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Immunosuppressive Effects of Two Probiotics, *Lactobacillus Paracasei* DSM 13434 and *Lactobacillus Plantarum* DSM 15312, on CD4$^+$ T Cells of Multiple Sclerosis Patients

Khadijeh Chakamian[1], Behrouz Robat-Jazi[2], Abdorreza Naser Moghadasi[3], Fatemeh Mansouri[2], Masoumeh Nodehi[4], Elahe motevaseli[5], Maryam Izad[3,6], Saeed Yekaninejad[7], Mahdieh Shirzad[8], Kiana Bidad[2], Mona Oraei[2], Bita Ansaripour[6], and Ali Akbar Saboor-Yaraghi[2]

[1] Food Microbiology Research Center, Tehran University of Medical Sciences, Tehran, Iran
[2] Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[3] Multiple Sclerosis Research Center, Neuroscience Institute, Tehran University of Medical Sciences, Tehran, Iran
[4] Department of Immunology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran
[5] Department of Molecular Medicine, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran
[6] Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[7] Department of Epidemiology and Biostatistics, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[8] Department of Microbial Biotechnology, School of Biology and Center of Excellence in Phylogeny of Living Organisms, College of Science, University of Tehran, Tehran, Iran

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ABSTRACT

T cells play an important role in the development and progression of multiple sclerosis (MS), an autoimmune disease of the central nervous system. In the present study, the immunomodulatory impacts of two *Lactobacillus* strains, *L paracasei* DSM 13434 and *L plantarum* DSM 15312, on the frequency and cytokine production of CD4$^+$ T cells in MS patients were explored.

Thirty MS patients were enrolled in this study. The CD4$^+$ T cells were isolated, cultured, and exposed to the media containing cell-free supernatants of *L plantarum* (group1), *L paracasei* (group 2), the mixture group of cell-free supernatants of both probiotics (group 3), and vehicle (control) group (group 4). The frequencies of T helper (Th) 1, Th17, Th2, and T regulatory type 1 (Tr1) cells and mean fluorescent intensity (MFI) of the associated cytokines were assessed using flow cytometry. The levels of interleukin 17 (IL-17), transforming growth factor β (TGF-β), and interferon-gamma (IFN-γ) cytokines in supernatants of all groups were measured by enzyme-linked immunosorbent assay.

The percentage of Th1 cells and the MFI of IFN-γ in Th1 cells (CD4+ IFN-γ+) in all three probiotic treatment groups were significantly decreased compared to the control group. However, no significant changes were observed in the proportion and MFI of Th2, Th17, and Tr1 cells. A

Corresponding Author: Ali Akbar Saboor-Yaraghi, PhD; Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. Tel: (+98 21) 4293 3168 Fax: (+98 21) 8895 4913 E-mail: asaboor@tums.ac.ir

significant decrease was observed in IL-17 secretion in the supernatant of cultured CD4+ T cells in all three treatment groups in comparison with control. The levels of TGF-β and IFN-γ were not significantly different among any of the study groups.

Collectively, cell-free supernatants of the lactobacilli showed an in vitro anti-inflammatory effect. However, further studies are needed to prove the real effects of probiotics on MS.

Keywords: Immunoregulation; *Lactobacillus paracasei*; *Lactobacillus plantarum*; Multiple sclerosis; Probiotics

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disorder of the central nervous system (CNS) that is characterized by axonal damage, demyelination, and inflammatory lesions. It has been suggested that activation of T helper (Th)1 (the interferon-gamma [IFN-γ]-producing cells), and Th17 cells (interleukin [IL]-17 producing cells) is involved in the pathogenesis of MS and experimental autoimmune encephalomyelitis (EAE).[1-4] Regulatory T (Treg) cells are an activated subset of CD4+ T cells that express high levels of CD25 and forkhead box transcription factor 3 (FOXP3). These cells modulate the function of the immune system by producing anti-inflammatory cytokines mainly transforming growth factor-beta (TGF-β) and interleukin-10 (IL-10), cell-to-cell contact, and impact on antigen-presenting cells (APC).[5] Previous studies demonstrated that in MS patients, the capacity of Treg cells to suppress the proliferation of other cells and the production of inflammatory cytokines had been downregulated.[6,7]

The outcomes of many studies have suggested that the gut microbiota contributes to the incidence and progression of several autoimmune disorders, such as MS, inflammatory bowel disease, and rheumatoid arthritis.[6-10] The effect of gut microbiota on MS progression has been demonstrated in EAE mice. The gut microbiota can regulate the blood-brain barrier (BBB) permeability, microglial activation, and myelin-related genes expression, mainly by secreting metabolites and bacterial antigens.[11-14]

According to the definition presented by Food and Agriculture Organization /World Health Organization (FAO/WHO) in 2001, probiotics are "live microorganisms or microbial mixtures that create a health condition in the host if administered in appropriate doses".[15] Gut microbiota, is the main source of probiotics inside the body. The probiotics exert their benefits through several mechanisms, such as pro- and anti-inflammatory balance, repairing the gastrointestinal barrier, and maintaining commensal bacteria homeostasis.[16-18] Immunoregulatory effects of probiotics, especially *Lactobacillus* strains, have previously been investigated in animal models and autoimmune diseases, such as arthritis,[19,20] colitis,[21,22] diabetes,[23] and MS.[4,10,18,24-29]

Two probiotic agents, including *L paracasei* DSM 13434 and *L plantarum* DSM 15312, are established inside the gastrointestinal tract after ingestion. The administration of these probiotics changes the microbial balance of the intestine, and by stimulating the goblet cells, they increase the secretion of mucin and reduce the growth of pathogens. Probiotics regulate the immune system in different ways. They decrease and increase the secretion of proinflammatory and anti-inflammatory cytokines, respectively, through their effect on the toll-like receptors, endothelial cells, dendritic cells, and macrophages, which drive the differentiation of immune cells toward regulatory cells. After reacting with the immune system, probiotics secrete the two anti-inflammatory cytokines, TGF-β and IL-10, into the gastrointestinal tract, which leads to the differentiation of Treg cells. In addition, probiotics inhibit the movement of pathogens from the intestine to the blood by increasing the efficiency of tight junction proteins on the surface of the intestinal lumen. All the above-mentioned factors induced by probiotics lead to an increase in the activity of Treg and Th2 cells and the release of anti-inflammatory cytokines IL-4, IL-10, TGF-β1, and on the other hand, they decrease the activity of Th1 and Th17 cells and also decrease Inflammatory cytokines, IL-17, TNF-α, and IFN-γ are secreted. As a result, these changes reduce the recruitment of an aggressive subset of $CD4^+$ T cells to destroy the CNS tissues.[30-32]

The present study is an in vitro investigation of the effect of two *Lactobacillus* strains on the frequency of $CD4^+$ T cell subsets and their cytokine production in

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peripheral blood mononuclear cells (PBMCs) of MS patients.

MATERIALS AND METHODS

Patients

Thirty well-documented relapsing-remitting MS patients, 6 men and 24 women with a mean age of 25.34±9.21; range 20–40 years) were enrolled in this study. Cases were selected from patients undergoing monthly follow-ups at Sina Hospital, Tehran, Iran. The diagnosis of relapsing-remitting MS was based on the McDonald criteria of 2017, and the EDSS (Expanded Disability Status Scale) score of the patients was between 0 and 5. EDSS provides a total score on a scale that ranges from 0 to 10. The initial levels of 1.0 to 4.5 and subsequent levels of 5.0 to 9.5 refer to people with a high degree of diability and the loss of ambulatory ability, respectively. The patients were introduced and enrolled in this study by neurologists according to their clinical characteristics and magnetic resonance imaging results. Pregnant women, patients with cancer, infectious diseases, endocrine disorders, or other autoimmune diseases and those who did not want to participate were excluded from the study. This study was approved by the Ethics Committee of Tehran University of Medical Sciences (approval code: IR.TUMS.SPH.REC.1395.1415). Before sampling, the study was explained to the patients, and all patients filled out an informed written consent form.

Cell-free Culture Supernatant Preparation

The probiotics, *L paracasei* DSM 13434 and *L plantarum* DSM 15312, were purchased from Biological Resource Center (BRC), Tehran, Iran. Prior to the experiment, the lyophilized cultures were activated in de Man, Rogosa, and Sharpe (MRS) broth at 37°C for 48 h. Bacteria were further grown on MRS agar. The bacterial cell concentration was standardized by enumeration as colony-forming units per milliliter (cfu/mL) by the pour plate technique. To prepare cell-free culture supernatants, each culture was centrifuged at 8000*g* for 5 min. Supernatants were isolated and filtered using 0.22-μm pore size filters (Millipore, Billerica, MA) to eliminate the possible presence of viable cells and debris.

Peripheral Blood Mononuclear Cell and $CD4^+$ T Cells Isolation

Heparinized venous blood was collected from all patients. The PBMCs were isolated by Ficoll density gradient centrifugation using Lymphodex (inno-train, Germany) and washed twice with phosphate-buffered saline (PBS) at pH 7.2. The $CD4^+$ T cells were isolated from PBMCs by immunomagnetic beads using magnetic-activated cell isolation (MACS) $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec, Germany) according to the manufacturer's instructions.

Cell Culture

The isolated $CD4^+$ T cells were cultured in RPMI (Sigma, Germany), containing 10% FBS(Sigma, Germany) for 72 h in 24-well plates ($1x10^6$ per well), stimulated with 4 μg/mL anti-CD3 and 2 μg/mL anti-CD28. The cells were cultured in media containing cell-free supernatants of *L plantarum* (group 1), *L paracasei* (group 2), the mixture group of cell-free supernatants of both probiotics (group 3), and the vehicle (control) group (group 4).

Flow Cytometry

After 72 h of culture, cells were treated with stimulation cocktail (eBioscience™ Cell Stimulation Cocktail (500X), USA). This contained 50 ng/mL, 1 μg/mL, 1.4 μg/mL, 3 μg/mL of phorbol myristate acetate, ionomycin, monensin and brefeldin, respectively. The cells were incubated for 6 h and then transferred to another tube, washed with PBS at pH 7.2, and then centrifuged at 400*g* for 5 min. Stimulated cells were then fixed and permeabilized by fixation and permeabilization buffers (eBioscience™ Intracellular Fixation & Permeabilization Buffer Set, USA). Then, cells were stained using the following antibodies: allophycocyanin anti-human IL-4 (eBioscience, USA), phycoerythrin anti-human IL-10 (eBioscience, USA), PerCP-Cy5.5 anti-human IL-17 (eBioscience, USA), and fluorescein isothiocyanate anti-human IFN-γ (eBioscience, USA) (Figure 1). Schematic representation of the method process has been shown in Figure 2).

Enzyme-linked Immunosorbent Assay (ELISA)

The cell-free supernatant concentration of secreted cytokines from stimulated $CD4^+$ T cells was assayed using IL-17 (eBioscience, USA), TGF-β (R&D Systems, USA), and IFN-γ (eBioscience, USA) ELISA kits.

Statistical Analysis

Statistical evaluation was performed using the SPSS software (IBM Analytics, USA). All statistical tests

were two-sided, and p values<0.05 were considered statistically significant. To compare the frequency and mean fluorescence intensity (MFI) of $CD4^+$ T cells in the 4 study groups using repeated measures Analysis of variance (ANOVA). The normality of outcome variables was evaluated by the Kolmogorov-Smirnov test.

RESULTS

Impact of *L plantarum* and *L paracasei* on the Frequency of $CD4^+$ T Cells Subsets

The flow cytometry results showed that the frequency of Th1 cells in the vehicle (control) group (group 4) was significantly higher than that in the other 3 intervention groups (Table 1 and Figure 3). There was no significant difference between the mixture of cell-free supernatants of both probiotics (group 3) and any of the two single-treatment groups (groups 1 and 2).

The percentage of Th17 cells in $CD4^+$ T cells of all treatment groups was lower than that of the vehicle (control) group (group 4); however, these differences were not statistically significant. Moreover, the effect of probiotic treatments on Th2 and Tr1 cell proportions was not significant in repeated measure ANOVA statistical analysis. (Table 1 and Figure 3).

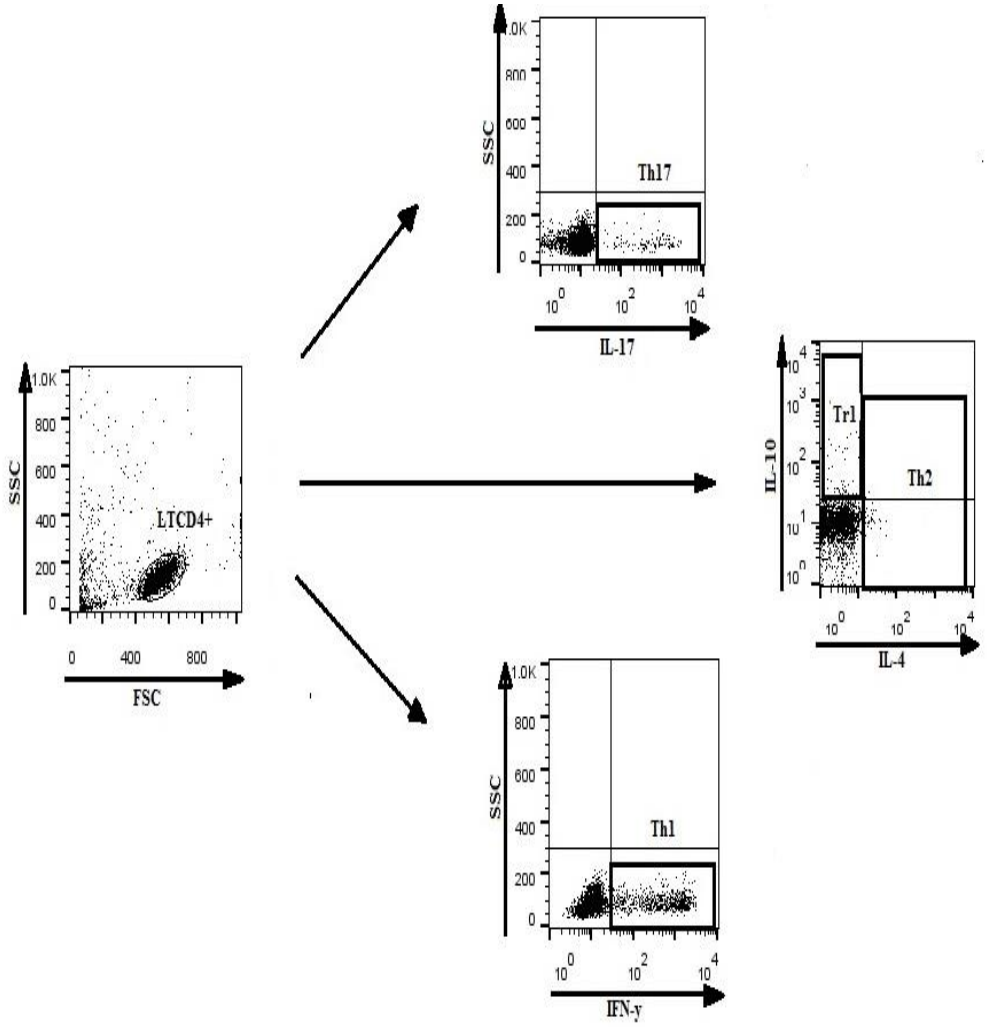

Figure 1. Gating strategy of T helper (Th) 1, Th2, Th17, and type 1 regulatory T cell (Tr1) populations. First, all the lymphocytes were selected according to the size and granularity of the cells, and then they were evaluated by staining with specific antibodies of the cell types of Th1 ($CD4^+$ IFN-γ^+), Th2 ($CD4^+$ IL-4^+), Th17 ($CD4^+$ IL-17^+) and Tr1 ($CD4^+$ IL-4^- IFN-γ^+ IL-10^+) cells.

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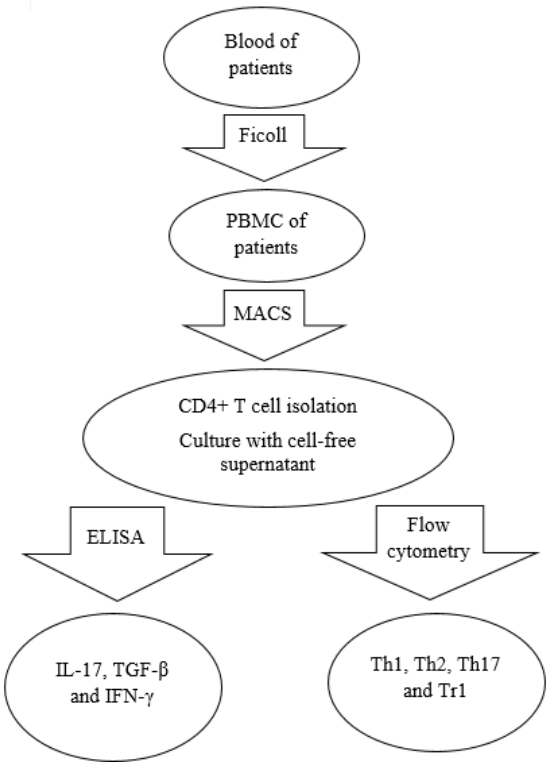

Figure 2. Schematic representation of the method process. First, blood was collected from the patients, and peripheral blood mononuclear cells (PBMC) were separated by Ficoll. Then, T cells were specifically isolated using the magnetic-activated cell isolation (MACS) kit. The isolated $CD4^+$ T cells were stimulated and cultured with the bacterial supernatant and cytokines. Cell phenotypes were evaluated using enzyme-linked immunosorbent assay (ELISA) and flow cytometry.

Effects of *L plantarum* and *L paracasei* on the MFI of IFN-γ, IL-4, IL-17, and IL-10 in CD4+ T Cells Subsets

As the intensity of expressed cytokines is more informative than the proportion of the cytokine-producing cells, we investigated the intensity of IFN-γ, IL-4, IL-17, and IL-10 in $CD4^+$ T cell subsets. The strength of expression of these cytokines was determined by calculating the MFI.

These results demonstrated that the MFI of IFN-γ in Th1 cells ($CD4^+$ IFN-γ^+) in the vehicle (control) group (group 4) was significantly higher than that in other treatment groups. These differences between the vehicle (control) group (group 4) and other treatment groups were significant (Table 1 and Figure 3). The MFI of IL-10 in Tr1 cells ($CD4^+$ IL-4^- IFN-γ^+ IL-10^+), the MFI for IL-17 in Th17 cells ($CD4^+$ IL-17^+), and the MFI for IL-4 in Th2 cells ($CD4^+$ IL-4^+) were not changed significantly in *L plantarum* group (group 1), *L paracasei* group (group 2), and the mixture group of cell-free supernatants of both probiotics (group 3) compared to vehicle (control) group (group 4) (Table 1 and Figure 3).

Effects of *L plantarum* and *L paracasei* on the Level of IL-17, TGF-β, and IFN-γ

The levels of IL-17, TGF-β, and IFN-γ cytokines in cell culture supernatants were determined by ELISA. The results demonstrated a significant decrease in the level of IL-17 in the *L plantarum* group (group 1) (p<0.0005), *L paracasei* group (group 2) (p<0.0005), and the group with cell-free supernatant of both probiotics (group 3) (p<0.0005) compared to the vehicle (control) group (group 4). The level of TGF β and IFN γ cytokines in the cell cultures supernatants of the studied groups was not significantly different from the control group. (Figure 3).

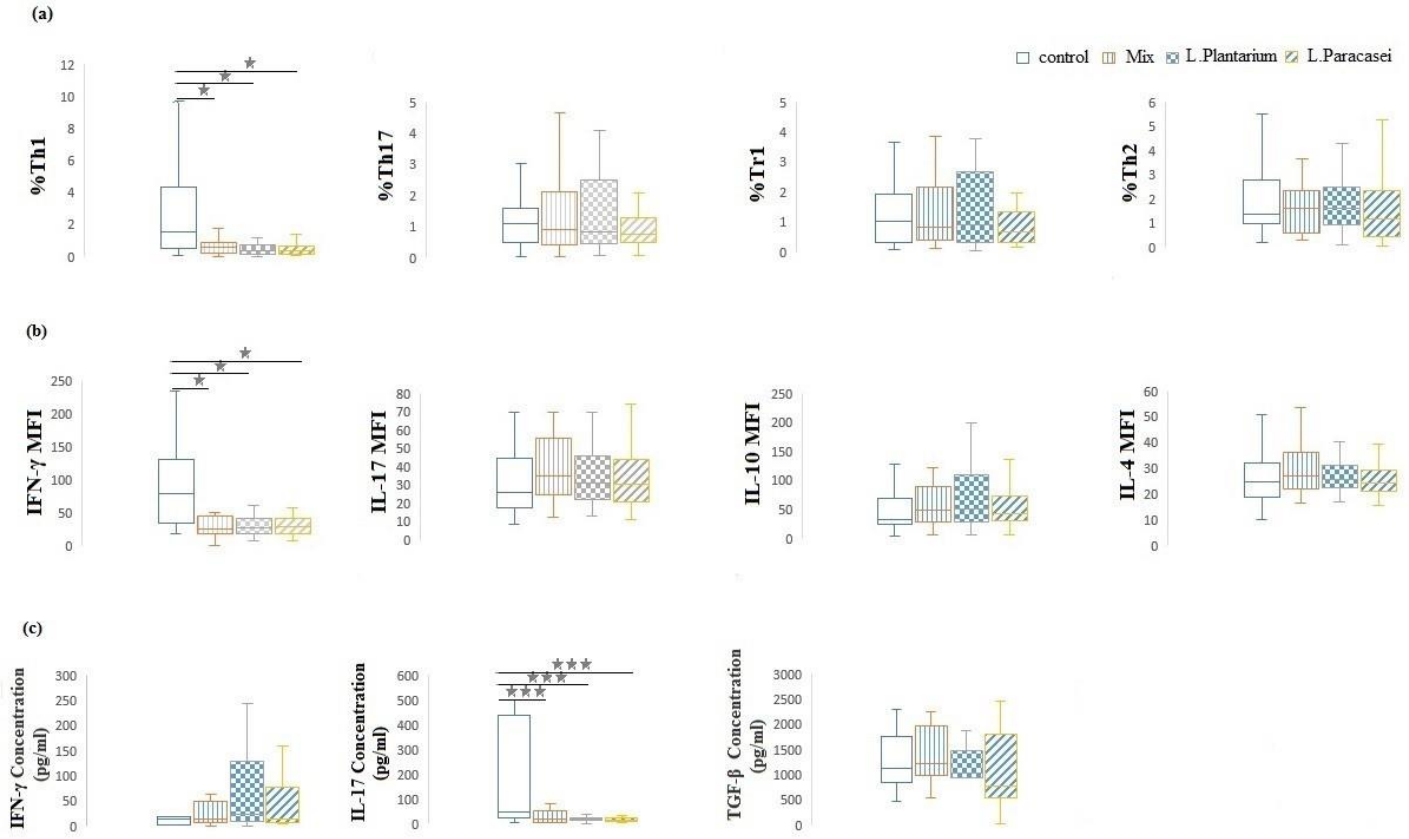

Figure 3. The effects of *L plantarum* and *L paracasei* on the population (a) and mean fluorescent intensity (MFI) (b) of cytokines of $CD4^+$ T-cell subclasses. Moreover, the effects of the probiotic on the level of interleukin (IL)-17, and transforming growth factor β (TGF-β) and interferon-gamma (IFN-γ) in the cell culture supernatant (c) of CD4+ T-cell subclasses. The $CD4^+$ T cells were treated by culture supernatant of *L plantarum, L paracasei,* and their mixture for 72 h. Then, they were stimulated with phorbol myristate acetate and ionomycin for 6 h. Finally, the frequencies of T helper (Th) 1, Th2, Th17, and type 1 regulatory T (Tr1) cells and the MFI of their cytokines were analyzed by flow cytometry, and the levels of IL-17, TGF-β, and IFN-γ in cell culture supernatant were measured by enzyme-linked immunosorbent assay. Th1, Th2, and Th17 cells were identified as IFN-γ, IL-4, and IL-17-secreting $CD4^+$ T cells respectively. Tr1 cells are identified with $CD4^+$ $IL\text{-}10^+$ IL-4.
***p*<0.05, ****p*<0.001 compared to the control group using repeated measures Analysis of variance (ANOVA). Statistical evaluations were performed using SPSS.**

Table 1. Changes in immunological outcomes of the treated groups

Variable	Vehicle (Control)	*L plantarum* and *L paracasei* (Group 3)	*L paracasei* (Group 2)	*L plantarum* (Group 1)	*p**
	Mean (SE)	Mean (SE)	Mean (SE)	Mean (SE)	
Th1 (%)	3.63 (0.94)	0.622 (0.092)	0.599 (0.211)	0.630 (0.189)	**0.004**
Th2 (%)	2.009 (0.316)	1.679 (0.248)	1.882 (0.296)	1.574 (0.272)	0.454
Th17 (%)	1.940 (0.752)	1.360 (0.254)	1.594 (0.354)	1.150 (0.241)	0.317
Tr1 (%)	1.198 (0.212)	1.530 (0.339)	1.696 (0.395)	1.167 (0.263)	0.190
CD4+ IL17+ IL4+ T (%)	0.239 (0.063)	0.178 (0.047)	0.137 (0.028)	0.125 (.040)	0.287
CD4+ IFN-γ+ IL17+ T (%)	0.216 (0.041)	0.185 (0.039)	0.124 (0.042)	0.152 (0.055)	0.323
CD4+ IFN-γ+ IL4+ (%)	0.082 (0.019)	0.051 (0.021)	0.032 (0.009)	0.026 (0.009)	0.084
CD4+ IL17+ IL10+ (%)	0.612 (0.140)	0.844 (0.202)	0.842 (0.246)	0.570 (0.153)	0.252
CD4+ IFN-γ+ IL10+ (%)	0.176 (0.042)	0.194 (0.044)	0.105 (0.033)	0.126 (0.052)	0.263
IFN-γ (MFI)	97.129 (18.328)	43.964 (10.001)	39.755 (7.36)	31.878 (3.853)	**0.002**
MeanIL-17 (MFI)	83.362 (22.990)	111.32 (31.991)	73.075 (22.654)	56.791 (12.433)	0.148
Mean IL-10 (MFI)	238.439 (74.105)	396.824 (177.498)	226.033 (62.685)	167.117 (38.089)	0.326
Mean IL-4 (MFI)	45.962 (10.161)	152.750 (79.775)	39.954 (5.793)	39.700 (7.605)	0.157

Comparing 1, 2 and 3 groups with control group, (p<0.05 was considered as significant).

DISCUSSION

An approximate 2 kg of human total body weight consists of about 1014 different populations of microorganisms living in the gastrointestinal tract.[33,34] To maintain gut homeostasis, a complex mutualistic relationship is required between the gut microbiota and the host.[35-40] Gut bacteria provide essential vitamins such as vitamin K, folate, and vitamin B12 and provide a regulated and proper immune response in the CNS.[41-47] The gut microbiota improves host metabolism by converting complex carbohydrates into short-chain fatty acids that can be absorbed by epithelial cells of the gastrointestinal tract and exert anti-inflammatory, anticancer, and neuroprotective functions. Moreover, short-chain fatty acids are involved in the frequency of Tregs in the circulation and intestine, the integrity and maintenance of the BBB, and regulating microglial activity in the CNS.[11,12,47,48] Therefore, microbiota plays a critical role in BBB permeability. As evidence, germ-free mice with defective tight junctions at the BBB were restored by colonization with conventional gut microbiota.[24,40] Another beneficial effect of gut microbiota on the host is the local and systematic stimulation of the innate and adaptive immune systems. Normal flora is necessary for the maturation of gut-associated lymphoid tissues (GALTs), which are the largest immunological structures and contain 80% of immune compartments. Regulatory and autoreactive T cells may mature in the GALT and exert their functions in the periphery.[34,49] In previous experiments, germ-free mice had underdeveloped GALT with decreased numbers of $CD4^+$ T cells, IgA-secreting plasma cells, and microbicidal peptides.[42,50-52] The spleen and lymph nodes in germ-free mice are abnormal in size and number of B and T cells in parafollicular regions and germinal centers.[47] Dysbiosis, a process of alteration in bacterial variation and function, can disturb the

mutualistic relationship between the host and gut microbiota and may contribute to the establishment of autoimmune or infectious diseases. Recent findings on dysbiosis in MS patients and animal models indicate a gut-brain connection. Studies in the EAE model have shown that intestinal dysbiosis involves in disease development and suggest the role of gut microbiota in the development of MS.[53] In addition, several studies demonstrated a dysbiosis of gut microbiota in MS patients compared to healthy controls.[6,8,9,47,54] Therefore, the manipulation of the gut microbiota of MS patients by probiotic administration may induce immunoregulation and attenuate the severity of the disease.

Probiotics as single or mixed cultures of microorganisms have shown some beneficial effects on maintaining the balance between pro- and anti-inflammatory cytokines and regulating the immune response. The preventive and therapeutic effects of probiotics have been shown in several studies on allergic and autoimmune diseases.[55-57] Many studies have affirmed the immunomodulatory effects of lactobacilli, as commonly used probiotics, in various immune-mediated diseases and animal models like food allergies,[58] arthritis,[19,20] diabetes,[23] and colitis.[21,22] Other studies have demonstrated the prophylactic and therapeutic effects of *Lactobacillus* strains, alone or along with other probiotics, in the EAE model.[4,24-29] According to some previous studies, oral supplementation with lactobacilli could attenuate EAE severity in a strain-specific manner by increasing Treg cells and anti-inflammatory cytokines.[24,25] More specifically, there have been studies conducted on the impacts of *L plantarum* and *L paracasei* on MS and EAE models. Libbey, et al, have shown that oral administration of *L paracasei* in EAE mice caused a significant decline in the prevalence and scoring of the disease.[29] In 2017, a study was conducted on the therapeutic effects of oral administration of *L plantarum* A7, *Bifidobacterium animalis* PTCC 1631, and their combination in EAE mice. The authors demonstrated that treatment with a mixture of two strains attenuated EAE progression and increased the population of Treg cells in the lymph nodes and the spleen.[57] Lavasani, et al. demonstrated some preventive effects of three Lactobacillus strains, *L plantarum* DSM 15312, *L plantarum* DSM 15313, and *L paracasei* DSM 13434, in EAE mice. A mixture of these lactobacilli slowed down the EAE's development and reversed the scoring and demyelination. Moreover, the suppressive activity of their mixture correlated with the reduction of proinflammatory Th1 and Th17 cytokines and IL-10 induction in the spleen, mesenteric lymph nodes, and blood. Moreover, *L paracasei* DSM 13434 and *L plantarum* DSM 15312 induced Treg cells in mesenteric lymph nodes and increased the production of serum TGF-β1.[24] Recently, Sanchez et al, investigated the impact of *L paracasei* products using EAE mouse models. Based on the results, the authors concluded that immune cell infiltration into the CNS might be prevented by local lactobacilli by releasing *Lactobacillus*-associated molecular patterns into the blood circulation.[59] Although the exact cause of MS is unknown, animal research in EAE models suggests that MS could be triggered by autoreactive T-cell activation by an external agent like a viral infection.[42] The activation may occur through bystander activation, molecular mimicry, or recognition of sequestered antigens. Infiltration of activated IFN-γ-producing Th1 cells and IL-17-producing Th17 cells into the CNS induces an uncontrollable inflammatory cascade which is followed by increased permeability of BBB and recruitment of other immune cells such as B cells and macrophages. Autoreactive B cells infiltrating the CNS can produce autoantibodies to myelin and induce macrophage- or complement-mediated cytotoxic reactions to attack the myelin sheath and cause axonal loss and progressive neurodegeneration. Other defective T-cell populations in MS patients are $Foxp3^+$ $CD25^+$ Treg cells and IL-10-producing Tr1. Frequency and immunosuppressive functions are impaired in these regulatory cells compared to healthy controls.[1,60]

In the present study, for the first time, the effects of two *Lactobacillus* strains, *L paracasei* DSM 13434 and *L plantarum* DSM 15312, and their combination on $CD4^+$ T cell subsets and cytokine production in PBMCs of relapsing-remitting MS patients were evaluated. In the flow cytometry assessment, our data revealed a significant reduction in the percentage of the Th1 cell subset amongst $CD4^+$ T cells in all the 3 groups of probiotic treatment compared to the vehicle (control) group (group 4). Moreover, $CD4^+$ T cells cultured in the presence of probiotic treatments contained a lower percentage of Th17 than the control group, but this discrepancy was not statistically significant.

As the intensity of expressed cytokines is more informative than the proportions of the cytokine-producing cells, we also examined the intensity of IFN-γ, IL-10, IL-4, and IL-17 in the CD4+ T cell

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subsets. The results demonstrated that the MFI for IFN-γ in Th1 cells ($CD4^+$ IFN-γ^+) is significantly decreased in all 3 probiotic-treated groups in comparison with the control group.

The levels of IL-17, TGF-β, and IFN-γ cytokines in supernatants of all treated and control groups were measured using ELISA, which indicated a significant decrease in IL-17 secretion in the supernatant of $CD4^+$ T-cell cultures in all 3 treatment groups in comparison with the control group.

Collectively, our findings have supported the anti-inflammatory effect of 2 *Lactobacillus* strains, as previously demonstrated in MS and EAE animal models for these *Lactobacillus* strains and other probiotics. The imbalance between inflammatory and regulatory cells and their functions, which leads to an inflammatory environment in the CNS, is thought to be the main cause of demyelination and axonal damage.[1,42,60,61]

Lavasani et al. found that *L paracasei* DSM 13434 and *L plantarum* DSM 15312 increased mesenteric lymph node Tregs and serum TGF-β levels in EAE mouse models.[24] Our findings are consistent with the results of this study supporting the anti-inflammatory effects of these two *Lactobacillus* species by decreasing the number of IFN-γ producing Th1 cells; however, this difference was not significant in Treg cell numbers and expression of IL-10 and TGF-β.

In 2017, Yamashita et al. found that intraperitoneal administration of *L helveticus* SBT2171 (LH2171) could significantly decrease the severity and incidence of EAE in mice. Moreover, LH2171 decreased the number of Th17 cells in the spinal cord and the ratio of Th17 cells to $CD4^+$ T cells in the inguinal lymph nodes of EAE mice.[4] All these data indicate the anti-inflammatory role of this *Lactobacillus* species, as we showed for *L plantarum* and *L paracasei* in our study.

A few previous studies investigated the anti-inflammatory effects of probiotics on immune cells in vitro. In 2017, Yamashita et al. demonstrated the anti-proliferative effect of *L helveticus* SBT2171 (LH2171) on primary murine immune cells and inhibited the production of Lipopolysaccharide (LPS)-stimulated inflammatory cytokines, IL-1-β and IL-6, from the immune cells in vitro.[20] In another study conducted in 2018, Yamashita et al. indicated the inhibitory effects of LH2171 on IL-6 production in 2 cell lines: DC2.4 and RAW264.7.[4] These studies substantiated the anti-inflammatory effects of LH2171 on murine immune cells in vitro.

Some recent studies have explored the benefit of probiotics on MS patients. In 2018, Tankou et al. investigated the effects of VSL3, a commercially sold probiotic containing *L plantarum*, *L paracasei*, and 6 other bacterial species, on the gut microbiome and immune function in relapsing-remitting MS patients and healthy controls. The findings demonstrated that the probiotic increased an anti-inflammatory response by decreasing the number of inflammatory monocytes, MFI of CD80 on classical monocytes, and HLA-DR MFI on dendritic cells. They suggested the potential synergistic effects of probiotics with current MS therapies.[10]

The results of the present in vitro study showed that the anti-inflammatory effects of probiotics *L paracasei* (group 1) and *L plantarum* (group 2) on $CD4^+$ T cells obtained from MS patients were not significantly different. Moreover, there was no significant difference between group 3 (treated with a mixture of 2 probiotics) and any of groups 1 or 2. Although we did not achieve the expected synergistic effect of mixing two probiotic groups, we assume that coprescription of a half-dose of both probiotics would be preferred to a complete dose of each one, considering the possible adverse effects.

Since the long-term use of the common treatments of MS, including oral steroids and immunosuppressive drugs, could result in several side effects, the identification of safe alternatives for the prevention or amelioration of disease severity would be of great value. The present study indicates that treatment with cell-free supernatant of *L plantarum* and *L paracasei* causes $CD4^+$ T cells of MS patients to shift toward an anti-inflammatory phenotype through inhibition of Th1 and Th17 and decreasing their cytokines. Although this study is a preliminary investigation, the results, along with previous studies, can suggest a potential preventive or therapeutic application of *L plantarum* and *L paracasei* for patients with MS and probably other autoimmune disorders. Additional studies are required to prove the efficacy and safety of the new probiotics for MS in the future.

STATEMENT OF ETHICS

Research was approved by approved by the Ethics Committee of Tehran University of Medical Sciences (approval code: IR.TUMS.SPH.REC.1395.1415).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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