

Modulation of cytokine gene expression in spleen and Peyer's patches by feeding dahi containing probiotic *Lactobacillus casei* in mice

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OBJECTIVE: In present study, feeding effect of probiotic dahi containing *Lactobacillus casei* on immune system in terms of cytokine gene expression in the spleen and Peyer's patches of mice was evaluated.

METHODS: Animals were divided into three groups and fed with; synthetic diet [control group (CD)], dahi containing mixed dahi culture [control dahi-fed group (CDF)]; and probiotic dahi fed group (PDF) for 28 days. The mRNA levels of IL-2, IL-4, IL-6 and IFN- γ were examined after 14 and 28 days. Total lactobacilli and lactococci counts were determined in the feces.

RESULTS: The mRNA levels of IFN- γ in both spleen and Peyer's patches was found to be significantly increased in PDF animals after 14 and 28 days ($P < 0.05$) compared with CD and CDF groups. The abundance of IL-2 mRNA also increased significantly

in the Peyer's patches of PDF-fed animals. No significant changes were observed in mRNA levels of IL-4 and IL-6 in both spleen and Peyer's patches during whole experimental period. Further, total fecal lactobacilli and lactococci counts in the PDF group were significantly increased during first 10 days, then remained higher up to day 28 compared to other two groups.

CONCLUSION: It is concluded that feeding probiotic dahi enhanced the expression of Th1 type cytokines (IFN- γ and IL-2), especially in the mucosal immune organ (Peyer's patches) rather than in systemic organs (the spleen). This indicates that feeding with probiotic dahi may strengthen the host immune system and protect against the progression of various immune-mediated diseases.

KEY WORDS: cytokine, gut immune system, lactobacilli, probiotic, T-helper cell.

INTRODUCTION

Immune-mediated health problems are increasing at a high rate worldwide.¹ One of the chief reasons for this is imbalanced nutrition.² This problem can be

eradicated on most levels by increasing consumption of natural and functional foods containing health beneficial ingredients. This created a strong need for development of new functional foods that can modulate consumer's immune system and may help to protect immune-mediated health threats. Probiotics are considered as superior nutraceuticals and are widely recognized for the various health benefits they produce by improving the gut environment.^{3–6} Generally, the lactic acid bacteria (LAB) family, i.e., lactobacilli and bifidobacteria has been considered to be probiotics. Several reports show that various strains of LAB stimulate the immune system in a beneficial way both

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Table 1. Composition of experimental diets used to feed mice

Constituent (s) (g/100 g)	Synthetic diet	Control dahi-supplemented diet	Probiotic dahi-supplemented diet
Starch	38.5	33.5	33.5
Casein	20.0	13.87	13.87
Sucrose	25.0	17.24	17.24
Refined oil	10.0	8.89	8.89
Vitamin mixture [†]	1.0	1.0	1.0
Mineral mixture [†]	5.0	5.0	5.0
Choline chloride	0.2	0.2	0.2
Methionine	0.3	0.3	0.3
Control dahi	0.0	20.0	0.0
Probiotic dahi	0.0	0.0	20.0

[†]The vitamin and mineral mixture were prepared and mixed according to AOAC (1984).³³

at mucosal and systemic levels.^{7,8} But the stimulation of the immune system by all LAB strains is obscure. Miettinen *et al.*⁹ reported that non-pathogenic *Lactobacillus rhamnosus* stimulate the T-helper (Th)1 type cytokines, i.e., IL-2 and IFN- γ in human peripheral blood mononuclear cells. Baken *et al.*¹⁰ reported that the administration of *L. casei* strain, shirota, had not shown any effects on the gene expression of Th1 type cytokines in the spleen, liver, thymus and mesenteric lymph nodes of rats. However Clancy and Pang¹¹ reported on the mucosal stimulation of IFN- γ by feeding different strains of *Bifidobacterium* and lactobacilli. So it is clear that the immunomodulatory potential of LAB is strictly strain dependent. This forces to identify new immunomodulatory LAB strains that can be used to develop new functional foods.

Dahi is an Indian fermented milk product (similar to yogurt) made by the lactic acid fermentation of milk.¹² Dahi has been considered as a functional food due to its various health benefits such as hypocholesterolemic, anti-diarrheal, anti-diabetic and anti-hypertensive.^{13–17} Preliminary studies conducted in our laboratory showed that dahi containing probiotic *L. casei* stimulates immune functions in terms of increased innate immunity.^{18,19} In the present study we evaluated the impact of probiotic dahi feeding on cytokine gene expression in systemic (spleen) and mucosal (Peyer's patches) immune organs in mice.

MATERIALS AND METHODS

Bacterial strains

Lactobacillus casei NCDC19 and a mixed dahi culture NCDC167 were obtained from the National Collection of Dairy Cultures (NCDC) of the National Dairy Research Institute, Karnal, India. *L. casei* and mixed dahi cultures were propagated in a deMan Rogosa

Sharpe media (Hi-Media Laboratories, Bombay, India) and M-17²⁰ broth as well as in sterilized skim milk during whole study period.

Preparation of probiotic dahi

Probiotic dahi was developed using various technological modifications described elsewhere.²¹ In brief, raw buffalo milk was procured from the experimental dairy, of the institute. The milk fat was adjusted to 2.5% by adding fresh skimmed milk, then boiled at 90°C for 15 min. After being cooled to 37°C, *L. casei* ($\sim 10^7$ cfu/mL) and a mixed dahi culture ($\sim 10^5$ cfu/mL) were inoculated and incubated for 12–14 h incubation at 37°C. The control dahi was prepared by inoculating mixed dahi cultures by 1% and incubation them at 30°C for 12–14 h.

Animals and feeding schedule

Six-week old Swiss albino male mice (25–30 g body-weight) were maintained in the small animal house of the institute and housed 2–3 mice per plastic polypropylene cage. The animals were divided into three groups ($n = 10$), i.e., (i) the control group (CD) fed with a synthetic diet alone (Table 1); (ii) the control dahi-fed group (CDF) fed with a control dahi-supplemented diet; and (iii) the probiotic dahi-fed group (PDF) fed with a probiotic dahi-supplemented diet. All the groups were allowed to take the experimental diet and water ad libitum, while 3–4 g dahi/day was fed to each. Food intake and bodyweight were recorded weekly. Five animals from each group were killed by cervical dislocation after 14 and 28 days, respectively. Their liver, kidney, spleen and Peyer's patches were excised and snap frozen in liquid nitrogen and stored at -80°C till further use. This study protocol was approved by the Institutional Animal Ethics Committee and the animals were maintained as per the rules of animals

Table 2. Physiological characteristics of mice fed with different experimental diets for 28 days

Variables	CD	CDF	PDF
Bodyweight gain (g)	4.49 ± 1.54	5.83 ± 1.86	5.84 ± 1.11
Feed intake (g/mouse/day) [†]	4.87 ± 0.82	5.01 ± 0.61	4.81 ± 0.93
Water intake (mL/mouse/day) [†]	7.98 ± 2.54	7.22 ± 1.66	7.71 ± 2.65
Urine volume (mL/mouse/day)	5.34 ± 0.78	4.98 ± 1.51	5.01 ± 2.05
Liver weight (g)	1.54 ± 0.45	1.66 ± 0.89	1.57 ± 0.91
Spleen weight (mg)	50.76 ± 12.76	56.98 ± 7.12	54.87 ± 8.94
Kidney weight (g)	0.42 ± 0.07	0.39 ± 0.09	0.44 ± 0.05

Values are mean ± SEM of 10 animals in each group.

[†]Feed and water intake was determined at weekly intervals.

Control group (CD); control dahi group (CDG); probiotic dahi group (PDG).

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RNA isolation and reverse transcription

Total RNA was isolated from the spleen and Peyer's patches using Tri-reagent (Sigma Chemical, St Louis, USA). RNA quality and quantity was analyzed before the reverse transcriptase polymerase chain reaction (PCR). Total RNA (1 µg) of each sample was reverse-transcribed by MuLV reverse transcriptase and random hexamer. The PCR from each sample was carried out in a total volume of 50 µL containing 2 µL of cDNA, 5 µL of 10X PCR buffer, 3 µL of 30 mmol/L dNTP mix, 1 µL of each forward, backward primer, 1 U Taq DNA polymerase and 33.5 µL water. The reaction was performed in a T-Thermocycler (Biometra, Analytik GmbH, Goettingen, Germany) using a 35-cycle program starting with denaturation at 95°C for 3 min followed by a three-step temperature cycling of (95°C for 1 min, 52°C for 1 min and 72°C for 3 min) and then terminated by a 72°C incubation for 10 min and by cooling at 4°C. The PCR products obtained were subjected to gel electrophoresis on 1.5% agarose gels containing 1X Tris borate ethylenediamine tetraacetic acid buffer and visualized by staining with ethidium bromide using a UV transilluminator. Primers for IFN γ are (forward: 5'-GCTCTGAGACAATGAACGCT-3') and (reverse: 3'-AAAGAGATAATCTGGCTCTGC-5'), IL-2 (forward: 5'-TCCACTTCAAGCTCTACAG-3') and (reverse: 3'-GAGTCAAATCCAGAACATGCC-5'), IL-4 (forward: 5'-GAATGTACCAGGAGCCATATC-3') and (reverse: 3'-CTCAGTAC-TACGAGTAATCCA-5'), IL-6 (forward: 5'-TTCCATCCAGTTGCCCTTCTTGG-3') and (reverse: 3'-CTTCATGTACTCCAGGTAG-5') and β -microglobulin (forward: 5'-TGACCGGCTTGATGCTATC-3') and (reverse: 3'-CAGTGTGAGCCAGGATAT-AG-5') used for PCR were taken from Zhou *et al.*²² and synthesized from Integrated DNA Technologies,

Coralville, IA, USA. Bands were quantified using gel analysis software (Bio-Rad Laboratories, Hercules, CA, USA). The density of each mRNA band was normalized to the expression of β -microglobulin. Results were expressed as means and standard error of means (SEM) of three independent estimations from each group.

Faecal microbial analysis

For the assay of the fecal lactobacilli and lactococci, fresh samples of feces were collected from all groups of animals at 0 (before experimental diet feeding), 2, 4, 6, 8, 10, 14, 21 and 28 days, by gently squeezing the rectal part of the mice. Mildly homogenized 100 mg of fecal samples were diluted in a final volume of 0.5 mL in 0.1% peptone water using a glass homogenizer, aseptically. The homogenate was serially diluted in peptone water and aliquot plated (in triplicate) on MRS and M-17 for enumeration of total lactobacilli and lactococci and the plates were incubated at 37°C for 48 and 24 h, respectively. The numbers of colonies (in CFU) were counted per gram of wet feces.

Statistical analysis

The data were subjected to analysis of variance using SPSS 10.0 (SPSS Inc, Chicago, IL) and significant differences among the means were analyzed using Duncan's test. Values were expressed as means ± SEM. Differences of *P*-value < 0.05 were considered significant.

RESULTS

Effect on physiological characteristics

The bodyweight gain, the weight of the organs, i.e., liver, spleen and kidney, and the food and water intake were not significantly different among all three groups (Table 2), which indicates that the feeding of dahi containing probiotic *L. casei* does not cause any health

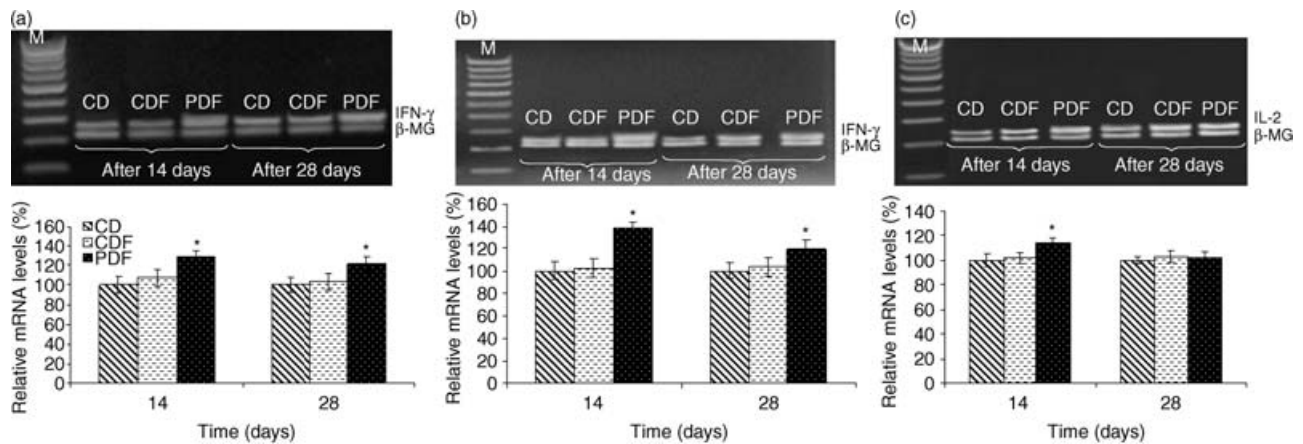


Figure 1. Relative mRNA abundance of IFN- γ in (a) spleen, (b) Peyer's patches and (c) mRNA levels of IL-2 in Peyer's patches collected from \blacksquare mice fed with synthetic diet (CD), \square control dahi-supplemented diet (CDF) and \blacksquare probiotic dahi-supplemented diet (PDF) after 14 and 28 days. The amount of mRNA is presented in terms of the relative mRNA abundance as determined by dividing the intensity of IFN- γ in all the groups of mice fed with different experimental diets by the intensity of the mRNA band of the CD group. Values are means \pm SEM of five animals. * $P < 0.05$.

complications to mice, and normal physiological characteristics during 28 days of experimental period were maintained in all the groups of mice.

Effect on cytokine gene expression in spleen

The mRNA expression of IFN- γ gene was significantly increased by 26 and 20% after 14 and 28 days, respectively, in the spleen collected from the PDF group compared with the CD group (Fig. 1a). A slight increase in the mRNA expression of IFN- γ was also observed in the CDF group after 14 days but it decreased thereafter and appeared closer to the CD group values after day 28. The expression of IL-2, IL-6 and IL-4 genes in the spleen collected from all groups did not change (data not shown). The mRNA levels of all studied cytokines did not change in the CD group during the whole experimental period.

Effect on cytokine gene expression in Peyer's patches

The mRNA levels of IFN- γ gene also increased by 33% in the Peyer's patches collected from the PDF mice as compared to the CDF and CD mice after day 14 and remained higher by 19% after 28 days than those of the CDF and CD groups ($P < 0.05$) (Fig. 1b). In addition, the mRNA levels of IL-2 also increased by 11% in the Peyer's patches collected from the PDF group than in the CDF and CD groups after day 14, but after 28 days its expression was decreased and became similar to the CDF and CD groups (Fig. 1c). The mRNA levels of IL-4 and IL-6 genes were also unchanged in the Peyer's patches of all groups during the whole experimental

period (data not shown). No significant changes were observed in cytokine expression in the Peyer's patches collected from the CDF and CD groups during whole experimental period.

Effect on fecal microbial counts

Total fecal lactobacilli and lactococci counts started to increase in the PDF group animals from day 2 and increased till day 10, after which the counts stabilized up to day 28 of the experimental period. Comparing the total fecal lactobacilli and lactococci counts in the PDF group, the total lactobacilli counts increased in significantly higher rates than those of total lactococci (Fig. 2). Though the total fecal lactococci counts also increased slightly in the CDF group in a manner similar to the PDF group, no significant changes were observed in the total lactobacilli count in this group. Moreover, no significant changes were observed in the total lactobacilli and lactococci counts in the fecal samples collected from the CD group.

DISCUSSION

In present study we investigated the *in vivo* effects of probiotic dahi feeding on basal cytokine mRNA expression in the spleen and Peyer's patches. It was observed that feeding on probiotic dahi significantly increased the expression of IFN- γ and IL-2 levels in the spleen and Peyer's patches and successfully implanted the probiotic lactic acid bacteria (*L. casei*) into the gut of mice. The spleen and Peyer's patches were chosen because they are representative of the systemic and gut

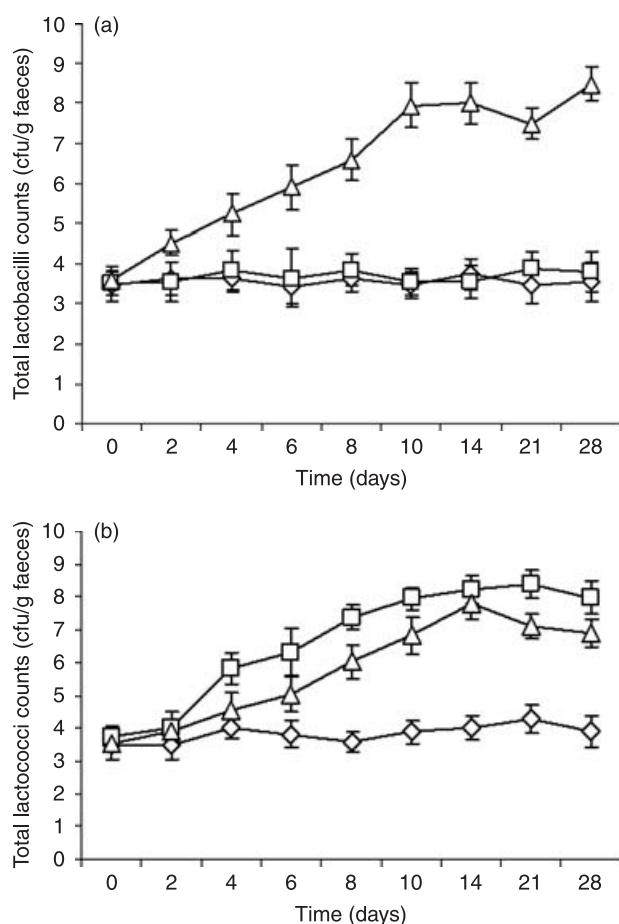


Figure 2. Total fecal (a) lactobacilli and (b) lactococci counts in animals fed with \diamond synthetic diet (CD), \square control dahi-supplemented diet (CDF) and \triangle probiotic dahi-supplemented diet (PDF) during 28 days. Values are mean \pm SEM (error bars) of five animals of each group.

mucosal immune system, respectively. The functioning of the immune system at the systemic as well as at the mucosal level could have been influenced by signals from these transient bacteria or de novo colonizers. Several studies^{3,23} reported that LAB may modulate various physiological proteins like cytokines in normal as well as diseased conditions in a beneficial manner to improve the immune status of the host, and they may protect against various immunological disorders such as cancer, autoimmune diseases and acute infections. But the exact mechanism of LAB in modulating cytokine production is not completely understood.

Probiotic bacteria in the small bowel are taken up into the Peyer's patches where activation, switching, proliferation and differentiation of B cells is under the control of T cells, cytokines and other accessory cells.²⁴ When the bacteria are engulfed, they enter in systemic

circulation through the M cells²⁵ and respond to the receptor system. The receptor system includes toll-like receptors on antigen presenting cells, which results in the secretion of Th1-specific responses characterized by IFN- γ .¹¹ Our results showed that feeding mice with probiotic dahi stimulated the mRNA expression of IFN- γ and IL-2 (Th1 type cytokine) in both their spleen and Peyer's patches. This indicates that probiotic dahi feeding stimulated a Th1-specific immune response in systemic as well as mucosal immune organs. These results are in agreement with observations of Ha *et al.*,²⁶ who reported that feeding with heat-killed yogurt containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* increased the IFN- γ mRNA after 14 days in mesenteric lymph nodes. Kawahara and Otani²⁷ reported that LAB (Nz8) isolated from *nozawana*, a traditional Japanese pickle, enhanced IFN- γ and IL-12 expression in mouse spleen cell cultures. This suggests that enhanced IFN- γ secretion by probiotic LAB feeding supports protective immunity, downregulates hypersensitivity at mucosal surfaces and enhances apoptosis retarding tumor formation.¹¹

The results of our study indicate that feeding of probiotic dahi stimulates the expression of IFN- γ in both Peyer's patches and the spleen compared to control dahi, implying that the effect was due to the involvement of the probiotic, instead of the normal dahi components, but the exact mechanism is not known. It has to be stated that the activation of the immune response by feeding probiotic dahi might be due to the higher availability and supplementation of immunomodulatory nutraceuticals. Our previous studies showed that probiotic dahi contains significantly higher amounts of oligosaccharides (probiotics), conjugated linolenic acid and bioactive peptides than control dahi,^{28–30} all of which are well known as immunomodulatory nutraceuticals. This suggests that probiotic *L. casei* might be responsible for the higher production of these beneficial nutraceuticals at the time of milk fermentation.

Moreover, as the immunostimulatory effect of probiotic dahi might be due to the modulation of gut flora, we also checked the microbial counts of the total lactobacilli and lactococci. We found that feeding with probiotic dahi significantly increased both total lactobacilli and lactococci counts in the fecal samples of mice. This indicates that feeding with probiotic dahi increased the population of good microbial flora in the gut, which is an indicator of the successful delivery and implantation of probiotic bacteria into the mouse gut. This may be one of the reasons for the immunomodulatory effect of probiotic dahi. It is well known that the gut is a main source of microbial population

consisting of beneficial and pathogenic bacteria. The increased ratio of beneficial bacteria (LAB) improves the host's health by various means, including strengthening the immune function.³¹ Vinderola *et al.*³² demonstrated that lactobacilli can stimulate the immune system by different pathways of internalization. Lactobacilli interact with epithelial cells through TLR-2 and release Th1-specific cytokines. In the present study, the increased population of the total lactobacilli into the gut might be the reason for increased interactions with epithelial cells which lead to enhanced mucosal immune cell activation in Peyer's patches, and these might further transmitted to the spleen through the cross-talking of circulatory immune cells. The results of our study agree with the statement that the effects of probiotic dahi feeding is prominent on the mucosal immune organ (Peyer's patches), in comparison with the systemic immune organ (spleen), for example, the expression of IL-2 increased only in Peyer's patches, with no change in the spleen.

In conclusion, we detected immunomodulation by orally administered probiotic dahi at the gene expression level, which confirms earlier indications that probiotic dahi can skew the immune system towards a Th1 type response.

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