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Short title: Intranasal *L. rhamnosus* GG prevents asthma in mice

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Abstract

Background: There is an increasing interest in targeted application of probiotic bacteria for prevention and treatment of airway diseases, including allergies. Here, we investigated the beneficial effects of preventive intranasal treatment with probiotics *Lactobacillus rhamnosus* GG and *L. rhamnosus* GR-1 in a mouse model of allergic asthma.

Methods: *L. rhamnosus* was administered intranasally eight times on days 1-4 and 8-11 at $5x10^8$ CFU/dose, followed by a two-week asthma induction protocol with birch pollen extract on alternating days. Effects of preventive treatment were analyzed based on serum antibody levels, bronchoalveolar lavage cell counts, lung histology, lung cytokine levels and airway hyperreactivity. Colonization and translocation of *L. rhamnosus* was assessed by bacterial cell counts in nasal mucosa, fecal samples, cervical lymph nodes and blood. Binding of fluorescent *L. rhamnosus* to fixed murine nasal mucosal cells and airway macrophages was visualized by fluorescence microscopy.

Results: Transient colonization of the murine upper airways by *L. rhamnosus* GG was demonstrated and was approximately ten times higher compared to *L. rhamnosus* GR-1. Marked binding of fluorescent *L. rhamnosus* GG to murine nasal mucosal cells and airway macrophages was visualized. Preventive treatment with *L. rhamnosus* GG (but not *L. rhamnosus* GR-1) resulted in a significant decrease in bronchoalveolar lavage eosinophil counts, lung interleukin-13 and interleukin-5 levels, and airway hyperreactivity. A tendency towards a decrease in serum Bet v 1-specific IgG1was likewise observed.

Conclusion: Intranasally administered *L. rhamnosus* GG prevents the development of cardinal features of birch pollen-induced allergic asthma in a strain-specific manner.

Abbreviations

AHR	Airway hyperreactivity
BALF	Bronchoalveolar lavage fluid
BP	Birch pollen
CFU	Colony forming units
LGG	*Lactobacillus rhamnosus* GG
LGR-1	*Lactobacillus rhamnosus* GR-1
MAMP	Microbe-associated molecular pattern
Rn	Airway resistance
Th	T helper
Treg	Regulatory T cell

Introduction

Allergic airway disease is a result of disregulated adaptive immune reactions to innocuous environmental stimuli, characterized by a shift towards local and systemic T helper 2 (Th2) responses. This typically results in systemic allergic sensitization, reflected by an increase in immunoglobulin E (IgE) levels, and Th2 type eosinophilic airway inflammation. In patients with severe allergic asthma, airway hyperreactivity (AHR), mucus hypersecretion and compromised airway function leads to an impaired quality of life and high socioeconomic costs (1). Birch pollen (BP) containing the major allergenic protein Bet v 1 represents a clinically relevant and highly potent source of aeroallergens with a strong sensitising capacity, capable of triggering or aggravating allergic disease development (2, 3).

Recent studies underline the importance of airway microbiota and the role of particular bacterial strains in regulation of immune functions and the maintenance of the Th1/Th2 balance (4, 5). The use of intranasal probiotics, or "live microorganisms that, when administered in adequate amounts,

confer a health benefit on the host" (6) represents a promising strategy for prevention and treatment of allergic sensitisation and disease. Interactions between mucosally applied probiotics and the host can modulate both local and systemic host immune responses (7, 8). Although probiotic bacteria were initially administered via the oral route, a wide range of non-oral probiotic applications have been described up to date (6). An example of their use in the nasal cavity involves nasal sprays containing lactic acid bacteria, which are increasingly being explored in clinical trials against diseases such as otitis media (9).

Lactobacillus rhamnosus GG and *Lactobacillus rhamnosus* GR-1 represent model probiotic strains with demonstrated immunomodulatory and immunostimulatory properties (10, 11, 12, 13, 14). Oral administration of *L. rhamnosus* GG was previously shown to offer benefits in the context of allergic disease prevention and treatment, both in animal models (15, 16, 17) and in human clinical trials (18, 19, 20). However, current knowledge on the systemic and mucosal impact of *L. rhamnosus* GG and *L. rhamnosus* GR-1 when they are applied via the nasal route is limited.

In this work, we explored the effects of intranasally administered *L. rhamnosus* GG and *L. rhamnosus* GR-1 as preventive treatment in a mouse model of BP-induced allergic airway disease. We also explored the potential of *L. rhamnosus* strains to colonize the nose or distribute from the nose to the gastrointestinal tract. In addition, we visualized their binding abilities to murine nasal mucosal cells and airway macrophages.

Materials and Methods

Animals

Five to six weeks old male BALB/cOlaHsd mice housed in specific-pathogen-free conditions (Envigo, The Netherlands) were used. Experiments were approved by the Ethical Committee for Animal Research (KU Leuven, project P063/2014).

Colonization and translocation potential of *L. rhamnosus*

To study translocation and colonization by *L. rhamnosus*, naïve mice were intranasally instilled with $5x10^8$ CFU of *L. rhamnosus* GG (strain CMPG11261) and *L. rhamnosus* GR-1 (strain CMPG11270) (21). The strains were cultured as described in (21). Bacterial suspensions were prepared and administered as described below for wild type *L. rhamnosus*. The phospate buffered saline (PBS) group received PBS (BioWhittaker® Lonza) intranasally. All intranasal instillations were performed after anaesthesia with isoflurane (Schering-Plough Animal Health). Blood, nasal mucosae, cervical lymph nodes and fecal samples were collected in sterile conditions 24 h and 72 h after intranasal *L. rhamnosus* instillation. Blood was collected by retro-orbital bleeding. Bronchoalveolar lavage fluid (BALF) was obtained by lavaging the lungs thrice with 0.7 ml of sterile saline (0.9% NaCl, B.Braun).

Single cell suspensions of nasal mucosa and cervical lymph nodes were prepared in 200 µl PBS using a 40 µm cell strainer (BD Bioscience). Fecal samples were dissolved in 500 µl PBS by vortexing. Hundred µl of blood, nasal mucosa, cervical lymph nodes or fecal suspensions were

plated out on selective de Man-Rogosa-Sharpe (MRS) agar with 5 µg/ml erythromycin and 10 µg/ml chloramphenicol for detection of live *L. rhamnosus*.

Adhesion of *L. rhamnosus* to murine nasal mucosal cells and airway macrophages

Single cell suspensions of nasal mucosa cells and BALF containing airway macrophages were collected from naïve mice as described above. Two hundred µl of single cell suspension were transferred onto glass slides using the Cytospin system (Shandon, TechGen, Zellik, Belgium). To visualize bacterial adhesion, fluorescent *L. rhamnosus* GG (strain CMPG11261) and *L. rhamnosus* GR-1 (strain CMPG11265) producing the red fluorescent protein mCherry, and recombinant *L. rhamnosus* GG (strain CMPG11260) and *L. rhamnosus* GR-1 (strain CMPG11264) producing the blue fluorescent protein mTagBFP2 were used. The strains were induced with nisin and cultured overnight as previously described (21). Adhesion assay was performed as previously described (14, 21). Briefly, mouse nasal mucosa or BALF cells were fixed on glass slides in glutaraldehyde/formaldehyde buffer for 1 h, and 10^7 CFU of nisin-induced fluorescent *L. rhamnosus* were subsequently added on top of the cells. Following 1 h of co-incubation at 37°C, the slides were washed in PBS and visualized using the Zeiss Axio Imager Z1 microscope with an AxioCam MRm and the AxioVision software.

Allergic sensitisation and provocation

Allergic sensitisation and airway inflammation was induced by intranasal instillations of 50 µl of BP extract (Greer, US) at 5 mg total protein/ml using a micropipette on days 14, 16, 18, 22, 24, 26 and 29, as depicted in figure 1. The negative control saline group received saline intranasally. All intranasal instillations were performed after anaesthesia with isoflurane. Mice were sacrificed on day 31 by a lethal intraperitoneal injection of pentobarbital (Nembutal, Sanofi Santé Animale, CEVA, Belgium).

Preventive treatment with *L. rhamnosus*

L. rhamnosus was administered as depicted in figure 1. Wild type *L. rhamnosus* GG (ATCC 53103) (22) and *L. rhamnosus* GR-1 (ATCC 5582) (23) were grown in MRS medium at 37°C in static conditions. Overnight cultures were pelleted (3000 g, 5 min) and washed twice in sterile PBS. The pellet was resuspended in PBS and $5x10^8$ CFU in 10 µl PBS were administered eight times by intranasal instillations using a micropipette on days 1-4 and 8-11. The saline and BP groups received PBS. All experiments were repeated at least twice.

Total IgE, Bet v 1-specific IgG1 and IgG2a determination

Total IgE, Bet v 1-specific IgG1 and IgG2a in serum were determined by ELISA. Plates were coated overnight with 5 µg Bet v 1/ml PBS for IgG1 and IgG2a, or with capture IgE antibody (553413) for total IgE, and blocked with 2% gelatin (for IgG1 and IgG2a) or bovine serum albumin (for total IgE) in PBS. After incubation with biotin-anti-mouse-IgG1 (553441), biotin-anti-mouse-IgG2a (553388) or biotin-anti-mouse-IgE (553414) antibodies diluted 1:1000 in PBS, detection was performed with streptavidin-horseradish peroxidase (Lucron), 3,3′,5,5′-tetramethylbenzidine (TMB) (Acros Organics) and H_2O_2. Absorbance at 450 nm was measured using a spectrophotometer (Multiskan RC, ThermoScientific) and converted to arbitrary units/ml based on a pool of Bet v 1-reactive sera. All antibodies were purchased from BD Pharmingen, BD Bioscience.

Total and differential cell counts in BALF

Total cell counts in BALF were performed in a Bürker hemocytometer using Trypan Blue coloring (BioWhittaker® Lonza). Differential cell counts were performed in duplicate on cytospins (Shandon, TechGen, Belgium) stained by the Diff-Quik method (Medical Diagnostics, Germany).

For each slide, the percentage of macrophages, eosinophils, neutrophils and lymphocytes was counted in 250 cells.

Lung histology

Lungs were fixed in 4% paraformaldehyde for 24 h and stored in 70% ethanol. After embedding in paraffin, 5 µm-thick sections were stained with haematoxylin and eosin (H&E), or with periodic acid-Schiff (PAS) staining. Light microscopic examination was performed at 40x magnification (Primo Star, Zeiss, Plan Achromat 40x/0.65 objective).

Lung cytokine analysis

Lungs were homogenized in 500 µl PBS and the supernatant was collected by centrifugation (10000 g, 10 min). Lung IL-13 cytokine analysis was performed using the BD CBA Mouse/Rat Soluble Protein Master Buffer Kit and BD CBA Mouse IL-13 Flex Set according to the manufacturer's instructions (detection limit 2.4 pg/ml). Acquisition was performed using FACSArray (BD Biosciences) and data was subsequently processed with FlowJo software (TreeStar, Inc.). Levels of other cytokines were determined with the MSD U-PLEX Custom Biomarker Mouse Multiplex assay combined with U-PLEX Mouse antibody sets for each cytokine. All cytokine levels were adjusted for lung weight.

Airway hyperreactivity (AHR) measurements

AHR was measured on day 31 using the Flexivent forced oscillation technique (Flexivent 7, SCIREQ, Montreal, Canada) as previously described (24, 25). Mice were anaesthetized by intraperitoneal injection of pentobarbital diluted in saline. Airway resistance (Rn) was recorded in response to increasing concentrations of methacholine (Sigma-Aldrich®) solution (0, 1.25, 2.5, 5, 10 and 20 mg/ml).

Statistical analysis

Statistical analysis was performed using GraphPad Prism v.6 (La Jolla, CA, USA). Mann–Whitney U test was used for pairwise comparisons between two groups and Kruskall-Wallis test with post-hoc analysis was implemented to compare between multiple groups. Two-way ANOVA with a Bonferroni post-hoc test was implemented for AHR data analysis. Differences between groups were considered significant when $p < 0.05$.

Results

Colonization and translocation potential of *L. rhamnosus* GG and *L. rhamnosus* GR-1 in naïve mice

Live *L. rhamnosus* cells were detected on the nasal mucosa of mice 24 h (Figure 2A) but not 72 h after intranasal instillation. Significantly more live *L. rhamnosus* GG cells ($5.2x10^4$ – $1.7x10^5$ CFU/mouse) were retained on the nasal mucosa after 24 h compared to *L. rhamnosus* GR-1 ($1.6x10^3$ – 10^4 CFU/mouse). Live *L. rhamnosus* cells were also detected in the gastrointestinal tract of the mice 24 h (Figure 2B), but not 72 h after intranasal instillaton. Likewise, significantly more live *L. rhamnosus* GG cells ($1.9x10^4$ – $8x10^5$ CFU/g feces) were present in the fecal samples compared to *L. rhamnosus* GR-1 (0 – $2.4x10^4$ CFU/g feces). A small fraction of live *L. rhamnosus* GG cells ($6x10^1$ – $3.6x10^2$ CFU/mouse) was found in the cervical lymph nodes of the mice 24 h after intranasal instillation (Figure 2C), however the bacterial cells were cleared 72 h after their administration. No live *L. rhamnosus* GR-1 could be detected in the cervical lymph nodes. Intranasal administration of either *L. rhamnosus* GG or *L. rhamnosus* GR-1 at the tested concentrations did not affect mouse weight (Figure S1) or behavior, and no live *L. rhamnosus* cells were detected in the blood of the mice.

Adherence of *L. rhamnosus* GG to isolated murine nasal mucosal cells was observed by visualization of *L. rhamnosus* GG-derived fluorescent strain CMPG11261 (expressing mCherry) that bound in large numbers to fixed nasal mucosal cells (Figure 2D). In comparison, only single *L. rhamnosus* GR-1-derived CMPG11265 (expressing mCherry) cells were bound to the fixed nasal mucosal cells (Figure 2D). In addition, the *L. rhamnosus* GG-derived fluorescent strains CMPG11261 (expressing mCherry) and CMPG11260 (expressing mTagBFP2) demonstrated higher binding to murine airway macrophages when compared to the *L. rhamnosus* GR-1-derived CMPG11265 (expressing mCherry) and CMPG11264 (expressing mTagBFP2) strains (Figure 2E).

Preventive effects of intranasally administered *L. rhamnosus* GG or *L. rhamnosus* GR-1 on allergic asthma

The effects of intranasal administration of *L. rhamnosus* GG and *L. rhamnosus* GR-1 were explored in a mouse model of BP-induced allergic asthma. The protocol is shown in figure 1. *L. rhamnosus* GG and *L. rhamnosus* GR-1 were administered on days 1-4 and 8-11 at $5x10^8$ CFU/dose, followed by allergic asthma induction with BP extract on days 14, 16, 18, 22, 24, 26 and 29. The effects on allergic sensitization were examined by measuring serum antibody levels against the major BP allergen Bet v 1 (Figure 3). A trend towards decrease in Bet v 1-specific IgG1 was observed after preventive treatment with *L. rhamnosus* GG ($p = 0.0649$) and was less pronounced with *L. rhamnosus* GR-1 ($p = 0.2381$) compared to the not-pretreated BP-exposed group (Figure 3A). No significant effects on total IgE (Figure 3B), Bet v 1-specific IgG2a or Bet v 1-specific IgG1 tot IgG2a ratio (Figure 3D) were found compared to the not pretreated BP-exposed mice.

Effects of preventive *L. rhamnosus* treatment on allergic airway inflammation were examined by BALF differential cell counts, lung histology and lung cytokine analysis. Preventive treatment with *L. rhamnosus* GG significantly decreased eosinophil counts in BALF compared to the not-pretreated BP-exposed group (Figure 4C) ($p = 0.0043$). Histological analysis of lung sections likewise demonstrated a decrease in regions of eosinophilic infiltration (Figure 4F) in mice pretreated with *L. rhamnosus* GG, as well as a moderate decrease in PAS-positive cells (Figure 4G) compared to the not-pretreated mice. No statistically significant effects could be detected on BALF total cell, macrophage, neutrophil or lymphocyte cell counts in any of the pretreated groups compared to the not-pretreated BP-exposed group.

The group pretreated with *L. rhamnosus* GG also had significantly lowered lung IL-5 ($p = 0.0390$) and IL-13 ($p = 0.0152$) levels (Figure 5A and B) compared to the not-pretreated BP-exposed mice. No significant effects of preventive treatment with *L. rhamnosus* GG or *L. rhamnosus* GR-1 could be demonstrated on lung IL-4 (Figure 5C), IFN-γ (Figure 5D), IL-10 (Figure 5E) or IL-17 (Figure 5F) levels. Furthermore, no significant difference was observed in expression of *Gata3*, *Tbx21*, *Rorc* or *Foxp3* mRNA (Figure S2), or granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1β or IL-33 levels in lung tissue homogenates (Figure 5G, H and I) between the mice pretreated with *L. rhamnosus* GG or *L. rhamnosus* GR-1 and the not-pretreated mice.

AHR is an essential hallmark of asthma. As shown in figure 6, repeated BP instillations induced AHR, as evaluated by airway resistance (Rn) after exposure to increasing doses of methacholine. Interestingly, we could observe a significantly lower Rn after exposure to methacholine as a result of *L. rhamnosus* GG preventive treatment (Figure 6A and B, $p = 0.0152$ for AUC of Rn), reflecting a decrease in AHR compared to the non-pretreated BP-exposed group. Intermediate levels of AHR

were observed in the *L. rhamnosus* GR-1 group, however the effect of preventive *L. rhamnosus* GR-1 treatment did not reach statistical significance.

Discussion

In this work we demonstrated that intranasal administration of live probiotic *L. rhamnosus* GG bacteria can decrease allergic airway inflammation, lung Th2 cytokine production and is even capable of preventing AHR, all induced by repeated intranasal application of BP extract in mice. AHR is a defining pathophysiological feature of allergic asthma in human patients (26), and the observed effect on AHR in our mouse model is therefore highly relevant for potential application in humans. *L. rhamnosus* GG pretreatment significantly lowered BALF eosinophil counts, as well as IL-5 and IL-13 levels measured in lung tissue. Lowering of IL-5 production can be responsible for the decrease in BALF eosinophils. These results are in line with the previously described prevention of pulmonary eosinophilia and AHR following oral *L. rhamnosus* GG administration in murine models of ovalbumin-induced asthma, which was likewise linked to a reduction in IL-5 production (16, 27). Also the significant decrease in IL-13 might be responsible for preventing AHR in this intranasal experimental set-up, as IL-13 has previously been shown to be the critical factor in the development of AHR in mouse models of allergic disease (28, 29).

Surprisingly, the effects of intranasal *L. rhamnosus* GG were not associated with a significant decrease in allergic sensitization at the level of antibody production, although a tendency towards lowered allergen-specific IgG1 was observed. Similar results have been reported with intranasally administered wild type *Lactobacillus plantarum* NCIMB8826 tested in models of allergic sensitization to house dust mite (30) and the major BP allergen Bet v 1 (31). More specifically, *L. plantarum* NCIMB8826 reduced airway inflammation, but did not significantly influence allergen-specific IgG1 or IgG2a antibody production, which is in accordance with our data. These and our

results thus indicate that effects on allergen-specific antibody induction are not always a prerequisite for the beneficial action of probiotics in allergic asthma. Of note, probiotic effects might differ depending on the implemented administration protocol, as no similar benefits on AHR or airway inflammation have so far been observed in a preliminary therapeutic set-up when *L. rhamnosus* GG was intranasally implemented together with BP exposure.

No Th1-related immunostimulation by intranasally instilled *L. rhamnosus* GG was observed, as we did not detect any change in IFN-γ cytokine or *Tbx21* mRNA levels in the lungs. Similarly, no Th17-related immune effects were detected, as the levels of IL-17 and *Rorc* mRNA in the lungs were not significantly altered by *L. rhamnosus* GG instillations. We also did not detect an increase in lung IL-10 cytokine or *Foxp3* mRNA levels, arguing against the induction of Treg cells as the mechanism of action. Of course, it can not be excluded that other regulatory cytokines are involved in mediating the observed beneficial effects. For example, stimulation of TGF-β production in mesenteric lymph nodes and in the airways as a result of oral *L. rhamnosus* GG application in experimental airway allergies was previously described (16, 27). These effects might also depend on the probiotic application route and the experimental model. For example, oral application of *L. rhamnosus* GG, *FoxP3* mRNA expression upregulation in peribronchial lymph node cells was demonstrated in a mouse model of ovalbumin-induced allergic asthma (16).

The mechanism of action of intranasal probiotic treatment remains open for further research. It is possible that local *L. rhamnosus* GG mechanisms of action previously unexplored in the nasal niche contribute to its protective effects. *L. rhamnosus* GG and its products have both anti-oxidative (32), as well as epithelial barrier-promoting properties in the gut (32, 33). Development of respiratory allergies is often associated with a disrupted epithelial barrier (34), therefore the ability of probiotics to preventively strengthen airway epithelial integrity could be one of the

potential protective mechanism of probiotic action, a possibility that certainly warrants further investigation.

In comparison to *L. rhamnosus* GG, the *L. rhamnosus* GR-1 strain had no clear effect in our experimental asthma set-up. Certain lactic acid bacteria strains are more effective at preventing allergic disease upon their intranasal administration, for example as demonstrated previously for *L. paracasei* NCC2461 compared to *L. plantarum* NCC1107 in an ovalbumin model of allergic asthma (35). The *in vivo* viability and transient colonization potential of probiotic strains might be an important consideration for their administration in the nasal niche, as up to 30% of intranasally instilled compounds in mice are cleared by nasal mucociliary clearance within the first hour (36). In this work, we for the first time demonstrated transient colonization of the nasal mucosa by both *L. rhamnosus* GG and *L. rhamnosus* GR-1. Interestingly, both *L. rhamnosus* GG- and *L. rhamnosus* GR-1-derived strains were also detected live in the mouse gut following their intranasal instillations. *L. rhamnosus* GG was retained at higher levels in the gut compared to *L. rhamnosus* GR-1, which can partially be attributed to the superior adherence of *L. rhamnosus* GG to intestinal epithelial cells compared to *L. rhamnosus* GR-1 demonstrated in our previous studies (21). These results suggest that the interaction of intranasally administered *L. rhamnosus* with the murine immune system and epithelium is not restricted to the upper airways.

Enhanced adherence abilities and strain-specificity of MAMPs might partially explain why intranasally instilled *L. rhamnosus* GG, but not *L. rhamnosus* GR-1, could prevent allergic asthma in our mouse model. For example, the SpaCBA pili structures are present only on the surface of *L. rhamnosus* GG and play an important role in adhesion to epithelial cells (12), as well as in immune interactions with dendritic cells (37) and macrophages (13). In fact, in this work we observed the translocation of intranasally instilled live *L. rhamnosus* GG, but not *L. rhamnosus*

GR-1, to cervical lymph nodes which drain the nasal cavity. The survival of *L. rhamnosus* GG in immune cells and its trafficking to mesenteric lymph nodes after oral administration has previously been shown to affect TNF-α and IL-12 gene expression in macrophages (38). Modulation of immune cell function and the local cytokine milieu is a key step in attenuation of allergic disease and the associated inflammation (39). Therefore, it is tempting to propose that the observed enhanced *L. rhamnosus* GG binding to innate immune cells (macrophages) and epithelial cells might promote enhanced probiotic-host immunomodulatory interactions, and similar binding to dendritic cells might modify their orchestrating function in immune regulation, although this requires further substantiation.

In conclusion, preventive intranasal administration of *L. rhamnosus* GG can offer a number of benefits in the context of allergic asthma induced by the clinically relevant BP aeroallergen. These strain-specific beneficial effects might be linked to superior binding of *L. rhamnosus* GG to host cells and its transient *in vivo* retention. As intranasal instillations of *L. rhamnosus* were shown to be innocuous in the tested conditions, they could therefore be considered as an effective alternative to the oral probiotic administration route. The ability of *L. rhamnosus* GG to prevent the hallmarks of allergic asthma typically observed in humans is especially promising, therefore the intranasal application of this strain deserves further exploration.

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Authors contributions

I.S., J.C., S.S., S.L. and M.P. contributed to the conception and design of the experimental work, as well as the writing and critical revision of the manuscript. I.S., A.F., M.P., L.P. and J.V. performed the experimental work and processed the data. All authors reviewed and corrected the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Figure legends

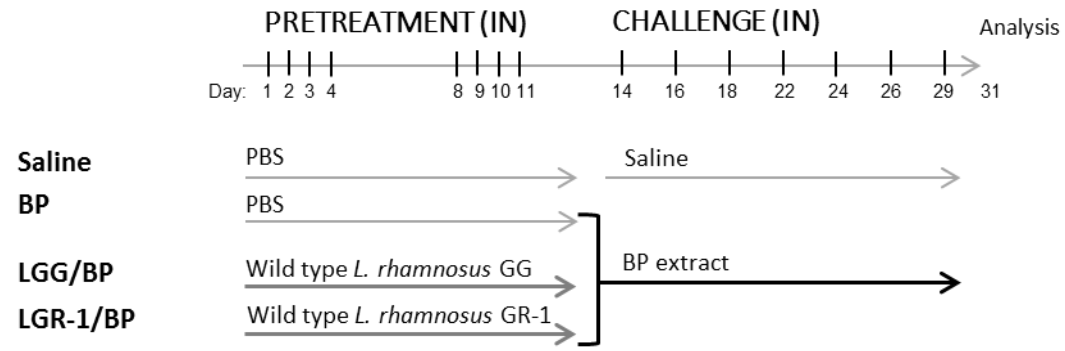

Figure 1. Protocol for testing the effects of intranasal *L. rhamnosus* GG (LGG) and *L. rhamnosus* GR-1 (LGR-1) preventive treatment on BP-induced allergic asthma. IN: intranasal; BP: birch pollen; PBS: phosphate buffered saline.

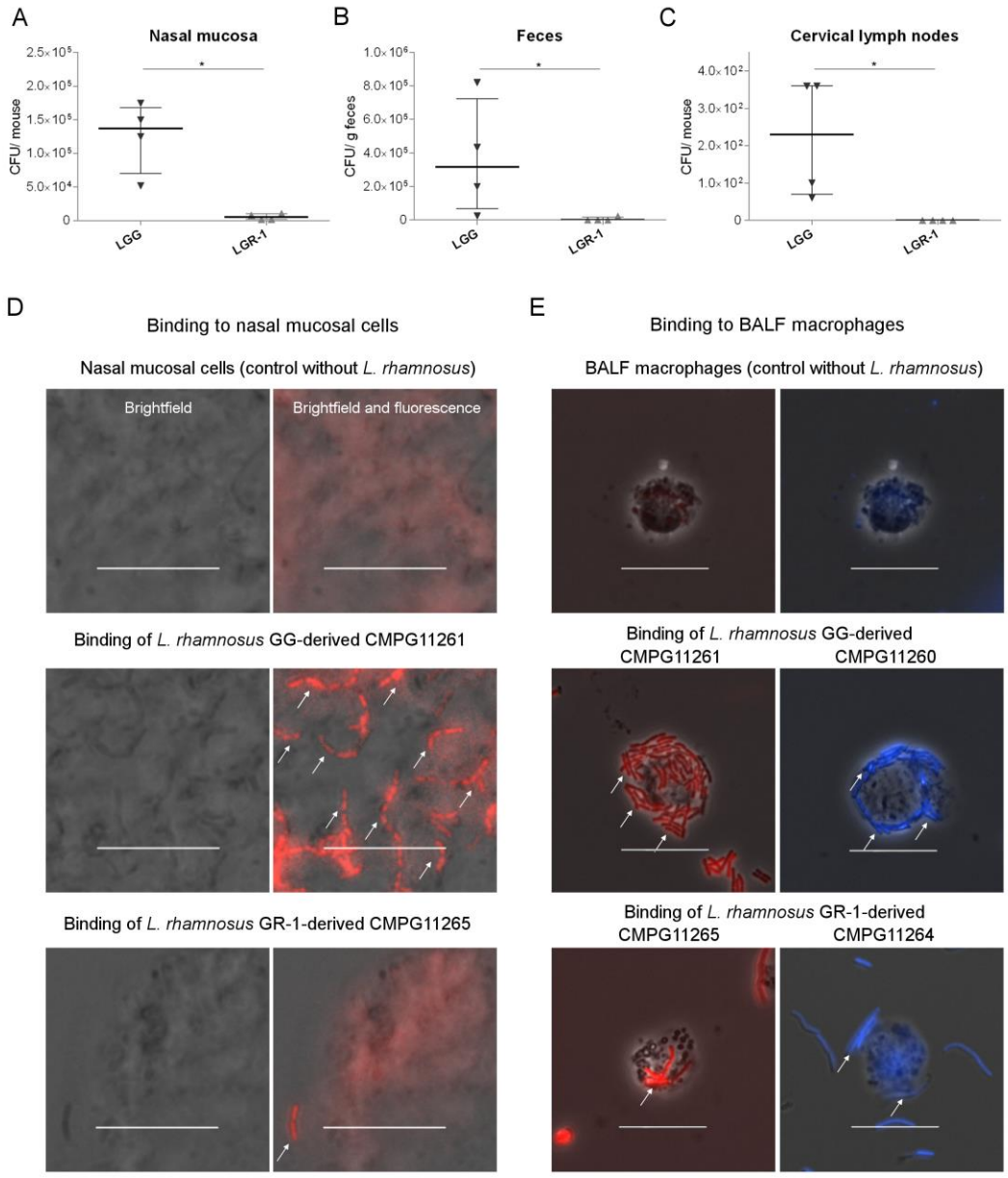

Figure 2. Detection of live *L. rhamnosus* GG and *L. rhamnosus* GR-1 in murine nasal mucosa (A), fecal samples (B) and cervical lymph nodes (C), and visualization of fluorescent *L. rhamnosus* strains binding to isolated murine nasal mucosal cells (D) and airway macrophages (E). Enumeration of live *L. rhamnosus* GG (LGG) and *L. rhamnosus* GR-1 (LGR-1) in (A) homogenized nasal mucosa, (B) feces and (C) cervical lymph nodes 24 h after intranasal instillation of bacteria. Data are depicted as CFUs per mouse or per g feces, and medians with

interquartile ranges per group. *p<0.05 compared to *L. rhamnosus* GG; n = 4 mice per group. (D) Visualization of adherence of fluorescent *L. rhamnosus* GG-derived strain CMPG112261 and *L. rhamnosus* GR-1-derived strain CMPG112265 expressing the red fluorescent mCherry protein (in red) to fixed murine nasal mucosal cells. Left panel represents brightfield and right panel represents a combination of brightfield with fluorescent microscopy. (E) Visualization of adherence of fluorescent *L. rhamnosus* GG-derived strain CMPG112261 and *L. rhamnosus* GR-1-derived strain CMPG112265 expressing the red fluorescent mCherry protein (in red), and *L. rhamnosus* GG-derived strain CMPG112260 and *L. rhamnosus* GR-1-derived strain CMPG112264 expressing the blue fluorescent mTagBFP2 protein (in blue) to fixed murine BALF macrophages. All panels represent a combination of phase contrast and fluorescent microsopy. White bars are equal to 20 μm. White arrows indicate bound fluorescent *L. rhamnosus* cells.

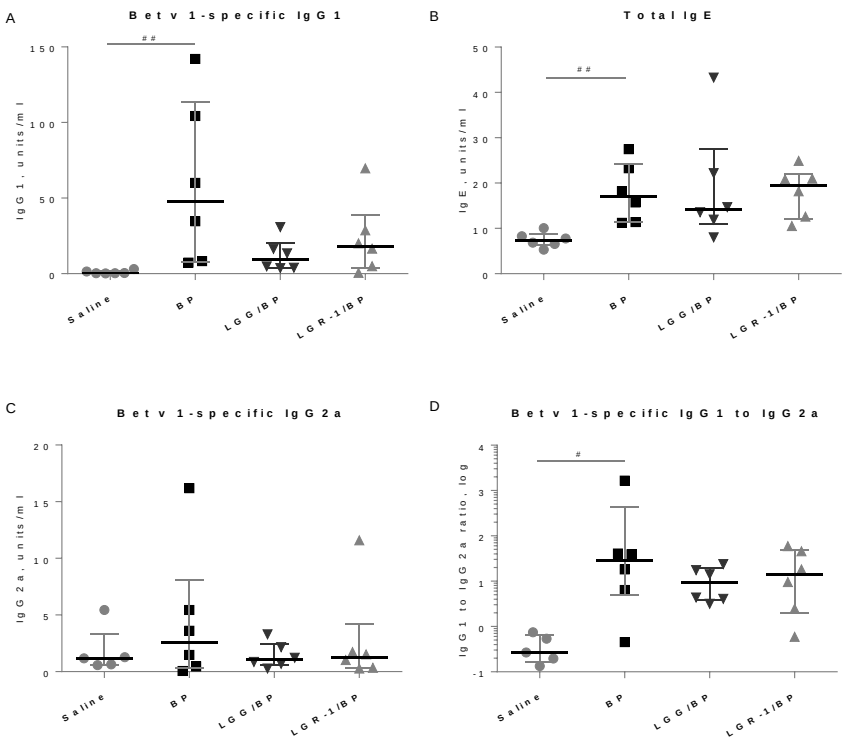

Figure 3. Serum total IgE and Bet v 1-specific antibody levels following intranasal preventive treatment with *L. rhamnosus* GG or *L. rhamnosus* GR-1 and exposure to BP. Levels of Bet v 1-specific IgG1 (A), total IgE (B), Bet v 1-specific IgG2a (C) and the ratio of Bet v 1-specific IgG1 to IgG2a (D) in serum.

Saline: no BP exposure, not pretreated; BP: BP-exposed, not pretreated; LGG/BP: BP-exposed, pretreated with wild type *L. rhamnosus* GG; LGR-1/BP: BP-exposed, pretreated with wild type *L. rhamnosus* GR-1. Data are expressed as arbitrary units/ml serum sample and depicted as values for individual mice with medians and interquartile ranges per group. ##$p < 0.01$ compared to the saline group; n = 5-6 mice per group.

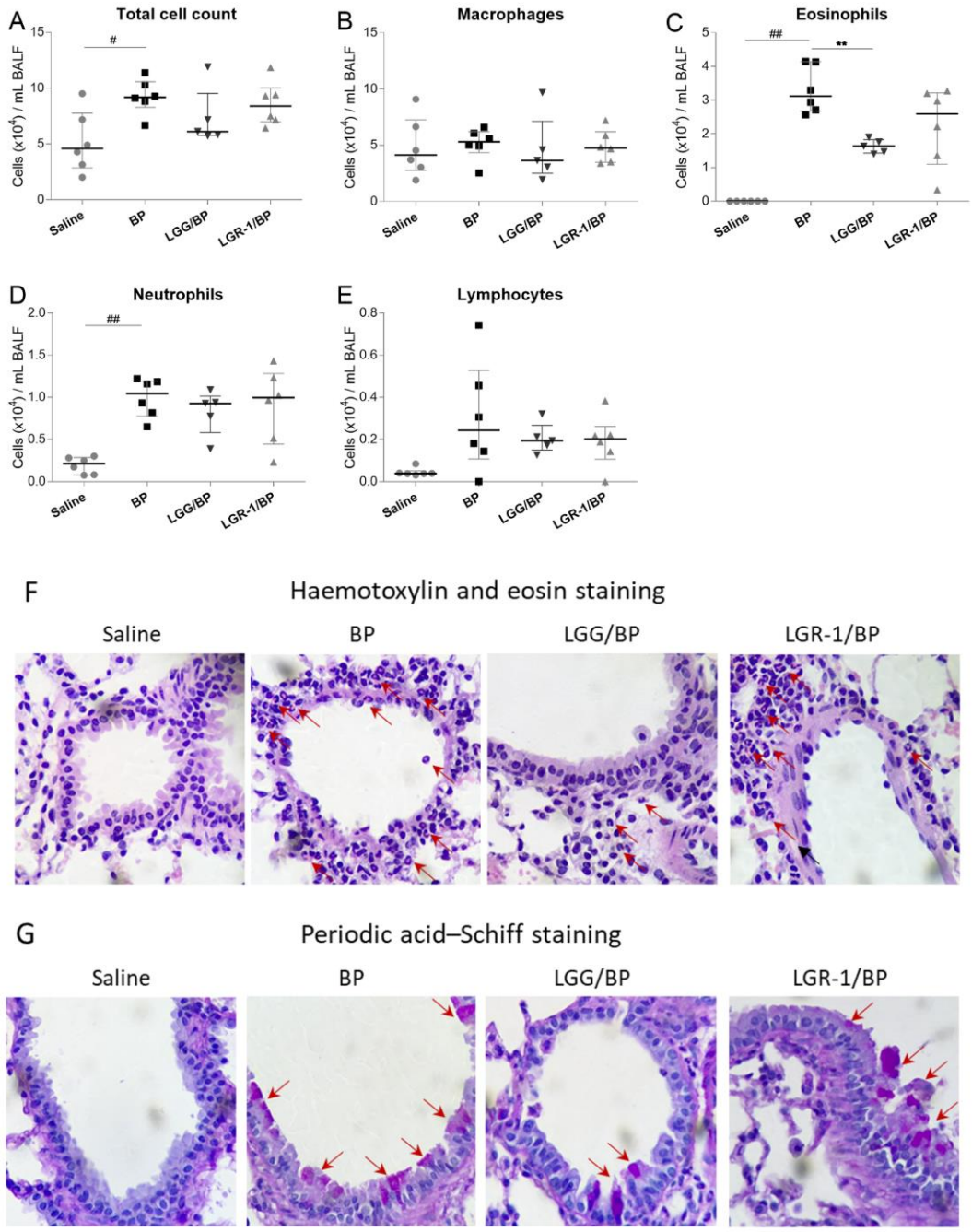

Figure 4. BALF total and differential cell counts following intranasal preventive treatment with *L. rhamnosus* GG or *L. rhamnosus* GR-1 and exposure to BP. Total number of cells (A), macrophages (B), eosinophils (C), neutrophils (D) and lymphocytes (E) per ml BALF. Representative sections of lungs stained with either H&E staining (F) or PAS staining (G) are

shown, with red arrows indicating respectively eosinophilic infiltration (F) or PAS-positive cells (G).

Saline: no BP exposure, not pretreated; BP: BP-exposed, not pretreated; LGG/BP: BP-exposed, pretreated with wild type *L. rhamnosus* GG; LGR-1/BP: BP-exposed, pretreated with wild type *L. rhamnosus* GR-1. Data are depicted as values for individual mice and medians with interquartile ranges per group. #p < 0.05 and ##p < 0.01 compared to the saline group; **p < 0.01 compared to the not-pretreated BP-exposed group; n = 5-6 mice per group.

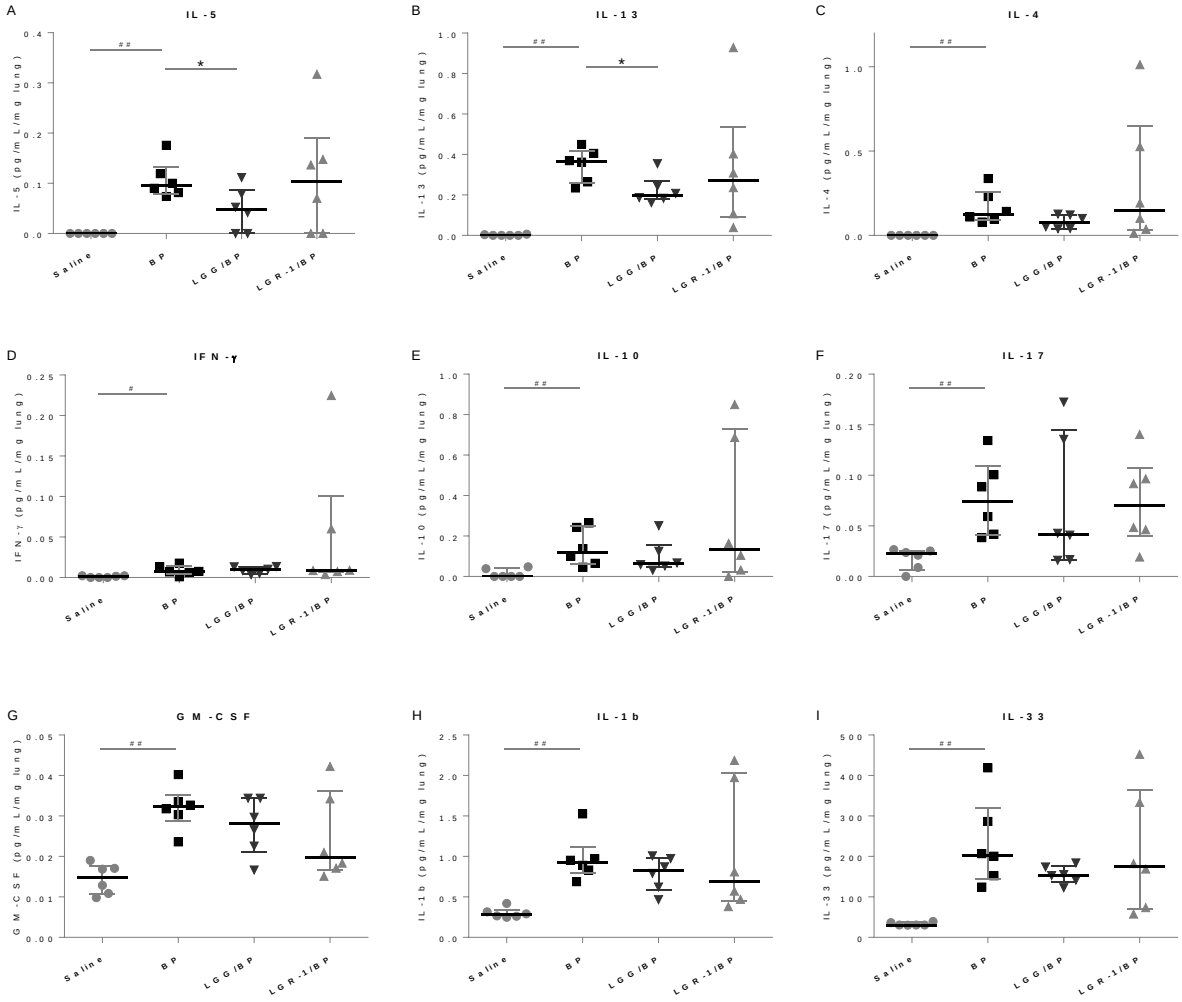

Figure 5. Cytokine levels in lung homogenates following intranasal preventive treatment with *L. rhamnosus* GG or *L. rhamnosus* GR-1 and exposure to BP. Th2-associated cytokines IL-5 (A), IL-13 (B), IL-4 (C), Th1-associated cytokine IFN-γ (D), Treg-associated cytokine IL-10 (E), Th17-associated cytokine IL-17 (F), granulocyte-macrophage colony-stimulating factor (GM-CSF) (G), IL-1β (H) and IL-33 (I).

Saline: no BP exposure, not pretreated; BP: BP-exposed, not pretreated; LGG/BP: BP-exposed, pretreated with wild type *L. rhamnosus* GG; LGR-1/BP: BP-exposed, pretreated with wild type *L. rhamnosus* GR-1. Data are depicted as values for individual mice and medians with interquartile ranges per group. #p < 0.05 and ##p < 0.01 compared to saline group; *p < 0.05 compared to not-pretreated BP-exposed group; n = 6 mice per group.

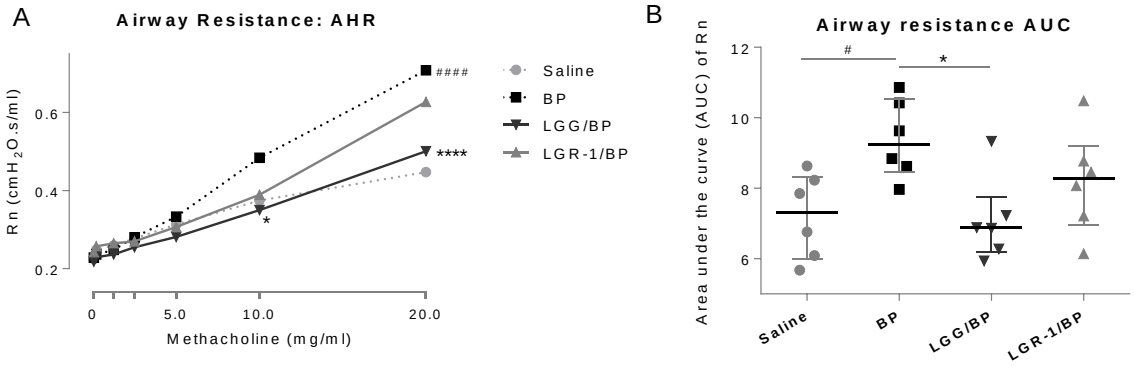

Figure 6. Airway hyperreactivity (AHR) measurements following intranasal preventive treatment with *L. rhamnosus* GG or *L. rhamnosus* GR-1 and exposure to BP. (A) AHR: Airway resistance (Rn) increase in response to methacholine inhalation (0-20 mg/ml) expressed as group means and (B) Rn area under the curve (AUC) for individual mice with group medians.

Saline: no BP exposure, not pretreated; BP: BP-exposed, not pretreated; LGG/BP: BP-exposed, pretreated with wild type *L. rhamnosus* GG; LGR-1/BP: BP-exposed, pretreated with wild type *L.*

rhamnosus GR-1. #p < 0.05 and ####p < 0.0001 compared to the saline group; *p < 0.05 and ****p < 0.0001 compared to the not-pretreated BP-exposed group; n = 6 mice per group.

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Figure 6. Airway hyperreactivity (AHR) measurements following intranasal preventive treatment with L. rhamnosus GG or L. rhamnosus GR-1 and exposure to BP. (A) AHR: Airway resistance (Rn) increase in response to methacholine inhalation (0-20 mg/ml) expressed as group means and (B) Rn area under the curve (AUC) for individual mice with group medians. Saline: no BP exposure, not pretreated; BP: BP-exposed, not pretreated; LGG/BP: BP-exposed, pretreated with wild type L. rhamnosus GG; LGR-1/BP: BP-exposed, pretreated with wild type L. rhamnosus GR-1. #p < 0.05 and ####p < 0.0001 compared to the saline group; *p < 0.05 and ****p < 0.0001 compared to the not-pretreated BP-exposed group; n = 6 mice per group.