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Research Article

The Effect of *Lactobacillus rhamnosus* and *Lactobacillus delbrueckii* on Fibroblast-like Synoviocytes (FLS) Isolated from the Synovium of Rheumatoid Arthritis Patients

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Abstract

Background: Rheumatoid arthritis (RA) is an autoimmune and inflammatory disorder and the most common type of autoimmune arthritis that causes joint inflammation and synovial membrane hypertrophy. Synovium of RA patients contains fibroblast-like synoviocytes (FLS) which contribute to cartilage degradation and inflammation through producing inflammatory cytokines. Recent researches have reported that probiotics can induce immunomodulatory activity in inflammatory disorders. Therefore, this study was conducted to investigate the effects of *Lactobacillus rhamnosus* and *Lactobacillus delbrueckii* on the FLS of RA patients.

Methods: The therapeutic effects of probiotic strains on FLS and their related molecules in RA patients were evaluated. FLS of patients with RA were cocultured for 48 h with *Lactobacillus rhamnosus, Lactobacillus delbrueckii* and a mixture of both probiotics. The supernatants of FLS cultured with probiotics were collected for quantification of IL-6 and TNF-α cytokines by ELISA. The mRNA expression of *IL-6, TNF-α, MMP3* and *RelA* was examined in FLS derived from RA patients by Real Time-PCR.

Results: Live *Lactobacillus rhamnosus and Lactobacillus delbrueckii*, alone and in combination, significantly increased the expression of *IL-6, TNF-α, MMP3* and *RelA* in FLS cells ($p<0.05$). However, a considerable difference was not observed among the groups that were treated with these two strains of *Lactobacillus*.

Conclusion: The current research may indicate these probiotics do not alleviate the inflammatory response of FLS cells in RA patients. Further investigation is needed to evaluate the probiotic effects of these bacteria on RA.

Introduction

Rheumatoid Arthritis (RA) is classified as a long-term autoimmune and inflammatory disorder affecting the synovial membranes of multiple joints.[1] The disease is estimated to affect about 0.8 percent of individuals worldwide and is more common in women.[2,3] In RA synovium, FLS cells, dendritic cells (DCs), macrophages, and infiltrating T lymphocytes are the most common and abundant cells that contribute to RA pathogenesis by producing inflammatory cytokines such as TNF-α, IL-1, and IL-6.[4,5] These mediators are associated with inflammatory processes, stimulation of FLS and activation of osteoclast activity.[4,6-8] Additionally, FLSs contain various matrix metalloproteinases (MMP), like MMP-3, contributing to the degradation of different types of collagen and proteoglycans and the activation of other MMPs.[9] It has been documented that nuclear factor kappa B (NF-κB) induces various pro-inflammatory factors and serves as a mediator of RA pathogenesis and inflammatory responses during early and late stages of inflammation in synovial tissues.[10] The NF-κB family is composed of five proteins, including RelA (p65), RelB, c-Rel, p50/p105, and p52/p100)[11] that RelA actively involves in the pathogenesis of RA.[12] Nonsteroidal anti-inflammatory

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drugs (NSAIDs), corticosteroids and disease-modifying antirheumatic (DMARDs) drugs are currently used to treat RA and reduce joint damage,[13] but these drugs have various side effects and are expensive.[14] In addition, some patients continue to suffer from persistent inflammation and progressive disability after using these drugs.[15] Hence, finding an efficient and cost-effective treatment is necessary to achieve better and more effective results.

Probiotics are non-pathogenic microorganisms that have recently been studied as a novel therapeutic option for the treatment or prevention of several diseases.[8,16,17] Researchers have documented that, probiotics can probably influence on balance between inflammatory and anti-inflammatory mediators.[18] Using probiotics as adjuvant therapy for RA has been suggested by several studies.[1] In animal model of RA, it has been documented that the outcome of disease was dependent on the mediators produced by different strains of probiotics.[19] Since the different strains of bacteria have the completely various probiotic effects, choosing the most suitable strain is essential. [1] Lactobacillus strains have been extensively studied for their probiotic potential in human and animals.[20] *Lactobacillus rhamnosus* (*L. rhamnosus* and *Lactobacillus delbrueckii* (*L. delbrueckii*) have potential probiotic effects, particularly through modulating immune system, pathogens inhibition, and anti-inflammatory effect.[21-23] In addition, several studies reported that lactobacillus strains represent an immunomodulatory effect on broad spectrum of autoimmune diseases, including multiple sclerosis, bowel disease, Celiac disease and RA.[13,24-26] Despite the immunomodulatory effect of lactobacillus by the mentioned studies, some researches have also reported that the strains may induce pro-inflammatory effect during immune responses.[27] Accordingly, this study aimed to evaluate the therapeutic potential of *L. rhamnosus* and *L.delbrueckii* on FLSs, by studying the expression of mediators such as IL-6, TNF-α, MMP3 and RelA to optimize the management of RA disease.

Methods

Study design and cell isolation

Five female RA patients were enrolled from Shafa Hospital, Sari, Mazandaran, Iran. RA was diagnosed according to the American College of Rheumatology criteria.[28] Patients with symptomatic RA of the knee were confirmed by a rheumatologist and synovial tissues were obtained during total knee replacement surgery. The characteristics of the patients were shown in Table 1. None of the RA patients were treated with biological DMARDs. However, they were treated with NSAIDs and non-biological DMARDs such as methotrexate, hydroxychloroquine and sulfasalazine. The experimental protocol was approved by the research ethics committee of the Kerman University of Medical Sciences (IR.KMU.AH.REC.1400.192). All patients gave informed consent via the statement "I am aware that my information is confidential, and I agree to participate in this study".

Table 1. Baseline characteristics of the study participants with rheumatoid arthritis.

Age (years) at diagnosis	54 ± 7.7
CRP (mg/L)	17.74±27.66
ESR (mm/h)	39.66±6.54
RF (%)	100
Anti CCP (%)	100
SJC28	7.8 ± 2.5
TJC28	5.3 ± 1.3

CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, SJC28: swollen join count for 28 joints, TJC28: tender- joint count for 28 joints.

Preparation and culture of fibroblast-like synoviocytes

The synovium tissue sample of RA patients was placed in a falcon tube containing 15 ml of DMEM medium (without FBS and antibiotics) by an expert surgeon during arthroplasty surgery. Then, fresh synovial tissues were washed with phosphate-buffered saline (PBS) containing 2% penicillin/ streptomycin and 2% amphotericin B. The tissues were then minced into 5mm^2 and digested with collagenase in high glucose Dulbecco's Modified Eagle's Medium (DMEM) (Biosera, South Korea) at 37 °C for 80 min using a shaking incubator. Then, the samples were centrifuged and resuspended in DMEM containing 10% FBS, 1% penicillin/streptomycin and subsequently were placed in a 25-cm^2 tissue culture flask (SLP, South Korea) at 37 °C in a humidified incubator with 5% CO_2 for all experiments. After 24 hours, non-adherent cells were removed and adherent cells were cultured in a complete medium. Medium was changed every three days. At confluence, cells were trypsinized and passaged in 25-cm^2 tissue culture flasks in a complete medium containing heat-inactivated FBS. Experiments were conducted between the third and the sixth passages that cultures had a homogenous fibroblastic cell population. Cell counting and viability were assessed using a hemocytometer and Trypan Blue dye (TB) (Merck, Germany).

Identification of FLS by light microscopy and flow cytometry

The morphology of FLS cells was evaluated by light microscope (Micros, Australia). In addition, the FLS cells were detected by flow cytometry. FLS cells were isolated from all patients and for detection of cell surface markers, the cells were washed and stained in staining buffer with the following fluorochrome labeled monoclonal antibodies: phycoerythrin (PE)-conjugated CD44 and fluorescein isothiocyanate (FITC)-conjugated CD68 antibodies or isotype control IgG2a PE at 4 °C for 30 min. Ultimately, all samples were washed and suspended in a staining buffer and then analyzed in a FACSCalibur system (BD Bioscience, San Diego, CA) and analyzed by flowJo software version 7.6.

Probiotic strains, culture medium and growth conditions

Two strains of *Lactobacillus*, including *rhamnosus* (ATCC: 9595) (American Type Culture Collection) and *delbrueckii subsp lactis* (PTCC: 1743(DSM 20072)) (Persian type

culture collection) were purchased from Pasteur Institute of Iran and Iranian Research Organization for Science and Technology (IROST), respectively. To activate the lyophilized Lactobacillus strains, they were suspended into MRS (de Man, Rogosa, and Sharpe) broth media (QUELAB, Canada) and incubated at 37°C for 1 h in anaerobic conditions, containing 5% CO_2. Subsequently, 1 µl of suspension was added to 4 µl MRS broth media and incubated at 37°C for 1 h. The suspension was vortexed and cultured in MRS Agar (QUELAB, Canada) at 37 °C for 48 h.

The concentration of the probiotic's population was measured using microbial growth curves. To do this, the suspension of both probiotics in MRS broth was incubated at 37 °C and the optical density (OD) of these suspensions was read at 0, 0.5, 1, 2, 4 and 6 hours by a spectrophotometer at 620 nm.[29]

Co-culture of FLS cells and probiotics

In order to investigate the effect of Lactobacillus strains on FLSs, the cells were stimulated with live probiotics at a ratio of 1:10. To do this, the isolated FLSs were adjusted to 3×10^4 cells in the presence of 3×10^5 probiotics /well and then the cells were divided into five groups as follows: The first group (control), FLS cells cultured without any intervention. The second group (LPS), FLS cells were treated with 100 ng/ml of lipopolysaccharide (LPS). In the other groups, FLS cells were treated with *L. delbrueckii* (FLS+Del), *L. rhamnosus* (FLS+Rham), and a mixture of both probiotics (FLS+Mix). FLS cells were cultured in DMEM media (BioSera, South Korea), for 48 h at 37 °C using the plastic adherence method. After 48 h, the supernatant as well as the cells were isolated for cytokine and gene expression assay, respectively.

Quantitative real-time PCR (qPCR)

The mRNA expression of *IL-6, TNF-α, MMP3* and *RelA* were determined by real-time PCR. To do this, FLS cells were trypsinized and isolated cells were transferred to a tube, centrifuged and pellet was washed twice in PBS, and total RNA was isolated from these cells using Tripure (Roche, Germany), according to manufacturer´s instructions. Then, the RNA was reverse-transcribed to cDNA using an Easy cDNA Synthesis kit (Parstous, Iran). The Real-Time PCR assay was conducted using RealQ Plus 2x Master Mix Green (Ampliqon, Denmark) in accordance with the manufacturer's instructions. The technique was performed by Roche Lightcycler 480 Real Time PCR system (Roche Diagnostics, Basel, Switzerland) following reaction conditions: 1 cycle at 95°C for 5 min (initial denaturation and enzyme activation), followed by 35 cycles amplification at 95°C for 30 s (denaturation), 57°C for 60 s (annealing and extension). The relative expression for each gene was calculated using the delta-delta Ct method. The *GAPDH* was used as a housekeeping gene for normalization the expression of studied genes. The tests were repeated in triplicate and the sequences of primers are depicted in Table 2.

Enzyme-linked immunosorbent assay (ELISA) assay

To determine the effect of probiotics on the cytokine levels, supernatants of FLS cells were collected after 48 h and cytokine contents were detected using ELISA kits for IL-6 and TNF-α (ZellBio, Germany) according to the manufacturer's protocol.

Statistical analysis

All results were reported as mean ±standard deviation and analyzed using one-way analysis of variance (ANOVA) followed by a Tukey *post-hoc* test. The statistical analysis was done by SPSS software version 16.0 (SPSS Inc, Chicago, IL, USA). P values < 0.05 were considered significant.

Results

Identification of FLS cells by light microscopy

To identify FLSs in RA joints, we isolated FLSs from synovial tissues of RA patients (Figure 1). The presence of the first cells took about 14 to 20 days of culture in the DMEM. In the third passage, a pure population of FLS cells was obtained. As was shown in Figure 1B, most cells are spindle-shaped, and a few cells have round to spherical shapes and stellate-shaped, so that after passaging further, cell populations have the spindle morphology (Figure 1C and 1D). In addition, the density and purity of FLS cells were increased during passaging (Figure 1A-D).

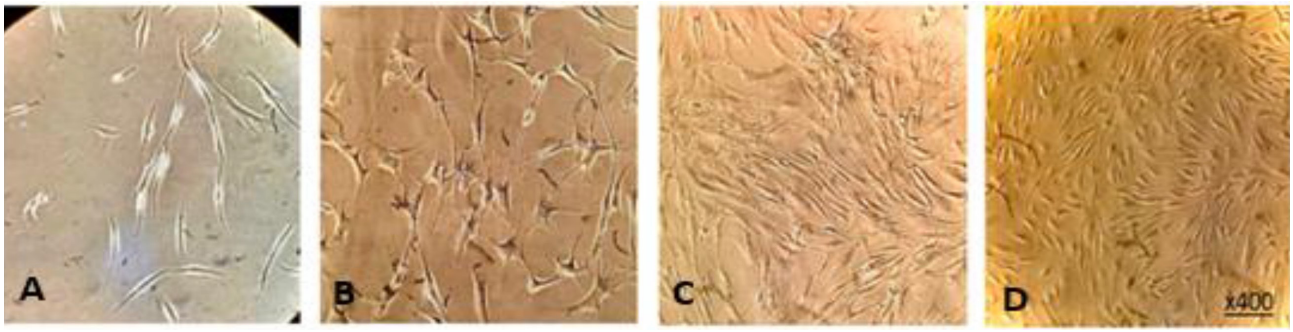

Figure 1. Light microscopic features of synovial tissue culture. Moving forward from (A) passage 0 toward (B) passage 1, (C) passage 2 and (D) passage 3, the density of FLS cells was increased.

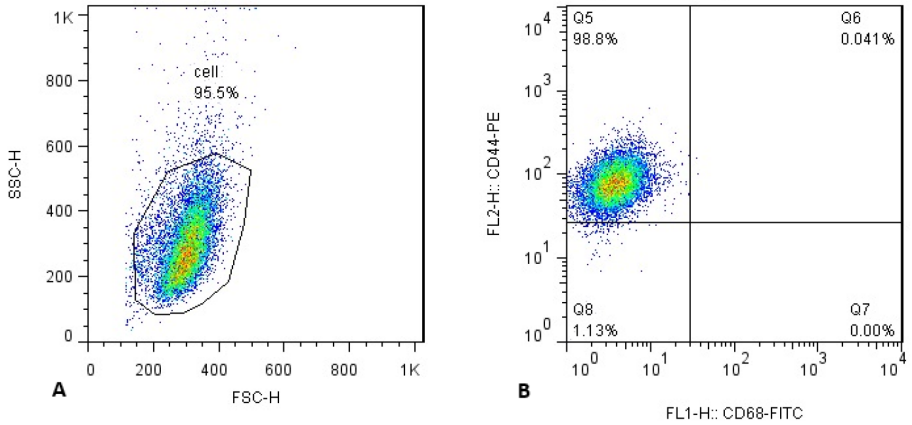

Figure 2. Confirmation of FLS cells using flow cytometry technique. The cells in passage 3 were gated by forward and side scatter. (B) FLS cells were then gated on the CD44 and CD68 population. Percent of CD44 positive and CD68 negative FLS cells in the samples was 98.8% and 0%, respectively.

Table 2. The sequences of primers which are used in the study.

Genes		Sequences
MMP3	F	5′- GGACAAAGGATACAACAGGGACCA -3′
	R	5′- TCATCTTGAGACAGGCGGAACC -3′
IL-6	F	5′- GACAAAGCCAGAGTCCTTCAGAGAG -3′
	R	5′- CTAGGTTTGCCGAGTAGATCTC -3′
TNF-α	F	5′- ATGAGCACAGAAAGCATGATC-3′
	R	5′- TACAGGCTTGTCACTCGAATT -3′
RelA	F	5′- CCCATCTTTGACAATCGTGCCC -3′
	R	5′- CAGCCTGGTCCCGTGAAATACA -3′
GAPDH	F	5′- AAGGTCGGAGTCAACGGATTT -3′
	R	5′- TGAAGGGGTCATTGATGGCA -3′

Identification of FLS cells by flow cytometry

In addition to light microscopy, flow cytometry was also used to identify FLS cells (Figure 2). The FLS cells are CD44+ (a specific marker of FLS cells), and $CD68^-$ (a marker of monocytes and macrophages). According to flow cytometry results, CD44 was expressed 98.8% and CD68 was not expressed (Figure 2B). Hence, cell lineage was confirmed by the presence of CD44 and the absence of CD68.

Effects of probiotics on the expression of genes related to inflammation by FLS cells

To investigate the effect of *L. rhamnosus* and *L. delbrueckii* on the expression of genes related to inflammation by FLS cells, Real Time PCR was performed. We selected some genes that contribute to inflammation, including *RelA, TNF-α, IL-6* and *MMP-3* to measure their expression in FLS cells that are exposed to probiotics. Probiotics significantly increased the mRNA expression of *IL-6* in FLSs ($p<0.01$) (Figure 3A). Although the level of *IL-6* expression in the Mix group (FLS+Mix) was higher than in the Rham and Del probiotic groups, this difference was not significant (Figure 3A). Our result also indicated the probiotics augmented the expression of the *TNF-α* in FLS cells that were exposed to probiotics, alone or in combination ($p<0.01$ for FLS+ Rham and $p<0.001$ for FLS+Del and FLS+Mix, Figure 3B). The data showed that the expression of *TNF-α* was lower in the FLS cells that were treated with *L. rhamnosus* than the other groups (FLS+ *L. delbrueckii* and FLS+mix probiotic), but this decrease was not significant between the three groups. There was also a statistically significant difference in the level of *RelA* and *MMP3* between FLS cells treated with probiotics and control (*P*<0.001 for *RelA* and P<0.01 for *MMP3,* Figures 3C and D). Furthermore, in FLS-treated with probiotics, the levels of *IL-6*, *TNF-α*, *RelA* and *MMP3* were increased (P<0.05, Figures 3A-D) when compared with the LPS group. Overall, our results indicated that *L. rhamnosus* and *L. delbrueckii* boost the level of *IL-6, TNF-α, RelA* and *MMP3* in FLS cells.

Effects of probiotics on IL-6 and TNF-α protein expression by FLS Cells

In order to evaluate the effect of probiotics on the production of inflammatory cytokines by FLS cells, the levels of IL-6 and TNF-α were measured in the supernatant of cell cultures. As shown in Figure 4, the production of IL-6 and TNF-α was significantly increased in the FLS cells which are treated with probiotics compared with the control group ($p<0.001$). *L. delbrueckii* stimulated IL-6 and TNF-α production more than *L. rhamnosus*, but this difference was not significant (Figure 4A and 4B). No significant differences were noted in IL-6 and TNF-α production following FLS exposure to both probiotics (FLS+Mix) when compared with probiotics alone.

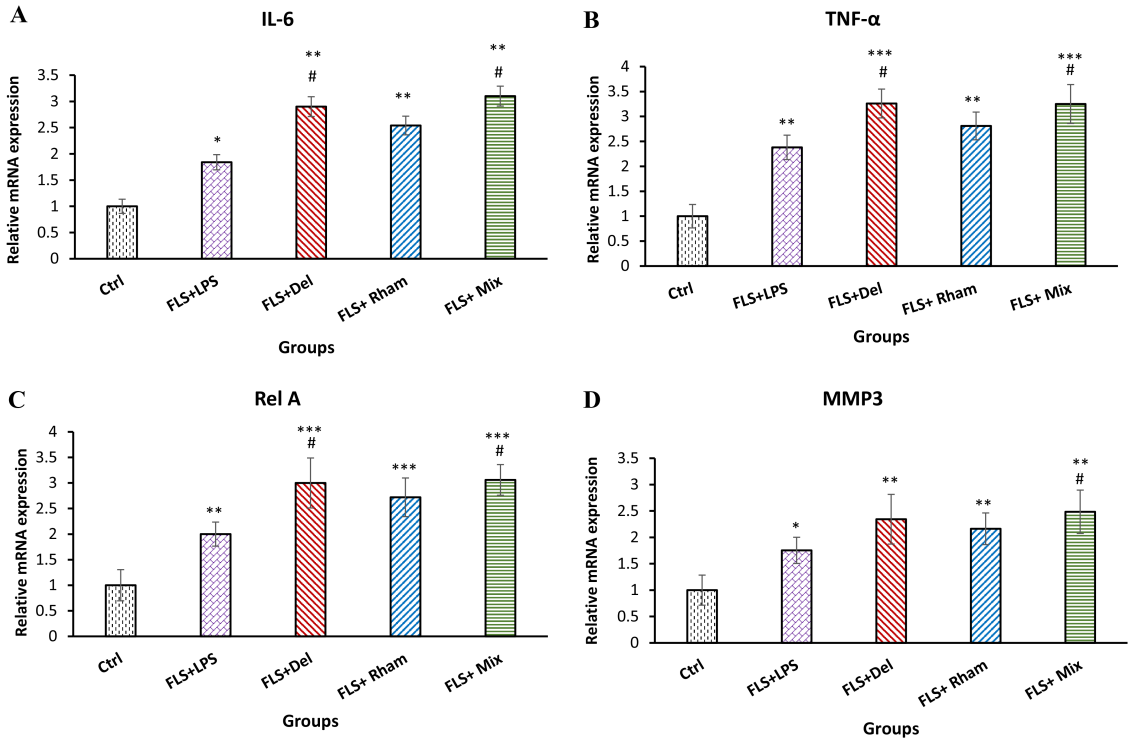

Figure 3. Effects of probiotics on the mRNA expression of pro-inflammatory factors by FLS cells. The mRNA expression of *IL-6* (A), *TNF-α* (B), *RelA* (C) and *MMP3* (D) were determined by real-time quantitative PCR. *GAPDH* was used as the housekeeping gene. Data are presented as mean±SEM of five independent experiments and analyzed by one-way ANOVA followed by Tukey's post hoc test. *p<0.05, **p< 0.01, ***p<0.001 versus control group and # p< 0.05 versus FLS+LPS group.

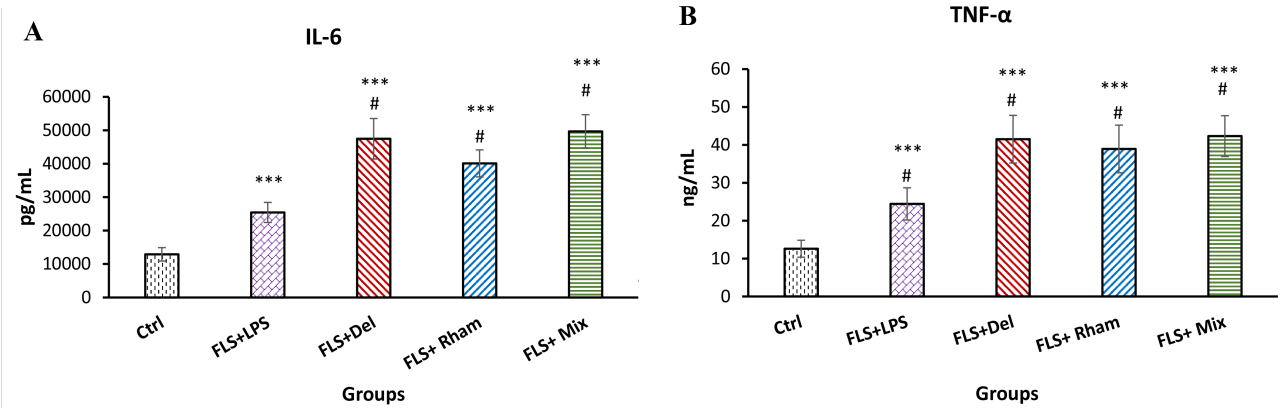

Figure 4. Effects of probiotics on the levels of key inflammatory mediators secreted by FLS Cells. The figure shows that *L. rhamnosus* and *L. delbrueckii* significantly increased the production of IL-6 (A) and TNF-α (B) by FLS cells. Data are presented as mean±SEM of five independent experiments and analyzed by one-way ANOVA followed by Tukey's *post hoc* test. ***p<0.001 versus control group and # p< 0.05 versus FLS+LPS group.

Discussion

RA is a chronic autoimmune disorder of the synovial tissues and the most common type of autoimmune arthritis. A variety of cells have an essential role in the pathogenesis of RA, including immune cells (T cells, B cells, plasma cells, dendritic cells, macrophages) and non-immune cells such as FLS cells. FLS contributes to RA pathogenesis by producing inflammatory mediators such as cytokines and matrix-degrading enzymes that boost local inflammation and disease progression.[30] FLS is the predominant cell type constituting 75–80% of all synoviocytes in normal human synovium[31] and used as a useful source for studying the pathogenesis of RA and assessing novel therapeutic targets.[11] Considering the side effects and low efficiency of currently available drugs in some patients,[32,33] the development of a novel and safe biomedical method needs to be considered for RA management.

Probiotics have been studied for their therapeutic effect in preventing or treating many diseases, such as infection, autoimmune and inflammatory diseases in animals and humans.[34] They exert their therapeutic properties in three ways, including immunomodulatory, antibacterial activity and competitive exclusion.[35] Researchers found that

specific probiotic supplements reduce inflammation, and alleviates symptoms associated with RA in randomized experimental trials.[13] Therefore, probiotics have the therapeutic potential for arthritis treatment. In this study, we addressed the effect of *L. rhamnosus* and *L. delbrueckii* on the FLS cells isolated from RA patients. The reason for selecting *L. rhamnosus* and *L. delbrueckii* was that these probiotics could modulate the immune and inflammatory responses in humans and animals.[29]

In the current study, *L. delbrueckii* and *L. rhamnosus*, alone or in combination, increased the expression of pro-inflammatory agents in FLS cells compared to the control and LPS-induced FLS groups. It was noticed that *L. delbrueckii* led to an increase in inflammatory genes greater than *L. rhamnosus*, which could be due to differences in the external structure or secreted metabolites between probiotics. Comparative genomics analyses revealed that *L. delbrueckii* strains interact with human proteins including *NF-κB, TLR4, TRAF6, RelA, NF-κB, FOS, JUN*, and *MAPK10* through their SLPs and extracellular proteins encoding genes. For example, it involves in the *NF-κB* signaling pathway with its PrtB protein.[22] It has also been reported in previous studies that different Lactobacillus strains have various inflammatory/anti-inflammatory effects.[36] In our experiment, treatment of FLS with *L. delbrueckii* and *L. rhamnosus* was accompanied by further production of RelA, IL-6 and TNF-α which was in line with the result of previous studies on the macrophages treated with the various strains of Lactobacillus.[37,38] Matsuguchi *et al.*[39] reported that the expression of NF-κB and AP-1 is necessary for the production of TNF-α in macrophages which is done through TLR-2. In fact, the protoplast of probiotics which contains 99% lipoteichoic acid (LTA), has the most effect on the production of TNF-α and NF-κB.[37] Specifically, peptidoglycan, which is present in abundance in the cell walls of all gram-positive bacteria plays a key role in the activation of these genes.

Many studies have demonstrated that treating cells with different forms of bacteria, such as dead, live or bacteria components, can have different effects on the cells. For example, a study by Ciszek-Lenda *et al.*[40] showed that dead *L. rhamnosus* stimulates more TNF-α production in comparison to LPS and extracellular polymeric substances (EPS)- derived from *L. rhamnosus*, while the live bacteria had the potential to activate the anti-inflammatory pathways. In another study on LPS-stimulated RAW264.7 macrophages, Qi *et al.*[41] showed that the SLP (surface layer protein, a component of *L. rhamnosus*) makes a more intense response than genomic DNA and unmethylated cytosine-phosphate-guanine-containing oligodeoxynucleotide, alone or in combination. Apparently, in our study, since whole bacteria were used, the expression level of *TNF-α* and *NF-κB* was higher than other inflammatory mediators, which suggests that the use of whole bacteria has a greater effect on the expression of these genes. Also, the live L. rhamnosus and *L. delbrueckii* probiotics in the current study demonstrated the increased expression of *MMP3* which is involved in cartilage destruction. The study on peptidoglycan and LPS-induced FLS in juvenile idiopathic arthritis showed that activation of *MMP3* occurred following TNF-α and IL-1β activation through TLR-2 and TLR-4, which has a similar result to our study.[42] However, another research showed that dead *L. rhamnosus* in synovium downregulates the expression of *MMP3*.[43] Therefore, the different inflammatory responses of our experiment with other studies[44-46] can be due to the difference in the Lactobacillus strains and the type of cells used, as most of the studies were done on macrophages. Moreover, the dead, live and different strains of Lactobacillus may have different pro-inflammatory responses.

Furthermore, it has been documented that probiotics have various biological effects in different doses. As shown by Zheng *et al.*,[47] treatment of Caco-2 cells with the different concentrations of *L. rhamnosus* revealed that the expression of *IL-6* and *TNF-α* is dose-dependent. However, the expression of *TNF-α* was significantly lower in 10^8 cfu/ml of *L. rhamnosus*.Hence, the concentration of probiotic bacteria strongly influences their therapeutic potential. The bacteria concentration per milliliter in our studies was much lower than 10^8 which may have a reverse effect on inflammatory cytokines. In addition, in studies that the cells were initially stimulated with LPS and then probiotics, probiotics have a beneficial effect on reducing the expression of inflammatory cytokines and alleviating the symptoms of the disease. For example, Cai *et al.*[27,48] showed that treatment of LPS-induced RAW 264.7 cells with SLP reduces the pro-inflammation genes, but the treatment of RAW 264.7 cells with SLP increases the inflammation. These results suggest that probiotics lack a synergistic effect with LPS in inducing inflammatory responses, and it can be assumed that probiotics or their products alone may have minor stimulatory effects. However, it seems that lactobacillus in the presence of an inflammatory stimulus such as LPS can act as an immunomodulator.

Conclusion

This study indicated that the live *L. rhamnosus* and *L. delbrueckii* induce the inflammatory response in FLS and speed up RA progression in the absence of pre-treatment with LPS. Hence, these live bacteria do not have an immunomodulatory effect in cells without exposure to LPS. However, the effect of live *L. rhamnosus* and *L. delbrueckii* on LPS-induced FLS could be completely different that it showed the need for further investigation. It is recommended that the effect of live and dead lactobacilli and cell-free supernatant on FLS cells and LPS-stimulated FLS will be investigated to understand more about the further probiotic's potential of these strains in the treatment of RA. On the other hand, although *L. rhamnosus* and *L. delbrueckii* have often shown inhibitory responses on other immune cells or *in vivo* studies, our results on FLS showed that further studies are needed to better clarify the mechanism of action of these probiotics.

Ethical Issues

The experimental protocol was approved by the research ethics committee of the Kerman University of Medical Sciences (IR.KMU.AH.REC.1400.192). All patients gave informed consent via the statement "I am aware that my information is confidential, and I agree to participate in this study".

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Author Contributions

Arezoo Babazadeh: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Data Curation, Funding Acquisition, Visualization, Writing – Original Draft, Writing – Review & Editing. **Reza Nosratabadi:** Conceptualization, Methodology, Validation, Formal Analysis, Resources, Project Administration, Visualization, Supervision, Writing – Review & Editing. **Hamid Daneshvar:** Methodology, Resources, Project Administration, Supervision. **Mahmoud Mahmoudi:** Validation, Formal Analysis, Visualization, Writing – Review & Editing. **Houshang Rafatpanah:** Conceptualization, Methodology, Resources, Project Administration. **Jalil Tavakol Afshari:** Validation, Formal Analysis, Project Administration, Visualization, Supervision, Writing – Review & Editing. **Parisa Zafari:** Validation, Formal Analysis, Project Administration, Visualization, Supervision, Writing – Review & Editing. **Seyed-Alireza Esmaeili:** Conceptualization, Methodology, Validation, Formal Analysis, Resources, Project Administration, Visualization, Supervision, Writing – Review & Editing.

Conflict of Interest

The authors report no conflicts of interest.

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