THE IMMUNOMODULATORY EFFECTS OF PROBIOTIC
BACTERIA ON PERIPHERAL BLOOD MONONUCLEAR CELLS
(PBMCs) OF ALLERGIC PATIENTS

Somaya M. El Sheikh, Mona A.M. Shalaby, Raghda A. Hafez, Wafaa S.A. Metwally and Yassin M. El-Ayoty

Department of Medical Microbiology and Immunology, Faculty of Medicine, Zagazig University, Egypt
Department of Botany, Faculty of Science, Zagazig University, Egypt

ABSTRACT

Allergic diseases represent major health burden. An allergic reaction is characterized by a disrupted T-helper 1/T-helper 2 balance toward a preferential allergen specifically induced TH2 cytokine profile, causing allergic inflammation. Probiotic bacteria have various beneficial effects in many pathologic situations. Studies have shown that the bacteria present in the intestinal micro flora play a role in the TH1/TH2 balance and its modulation can promote the control of infectious and immune processes. Testing the effects of probiotic bacteria on TH1/TH2 cytokine production by peripheral blood mononuclear cells of allergic patients and control subjects. This study included 24 patients allergic to date pollen and 16 healthy control subjects. PBMC of both groups were separated and cultured for 72 h with date pollen allergen (home-made) in the presence or absence of Lactobacillus rhamnosus ATCC 7469 (Living and dead) and C-phycocyanin (extracted from Spirulina platensis). The cell culture supernatants were collected to measure Interleukin 4 and Interferon gamma by quantitative ELISA. Incubation of PBMCs of allergic patients with living Lactobacillus rhamnosus ATCC 7469 showed marked reduction in IL4 production (median IL4 concentration = 3.9 pg.) compared to PBMCs challenged with pollen alone (median IL4 concentration = 52.6 pg). When PBMCs were incubated with living Lactobacillus rhamnosus in absence of allergen significant increase in IFNγ (median concentration = 42.75 pg.) was obtained, compared to PBMC challenged with allergen alone (median = 22.8 pg). When PBMCs incubated with heat killed Lactobacillus rhamnosus either in presence or absence of the offending allergen, marked reduction in IL4 production was obtained (median = 10.6, 3.6 pg respectively) compared to PBMC incubated with allergen alone (median = 52.6 pg). When PBMCs incubated with dead Lactobacillus rhamnosus, marked increase in IFNγ production was obtained (median = 49 pg) when compared to IFNγ production by PBMC challenged with allergen (median 22.8 pg). PBMCs challenged with PC in the presence or absence of allergen showed marked decrease of IL4 production (median = 19.8, 17 pg respectively) when compared to PBMC incubated with the offending allergen alone (median = 52.6 pg). PBMCs incubated with PC showed significant increase of and IFNγ production (median= 319.6 pg) when compared to PBMC incubated with the offending allergen alone (median = 22.8 pg). Conclusion Lactobacillus rhamnosus ATCC 7469 and C-phycocyanin (extracted from Spirulina platensis) reversed the TH1: TH2 polarization in allergic patients and could be a promising line of treatment.

Keywords: TH: T Helper Cells, PC: Phycocyanin, IL 4: Interlukine 4, IFNγ: Interferon Gamma, PBMCs: Peripheral Blood Mononuclear Cells

Corresponding Author: Raghda A. Hafiz, Department of Medical Microbiology and Immunology, Faculty of Medicine, Zagazig University, Egypt
1. INTRODUCTION

Allergy is a reaction characterized by a disrupted TH1/TH2 balance with predominance of TH2 cytokines (IL-4, IL-5, IL-9 and IL-13). These cytokines induce IgE antibody formation, promote eosinophil development and recruitment and increase the production of mucus in the gut and airways (Vissers et al., 2011).

Studies have shown that the bacteria present in the intestinal micro flora play a role in the TH1/TH2 balance and its modulation can promote the control of infectious and immune processes (Huang et al., 2014).

Intestinal flora differs in both atopic and healthy subjects. Atopic children were found to have higher levels of Clostridia and lower levels of Bifidobacterium (Ozdemir et al., 2010).

Administration of probiotics, defined as living microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Rijkers et al., 2010), has shown to be able to reduce the incidence of many atopic disorders (Niers et al., 2009; Kalliomäki et al., 2010). The most popular probiotic strains are represented by the following genera: Lactobacillus, Streptococcus and Bifidobacterium. Enterococci and yeasts have also been included (Chow, 2002; Shah, 2007).

Spirulina platensis is symbiotic, multicellular and filamentous blue-green bacteria with various health benefits (Ali and Saleh, 2012). C Phycocyanin (C-PC) is the major photosynthetic pigment of Spirulina platensis. C-PC has shown potential therapeutic benefits for immunostimulation. It was proved to enhance proliferation and differentiation of bone marrow hematopoietic cells (Hayashi et al., 2006).

Taurine is a free amino acid with many probiotic actions, (Abdel-Rahman, 2014) found that co-administration of taurine protects hepatic and cardiac tissues against histopathological changes and apoptosis induced by hypercholesterolemia.

This study tried to investigate the effects of Lactobacillus rhamnosus ATCC 7469 (living and dead) and phycocyanin extracted from Spirulina platensis on TH1/TH2 paradigm. The peripheral blood mononuclear cells system was used to evaluate these effects.

2. MATERIALS AND METHODS

This study included 24 allergic patients and 16 control subjects. Patient group: They were 10 males (41.6%) and 14 females (58.4%). Their ages ranged from 20-32 years with a mean age (26.5±0.7) years. 20 of them had positive family history (83%). They were referred to Allergy and Immunotherapy unit, Faculty of medicine, Zagazig University. All selected patients suffered from allergic rhinitis and diagnosed by an ENT specialist and were monosensitized to date pollen allergen.

Control group: They were 6 males (37.5%) and 10 females (62.5%). Their ages ranged from 20-31 years with a mean age (25.7±0.9) years. They had never suffered from allergy symptoms (asthma, sneezing, itching). They showed negative skin test against the allergen panel used in the unit.

2.1. Allergen Extraction

2.1.1. Method (Home Made, According to the Protocol Used in the Unit of Immunotherapy, Zagazig University)

Addition of crude pollen (50 gm) to the Coca’s solution (500 mL) at a concentration of 1:10 in a flask with shaking in a shaker for 48 h at room temperature.

The extract was filtered through Whatman No1 filter paper. Then, through a sterilized Sefitex filter using membrane filter pore size 0.45 um. Lastly filtration through syringe filter pore size: 0.22 um.

The sterility of the extract was checked by cultivation on nutrient and blood agar both aerobically and anaerobically to exclude bacterial contamination.

2.2. Preparation of Bacteria

The Lactobacillus rhamnosus ATCC 7469 were provided in MRS broth (de Man, Rogosa, Sharpe (MRS broth with tween 80); biolife, Italy. Selective medium for lactobacilli) with 30% glycrol. First, they were grown anaerobically (Oxoid anaerobic gas generating system) in MRS agar medium at 37°C for 48 h. Subculture was successfully achieved in Candle jar.

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The growth was examined by Gram staining, catalase test, oxidase test and triple sugar iron test and it confirmed to be Gram positive bacillus, Catalase and oxidase negative, ferment glucose, sucrose and lactose with production of acid only.

After identification, the bacterial strain was subcultured on MRS broth at 37°C either in anaerobic jar or in candle jar (CO2 rich atmosphere) for 24 h. Serial dilutions of freshly prepared broth cultures were plated onto MRS-agar and cultured for enumeration. Colony Forming Units (CFU/mL) were determined by plating serial 10-fold dilutions. Plates were
anaerobically incubated at 37°C for 24-48 h. Bacteria were then washed three times with Phosphate Buffered Saline (PBS) and adjusted to final concentrations of $3 \times 10^8$ CFU/mL. An aliquot of the bacterial samples were also stored at 20°C in MRS broth containing 20% glycerol for subsequent experiments. Each aliquot contains $3 \times 10^8$ CFU/mL.

2.3. Extraction of C-Phycocyanin from Spirulina Platensis

C-PC was extracted from *S. platensis* according to (Boussiba and Richmond 1979; Silva et al., 2009) as follow:

- 20 gm of cultured *S. platensis* was suspended in 200 ml of 0.1 sodium phosphate buffer pH 7.2, containing 100 ug mL$^{-1}$ lysozome and 10 ml EDTA
- The enzymatic disintegration of the cell wall occurred by placing the algae in shaking water path at 30°C for 24 h
- The resultant slurry centrifuged for 1 h at 10,000 rpm to remove cell debris, yielding a clear supernatant of crude C-PC
- Ammonium sulfate was gradually added to the crude extract to achieve 25 and 50% saturation with continuous stirring. The resulting solution was kept for 2 h and centrifuged at 12,000 g for 30 min
- The obtained precipitate was dissolved in Na-phosphate buffer and dialyzed over night at 4°C against the same

According to (Bennett and Bogorad, 1973), the C-Phycocyanin Concentration (PC) was defined as:

$$PC = \frac{[OD_{615} - 0.474 \times OD_{652}]}{5.34}$$

where, PC is the C-phycocyanin concentration (mg mL$^{-1}$), $OD_{615}$ is the optical density of the sample at 615 nm and $OD_{652}$ is the optical density of the sample at 652 nm. OD was measures by spectrophotometer at 615 nm and at 652 nm $0.73-(0.47\times0.47)/5.34 = 0.09$ mg mL$^{-1} = 90$ ug mL$^{-1}$.

Filtration of phycocyanin extract via syringe filter pore size: 0.22 um to ensure sterility.

2.4. Skin Testing and Selection of Patients Involved in the Study

2.4.1. Skin Tests

Disinfection of forearm skin by 70% ethyle alcohol.

Intradermal injection of 100 microliter of home made extracts of: House dust, smoke, wool, cotton, mixed fungi, date pollen and hey dust, in addition to normal saline as negative control. The reaction is observed within 20 min.

2.4.2. Patient Selection

Allergic subjects sensitive date palm pollen (*Phoenix dactylifera; Pho d*) allergen. The allergic patients presented with a history of allergic rhinitis and referred diagnosed by a physician and showed positive skin prick test responses. Consent was taken from each individual after explaining the nature of investigation and the purpose of the study in accordance with the ethical standards of the responsible regional committee.

2.5. Blood Sampling

About 5 mL of peripheral blood were obtained from the study participants by venous puncture and collected into preservative-free heparin containing tubes at 10 unit mL$^{-1}$ final concentration.

2.6. Separation of Peripheral Blood Mononuclear Cells

Heparinized blood was diluted 1:1 with normal saline in 15 mL conical centrifuge tubes (Falcon tubes) and mixed gently by inversion. Diluted blood (5 mL) was completely layered on an identical volume of the density gradient which contained 5.6% Ficoll and 9.6% diactrizoate with a density of 1.077 g mL$^{-1}$ and an osmolarity of 300 mOsm. The tubes were kept at a 45° angle and the diluted blood was allowed to run down side of tubes without allowing the two solutions to mix. The tubes were transferred to the centrifuge without disturbing the interface. Samples were centrifuged for 30 min at 400× g at room temperature without applying a brake.

The PBMCs interface (Buffy coat) was carefully removed by Pasteur pipettes and was washed twice ; the first wash with normal saline supplemented with 2% heat-inactivated fetal calf serum and the second wash with RPMI 1640 complete medium (RPMI 1640 medium that contained 10% heat-inactivated fetal bovine serum, penicillin/streptomycin 1%) by centrifugation for 10 min at 400× g.

2.7. Activation of Mononuclear Cells

PBMCs (2×10$^6$ mL) from each participant were cultured in 300 mL/well in flat-bottomed 96-well microtiter (Pochard et al., 2002).

About 180 uL of PBMCs in complete medium (RPMI 1640 medium that contained 10% heat-inactivated fetal

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bovine serum penicillin/streptomycin (1%) were added to wells from A-F and other components were added as the following protocol:

A: +20 uL of complete medium as negative control
B: +20 uL of bacterial suspension (Thawed, washed and resuspended in complete medium)
C: +20 uL of bacterial suspension (Thawed, washed and resuspended in complete medium) +20 uL of date pollen allergen (1:1000)
D: +20 uL of phycoerythrin 90 mg mL$^{-1}$
E: +20 uL of phycoerythrin 90 mg mL$^{-1}$ + 20 uL of date pollen allergen (1:1000)
F: +20 uL of date pollen allergen (1:1000)
G: +20 uL of killed bacterial suspension (1 h incubation at 70°C)
H: +20 uL of killed bacterial suspension + 20 uL of date pollen allergen (1:1000)

The microtitre plate then covered by its lid and incubated in CO2 incubator providing 5% CO2 for 72 h. Cell-free supernatants were harvested and centrifuged. The supernatants were stored at -20°C until analysis of cytokines.

2.8. Cytokine Assays

IL-4 and IFN-γ were quantified in the supernatants by means of specific ELISA (e Bioscience) according to the manufacturer’s recommendations.

2.9. Statistical Analysis

Because of a non-normal distribution of most of the data the nonparametric Wilcoxon signed-rank test was used. This test allowed to compare data from cultures in the absence and presence of lactobacillus rhamnosus and to compare data from cultures of phycocyanin. When p≤0.05, the difference was considered to be statistically significant The statistical analysis was performed using SPSS software (version 15.0; SPSS Inc., Chicago).

3. RESULTS

3.1. Standardization Experiments

As multiple factors were involved in the study, standardization experiments were done on 3 cases to select the optimum conditions:

- Concentration of the allergen: 1:10, 1:100 and 1:1000 concentration s were examined, the concentration 1:1000 were the optimum one in terms cytokine induction
- Time of incubation: 48, 72, 96 h of incubation were examined. Incubation for 72 h were the optimum regarding cytokine production and cell viability
- Phycoerythrin concentration: We examined 90 and 45 ug per mL and found that 90 ug per mL were the optimum in terms of cytokine production.

3.1.1. Effects Date Pollen Allergen on Cytokine Production by PBMCs of Allergic Patients. (Table 1, Fig 1)

Significant increase in IL4 level occurred when PBMCs incubated with the allergen compared to the basal level. Significant reduction in IFN gamma occurred when PBMCs incubated with the allergen compared to the basal level.

3.1.2. Effects of Living Lactobacillus Rhamnosus on Cytokine Production by PBMCs of Allergic Patients (Table 3, 4, Fig 1)

Significant reduction in IL4 production obtained when PBMCs of allergic patients were incubated with living lactobacilli rhamnosus in the presence or absence of the offending allergen. Interferon gamma significantly increased when PBMCs incubated with living lactobacilli alone. However, PBMCs incubated with living lactobacilli in the presence of the allergen showed insignificant difference in interferon gamma production.

3.1.3. Effects of Dead Lactobacillus Rhamnosus on Cytokine Production by PBMCs of Allergic Patients (Table 5, 6 and Fig. 1)

Significant reduction in IL4 production occurred when PBMCs of allergic patients were incubated with dead lactobacilli in the presence or absence of the offending allergen. Interferon gamma significantly increased when PBMCs incubated with dead lactobacilli in the presence or absence of the allergen.

3.1.4. Effects of PC on Cytokine Production by PBMCs of Allergic Patients. (Table 7, 8 and Fig. 1)

Significant reduction in IL4 production happened when PBMCs of allergic patients were incubated with PC in the presence or absence of the offending allergen. Interferon gamma significantly increased when PBMCs incubated with PC in the presence or absence of allergen.
### Table 1. Effect of date pollen allergen on IL4 production by allergic patients PBMCS

| Condition 1                  | Condition 2                  | W for N = 24 | Critical value at p≤0.05 | Significance |
|------------------------------|------------------------------|--------------|--------------------------|--------------|
| Basal IL4                    | PBMCs+ allergen              | 0            | 81                       | Sig.         |
| (median: 1.25)               | (median: 52.6)               |              |                          |              |

### Table 2. Effect of date pollen allergen on IFN gamma production by allergic patients PBMCS

| Condition 1                  | Condition 2                  | W for N = 24 | Critical value at p≤0.05 | Significance |
|------------------------------|------------------------------|--------------|--------------------------|--------------|
| Basal IFNγ                   | PBMCs+ allergen              | 34           | 81                       | Sig.         |
| (median: 22.8)               | (median: 39.5)               |              |                          |              |

### Table 3. Effect of Living *Lactobacillus rhamnosus* on IL4 production by allergic patients PBMCS

| Condition 1                  | Condition 2                  | W for N = 24 | Critical value at p≤0.05 | Significance |
|------------------------------|------------------------------|--------------|--------------------------|--------------|
| PBMCs+allergen               | PBMCs+ Living LAB            | 11           | 81                       | Sig.         |
| (median: 52.6)               | (median: 3.9)                |              |                          |              |
| PBMCs+allergen               | PBMCs+ Living LAB+ allergen   | 50           | 81                       | Sig.         |
| (median: 52.6)               | (median: 18.5)               |              |                          |              |

### Table 4. Effect of living *Lactobacillus rhamnosus* on IFN gamma production by allergic patients PBMCS

| Condition 1                  | Condition 2                  | W for N = 24 | Critical value at p≤0.05 | Significance |
|------------------------------|------------------------------|--------------|--------------------------|--------------|
| PBMCs+allergen               | PBMCs+ Living LAB            | 27           | 81                       | Sig.         |
| (median: 22.8)               | (median: 42.75)              |              |                          |              |
| PBMCs+allergen               | PBMCs+ Living LAB+ allergen   | 90           | 81                       | Non Sig.     |
| (median: 22.8)               | (median: 28.7)               |              |                          |              |

### Table 5. Effect of dead *Lactobacillus rhamnosus* on IL4 production by allergic patients PBMCS

| Condition 1                  | Condition 2                  | W for N = 24 | Critical value at p≤0.05 | Significance |
|------------------------------|------------------------------|--------------|--------------------------|--------------|
| PBMCs+allergen               | PBMCs+ Dead LAB              | 3            | 81                       | Sig.         |
| (median: 52.6)               | (median: 3.6)                |              |                          |              |
| PBMCs+allergen               | PBMCs+ Dead LAB+ allergen     | 26           | 81                       | Sig.         |
| (median: 52.6)               | (median: 10.6)               |              |                          |              |

### Table 6. Effect of dead *Lactobacillus rhamnosus* on IFN gamma production by allergic patients PBMCSs

| Condition 1                  | Condition 2                  | W for N = 24 | Critical value at p≤0.05 | Significance |
|------------------------------|------------------------------|--------------|--------------------------|--------------|
| PBMCs+allergen               | PBMCs+ Dead LAB              | 7            | 81                       | Sig.         |
| (median: 22.8)               | (median: 49)                 |              |                          |              |
| PBMCs+allergen               | PBMCs+ Dead LAB+ allergen     | 72           | 81                       | Sig.         |
| (median: 22.8)               | (median: 36.4)               |              |                          |              |

### Table 7. Effect of PC on IL4 production by allergic patients PBMCSs

| Condition 1                  | Condition 2                  | W for N = 24 | Critical value at p≤0.05 | Significance |
|------------------------------|------------------------------|--------------|--------------------------|--------------|
| PBMCs+allergen               | PBMCs+ PC                    | 20           | 81                       | Sig.         |
| (median: 52.6)               | (median: 17)                 |              |                          |              |
| PBMCs+allergen               | PBMCs+PC+allergen            | 37           | 81                       | Sig.         |
| (median: 52.6)               | (median: 19.8)               |              |                          |              |

### Table 8. Effect of PC on IFN gamma production by allergic patients PBMCSs

| Condition 1                  | Condition 2                  | W for N = 24 | Critical value at p≤0.05 | Significance |
|------------------------------|------------------------------|--------------|--------------------------|--------------|
| PBMCs+allergen               | PBMCs+ PC                    | 0            | 81                       | Sig.         |
| (median: 22.8)               | (median: 319.6)              |              |                          |              |
| PBMCs+allergen               | PBMCs+PC+allergen            | 73           | 81                       | Sig.         |
| (median: 22.8)               | (median: 50.6)               |              |                          |              |
Fig. 1. Median IL-4 concentration (in picograms) in supernatant after 72 h in allergic patients

Fig. 2. Median IFN gamma concentration (in picograms) in supernatant after 72 h in allergic patients
Fig. 3. Median IL-4 concentration (in picograms) in supernatant after 72 h in control subjects.

Fig. 4. Median IFN gamma concentration (in picograms) in supernatant after 72 h in control subjects.
3.1.5. Effects of living *Lactobacillus Rhamnosus* on Cytokine Production by PBMCs of Control Subjects. (Table 9, 10 and Fig. 3, 4)

IL4 was significantly decreased when PBMCs incubated with living lactobacilli alone, however, PBMCs incubated with living lactobacilli in the presence of the allergen showed insignificant difference in IL4.

Interferon gamma significantly increased when PBMCs incubated with living lactobacilli alone. Incubation of PBMCs with living lactobacilli and the allergen showed significant IFN gamma reduction.

3.1.6. Effects of dead *Lactobacillus Rhamnosus* on Cytokine Production by PBMCs of Control Subjects. (Table 11, 12 and Fig. 3, 4)

IL4 was significantly decreased when PBMCs incubated with dead lactobacilli in the presence or absence of the allergen.

Interferon gamma significantly increased when PBMCs incubated with dead lactobacilli alone. Simultaneous incubation of PBMCs with dead lactobacilli and the allergen revealed insignificant difference in IFN gamma production.

3.1.7. Effects of PC on Cytokine Production by PBMCs of Control Subjects. (Table 13, 14 and Fig. 5)

PC induced significant IL4 increase in the presence or absence of pollen allergen. PBMCs incubated with PC produced significantly increased IFN $\gamma$ while PBMCs stimulated with both PC and pollen allergen showed significant decrease of IFN $\gamma$. However, the overall IFN$\gamma$/IL4 (Fig. 5) ratio showed significant increase (median 28.8) when PBMCs incubated with PC compared to PBMCs challenged with allergen (median 10)(W = 0, $p \leq 0.05$) Fig. 5.

Table 9. Effect of living lactobacilli on IL4 production by control subject PBMCs

| Condition 1       | Condition 2                  | W for N = 16 | Critical value at $p \leq 0.05$ | Significance |
|-------------------|-------------------------------|--------------|---------------------------------|--------------|
| PBMCs + allergen  | PBMCs + Living LAB            | 0            | 29                              | Sig.         |
| (median: 6.2)     | (median: 1.2)                 |              |                                 |              |
| PBMCs + allergen  | PBMCs + Living LAB + allergen | 31           | 29                              | Non Sig.     |
| (median: 6.2)     | (median: 3.1)                 |              |                                 |              |

Table 10. Effect of living lactobacilli on IFN gamma production by control subject PBMCs

| Condition 1       | Condition 2                  | W for N = 16 | Critical value at $p \leq 0.05$ | Significance |
|-------------------|-------------------------------|--------------|---------------------------------|--------------|
| PBMCs + allergen  | PBMCs + Living LAB            | 0            | 29                              | Sig.         |
| (median: 58.5)    | (median: 251.2)               |              |                                 |              |
| PBMCs + allergen  | PBMCs + Living LAB + allergen | 0            | 29                              | Sig.         |
| (median: 58.5)    | (median: 30.5)                |              |                                 |              |
| Basal PBMCs       | Basal PBMCs                  | 0            | 29                              | Sig.         |
| (median: 397.4)   | (median: 251.2)               |              |                                 |              |

Table 11. Effect of dead lactobacilli on IL4 production by control subject PBMCs

| Condition 1       | Condition 2                  | W for N = 16 | Critical value at $p \leq 0.05$ | Significance |
|-------------------|-------------------------------|--------------|---------------------------------|--------------|
| PBMCs + allergen  | PBMCs + Dead LAB              | 0            | 29                              | Sig.         |
| (median: 6.2)     | (median: 2.17)                |              |                                 |              |
| PBMCs + allergen  | PBMCs + Dead LAB + allergen   | 1            | 29                              | Sig.         |
| (median: 6.2)     | (median: 2.6)                 |              |                                 |              |

Table 12. Effect of dead lactobacilli on IFN gamma production by control subject PBMCs

| Condition 1       | Condition 2                  | W for N = 16 | Critical value at $p \leq 0.05$ | Significance |
|-------------------|-------------------------------|--------------|---------------------------------|--------------|
| PBMCs + allergen  | PBMCs + Dead LAB              | 0            | 29                              | Sig.         |
| (median: 58.5)    | (median: 345.2)               |              |                                 |              |
| PBMCs + allergen  | PBMCs + Dead LAB + allergen   | 31           | 29                              | Non Sig.     |
| (median: 58.5)    | (median: 30.3)                |              |                                 |              |
Table 13. Effect of PC on IL4 production by control subject PBMCS

| Condition 1       | Condition 2          | W for N = 16 | Critical value at p≤0.05 | Significance |
|-------------------|----------------------|--------------|--------------------------|--------------|
| PBMCS+allergen    | PBMCS+ PC            | 0            | 29                       | Sig.         |
| (median:6.2)      | (median: 14.7)       |              |                          |              |
| PBMCS+allergen    | PBMCS+PC+allergen    | 0            | 29                       | Sig.         |
| (median:6.2)      | (median: 28.4)       |              |                          |              |

Table 14. Testing of significant differences IFN gamma production by control subjects PBMCS at different conditions

| Condition 1       | Condition 2          | W for N = 16 | Critical value at p≤0.05 | Significance |
|-------------------|----------------------|--------------|--------------------------|--------------|
| PBMCS+allergen    | PBMCS+ PC            | 0            | 29                       | Sig.         |
| (median:58.5)     | (median: 414)        |              |                          |              |
| PBMCS+allergen    | PBMCS+PC+allergen    | 0            | 29                       | Sig.         |
| (median:58.5)     | (median: 28.2)       |              |                          |              |

Fig. 5. IFN γ/IL4 ratio. after 72 h incubation (PC, control subjects)

4. DISCUSSION

This study tried to investigate the effects of *Lactobacillus rhamnosus* ATCC 7469 (living and dead) and phycocyanin extracted from *Spirulina platensis* on TH1/TH2 paradigm. The peripheral blood mononuclear cells system was used to evaluate theses effects.

The current study revealed that simulation of PBMCs of allergic patients with the offending allergen (date pollen) induced significant increase of IL4 level and significant reduction of IFNγ level when compared to the basal levels (unstimulated PBMCs), this could be explained by polarization of immune system of allergic patients toward TH2, more IL4 and less IFNγ (*Table 1 and 2*). This observation was previously documented by Imada *et al.* (1995), they found that IL-4 production was more significantly increased among atopic individuals, than normal subjects when their PBMCs stimulated by pollen allergen.

The resultant TH2 shifted response of allergic patients indicates the role of impaired TH1/TH2 balance in allergic diseases and was proved by (Pochard *et al.*, 2002; Ghadimi *et al.*, 2008), their experiments included challenging of PBMCs of patients allergic to house dust mite (Dpt allergen). IFN-γ mRNA expression and production was reported to be reduced in atopic subjects (Parronchi *et al.*, 1991) during *in vitro* stimulation experiments.

Bullens *et al.* (2005) showed that challenge with rDer p 2 resulted in TH2 cytokine production in
cultures of PBMC from allergic but not from healthy children. In contrast, IL-10 and IFN-γ were induced in cultures from both allergic and nonallergic children.

There was significant reduction of IL4 production when PBMCs of allergic patients incubated with living *Lactobacillus rhamnosus* in the presence or absence of the allergen. The reduction was more significant when PBMC incubated with *lactobacillus rhamnosus* in absence of the offending allergen (Table 3).

Interferon gamma significantly increased when PBMCs incubated with living lactobacilli alone. However, PBMCs incubated with living lactobacilli in the presence of the allergen showed insignificant difference in interferon gamma production (Table 4). This may be due to the TH2 shift of the allergic patients when their PBMC challenged with this probiotic bacterial strain in the presence of the offending allergen, meaning, the presence of the specific allergen prevents *Lactobacillus rhamnosus* from exerting their effect on IFN gamma, the predominant cytokine (IL4) downregulates IFNγ production. This was in accordance with Pohjavuori et al. (2004), they observed that that peripheral blood mononuclear cells in patients with atopic disease have a reduced TH1 cytokine IFNγ secretion capacity.

Heat-killed *Lactobacillus rhamnosus* was examined to evaluate the immunomodulatory effects of the dead strain, the results obtained were more or less similar to those of living bacteria. Significant reduction in IL4 production when PBMCs of allergic patients were incubated with dead lactobacilli in the presence or absence of the offending allergen (Table 5). Interferon gamma significantly increased when PBMCs incubated with dead lactobacilli in the presence or absence of the allergen (Table 6).

These results are quite similar to those obtained by Pochard et al. (2002), they found that the level of IL4 inhibition was not affected by the physiologic state of the bacteria because live bacteria induced the same effect as heat-killed or paraformaldehyde-treated LAB. This observation may point to the ability of lactobacilli to modulate the immune response via their cell wall composition, namely by their recognition by PRR. Toll-Like Receptors (TLR) are important PRRs that recognize a range of MAMPs such as Lipoteichoic acid (TLR2) and Lipopolysaccharide (TLR4) on Gram-positive and Gram-negative bacteria, respectively. Taylor et al. (2006) found that TLR2 ligands had the capacity to inhibit TH2 cytokine production by mononuclear cells stimulated with mite allergen. The inhibition of TH2 cytokine production was observed with a variety of TLR2 ligands, including high and low concentrations of heat-killed *S. aureus*, LTA and Pam3CSK4. Pinto et al. (2009) reported that *L. rhamnosus* GG and *L. plantarum* BFE 1685, enhanced TLR2 at both the mRNA and the protein level in human intestinal cells.

The ability of *L. lactis* subsp. Lactis G50 killed by heat to induce cytokine production of macrophages in culture, was previously proved by Kimoto et al. (2004), it was observed that this strain continued to induce cytokine production, suggesting that such activity is associated with elements on the bacterial wall.

Ghadimi et al. (2008) proved that *Lactobacillus rhamnosus* GG, *Lactobacillus gaseri* (PA16/6), *Bifidobacterium bifidum* (MP20/S) and *Bifidobacterium longum* (SP07/3), as well as their genomic DNA, dose-dependently modulated the TH1/TH2 response to allergens. DNA seemed to contribute to about 50% of the immunomodulatory effects exerted by live bacteria.

Lactobacilli can also affect cytokine production by PBMCs of control subjects, when PBMCs of the controls challenged only by living lactobacilli IL4 was significantly decreased compared to its level after stimulation with the tested allergen. This finding was in accordance with Rutten et al. (2011), they reported that IL4 was significantly reduced when healthy PBMCs stimulated by different lactobacillus strain compared to cultures stimulated with Phytoheamagglutinin (PHA).

Simultaneous stimulation of control PBMCs with living lactobacilli and the tested allergen (pollen allergen) resulted in insignificant IL4 difference.

Interferon gamma significantly increased when PBMCs incubated with living lactobacilli alone. Incubation of PBMCs with living lactobacilli and the allergen showed significant IFN gamma reduction. These findings were similar to those obtained by Rasche et al. (2007), they co-stimulated peripheral mononuclear cells of individuals allergic to grass pollen and those non-allergic with inactive *Lactobacillus acidophilus* and the non-pathogenic Nissle strain of *Escherichia*, they reported that stimulation with lactobacilli plus allergen resulted in a TH2-like response in allergic and non-allergic individuals.

Other studies, Rutten et al. (2011), found that probiotic mixtures were able to induce significant amounts of IFN-γ compared to PBMCs which were cultured in medium only (for the four mixtures together compared to unstimulated medium).

So, the effect of lactobacilli on PBMC of healthy controls seems to be strain specific and could be really
affected by the PBMC: Bacteria ratio and the type of used antigen during stimulation experiments.

IL-4 was significantly decreased when PBMCs incubated with dead lactobacilli in the presence or absence of the allergen (Table 11). Interferon gamma significantly increased when PBMCs incubated with dead lactobacilli alone. Simultaneous incubation of PBMCs with dead lactobacilli and the allergen revealed insignificant difference in IFN gamma production (Table 12).

To date, the real pathways of probiotic immunomodulatory effects are not fully understood and some types of immune cells that are primed by probiotics might be the connection between in vivo and in vitro stimulation.

The overall effect of Lactobacillus rhamnosus was skewing of the cytokine production toward TH1. More than one mechanism could be involved, PBMCs have been used widely for screening of the ability of probiotics to induce cytokine production. In vitro studies using PBMCs from allergic patients have shown reduced expression of TH2-associated cytokines (IL-4 and IL-5) on stimulation with total extract of Dermatophagoides pteronyssinus (house dust mite) and prior treatment with lactic acid bacteria strains such as Lactobacillus plantarum (Pochard et al., 2002). Both Lactococcus lactis and Lactobacillus plantarum, induce high levels of IL-12 and IFNγ, suppressing Th2 differentiation (Ghadimi et al., 2008). In contrast, suppression of contact dermatitis in mice has been shown to be mediated by a Lactobacillus acidophilus L92-dependent generation of Tregs (Shah et al., 2012). This effect is thought to result from strain-dependent tolerisation of DCs, increasing suppressor activity of natural Tregs as well as inducing Foxp3+conversion through high expression of IL-10, TGFβ, COX-2 and indoleamine 2,3-dioxygenase (Kwon et al., 2010).

Several studies, focussing on probiotic administration in type-1 hypersensitivity responses, revealed inhibitory characteristics for both Lactobacillus and Bifidobacterium strains. Mouse models have demonstrated the ability of L. casei and B. longum to inhibit IgE production (Schiffer et al., 2009). Inhibition of antibody production prevents binding to Fc receptors on mast cells, thus inhibiting the secretion of vasoactive amines, such as histamines and other inflammatory mediators, such as TNF-α. TNF-α production was found to be inhibited by L. reuteri (Thomas et al., 2012).

The inhibition of IgE production is thought to be a consequence of direct action by probiotics on TH2 cells or APCs, which prime B-cell activation and class-switching. A large body of evidence demonstrates a role for Lactobacillus and Bifidobacterium strains in decreasing the levels of secreted IL-4 and IL-5. Both cytokines are TH2-derived, with IL-4 acting on B-cells to induce class-switching and on mast cells to induce degranulation and further cytokine production and IL-5 inducing eosinophil degranulation. Specific strains found to inhibit IL-4 and IL-5 production include L. casei (Schiffer et al., 2009), L. rhamnosus (Ghadimi et al., 2008), B. longum (Takahashi et al., 2006) and B. infantis (Dev et al., 2008).

L. casei treatment in mice inhibited IgE production by inhibition of Syk/Lyn and MAPK signalling (Schiffer et al., 2009).

Evrard et al. (2011) found that the probiotic L. rhamnosus Lcr35 induces a dose-dependent immunomodulation of human DCs leading, at high bacterial doses, to the semi-maturation of the cells and a strong synthesis of pro-TH1/TH17 cytokines.

The current present study also evaluated the effect of Spirulina platensis on cytokine production of PBMCs of allergic patients and control subjects. Previous studies on immunomodulatory effects of spirulina proved that the photosynthetic pigment phycocyanin has a part to play in modulating the immune system.

Allergic PBMCs challenged with PC in the presence or absence of the offending allergen resulted in significant IL4 reduction and IFNγ increase, denoting TH1 polarizing effect of this compound (Table 7 and 8). Mao et al. (2005) had shown that allergic patients consuming 2,000 mg of Spirulina daily can reduce the production of IL-4 from PHA stimulated PBMCs by 32%. Another double-blind, placebo-controlled study from Turkey evaluating the effectiveness and tolerability of Spirulina for treating patients with allergic rhinitis, Spirulina consumption significantly improved the symptoms and physical findings compared with placebo (p<0.001), including nasal discharge, sneezing, nasal congestion and itching (Cingi et al., 2008).

The effect of phycocyanin was quite different on PBMCs of the control subjects, challenging of these PBMCs with PC resulted in significant increase of both IL 4 and levels IFNγ (Table 13 and 14). However, the overall IFNγ/IL4 ratio showed significant increase.
(median 28.8) when PBMC incubated with PC compared to PBMC challenged with allergen (Fig. 5).

Mao et al. (2000) reported that in vitro culture of resting and PHA-stimulated PBMCs of healthy individuals with Spirulina platensis extract significantly increased the levels of IL-4. Although Spirulina platensis stimulates several cytokines, it is clearly more effective in the generation of a Th1-type response. Basha et al. (2009), also proved that PC had induced significant elevation of IFN γ level from PBMC of healthy subjects and chronic HCV patients.

Subhashini et al. (2004) found that PC had an inhibitory effect on the release of histamine from mast cells during an allergic inflammatory response. Lactobacilli and bifidobacteria are generally considered to be safe (FAO/WHO, 2002). They have been used in various types of foods for a long time and they rarely cause infections in humans. The numbers of reported lactobacilli-induced bacteremia have not increased, despite the rapid increase of probiotic use the last decade (Salminen et al., 2002).

In a Swedish study, the incidence of lactobacilli-induced bacteremia and the presence in blood cultures of three commercially available probiotic strains were followed for five years. The incidence of bacteremia caused by lactobacilli constituted <1% of the total number of bacteremia cases with no increase during this five year-period. Lactobacilli-induced bacteremia was not caused by any of the three commercially available strains in any of the reported cases (Sullivan and Nord, 2006).

It has been suggested that probiotics should be used with caution in patients who are immunocompromised, have cardiac valvular disease, short bowel, jejunostomy or a central venous catheter (Besselink et al., 2008). No severe adverse events have been reported in any of the intervention studies performed in full term neonates and healthy infants (Boyle et al., 2008).

Although the effective dosage range of Phycocyanin in various animal models of inflammation was from 25 to 300 mg kg⁻¹ p.o, the safety of the phycobiliprotein is good. The measured LD50 values were estimated to be greater than 3 g kg⁻¹ for rats and mice (Romay et al., 1998).

5. CONCLUSION

In this study we showed that Lactobacillus rhamnosus ATCC 7469 strain can modulate the Th1/Th2 balance by reducing TH2 cytokine (IL4) release and enhancing TH1 cytokine production (IFN-γ). The same effect was also obtained by the phycocyanin, pigmented extract from the cyanobacterium Spirulina platensis. The mechanism by which Lactobacillus rhamnosus exerted these effect seemed to be their cell wall composition, as the results obtained by dead bacteria were similar to those obtained by the living ones.

As the best anti-allergic effects of these bacteria were obtained when acting in absence of the allergen, it is better to use this bacteria in the prophylaxis rather than in treatment of allergic attacks. As the bacteria gave quite similar results in patients and healthy subjects, we assume that it could be a permissive line of prevention of allergy.

The safety of lactobacilli is very good, it could be the future treatment for allergy.

PC is good IFN-γ inducer in both allergic and control subjects. As Spirulina and its content C-phycocyanin are generally safe, in vivo expirment to evaluate their effect on allergic patients may be a new promising anti allergic therapy.

6. RECOMMENDATIONS

Further studies are needed to determine the exact source of IFN γ, using ELISpot or flowcytometry. Both living and dead Lactobacillus rhamnosus ATCC 7469 should be considered as new line of allergy prevention.

C-phycocyanin gave promising results in allergic patients and could be a new line of treatment of allergy.

Further studies on animals and human subjects are needed.

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