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Oral administration of *Lactobacillus acidophilus* induces IL-12 production in spleen cell culture of BALB/c mice bearing transplanted breast tumour

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Lactic acid bacteria can affect the maturation of immune cells and their products not only in the gut but also on the systemic immune organs such as lymph nodes and spleen. In the present work, we studied the effects of oral administration of *Lactobacillus acidophilus* on the immune responses of BALB/c mice bearing transplanted breast tumour. Two groups of female inbred BALB/c mice, each containing nine mice as test and control, were used. The *L. acidophilus* ATCC4356 strain was inoculated in DeMan–Rogosa–Sharpe broth and cultivated for 24 h at 37°C. Then, it was collected by centrifugation, and was washed and suspended in PBS. Afterwards, 0·5 ml/d of this suspension, which contained $2{\cdot}7 \times 10^8$ colony forming units/ml of bacteria, was orally administered to the mice by gavage, 14 d before tumour transplantation and 30 d after that with 3-d intervals. Similar to the test mice, the control mice received an equal volume of PBS. The results showed that oral administration of *L. acidophilus* increased the production of IL-12 ($P < 0{\cdot}05$) and decreased the level of transforming growth factor β ($P = 0{\cdot}05$) in the splenocyte culture. Moreover, the growth rate of tumour in the test mice decreased ($P < 0{\cdot}01$), and the results of delayed-type hypersensitivity assay after 48 h were risen ($P < 0{\cdot}05$) in comparison with the controls. Results suggest that daily consumption of *L. acidophilus* can improve the production of immunomodulatory cytokine IL-12 in the splenocyte culture, which was stimulated by tumour antigen in BALB/c mice bearing transplanted breast tumour. But further studies are needed to find out some other possible mechanisms of this effect.

***Lactobacillus acidophilus*: Probiotics: Immunomodulation: Breast cancer**

Lactic acid bacteria (LAB), defined as probiotics, are non-pathogenic microbial flora. There are many reports demonstrating the beneficial effects of consumption of these bacteria for health maintenance[1]. The immunomodulatory effects of these probiotics have also been reported in several studies[2,3]. Results of such investigations have shown improvement of the intestinal micro-environment and modulation of the immune system by the consumption of probiotic LAB[4]. In addition, some studies which were performed on animal models have shown that oral administrations of some lactobacillus species have regulative effects on the host's immune responses (e.g. tumour, infection and allergy models). Recent studies on the anti-tumour effect of *Lactobacillus casei* and *Lactobacillus acidophilus* have shown the potency of these bacteria to inhibit the growth of transplantable tumour cells in experimental animals[5–7]. It is known that oral administration of certain strains of these bacteria not only can modulate the local immune response in the intestine, but is also effective on the systemic immune responses such as macrophage function and Ig concentration in serum[8] and also on the immune responses in other mucosal areas including bronchus and mammary glands[9]. One of the major products of macrophages and dendritic cells (DC) is IL-12, which is an important T-helper 1 (Th1) cytokine not only for stimulating the growth and function of T cells, but also for activating the natural killer cells which have an important role in triggering immune responses against solid tumours[10]. Another important cytokine which is worth evaluating especially in cancer patients is the transforming growth factor β (TGF-β). TGF-β is one of the Th2 cytokines, and unlike IL-12, it has an anti-inflammatory property. TGF-β can also cause immunosuppression and angiogenesis, which can help the tumour to grow and be more invasive[11]. Results of some studies have demonstrated that orally administered lactobacilli can affect the production of IL-12[9,12]. It has also been indicated previously that oral administration of some strains of *Lactobacillus* augmented the interferon-γ and IL-12 production in spleen cell culture of BALB/c mice, and subsequently enhanced the functions of Th1 cells in them[13]. Regarding this Th1 activity skewing property, lactobacilli have gained a lot of interest in many research fields such as immunomodulation in cancer, allergic disorders and even as

Abbreviations: DC, dendritic cells; Th1, T-helper 1; TLR, Toll-like receptor; LAB, lactic acid bacteria; TGF-β, transforming growth factor β.

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an oral vaccine[14]. Therefore, using LAB for stimulating the spleen cells to produce the Th1 cytokines, which are needed specially to launch the anti-tumour immune responses, may seem to be rational, but there are only a few reports about probiotics and their capacity for triggering immune responses against breast tumour; hence, in the present study, we have tried to evaluate the direct effect of oral administration of *L. acidophilus* on the transplantable breast tumour in BALB/c mice model.

Materials and methods

Animals

Six- to eight-week-old female inbred BALB/c mice, each weighing 25–30 g, were obtained from the Pasture Institute of Iran (Tehran, Iran). They were divided into two groups of test and control, each containing nine mice. The mice were kept in plastic cages, allowed free access to water and maintained under a 12 h light–12 h dark cycle during the study period. The temperature and humidity were controlled at 23 ± 1°C and 55 ± 10 %, respectively, and all the mice were fed a pellet diet which contained 4 % fibre and 3·5 % fats. It was supplied by the Pasture Institute of Iran. Although the control mice in the present study were kept separated from the test group, they were maintained at the same temperature and humidity, and fed with the same food.

Micro-organisms and feeding procedure

The *L. acidophilus* ATCC4356 strain was purchased from the Persian type culture collection (Iranian Research Organization for Science and Technology, Tehran, Iran). It was inoculated in 10 ml of DeMan–Rogosa–Sharpe broth (Merck, Darmstadt, Germany), and was cultivated overnight at 37°C under anaerobic conditions. Then, it was collected by centrifugation at 4000 ***g*** for 30 min at 4°C, washed three times with sterile PBS and resuspended in PBS. In order to evaluate the total count of bacteria in this suspension, the colony-forming unit counting method was used, and after preparation of different dilutions, 100 μl from 10^6 dilution were cultivated in DeMan–Rogosa–Sharpe agar and incubated at 37°C. Then, on the next day, colonies that grew in the DeMan–Rogosa–Sharpe agar were counted using a colony counter. There were approximately twenty-seven colonies. Thus, the total count of bacteria in 10^8 dilution was calculated as $2{\cdot}7 \times 10^8$ colony forming units/ml. Finally, 0·5 ml/d from 10^8 dilution was orally administered to the mice using a standard gastric feeding tube according to the procedure described below.

The test group mice (*L. acidophilus*-treated mice) were given 0·5 ml of this suspension daily for fourteen consecutive days before tumour transplantation. Then, the administration was continued with 3-d intervals and 7-d consecutive feedings until day 30 after the tumour transplantation. The control mice (PBS-treated mice) were given an equal volume of PBS according to the same procedure. To confirm the colonisation of the LAB in mice, their stools were used after 3 d of the first administration, and this strain was isolated and confirmed using the *Lactobacillus* API biochemical kit.

Tumour transplantation

Spontaneous adenocarcinoma breast tumour mice[15] were used as tumour stock. After cervical dislocation, the tumours of these mice were removed aseptically, dissected into 0·5 cm^3 pieces with scalpel and washed three times with sterile PBS. The test and control mice were anaesthetised with intraperitoneal injection of ketamine and xylazine (10 mg/kg of the body weight), and the tumour pieces were transplanted subcutaneously into their right flank.

Measurement of the tumour growth

Tumour growth was measured twice a week, and was evaluated by calliper measurement of the tumour length. The volume was determined using the formula: $V = 0{\cdot}5_d2_D$ [16], where V is the tumour volume (cm^3), d is the shorter diameter and D is the longer diameter.

Preparation of the tumour antigen

In order to obtain the tumour antigen, one of the tumour stock mice was used, and the tumour was extracted from its body and dissected into small sections (3 mm^3). After that, the tumour sections were washed with sterile PBS and fragmented via sonication. Then, the protein dialysis method was used in order to extract the proteins from the lysate suspension, and the concentration of protein in that was measured by the Bradford method. Finally, it was used as a stimulator to stimulate the splenocytes in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and cytokine production tests and in delayed-type hypersensitivity assay as well. Actually, 20 μg/ml of tumour antigen as stimulator were shown to induce optimum splenocyte proliferation under our assay conditions. (ZM Hassan and M Yazdi, unpublished results).

Evaluation of delayed-type hypersensitivity response

Evaluation of delayed-type hypersensitivity response was carried out according to the method of Jin *et al.* [17]. Briefly, 14 d after the tumour transplantation, five mice from each group were randomly selected and were challenged with the tumour antigen in the left footpad and with PBS in the right footpad. Footpad thickness and swelling were measured 48 h later using an Engineer's micrometer.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide spleen cell culture assay

The spleens were removed aseptically and resuspended in Hanks' balanced salt solution (Sigma, Munich, Germany). Erythrocytes were lysed with lysing buffer (Sigma) for 5 min, washed and resuspended in Roswell Park Memorial Institute 1640 medium (Gibco, Rockville, MD, USA), supplemented with 10 % fetal bovine serum, 100 μg/ml penicillin and streptomycin (Sigma). The cell suspensions containing $1{\cdot}5 \times 10^6$ of viable cells/ml were distributed at 100 μl/well into ninety-six-well tissue culture plates with flat-bottomed wells (Costar, Cambridge, MA, USA). Tumour antigen at 20 μg/ml was added as a stimulator into

each well except for the negative control wells and blank wells. After incubation in humidified 5 % CO_2 at 37°C for 72 h, the proliferation of spleen cells was measured by standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay method, and reported as the stimulation index[18].

Cytokine determination in spleen cell culture

The spleen cells were collected just after the mice were sacrificed. The spleen cells were counted to $2\cdot5\times10^6$ cells/ml, and were incubated alone or treated with 20 μg/ml of the tumour antigen for 72 h in Roswell Park Memorial Institute 1640 medium (Gibco Life Technologies, Eggenstein, Germany), supplemented with 10 % fetal bovine serum (Invitrogen, Paisley, Scotland, UK) and 1 % penicillin/streptomycin (Sigma), at 37°C in a humidified atmosphere of 5 % CO_2. IL-12 and TGF-β levels in the spleen cell culture supernatants were detected using ELISA (R&D System, Minneapolis, MN, USA) as described by the manufacturer (R&D Kit instruction).

Statistical analysis

All the statistical analyses were conducted by SPSS 15.0 (SPSS, Chicago, IL, USA), using one-way ANOVA. The values are presented as means and standard deviations. The number of mice used in the present study was calculated according to the following formula:

$$1-\beta=\phi\left(-1.64+\frac{\Delta}{\sqrt{s_1^2+s_2^2}}\sqrt{n}\right)\ (\alpha=0.05,\ \beta=10\,\%).$$

Ethical approval

All experimental procedures involving animals were approved by the Ethics Committee of Tehran University of Medical Sciences.

Results

Measurement of primary tumour growth

To evaluate the growth of tumour, nine mice of each group were used, and tumour growth was measured twice a week with a calliper. The tumour-related volume of each mouse was calculated using the formula mentioned previously. The data analysis showed a significant ($P<0\cdot01$) decrease in the growth rate of tumour in the test mice in comparison with the control mice (Table 1). This effect could be the result of the promotion of immune responses through Th1 and enhancement of the cellular immunity via *L. acidophilus* administration.

Evaluation of delayed-type hypersensitivity response

To asses the antigen-specific Th1 response, delayed-type hypersensitivity reaction was evaluated in the tumour antigen-rechallenged mice[17]. They were challenged with the tumour antigen in the left footpad and with PBS in the right footpad 14 d after the tumour transplantation. The results showed a significant ($P<0\cdot05$) increase in the swelling and thickness in the left footpad of the test group after 48 h of the tumour antigen challenge compared with the control group (Table 1).

Effect of tumour antigen on proliferation of spleen cells

The results of the effects of *L. acidophilus* administration on tumour antigen-stimulated spleen cell proliferation in the tumour-bearing mice are given in Table 1. Tumour-specific antigen was prepared before the test, and was used as a stimulator. Spleen cell proliferation in the test mice was significantly higher than that in the control mice ($P<0\cdot05$).

Cytokine assay in spleen cell culture

The spleen cells were cultured in Roswell Park Memorial Institute 1640 medium with 10 % fetal bovine serum and penicillin/streptomycin ($2\cdot5\times10^6$ cell/ml), and were incubated alone or treated with 20 μg/ml of the tumour antigen for

Table 1. Relative tumour volume, delayed-type hypersensitivity (DTH) and lymphocyte proliferation results
(Mean values and standard deviations)

	Relative tumour volume†		Swelling in DTH assay (%)‡		Lymphocyte proliferation (SI)§	
Groups	Mean	SD	Mean	SD	Mean	SD
*Lactobacillus acidophi*lus	3·6***	0·86	24·09 %*	5·6	1·3*	0·144
PBS	5·47	1·4	12·06 %	4·1	1·05	0·024

SI, stimulation index.
† Related tumour volume: Tumour growth was measured twice a week and was evaluated by calliper measurement of the tumour length, and the tumour volume was calculated with the formula given in the text. The data are shown with ***($P<0\cdot01$ significance). Data represent the means and standard deviations for nine animals per group.
‡ DTH responses of the mice 48 h after tumour antigen rechallenge. The mice were challenged with the tumour antigen in the left footpad and with PBS in the right footpad. The results showed a significant *($P<0\cdot05$) difference in the left footpad thickness and swelling between the test and control mice. Data represent the means and standard deviations for five animals per group.
§ Proliferation of the spleen cells from the untreated controls and the mice treated with *L. acidophilus* in response to tumour antigen stimulation. Daily intragastric administration of *L. acidophilus* was started 14 d before the tumour transplantation and was continued to the day the sacrifice was done. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was conducted using the procedure explained in the materials and methods. Tumour antigen was used at 20 μg/ml as a mitogen. Significance of the data was * ($P<0\cdot05$) in this test. Data represent the means and standard deviations for triplicate cultures of nine animals per group.

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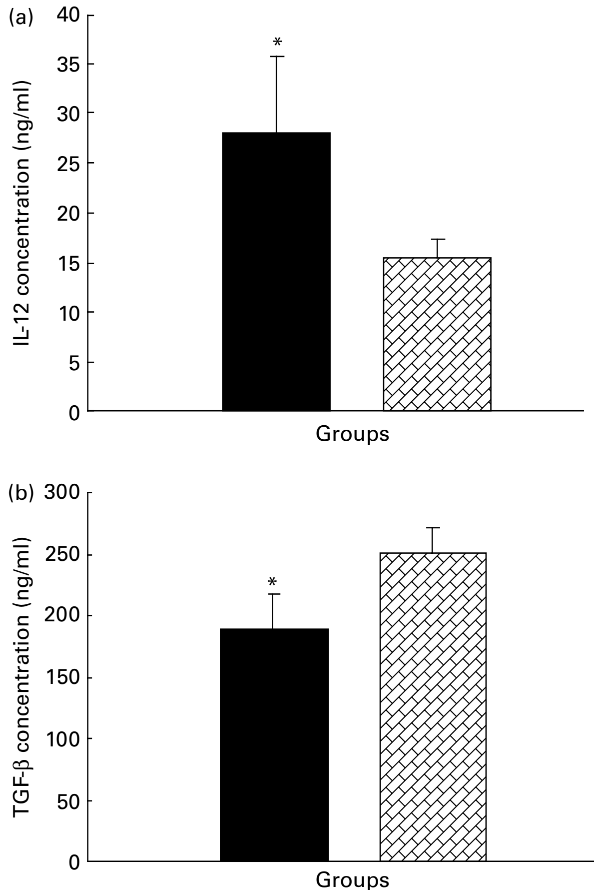

Fig. 1. IL-12 (a) and transforming growth factor β (TGF-β) (b) induction by *Lactobacillus acidophilus* administration in the spleen cell culture. The spleen cells were cultured for 72 h with tumour antigen stimulation, and the levels of IL-12 and TGF-β in the supernatants were determined with ELISA. Data represent the means and standard deviations for triplicate cultures of nine animals per group (* $P < 0.05$). ■, *L. acidophilus*; ▨, control.

72 h at 37°C in a humidified atmosphere of 5 % CO_2. Then, the levels of IL-12 and TGF-β in the spleen cell culture supernatants were measured using a sandwich ELISA (R&D System). As shown in Fig. 1, the level of IL-12 was significantly higher in the test group than in the control group ($P<0.05$). On the other hand, the TGF-β level was lower in this group than in the control group ($P=0.05$).

Discussion

There is a growing interest in the use of LAB as immunomodulator agents in different immunological disorders such as atopic diseases, allergies, infectious diseases and cancers. The effects of LAB in stimulating the Th1 cytokine production have been reported in some studies as well[13]. Although Takahashi *et al.* [19] have demonstrated that heat-killed lactobacilli may be more potent and safer as a therapeutic agent than bacillus Calmette–guérin for superficial bladder tumours, many reports are not there about using oral administration of LAB for triggering the immune responses against solid tumours and effects of lactobacillus on the local tumour environment. De Moreno *et al.* [9] have also shown only the effects of *Lactobacillus helveticus* fermentative products on the local tumour environment in murine breast cancer. In the present work, our findings have demonstrated that oral administration of *L. acidophilus* can induce the production of IL-12 by mouse splenocytes in the mice bearing transplanted breast tumour. IL-12 is produced by the antigen-presenting cells, such as DC and macrophages, and it is well known due to its critical role in polarising immune responses skewed by Th1 balance and also in enhancing cellular immunity[20]. Murosaki *et al.* [21] have also shown the increase in IL-12 production via administration of some lactobacilli in the tumour-bearing mice, and their results have shown that daily consumption of lactobacillus is required to exert an anti-tumour effect at the late stage of tumour development when the IL-12 production is considerably impaired. IL-12 also contributes to the stimulation of both natural killer and Th1 cells to produce interferon-γ[22]. Interferon-γ affects the up-regulation of the MHC expression in tumour cells. Furthermore, it has an ability to inhibit tumour angiogenesis[23] and consequently suppress tumour growth. In fact, according to the results of the present work, oral administration of *L. acidophilus* exerts its anti-tumour property by promoting Th1-dominant responses via IL-12 induction. Some studies on the structural components of LAB have demonstrated the cell wall components of these probiotics as an excellent inducer of inflammatory cytokine[24–26]. Lipoteichoic acid is the major component of LAB that is recognised by Toll-like receptor (TLR) 2, especially via DC in Peyer's patches[27,28], and DC are the major source of IL-12 production among the immune cells. It has been demonstrated that the stimulation of DC *in vivo* with microbial extracts causes them to rapidly begin producing IL-12[29]. Therefore, IL-12 is more likely responsible for the immunomodulative function of *L. acidophilus*, especially in enhancing Th1 responses and cellular immunity. Some investigations on the signalling pathways between the LAB and gut immune cells imply that these micro-organisms can use pattern recognition receptors other than TLR2 to send the immune signals, which are closer to promote the inflammatory responses[30]. LAB contain CpG-like immunostimulatory oligonucleotides as well[31,32], which are recognised via TLR9. There are some studies showing the ligation of TLR9 directly by some LAB species[33,34]. According to these reports, stimulation of TLR2 and TLR9 could induce Th1-type responses that are more effective under tumour conditions. Tumour-induced immunosuppression has been reported previously. For instance, O'Hara *et al.* [35] have shown a decrease in IL-12 production and an increase in the serum level of IL-10 as an anti-inflammatory Th2 cytokine in the patients with colorectal cancer. Like IL-10, TGF-β is another Th2 cytokine with an anti-inflammatory property. When tumour cells are in the growing conditions, TGF-β acts on the surrounding stromal cells, immune cells, endothelial cells and smooth muscle cells, and it causes immunosuppression and angiogenesis, consequently making the cancer more invasive[11]. It has also been reported that administration of some LAB species can inhibit the production of Th2 cytokine such as TGF-β[36]. According to the results of the present work, the decrease in TGF-β level in the spleen cell culture of the *L. acidophilus*-treated group can support the increase in Th1 immune responses and thus the decrease in the tumour growth rate and tumour-related volume compared with

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the control mice. Moreover, induction of a high level of IL-12 can promote the Th1-dominant immune responses, which have a critical role in cancer remedy. In addition, the results of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay showed that *L. acidophilus* when administered orally can act as an immunostimulatory agent, which affects the immune cell proliferation. Result of delayed-type hypersensitivity assay in this work has also certified that the administration of *L. acidophilus* can stimulate Th1 response and enhance cellular immunity.

Conclusion

The present results suggest that daily consumption of *L. acidophilus* can modulate immune responses via stimulation of IL-12 production in spleen cell culture, and may promote the Th1 responses that will be more effective under tumour conditions. But further studies are needed to investigate the other mechanisms of this effect.

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