

Effect of *Lactobacillus rhamnosus* GG and vitamin D supplementation on the immunologic effectiveness of grass-specific sublingual immunotherapy in children with allergy

Joanna Jerzynska, M.D., Ph.D.,¹ Włodzimierz Stelmach, M.D., Ph.D.,² Joanna Balcerak, M.D.,¹ Katarzyna Woicka-Kolejwa, M.D.,¹ Blazej Rychlik, Ph.D.,³ Andrzej Blauz, Ph.D.,³ Marcin Wachulec, Ph.D.,³ Piotr Stelmach, M.D.,⁴ Pawel Majak, M.D., Ph.D.,⁵ and Iwona Stelmach, M.D., Ph.D.¹

ABSTRACT

Background: An important issue in sublingual immunotherapy (SLIT) is how to improve efficacy.

Objective: To compare the clinical and immunologic efficacy of SLIT given alone and, to enhance clinical efficacy, given with probiotic or vitamin D supplementation.

Methods: One hundred children, ages 5–12 years, sensitive to grass pollen, with allergic rhinitis participated in a 5-month prospective, randomized, double-blind, placebo-controlled trial. Children received 5-grass SLIT 300 IR tablets with either vitamin D 1000 IU daily supplementation, probiotic, or placebo. The control group included children with allergy who did not qualify for immunotherapy. Primary end points included a symptom-medication score, lung function, and exhaled nitric oxide concentration. The secondary end point was the immunologic efficacy measured by the following: CD4⁺CD25⁺Foxp3⁺ (forkhead box P3) cells, Toll-like receptor (TLR) 4, interleukin (IL) 1, IL-6, tumor necrosis factor, IL-10, and transforming growth factor β -1 levels in cell culture supernatants.

Results: Reduction in the symptom-medication score and improvement in lung function as well as a significant increase in the percentage of CD4⁺CD25⁺Foxp3⁺ in children who received SLIT in all the groups were observed compared with control group. In the SLIT-probiotic group, between-group analysis showed significantly higher CD4⁺CD25⁺Foxp3⁺ induction compared with the SLIT group and higher reduction in the percentage of TLR-positive cell group compared with the SLIT-vitamin D group (Fig. 1). An increase in CD4⁺CD25⁺Foxp3⁺ induction, reduction in TLR-positive cells recruitment and an increase in transforming growth factor β -1 production were independently associated with a better clinical effect of SLIT in children.

Conclusions: We demonstrated the clinical and immunologic effect of probiotic and vitamin D supplementation on SLIT. Probiotic supplementation showed better clinical and immunologic response in children with allergic rhinitis.

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Allergen immunotherapy (AIT) is the only treatment of allergy that modifies the immune response with the potential to change the natural history of allergic diseases in many children who are atopic. Mucosal immunotherapy is one advantageous choice because of its noninjection routes of administration and lower incidence of systemic reactions.^{1,2} Sublin-

gual immunotherapy (SLIT) is safe, effective, and well tolerated in children with allergic rhinitis, including very young patients.³ After SLIT, allergic disease-promoting T-helper (Th) cell type 2 responses shift to a Th1 inflammatory response, and interleukin (IL) 10 and transforming growth factor (TGF) β production by regulatory T (Treg) cells and tolerogenic dendritic cells suppress allergen-specific T-cell responses in the oral immune system.⁴ SLIT also decreases Toll-like receptor (TLR) induced IL-6 secretion by myeloid dendritic cells.⁵ An important issue in mucosal immunotherapy is how to improve efficacy. Adjuvants to be included in products for allergen-specific immunotherapy should enhance Th1 and CD4⁺ Treg cell responses. The emerging class of potential adjuvants for the sublingual routes include probiotics and vitamin D.

Probiotics are dietary supplements that contain beneficial bacteria, e.g., *Lactobacillus rhamnosus* GG and are effective in the modulation of local immune response. Oral administration of lactic acid bacteria reduces

From the ¹Department of Pediatrics and Allergy, N Copernicus Hospital, Medical University of Lodz, Lodz, Poland, ²Department of Social and Preventive Medicine, Medical University of Lodz, Lodz, Poland, ³Department of Molecular Biophysics, University of Lodz, Lodz, Poland, ⁴Medical University of Lodz, Poland, and ⁵Department of Internal Medicine, Asthma and Allergy, Medical University of Lodz, Lodz, Poland

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Address correspondence to I. Stelmach, M.D., Department of Pediatrics and Allergy, N. Copernicus Memorial Hospital, Medical University of Lodz, Poland, 71 Al. Pilsudskiego, 90–329, Lodz, Poland

E-mail address: alergol@kopernik.lodz.pl

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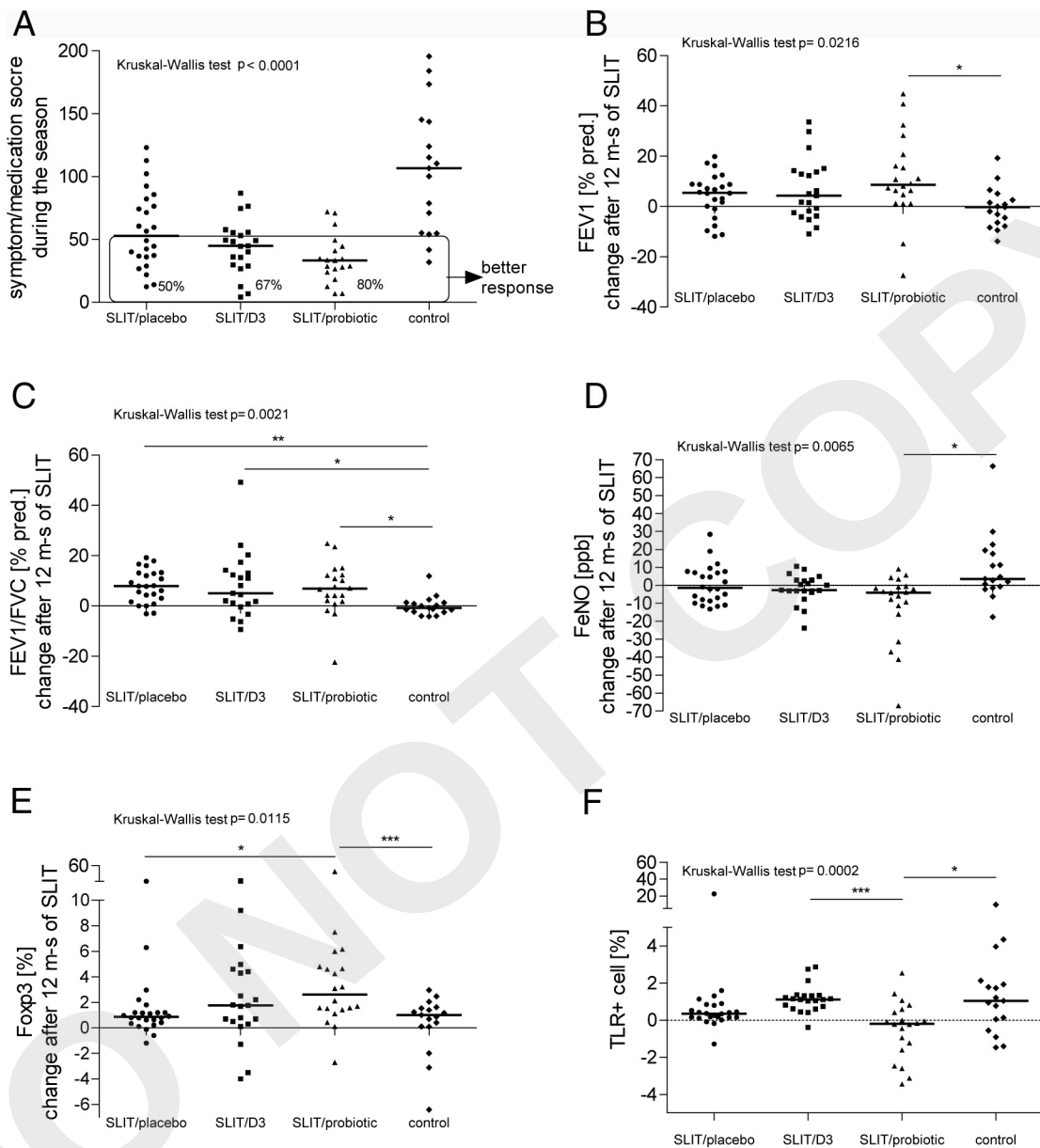


Figure 1. Changes in symptom-medication score, lung function (FEV_1 , $FEV_1\%VC$), FeNO level, $CD4^+CD25^+Foxp3^+$ induction, percentage of TLR-positive cell in children from the SLIT-probiotic group compared with children from the SLIT-vitamin D, the SLIT-placebo group, and the control group; between-group analysis. FEV_1 = forced expiratory volume in 1 second; FVC = forced vital capacity; FeNO = fractional exhaled nitric oxide; Foxp3 = forkhead box P3; TLR = toll-like receptor; SLIT = sublingual immunotherapy. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

atopic dermatitis in children with a positive family history of type I allergy.⁶ There is insufficient but promising evidence to recommend the addition of probiotics to foods for prevention and treatment of allergic diseases, and atopic dermatitis clinical improvement, especially in allergic rhinitis and atopic eczema, has been reported.⁷ Probiotics are able to induce an antigenic stimulation that leads to release of IL-12 by antigen-presenting cells, which promotes the maturation of the immune system and the Th1 response. Probiotics have anti-inflammatory properties related to the stim-

ulation of secretory immunoglobulin A synthesis in mucous membranes and the production of cytokines.

Probiotics have been shown to be efficacious in reducing allergic inflammation in murine models. Bacteria selected specifically for their capacity to induce a strong production of IL-10 and IL-12 by mucosal dendritic cells, such as *Lactobacillus*, were shown to enhance tolerance after SLIT in mice with induced asthma to ovalbumin, at least in part by strengthening allergen-specific Th1 and Treg responses.^{5,8} Recent studies showed that oral administration of a certain

probiotic exerts therapeutic effects in the treatment of allergic respiratory diseases, such as asthma and rhinitis as well as in the primary prevention of allergic disease.^{9–13} Such observations indicated that selected probiotics could be used as adjuvants for vaccines. As of today, probiotics have not yet been tested in combination with allergens in patients with allergy.

Vitamin D deficiency has been proposed as a potential contributing factor in patients with allergic diseases.^{14–16} Vitamin D promotes different tolerogenic processes of the immune system, which leads to the inhibition of both Th1 and Th2 responses in a dose-dependent manner.¹⁷ Vitamin D enhances IL-10 production by CD4⁺ T cells and enhances SLIT efficacy in a murine asthma model.⁵ Recently, Heine *et al.*¹⁸ showed that 25-hydroxyvitamin D₃ promotes the long-term effect of specific immunotherapy in a murine allergy model; their findings were paralleled by reduced Th2 cytokine expression in the lungs. They observed that vitamin D deficiency promotes the development of type I sensitization and correction of its serum concentrations enhances the benefit of specific immunotherapy.

Some favorable clinical (lower total symptom score) and immunologic (higher induction of IL-10 and transforming growth factor [TGF] β 1) outcomes of vitamin D as an adjunct to subcutaneous AIT in children with asthma sensitized to house-dust mite has also been shown.¹⁹ Also, a serum level of 25-hydroxyvitamin D of >30 ng/mL was shown to facilitate the optimal effect of AIT, and lower levels of vitamin D were associated with reduced lung function and increased reactivity to exercise.^{20,21} Natural activity of vitamin D seems to be highly attractive in the context of the mechanism and clinical effect of subcutaneous immunotherapy; however, its role in SLIT is still undefined.

The aim of the present study was to compare the clinical and immunologic effectiveness of SLIT given alone and, to enhance clinical efficacy, given with *L. rhamnosus* GG or vitamin D supplementation in children with allergic rhinitis. Grass-pollen SLIT combined with probiotic or vitamin D or placebo were given pre-coseasonally (starting at least 8 weeks before the pollen season and continuing until the end of the pollen season) in children allergic to grass pollen.

METHODS

Subjects

We studied 100 children, ages 5–12 years, who lived in the same geographic location and who were sensitive only to grass pollen, with a clinical diagnosis of grass-related moderate-to-severe rhinoconjunctivitis (according to Allergic Rhinitis and its Impact on Asthma 2014 recommendation²²), with a duration of at least 1 year before the first study visit; 12 patients had

concomitant asthma (intermittent or mild-to-moderate persistent asthma according to the Global Initiative for Asthma 2014 criteria²³). All the subjects with an asthma diagnosis were treated by their physicians according to Global Initiative for Asthma guidelines²³ for a minimum of 6 months, and their asthma was well controlled.

The subjects allergic to perennial allergens or with severe or unstable asthma were excluded from the study. Exclusion criteria also were active upper respiratory tract infection within 1 month before the first study visit and the known contraindications of SLIT according to the European Academy of Allergy and Clinical Immunology.²⁴ The patients with any previous immunotherapy or those who had clinically significant pulmonary, hematologic, hepatic, gastrointestinal, renal, endocrine, neuronal, cardiovascular, and/or psychiatric diseases or malignancies, which could either put them at risk during their participation in the study or might have influenced the results of the study (as judged by the investigator, P. S.), were also excluded. Also, excluded medications were systemic corticosteroids or immune suppressive drugs as well as supplementary vitamin D (or any vitamin supplements) used within 4 weeks before the study. The study was approved by the medical ethics committee of the Medical University of Lodz. All the parents or guardians of the patients gave their oral and written consent for the evaluation of data from medical documentation of their children.

Study Design

We performed a 5-month (between March 2014 and July 2014), prospective, randomized, double-blind, placebo-controlled study of children to compare the efficacy of pre-coseasonal SLIT combined with a probiotic (*L. rhamnosus* GG) or vitamin D 1000 IU daily or placebo supplementation with the control group (no SLIT) in children allergic to grass pollen. All suitable subjects were randomized according to a computer-generated allocation schedule into the three treatment arms: the SLIT group combined with probiotic supplementation (SLIT-probiotic, $n = 25$), the SLIT group combined with vitamin D 1000 IU supplementation (SLIT-vitamin D, $n = 25$), and the SLIT group with placebo (SLIT-placebo, $n = 25$). The control group included children allergic to grass pollen who were not qualified for AIT (control, $n = 25$). All the subjects were recruited from our specialty clinic setting.

The primary end points included clinical efficacy based on the reduction in symptom- medication score and in symptoms (nasal, ocular, asthma) and medication use separately as well as lung function, exhaled nitric oxide concentration, and methacholine bronchial provocation test. The safety was estimated based on

adverse symptoms. The secondary end point of the study was the immunologic efficacy. The immunologic parameters included the measurement of following: (a) surface molecules (CD4⁺CD25⁺Foxp3⁺ cells, TLR4), IL-1, IL-6, TNF, direct evidence of TLR activation, and (b) IL-10 and TGF- β 1 levels in cell culture supernatants of grass-stimulated peripheral blood mononuclear cells (PBMC).

The clinical and immunologic efficacy was evaluated for 5 months during SLIT. The patients attended one prestudy and two study visits at the clinic. During the prestudy visit, before March 2014, all the participants were informed about the purpose of the study, were told how to score asthma and/or allergic symptoms, and underwent skin testing with standard allergens. The patients were classified as being atopic based on history and positive skin-prick testing results. In patients unable to undergo skin testing, a serum-specific immunoglobulin E for standard allergens was used. During the prestudy visit, a diet with similar vitamin D intake was recommended for all the patients. All the children remained in the same geographic area during the study, so that they all were under the same sunlight exposure. During the first study visit (first week of March 2014), the patients received the first 5-grass SLIT tablet (Oralair; Stallergenes, Antony, France) (100 IR) combined with either probiotic supplementation or vitamin D supplementation, or placebo; the control group did not receive any treatment. The second visit took place 5 months after the first visit, at the end of the grass-pollen season. During each study visit, blood samples were obtained from all the patients to assess immunologic parameters. Lung function, exhaled nitric oxide concentration, and methacholine bronchial provocation test were measured, and the patients' diaries were also evaluated. Both study visits were scheduled in such a way that blood sampling in individual patients always took place at the same time and on the same day of each week.

Treatment

All three SLIT groups were treated once daily with a 5-grass SLIT tablet as a standardized extract of 5 grass pollens (*Dactylis glomerata*, *Anthoxanthum odoratum*, *Lolium perenne*, *Poa pratensis*, *Phleum pratense*) and either probiotic that contained *L. rhamnosus* GG (Dicoflor 30, which contained 3 billion live cultures of bacteria in 1 dose; Vitis Pharma, Warsaw, Poland), or vitamin D that contained 1000 IU (25 μ g of cholecalciferol, Vigan-toletten 1000; Merck KGaA, Darmstadt, Germany) or an identically looking placebo once daily. To blind studied drugs, probiotic, vitamin D, and placebo were prepared in wafers with 0.3 mg of lactose by the hospital pharmacy. The first SLIT dose (100 IR) was administered at the physician's office due to the manu-

facturer's recommendations, together with probiotic, vitamin D, or placebo. All subsequent doses of 5-grass SLIT tablets were self-administered by children or their guardians at home (together with probiotic or vitamin D, or placebo) as follows: the second dose of 200 IR, and the rest were consecutive daily doses of 300 IR. Every morning before breakfast, all the patients received a wafer with probiotic or vitamin D, or placebo, followed by administration of a 5-grass SLIT tablet. Each wafer or tablet was placed in an empty mouth, under the tongue, and held until complete dissolution (at least 1 minute), and then, swallowed. Children were treated at least 8 weeks before the grass-pollen season and through the entire season, for a total of at least 20 weeks after randomization. To assess adherence to the treatment, all the subjects treated with SLIT were asked to bring all empty packages to the last visit.

The onset of the pollen season was defined as the first of 3 consecutive days with grass pollen counts of at least 10 grains/m³ and its end was the last day before 3 consecutive days with a grass-pollen count of <10 grains/m³. The pollen count was performed throughout the study for the region of interest.²⁵ During the pollen season, all the children received, at baseline, the standard treatments for the allergic rhinoconjunctivitis: systemic antihistamines. Other permissible treatments included nasal steroids, local antihistamines, or cromones for eyes; inhaled corticosteroids in minimal doses necessary to control the symptoms; short-acting β 2-agonist as a quick-reliever; and the standard treatments of the infections and exacerbations of asthma.

Assessment of the Clinical Effects of SLIT

Symptom-Medication Score. The combined symptom-medication score was calculated as a sum of the symptom score and the medication score recorded by the patients on a daily basis during the season from March to July. The symptom score was recorded with the use of a point score system for the most common symptoms of rhinitis (rhinorrhea, sneezing, itching, and nasal congestion), conjunctivitis (ocular pruritus, watery eyes, and itching), and respiratory disorders (cough, wheeze, and dyspnea). In addition, the amount of as-needed β 2-agonist (salbutamol) was recorded daily as the number of puffs: 0, no use; 1, used once a day; 2, used 2–3 times a day; 3, used >3 times a day. A symptom score that ranged from 0 to 3 was used for each symptom: 0, no symptoms; 1, mild symptoms (symptoms clearly present but easily tolerated); 2, moderate symptoms (definite awareness of bothersome but tolerable symptoms); 3, severe symptoms (symptoms hard to tolerate and that cause interference with daily activities and/or sleeping), and ranged from a score of 0 (minimum) to 27 (maximum). The mini-

imum combined symptom-medication score for each day was 0 (no symptoms during the day, no symptoms at night, no use of medication), and the maximum score was 30 (severe symptoms during the day and at night, and the use of rescue medication of >3 times daily).²⁶ The points were multiplied by the number of days during the season from March to July. The total score for symptom and medication were adjusted to 1000 pollen grains/m³ during grass-pollen season. The adjustment was calculated according to the following rule: (total of seasonal score)/(cumulative concentration of grass pollen during the season) × 1000.

Lung Function Test. Pulmonary function testing was performed with a Master Screen unit (Erich Jaeger GmbH-Hochberg, Hochberg, Germany). Predicted values for all lung function variables were based on a previous study of healthy controls provided by the manufacturer of the lung function test equipment.²⁷ Flow-volume curves were performed according to American Thoracic Society standards.²⁷ The highest of three successful measurements was used. The results were expressed as the percentage of a predicted value. We evaluated the following lung function parameters: forced expiratory volume in 1 second (FEV₁), and the proportion of a person's vital capacity that they are able to expire in the first second of forced expiration (FEV₁%VC), also called the Tiffeneau-Pinelli index.

Nitric Oxide Measurement. The nitric oxide (NO) measurements were performed according to European Respiratory Society/American Thoracic Society recommendations²⁸ with a chemiluminescence analyzer (model 280i nitric oxide analyzer; Sievers, Boulder, CO) and defined in parts per billion. The analyzer provides an online continuous measurement of NO in a single exhalation with a detection range of 0.1–500 ppb. Environmental NO was measured before and after each test, and never exceeded 5 ppb. All the subjects were studied with them in the sitting position, without wearing a nose clip. The subjects exhaled at a constant flow rate (50 mL/s) from total lung capacity to residual volume without breath-holding. They maintained a constant mouth pressure (17 cm H₂O) by monitoring a visual display to eliminate contamination from nasal NO. Dead space and nasal NO (which are reflected by the NO concentration peak during exhalation), and NO from the lower respiratory tract (determined by the plateau value after the peak) were recorded automatically by using the manufacturer's software. Three fractional exhaled nitric oxide (FeNO) measurements of the plateau phase were obtained, with <10% variation. The mean value of three successive, reproducible recordings was retained for statistical analysis.

Methacholine Bronchial Provocation Test. The methacholine bronchial provocation test was performed at the beginning and at the end of the study, before and after immunotherapy (March and August 2014) by using the dosimeter technique APS Pro (Erich Jaeger) with controlled tidal breathing. After adding physiologic diluent (0.9% NaCl) to a nebulizer, methacholine was delivered in four cumulative doses: 0.015 mg, 0.045 mg, 0.18 mg, and 0.72 mg. The methacholine bronchial provocation test was continued with 2-minute intervals between the inhalations until a fall in the FEV₁ of ≥20% was obtained. PD₂₀ (provocation dose causing a 20% decline in FEV₁) was calculated by linear interpolation on a log-dose-response curve.²⁹

Vitamin D Measurement. Serum 25-hydroxvitamin D₃ was measured by radioimmunoassay (Biosource, Camarillo, CA) according to the manufacturer's instructions. Calcium and phosphorus were measured by standard methods.

Assessment of the Immunologic Effects of SLIT

One sample of PBMC was isolated from whole blood by density gradient centrifugation by using an Histopaque-1077 (Sigma-Aldrich, Highland, IL). Surface expression of TLR4 (eBioscience, San Diego, CA) was determined by using a flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and was analyzed by using CellQuest software (Becton Dickinson). CD14⁺ cells only were used for the analysis of TLR.

Another sample of PBMCs was isolated from whole blood by density gradient centrifugation by using Histopaque-1077 (Sigma-Aldrich) and was cultured in standard mammalian cell culture conditions (37°C, 5% carbon dioxide, 80% relative humidity) in the presence of grass allergen. Surface molecule CD4⁺CD25⁺Foxp3⁺ expression on cultured PBMCs was estimated by flow cytometry (LSRII instrument; BD Biosciences, San Jose, CA) by using appropriate fluorophore-conjugated monoclonal antibodies. The IL-1, IL-6, IL-10, and TGF-β1, TNF levels in cell culture supernatants were determined by using enzyme-linked immunosorbent assay-based specific tests (R&D, Minneapolis, MN) according to the manufacturer's instructions.

Safety

During each visit, an interview and a physical examination were performed. The physician assessed changes in asthma scores, drug use, and the lung function test in comparison with the baseline. All adverse events were recorded.

Statistical Analysis

Analysis was planned in three steps. First, within-group comparisons were made by using the *t*-test with

Table 1 Clinical characteristics of all study participants; symptom-medication score evaluated 5 months during SLIT, lung function, FeNO level, and vitamin D serum level measured at baseline

	SLIT-Placebo Group (N = 24)	SLIT-Vitamin D Group (N = 21)	SLIT-Probiotic Group (N = 20)	Control Group (N = 17)
Symptom-medication score, median (quartile range)*	52.8 (36.4–79.4)	44.9 (29.7–55.4)	33.6 (25.3–47.0)	106 (55–143.5)
FEV ₁ , mean (SD), % pred.	104.3 ± 10.7	104.4 ± 15.0	105.4 ± 15.6	103.7 ± 10.3
FEV ₁ %VC, mean (SD), % pred.	102.9 ± 5.2	104.3 ± 8.6	101.7 ± 7.5	100.8 ± 6.4
FeNO, median (quartile range), ppb	23.1 (16.3–28.8)	19.3 (13.4–21.9)	20.3 (9.1–31.5)	15.6 (9.2–21.8)
Vitamin D ₃ serum level, median (quartile range), pg/mL	39.0 (30.6–49.3)	41.2 (30.2–66.2)	67.7 (39.3–89.9)	82.9 (36.7–87.1)

SLIT = sublingual immunotherapy; FeNO = fractional exhaled nitric oxide; FEV₁ = forced expiratory volume in 1 second; SD = standard deviation; pred. = predicted; FVC = forced vital capacity.

*Adjusted for natural allergen exposure (points).

Bonferroni correction. This analysis was preceded by the Levene test of equality of error variances and after log-transformation when appropriate. Second, changes before and after in each variable were compared among the groups by using Kruskal-Wallis test, followed by the *post hoc* Dunnett test for multiple comparisons adjustment. Also, logistic regression was used to point out the best predictors of SLIT effectiveness. First, logistic regression was used to assess the relationship between dependent variables and each of the independent variables. A stepwise forward procedure was then used to select variables. Predictors with *p* levels of at least 0.1 estimated in univariate models were included in multivariate regression analyses. All of the statistical analyses were performed by using Statistica for Windows version 8.0 (StatSoft Inc, Tulsa, OK). The null hypothesis was rejected if *p* < 0.05.

RESULTS

Subjects

Eighty-two patients completed the study. Baseline characteristics are shown in Table 1. There was no difference in asthma prevalence among the groups. From among them, 20 study participants received SLIT with probiotic doses, whereas 24 children received SLIT and placebo, 21 study participants received SLIT with vitamin D, and 17 study participants were assigned to a control group; all exclusions were due to poor adherence. Significant differences in the vitamin D serum level were observed among the groups at baseline; patients from SLIT-probiotic and control groups compared with other groups had higher vitamin D serum levels before the intervention (Table 1). Other clinical measures were comparable among the study groups (Table 1). There were no differences in dose of allergy medications among the groups.

Grass-Pollen Season

The 2014 grass-pollen season for the region of interest had a mean pollen count of ~53 grains/m³ over the entire season, which lasted 77 days. The season was characterized by few pollen peaks, with an average peak season count of 144 grains/m³. Diaries were available for evaluations for the 2014 true grass-pollen season (pollen counts per m³ per 24 hours).

Effect of SLIT

In children from the SLIT-placebo group, significant improvement in lung function measured by the FEV₁%VC ratio was seen and an increase in CD4⁺CD25⁺Foxp3⁺ cells induction and IL-10 production from stimulated PBMC culture were close to reaching the level of significance after Bonferroni correction (Table 2). Between-groups analysis did not show any significant differences between SLIT-placebo and the control group, except the symptom-medication score and FEV₁%VC of predicted value (Fig. 1).

Effect of Vitamin D Supplementation Combined with SLIT

Within-group analysis revealed significant improvement in lung function measured by FEV₁%VC and the increased percentage of CD4⁺CD25⁺Foxp3⁺ in children given supplements of vitamin D during pre-co-seasonal SLIT (Table 2). Also, a significant increase in TLR-positive cells was observed in these children (Table 2). These changes were irrespective of vitamin D serum level. The symptom-medication score was lower in children from the SLIT-vitamin D group compared with children in the control group (Fig. 1). Between-groups analysis did not show any significant differences between the SLIT-vitamin D group and the other active groups.

Table 2 Before and after comparisons of study end points

	Visit 1, mean $\pm$ SE	Visit 2, mean $\pm$ SE	<i>p</i> Level with Bonferroni Correction
SLIT -placebo group			
IL-10, pg/mL*	0.39 $\pm$ 0.12	0.54 $\pm$ 0.17	0.4641
IL-10, pg/mL#	0.65 $\pm$ 0.14	1.05 $\pm$ 0.16	0.0626
TGF, pg/mL*	569.6 $\pm$ 60.1	492.6 $\pm$ 62.4	0.3792
TGF, pg/mL#	784.5 $\pm$ 78.8	911.5 $\pm$ 112.8	0.3611
CD4 ⁺ CD25 ⁺ FoxP3 ⁺ , %	7.11 $\pm$ 0.46	8.54 $\pm$ 0.61	0.0685
IL-1-positive cells, %	0.43 $\pm$ 0.04	0.37 $\pm$ 0.09	0.5957
TNF-positive cells, %	0.77 $\pm$ 0.08	0.81 $\pm$ 0.12	0.7950
IL-6-positive cells, %	1.30 $\pm$ 0.14	1.06 $\pm$ 0.12	0.1964
TLR-positive cells, %	1.47 $\pm$ 0.37	2.77 $\pm$ 1.04	0.2464
FEV ₁ , % pred.	104.3 $\pm$ 2.2	108.7 $\pm$ 1.9	0.1422
FEV ₁ %VC, % pred.	102.9 $\pm$ 1.1	110.6 $\pm$ 1.9	0.0009
FeNO, ppb	23.85 $\pm$ 1.87	24.35 $\pm$ 2.17	0.8610
Vitamin D serum level, pg/mL	43.30 $\pm$ 3.60	43.88 $\pm$ 3.55	0.9086
SLIT-vitamin D ₃ group			
IL-10, pg/mL*	0.66 $\pm$ 0.21	0.52 $\pm$ 0.14	0.5813
IL-10, pg/mL#	1.09 $\pm$ 0.31	1.28 $\pm$ 0.31	0.6529
TGF, pg/mL*	711.8 $\pm$ 70.1	560.3 $\pm$ 80.7	0.1640
TGF, pg/mL#	806.3 $\pm$ 74.4	927.8 $\pm$ 119.9	0.3945
CD4 ⁺ CD25 ⁺ FoxP3 ⁺ , %	7.56 $\pm$ 0.56	9.94 $\pm$ 0.78	0.0183
IL-1-positive cells, %	0.45 $\pm$ 0.04	0.35 $\pm$ 0.07	0.2113
TNF-positive cells, %	0.80 $\pm$ 0.09	0.64 $\pm$ 0.10	0.2324
IL-6 positive cells, %	1.19 $\pm$ 0.16	1.17 $\pm$ 0.13	0.9136
TLR-positive cells, %	1.07 $\pm$ 0.35	2.22 $\pm$ 0.38	0.0319
FEV ₁ , % pred.	104.4 $\pm$ 3.3	110.9 $\pm$ 3.1	0.1555
FEV ₁ %VC, % pred.	104.3 $\pm$ 1.9	112.5 $\pm$ 1.6	0.0019
FeNO, ppb	21.07 $\pm$ 3.22	19.17 $\pm$ 2.58	0.6487
Vitamin D serum level, pg/mL	48.84 $\pm$ 5.31	51.55 $\pm$ 5.18	0.7169
SLIT-probiotic group			
IL-10, pg/mL*	3.31 $\pm$ 2.70	0.98 $\pm$ 0.19	0.3963
IL-10, pg/mL#	3.10 $\pm$ 1.77	2.01 $\pm$ 0.68	0.5725
TGF, pg/mL*	688.5 $\pm$ 79.2	576.5 $\pm$ 74.9	0.3109
TGF, pg/mL#	775.7 $\pm$ 95.3	910.0 $\pm$ 137.0	0.4259
CD4 ⁺ CD25 ⁺ FoxP3 ⁺ , %	7.91 $\pm$ 0.60	12.65 $\pm$ 1.99	0.0281
IL-1-positive cells, %	0.56 $\pm$ 0.05	0.48 $\pm$ 0.08	0.4057
TNF-positive cells, %	1.00 $\pm$ 0.08	0.92 $\pm$ 0.12	0.5982
IL-6-positive cells, %	1.51 $\pm$ 0.15	1.28 $\pm$ 0.22	0.3962
TLR-positive cells, %	1.44 $\pm$ 0.31	0.95 $\pm$ 0.21	0.1964
FEV ₁ , % pred.	105.4 $\pm$ 3.5	116.6 $\pm$ 3.3	0.0256
FEV ₁ %VC, % pred.	101.7 $\pm$ 1.7	108.4 $\pm$ 1.5	0.0043
FeNO, ppb	24.85 $\pm$ 4.38	13.79 $\pm$ 2.17	0.0294
Vitamin D serum level, pg/mL	65.26 $\pm$ 6.20	85.64 $\pm$ 6.65	0.0308
Control group			
IL-10, pg/mL*	1.50 $\pm$ 0.71	0.66 $\pm$ 0.17	0.2820
IL-10, pg/mL#	1.48 $\pm$ 0.37	0.75 $\pm$ 0.15	0.0909
TGF, pg/mL*	712.7 $\pm$ 123.2	903.8 $\pm$ 181.6	0.4216
TGF, pg/mL#	1009.0 $\pm$ 198.2	1029.1 $\pm$ 177.9	0.9881
CD4 ⁺ CD25 ⁺ FoxP3 ⁺ , %	7.84 $\pm$ 0.55	8.19 $\pm$ 0.62	0.8306
IL-1-positive cells, %	0.58 $\pm$ 0.08	0.57 $\pm$ 0.13	0.9174
TNF-positive cells, %	0.88 $\pm$ 0.11	0.96 $\pm$ 0.25	0.8620
IL-6-positive cells, %	0.98 $\pm$ 0.11	1.07 $\pm$ 0.29	0.7590
TLR-positive cells, %	1.04 $\pm$ 0.32	2.55 $\pm$ 0.57	0.0287
FEV ₁ , % pred.	103.7 $\pm$ 2.5	103.2 $\pm$ 2.7	0.9704
FEV ₁ %VC, % pred.	100.8 $\pm$ 1.6	100.8 $\pm$ 1.6	0.8952
FeNO, ppb	19.82 $\pm$ 4.14	29.44 $\pm$ 6.04	0.1881
Vitamin D serum level, pg/mL	67.50 $\pm$ 7.19	101.90 $\pm$ 4.77	0.0005

SE = standard error; SLIT = sublingual immunotherapy; IL = interleukin; TGF = transforming growth factor; Foxp3 = forkhead box P3; TNF = tumor necrosis factor; TLR = Toll-like receptor; FEV₁ = forced expiratory volume in 1 second; pred. = predicted; FVC = forced vital capacity; FeNO = fractional exhaled nitric oxide; PBMC = peripheral blood mononuclear cell. The data in bold represent statistically significant values $p > 0.05$.

*Cytokine concentration in supernatant from nonstimulated PBMC culture.

#Cytokine concentration in supernatant from PBMC culture stimulated with sensitizing allergen.

Effect of *L. rhamnosus* GG Supplementation Combined with SLIT

Within-group analysis revealed significant improvement in FEV₁, FEV₁%VC, reduction in the FeNO level, higher CD4⁺CD25⁺Foxp3⁺ cells induction and an increase in vitamin D serum level in children treated with probiotic during pre-coseasonal SLIT (Table 2). Symptoms and/or medication scores were lower in children from the SLIT-probiotic group compared with children from the placebo control group (Fig. 1). Improvement in FEV₁ was higher in the SLIT-probiotic group compared with the control group. Between-groups analysis showed significantly higher CD4⁺CD25⁺Foxp3⁺ induction in the SLIT-probiotic group compared with the control group and higher reduction in percentage of TLR-positive cell compared with the SLIT-vitamin D group (Fig. 1).

Control Group

In children from the control group, an increase in the vitamin D serum level and increase in percentage of TLR-positive cells were observed during the spring-summer season of natural allergen exposure.

Predictors of a Better Response to SLIT

In the next step, the participants were separated according to the median value of the symptom-medication score for the SLIT-placebo group. The median symptom-medication score served as a reference category that represented the isolated effect of SLIT. Children from all the groups who received SLIT with a lower than median symptom-medication score were defined as having a better response to SLIT. According to this definition, 67% children from the SLIT-vitamin D group and 80% from the SLIT-probiotic group experienced a better response to SLIT.

Also, a better versus worse response to SLIT was defined as a dependent variable and was included in the logistic regression analysis. Multivariate logistic regression analysis showed that an increase in TGF- β 1 production (odds ratio, 116; $p = 0.02$), increase in CD4⁺CD25⁺Foxp3⁺ induction (odds ratio, 102; $p = 0.03$), and reduction in TLR-positive cells recruitment (odds ratio, 0.053; $p = 0.046$) were independently associated with better clinical effect of SLIT in children (Fig. 1). These associations were adjusted for the effect of vitamin D or probiotic because variables that define the group allocation were included in the final model.

Safety Results

All treatments were well tolerated in the patients, with treatment-related adverse reactions that were mild to moderate in severity. Predominantly local reactions in the SLIT groups (versus the control group), such as sublingual itching (48% versus 16.1%), were reported. Other

adverse events possibly not associated with SLIT treatment (versus the control group) were the following: headache, 4.9% versus 4%; and stomachache, 3.1% versus 5.2%. There were no cases of termination of the study due to adverse events in any of the groups. All the subjects brought the empty packages of drugs to the last visit according to the study design. Adherence was the same in both SLIT-probiotic and SLIT-vitamin D protocols.

DISCUSSION

New adjuvants are needed to improve the efficacy of allergy vaccines. To our knowledge, as far as pollen allergens are concerned, there were no immunologic studies that assessed the effects of *L. rhamnosus* GG or vitamin D supplementation on efficacy of SLIT in children. We performed a head-to-head comparison between probiotic and vitamin D adjuvants combined with SLIT and with SLIT alone. In the present study, a pediatric population with grass pollen-related allergic rhinitis with or without asthma was treated with the 300 IR dose of the 5-grass-pollen SLIT tablet, with supplementation of probiotic *L. rhamnosus* GG or vitamin D 1000 IU daily or placebo. Reductions in the symptom-medication score and improvement in lung function in children who received SLIT in all the groups (supplemented with probiotic or vitamin D or placebo) were observed compared with those who received no SLIT treatment (placebo control group); in addition, a reduction in the FeNO level was observed in the SLIT-probiotic group.

A significant increase in the percentage of CD4⁺CD25⁺Foxp3⁺ in children who received SLIT in all the groups was observed. In the SLIT-probiotic group, between-groups analysis showed significantly higher CD4⁺CD25⁺Foxp3⁺ induction compared with the SLIT-placebo group and a higher reduction in the percentage of the TLR-positive cell group compared with the SLIT-vitamin D group. Probiotic supplementation combined with SLIT showed a better immunologic response in children with allergic rhinitis. Increase in CD4⁺CD25⁺Foxp3⁺ induction, reduction in TLR-positive cells recruitment and an increase in TGF- β 1 production were independently associated with better clinical effect of SLIT in children. Interestingly, in children from the control group, an increase in the vitamin D serum level and an increase in the percentage of TLR-positive cell were observed during the spring-summer season of natural allergen exposure.

The oral immune system is composed of tolerogenic dendritic cells that, during SLIT, support the differentiation of Th1 and the induction of IL-10-producing Treg cells. The ultimate goal for the therapy of allergy is to induce immune tolerance, and peripheral T-cell tolerance is crucial for such benefits. Peripheral tolerance to allergens involves multiple suppressive factors, *e.g.*, IL-10 and TGF- β .³⁰ In contrast, breaking of peripheral T-cell toler-

ance to allergens can lead to the development of allergies, and these mechanisms can include activity of TLR 4, and the proinflammatory cytokines IL-1 β and IL-6.³¹ Grass-pollen SLIT was shown to be associated with an increase in sublingual Foxp3 expressing cell numbers.³² In human subjects, CD4⁺CD25⁺Foxp3⁺ (Treg) cells seem to play a major role in inhibiting allergic disorders. In patients who underwent AIT with grass pollen, an increase in IL-10 expression and interferon γ levels has been shown.³³ We studied TGF- β 1 and IL-10-secreting allergen-specific CD4⁺ T cells that resemble Treg cells, to investigate allergen-specific T-cell responses. In children from the SLIT-placebo group with no supplementation at all, a significant increase in TGF- β 1 and IL-10 production was observed. Importantly, a significant increase in the percentage of CD4⁺CD25⁺Foxp3⁺ in children who received SLIT in all the groups was observed. Significantly higher CD4⁺CD25⁺Foxp3⁺ induction and a greater reduction in the percentage of TLR-positive cells were observed in the SLIT-probiotic group compared with the SLIT-vitamin D group. We showed that the effect of SLIT correlated with an early immunologic response measured by TGF- β 1 and IL-10 production and CD4⁺CD25⁺Foxp3⁺ induction of patients after 5 months of SLIT.

It was previously reported that the prophylactic use of a probiotic milk reduced the number and the severity of respiratory infections among children who attended day-care centers.³⁴ It was demonstrated that probiotics stimulate Th1 and Treg immunity, which promotes IL-12, interferon γ , IL-10, and TGF- β synthesis, and downregulates Th2 response, which inhibits IL-4 production in children with allergic rhinitis.³⁵ In other studies, probiotics were found to enhance the function of mucosal epithelial cells shown as decreased permeability of gut mucosa in children who were atopic, to increase mucosal immunoglobulin A production and reduce systemic production of Th2 cytokines after allergen challenge, both of which are immunomodulatory effects, and further can strengthen tolerance against environmental allergens.³⁶ In our study, we observed an increase in IL-10 production after probiotic supplementation. What is more important, significantly higher CD4⁺CD25⁺Foxp3⁺ induction and a higher reduction in the percentage of TLR-positive cells were observed in the SLIT-probiotic group compared with the other SLIT groups.

Similar results were obtained in a study by Konieczna *et al.*,³⁷ who showed that administration of *Bifidobacterium infantis* in healthy volunteers increased the amount of IL-10 produced by PBMCs and the expression of the transcription factor Foxp3 by Treg cells. These findings are of great importance in the light of a recent review by Scheurer *et al.*³⁸ The investigators present the allergenic properties of certain protein families and the underlying mechanisms of how allergens can stimulate innate immune responses and subse-

quently contribute to Th2-biased adaptive immune responses. Can some adjuvants (possibly probiotics) have an effect on the innate immune cells, in particular, antigen-presenting cells and epithelial cells?

The clinical efficacy of SLIT is well established for both rhinitis and asthma, including a recent meta-analysis by Devillier *et al.*³⁹ In our study, efficacy was obtained after 2 months before the season allergen administration, similar to that found in recent studies that used SLIT tablets and showed that a pre-seasonal regimen, starting at least 8 weeks before the pollen season would be the best choice for pollen SLIT.⁴⁰ In the present study, treatment with SLIT in all the groups resulted in overall clinical improvement compared with those children who received no SLIT treatment; however, more children with probiotic supplementation combined with SLIT responded better to SLIT treatment based on the reduction in the symptom-medication score.

The level of improvement of the combined symptom-medication score compared with the control exceeded the 20% threshold, which was indicated as the threshold for clinically relevant efficacy.⁴¹ The 5-grass SLIT tablet was also highly effective in children with comorbid mild asthma, as seen in a recent study.⁴² This magnitude of effect is impressive and occurred despite the use of rescue medication. We observed significant improvement in lung function in children who received SLIT in all the groups compared with those who received no SLIT treatment. In addition, a FeNO level reduction was observed only in the SLIT-probiotic group, which could be explained by the small number of patients with asthma distributed across all the groups. All these changes were irrespective of the vitamin D serum level, which increased in all the study groups.

There were no cases of the termination of the study due to adverse events in any of the groups. The rate and severity of the adverse effects observed in this study were similar to those reported in other tablet SLIT studies^{43,44}; however, local reactions were relatively common (up to 55% of the patients). The global assessment of tolerability made by the subjects and physicians was very good.

Our study had some limitations. Due to ethical reasons, methacholine challenge was performed outside the pollen season. Nevertheless, we recognized the assessment of bronchial hyperresponsiveness just before and after the pollen season as a valuable study end point. Also, due to ethical reasons, we could not give 5-grass SLIT placebo tablets to the control group, so this arm of the study was not blinded. This was a study based on correlation analyses in a relatively small sample size, with a low number of patients with asthma. However, the sample size was determined based on the recommendations to reach the necessary power.⁴⁵ A high level of odds ratios in logistic regression analysis is probably the result of the relatively low sample size. Although the degree of associations

could be overestimated, the independence of estimated predictors should be taken into consideration. The methods used in our study did not allow assessing the cause and effect relationship between probiotic or vitamin D and SLIT effectiveness. To assess this relationship, all the groups should be prospectively followed up. The other limitation of the study was the fact that there was only one season of observation. Therefore, conclusions should be verified in a well-designed randomized control trial.

While keeping in mind a general rule that the effectiveness of immunotherapy depends on the modification of immune regulation, probiotics and vitamin D are highly attractive. In our study, adding probiotic or vitamin D to SLIT improved clinical and immunologic effectiveness of SLIT without increasing its allergenicity, thereby improving the risk-benefit profile. Probiotic was shown to provide better clinical and immunologic benefits than vitamin D after a relatively short course of treatment compared with the currently available AIT treatments. Our finding seemed to be attractive in light of the latest study by Schuijs *et al.*,⁴⁶ recently published in *Science*, that showed that chronic exposure to low-dose endotoxin or farm dust protects mice from developing house-dust mite induced asthma. Endotoxin reduced epithelial cell cytokines that activate dendritic cells, which thus suppress type 2 immunity to house-dust mites.⁴⁶ Their findings demonstrate that lipopolysaccharides protection blunts the innate immune response to house-dust mites, which these investigators and others previously found to be driven by TLR 4 on epithelial cells.⁴⁷

Yenigun *et al.*⁴⁸ found lower plasma vitamin D levels in patients with allergic rhinitis when compared with the control group. In our previous study, vitamin D supplementation combined with grass-specific SLIT therapy was more effective in the reduction of nasal and asthma symptoms when compared with a SLIT-placebo group.⁴⁹ Also, vitamin D has been shown to have several beneficial effects on the innate and adaptive immune system that might modulate the response to AIT through its effects on dendritic cell maturation and Treg cell induction.^{50,51} Vitamin D has been reported to increase serum levels of the immunomodulatory cytokines TGF- β and IL-10 in humans¹⁷ and to enhance the benefits of AIT in a murine model by IL-10 and TGF- β dependent mechanisms.⁵² In our study, we showed the effect of vitamin D combined with SLIT on early immunologic response measured by CD4⁺CD25⁺Foxp3⁺ induction in patients after 5 months of SLIT.

CONCLUSION

In our study, we were able to demonstrate correlations between probiotic or vitamin D and outcomes such as symptom scores, but one could conclude that these effects are related to probiotic independent of

SLIT. Our results delivered direct evidence for the effectiveness of probiotic and vitamin D supplementation combined with SLIT as a recommended treatment of grass-pollen allergy in children. However, based on our results, we can only speculate that vitamin D and probiotic may boost the immunologic effect of SLIT *via* modulation of the cytokine microenvironment or direct modulation of Foxp3 cell induction.

We also showed that an increase in CD4⁺CD25⁺Foxp3⁺ induction, reduction in TLR-positive cell recruitment and an increase in TGF- β 1 production were independently associated with a better clinical effect of SLIT in children. Interestingly, we noticed TLR-positive cells induction in children from the control group as a result of natural allergen exposure. Nevertheless, additional studies that evaluate the long-term efficacy of *L. rhamnosus* GG or vitamin D in large cohorts of patients with allergy are needed to understand their true potential in the field of allergen-specific immunotherapy.

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