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Original article

Modulation of expression of Programmed Death-1 by administration of probiotic Dahi in DMH-induced colorectal carcinogenesis in rats

Dheeraj Mohania[1,2], Vinod K. Kansal[2], Manoj Kumar[3], Ravinder Nagpal[4], Yuichiro Yamashiro[4], Francesco Marotta[5]

[1] Department of Research, Sir Ganga Ram Hospital, New Delhi, India; [2] Animal Biochemistry Division, National Dairy Research Institute, Karnal, Haryana, India; [3] Department of Microbiology & Immunology, National Institute of Nutrition, Hyderabad, India; [4] Probiotic Research Laboratory, Juntendo University Graduate School of Medicine, Tokyo, Japan; [5] ReGenera Research Group for Aging Intervention, Milan, Italy

Abstract. *Background and Aim:* Interaction of probiotic bacteria with the host immune system elicits beneficial immune modulating effects. Although, there are many published studies on interaction of probiotics with immune system focusing on activation of immune system by bacterial cell wall through the engagement of Toll-like receptor family, very few studies have focused on molecules involved in the T-cell activation, and not much work has been executed to study the correlation of probiotics and programmed death-1 in colorectal carcinogenesis in animal models. Hence, the present study was carried out to assess the effect of probiotic Dahi on expression of programmed death (PD-1) in colorectum of 1, 2-dimethylhydrazine treated Wistar rats. *Methods:* DMH was injected subcutaneously at the rate of 40 mg/kg body weight per animal twice a week for 2 weeks. A total of 168 male Wistar rats were randomly allocated to seven groups, each group having twenty-four animals. The rats were euthanized at the 8th, 16th and 32nd week of the experiment and examined for the expression of PD-1 in colorectal tissues by immunohistochemical staining. *Results:* PD-1 expression was observed in colorectal tissues of normal and DMH-treated rats. An increase in PD-1 expression upon DMH treatment was observed and its reversion by two different preparations of the probiotic Dahi, an effect also observed with the NSAID piroxicam. The effects of probiotic Dahi and piroxicam were synergistic. Feeding rats with probiotic Dahi or piroxicam treatment decreased the expression of PD-1 in DMH-induced colorectal mucosa. Combined treatment with probiotic Dahi and piroxicam was significantly more effective in reducing the expression of PD-1. *Conclusion:* PD-1 is expressed independently of carcinogen administration in normal colonic mucosa and may play a role in immune response modulation in DMH-induced colorectal carcinogenesis. The present study suggests that probiotic Dahi can be used as an effective chemopreventive agent in the management of colorectal cancer (www.actabiomedica.it)

Key words: colorectal cancer, 1,2-dimethylhydrazine, piroxicam, programmed death-1, probiotics

Introduction

Gastrointestinal tract of humans and mammals are the biggest reservoir of large number of commensal microbial flora that contributes to the beneficial health of the host via immunomodulation. The reservoir of these symbiotic bacteria participates in nutrient assimilation, production of amino acids, vitamins for their host and plays a key role in the development of healthy immune system. Immunomodulatory compo-

nents such as cell surface components and peptidoglycan components of these bacteria may play an important role in activating immune-proficient cells in the gastrointestinal (GI) tract (1). Additionally, functional association of the intestinal microflora in immunomodulatory responses and maintenance of homeostasis accentuate the important role of the microbiota in the GI tract (2, 3). Furthermore, intestinal microbiota, which includes various species of Lactobacillus, interacts regularly with colonic epithelial cells (4-6). A growing body of evidence suggests that modulations of the immune system could be mediated by ingestion of probiotics which confer a health benefit upon the host by promoting humoral and cell mediated immunity (7). However, little is known about the relationship between the probiotics and proteins involved in the regulation of T-cell activation in 1, 2-dimethylhydrazine (DMH)-induced colorectal carcinogenesis. Programmed cell death 1 (PD-1) is one among these proteins, a homolog of CD28 and CTLA-4, involved in the regulation of T-cell activation (8). PD-1 is expressed on T cells, B cells and myeloid cells. It, plays an important role in lymphocyte activation at tissue level based upon the expression of PDL-1 in non-lymphoid organs (9-13). Furthermore, PD-1 mediates immune regulation via not only on activated T cells, but also on B cells and monocytes (11, 14). Up-regulation of PD-1 might be associated with immune evasion and inhibition in tumor-bearing hosts (14).

Probiotic Dahi, an Indian fermented milk product, has been studied in tumor-bearing animals; however, the relationship between probiotics and expression of PD-1 in colorectal cancer is not known. Consequently, in the present investigation, we prepared the buffalo milk-based probiotic Dahi by co-culturing two combinations of selected strains of lactobacillus with Dahi: (A) *Lactobacillus acidophilus* (LaVK2) along with *Lactobacillus plantarum* (Lp9) and Dahi culture (B) *Lactobacillus acidophilus* (LaVK2) along with *Bifidobacterium bifidum* BbVK3 and Dahi culture. Consumption of the fermented product was then evaluated for its effects on the expression of programme death-1 (PD-1) in DMH-induced colorectal carcinogenesis in rats.

Materials and methods

Bacterial strains

Lactococcus lactis ssp. *cremoris* NCDC-86 and *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* NCDC-60 were obtained from National Collection of Dairy Cultures, National Dairy Research Institute , Karnal, India. *Lactobacillus acidophilus* LaVK2 and *Bifidobacterium bifidum* BbVK3 are our laboratory isolates with probiotic attributes tested through *in vitro* tests as per FAO/WHO guidelines. *Lactobacillus plantarum* (Lp9) was a generous gift from Dr. V. K. Batish, Scientist Emeritus, Dairy Microbiology Division, NDRI, Karnal, India. Lactobacilli and lactococci were propagated and maintained in MRS-broth and M17 broth (Himedia Laboratories Pvt. Ltd., Mumbai, India) at 37° and 30°C, respectively, and were stored at 4-8°C between transfers. *B. bifidum* BbVK3 was cultured and propagated under anaerobic conditions at 37°C.

Probiotic Dahi and Dahi preparation

Bacterial cultures were revitalized three times in reconstituted and autoclaved skim milk prior to use for preparation of fermented milk. Buffalo milk obtained from the cattle yard of the institute and standardized to 3.0% fat was heated to 90°C for 15 min and then cooled to 37°C. Dahi was prepared by culturing standardized buffalo milk with Dahi starter (*Lactococcus lactis* ssp. *cremoris* and *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis,* 1% each) at 30°C for 8 h. Probiotic LaLp-Dahi was prepared by co-culturing standardized buffalo milk with *L. acidophilus* LaVK2, *L. plantarum* Lp9 and Dahi starter under aseptic condition. The final product contained lactococci, $20x10^8$ cfu/g, *L. acidophilus* LaVK2, $2x10^8$ cfu/g and *L. plantarum* Lp9, $2x10^8$ cfu/g. Probiotic LaBb-Dahi was prepared by culturing standardized buffalo milk with *L. acidophilus* LaVK2, *B. bifidum* BbVK3 and Dahi starter. The final product contained lactococci, $1\text{-}2x10^9$ cfu/g, *L. acidophilus,* $2\text{-}20x10^8$ cfu/g and *B. bifidus*, $2\text{-}20x10^8$cfu/g.

Chemicals

1, 2-dimethylhydrazine dihydrochloride (DMH), piroxicam (PXC), Harris' haematoxylin and 3,3-di-

aminobenzidine were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The source of primary antibody and Ultravision Kit was Abcam (Cambridge, UK). All other chemicals were obtained from S.D. Fine Chemicals Ltd., Mumbai, India, or Hi-Media Lab. Ltd., Mumbai, India.

Animals and Diet

Male Wistar rats (21 d old) were obtained from Small Animal House of National Dairy Research Institute, Karnal, India and maintained in a small animal house. Animals were housed in stainless steel cages (three animals per cage) throughout the study and room temperature was maintained at 25 ± 2°C with 55 ± 5% humidity and at a 12-h light/12-h dark cycle and allowed water *ad libitum*. The animals were used and cared for in accordance with the principles and guidelines for humane use and protocols were approved the Institutional Ethics Committee. Basal diet composition is given in Table 1.

Experimental design

Animals randomly distributed into 7 groups (24 in each group) were given the following dietary treatment in addition to basal diet for 32 weeks: 1) Milk group, fed buffalo milk; 2) Milk-DMH group, fed buffalo milk (3% fat adjusted) and administered 1,2-dimethylhydrazine dihydrochloride (DMH); 3) Milk-DMH-PXC group, fed buffalo milk (3% fat adjusted) and administered DMH and piroxicam; 4) LaLp Dahi-DMH group, fed LaLp Dahi and administered DMH; 5) LaLp Dahi-DMH-PXC group, fed LaLp Dahi and administered DMH and piroxicam; 6) LaBb Dahi-DMH group, fed LaBb Dahi and administered DMH; 7) LaBb Dahi-DMH-PXC group, fed LaBb Dahi and administered DMH and piroxicam.Each rat was fed 20 g supplements of buffalo milk (3% fat adjusted) or probiotic Dahi, followed by basal diet *ad libitum*. Following 28 day feeding, each animal was injected (s.c.) DMH (40 mg/kg body weight) twice a week for 2 weeks except for the Milk group. Piroxicam (4 mg/day/rat) was delivered through milk/probiotic Dahi, start one week after the last DMH dose till termination of the experiment. Eight rats from each group were sacrificed by cervical dislocation at 8, 16 and 32 weeks, and their colorectal tissues were examined for PD-1 expression. These periods correspond to 2, 10 and 26 weeks past DMH administration, and 1, 9 and 25 weeks from piroxicam introduction in the drug treatment groups. Colorectal tissue specimens were fixed in formalin and embedded in paraffin for routine histological diagnosis and immunohistochemical analysis.

Table 1. Composition of hypercholesterolaemic basal diet.[a]

Ingredients	Amount
Casein	20.0%
Hydrogenated vegetable oil	20.0%
Cellulose	5.0%
Choline chloride	0.2%
Starch	19.25%
Sucrose	30.0%
D, L-methionine	0.3%
Salt mixture	5.3%
Vitamin mixture	1.0%

[a] Salt mixture (AOAC, 2005) required for 10 kg diet (500 g) contained $CaCO_3$, 190.7 g; $CoCl_2.6H_2O$, 0.0115 g; $CuSO_4.5H_2O$, 0.238 g; $FeSO_4.7H2O$, 13.5 g; KH_2PO_4, 194.5 g; KI, 0.4 g; $MgSO_4.7H_2O$, 58.62 g; $MnSO_4.H_2O$, 2.005 g; NaCl, 69.65 g; and $ZnSO_4.7H2O$, 0.274 g. Vitamin mixture (100 g) comprised of biotin, 4 mg; folic acid, 20 mg; vitamin B_{12}, 0.3 mg; menadione, 50 mg; para aminobezoic acid, 1 g; meso-inositol, 1 g; thiamine, 50 mg; riboflavin, 80 mg; pyridoxine, 50 mg; calcium pentothenate, 0.4g and starch, 76.946 g. Vitamin A (2×10^5 IU), vitamin E (10^3 IU) and vitamin D (2×10^4 IU) were administered to the diet through oil / fat (for 10Kg diet)

Immunohistochemical staining

Four microns sections of the specimens were cut from paraffin-embedded tissue, mounted on poly-L-lysine coated slides, air dried for 10 min and fixed at 65°C for 15 min. Endogenous peroxidase activity was blocked with 1.0% hydrogen peroxide in PBS for 30 min at room temperature. Sections were then washed three times in PBS. Non-specific background staining was blocked with protein blocking agent (PBA) for 20 min at room temperature. Sections were incubated with primary antibody (Anti-PD-1) at the final concentration of 1:100 in a humidified chamber at 37°C for 2 h. Bound primary antibodies were detected with Ultravision Kit according to the manufacturer's instructions. Sections were washed with deoinized wa-

Table 2. Effect of feeding probiotic Dahi and piroxicam (PXC) treatment on feed intake and body weight of dimethylhydrazine (DMH) treated rats. Values are mean ± SD for n=8

Groups	Average Feed Intake (g/d/rat)	Body weight (g) Initial	Body weight (g) Initial
Milk	17.8[a] ± 1.9	22.2[a] ± 0.3	325.2[a] ± 1.0
Milk-DMH	11.4[b] ± 2.0	22.8[a] ± 0.4	237.0[b] ± 3.5
Milk-DMH-PXC	16.8[a] ± 2.0	23.2[a] ± 0.4	304.5[c] ± 3.1
LaLp Dahi-DMH	17.8[a] ± 1.5	21.7[a] ± 0.3	342.0[d] ± 3.8
LaLp Dahi-DMH-PXC	18.9[a] ± 1.8	24.2[a] ± 0.3	345.8[d] ± 3.3
LaBb Dahi-DMH	17.7[a] ± 1.3	22.7[a] ± 0.3	338.0[d] ± 4.8
LaBb Dahi-DMH-PXC	18.6[a] ± 2.0	23.2[a] ± 0.3	340.8[d] ± 2.9

DMH, 1,2-dimethylhydrazine dihydrochloride; PXC, piroxicam.
[a,b,c,d] Mean values within a column with unlike superscript letters were significantly different (P<0.05).

ter for 5 min and counterstained with Harris' haematoxylin. The expression levels of PD-1 were calculated according to Detre S' method (15).

Statistical analysis

The results were expressed as mean ± SD for each group (n=8) and analyzed by 1-way analysis of variance (ANOVA) followed by the Tukey post hoc test (SYSTAT version 6.0.1, SPSS Inc, Chicago, IL, USA). Differences were considered significant at $P < 0.05$.

Results

A significant decline in feed intake as well as in body weight gain was observed in rats treated with DMH (Table 2). Treatment of DMH induced rats with either piroxicam (PXC), or LaBb Dahi, or LaLp Dahi, or piroxicam plus probiotic Dahi combined (LaBb or LaLp), restored feed intake to normal levels and increased weight gain significantly ($P< 0.05$). The DMH induced animals treated with LaBb Dahi or piroxicam and LaBb Dahi grew at rates even faster than the control rats. Typically, lymphocyte infiltration occurred in colorectal tissues of DMH-treated rats (Fig. 1). In these tissues, mononuclear infiltration of lymphocytes showed positive membrane staining with anti-PD-1 (Fig. 2). The pattern of staining was consistent with the fact that PD-1 molecules are associated with cell membranes. PD-1 positive cells showed a U-shaped pattern throughout colonic epithelial membrane in colorectal mucosa. The expression of PD-1 in colorectal tissues of DMH-treated rats varied from 8 wk to 32 wk of the experimental study (Table 3).

In the present study, PD-1 expression in the colorectal mucosa of normal and DMH-treated animals supplemented with buffalo milk and different probiotic Dahi preparations was detected and measured (Fig. 2). PD-1 expression was significantly greater in milk fed and DMH-treated rats when compared to all other groups from 8 wk to 32 wk (Table 3). When animals milk-fed animals were injected with DMH, the expression of PD-1 was substantially enhanced at 32 wk reaching 7-fold of that at 0 d level (Table 3). Treatment with piroxicam or probiotic Dahi significantly decreased DMH-induced expression of PD-1 in epithelial cells of colorectum. Piroxicam and both probiotic Dahi (LaLp and LaBb Dahi) were almost equally effective in reducing DMH-induced accumulation of PD-1 in epithelial cells of colorectum. The reductions in DMH-induced accumulation of PD-1 in epithelial cells of colorectum were more pronounced in animals treated with the combination of piroxicam and probiotic Dahi. In animals treated with LaBb Dahi along with piroxicam, PD-1 expression in epithelial cells of colorectum at 32 wk of experimental period reached levels similar to untreated normal animals.

Discussion

Probiotic microorganisms have a long history of consumption in the form of fermented foods and are

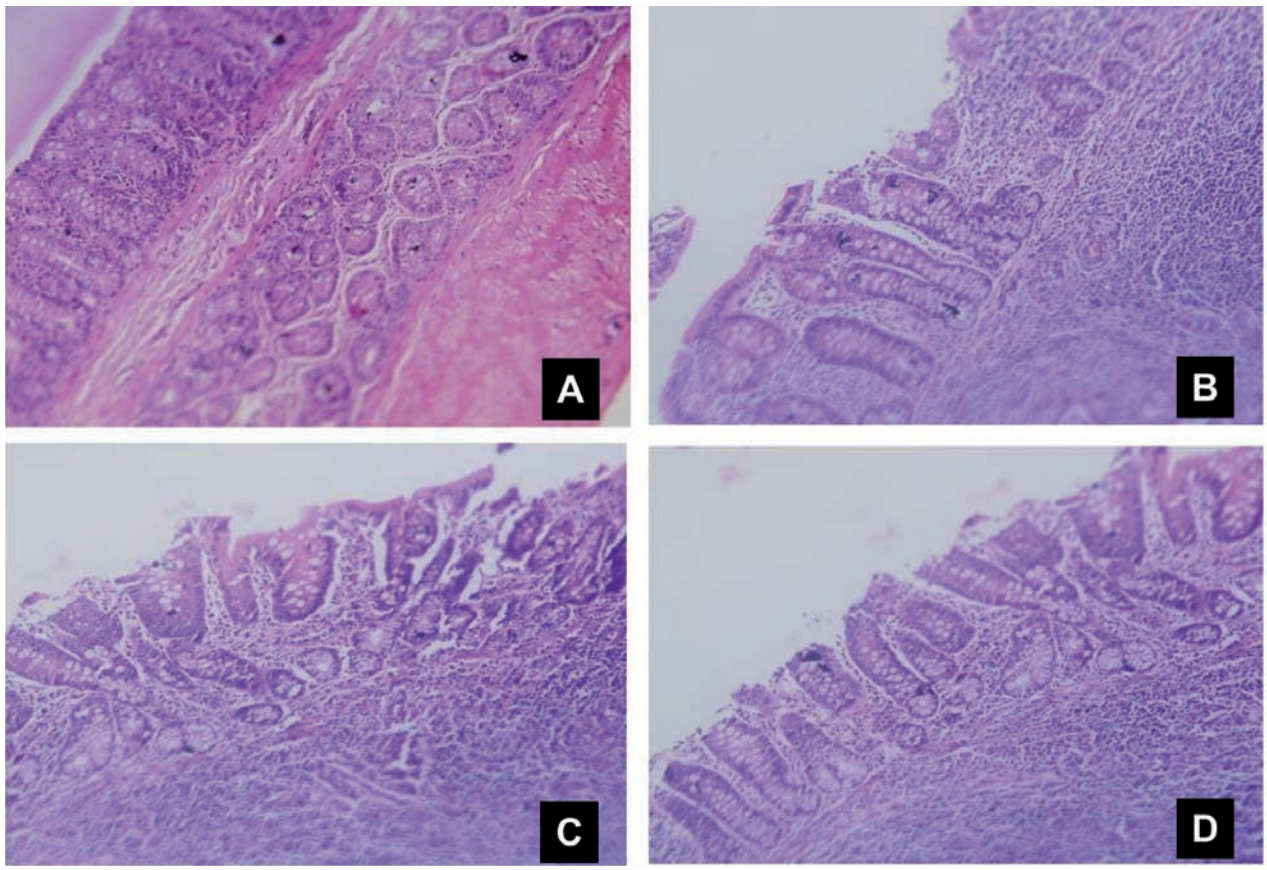

Figure 1. Longitudinal section of colonic mucosa showing aberrant crypt focus stained with haematoxylin and eosin (200X) at 32 week. (A) Normal looking colonic mucosa; (B, C, D) Colonic mucosa showing crypt disarray and inflammatory cell infiltration in lamina propria in DMH-treated rats. Crypts are smaller than normal, variable in shape and show branching. There is loss of normal crypt architecture with widening of lamina propria. Submucosa reveals dense lymphomononuclear infiltrate

known to interact with the immune system and elicit beneficial immune modulating effects (1, 16-18). In line of these evidences, we have prepared probiotic fermented milk namely probiotic Dahi (LaLp Dahi or LaBb Dahi) along with mixed Dahi cultures of lactococci for the delivery of probiotic strains that can provide protection against colorectal cancer in animal models. In this study, we examined the expression of PD-1 in normal colorectal mucosa and investigated the effects of probiotic Dahi (LaLp or LaBb Dahi) or their combination with piroxicam on the expression of PD-1 in colorectal tissues of DMH-treated rats. Both probiotic Dahi (LaBb Dahi or LaLp Dahi) were equally effective, and were even more effective than piroxicam in reducing PD-1 expression in the colorectum of DMH-treated rats. Furthermore, the combination of piroxicam and probiotic Dahi treatment decreased DMH-induced initiation and progression of neoplastic lesions more effectively, suggesting that this treatment combination is effective in preventing the initiation and progression of carcinogenesis. Hence, the role of probiotic Dahi (LaBb Dahi or LaLp Dahi) as an alternate biotherapeutic agent in the treatment of colorectal cancer may also be explored. Probiotic intervention may decrease exposure of the colonic epithelial cells to cytotoxic and genotoxic agents or may modulate the balance of colonic cell proliferation and apoptosis, and/or enhance the production of butyrate acetate, thereby improving immunomodulation of colorectal mucosa (19-21). Intestinal bacteria are capable of activating or deactivating proximal carcinogens, behaving as promoters or

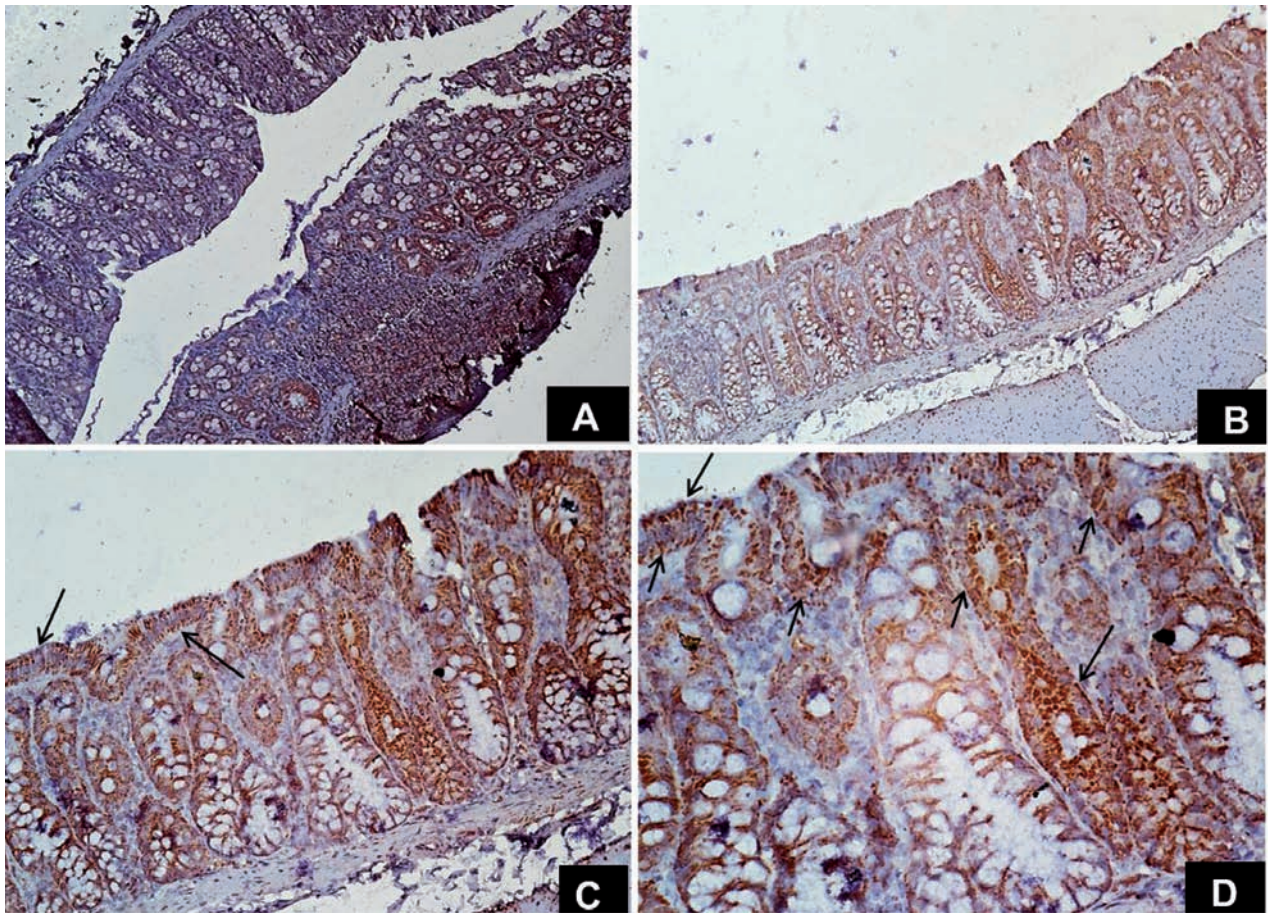

Figure 2. Longitudinal section of colonic mucosa showing immunohistochemical staining of normal and DMH treated colorectal tissues for programmed death-1 (PD-1) at 32 week. A: PD-1 expression in normal colonic mucosa (200X); (B, 200X; C, 400X; D, 600X): Immunohistochemical staining of colonic mucosa showing positive staining (arrow head) for PD-1 treated with DMH showing crypt disarray and inflammatory cell infiltration in lamina propria

anti-promoters in colon carcinogenesis (22). Recently, the author's laboratory has shown that probiotic LaBb Dahi down regulate carcinogen activating cytochrome P450 enzymes CYP1A1, CYP1A2 and CYP1B1 in liver, and up regulate carcinogen detoxifying γ-glutamyltranspeptidase, UDP-glucuronosyl transferase and quinone reductase activities in liver as well as in colon (23). The potential of LaBb Dahi to improve macrophage and lymphocyte functions (24) and antioxidative status (25) has been also established. The results presented herein could be due to the maintenance of gut homeostasis by balancing proinflammatory (secretion of IL-12, a critical factor in switching memory T cells to Th1 response) and anti-inflammatory (secretion of IL-10 and IL-14 which promote the generation of a Th2 response) mucosal responses with the help of intestinal bacteria and probiotics, including lactobacilli and bifidobacteria (26).

Conclusion

From the results obtained, it could be concluded that PD-1 is expressed independent of carcinogen administration and is upregulated by DMH administration in colorectal carcinogenesis. The findings of this study indicate that consumption of probiotic Dahi (LaLp or LaBb Dahi) or piroxicam or probiotic Dahi combined results in the decreased expression of PD-1 antigen. Moreover, the effects of probiotic Dahi and

Table 3. Effect of feeding probiotic Dahi and piroxicam (PXC) treatment on programmed death 1 (PD-1) expression in colorectal tissue of dimethylhydrazine (DMH) treated rats

Group	0 day	8 week	16 week	32 week
Buffalo milk	$1.75^{a} \pm 1.04$	$1.63^{a} \pm 0.52$	$1.50^{a} \pm 0.54$	$1.63^{a} \pm 0.52$
Buffalo milk-DMH	$1.75^{a} \pm 1.04$	$8.50^{bc} \pm 2.00$	$13.88^{b} \pm 2.23$	$14.33^{b} \pm 3.29$
Buffalo milk-DMH-PXC	$1.75^{a} \pm 1.04$	$9.75^{c} \pm 2.55$	$9.00^{cd} \pm 3.42$	$7.63^{cd} \pm 2.62$
LaLp Dahi-DMH	$1.75^{a} \pm 1.04$	$6.38^{bde} \pm 1.85$	$7.00^{de} \pm 2.56$	$6.50^{d} \pm 2.98$
LaLp Dahi-DMH-PXC	$1.75^{a} \pm 1.04$	$3.50^{a} \pm 1.69$	$3.63^{ae} \pm 1.92$	$3.00^{a} \pm 0.76$
LaBb Dahi-DMH	$1.75^{a} \pm 1.04$	$4.00^{ae} \pm 2.07$	$3.63^{ae} \pm 1.92$	$3.13^{a} \pm 0.99$
LaBb Dahi -DMH-PXC	$1.75^{a} \pm 1.04$	$1.63^{a} \pm 0.52$	$3.38^{a} \pm 1.77$	$2.00^{a} \pm 0.93$

Values are mean ± SD for n=8.
^{a,b,c,d,e,f} Values within column with different superscripts letters are significantly different ($P<0.05$).

piroxicam were synergistic. Furthermore, the PD-1expression is significantly higher in DMH-induced adenocarcinoma than in normal colonic mucosa and correlated with the number of infiltrating lymphocytes, indicating the importance of PD-1 in tumor development. The study has demonstrated that traditionally used dairy based fermented foods could be used as a good medium or potential nutraceutical intervention for the delivery of probiotic strains of bacteria to achieve health-benefits to the consumers.

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Correspondence: Dr. Dheeraj Mohania,
Associate Scientist Consultant,
Department of Research, Sir Ganga Ram Hospital,
Rajinder Nagar, New Delhi 110060, India.
Tel. + 91-011-42251624
Fax: +91-011-42251633
E-mail: dmohania@gmail.com