Allergic asthma: Environmental factors challenging the immune system
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Synbiotics reduce allergen-induced T-helper 2 response and improve peak expiratory flow in allergic asthmatics

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ABSTRACT
Background: Previous studies suggest that pre-/probiotics can be used in prevention and treatment of early allergic disease in newborns and young children.
Objective: To determine effect of treatment with synbiotics (90% short-chain galacto-oligosaccharides, 10% long-chain fructo-oligosaccharides: Immunofortis® and Bifidobacterium breve M-16V) on allergic responses in adults with established allergic asthma. Primary outcome was allergen-induced bronchial inflammation as represented by eosinophil counts.
Methods: Twenty-nine patients with asthma and house dust mite (HDM) allergy were randomized in a double-blind parallel design to receive placebo or synbiotics for 4 weeks. At study entry and after treatment a bronchial allergen challenge with HDM was performed, followed by lung function tests, collection of blood (in/ex vivo IL-5) and induced sputum (inflammatory parameters). During treatment a diary was kept with peak expiratory flow (PEF) and asthma scores.
Results: Treatment did not affect the allergen-induced increase in sputum eosinophils at 6 and 24 hrs after challenge. Likewise, other parameters for bronchial inflammation and early and late changes in lung function did not differ upon treatment. Both the morning and evening PEF, however, significantly increased during synbiotics treatment (morning p = 0.003, evening p = 0.011). Also, the increase in serum IL-5 after allergen challenge was significantly inhibited by synbiotics (p = 0.034), as was ex vivo allergen-induced Th2-cytokine (IL-5 and IL-4 + IL-13) production by PBMCs (p = 0.046). In vivo (24 hrs) and ex vivo IL-5 production were associated.
Conclusion: Four-week treatment with synbiotics had no effect on bronchial inflammation and LAR, but did significantly reduce systemic production of Th2-cytokines after allergen challenge and improved PEF

INTRODUCTION
Asthma is a chronic inflammatory disorder of the airways in which many inflammatory and immune cells and mediators are implicated. The chronic airway inflammation is associated with airway hyperresponsiveness (AHR) which leads to recurrent episodes of shortness of breath with variable airflow obstruction. Activated mast cells, eosinophils and T-helper 2 lymphocytes (Th2), producing inflammatory mediators like IL-4, IL-5 and IL-13, contribute to persistent airway inflammation, leading to the pathology characteristic of asthma. Nowadays, treatment is based mainly on suppression of airway inflammation with (inhaled) corticosteroids and relief of symptoms with bronchodilators. Nevertheless, these therapies do not consistently abrogate airway inflammation in patients with
Effect of synbiotics in allergic asthmatics

asthma. Other approaches to modulate the immune-mediated inflammatory responses are under development, including treatment with probiotics.

The composition of the intestinal microbiota may contribute to the genesis of immunological diseases, including allergy and asthma. The bacterial composition has been implicated in the development of gut-associated lymphoid tissue (GALT), which largely regulates Th1- and Th2-type immunity and immune tolerance from early life on. In line herewith, the composition of the intestinal microbiota differs between healthy infants in countries with a low and high prevalence of allergy and between allergic and non-allergic infants in both environments. Therefore, modulation of the gut microbiota through the use of probiotics may, directly or indirectly, modulate the immune system. The reported modes of action of pre/probiotics are strain dependent and range from modulating local innate and adaptive mucosal immune responses to systemic effects. The latter may comprise modulation of systemic cytokine responses and of regulatory T cells. Together these multiple actions may explain why effects of pre/probiotics are not limited to the developing immune responses in the gut, and also why mucosa other than that of the gut can be affected.

Some clinical studies suggested that certain probiotic strains, mostly Lactobacilli and Bifidobacteria, promote early immune system maturation and may alleviate symptoms of (IgE-associated) eczema in young children. Conversely, studies with Lactobacilli in older individuals with already established respiratory disease have failed to show improvement in symptoms of asthma or allergic rhinitis. In addition, although some studies have reported benefit in the treatment and prevention of atopic eczema, none has had any clear effects on the development of Th2-mediated allergic responses.

Prebiotics are indigestible oligosaccharides that promote the growth and activity of commensal bacteria, mainly of Bifidobacteria. It has been demonstrated that a specific mixture of short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS) in a ratio of 9:1 (Immunofortis) normalized the intestinal microbiota of bottle-fed infants to that of breast-fed infants. In a recent double-blind, placebo-controlled multicentre trial with 90 infants with atopic dermatitis, it was shown that Bifidobacteria with the specific mixture of scGOS/lcFOS affected the microflora during the 12 weeks of treatment. In addition, in a murine model for allergic hypersensitivity, using ovalbumin as allergen, dietary scGOS/lcFOS reduced the allergic inflammation.

In an ovalbumin allergic mouse model, oral administration of Bifidobacterium breve M-16V significantly decreased the influx of inflammatory cells in lungs after allergen challenge (lymphocytes (-61%) and eosinophils (-46%)), and reduced airway hyperresponsiveness (AHR) and the level of Th2-type cytokines (IL-4, IL-5 and IL-10).
bronchoalveolar lavage fluid (BALF). A combination of this probiotic strain and Immunofortis® (synbiotics) demonstrated synergistic effects in suppressing the AHR.

In a double-blind, placebo-controlled study, we determined whether supplementing these synbiotics for four weeks had similar results, as observed in the animal model, on the allergic asthmatic responses of adult patients with house dust mite (HDM)-IgE-mediated allergic asthma. Primary outcome was change in allergen-induced bronchial inflammation as determined by eosinophil counts in sputum after allergen challenge. Secondary outcomes included changes in lung function (FEV1) during the late asthmatic reaction (LAR) and allergen-induced bronchial inflammation as determined by neutrophil counts and the level of myeloperoxidase (MPO) and eosinophil cationic protein (ECP) in sputum after allergen challenge. In addition, bronchial hyperresponsiveness, determined by PC_{20} methacholine, peak expiratory flow (PEF), measured during intervention, and immunological parameters, determined in serum and ex vivo HDM-stimulated peripheral blood mononuclear cells (PBMCs) were evaluated as secondary outcomes.

This is the first study to show that treatment with synbiotics reduces the allergen-induced systemic Th2 response and improves the PEF in patients with allergic asthma.

METHODS

Patients

We included 29 adult allergic patients with intermittent to mild persistent asthma, according to American Thoracic Society criteria. All patients were sensitized to HDM, as determined by skin prick test and IgE to D. pteronyssinus > 0.5 kU/L, and were able to stop short-acting β2-agonist agonists for ≥12 h before each visit, and long acting β2-agonists, oral antihistamines and inhaled corticosteroids for 4 weeks prior to and during the study.

Patients with a history of allergen-specific immunotherapy or a respiratory tract infection within 6 weeks prior to the study were excluded. Other exclusion criteria were current smoking, within 6 months prior to the study or a smoking history of ≥10 pack years, and significant changes in environmental allergen exposure 6 months prior to and during the study. Patients co-sensitized to pollen participated only outside the relevant pollen season. In addition, patients were not allowed to use probiotic bacteria, antibiotics and immunosuppressive medication 4 weeks prior to and during the study. Subjects were recruited via advertisement in the Academic Medical Center (AMC) in Amsterdam and gave written informed consent. The study was approved by the AMC Medical Ethics Committee.
Figure 1. Study schedule. After a medication wash-out period of 4 weeks, baseline values were obtained during 3 consecutive days: on day one, blood and sputum samples were collected and PC20 methacholine was determined; on day two, patients were challenged with HDM and blood was collected after 1, 6 and 24 h (day 3), induced sputum samples were collected after 6 and 24 h (day 3) and PC20 methacholine was repeated after 24 h (day 3). Then, subjects were randomized to receive placebo or synbiotics twice daily for 4 weeks, followed by a second period of 3 days involving the same procedures.

Study design
The intervention study had a double-blind, placebo-controlled, parallel design (fig. 1). If necessary, a medication-wash-out period of ≥4 weeks was included. The study started with a baseline visit: on day one, blood and sputum samples were collected and baseline bronchial hyperresponsiveness was determined with PC20 methacholine; on day two, patients were challenged with HDM, blood was collected at 1, 6 and 24 h (day 3) after HDM challenge, induced sputum samples at 6 and 24 h (day 3) and PC20 methacholine at 24 h (day 3). Subsequently, subjects were randomly allocated to receive a food supplement with or without synbiotics (probiotic strain: Bifidobacterium breve M-16V \((10^{10} \text{ cfu})\) combined with a specific prebiotic scGOS/lcFOS mixture (7.2:0.8 gram) (Immunofortis®), Danone Research, Wageningen, The Netherlands) twice daily for a period of 4 weeks. The placebo was also produced by Danone, consisted of maltodextrin only and was indistinguishable from the verum. During the intervention period, study subjects measured their PEF twice daily and kept a diary, including asthma scores, questions on tolerance and acceptance of the product, gastrointestinal complaints, possible illnesses and medication. After intervention, patients were subjected to the same schedule as for baseline visit. During the study, patients were provided with rescue salbutamol (Airomir, TevaPharma, Haarlem, The Netherlands). Safety parameters included tolerance of the product as recorded by patients in diaries, haemocytometric values (hemoglobin and hematocrit), liver function parameters (alanine aminotransferase and aspartate aminotransferase in plasma) and renal function (urea, creatinine and albumine in plasma).
Lung function and Allergen challenge  
Spirometry, PC_{20} methacholine and bronchial allergen challenge were performed according to standardized procedures.\textsuperscript{28} Forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC) were measured with a Vmax 22 spirometer (SensorMedics) and with a portable spirometer (Micromedical diarycard, Sensor Medics). Peak expiratory flow was measured with a portable peak flow meter (Respironics HealthScan Inc., NJ, USA).  
Allergen challenge was performed as described earlier\textsuperscript{29}. The LAR was defined by maximal drop in FEV1 and area under the curve (AUC) from 2-6 h after allergen challenge.

Sputum induction, processing and analysis.
Sputum induction and processing, with DTT to liquefy the whole sample, was performed with minor modifications as described elsewhere\textsuperscript{30}. Differential cell counts were expressed as number and percentage of cells excluding squamous epithelial cells. Sputum samples containing >80% squamous cells on differential cell counting were excluded from analysis. Sputum ECP and MPO were determined as described\textsuperscript{31,32}.

Processing and analysis of blood
Total and differential leukocyte counts were determined in EDTA-blood. Serum samples (for IgE and IL-5) were stored at -80°C until analysis. IgE was determined by ImmunoCAP (Phadia AB, Uppsala, Sweden) and IL-5 was determined by ELISA\textsuperscript{33}. Haemoglobin, hematocrit, ALAT, ASAT, urea, creatinine and albumin were assessed as safety parameters in heparinized blood. Peripheral blood mononuclear cells (PBMCs) were isolated within 2 h after collection from heparinized blood by standard density gradient techniques and stored in liquid-N\textsubscript{2} until analysis.

ELISpot assay
Numbers of cytokine-producing cells after HDM-specific stimulation were determined in ELISpot assay (Mabtech AB, Sweden). Briefly, cryopreserved PBMCs were thawed, washed and preincubated for 4 h in round-bottom tubes (Micronic, McMurray, PA, USA) at 4x10\textsuperscript{5} per ml culture medium (RPMI 1640 (Gibco, Invitrogen Ltd, Paisley, UK), containing 10% heat-inactivated FCS (Gibco), antibiotics and L-glutamine (Gibco) in the absence or presence of HDM (5 \mu g/ml, kindly provided by Sanquin, Amsterdam, The Netherlands). Culture medium and phytohemaglutinin (PHA, 0.1 \mu g/ml) were used respectively as negative and positive control. Then, cells were diluted to a final 2x10\textsuperscript{5} (for IL-4), 1x10\textsuperscript{5} (for IL-12, IL-13), 5x10\textsuperscript{4} (for IL-10) and 2.5x10\textsuperscript{4} (for IFN-\gamma) cells per well and incubated in duplicate at 37°C and 5% CO\textsubscript{2} for 72 h in 96-well PVDF-based membrane plates (Millipore, Billerica, MA, USA), which were coated with monoclonal antibodies to either IL-4, IL-10, IL-
Effect of synbiotics in allergic asthmatics

12p40, IL-13 or IFN-γ (Mabtech). After 72 h, plates were washed and cytokine production by individual cells was visualized using biotinylated detection antibodies, streptavidin-ALP and BCIP/NBT substrate (Sigma-Aldrich, St. Louis, MO, USA) successively. Numbers of cytokine-producing cells were determined by software from A.EL.VIS GmbH (Hannover, Germany) and were expressed as positive cells per 1x10⁵ stimulated PBMCs. Number of positive cells in stimulated cultures was adjusted for background by subtracting number of positive cells from medium-only culture. Analyses were performed batchwise to limit inter-assay variation. In addition, we took along control PBMC, which yielded similar results each time.

**Luminex assay**

For cytokine production by PBMCs after HDM-specific stimulation, PBMCs were cultured for 24 h in round-bottom microtiter plates with the same culture medium and stimuli used in the ELISpot assay. IL-4 (lower limit of detection in pg/ml: 0.56), IL-5 (0.19), IL-6 (0.26), IL-10 (0.4), IL-12p40/p70 (1.51), IL-12p70 (0.42), IL-13 (0.98), IL-17 (0.21) and IFN-γ (0.13) were determined in Luminex assay (Biosource, Camarillo, CA, USA). Apart from the IL-17 data, none of the values were below the lower limit of detection. The production of each cytokine was adjusted for background by subtracting the production in the medium-only supernatant. All samples were analysed in parallel, and for repeat measurements (above higher limit of detection), samples that were determined in the first run were taken along and yielded similar results.

**Immunofluorescent staining and flow cytometry**

To determine percentage of CD4⁺/CD25⁺/IL7R⁻ regulatory T cells, 500,000 PBMCs were incubated for 30 min at 4°C with anti-CD3-FITC, anti-CD4-PerCP, anti-CD25-APC and anti-CD127-PE (BD Biosciences, San Jose, CA, USA; according to manufacturer’s protocols). For intracellular FoxP3 staining, PBMCs were first stained with anti-CD3-FITC, anti-CD4-PerCP and anti-CD25-PE (BD Biosciences), then fixed, permeabilized and stained with anti-FoxP3-APC (eBioscience, San Diego, CA, USA). Cells were washed and analyzed using a FACSCalibur flow cytometer and FlowJo software.

**Statistical analysis**

The sample size was based on the outcome of an intervention study with the probiotic strain *B. breve* M16-V in an ovalbumin allergic mouse model, that resulted in a consistently lower AHR to methacholine and a 50% reduction in number of eosinophils in BALF after allergen challenge. With an average eosinophil count of 15.1 ± 6.6 x10⁴ per gram sputum in asthmatic patients after allergen challenge aiming to detect a 50%
reduction, testing two sided with 5% significance and 80% power, a group size of minimal 12 persons per arm was required.

Randomization was carried out prior to the study by the logistics manager of Danone Research, using a 4-block design. Subjects who were not-compliant, i.e. used study medication less than 2 weeks, terminated the study early or violated the protocol, were excluded from statistical analyses.

When data were normally distributed and the variance was homogeneous, differences between treatment groups were analysed using ANOVA, including the factor treatment. Special attention was given to confounding factors and covariates that could be related to primary efficacy parameters such as baseline values. When data were not normally distributed, either data transformation was performed to get normal distribution or the non-parametric Mann-Whitney test was used. Differences, which occurred over time between treatment groups were analysed using a mixed model. In the mixed linear model evaluating the effect on PEF the factor “day” was included as a repeated measure and baseline PEF was included as a covariate. Treatment and the interaction term treatment by day were included in the model. Two-sided p-values of less than 0.05 were considered statistically significant. The statistical analysis was performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA).

RESULTS
Subject characteristics
At baseline, subject characteristics were not significantly different between both study groups (table 1). From 29 randomized patients, 3 subjects were excluded from all analyses: 2 subjects in the synbiotics group were considered drop-outs, as they stopped in the first week of intervention for personal reasons; 1 subject from the placebo group was not-compliant, as she did not consume the study product for at least 2 weeks of intervention period, because of intestinal side-effects. Post hoc analysis with inclusion of the data of this patient did not change the results for the primary and secondary outcomes. From another subject in the placebo group, sputum, serum and lung function data obtained after the second HDM challenge were excluded from analyses as she accidentally received half the cumulative dose of allergen given during the first challenge. Mild gastrointestinal complaints (diarrhoea or obstipation) were reported most frequently and slightly more in the placebo group than in the verum group (3/14 in the verum group and 7/15 in the placebo group respectively). No long-term adverse effects were experienced. All blood safety parameters showed normal values before and after intervention (not shown).
Table 1. Subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Synbiotics</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (drop-outs/not-compliant)</td>
<td>15 (0 / 1)</td>
<td>14 (2 / 0)</td>
<td>0.473</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>3 / 11</td>
<td>2 / 10</td>
<td>0.578</td>
</tr>
<tr>
<td>Age (years) *</td>
<td>25.0 (18; 51)</td>
<td>28.0 (21; 51)</td>
<td>0.624</td>
</tr>
<tr>
<td>FEV1 (% predicted) †</td>
<td>93.9 (2.5)</td>
<td>97.1 (2.8)</td>
<td>0.226</td>
</tr>
<tr>
<td>PC_{20} methacholine (mg/ml) ‡</td>
<td>1.72 (1.4)</td>
<td>1.27 (1.4)</td>
<td>0.504</td>
</tr>
<tr>
<td>AUC EAR (ΔFEV1*hour) †</td>
<td>20.9 (1.9)</td>
<td>18.8 (1.3)</td>
<td>0.390</td>
</tr>
<tr>
<td>AUC LAR (ΔFEV1*hour) †</td>
<td>39.0 (4.8)</td>
<td>38.7 (10.5)</td>
<td>0.980</td>
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<tr>
<td>PEF_{morning} (L/min) † / [median]</td>
<td>425 (24) / [399]</td>
<td>382 (20) / [383]</td>
<td>0.195</td>
</tr>
<tr>
<td>PEF_{evening} (L/min) † / [median]</td>
<td>445 (24) / [396]</td>
<td>398 (19) / [402]</td>
<td>0.149</td>
</tr>
<tr>
<td>Total IgE (kU/L) ‡</td>
<td>182 (1.4)</td>
<td>257 (1.3)</td>
<td>0.537</td>
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<tr>
<td>HDM-specific IgE (kU/L) ‡</td>
<td>20.8 (1.5)</td>
<td>33.3 (1.4)</td>
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<td>Previous use of LABA</td>
<td>3/15</td>
<td>4/14</td>
<td>0.68</td>
</tr>
<tr>
<td>Previous use of ICS</td>
<td>6/15</td>
<td>5/14</td>
<td>1.0</td>
</tr>
<tr>
<td>Diagnosis of asthma (years)§</td>
<td>11.1 (7.5)</td>
<td>18.5 (14.6)</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Values are expressed as *median (range), †mean (SE), ‡geometric mean (SE), §mean (SD).

FEV1, forced expiratory volume in 1 second; PC_{20} methacholine, provocative concentration of methacholine causing a decline in FEV1 of 20% from baseline; AUC, area under the curve during the first hour after allergen challenge (early asthmatic reaction) and from 2-6 hours after allergen challenge (late asthmatic reaction); PEF, peak expiratory flow; IgE, Immunoglobulin E; LABA, long-acting beta-agonist; ICS, inhaled corticosteroids.

**Primary outcome**

**HDM-induced bronchial inflammation determined by eosinophil counts in sputum**

We observed a significant increase at 6 and 24 h after HDM challenge in sputum eosinophil counts (table 2). The increase in sputum eosinophils during the LAR did not differ significantly between both groups after intervention.

**Secondary outcomes**

**Changes in lung function (FEV1) after HDM challenge**

Bronchial exposure to HDM (geometric mean cumulative dose (range): 313 BU (20-1718)) caused an immediate decline in FEV1 of ≥ 20% (EAR) in all subjects (fig. 2A). Most subjects also experienced a LAR several hours after inhalation of the last dose of HDM (mean maximum decline in FEV1 from 2 to 6 h after challenge (SE): -18.9 % (2.3)). We found no significant differences in lung function between both groups during
Table 2. Sputum inflammatory parameters before and after treatment

<table>
<thead>
<tr>
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<th>Before treatment</th>
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<td>p-value</td>
<td>Placebo</td>
<td>Synbiotics</td>
<td>p-value</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>-24</td>
<td>0.8 (1.5)</td>
<td>2.0 (1.5)</td>
<td>0.103</td>
<td>1.8 (1.5)</td>
<td>1.4 (1.9)</td>
<td>0.756</td>
</tr>
<tr>
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<td>14.2 (1.3)***</td>
<td>14.3 (1.5)***</td>
<td>0.981</td>
<td>19.3 (1.5)***</td>
<td>10.7 (1.7)***</td>
<td>0.364</td>
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<tr>
<td></td>
<td>24</td>
<td>8.5 (1.6)***</td>
<td>7.0 (2.1)*</td>
<td>0.826</td>
<td>9.5 (1.7)***</td>
<td>9.5 (1.7)***</td>
<td>0.998</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>-24</td>
<td>15.8 (1.3)</td>
<td>17.0 (1.3)</td>
<td>0.839</td>
<td>20.6 (1.3)</td>
<td>18.8 (1.4)</td>
<td>0.817</td>
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<tr>
<td></td>
<td>6</td>
<td>38.7 (1.3)*</td>
<td>36.7 (1.4)***</td>
<td>0.894</td>
<td>42.3 (1.4)*</td>
<td>32.2 (1.4)*</td>
<td>0.571</td>
</tr>
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<td></td>
<td>24</td>
<td>29.0 (1.4)</td>
<td>35.2 (1.5)*</td>
<td>0.724</td>
<td>26.4 (1.3)</td>
<td>44.0 (1.5)***</td>
<td>0.295</td>
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<tr>
<td>ECP (ng/gr)</td>
<td>-24</td>
<td>7 (1.6)</td>
<td>18 (1.5)</td>
<td>0.114</td>
<td>15 (1.6)</td>
<td>19 (1.5)</td>
<td>0.685</td>
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<td></td>
<td>6</td>
<td>83 (1.7)***</td>
<td>114 (1.7)***</td>
<td>0.667</td>
<td>87 (1.7)***</td>
<td>73 (1.7)***</td>
<td>0.825</td>
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<tr>
<td></td>
<td>24</td>
<td>62 (1.9)***</td>
<td>114 (2.0)***</td>
<td>0.526</td>
<td>65 (1.9)***</td>
<td>138 (1.9)***</td>
<td>0.403</td>
</tr>
<tr>
<td>MPO (ng/gr)</td>
<td>-24</td>
<td>261 (1.8)</td>
<td>367 (1.4)</td>
<td>0.637</td>
<td>356 (1.8)</td>
<td>323 (1.4)</td>
<td>0.895</td>
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<tr>
<td></td>
<td>6</td>
<td>621 (1.5)</td>
<td>756 (1.4)***</td>
<td>0.718</td>
<td>636 (1.6)</td>
<td>562 (1.5)</td>
<td>0.837</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>541 (1.6)</td>
<td>889 (1.6)*</td>
<td>0.479</td>
<td>582 (1.6)</td>
<td>884 (1.4)***</td>
<td>0.500</td>
</tr>
</tbody>
</table>

Values are expressed as geometric mean (SE) per gram sputum. ECP, eosinophil cationic protein; MPO, myeloperoxidase. Significant changes from prechallenge baseline values (-24 hr) are marked with *p<0.05, **p<0.01, ***p<0.001.
Effect of synbiotics in allergic asthmatics

**Figure 2.** Changes in FEV1 after allergen challenge before (open symbols/bars) and after (filled symbols/bars) treatment for the placebo (□) and synbiotics (○) group. (A) Decline in FEV1 (%) from baseline. (B) Area under the curve during the late asthmatic reaction (ΔFEV1*hour). No significant differences in changes in FEV1 after allergen challenge were observed between the two treatment groups. Mean values (SE) are shown.

**Figure 3.** Changes from baseline in morning (A) and evening (B) peak expiratory flow (PEF) during treatment for the placebo (■) and synbiotics (○) group. Significant differences between both treatment groups were found from 3 weeks of treatment with synbiotics. Mean values per week (SE) are shown. *p<0.05, **p<0.01.

the LAR after intervention (difference in mean AUC from 2 to 6 h after challenge (SE): +2.3 (7.9) after placebo vs -3.5 (7.8) after synbiotics, p = 0.603, fig. 2B). Likewise, the calculated provocative dose of allergen for causing an early phase 20% decline in FEV1 and the maximal fall in FEV1 during the late phase did not differ between groups.

**HDM-induced bronchial inflammation**

We observed a significant increase at 6 and 24 h after HDM challenge in sputum neutrophil numbers as well as amounts of ECP and MPO (table 2). The increase in sputum inflammatory parameters during the LAR after intervention did not differ significantly between both groups.
Lung function parameters
The peak expiratory flow (PEF), measured twice daily during 4 weeks of intervention, significantly improved over time in the synbiotics group (morning p = 0.003, evening p = 0.011), compared to placebo (fig. 3). The change in morning and evening PEF differed significantly between both groups from week 3 on. No significant differences between both groups were observed in asthma symptom scores, short-acting β2-agonist usage and PC_{20} methacholine (pre- and post-challenge), during and after intervention.

HDM-induced cytokine production
Serum IL-5 was significantly elevated at 6 and 24 h after baseline HDM challenge (fig. 4A). The use of synbiotics, however, was associated with a significant lower increase in serum IL-5 at 24 h after HDM challenge compared to that for placebo (mean increase in serum IL-5 at 24 h after challenge (SE): from 2.5 (1.5) to 74.1 (28.4) pg/ml after placebo vs from 1.4 (0.4) to 36.3 (18.7) pg/ml after synbiotics, p = 0.034; fig. 4B). At 6 h after challenge we found trendwise reduced serum IL-5 levels in the synbiotics group (p = 0.092).

In line with serum IL-5, ex vivo production of IL-5 was increased after placebo (p = 0.055), but not after synbiotics (mean increase in IL-5 (SE): 16.5 (7.8) pg/ml after placebo vs 2.4 (3.3) pg/ml after synbiotics; fig. 4C). Interestingly, serum IL-5 at 24 h after HDM challenge correlated with IL-5 production by HDM-stimulated PBMCs (Rs = 0.845, p < 0.001; fig. 4D).

Consistent with changes for IL-5, ex vivo-production of other Th2 cytokines (IL-4 and IL-13; fig. 4E) by HDM-stimulated PBMCs after placebo was also enhanced as opposed to that after synbiotics (p = 0.046). The number of IL-4 and IL-13-producing cells showed a small, but non-significant increase after intervention in both groups (data not shown). No significant differences were found for numbers of IL-10-, IL-12- and IFN-γ-producing cells nor for the production of these cytokines by HDM-stimulated PBMCs (fig. 4F).

FACS analysis
The percentage of regulatory T cells, determined by CD4^+/CD25^+/IL7R^- T cells (mean percentage (SE): 4.9 (0.3)% of CD3^+ -lymphocytes) or the expression of FoxP3 in CD4^+/CD25^hi T cells (mean MFI (SE): 12.3 (0.4)), did not change after intervention for both study groups (not shown).
DISCUSSION

Modulation of the intestinal microbiota by prebiotics, probiotics or a combination (synbiotics) is being investigated as a potential anti-allergic treatment strategy. In the present double-blind, placebo-controlled clinical study, we investigated the potential therapeutic effect of a specific synbiotic mixture to target established allergic asthma. We show that treatment with synbiotics for four weeks did not significantly reduce the allergen-induced eosinophilic airway inflammation in allergic asthmatics during the LAR after bronchial challenge with HDM. Interestingly, treatment with synbiotics is associated with a significant improvement in the PEF and with a reduced production of IL-5 after allergen exposure, both in vivo and ex vivo. Moreover, the increased ex vivo production of...
other Th2-type cytokines (IL-4 and IL-13) by HDM-stimulated PBMCs in the placebo group is absent in the synbiotics group.

Based on the suppression of HDM-induced Th2-cytokine production by synbiotics, a reduction in allergic airway inflammation after allergen challenge as observed in the mouse model was expected, but not found. The chronology of sensitization and subsequent airway allergen challenge in the mouse model is different from that in allergic asthmatics and this may explain observed differences. Also, the reduction in airway inflammation by synbiotics in allergic asthmatics may have been less and therefore the power of the study insufficient. In addition, the large variation of inflammatory parameters in induced sputum samples may have hindered the detection of small changes in inflammation in asthmatics.

The finding that both morning and evening PEF improved significantly during treatment with synbiotics when compared to that with placebo may indicate that the change in HDM-induced systemic Th2 response did ameliorate the clinical course of asthma. In concordance with the mechanism proposed by Kukkonen, synbiotics may modulate the course of sensitization to clinical disease by suppressing the production of Th2-type cytokines.

After placebo treatment, the HDM-induced production of IL-5 in vivo and production of IL-4, IL-5 and IL-13 by PBMCs ex vivo was increased. This may be because of the baseline HDM challenge in the absence of treatment with anti-inflammatory drugs. As we observed no significant differences in the number of Th2-cytokine-producing cells between both treatment groups, the augmented Th2-cytokine production in the placebo group may relate to enhanced activation of PBMCs by HDM, which is suppressed by treatment with synbiotics. Van der Veen et al. reported significant increases of serum IL-5 at 6 and 24 hours after bronchial allergen challenge which correlated with the allergen-specific proliferative response of peripheral T lymphocytes in vitro. The association of these systemic Th2 phenomena with the magnitude of the LAR emphasizes the relevance for the clinical course of allergic asthma.

In contrast to several human studies, suggesting an increase in Th1-cytokines (IFN-γ) regulatory cytokines (IL-10) or regulatory T cells after probiotic treatment, the immune-modulatory effect of synbiotics was not mediated by the induction of or a change in Th1- or regulatory T-cell cytokines or cell numbers.

To determine whether reduction in Th2-cytokines by treatment with synbiotics will eventually lead to a clinical relevant reduction in bronchial inflammation and improvement in asthmatic symptoms, this study should be repeated with a larger number of patients to increase the power of the study. Moreover, prolonged administration of
synbiotics should be investigated to study the long-term effect on Th2 suppression and allergen-induced inflammatory responses.

To our knowledge, this is the first study to show that synbiotics improve the peak expiratory flow and reduce the systemic production of Th2-type cytokines like IL-4, IL-5 and IL-13 in allergic asthmatics. Thus, treatment with synbiotics may gradually decrease the allergen-induced immunological response in allergic patients with established asthma.

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