell system (Fig. 6A and B), we further investigated the impact of *L. paracasei* NCC 2461 or *B. longum* NCC 3001 on the expression of these receptors *in vivo*. The data show that the treatment with *B. longum* NCC 3001 leads to up-regulation of the TLR2 mRNA both in submandibular and bronchial lymph nodes, suggesting the role of TLR2 in recognition of this strain *in vivo* (Fig. 7B and E). Accordingly, the *L. paracasei* NCC 2461 increased the expression of TLR4 mRNA in those lymph nodes (Fig. 7C and F).

4. Discussion

In this study we show that intranasal application of *L. paracasei* NCC 2461 and *B. longum* NCC 3001 during sensitization and

challenge suppressed allergen-specific immune responses and airway inflammation in poly-sensitized mice. However, when applied prior to sensitization and challenge, only *B. longum* NCC 3001 had a beneficial effect.

Several experimental models of allergic disease have been used to test the immunomodulatory potential of probiotic bacteria *in vivo*, the majority of them based on mono-sensitization with antigens such as ovalbumin [43–46]. According to clinical data, the prevalence of asthma is significantly higher in poly-sensitized patients compared to mono-sensitized patients [47,48]. Moreover, allergen-specific immunotherapy, which is so far the only causative treatment for allergic disorders, is not recommended for poly-sensitized patients [49]. This is highly relevant for birch pollen allergic patients in particular as they become frequently sensitized to other classes of aeroallergens, such as grass pollen allergens [50,51]. In order to mimic the human situation and thus to develop alternative strategies for prevention or treatment of allergic multi-sensitizations, we have established a mouse model of allergic poly-sensitization to the major birch and grass pollen allergens [36].

Although the oral route is evidently the preferred route for mucosal interventions over the nasal route in humans, mainly with respect to safety [52], our previous experimental studies have shown that the intranasal route gives more robust outcomes [53].

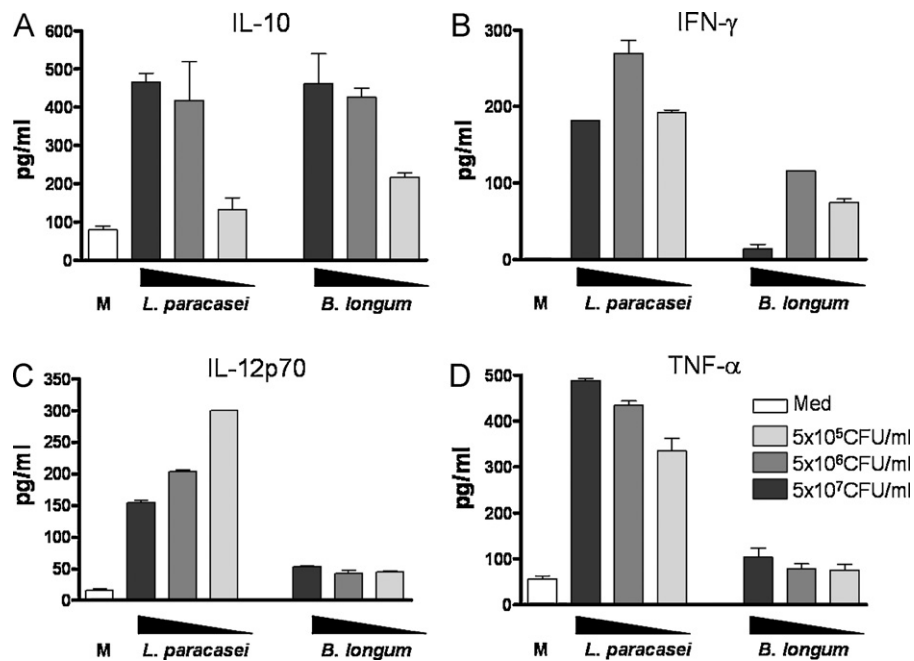


Fig. 5. Immunomodulation activity of *L. paracasei* NCC 2461 and *B. longum* NCC 3001 on murine mononuclear spleen cells. Spleen cells isolated from naïve BALB/c mice were stimulated with live *L. paracasei* NCC 2461 or *B. longum* NCC 3001 at concentrations displayed in the figure. Cytokine levels of IL-10 (A), IFN- γ (B), IL-12p70 (C) and TNF- α (D) were evaluated in cultures after 48 h by ELISA (eBioscience, San Diego, CA, USA). Results are expressed as mean $\pm$ SEM of duplicate measures. One representative out of three experiments is shown.

Therefore, to study the effects of probiotic bacteria in mice, we decided to use the intranasal route as a proof of concept.

Here we show that intranasal application of *L. paracasei* NCC 2461 or *B. longum* NCC 3001 at the time of sensitization and challenge reduced IgE-dependent basophil degranulation to all three allergens, a significant reduction occurring only against Bet v 1

and Phl p 5, but not Phl p 1. Interestingly, clinical studies have shown that the beneficial effect of probiotic treatment regime may also occur in the absence of IgE reduction [12,13]. The finding that IgG1 and IgG2a remained unaffected is in agreement with work by Ohno et al., showing that oral administration of *Bifidobacterium bifidum* G9-1 to mice also reduced total and allergen-specific IgE in

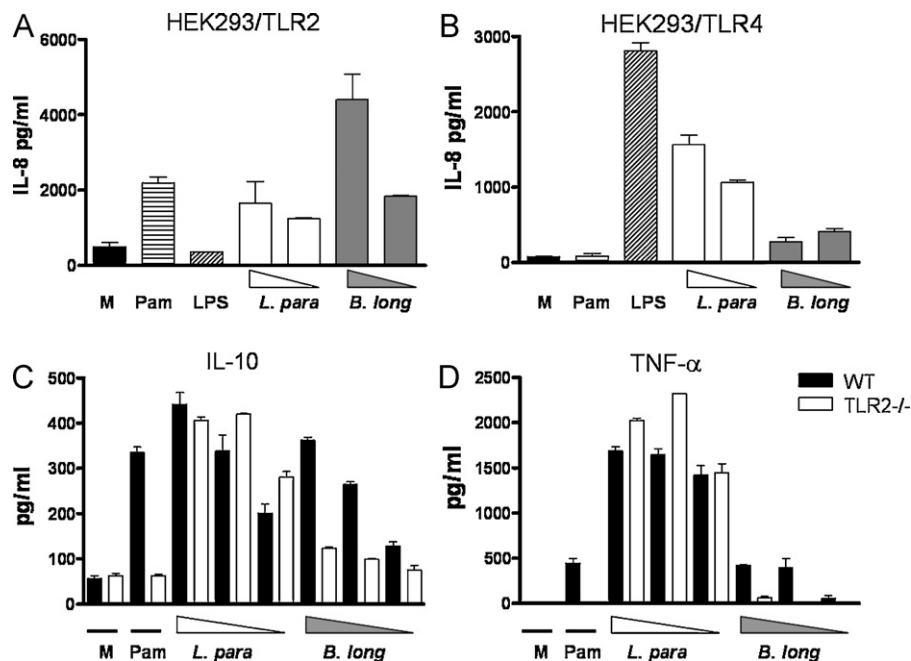


Fig. 6. Activation of TLR by *L. paracasei* NCC 2461 and *B. longum* NCC 3001. Human embryonic kidney cells (HEK293) stably transfected with an expression vector for human TLR2 (293-hTLR2/CD14; A) or TLR4 (293-hTLR4/MD2/CD14; B), respectively, were stimulated with medium alone, live *L. paracasei* NCC 2461 (*L. para*), or *B. longum* NCC 3001 (*B. long*) for 20 h. Stimulation was done at concentrations of 5×10^8 and 5×10^7 CFU/ml. Ultra pure lipopolysaccharide from *E. coli* (LPS; 100 ng/ml) and Pam3CSK4 (Pam; 100 ng/ml) were used as positive controls for TLR4 or TLR2, respectively. Unstimulated cells (M) were used as negative control. (C and D) Spleen cells from wild-type (black bars) or TLR2-deficient (white bars) mice were cultured with *L. paracasei* NCC 2461 (*L. para*) or *B. longum* NCC 3001 (*B. long*) (10^7 , 10^6 and 10^5 CFU/ml) for 48 h. Pam3CSK4 (Pam; 100 ng/ml) and unstimulated cells (M) were used as controls. The concentration of IL-8, or IL-10 and TNF- α in the supernatants was measured by ELISA. Results are expressed as mean $\pm$ SEM of duplicate measures. One representative out of three experiments is shown.

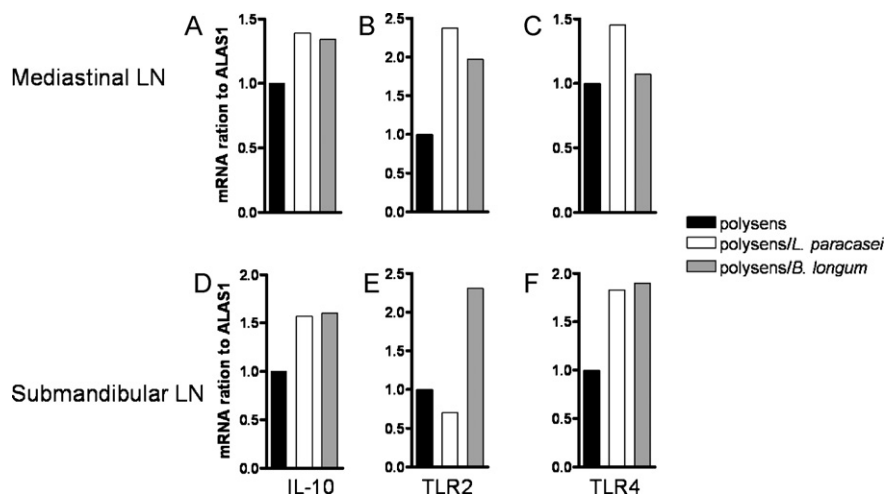


Fig. 7. *L. paracasei* NCC 2461 and *B. longum* NCC 3001 induce regulatory cytokine IL-10 and increase the mRNA expression of specific TLR *in vivo*. Relative expression of IL-10 (A and D); TLR2 (B and E) or TLR4 (C and F) to the housekeeping gene ALAS1 was determined by quantitative real-time RT-PCR in cells from pooled submandibular and bronchial lymph nodes of mice treated with probiotic bacteria at the time of sensitization and challenge. One representative out of two independent experiments is shown.

serum, but had no effect on allergen-specific IgG1 or IgG2a levels [54].

We also focused on the magnitude of eosinophilic infiltration in lungs after allergen aerosol challenge, which represents a key feature of the allergic inflammation. Repeated intranasal application of *L. paracasei* NCC 2461 or *B. longum* NCC 3001 resulted in significant decrease in eosinophils in the BAL fluid. Notably, none of the strains induced neutrophilic inflammation within the lungs but a modest increase in numbers of macrophages was observed in BAL fluid of probiotic treated mice. There is increasing evidence that macrophages are recruited to a variety of inflammation sites where they participate in the regulation of inflammation [55]. In the lungs, alveolar macrophages have been shown to participate in limiting the pathogenesis of pulmonary inflammation and the subsequent tissue repair process [55].

The probiotic treatment led to induction of high levels of secretory IgA in BAL fluids. These data are in agreement with our previous work, where intranasal application of *L. plantarum* enhanced IgA levels in BAL fluids [25], indicating that specific lactic acid bacteria possess classical characteristics of mucosal adjuvants. With respect to allergy, the role of secretory IgA has often been a matter of debate. In favour of a protective role, recent data by Smits et al. have shown that mucosal IgA plays a prominent role in protection from allergic disease in a murine model [56]. In contrast, Taylor et al. reported that patients with severe asthma have high IgA levels and IgA deficiency was not associated with atopic conditions [57].

Similar to the local suppression of Th1 and Th2 responses, systemic cytokine production of IL-5, IFN- γ and IL-10 was also markedly reduced in re-stimulated spleen cells. In accordance with our data, others showed that certain probiotic strains suppress both Th1 and Th2 systemic responses. For example, *L. rhamnosus* GG led to reduction of antigen-specific Th2 cytokines as well as of IFN- γ and IL-10 in allergen re-stimulated spleen cells [15]. Likewise, the application of *B. bifidum* G9-1 significantly reduced Th1 and Th2 cytokines in allergen re-stimulated spleen cultures [54]. Our data therefore suggest that the application of *L. paracasei* NCC 2461 or *B. longum* NCC 3001 to the nasal mucosa induces a general immunosuppression of T cell responses, rather than a shift of the allergen-specific Th2 responses towards the Th1 phenotype.

Regulatory cytokine IL-10 is a key cytokine in tolerance induction via the mucosa [58]. Indeed, here we have shown that IL-10 mRNA expression was elevated in the probiotic treated compared to sham treated mice, however, protein levels of IL-10 measured in allergen-re-stimulated lung and spleen cell cultures were reduced

in these animals. We observed similar phenomenon in our previous study, where the decreased IL-10 levels could be explained by an increased consumption of this cytokine as a consequent of high expression IL-10 receptor [59]. Indeed, blocking of this receptor abrogated the suppression of IL-10 as well as IL-4 and IFN- γ in the cell cultures [59]. Still, the precise role of IL-10 as a mediator of the immuno-suppression in our model remains to be evaluated.

Taken together, our data indicate that both *L. paracasei* NCC 2461 and *B. longum* NCC 3001, applied at the time of allergic sensitization and challenge have the intrinsic capacity to reduce systemic allergic immune responses and, importantly, prevent inflammation in the lungs, a major target organ of allergic disease.

Interestingly, the immunosuppressive properties of *L. paracasei* NCC 2461 and *B. longum* NCC 3001 were dependent of the time of their application. In the experiments where these probiotics were applied prior to sensitization and challenge, distinct outcomes were observed. The effect of *B. longum* NCC 3001 was similar when used prior or at the time of sensitization, while *L. paracasei* NCC 2461 only protected when given at time of sensitization. Thus, the protective effect of *B. longum* NCC 3001 lasted for at least 5 weeks beyond its last application. Similar long lasting effects have been described after the use of a other bacterial adjuvant system, such as DNA containing CpG motifs. Intratracheal application of synthetic CpG ODN reduced the manifestation of allergic disease if delivered 6 weeks before allergen challenge, however, no benefit was observed when administered at the same time as the allergen [60]. The long lasting effect of *B. longum* NCC 3001 but not of *L. paracasei* NCC 2461 in our experiment strongly suggests the existence of different mechanisms in their action. In addition, we cannot exclude that the persistence or the fate of the strains differ in the nasal cavity of mice. The question arising from our data is how we can explain the differences in the treatment efficacy of the two strains in relation to the intervention time?

As discussed above, secretory IgA is an important component of immune responses at mucosal surfaces and might have the capacity to suppress allergic responses by acting as “blocking antibody” [56]. However, as high IgA antibody levels were observed after prophylactic regimes with both probiotic strains, IgA does not appear to be one of the key factors for long time protection in our model.

In an attempt to further analyse the cause of different immunomodulatory properties of *L. paracasei* NCC 2461 and *B. longum* NCC 3001 observed *in vivo*, the immunomodulatory capacity of these two strains was investigated *in vitro*. Even if both strains induced similar levels of IL-10, they exhibited different abilities to

induce inflammatory cytokine production, with *B. longum* being the less pro-inflammatory strain. Accordingly, it has been shown that the probiotic strains displaying higher IL-10/IL-12 ratio *in vitro* offered the best protection in the murine model of colitis [61].

Recent data on the molecular mechanisms related to the beneficial effects of different bacterial products highlight the role of TLR ligands in the modulation of allergic inflammation [62,63]. Here, we have shown that stimulation of HEK293 cells transfected with TLR4 by *L. paracasei* NCC 2461 led to high production of IL-8, suggesting involvement of TLR4 in recognition of this strain. In contrast, TLR2 but not TLR4 was essential for recognition of *B. longum* NCC 3001. Additional experiments with spleen cells from TLR-deficient mice further support that TLR2 plays a crucial role in induction of IL-10 and TNF- α by *B. longum* NCC 3001 but not by *L. paracasei* NCC 2461. We confirmed the *in vitro* data by showing that *B. longum* NCC 3001 led to increased mRNA expression of TLR2, while *L. paracasei* NCC 2461 treatment led to increased TLR4 mRNA expression in the draining lymph nodes (submandibular/bronchial) of mucosally treated mice. Along these lines, stimulation of TLR2 with peptidoglycan (a natural TLR2 ligand) was shown to decrease pulmonary eosinophilia in a murine model of asthma [62]. The relevance of TLR2 in allergy has been previously confirmed in clinical studies in children of farmers showing an inverse correlation between increased levels of TLR2 mRNA and risk of allergy development [64].

Finally, we have shown that both *L. paracasei* NCC 2461 and *B. longum* NCC 3001 are interesting candidates for seasonal treatment protocols to reduce allergic symptoms in poly-sensitized individuals. The results obtained with *B. longum* NCC 3001 in this unique mouse model of poly-sensitization fit quite well with the clinical data obtained by Xiao et al. in humans suffering from cedar pollen allergy [65]. However, the beneficial effects of *B. longum* NCC 3001 may be maintained for a more prolonged period of time which is of particular relevance for multi-sensitized patients, who may be perennially exposed to a series of different allergens.

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