Research Article

Formulation and Design of Probiotic Supplements for Rheumatoid Arthritis Patients

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ABSTRACT

Background: Probiotics are live microorganisms with immune-regulatory properties and may be useful for patients suffering from rheumatoid arthritis (RA), an autoimmune inflammatory disorder. The aim of the present study was to formulate *L. casei* 01 capsules at laboratory scale, and evaluate its effects on the proportion of T-helper type 2 (Th2) anti-inflammatory cytokines to T-helper type 1 (Th1) pro-inflammatory cytokines (Th2/Th1), in RA patients.

Methods: After blending the probiotic and excipient (maltodextrin) based on the relevant calculations, the content uniformity of the mixture was evaluated. Furthermore, viability of the probiotic bacteria was assessed during capsules production and throughout three months of storage. In a randomized double-blind placebo-controlled trial, 46 RA patients were supplemented with either the capsules (containing at least 10^8 CFU of *Lactobacillus. casei* 01) or placebo (maltodextrin), for eight weeks; DAS28 (Disease activity score 28) as well as serum inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-10 and IL-12) were measured at baseline and the end of study. IL-10/IL-1β, IL-10/IL-6, IL-10/IL-12, IL10/TNF-α and IL-10/(IL-1β+ IL-6+ IL-12+ TNF-α) were calculated, the latter being expressed as IL-10/total Th1, and compared for the groups. Paired samples *t* test, Wilcoxon signed-rank test and ANCOVA tests were applied.

Results: Probiotic powder had been uniformly mixed with the excipient and the bacteria had acceptable viability throughout the study course. Supplementation of RA patients with the capsules resulted in a significant decrease in disease activity (DAS28, *P*=0.039) and increase in IL-10/TNF-α, IL-10/IL-12 and IL-10/total Th1 (*P*=0.039, *P*=0.012 and *P*=0.014, respectively). At the end of the study, there was a significant difference between the two groups in terms of IL-10/IL-12 and IL-10/total Th1 (*P*= 0.038 and *P*= 0.006, respectively).

Conclusion: *L. casei* 01 supplements may have the expected desired anti-inflammatory effects in RA patients. Further clinical trials are warranted to confirm these results.

Introduction

Probiotics have been defined by FAO/WHO as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host".[1] These microorganisms have been reported to be effective against a substantial number of disorders; regulation of the immune system function is a privilege that has been frequently documented.[2] Different probiotics (even the strains of the same species) influence immune system responses in a strain-specific manner. For instance, some probiotics have been found to increase T-helper type 1 (Th1) cytokines; this apparently makes them a better choice as beneficial adjunct therapy for patients suffering from immuno-deficiencies like acquired immune deficiency syndrome (AIDS). Other strains have been shown to increase T-helper type 2 (Th2) cytokines, and may act as effective agents for alleviating symptoms in patients with auto-immunities such as rheumatoid arthritis (RA). Deciding on an appropriate dosage is as critical, to obtain the expected benefits for probiotics.[3-5]

RA is an autoimmune inflammatory disease which predominantly affects the joints and causes severe pain and a great extent of disability.[6] Results from observational and animal studies have suggested a causal

effect of gut microbiota changes in the development of RA.[7-12] Therefore, it has been attempted to alleviate the symptoms of the disease through normalizing the gut bacterial pattern of the patients; administering probiotic preparations is one possible way to achieve this goal.[13-15] Animal studies have shown that various strains of *Lactobacillus casei* (*L. casei*) can efficiently decrease inflammatory cytokines in RA.[16-19] However, it is unclear whether this species can be efficacious for RA patients; the optimum dosage of this probiotic for presenting the consumer with the desired effects has not been determined either. Thus, the aims of the present study were formulating probiotic capsules containing 10^8 colony forming units (CFU) of pure *L. casei* 01, and studying its effects on inflammatory pattern of RA patients in a clinical trial.

Material and Methods

L. casei 01 capsule preparation

Gelatin yellow size four (volume: 210 mm^3) capsules were used for capsulation of the probiotic bacteria, in the present study. *L. casei* 01 was the active agent of the capsules, and maltodextrin was used as the excipient. The capsules were prepared using a self-designed laboratory scale capsule filling device. The capsule filling device was made up of three parts: 1) the main board on which, 144 (12×12) holes were pierced by laser, 2) the lower board which rested on the screws dipped through the legs, and was thus mobile, and 3) the legs, into which the screws were dipped. To use the device, the capsules were uncapped; then the bodies of the capsules were placed in the device in a way that their ends rested on the lower board and their tops were tangent to the main board. The powder (mixture of probiotic and excipient) was then spread over the main board by a sterilized spatula. Then the device was moved onto a laboratory plate to let the mobile lower board move upward; this allowed for capping of the capsules. The capsules were locked.

L. casei 01 as the active agent of the capsules

Lyophilized *L. casei* 01 bacteria, with a granular yellow appearance, were purchased from Chr. Hansen (Denmark). The granules were pounded into powder. The powder was cultured using MRS agar (Liofilmchem, Italy) to make sure it contained the minimum of 10^{12} CFU of the bacteria per gram; serial dilution and pour plate technique were used. The density of *L. casei* 01 was calculated by dividing the mass of the probiotic powder filled in a capsule, by the volume of the capsule (210 mm^3). To calculate the bacteria mass in a capsule, three capsules were weighed before and after filling them with the powder. The mean of the weight differences (weight after filling minus weight before filling) for the three capsules was considered the mass of the *L. casei* 01 powder within them.

Maltodextrin as the excipient of the capsules

Food grade maltodextrin (Shandong, China) was used as the excipient in the capsules. Since the bacterial count of the excipient could have confounded the outcome of the study, the powder was cultured to ensure it was free of pathogens. Plate count agar (Merck, Germany) and MacConkey agar (Merck, Germany) were used for total, and gram-negative bacteria enumeration, respectively; serial dilution and pour plate technique were used. To ensure the desired bacterial count of the final capsules, the accurate density of the excipient was required for proper estimation of the proportion at which the excipient had to be mixed with the active agent (*L. casei* 01)., The density of the maltodextrin sample was calculated in a similar way to the probiotic powder.

To check if the excipient smoothly dropped into the capsule bodies and even amounts of the powder were distributed into the capsules, defined as proper flowing property, 40 capsules filled with maltodextrin, were weighed and the data were analyzed by SPSS version 20.0 software (SPSS Inc, Chicago, IL, USA). The mean weight, standard deviation (SD), skewness and kurtosis were checked and the relative standard deviation (RSD) was calculated by dividing SD by mean, multiplied by 100 and reported as percent. The lower the RSD, the more appropriate the flowing property is assumed to be; acceptable RSD is ≤5%.[20]

Preparation of the mixture

Accurate calculations were performed to estimate the proper proportion with which the probiotic powder and the excipient had to be mixed. The final capsules were planned to have a minimum of 10^8 CFU of *L. casei* 01. Considering the probable decrease in the bacterial counts during the capsule preparation procedures, the calculations were based on 10^{10} CFU of the probiotic bacteria per capsule. As aforementioned, the probiotic powder had an average of 10^{12} CFU of bacteria per gram. Thus, the mass of probiotic powder which had 10^{10} CFU of bacteria was obtained to be 0.01 g:

$$m(pro) = \frac{10(10)}{10(12)} = 0.01 \text{ g}$$

$$\rho(pro) = \frac{m(pro)}{V(pro)} \qquad V(pro) = \frac{0.01}{\rho(pro)}$$

$$V(exc) = V(cap) - V(pro)$$

$$\rho(exc) = \frac{m(exc)}{V(exc)} \qquad m(exc) = \rho(exc) \times V(exc)$$

$$m(cap) = m(pro) + m(exc)$$

[m: mass, ρ: density, V: volume, cap: capsule, pro: probiotic powder, exc: excipient powder]

To assess whether the final mixture had content uniformity and check if the probiotic bacteria were properly mixed with the excipient and evenly dispersed into the capsules, 10% of the final batch was prepared, in a pilot study. About 2400 probiotic capsules were to be prepared for the clinical trial; this was equal to 24 g of the *L. casei* 01 powder. Thus, 2.4 g (10%×24 gr) of *L. casei* 01 was weighed and mixed withmaltodextrin based on the calculations. The mixture was transferred into a mixer. At 15, 25 and 35 minutes from the beginning of the mixing process, a capsule was filled with the mixture.. The three capsules were then cultured using MRS agar, by serial

dilution and pour plate technique. Equal bacterial count of the three capsules was indicative of the uniformity of the mixture. Moreover, this procedure tested whether the bacterial content of each capsule conformed to the minimum count required (10^8 CFU/capsule).

Bacterial count of the capsules

Since the bacterial count of the capsules was of great significance and a minimum of 10^8 CFU of the probiotic was expected to retain by the end of the study period, the capsules were cultured three times; at baseline, in the middle and at the end of the clinical trial. The content of the capsule was thoroughly dissolved in 10 ml of physiological saline; then, serial dilution and pour plate technique were applied; the plates were checked for the number of colonies, 24 and 48 hours after culturing. The average of the colony counts for the three capsules was considered as the probiotic count per capsule.

Clinical assessment of the capsules

This is a secondary analysis from a previously published paper.[21] No details were presented on the formulation and preparation of the capsules in that paper; furthermore, the ratios of the inflammatory cytokines, which are of great importance in the treatment-response evaluation of inflammatory diseases, were not published in that article. The target population of the study was the women with inactive or moderate RA, diagnosed in the rheumatology clinic of Sina Hospital (Tabriz) or Specialized and Sub-specialized Sheykholrais Poly-Clinic (Tabriz). In a randomized double-blind, placebo-controlled clinical trial, 22 RA patients received *L. casei* 01 capsules and 24 RA women took identical capsules containing only maltodextrin, for eight weeks. The patients were non-pregnant,– non-lactating women who had established RA according to ACR criteria for more than one year, had no coexisting metabolic diseases or gastrointestinal disorders, consumed no dietary supplements, antibiotics or other probiotic products, were not exposed to cigarette smoke and were willing to participate in the study. At baseline, a demographic questionnaire was filled, and anthropometric measurements were performed for the patients. At baseline and the end of the study, eight ml of fasting blood sample was drawn, and the sera of the samples were separated and stored at -70ºC until analysis. At the end of the study, anthropometric measures were repeated. The participants were asked not to change their habitual dietary intake and physical activities through the study course. Informed consent was obtained from the patients and the procedures followed in the present clinical trial were in accord with the Helsinki Declaration and approved by the ethics committee of Tabriz University of Medical Sciences (no. 9149). The study was also registered in IRCT (IRCT201206234105N9).

Disease activity score 28 (DAS28) which is a measure of disease activity in RA patients, was calculated based on the count of swollen and tender joints, serum highly sensitive C reactive protein (hs-CRP) and visual analogue scale (VAS) scores (a 100 mm line, one end representing "Good health" and the other indicative of "The worst health status possible"), at baseline and end of the study; the tender and swollen joints were detected by a rheumatologist, VAS scores were reported by the patients based on their conception of their present disease state, and Turbidometric assay and commercial kits (Parsazmun, Iran) were used to measure hs-CRP. Serum levels of Th1 cytokines (IL-1β, IL-6, IL-12 and TNF-α) and Th2 cytokine (IL-10) were measured by Enzyme-Linked Immunosorbent Assay (ELISA) method using commercial kits (DIASource, Belgium). The IL-10/IL-1β, IL-10/IL-6, IL-10/IL-12 and IL-10/TNF-α as well as IL-10/(IL-1β+IL-6+IL-12+TNF-α) (expressed as IL-10/total Th1) were calculated at baseline and the end of the study as indicators of Th2/Th1 ratio. These results were compared for the two intervention groups.

Statistical analyses

SPSS software version 20.0 (SPSS Inc, Chicago, IL, USA) was used to perform the study analyses. Results are presented as Mean (SD) for normally distributed quantitative data, Median (percentiles 25 and 75) for quantitative data not normally distributed, and frequency (percentage) for qualitative data. To compare the two groups, Independent samples *t* test, Mann-Whitney U test, Chi square and Fisher's Exact Test were applied. The within group changes were assessed by Paired samples *t* test and Wilcoxon signed-rank test. ANCOVA was used to compare the study groups at the end of the study, adjusting the results for baseline measures and menopausal status. *P* values less than 0.05 indicated statistically significant differences.

Results and Discussion

L. casei 01 capsules evaluation

The density of *L. casei* 01 powder was 0.37 g/ml (0.00037 g/μl) and culturing results confirmed that each gram of the purchased probiotic powder contained a minimum of 10^{12} CFU of bacteria. The density of maltodextrin was 0.86 g/ml (0.00086 g/μl). Plate count agar and MacConkey agar culturing results showed that the powder was devoid of pathogenic bacteria.

The mean weight, SD and RSD of maltodextrin filled in the capsules, was 0.18 g, 0.003 and 1.62%, respectively; thus the flowing property of maltodextrin was acceptable. The proportion of probiotic powder and maltodextrin was calculated as follows:

$$m(pro) = \frac{10(10)}{10(12)} = 0.01 \text{ g}$$

$$V(pro) = \frac{0.01}{0.00037} = 27 \text{ μl}$$

$$V(exc) = 210 - 27 = 183 \text{ μl}$$

$$m(exc) = 0.00086 \times 183 \sim 0.15 \text{ g}$$

$$m(cap) = 0.01 + 0.15 = 0.16 \text{ g}$$

The pilot batch was prepared accordingly; 36 g (10% of the total amount required for the capsules prepared for the clinical trial) of maltodextrin was mixed with 2.4 g *L. casei* 01 powder. Content uniformity of the probiotic bacteria was assessed as described in section 2-1-3. The

bacterial count in all the three capsules was 10^8 CFU, indicating proper intermixture of the probiotic powder and the excipient.

Bacterial enumeration of the capsules at baseline, in the middle and at the end of the intervention period showed that the capsules contained a minimum of 10^8 CFU of *L. casei* 01 at the three time sections.

The majority of the probiotic supplements available in the market contain a combination of various bacterial strains due to the belief that a synergistic effect of different probiotics consumed together may result in more significant clinical effects.[22] However, when probiotic supplements are to be administered to patients with immune-related disorders, it is crucial to choose a specific single or combination of bacterial strains with particular immune-regulatory properties.[5] To the best of our knowledge, no probiotic supplements have been claimed to be efficacious for RA subjects, thus far.

L. casei being a species of genus *Lactobacillus*, is among the few known lactic acid bacteria (LAB) that are used as probiotics.[23] *L. casei* 01 has been shown to sufficiently resist gastric and intestinal pH and properly adhere to IEC-6 epithelial cells (which is an *in vitro* indicator of proper colonization in the gut).[24-26] *L. casei* strains have been frequently shown to down-regulate immune system function,[27-33] which is desired for rheumatoid arthritis patients in whom excessive immune system activity results in the disease manifestations. Thus, *L. casei* 01 was opted as the active agent in the probiotic capsules aimed for RA patients.

Generally, it is believed that a probiotic product should provide the consumers with $\geq 10^8$-10^{10} CFU/day of live microorganisms to be clinically effective.[34] Most probiotic supplements contain 5-25 billion of the bacteria to meet these criteria; even much higher dosages are used in clinical trials to guarantee the expected health benefit.[35] Some studies have shown that lower dosages of probiotics are more effective in exerting immune-regulatory properties. It is proposed that these bacteria might activate other pathways in dendritic cells at high dosages. These pathways may interfere with the cross-talk of Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) signaling, which might consequently prevent development of T-regulatory cell, to some extent.[4,36-38]

Taking all the above mentioned issues into account, the *L. casei* 01 capsules were designed to contain the dose of 10^8 CFU; this dosage was safe for RA patients.

Effects of L. casei 01 supplementation on disease activity and Th2/Th1 cytokines proportion in RA patients

Probiotic supplementation significantly decreased serum hs-CRP level ($P=0.009$), tender ($P=0.003$) and swollen ($P=0.003$) joint counts, VAS ($P<0.001$) and DAS28 ($P=0.039$), compared to the placebo group. Table 1 presents the results for demographic characteristics and anthropometric measures of the study patients. No significant difference was observed between the two groups for these variables. Also, weight and body mass index (BMI) of the participants showed no significant changes throughout the study course ($P>0.05$)
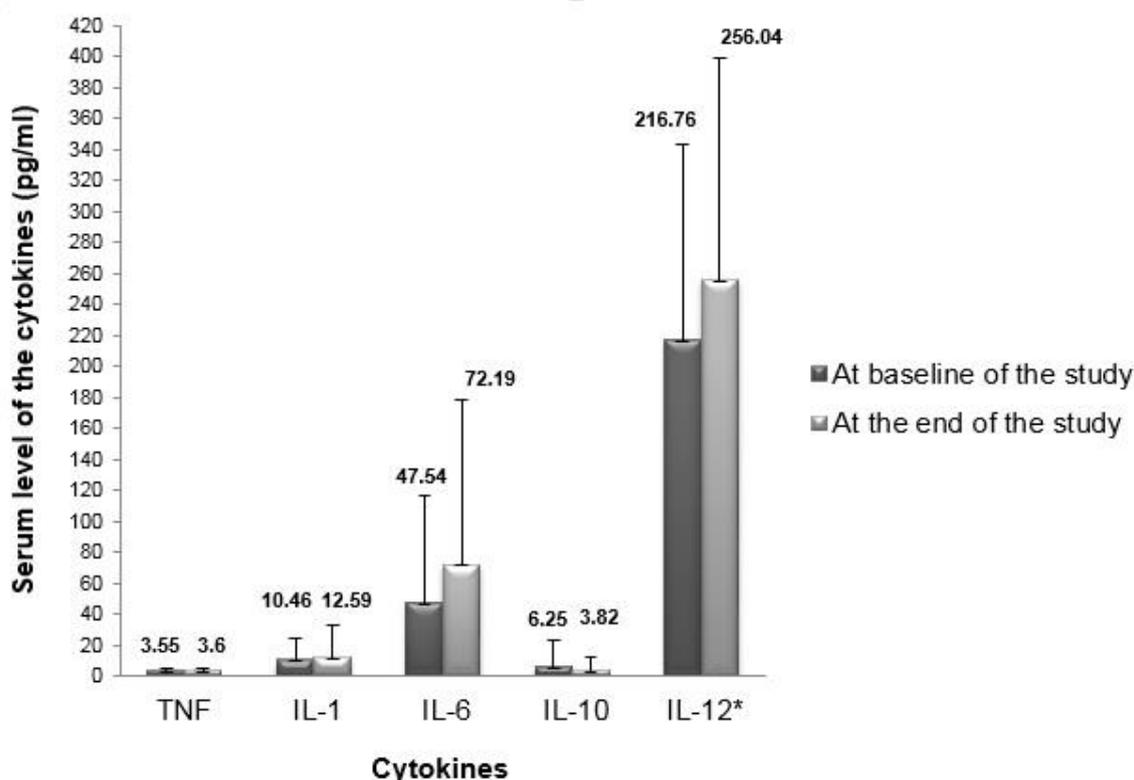

Figure 1. Mean concentration (pg/ml) of serum cytokines throughout the study course in the placebo group. Wilcoxon signed-rank test; ● statistically significant change within the group ($P<0.05$).
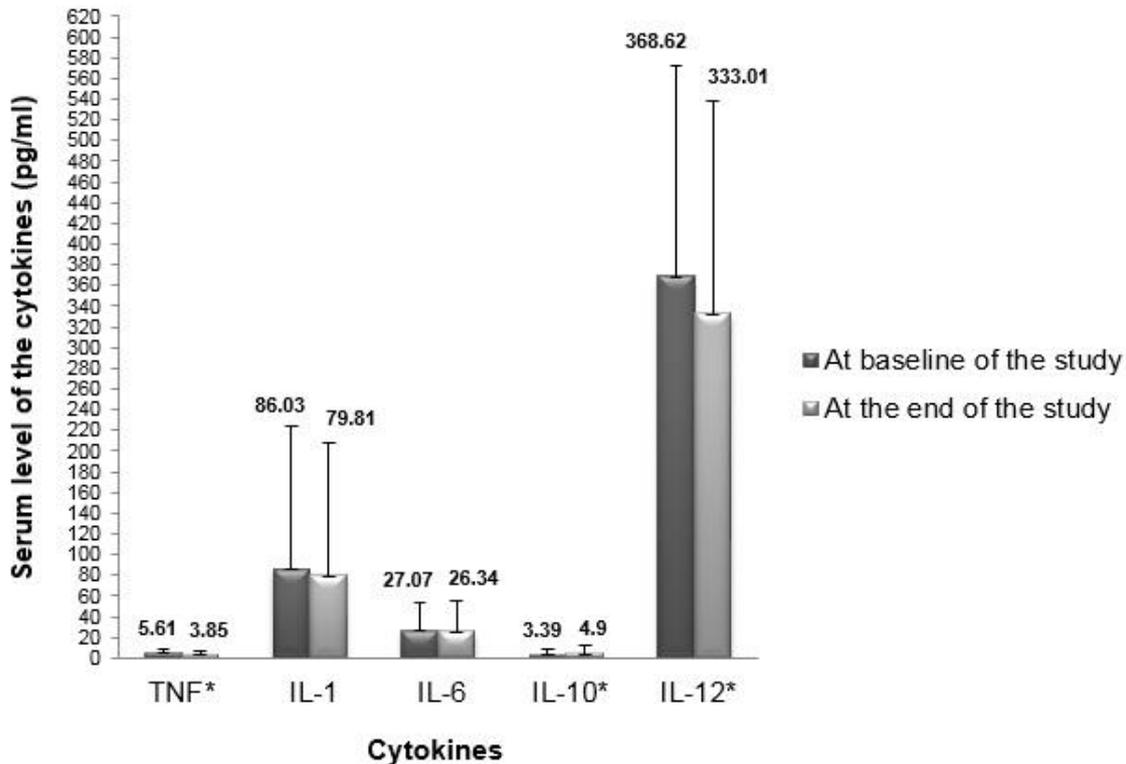
Figure 2. Mean concentration (pg/ml) of serum cytokines throughout the study course in the probiotic group. Wilcoxon signed-rank test; ● statistically significant change within the group (*P*<0.05).

In Figure 1 and 2, the mean results (pg/ml) for the serum cytokines are presented in the placebo and probiotic groups, respectively. There was a significant difference between the two groups for baseline IL-12 (*P*= 0.006).

Table 1. Demographic characteristics and anthropometric measures of the RA patients at baseline.

	Placebo group (n=24)	Probiotic group (n=22)
Age (yrs)[†]	44.29 (9.77)	41.14 (12.65)
Duration of RA (yrs) [‡]	4.75 (3.0, 9.0)	5.25 (3.75, 10.0)
Menopausal status[‡]		
Premenopausal	17 (70.8)	15 (68.2)
Postmenopausal	7 (29.2)	7 (31.8)
Current medication		
Methotrexate [§]	20 (83.3)	15 (68.2)
Hydroxychloroquine [*]	18 (75.0)	18 (81.8)
Prednisolone [*]	23 (95.8)	21 (95.5)
Height (cm) [†]	156.02 (6.40)	158.16 (6.78)
Weight (Kg) [†]	68.56 (11.96)	69.29 (11.47)
BMI (Kg/m²) [†]	28.08 (4.03)	27.70 (4.16)

RA: Rheumatoid arthritis; BMI: Body mass index.
Mean (SD) are presented for age, height, weight and BMI, median (percentiles 25 and 75) is presented for duration of RA, frequency (percent) is reported for menopausal status and current medication.
[†] Independent t test
[‡] Mann-Whitney U test
[§] Chi-square test
[*] Fisher's exact test

Table 2 presents the results for the interleukin proportions. In the placebo group, a significant decrease was observed for IL-10/IL-6 and IL-10/total Th1 (*P*= 0.008 and *P*= 0.004, respectively) by the end of the study

period. IL-10/IL12, IL10/TNF-α and IL-10/total Th1 significantly increased in the probiotic group (*P*= 0.012, *P*= 0.039 and *P*= 0.014, respectively) throughout the study course. There was a significant difference between the two study groups for IL-10/IL-12 and IL-10/total Th1 at the end of the study, in favor of the probiotic group (*P*= 0.038 and *P*= 0.006, respectively).

Table 2. Effect of eight weeks of probiotic supplementation on cytokine ratios in RA female patients.

	Placebo group (n=24)	Probiotic group (n=22)
IL-10/IL-1β		
Baseline	0.17 (0.04, 1.09)	0.03 (0.00, 0.24)[*]
End of study	0.12 (0.00, 0.70)	0.06 (0.00, 0.38)
IL-10/IL-6		
Baseline	0.09 (0.00, 0.21)	0.04 (0.00, 0.17)
End of study	0.02 (0.00, 0.12) [†]	0.03 (0.00, 0.30)
IL-10/IL-12		
Baseline	0.01 (0.00, 0.02)	0.00 (0.00, 0.01)
End of study	0.02 (0.00, 0.02)	0.00 (0.00, 0.03) [†‡]
IL-10/TNF-α		
Baseline	0.41 (0.06, 1.25)	0.22 (0.00, 0.55)
End of study	0.19 (0.00, 1.15)	0.17 (0.00, 2.66) [†]
IL10/total Th1[a]		
Baseline	0.01 (0.00, 0.02)	0.00 (0.00, 0.01)
End of study	0.00 (0.00, 0.02) [†]	0.00 (0.00, 0.02) [†‡]

[a] IL-10/total Th1: IL-10/(IL-1β+ IL-6+ IL-12+ TNF-α)
IL: Interleukin; TNF: Tumor necrosis factor
[*] Significant difference between groups at baseline (*P*< 0.05, Mann-Whitney U test)
[†] Significant change within group throughout the study (*P*< 0.05, Wilcoxon signed-rank test)
[‡] Significant difference between groups after intervention (*P*< 0.05, ANCOVA, adjusted for baseline measures and menopausal status)
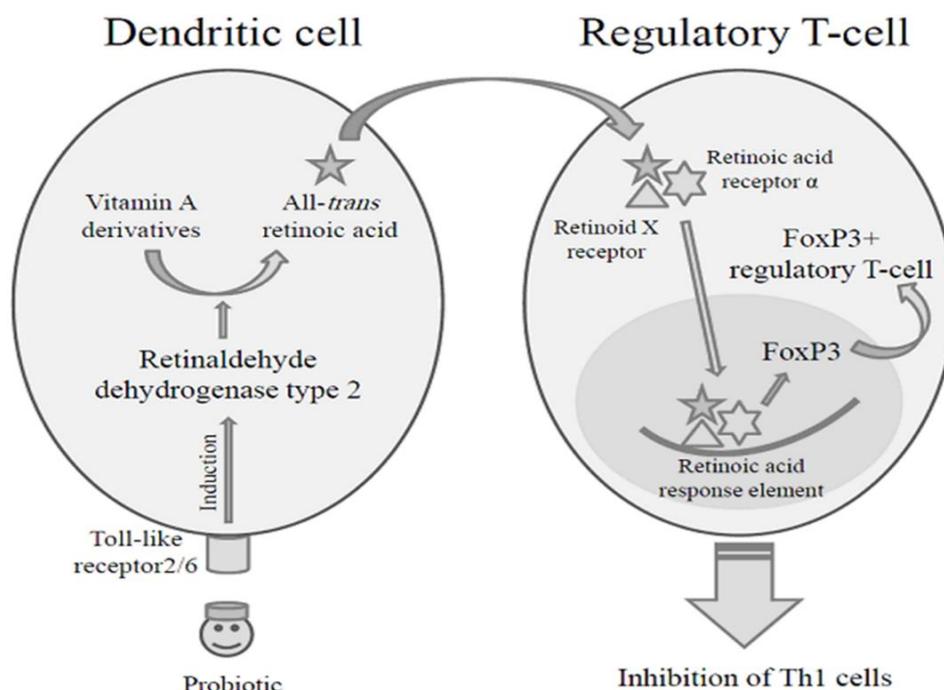
Figure 3. Induction of regulatory T-cells by probiotics.

In animal models of RA, feeding *L. casei* strains has shown great benefits by reducing arthritis score and improving serum inflammatory cytokines.[16-19] No clinical trials had assessed the effects of pure *L. casei* administration in RA patients, though. Probiotics are generally believed to affect the systemic immune system by triggering dendritic cells present in the lamina propria of the gut.[39] The mechanism by which *L. casei* induces the regulatory T-cell and down-regulates the Th1 cells, is presented in Figure 3. *L. casei* by binding to toll-like receptor2/6 in dendritic cells, induces production of retinaldehyde dehydrogenase type 2 enzyme, which in turn increases conversion of vitamin A derivatives to all-*trans* retinoic acid (ATRA). ATRA forms a complex with Retinoid X receptor and Retinoic acid receptor α, travels into the nucleus of naive FoxP3⁻CD4⁺ cells and contributes to its maturation towards FoxP3 regulatory T-cells. These regulatory cells dominantly produce IL-10. IL-10 has inhibitory effects on Th1 cells which produce pro-inflammatory cytokines.[40]

To the best of our knowledge, this was the first study in which a particular probiotic capsule was formulated for RA patients. The double-blind and placebo-controlled design of the clinical trial also allowed for unbiased interpretation of the results obtained. Moreover, unlike many other studies conducted in Iran, we did not rely only on the labels of the probiotic powder we purchased, but checked for its colony count ourselves. There were some limitations in our study as well. We could not obtain feces samples from the patients to check for the adequate colonization of the probiotic in all subjects. We also failed to assess the effects of the probiotic supplementation on CD4 cells. Both these shortcomings were due to financial constraints in our study. Further studies are encouraged to investigate the effects of other probiotic strains and also prebiotics on inflammatory biomarkers of RA patients.

Conclusion

L. casei 01 at a dosage of 10^8 CFU might be used to produce supplements specifically for RA patients. These capsules can efficiently improve the proportion of Th2/Th1 cytokines in these subjects, and may be appropriate to be introduced to this group of patients. Further studies are encouraged to confirm these results.

Conflict of interests

The authors claim that there is no conflict of interest.

References

1. Homayouni A, Azizi A, Ehsani MR, Yarmand MS, Razavi SH. Effect of microencapsulation and resistant starch on the probiotic survival and sensory properties of synbiotic ice cream. Food Chem. 2008;111(1):50-5. doi:10.1016/j.foodchem.2008.03.036

2. Homayouni Rad A, Vaghef Mehrabany E, Alipoor B, Vaghef Mehrabany L, Javadi M. Do probiotics act more efficiently in foods than in supplements? Nutrition. 2012;28(7-8):733-6. doi:10.1016/j.nut.2012.01.012

3. Weng M, Walker WA. Bacterial colonization, probiotics and clinical disease. J Pediatr. 2006;149(5):S107-14. doi:10.1016/j.jpeds.2006.06.061

4. Borchers AT, Selmi C, Meyers FJ, Keen CL, Gershwin ME. Probiotics and immunity. J Gastroenterol. 2009;44(1):26-46. doi:10.1007/s00535-008-2296-0

5. Homayouni Rad A, Torab R, Ghalibaf M, Norouzi S, Vaghef Mehrabany E. Might patients with immune-related diseases benefit from probiotics? Nutrition. 2013;29(3):583-6. doi:10.1016/j.nut.2012.10.008

6. O'Dell JR. Rheumatoid Arthritis. In: Goldman L, Ausiello D, editors. Cecil Medicine. 23rd ed. Philadelphia: Saunders; 2008. p. 2003-11.

7. Shinebaum R, Neumann VC, Cooke EM, Wright V. Comparison of faecal flora in patients with rheumatoid arthritis and controls. Rheumatology. 1987;26(5):329-33. doi:10.1093/rheumatology/26.5.329

8. McCulloch J, Lydyard PM, Rook GAW. Rheumatoid arthritis: How well do the theories fit the evidence? Clin Exp Immunol. 1993;92(1):1-6. doi:10.1111/j.1365-2249.1993.tb05938.x

9. Eerola E, Mottonen T, Hannonen P, Luukkainen R, Kantola I, Vuori K, et al. Intestinal flora in early rheumatoid arthritis. Rheumatology. 1994;33(11):1030-8. doi:10.1093/rheumatology/33.11.1030

10. Malin M, Verronen P, Mykkänen H, Salininen S, Isolauri E. Increased bacterial urease activity in faeces in juvenile chronic arthritis: evidence of altered intestinal microflora? Rheumatology. 1996;35(7):689-94. doi:10.1.1.495.8529

11. Peltonen R, Nenonen M, Helve T, Hanninen O, Toivanen P, Eerola E. Faecal microbial flora and disease activity in rheumatoid arthritis during a vegan diet. Rheumatology. 1997;36(1):64-8. doi:10.1093/rheumatology/36.1.64

12. Saarela M, Lahteenmaki L, Crittenden R, Salminen S, Mattila Sandholm T. Gut bacteria and health foods-the European perspective. Int J Food Microbiol. 2002;78(1-2):99-117. doi:10.1016/S0168-1605(02)00235-0

13. Hatakka K, Martio J, Korpela M, Herranen M, Poussa T, Laasanen T, et al. Effects of probiotic therapy on the activity and activation of mild rheumatoid arthritis- a pilot study. Scand J Rheumatol. 2003;32(4):211-5. doi:10.1080/03009740310003695

14. Mandel DR, Eichas K, Holmes J. Bacillus coagulans: a viable adjunct therapy for relieving symptoms of rheumatoid arthritis according to a randomized, controlled trial. BMC Complement Altern Med. 2010;10(1):1-7. doi:10.1186/1472-6882-10-1

15. Pineda MA, Thompson SF, Summers K, Leon F, Pope J, Reid G. A randomized, double-blinded, placebo-controlled pilot study of probiotics in active rheumatoid arthritis. Med Sci Monit. 2011;17(6):CR347-54. doi:10.12659/MSM.881808

16. Kato I, Endo Tanaka K, Yokokura T. Suppressive effects of the oral administration of Lactobacillus casei on type II collagen-induced arthritis in DBA/1 mice. Life Sci. 1998;63(8):635-44. doi:10.1016/S0024-3205(98)00315-4

17. So JS, Lee CG, Kwon HK, Yi HJ, Chae CS, Park JA, et al. Lactobacillus casei potentiates induction of oral tolerance in experimental arthritis. Mol Immunol. 2008;46(1):172-80. doi:10.1016/j.molimm.2008.07.038

18. So JS, Kwon HK, Lee CG, Yi HJ, Park JA, Lim SY, et al. Lactobacillus casei suppresses experimental arthritis by down-regulating T helper 1effector functions. Mol Immunol. 2008;45(9):2690-9. doi:10.1016/j.molimm.2007.12.010

19. Amdekar S, Singh V, Singh R, Sharma P, Keshav P, Kumar A. Lactobacillus casei reduces the inflammatory joint damage associated with collagen-induced arthritis (CIA) by reducing the pro-inflammatory cytokines. J Clin Immunol. 2011;31(2):147-54. doi:10.1007/s10875-010-9457-7

20. Kukkar V, Anand V, Kataria M, Gera M, Choudhury PK. Mixing and formulation of low dose drugs: underlying problems and solutions. Thai J Pharm Sci. 2008;32(3-4):43-58.

21. Vaghef Mehrabany E, Alipour B, Homayouni Rad A, Sharif SK, Asghari Jafarabadi M, Zavvari S. Probiotic supplementation improves inflammatory status in patients with rheumatoid arthritis. Nutrition. 2014;30(4):430-5. doi:10.1016/j.nut.2013.09.007

22. Timmerman HM, Koning CJ, Mulder L, Rombouts FM, Beynen AC. Monostrain, multistrain and multispecies probiotics-A comparison of functionality and efficacy. Int J Food Microbiol. 2004;96(3):219-33. doi:10.1016/j.ijfoodmicro.2004.05.012

23. Anal AK, Singh H. Recent advances in microencapsulation of probiotics for industrial applications and targeted delivery. Trends Food Sci Technol. 2007;18(5):240-51. doi:10.1016/j.tifs.2007.01.004

24. Homayouni Rad A. A survey on the effect of microencapsulation on probiotic survival in functional ice cream [dissertation]. Tehran: University of Tehran; 2009.

25. Chan ES, Lee PP, Ravindra P, Krishnaiah K, Voo WP. A standard quantitative method to measure acid tolerance of probiotic cells. Appl Microbiol Biotechnol. 2010;86(1):385-91. doi:10.1007/s00253-009-2384-y

26. Both E, Gyorgy E, Kibedi Szabo CZ, Tamas E, Abraham B, Miklossy I, et al. Acid and bile tolerance, adhesion to epithelial cells of probiotic microorganisms. Sci. Bull. B Chem. Mater. Sci. UPB. 2010;72(2):37-44.

27. Perdigon G, Maldonado Galdeano C, Valdez JC, Medici M. Interaction of lactic acid bacteria with the gut immune system. Eur J Clin Nutr. 2002;56(S4):S21-6. doi:10.1038/sj.ejcn.1601658

28. Matsumoto S, Hara T, Hori T, Mitsuyama K, Nagaoka M, Tomiyasu N, et al. Probiotic Lactobacillus-induced improvement in murine chronic inflammatory bowel disease is associated with the down-regulation of pro-inflammatory cytokines in lamina propria mononuclear cells. Clin Exp Immunol. 2005;140(3):417-26. doi:10.1111/j.1365-2249.2005.02790.x

29. Aguero G, Villena J, Racedo S, Haro C, Alvarez S. Beneficial immunomodulatory activity of Lactobacillus casei in malnourished mice pneumonia: effect on inflammation and coagulation. Nutrition. 2006;22(7-8):810-9. doi:10.1016/j.nut.2006.03.013

30. Niers LEM, Timmerman HM, Rijkers GT, Bleek GM, Uden NOP, Knol EF, et al. Identification of strong interleukin-10 inducing lactic acid bacteria which down-regulate T helper type 2 cytokines. Clin Exp Allergy. 2005;35(11):1481-9. doi:10.1111/j.1365-2222.2005.02375.x

31. Carol M, Borruel N, Antolin M, Llopis M, Casellas F, Guarner F, et al. Modulation of apoptosis in intestinal lymphocytes by a probiotic bacteria in Crohn's disease. J Leukoc Biol. 2006;79(5):917-22. doi:10.1189/jlb.0405188

32. Haro C, Villena J, Zelaya H, Alvarez S, Aguero G. Lactobacillus casei modulates the inflammation-coagulation interaction in a pneumococcal pneumonia experimental model. J Inflamm. 2009;6(1):28-37. doi:10.1186/1476-9255-6-28

33. Chiu YH, Hsieh YJ, Liao KW, Peng KC. Preferential promotion of apoptosis of monocytes by Lactobacillus casei rhamnosus soluble factors. Clin Nutr. 2010;29(1):131-40. doi:10.1016/j.clnu.2009.07.004

34. Champagne CP, Ross RP, Saarela M, Hansen KF, Charalampopoulos D. Recommendations for the viability assessment of probiotics as concentrated cultures and in food matrices. Int J Food Microbiol. 2011;149(3):185-93. doi:10.1016/j.ijfoodmicro.2011.07.005

35. An Emerging Trend of High Dose Probiotic Use in Clinical Practice-A Brief Survey. http://www.pointinstitute.org/wp-content/uploads/2012/10/High-Dose-Probiotics-in-Clinical-Practice.pdf. Accessed 14 Jan 2014.

36. Gill HS, Rutherfurd KJ, Cross ML, Gopal PK. Enhancement of immunity in the elderly by dietary supplementation with the probiotic Bifidobacterium lactis HN019. Am J Clin Nutr. 2001;74(6):833-9.

37. Smits HH, Engering A, van der Kleij D, de Jong EC, Schipper K, van Capel TMM, et al. Selective probiotic bacteria induce IL-10 producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3 grabbing nonintegrin. J Allergy Clin Immunol. 2005;115(6);1260-7. doi:10.1016/j.jaci.2005.03.036

38. Zhang L, Li N, Caicedo R, Neu J. Alive and dead Lactobacillus rhamnosus GG decrease tumor necrosis factor-alpha-induced interleukin-8 production in Caco-2 cells. J Nutr. 2005;135(7):1752-6. doi:10.1093/jn/135.7.1752

39. Delcenserie V, Martel D, Lamoureux M, Amiot J, Boutin Y, Roy D. Immunomodulatory effects of probiotics in the intestinal tract. Curr Issues Mol Biol. 2008;10(1-2):37-54.

40. Issazadeh Navikas S, Teimer R, Bockermann R. Influence of dietary components on regulatory T cells. Mol Med. 2012;18(1):95-110. doi:10.2119/molmed.2011.00311