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## Effects of synbiotic supplementation on regulatory T cells' response in patients with axial spondyloarthritis: a randomized double-masked placebo-controlled trial†

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This study was conducted on samples from patients enrolled in a randomized double-masked placebo-controlled trial on the effect of synbiotic supplementation on the IL-17/IL-23 pathway and disease activity in patients with axial spondyloarthritis (axSpA) to investigate the effects of synbiotic supplementation on regulatory T (Treg) cells' response in these patients. Forty-eight axSpA patients were randomized to take one synbiotic capsule or placebo daily for 12 weeks. Treg cell proportion, gene expression of forkhead box protein P3 (Foxp3), microRNA (miRNA)-25, miRNA-106b, miRNA-146a, interleukin (IL)-10, and transforming growth factor (TGF)- $\beta$  as well as serum IL-10 and TGF- $\beta$  levels were assessed before and after the trial. Thirty-eight patients (19 in each group) completed the trial. The proportion of Treg cells ( $P < 0.001$ ), the gene expression of Foxp3 ( $P < 0.001$ ), IL-10 ( $P = 0.001$ ), TGF- $\beta$  ( $P < 0.001$ ), and miRNA-146a ( $P < 0.001$ ) and serum IL-10 ( $P = 0.003$ ) and TGF- $\beta$  ( $P = 0.002$ ) levels significantly increased compared to the baseline in the synbiotic group. Additionally, a significant reduction in the gene expression of miRNA-25 ( $P < 0.001$ ) and miRNA-106b ( $P < 0.001$ ) was observed in the synbiotic group. Significant between-group differences were observed in the proportion of Treg cells ( $P = 0.024$ ) and the gene expression of Foxp3 ( $P = 0.010$ ), IL-10 ( $P = 0.002$ ), TGF- $\beta$  ( $P = 0.016$ ), miRNA-25 ( $P = 0.008$ ), miRNA-106b ( $P = 0.001$ ), and miRNA-146a ( $P = 0.010$ ). Differences in the serum levels of IL-10 and TGF- $\beta$  between the groups were not significant. As a conclusion, synbiotic supplementation could modulate Treg cells' response in axSpA patients and thus can be promising as an adjunctive therapy. Additional investigations would help in further clarifying the subject.

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## 1. Introduction

Axial spondyloarthritis (axSpA) is an inflammatory ailment that influences the spine and sacroiliac joints and leads to back pain, incapacitation and poor quality of life.<sup>1</sup> In addition to musculoskeletal involvement, extra-articular involvement like uveitis, enthesitis and inflammatory bowel disease (IBD) can be noticed in axSpA.<sup>1</sup> Commonly, the disease initiates in the third decade of life and affects males more than females.<sup>2</sup>

The global prevalence of axSpA ranges from 0.1% to 1.4%.<sup>3</sup> Although the cause of axSpA is not well understood, genetic susceptibility, environmental factors (e.g., microbial infections) and immune reaction are related to disease pathogenesis.<sup>4</sup> It is also suggested that intestinal microbiota and dysbiosis may play a role in axSpA.<sup>5</sup> Variation in intestinal permeability and induction of immune responses are mechanisms that have been suggested to explicate the plausible function of the microbiome in axSpA progression.<sup>5</sup>

T lymphocytes are the main cells involved in axSpA pathogenesis. Alteration in the proportion of peripheral blood CD4 $^{+}$  T cells including higher T-helper 17 (Th17) cells and lower regulatory T (Treg) cells has been noticed in axSpA.<sup>6-8</sup> Treg cells are a subpopulation of CD4 $^{+}$  T cells that are involved in controlling immune tolerance and sustaining immune homeostasis.<sup>9,10</sup> These cells are responsible for expressing forkhead box protein P3 (Foxp3), a transcription factor that suppresses the proliferation and activity of a variety of immune cells.<sup>11,12</sup> Moreover, Treg cells generate interleukin

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(IL)-10 and transforming growth factor (TGF)- $\beta$ , which are immunosuppressive agents.<sup>13</sup> MicroRNAs (miRNAs) are non-coding ribonucleic acids that have a significant role in several biological processes *via* acting at the post-transcriptional level.<sup>14</sup> It has been proposed that the microbiota–miRNA interplay influences host immunity.<sup>15</sup> Dysbiosis can alter the expression of miRNAs.<sup>15</sup> An altered expression of miRNAs occurs in autoimmune inflammatory conditions including rheumatic diseases.<sup>16</sup> It is indicated that miRNA-25 and miRNA-106b have a role in regulating the TGF- $\beta$  pathway and enhanced expression of these miRNAs disrupts the TGF- $\beta$  signaling pathway and affects Tregs.<sup>17,18</sup> It has also been reported that miRNA-146a down-regulation in monocytes and its level have a negative correlation with the axSpA disease activity score.<sup>19</sup> Thus, these miRNAs modulate the biology and function of Treg cells and can be considered as potential therapeutic targets.

Non-steroidal anti-inflammatory drugs (NSAIDs), conventional synthetic disease-modifying anti-rheumatic drugs (csDMARDs) and biological DMARDs (bDMARDs) are common medications used for treating axSpA.<sup>20</sup> Because of undesirable effects, limited efficacy and sometimes high expenses of these medications, research on safer and inexpensive alternative treatments appears to be indispensable. Interest in complementary treatments including nutritional supplements has been lately increasing to identify safer alternatives for disease management.

Synbiotics are nutritional supplements containing both probiotics and prebiotics.<sup>21</sup> It has been indicated that synbiotic supplementation has a notably more synergistic impact on the intestinal and fecal microflora and the immune system than either prebiotic or probiotic supplementation alone.<sup>22,23</sup> Limited studies are available regarding the syn/probiotics supplementation in autoimmune inflammatory diseases including rheumatic disorders, which indicate conflicting results.<sup>24–32</sup> To the authors' knowledge, no study evaluates the effect of synbiotic supplementation on Treg cells in axSpA patients; therefore, the current study was conducted to assess the effect of synbiotic supplementation on Treg cells' response in patients with axSpA.

## 2. Methods

### 2.1. Participants

This study was conducted on samples collected in our previous study about the effect of synbiotic supplementation on the IL-17/IL-23 pathway and disease activity in patients with axSpA.<sup>33</sup> The protocol of the current study was approved by the Ethics Committee of Tabriz University of Medical Sciences (ethics code: IR.TBZMED.REC.1399.152) and the Iranian Registry of Clinical Trials (code: IRCT20190917044794N2). Inclusion criteria were determined as: (i) diagnosis of axSpA based on the international criteria<sup>34</sup> and (ii) age range between 20–60 years. Exclusion criteria were determined as: taking nutritional supplements or antioxidants one month

before and during the trial, use of antibiotics, pregnancy and breast-feeding, having other autoimmune diseases, diabetes mellitus, IBD, gastrointestinal infection and other chronic diseases. Sample size determination was performed considering the information from the previous research.<sup>30</sup> Assuming 95% confidence level and 80% power, the sample was minimum 19 subjects per group, which was increased to 24 subjects in each group for a potential 25% dropout. Patients were aware of the research and signed written informed consents.

### 2.2. Design and measurements

Forty-eight axSpA patients were designated from the rheumatology outpatient clinic of Tabriz University of Medical Sciences and divided into intervention and placebo groups randomly *via* a block randomization method (Random Allocation Software), which matched subjects according to the gender and age per block. The intervention group ( $n = 24$ ) received one synbiotic capsule (Zist-Takhmir Company, Tehran, Iran) per day for 12 weeks. This capsule contains  $10^9$  colony-forming units (CFUs) of 7 beneficial bacteria including *L. casei*, *L. acidophilus*, *L. rhamnosus*, *L. bulgaricus*, *B. longum*, *B. breve*, and *S. thermophiles* together with fructooligosaccharide and other constituents (lactose-magnesium acetate-talc). The control group ( $n = 24$ ) received one placebo capsule (Zist-Takhmir Company, Tehran, Iran) per day for 12 weeks. The placebo capsules were identical in shape and color and contained a non-therapeutic substance, cornstarch. Patients took the supplements with lunch and they were asked not to modify diet and physical activity. Patients were monitored every two weeks. The study flowchart was presented in our previous report.<sup>33</sup>

8 mL of fasting blood samples were gathered at the baseline and at the end of the trial. In order to separate peripheral blood mononuclear cells (PBMCs), the Ficoll separation method (Biosera, East Sussex, UK) and centrifugation (25 minutes, 450 g) were used. Then,  $5 \times 10^6$  of cells were cultured in a 5 mL medium (fetal bovine serum, penicillin, and L-glutamine) in the presence of 10 ng mL<sup>-1</sup> of phorbol myristate acetate (eBioscience, San Diego, CA) and incubated (37 °C, 5% CO<sub>2</sub>) for 48 hours. In order to assess Treg cells, the proportion of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> T cells was determined by flow cytometry. Overall, incubation of  $1 \times 10^6$  of PBMCs was performed at 4 °C for 15 minutes using anti-human CD4, anti-human CD25 and anti-human CD127 (eBioscience). Stained cells were assessed using a FACSCalibur flow cytometer (BD Biosciences) and Flowing Software 2.4.1. Then, gating of CD4-positive lymphocytes was performed for analyzing the expression of CD25 and CD127. In order to analyze the mRNA expression of FoxP3, TGF- $\beta$ , and IL-10, the real-time polymerase chain reaction (PCR) was performed. Homogenization was performed on cultured PBMCs and extraction of total RNA was conducted using RNX-PLUS Solution (SinaClon, Tehran, Iran). The complementary DNA (cDNA) was produced using the RevertAid Reverse Transcriptase kit (Thermo Fisher, Waltham, MA). miRNA-25, miRNA-106b and miRNA-146a were extracted by TaqMan RT-PCR. The reverse transcription of RNA was

conducted using the TaqMan MicroRNA Reverse Transcription Kit and Master Mix. Quantification of transcripts of  $\beta$ -actin and RNA, U6 small nuclear (RNU6) was performed as the endogenous RNA control.  $\beta$ -Actin was assumed as a housekeeping control gene for FoxP3, TGF- $\beta$ , and IL-10 genes and RNU6 was assumed as an endogenous control for miRNAs. In order to analyze the results, the  $2^{-\Delta\Delta Ct}$  procedure was used. Furthermore, enzyme-linked immunosorbent assay (ELISA) kits (Biosource, Nivelles, Belgium) and a Medgenix ELISA reader (BP-800; Biohit, Helsinki, Finland) were used for measuring IL-10 and TGF- $\beta$  in the supernatant of cultured PBMCs.

### 2.3. Statistical analysis

For analyzing data, SPSS 16.0 software (SPSS Inc., Chicago, IL) was used. The Kolmogorov-Smirnov test was performed to evaluate the normality of variable distribution. Categorical and continuous variables were displayed as frequency (percentage) and mean  $\pm$  standard deviation (SD), respectively. Differences in categorical variables between two groups were compared by the Chi-square test. Intragroup differences were assessed by a paired *t*-test. At the baseline, intergroup comparisons were made with an independent sample *t*-test. After research, analysis of covariance (ANCOVA) was performed for intergroup comparisons and considering adjustment for baseline measures and axSpA duration.  $P < 0.05$  was assumed to be statistically significant.

## 3. Results

A total of 48 patients were randomized into the synbiotic and placebo groups. Five patients were excluded from each study group. Therefore, thirty-eight patients completed the study. Baseline characteristics of the studied patients were presented in our previous report.<sup>33</sup>

As illustrated in Fig. 1, the baseline proportion of Treg cells did not differ between the two groups ( $P > 0.05$ ). The proportion of Treg cells increased considerably in the synbiotic group compared with the baseline ( $4.68 \pm 2.28$  vs.  $2.57 \pm 1.44$ ,  $P < 0.001$ ), whilst no considerable changes occurred in the placebo group ( $3.06 \pm 1.80$  vs.  $2.62 \pm 1.40$ ,  $P > 0.05$ ). After the study, significant between-group differences in the proportion of Treg cells, adjusted baseline measures and the disease duration were observed ( $P = 0.024$ ).

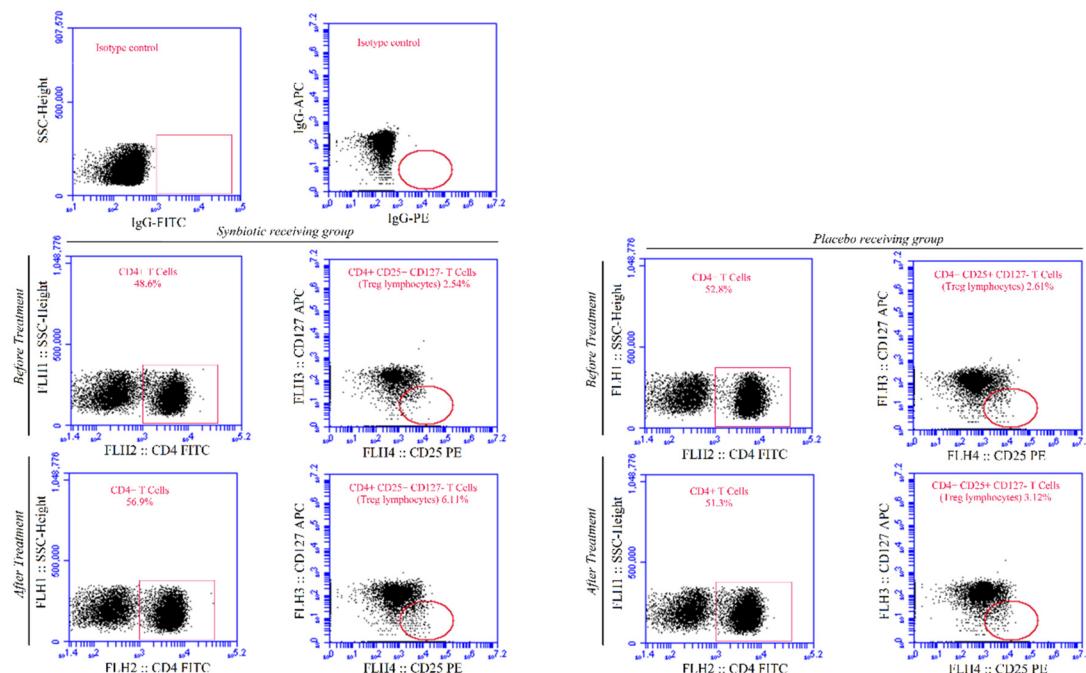
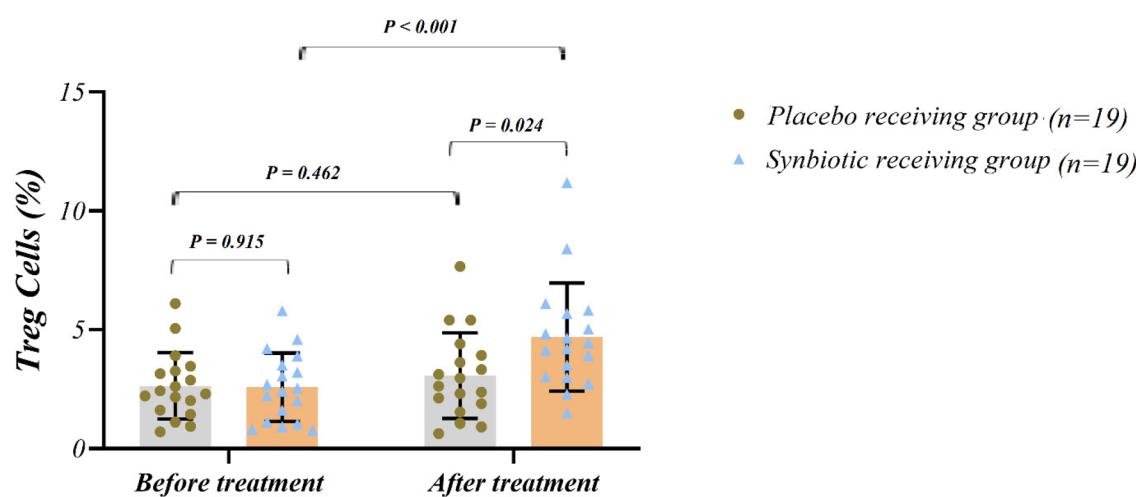
As depicted in Fig. 2, the baseline mRNA expression of FoxP3, IL-10 and TGF- $\beta$  did not vary notably between the two groups ( $P > 0.05$ ). The mRNA expression of FoxP3, IL-10, and TGF- $\beta$  increased remarkably in the synbiotic group compared with the baseline ( $P < 0.001$ ,  $P = 0.001$ , and  $P < 0.001$ , respectively); whereas in the placebo group, no changes occurred in the mRNA expression of FoxP3, IL-10, and TGF- $\beta$  ( $P > 0.05$ ). After the research, the results of the ANCOVA test indicated significant between-group differences in the mRNA expression of FoxP3, IL-10 and TGF- $\beta$ , adjusted baseline values and the disease duration ( $P = 0.010$ ,  $P = 0.002$ , and  $P = 0.016$ , respectively).

According to Fig. 3, there were no significant differences in the mRNA expression of miRNA-25, miRNA-106b and miRNA-146a between the two groups at the baseline ( $P > 0.05$ ). Compared to the baseline, significant reduction in the mRNA expression of miRNA-106b and a significant increase in the mRNA expression of miRNA-146a were observed in both synbiotic ( $P < 0.001$  and  $P < 0.001$ ) and placebo ( $P = 0.027$  and  $P = 0.007$ ) groups. Furthermore, a significant reduction in the mRNA expression of miRNA-25 was observed in the synbiotic group compared to the baseline ( $P < 0.001$ ); however, no remarkable change was noted ( $P > 0.05$ ) in the placebo group. After the research, the results of the ANCOVA test showed significant between-group differences in the mRNA expression of miRNA-25, miRNA-106b and miRNA-146a adjusted baseline measures and the disease duration ( $P = 0.008$ ,  $P = 0.001$ , and  $P = 0.010$ , respectively).

In addition, we assessed the serum levels of IL-10 and TGF- $\beta$  and disease activity in the studied groups (Table 1). No significant differences existed in the baseline serum IL-10 and TGF- $\beta$  between groups ( $P > 0.05$ ). Compared with the baseline, serum IL-10 and TGF- $\beta$  levels enhanced notably in the synbiotic group ( $P = 0.003$  and  $P = 0.002$ , respectively), whilst in the placebo group, no significant changes occurred in serum IL-10 and TGF- $\beta$  levels ( $P > 0.05$ ). After the research, no significant differences were observed between the two groups in serum IL-10 and TGF- $\beta$ , adjusted baseline measures and the disease duration ( $P > 0.05$ ).

## 4. Discussion

Considering the importance of Tregs in modulating immune responses as well as the abnormalities occurred in the proportion and activity of these cells in autoimmune inflammatory diseases,<sup>35</sup> it is supposed that Treg cell-inducing therapies are possibly an innovative modality to treat axSpA. Synbiotics are nutritional supplements with immunomodulatory properties<sup>36</sup> that have been shown to be useful in the management of autoimmune inflammatory disorders.<sup>37</sup> The present randomized double-masked placebo-controlled trial was the first investigation assessing the effects of synbiotic supplementation on Tregs in patients with axSpA. Our research demonstrated that synbiotic supplementation led to a significant elevation in Treg cells' proportion in comparison with the baseline and placebo. In agreement with our results, Lorea Baroja *et al.*<sup>38</sup> indicated that consumption of probiotic yogurt increases the CD4 $^{+}$  CD25 $^{+}$  Tregs proportion in IBD patients. López *et al.*<sup>39</sup> and Chen *et al.*<sup>40</sup> reported that treatment with probiotics could stimulate Treg CD4 $^{+}$  CD25 $^{+}$  FoxP3 $^{+}$  differentiation. However, differences were not significant. Inconsistent with our research, van der Aa *et al.*<sup>41</sup> did not show a significant difference in Treg cell percentage between synbiotic and placebo receiving infants with atopic dermatitis. In addition, Shukla *et al.*<sup>42</sup> declared no significant variation in Treg cell percentage between probiotic and placebo receiving children with active enthesitis-related arthritis. Although the mecha-

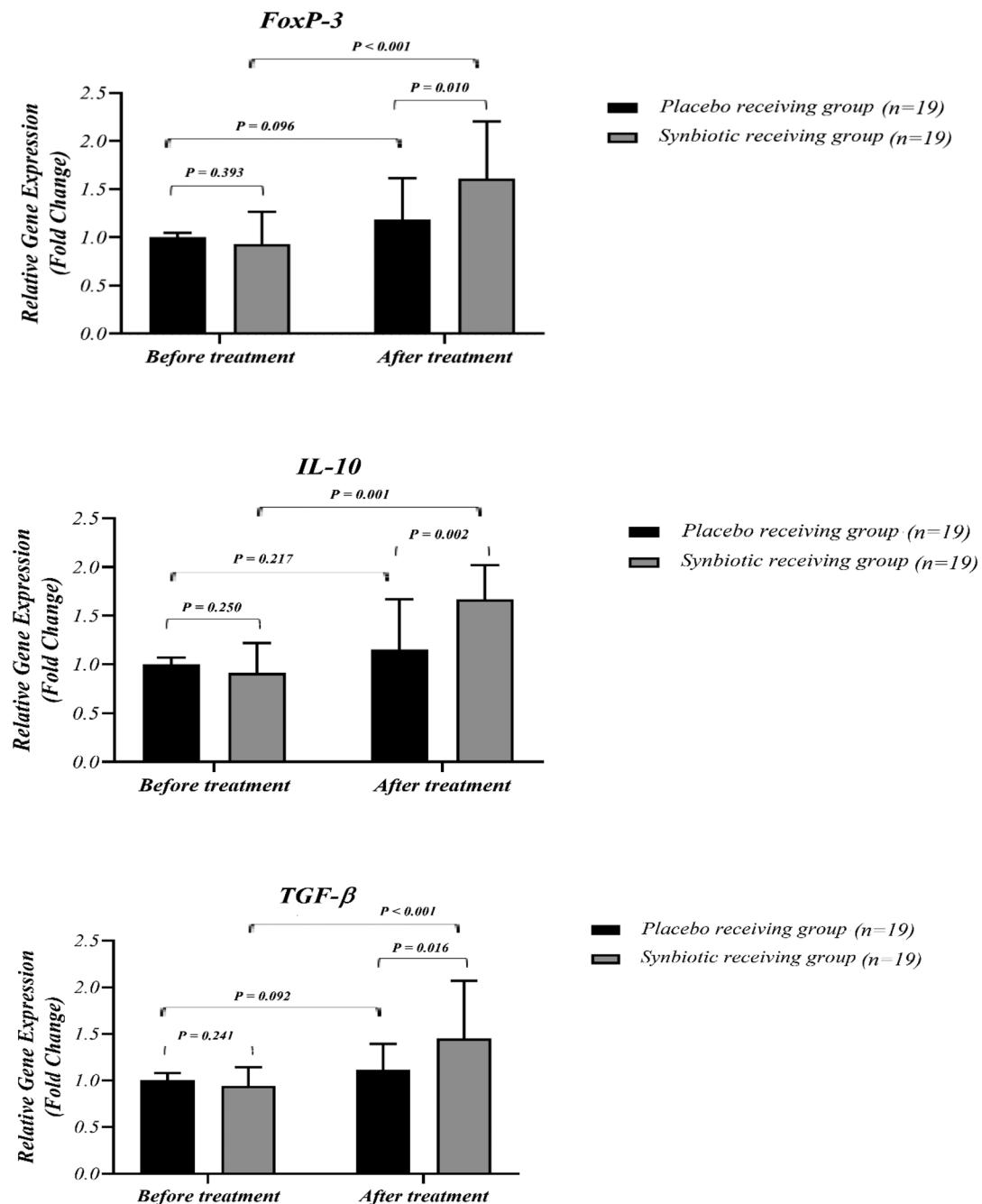
**A****B**

**Fig. 1** (A) Flow cytometry plots for identifying regulatory T cells in whole blood. (B) Percentage of circulating regulatory T cells in study groups. Values are mean  $\pm$  SD. Intragroup differences were assessed by a paired *t*-test. Intergroup differences were assessed by an independent sample *t*-test at the baseline or the ANCOVA test, adjusted baseline values and the disease duration after 12 weeks.  $P < 0.05$  is considered significant.

nism of increasing Treg cells by syn/probiotics is not well understood, several mechanisms have been proposed, including (i) activation of tolerogenic dendritic cells (DCs) *via* binding to the lectin dendritic cell (DC)-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), which in turn promotes Foxp3<sup>+</sup> Treg cells' production,<sup>43,44</sup> (ii) stimulation of Toll like receptors (TLR) like TLR9 which led to release of immunosuppressive cytokines such as TGF- $\beta$  and

IL-10,<sup>43</sup> and (iii) direct activation of Tregs by producing short chain fatty acids (SVFAs).<sup>43</sup> Short-chain fatty acids (SCFAs) bind to G protein-coupled receptors (GPCRs) on Tregs and activate them to suppress CD8 and Th17 cells,<sup>43</sup> (iv) modulating the host miRNA expression<sup>15</sup> and (v) interfering with nuclear factor- $\kappa$ B degradation.<sup>44</sup>

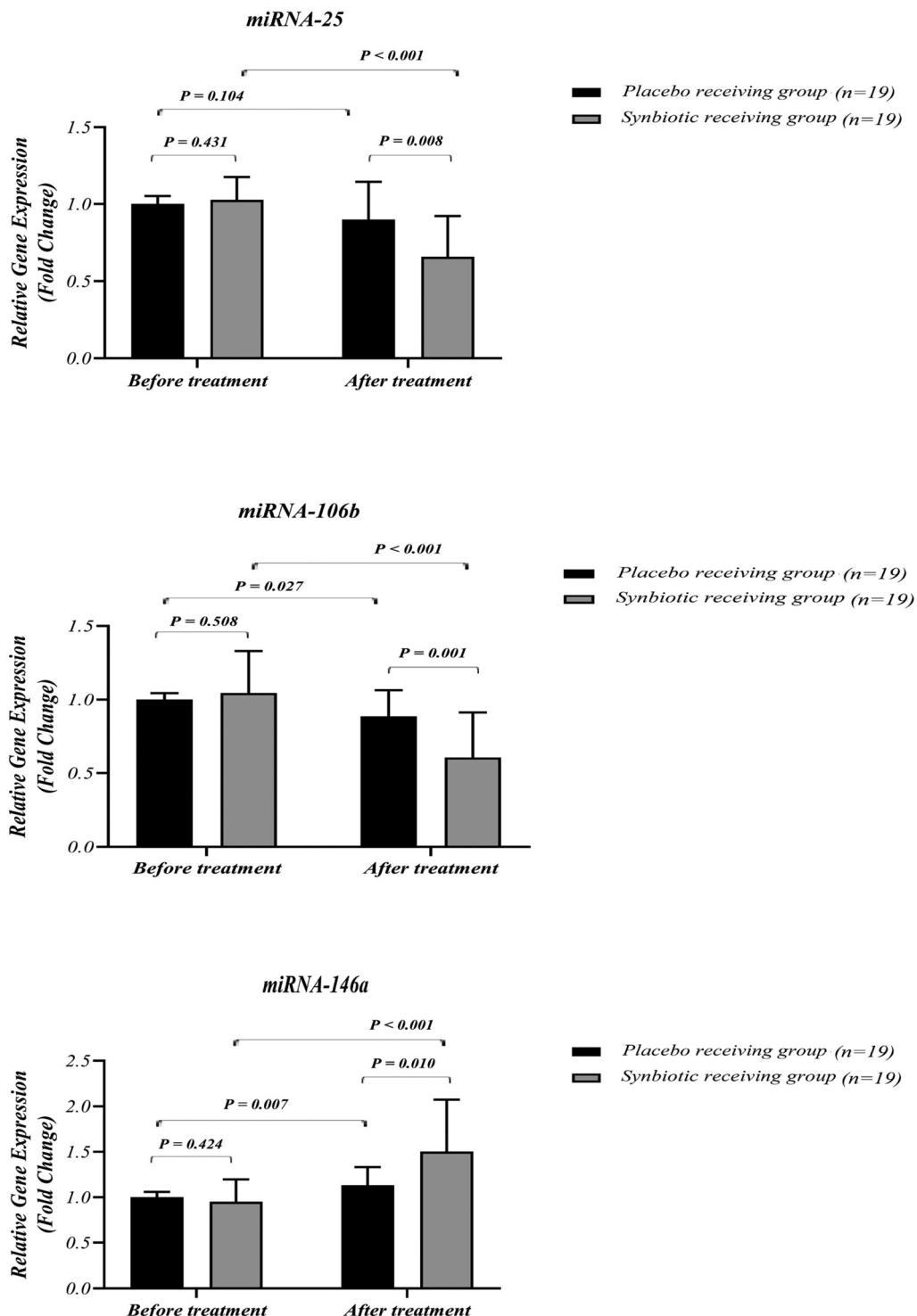
This study demonstrated a significant increase in the expression of FoxP3, IL-10 and TGF- $\beta$  with symbiotic



**Fig. 2** The mRNA expression level of regulatory T cells related to transcription factor FoxP3 and cytokines IL-10 and TGF- $\beta$  in study groups. Values are mean  $\pm$  SD. Intragroup differences were assessed by a paired *t*-test. Intergroup differences were assessed by an independent sample *t*-test at the baseline or the ANCOVA test, adjusted baseline values and the disease duration after 12 weeks.  $P < 0.05$  is considered significant.

supplementation. Inconsistent with our research, Dehnavi *et al.*<sup>45</sup> did not report a significant change in the gene expression of FoxP3, IL-10, and TGF- $\beta$  after synbiotic supplementation in patients with allergic rhinitis. In addition, studies in patients with IBD<sup>46</sup> and recurrent aphthous stomatitis<sup>47</sup> indicated no significant differences in the serum IL-10 level between synbiotic and placebo groups. Pineda *et al.*<sup>27</sup> did not report a significant change in serum IL-10 with probiotic consumption in RA patients. Similarly, Inoue *et al.*,<sup>48</sup> Wang *et al.*<sup>49</sup> and Smecoul

*et al.*<sup>50</sup> demonstrated that oral administration of probiotics did not lead to a significant change in serum IL-10 concentrations compared with placebo in subjects with atopic dermatitis, peritoneal dialysis and celiac disease, respectively. Additionally, our study was different from Shukla *et al.*<sup>42</sup> and Vaghef-Mehraban *et al.*<sup>51</sup> studies that reported a substantial difference in serum IL-10 levels between probiotic and placebo receiving patients with active enthesitis-related arthritis and rheumatoid arthritis (RA). Furthermore, Inoue *et al.*<sup>48</sup> reported



**Fig. 3** The mRNA expression level of miRNA-25, miRNA-106b, and miRNA-146a in study groups. Values are mean  $\pm$  SD. Intragroup differences were assessed by a paired t-test. Intergroup differences were assessed by an independent sample t-test at the baseline or the ANCOVA test, adjusted baseline values and the disease duration after 12 weeks.  $P < 0.05$  is considered significant.

that oral administration of probiotics significantly increases the serum TGF- $\beta$  level compared with placebo in subjects with atopic dermatitis. Discrepancy between the findings of this study with previous studies on other diseases may be related

to variations in pathophysiological conditions (e.g., various ailments), the baseline Treg cell proportion, the baseline FoxP3, IL-10 and TGF  $\beta$  status, the dosage and type of syn/probiotic supplements and the duration of supplementation.

**Table 1** Comparison of biochemical and clinical parameters before and after the study

Variable		Synbiotic group ( <i>n</i> = 19)	Placebo group ( <i>n</i> = 19)	<i>P</i> -value*
IL-10 (pg mL <sup>-1</sup> )	Baseline	693.20 ± 291.09	819.61 ± 346.33	0.223
	After 12 weeks	1139.30 ± 401.28	922.22 ± 349.19	0.074
	<i>P</i> -value**	0.003	0.369	
TGF- $\beta$ (pg mL <sup>-1</sup> )	Baseline	346.11 ± 158.99	337.28 ± 142.56	0.862
	After 12 weeks	513.28 ± 194.62	402.50 ± 166.34	0.105
	<i>P</i> -value**	0.002	0.174	

IL, interleukin; TGF, transforming growth factor. Variables are reported as mean ± SD. *P* < 0.05 is considered significant. \**P* values indicate a comparison between groups (an independent sample *t*-test at the baseline or the ANCOVA test, adjusted baseline values and disease duration after 12 weeks). \*\*A paired *t*-test.

This study demonstrated a significant increase in the expression of miRNA-146a genes with synbiotic supplementation. Moreover, synbiotic supplementation led to a significant decrease in the expression of miRNA-25 and miRNA-106b genes in comparison with the baseline and placebo. MiR-146a is usually expressed in Treg cells and has a critical function in Treg-mediated inhibition. The lack of miR-146a in Treg cells causes an enhanced expression of STAT1, a target gene of miR-146a that stimulates the proinflammatory phenotype of Tregs. Up-regulation of miR-146a can inhibit Th1 responses derived from Tregs function, thereby maintaining the optimal range of STAT1 activity and decreasing severe failure of immunologic tolerance.<sup>52,53</sup> Furthermore, miRNA-25 and miRNA-106b have a role in regulating the TGF- $\beta$  pathway and enhanced expression of these miRNAs disrupts the TGF- $\beta$  signaling pathway and affects Tregs.<sup>17,18</sup> Thus, miR-25 and miR-106b dysregulation can alter Treg cells' action principally *via* changing the TGF- $\beta$  actions.<sup>17</sup> Down-regulation of miR-25 and miR-106b can induce the TGF- $\beta$  pathway as well as Treg cell differentiation and maturation.

The weak points of the study include a relatively small number of patients. The strengths of the present study were regular monitoring of patients by a phone call and a relatively favorable acceptance of interventions.

## 5. Conclusion

As a conclusion, synbiotic supplementation could modulate Treg cells' response in axSpA patients and thus can be promising as an adjunctive therapy. Additional investigations would help in further clarifying the subject.

## Conflicts of interest

The authors have no conflicts to declare.

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