

Probiotic Yogurt Culture *Bifidobacterium Animalis* Subsp. *Lactis* BB-12 and *Lactobacillus Acidophilus* LA-5 Modulate the Cytokine Secretion by Peripheral Blood Mononuclear Cells from Patients with Ulcerative Colitis

Authors

A. Sheikhi^{1,2}, M. Shakerian³, H. Giti⁴, M. Baghaeifar³, A. Jafarzadeh⁵, V. Ghaed¹, M. R. Heibor¹, N. Baharifar⁶, Z. Dadafarin⁷, G. Bashirpour⁸

Affiliations

Affiliation addresses are listed at the end of the article

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Correspondence

A. Sheikhi

Associate Professor of Immunology
Department of Immunology
Faculty of Medicine
Dezful University of Medical Sciences
Azadegan, Dezful
Iran
Post code: 6461945914
Tel.: +98/61/42429 733
Fax: +98/61/42429 538
sheikhi.a@dums.ac.ir
sheikhi@queensu.ca

Abstract



Background: There are some evidences for the immunomodulation disorders in the response to intestinal microbiota in inflammatory bowel disease. Yogurt is a fermented milk product made with a starter culture consisting of different probiotics which could be colonized in intestine. However, the role of probiotics in the aetiopathogenesis of ulcerative colitis (UC) has not been clarified. To determine how the immune system responds to these bacteria this study was planned.

Methods: *Bifidobacterium lactis* BB-12 (*B. lactis*) and *Lactobacillus acidophilus* LA-5 (*L. acidophilus*) were cultivated on MRS broth. PBMCs of 36 UC patients were separated by Ficoll-Hypaque centrifugation and co-cultured with different concentrations of UV killed bacteria in RPMI-1640 plus 10% FCS for 48/72h. IL-10, TGF-

β, IFN-γ and TNF-α were measured in supernatant of PBMCs by ELISA.

Results: Both bacteria significantly augmented IL-10, TGF-β, IFN-γ and TNF-α compared to control ($p < 0.001$). The secretion levels of IL-10 and TGF-β by *B. lactis*- compared to *L. acidophilus*-stimulated PBMCs were significantly higher ($p < 0.05$, $p < 0.01$ respectively). The secretion levels of TNF-α and IFN-γ by PBMCs after 72 h were significantly lower compared to 48 h stimulation by *B. lactis* ($p < 0.001$, $p < 0.035$ respectively).

Conclusion: These data show that both probiotics may trigger the pro- and anti-inflammatory immune response of UC patients. It seems that IL-10/TGF-β uprising by *B. lactis* could be the reason of TNF-α/IFN-γ reduction. Therefore albeit *B. lactis* still stimulates the effector Th cells but because of more stimulatory effect on Tregs, it could be a good potential therapeutic candidate for further investigation.

Introduction



Ulcerative colitis (UC) as one of the 2 major forms of inflammatory bowel disease (IBD) is a chronic and relapsing inflammatory condition that is characterized by colonic tissue edema, increased colonic epithelial permeability and extensive infiltration of leukocytes in the colon and rectum [1]. It is a cause of significant morbidity worldwide and its incidence and prevalence appear to be increasing with time. Patients with UC frequently experience episodes of bloody diarrhea with or without mucous, abdominal pain, fever and weight loss [2].

UC is assumed to be a result of a breakdown of tolerance to intestinal environmental antigens such as resident enteric bacteria. Several species of bacteria are living in human gastrointestinal lumen [3], which main sources of them are fermented foods containing probiotics, such as

yogurt [4]. Some studies suggest that different species have variable potential to stimulate regulatory or effector T cells in the mucosal immune system [5, 6]. Indeed, cytokine profiles in co-cultures of the probiotic bacteria with peripheral blood mononuclear cells (PBMC) show marked differences between strains [7].

Upon antigenic stimulation, the T CD4+ cells differentiate into several subsets such as Th1, Th2, Th17 and regulatory T (Treg) cells which are characterized by distinct cytokine profile [8,9]. Treg cells are poised with regulatory activity limiting the development of T-cell responses to intestinal commensal bacteria and the development of pathologic intestinal inflammation. Evidence indicate the Th1 (such as IFN-γ and TNF-α), Th2 (IL-4, IL-5, IL-13) and Th17 (IL-17A) (all effector Th)-related cytokines could lead to UC disease progression and worsening of symptoms whereas the Treg-related cytokines (TGF-β and IL-10)

have been associated with the reduction and improvement of symptoms in UC patients [10,11]. Recent evidences have indicated that probiotic treatment has a great influence on the host intestinal immune system to control inflammation [12,13]. The aim of this study is to find whether *B. lactis* BB-12 and *L. acidophilus* LA-5, as probiotic strains used as dietary supplements in yogurt, differ in their capacity to induce Treg- or effector Th related cytokines in PBMCs from UC patients.

Materials and Methods

Patients

The study included 36 inactive UC patients (21 male and 15 female; 32.5 ± 10.4 years of age). Diagnosis of UC had been established based upon endoscopic and histological criteria as well as clinical symptoms. The patients have no complications or history of immunological disorders other than UC. None of the patients had received glucocorticoids, cyclosporine, or tacrolimus for at least 2 months prior to the study. The study was approved by Ethical Committee of Dezful University of Medical Sciences.

Isolation of peripheral blood mononuclear cells

After informed consent was obtained, 10ml of heparinized venous blood was taken from patients with UC. The heparinized blood was loaded on 3 ml of Ficoll-Hypaque (Pharmacia), centrifuged at $1300 \times g$ for 20min, and PBMCs were separated as described previously [14–17].

Briefly, the cells were washed 3 times with phosphate buffered saline (PBS), resuspended in RPMI 1640 media (Gibco), supplemented with 10% heat-inactivated fetal calf serum (FCS), 100U/mL penicillin, 100 μ g/mL streptomycin (Gibco), and cultured at 37°C in a humidified 5% CO₂ incubator to a final density of 2×10^6 cells/ml.

Preparation of bacterial strains

Lactobacillus (*L.*) *acidophilus* LA-5 and *Bifidobacterium* (*B.*) *lactis* BB-12 were kindly provided by Sherkat Pegah Khuzestan, Dezful, Iran. Strains were cultured in de Man, Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany) at 37°C under anaerobic jars for 48h. All bacteria were harvested by centrifugation (3000g for 15min) during stationary growth phase.

Pelleted bacteria were then washed 3 times in PBS, concentration was determined by colony-forming unit (CFU) counting, and diluted to a final working concentration of 10^8 CFU/mL in RPMI 1640. This stock suspension was aliquoted and stored

at -20°C . To inhibit uncontrolled bacterial growth, ultraviolet (UV) inactivation was done by placing an aliquot in wells of a 48-well culture plate and placing the plate 4cm from a UV light source for 40min. Samples were then tested for inactivity by culturing on MRS agar compared to live bacteria sample. UV-killed bacteria have a preserved structural integrity, in contrast to heat-inactivated bacteria.

The measurement of TNF- α , IFN- γ , IL-10 and IL-1 β secretion

After the incubation of PBMCs (2×10^5 cells/well) with different concentrations of (in a cell:bacteria ratio of 1:100 and 1:50) killed *L. acidophilus* LA-5 and *B. lactis* BB-12 strains (or RPMI-1640 as control) for 48 and 72h, the supernatant was aspirated and the TNF- α , IFN- γ , IL-10 and TGF- β levels were measured using sandwich ELISA (Quantikine, R&D Systems, Minneapolis, MN).

Statistical analysis

Results were expressed as mean \pm SD. Differences in mean values between groups were analyzed with Student's t-test to reveal significant differences between cytokine production in response to different strains of bacteria. Differences were considered to be significant at $P < 0.05$. Statistical calculations were performed with SPSS 19 (SPSS Inc., Chicago, IL, USA).

Results

Effect of *B. lactis* on pro-inflammatory and regulatory cytokines

As shown in **Table 1**, *B. lactis* significantly upraised IL-10 and TGF- β secretion by PBMCs in a dose- and time dependent-manner compared to control cultures ($p < 0.001$) (**Fig. 1a–d**). The secretion levels of TNF- α and IFN- γ by *B. lactis* stimulated PBMCs at 72h was significantly lower as compared to 48h time (TNF- α : $p < 0.001$ IFN- γ : $p < 0.035$) (**Fig. 2a–d**). This reduction could be due to immunomodulatory effects of the augmented IL-10 and TGF- β after 72h compared to 48h incubation time (**Fig. 1a–d**). The secretion levels of TNF- α and IFN- γ after stimulation of PBMCs by *B. lactis* at 100:1 bacteria:cells ratio was significantly lower than 50:1 bacteria:cells ratio ($p < 0.001$) (**Fig. 2a–d**). As it is shown in **Fig. 1a–d** this reduction could be due to the immunomodulatory effects of the augmented IL-10 and TGF- β at 100:1 compared to 50:1 bacteria:cells ratio.

At 48h of incubation time, the secretion levels of IFN- γ by PBMCs in the presence of the 50:1 bacteria:cells ratio of *B. lactis* were

Table 1 Evaluation of TNF α , IFN- γ , TGF- β and IL-10 secretion by PBMCs of Ulcerative Colitis patients by ELISA method.

	Bacteria: Cell	IFN- γ (pg/ml)		TNF- α (pg/ml)		IL-10 (pg/ml)		TGF- β (pg/ml)	
		Mean \pm SD		Mean \pm SD		Mean \pm SD		Mean \pm SD	
		48h	72h	48h	72h	48h	72h	48h	72h
Bifidobacterium	100:1	1320 \pm 341	1556 \pm 382	2071 \pm 452	2463 \pm 484	510 \pm 253	824 \pm 275	611 \pm 284	711 \pm 290
<i>lactis</i> + <i>Lactobacillus acidophilus</i>	50:1	1046 \pm 322	1177 \pm 292	1561 \pm 381	2072 \pm 426	317 \pm 171	498 \pm 268	472 \pm 164	566 \pm 168
Bifidobacterium	100:1	588 \pm 228	505 \pm 213	1486 \pm 380	822 \pm 366	756 \pm 191	1198 \pm 487	1422 \pm 401	1777 \pm 409
<i>lactis</i>	50:1	833 \pm 270	648 \pm 177	1888 \pm 561	1067 \pm 392	630 \pm 254	953 \pm 303	923 \pm 320	1195 \pm 398
<i>Lactobacillus</i>	100:1	1444 \pm 365	2061 \pm 388	2171 \pm 531	2687 \pm 553	473 \pm 214	563 \pm 250	276 \pm 165	341 \pm 164
<i>acidophilus</i>	50:1	1073 \pm 305	1419 \pm 438	1754 \pm 476	2173 \pm 546	315 \pm 167	416 \pm 233	228 \pm 165	284 \pm 163
Control	–	152 \pm 59	216 \pm 91	229 \pm 109	335 \pm 118	97 \pm 61	132 \pm 76	59 \pm 35	73 \pm 38

SD=standard deviation

significantly lower ($P<0.05$) whereas the secretion levels of IL-10 and TGF- β were significantly higher ($P<0.001$ and $P<0.0001$, respectively) as compared with the stimulation of PBMCs with *L. acidophilus* in the same time and same bacteria:cells ratio.

Similarly, at 48 h of incubation time, the secretion levels of IFN- γ by PBMCs in the presence of the 50:1 bacteria:cells ratio of *B. lactis* were significantly lower ($P<0.05$) whereas the secretion levels of IL-10 and TGF- β were significantly higher ($P<0.002$ and $P<0.001$, respectively) as compared with the stimulation of PBMCs with mixed probiotics in the same time and same bacteria:cells ratio.

At 48 h of incubation time, the secretion levels of IFN- γ and TNF- α by PBMCs in the presence of the 100:1 bacteria:cells ratio of *B. lactis* were significantly lower ($P<0.0001$ and $P<0.001$, respectively) whereas the secretion levels of IL-10 and TGF- β were significantly higher ($P<0.003$ and $P<0.0001$, respectively) as compared with the stimulation of PBMCs with *L. acidophilus* in the same time and same bacteria:cells ratio.

The same results were obtained with mixed probiotics in the same time and same bacteria:cells ratio.

At 72 h of incubation time, the secretion levels of IFN- γ by PBMCs in the presence of the 50:1 bacteria:cells ratio of *B. lactis* were significantly lower ($P<0.0001$) whereas the secretion levels of

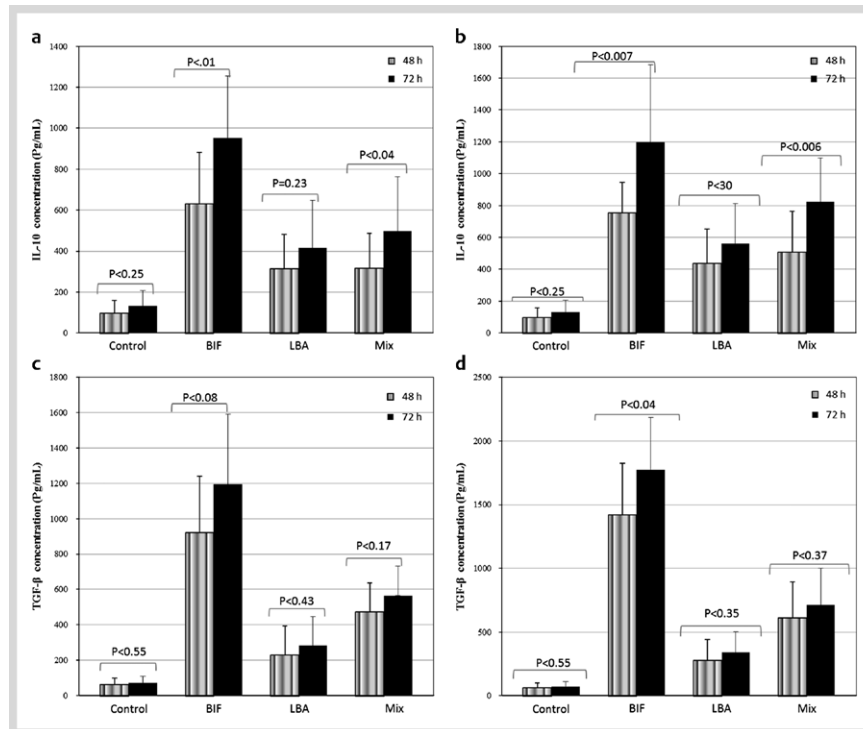


Fig. 1 a Effects of probiotics on the IL-10 secretion by PBMC the cell:bacteria ratio of 1:50. At 48 or 72 h of incubation times and in the cell:bacteria ratio of 1:50 *B. lactis*, *L. acidophilus* or both probiotics significantly enhance the production of IL-10 by PBMCs compared to control ($P<0.001$). b Effects of probiotics on the IL-10 secretion by PBMC the cell:bacteria ratio of 1:100. At 48 or 72 h of incubation times and in the cell:bacteria ratio of 1:100 *B. lactis*, *L. acidophilus* or both probiotics significantly enhance the production of IL-10 by PBMCs compared to control ($P<0.001$). c Effects of probiotics on the TGF- β secretion by PBMC the cell:bacteria ratio of 1:50. At 48 or 72 h of incubation times and in the cell:bacteria ratio of 1:50 *B. lactis*, *L. acidophilus*, or both probiotics significantly enhance the production of TGF- β by PBMCs compared to control ($P<0.001$). d Effects of probiotics on the TGF- β secretion by PBMC the cell:bacteria ratio of 1:100. At 48 or 72 h of incubation times and in the cell:bacteria density of 1:100 *B. lactis*, *L. acidophilus*, or both probiotics significantly enhance the production of TGF- β by PBMCs compared to control ($P<0.001$).

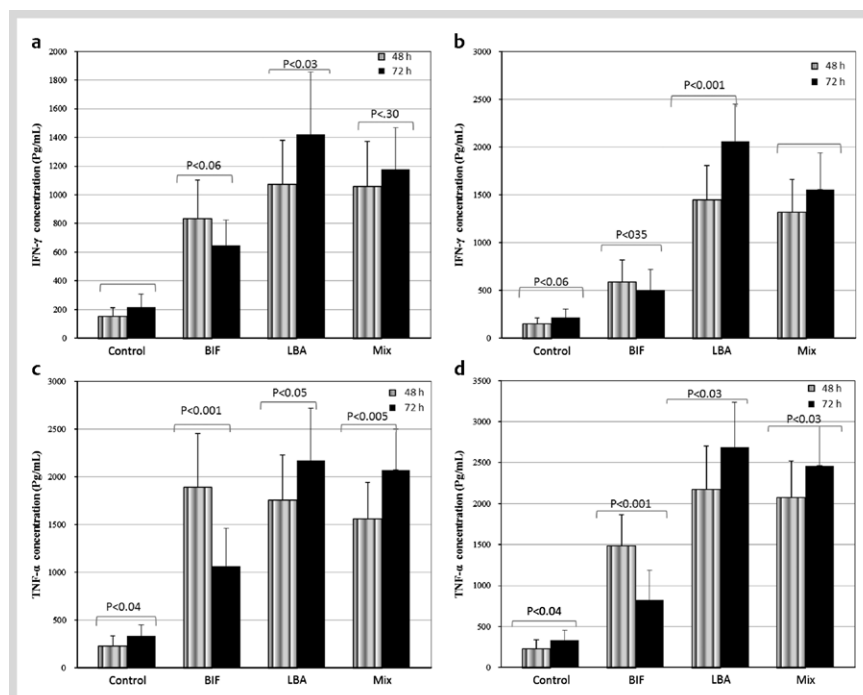


Fig. 2 a Effects of probiotics on the IFN- γ secretion by PBMC the cell:bacteria ratio of 1:50. At 48 or 72 h of incubation times and in the cell:bacteria ratio of 1:50 *B. lactis*, *L. acidophilus* or both probiotics significantly enhance the production of IFN- γ by PBMCs compared to control ($P<0.001$). b Effects of probiotics on the IFN- γ secretion by PBMC the cell:bacteria ratio of 1:100. At 48 or 72 h of incubation times and in the cell:bacteria ratio of 1:100 *B. lactis*, *L. acidophilus* or both probiotics significantly enhance the production of IFN- γ by PBMCs compared to control ($P<0.001$). c Effects of probiotics on the TNF- α secretion by PBMC the cell:bacteria ratio of 1:50. At 48 or 72 h of incubation times and in the cell:bacteria ratio of 1:50 *B. lactis*, *L. acidophilus* or both probiotics significantly enhance the production of TNF- α by PBMCs compared to control ($P<0.001$). d Effects of probiotics on the TNF- α secretion by PBMC the cell:bacteria ratio of 1:100. At 48 or 72 h of incubation times and in the cell:bacteria ratio of 1:100 *B. lactis*, *L. acidophilus* or both probiotics significantly enhance the production of TNF- α by PBMCs compared to control ($P<0.001$).

IL-10 and TGF- β were significantly higher ($P < 0.0001$) as compared with the stimulation of PBMCs with *L. acidophilus* or mixed probiotics in the same time and same bacteria:cells ratio. The same results were obtained in the presence of the 100:1 bacteria:cells (\bullet Fig. 1,2).

Effect of *L. acidophilus* on pro-inflammatory and regulatory cytokines

L. acidophilus upraised TNF- α , IFN- γ , IL-10 and TGF- β by PBMCs in a dose- and time-dependent manner in comparison with control cultures ($p < 0.001$) (\bullet Table 1). The secretion levels of IL-10 and TGF- β after stimulation of PBMCs by *L. acidophilus* were significantly lower as compared to *B. lactis* and mixed bacteria, totally (at 2 different incubation times and 2 different cells:bacteria ratios) ($p < 0.05$) (\bullet Fig. 1a–d). The secretion levels of TNF- α and IFN- γ after stimulation of PBMCs by *L. acidophilus* were significantly higher in comparison to *B. lactis* and mixed bacteria, totally (at 2 different incubation times and 2 different cells:bacteria ratios) ($p < 0.05$) (\bullet Fig. 2a–d). This difference could be a result of the lower secretion of the mentioned immunoregulatory cytokines by *L. acidophilus* compared to *B. lactis*.

Effect of mixed bacteria on pro-inflammatory and regulatory cytokines

The mixed bacteria significantly influenced the secretion of TNF- α , IFN- γ , IL-10 and TGF- β by PBMCs in a manner of dose- and time-dependent manner in comparison with control cultures ($p < 0.001$) (\bullet Table 1). The secretion levels of TNF- α and IFN- γ were significantly higher after stimulation of PBMCs by mixed bacteria, as compared to *B. lactis*, totally (at 2 different incubation times and 2 different cells:bacteria ratios) were significantly lower as compared to *L. acidophilus* ($p < 0.05$ and $p < 0.05$ respectively) (\bullet Fig. 2a–d). Furthermore, the secretion levels of IL-10 and TGF- β after stimulation of PBMCs by mixed bacteria was significantly lower in comparison to *B. lactis*, but were higher as compared to *L. acidophilus* ($p < 0.05$ and $p < 0.05$ respectively) (\bullet Fig. 1a–d).

Discussion

Although the immunopathogenesis of ulcerative colitis (UC) remains unclear, current evidence indicates that altered T cell response to intestinal environmental antigens such as resident commensal bacteria plays an important role [18–20]. So that an increased mucosal infiltration of activated CD4+ lymphocytes, dysfunctional dendritic cells, dysregulated macrophage-induced immune responses and abnormalities in regulatory pathways have been demonstrated [21,22]. Conventional therapies of UC, including corticosteroids, 5-ASA and thiopurines [23], were performed to reduce inflammation but do not change the natural course of disease, and many patients become refractory to conventional therapies during the course of the disease.

Recently, probiotic therapy has been considered to be potentially effective and safe in patients with UC. It's been reported that individual probiotic species have variable potential to stimulate regulatory or effector T cells in the mucosal immune system. Indeed, cytokine profiles in co-cultures of the probiotic bacteria with peripheral blood mononuclear cells (PBMC) show marked differences between strains [24].

In the present study we found that the mixed (*B. lactis* plus *L. acidophilus*) bacteria strongly modulate the secretion of IL-10,

TGF- β , IFN- γ and TNF- α in a manner of dose and time-dependent in comparison with control PBMCs. The amount of secreted TNF- α and IFN- γ by PBMCs after stimulation by mixed bacteria were less than *L. acidophilus* but more than *B. lactis*. These results indicate that *B. lactis* acts as a weak stimulator of PBMCs as compared to *L. acidophilus*.

Although the secretion of IL-10 and TGF- β after stimulation by *L. acidophilus* were upraised, however, the secretion of IL-10 and TGF- β by *B. lactis* stimulated- PBMCs were significantly higher in comparison to *L. acidophilus* and mixed bacteria. Higher levels of IL-10 and TGF- β may be a reason for the lower secretion levels of TNF- α and IFN- γ after stimulation of PBMCs by *B. lactis*, compared to *L. acidophilus* and mixed bacteria. Although we did not use blocking antibodies to IL-10 and/or TGF to demonstrate whether these cytokines are suppressing IFN and TNF production, these results still as a valuable data indicate that *B. lactis* could be a better candidate for Treg stimulation in comparison with *L. acidophilus* and mixed bacteria in UC patients.

Our results are in accordance with some previous studies. Akemi Imaoka et al. showed the secretion levels of IL-10 in the cultures of PBMCs isolated from the UC patients with heat-killed Bifidobacterium were higher as compared control cultures in the absence of probiotic bacteria [25]. Kato et al. showed that administration of Bifidobacterium-fermented milk for patients significantly reduced the clinical activity index after 12 weeks as compared with the control group [26]. Moreover, Peran L. et al. in an experimental study to compare the preventative effects exerted by *B. lactis* and *L. acidophilus*, in the trinitrobenzenesulphonic acid model of rat colitis showed when *B. lactis* was administered to colitic rats, a significant reduction in colonic TNF- α production was observed, whereas *L. acidophilus* administration significantly reduced colonic LTb4 production in the inflamed colon in comparison with untreated colitic rats [27]. Although still incompletely characterized, several reports indicate that the cytokine profile in UC patients is complex and seems to involve a variety of cytokines, as demonstrated by increased mRNA expression of IFN- γ , IL-13, IL-17A, IL-4, IL-5, IL-1b, IL-6, IL-8 and TNF- α which are categorized under 3 kinds of effector Th: Th1 (IFN- γ , TNF- α), Th2 (IL-4, IL-5, IL-13) and Th17 (IL-17A) and innate immune responses (TNF, IL-1b, IL-6 and IL-8) [28–32].

Studies have demonstrated that anti-TNF therapy resulted in down-regulation of mucosal TNF- α and IFN- γ mRNA expression in UC patients but therapy failure was positively associated with high mucosal mRNA expression of IFN- γ [33,34]. Increased expression of TNF- α and IFN- γ mRNA has been detected not only in active UC but also in inactive UC, suggesting that these cytokines play a pivotal role in relapse of UC [11,35].

Thus, it seems that high IFN- γ and TNF- α as pro-inflammatory cytokines have important role in mucosal inflammation and it suggests that suppression of them could have important role in the successful treatment of UC. So, in the present study we measured these 2 pro-inflammatory cytokines as the sign of effector Th stimulatory potential of *B. lactis* and *L. acidophilus*. On the other hand, evidence has shown that non-functional, absent Tregs or genetic mutations in Foxp3 induces hypersensitivity to intestinal bacterial antigens [36,37], and impairs the balance of intestinal mucosal immunity to inflammatory injury followed by lymphocytic infiltration of the intestinal mucosa [38,39]. CD4+CD25+Foxp3+ T cells increased in the colon of patients with UC [40]. These data indicated that Tregs would be a promising target for treating UC, because increasing the activ-

ity of appropriate Tregs in the gut should help to restore inflamed colonic tissues [41]. Then, although IL-10 and TGF- β are good representative of Tregs, to support the conclusions concerning the effects of the mentioned probiotics on Treg cells in this study it could be good to show increasing the frequency of the Treg population by known markers, such as Foxp3.

Furthermore, evidence has suggested that probiotic bacteria may have, in a species- or even strain-dependent manner, a potential use as anti-inflammatory agents which exert their effect by stimulation of Tregs [42]. Hence, in this study we tried to check if *B. lactis*, *L. acidophilus* or both have any effect on the Tregs of UC patients by measuring IL-10 and TGF- β . Although the results indicate that *B. lactis*, compared to *L. acidophilus*, had more stimulatory effect on Tregs of UC patients but it still stimulates, even less than *Lactobacillus acidophilus*, the effector Th cells (by augmenting TNF- α and IFN- γ) compared to control. Since it has been shown that each metabolic product or body fraction of probiotic bacteria could have its individual effect on immune system [43], it is suggested to fractionate *B. lactis* and check the individual effect of each metabolic product or trunk of the bacterium on Tregs of UC patients.

In conclusion, each of these 2 probiotics or their mixed could have individual modulatory impact on immune system including Treg/effector Th of UC patients. Because UC patients suffer from an aggressive intestinal mucosal inflammatory response, researchers are seeking for probiotic bacteria which potentiate Treg activity to suppress the effector Th cells in UC patients. Thus from these 2 selected probiotics in this study, *B. lactis* as one of the probiotic sources of yogurt, which had more stimulatory effect on Tregs of UC patients, albeit still stimulates the effector Th cells could be a good potential therapeutic candidate for further investigation.

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Declaration of Interest

The authors declare no conflicts of interest.

Affiliations

¹ Department of Immunology, Faculty of Medicine, Dezful University of Medical Sciences, Dezful, Iran

² Cellular and Molecular Immunology Research laboratory, Faculty of Medicine, Dezful University of Medical Sciences, Dezful, Iran

³ Sherkat Pegah Khoozestan, Dezful, Iran

⁴ Tehran Chemie Pharmaceutical Company, Tehran, Iran

⁵ Department of Immunology, School of Medicine, Kerman University of Medical Sciences, Kerman, Iran

⁶ Department of Microbiology, Azad University, Boroujerd, Iran

⁷ Department of Microbiology, Azad University, Arak, Iran

⁸ Department of Internal Medicine, School of Medicine, Dezful University of Medical Sciences, Dezful, Iran

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