Effect of orally administered probiotic strains *Lactobacillus* and *Bifidobacterium* in children with atopic asthma

PIOTR GUTKOWSKI[1], KAZIMIERZ MADALIŃSKI[2], MARTYNA GREK[2], HANNA DMEŃSKA[1], MAŁGORZATA SYCZEWSKA[1], JACEK MICHAŁKIEWICZ[3]

[1]Pulmonology Department, Child Health Memorial Institute, Warsaw, Poland
[2]Virology Department, Laboratory of Immunopathology of Hepatotropic Infections, National Institute of Public Health – National Institute of Hygiene, Warsaw, Poland
[3]Microbiology and Clinical Immunology Department, Child Health Memorial Institute, Warsaw, Poland

Abstract

Children with mild to moderate asthma (n = 46), aged 4-10 years, were randomly allocated into groups matched according to age, height and body weight (Group 1 and Group 2). Group 1 (n = 22) received Trilac capsules (1.6 × 10^9 lactic acid bacteria cells: Lactobacillus acidophilus – 37.5%, Bifidobacterium bifidum – 37.5% and Lactobacillus delbrueckii subsp. bulgaricus – 25%), whilst Group 2 (n = 24) received placebo. The study was performed over 12 weeks and the results were evaluated using lung function and immunological tests. During the study period, the patients continued treatment with inhaled beta-mimetics and inhaled corticosteroids.

The children receiving Trilac significantly improved their lung function and presented with less episodes of asthma exacerbations during the study period than the children receiving placebo. Similarly, the amount of bronchodilatators used was significantly reduced in the children receiving Trilac than the children on placebo. The differences were also observed in the immunological parameters studied by flow cytometry. The statistically significant increase in the expression of HLA-DR on monocytes and decrease of CD8CD45RA+ lymphocytes were observed in Trilac group, but not in the placebo group. The levels of IFN-γ (Th1) and IL-10 (Treg) cytokines were increased in both examined groups.

In conclusion, a positive effect of 12-weeks administration of Trilac on the clinical course and lung function of the children with mild to moderate asthma was observed. Changes of immunological markers (increase of HLA-DR expression and decrease of CD8CD45RA+ cells) in the children treated with Trilac were also noted.

Key words: *probiotics, atopic asthma, children, lung function, immunologic parameters.*

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Introduction

The explanation of probiotics influence on immune system from the early hypothesis that the ingestion of fermented milk products had a beneficial impact on human health [1]. Later, with the advent of refrigeration and a tendency toward processed and a sterile food supply, the ingestion of probiotics has become much more limited. Probiotics have recently gained more recognition in conjunction with the knowledge of the detrimental effects of antibiotic overuse. Today probiotics are defined as live microorganisms that, when administered, produce some preventive or therapeutic health benefit to the host [2].

Early studies draw attention to the potential use of probiotic supplementationin infant nutrition to control atopic eczema and local and systemic inflammatory responses, as well as to prevent the onset ofatopic diseases [3-6]. The use of probiotics in the prevention or treatment of allergic disease is a relatively new concept [7].

Correspondence to: Prof. Kazimierz Madaliński, Department of Virology Laboratory of Immunopathology of Hepatotropic Infections, National Institute of Public Health – National Institute of Hygiene, Chocimska 24, 00-791 Warsaw, Poland, phone: +48 22 54 21 326, fax: +48 22 54 21 237, e-mail: kmadalinski@pzh.gov.pl

The last decades have seen an unprecedented increase in the diagnosis of asthma and allergic diseases among both children and adults. The rise in the prevalence of atopic diseases has been linked to the decline of infectious diseases [8-10].

The associations between exposure to microbes or microbial products and sensitization to allergens has led to renewed interest in intestinal flora, particularly in the stimulation of the developing immune system during the first year of life [3, 4, 11]. All these findings, although well documented, are preliminary but suggest the possibility of modifying immune responses in early life. Probiotic supplementation is particularly appealing as it could be widely introduced as public health early prevention strategy.

The primary aim of this study was to examine whether children with mild to moderate atopic asthma receiving probiotic strains or placebo in addition to the conventional treatment, differ in the clinical course of the disease, number of asthma exacerbations, the dose of bronchodilators, lung functions and laboratory parameters.

Material and methods

Subjects

Patients were recruited from the Pulmonary Outpatient Clinic of The Child Memorial Health Institute in Warsaw, Poland. Forty-six children aged 4-10 years with mild to moderate atopic asthma diagnosed by a physician at least one year prior to inclusion were asked to participate in the study. The inclusion criteria for the children were following:

- age 4-10 years old,
- diagnosed mild to moderate atopic asthma,
- positive skin prick test,
- elevated IgE level,
- positive history of atopic asthma symptoms,
- informed consent signed by parent or legal guardian.

The study was performed between 2002 and 2005. The Ethics Committee of the Child Memorial Health Institute in Warsaw approved the study protocol with the included information and written informed consent to be signed by the parents or guardians of the participating patients.

Study design

The patients with mild to moderate atopic asthma were randomized in a double-blind design to receive Trilac or placebo. Trilac and placebo capsules, both manufactured by Allergon AB, Sweden, were administered b.i.d. Trilac capsules contain 1.6×10^9 lactic acid bacteria cells: *Lactobacillus acidophilus* – 37.5%, *Bifidobacterium bifidum* – 37.5% and *Lactobacillus delbrueckii* subsp. *bulgaricus* – 25%. The daily dose of lactic acid bacteria was 3.2×10^9. The study was designed for 12 weeks per patient.

During the study period, the patients continued treatment with inhaled beta-mimetics and inhaled corticosteroids. A quantitative estimate of the use of medication was done at each visit (V1 – V4) on the basis of a diary, filled out by the patient's parents/guardians. The clinical severity of atopic asthma was evaluated at inclusion and every four weeks after the initiation of Trilac administration. At each visit a spirometry was performed in children aged 7-10 years old. The distribution of lymphocytes subpopulations and the level of cytokines in peripheral blood mononuclear cells were assessed at inclusion and at the end of the study.

Isolation of cells for immunological studies

Peripheral blood mononuclear cells (PBMC) were obtained from the heparinized blood of patients by Isopaque-Ficoll (Lymphoprep; Nycomed Pharma AS, Oslo, Norway) gradient centrifugation [11]. Mononuclear cells were re-suspended in a culture medium made up of RPMI 1640 (Gibco, Paisley, UK) supplemented with a 5% heat-inactivated fetal calf serum (Gibco) and gentamicin (40 μg/ml).

Purification of lymphocyte subpopulations

PBMC were labeled with mouse anti-human CD4, CD8, and CD3 monoclonal antibodies (IgG2a, Ortho-Diagnostic, Raritan NJ, USA), then incubated for 30 min with rabbit complement, washed twice in cold PBS and re-suspended in a culture medium at a concentration of 2×10^6/ml. The purity of obtained lymphocyte subsets enriched either for CD8+ or CD4+ cells as well as the extent of depletion of CD3+ lymphocytes from the PBMC population were controlled by flow cytometry analysis and was always about 90%.

Flow cytometry analysis

Freshly isolated PBMC (4×10^6/ml) were washed in cold PBS and labeled with FITC (fluorescein) and PE (phycoerithrin) conjugated mouse anti-human monoclonal antibodies (Becton-Dickinson), specific for different cell membrane receptors of monocyte and lymphocyte. The panel of FITC-labeled monoclonals involved: anti-HLA-DR, anti-ICAM-1 (CD54), anti-CD86, anti-LFA-1 (CD11a). The panel of PE conjugated monoclonals involved: anti-CD3, –CD4, –CD8, –CD14, –CD19, –CD25, –CD45RA (–CD4, –CD8); anti-CD45RO (–CD4, –CD8), –CD69, –CD80 and –CD86. Simultest LeucoGate, CD45-FITC/CD14-PE, and simultest control γ_1-FITC/γ_{2a}-PE (Becton-Dickinson) were included for each staining panel. Fluorescence intensity measurements of FITC and PE staining were standardized daily by using 10 mm diameter fluorescent beads (Ortho Diagnostic Systems, Raritan, NJ, USA). The peak of green fluorescence histogram was placed approximately in channel 78 by adjusting the green fluorescence voltage gain to 850 ±30. The peak of the red fluorescence histogram was

placed in channel 156 by adjusting the red high-voltage gain to 700 ±20. The mean of the relative fluorescence intensity (RFI) was calculated as the mean channel number of evaluated surface molecules. Analysis of surface markers expression was performed on cells gated as monocytes and lymphocytes, using double color flow cytometry (EPICS XL-MCL).

Statistics

The distribution type was determined using several tests. The comparison of the variable's distribution and theoretical normal distribution was carried out using Kolmogorov-Smirnov and Shapiro-Wilk tests. The comparison of the variable's distribution and theoretical log – normal distribution was completed using Kolmogorov-Smirnov and chi-square test.

Variables with normal distribution were summarized by mean and standard deviation, these with log – normal distribution by geometric mean and range, these with other distributions by median and range.

Comparison between the groups in the case of normally distributed variables was carried out using *t*-Student test (after checking of the variance homogenity with Levene's test), in other variables using Kolmogorov-Smirnov and Mann-Whitney test. Comparison of groups between variables obtained in different time points was done using ANOVA Friedman test.

The frequency variables were assessed using Pearson's χ^2 test.

For all tests $p < 0.05$ was considered as a significant difference.

Results

Baseline characteristics of the patients

The summary of characteristics of the studied subjects in both groups, which were sex and age matched, are illustrated in Table 1. The asthma exacerbations were statistically more frequent and bronchodilators use was statistically higher in placebo group (V1/V2 and V3/V4) than in Trilac group (V3/V4 only) – Table 2 and Table 3.

Lung function assessed by the Tiffeneau index in the Trilac group of children during the observation period was statistically improved, but was unchanged in the placebo group – Table 4.

No serious undesired side effects were observed in either group of patients.

Clinical course and lung function

The groups differed from visit to visit in respect of asthma exacerbations and amount of bronchodilators administered (Tables 2-4). In the placebo group exacerbations were statistically more frequent (V1/V2 and V3/V4) than in the

Table 1. Characteristics of Trilac and placebo groups of children in whom lung function studies were performed

Studied Group	Age (years)	Height (cm)	Weight (kg)	Gender (M/F)
Trilac (n = 22)	6.93 4.3-9.9	124.8 108-152	24.6 16.5-39.0	16/6
placebo (n = 24)	6.65 4.2-9.7	125.3 102-136	24.3 15-40	10/14

Table 2. Asthma exacerbations from visit to visit in both the Trilac and placebo groups

Visit (V)	Pearson's χ^2 test		Degrees of freedom
	Trilac	placebo	
V1/V2	12.925 p = 0.115	20.473 p = 0.009	8
V2/V3	21.175 p = 0.172	23.339 p = 0.105	16
V3/V4	34.937 p = 0.0005	27.070 p = 0.008	12

Table 3. Bronchodilators used from visit to visit in both the Trilac and placebo groups

Visit (V)	Pearson's χ^2 test		Degrees of freedom
	Trilac	placebo	
V1/V2	7.050 p = 0.133	20.184 p = 0.0005	4
V2/V3	5.848 p = 0.211	12.851 p = 0.120	4
V3/V4	16.416 p = 0.003	18.464 p = 0.001	4

Table 4. FEV_1 %VC in both the Trilac and placebo groups

Visit	Value (geometric mean) and range	
	Trilac (n = 17)	placebo (n = 13)
V1	81.42 52.55- 98.58	84.84 62.32-100.00
V2	80.59 55.71-99.46	81.05 50.43-93.23
V3	83.71 57.11-97.77	81.26 51.43-95.43
V4	83.54 64.02-97.99	81.38 71.57-90.37
χ	10.2	3.993
p	0.017	0.262

Trilac group (only V3/V4). In the placebo group bronchodilators were used more frequently (V1/V2, V2/V3 and V3/V4) in comparison with the Trilac group (only V3/V4).

The evaluation of the immunologic parameters

The distribution of lymphocytes subpopulations and levels of cytokines (IFN-γ, IL-10) was performed at the beginning of the study and after the administration of Trilac or placebo (12 weeks). The treatment with Trilac affected both parameters (Table 5). Flow-cytometric analysis revealed a statistically significant decrease in the percentages of CD8/CD45RA+ cells after Trilac administration. The Trilac group also showed higher percentage of monocytes with the expression of HLA-DR receptors at the end of the study. All other evaluated parameters although had changed after 12 weeks of administration of the placebo or Trilac, but the differences were not significant between Trilac and placebo group.

Discussion

The prevalence of allergic diseases, including atopic asthma increased recently among children living in industrialized countries and continues to rise [12]. Asthma is a heterogeneous disease, of different origins (genetic and environmental factors may be involved), seasonal appearance and various responses to treatment [13-15]. In particular it was pointed out that genetic background as well as environmental factors, but also the age of the children are important in the development of asthma [14]. It was further suggested that the allergic diseases might arise from a reduced microbial exposure at an early age [16]. Well documented studies demonstrated the role of probiotics in the prevention and treatment of the allergic diseases [17]. The main objective of our study was to determine the differences in the clinical course of the disease, number of attacks, the dose of bronchodilatators used, lung function and immunologic parameters in children with atopic asthma, receiving a mixture of three strains of lactic acid bacteria (*Lactobacillus acidophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Bifidobacterium bifidum*) – Trilac, in comparison to children receiving a placebo.

The decrease of asthma exacerbations and therefore decreased use of bronchodilatators and improvement of lung function were observed in the group treated with Trilac compared to placebo group.

It is possible that the positive effect of Trilac on pulmonary function in the asthmatic children, observed in our study, was caused by at least two factors: the use of three strains of lactobacilli in the preparation given to the children (*Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Lactobacillus delbrueckii* subsp. *bulgaricus*) which may be very effective or the lack of severe asthma cases. The mild to moderate asthma exacerbation fluctuates and this may have a potential impact on the course of the disease.

There is a number of reports, concerning the effect of probiotics (synbiotics) on lung function and immunological parameters in children with atopic asthma and atopic diseases. Our observations are in agreement with two Dutch double-blind studies. The authors showed that synbiotics administration to 29 children with asthma for 4 weeks, resulted in the increase of peak expiratory flow and inhibition of pro-allergic IL-5 [18]. The other study, performed in the group of 90 infants with atopic dermatitis, aged < 7 years, who received synbiotics or placebo during 12 weeks, documented the decrease of wheezing in the synbiotic treated group in comparison to control [19]. The positive effect of six lactobacilli strains on the prevention of allergic disorders in mice was also observed [20].

However, other studies were unequivocal: authors obtained only weak effects in asthmatic children, $n = 131$, treated with LGG preparation [21]; or the effect was limited only to pregnant women with atopic dermatitis, treated with 3 strains of *Lactobacilli* [22]. There were also studies, which obtained negative or weak positive data as to the effect of probiotics on allergic diseases [23, 24]. Summary of negative results, as to the action of probiotics on prevention of allergic disease was reviewed by Kopp and Salfeld [25].

As already known, one of the features of atopic diseases is the dominance of Th2 type cytokines over those of Th 1. There are many reports describing the possibility of reverting this unfavourable ratio by using *Lactobacilli* or other immunomodulators [26-30]. E.g., strains of Lactobacillus sp. were shown to polarize human dendritic cells towards Th1 cytokines secretion, i.e. IFN-γ, IL-12 and IL-18, but not IL-4 or IL-13 [26]. Very interesting findings were also obtained, showing that cytosine-guanine (CpG) oligodeoxynucleotides modulate the course of the murine asthma. It was shown that suppression of IL-4 in the BAL, in the presence of increased levels of IL-12 and IFN-γ was not the leading factor; it was further proven that the induction in T cells and APC's of regulatory type response was involved in the protection of asthma development [27]. The other authors showed the immunomodulatory effects of Lactobacilli, with the obvious trend to support cytokines of Th1 type [28-30].

Table 5. Percentage of HLA-DR - positive monocytes and CD8/CD45RA cells in the Trilac groups before and after treatment

Lymphocyte subpopulations	Trilac (Visit 1)	Trilac (Visit 4)	*p*
HLA DR %	90 (72-98)	96 (89-99)	0.00041
CD8/CD45RA %	28 ±4	25 ±4	0.03887

It was shown that probiotics have immunomodulatory properties, including the suppression of IgE production and activation of Th1 response [17]. In the present study, the subset of CD8/CD45RA+ cells and the expression of monocyte surface receptor (HLA-DR) significantly differ after the administration of Trilac. The statistically significant increase in the expression of HLA-DR and decrease in the subset of CD8/CD45RA+ cells was observed in peripheral blood mononuclear cells from children with atopic asthma after Trilac treatment. Since HLA-DR is considered as the monocyte activation marker and CD45RA as the naive cells marker, our results suggest that Trilac administration leads to the activation of monocytes and to the suppression of lymphocytes proliferation. This is in line with our earlier *in vitro* studies, showing that stimulation of human PBMC by Trilac causes an increase in the expression of HLA-DR receptor on these cells. The same immunogenicity studies of Trilac revealed it to be a weak inductor of T cells proliferation *in vitro* [30]. Our results have confirmed a weak immunogenicity of the *Lactobacillus* strains, included by Trilac. We have also found that in both analyzed groups other evaluated cellular parameters were significantly higher after treatment. Similarly, the level of IFN-γ and IL-10 was increased after administration of both Trilac and the placebo. In contrast to the results of our study, Hua *et al*. demonstrated enhanced expression of CD45RA in response to probiotics. However, this group used Bio-Three, a mixture of different probiotic strains (*B. mesentericus*, *C. butyricum* and *E. faecalis*) [31]. It was earlier demonstrated that lactic acid bacteria have a different ability to activate the immune response [32].

To summarise – we have found that the administration of Trilac over a 12-week period had a positive effect on the clinical course and on the lung function in children with mild to moderate asthma. This effect was clear. The question is how stable this effect is and how reproducible are the results. However, a number of other researchers were trying to use probiotics or synbiotics as supportive/supplementary treatment in asthma and other atopic diseases.

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