



## Research Article

# Probiotic Dahi Containing *Lactobacillus Acidophilus* and *Lactobacillus Plantarum* Suppresses DMH Induced Preneoplastic lesions in early Colorectal Carcinogenesis in Wistar Rats

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This study evaluated the chemopreventive effects of probiotic LaLp Dahi containing *Lactobacillus acidophilus* LaVK2 and *Lactobacillus plantarum* Lp9 alone or as an adjunct with piroxicam (PXC) in male Wistar rats administered 1,2-dimethylhydrazine dihydrochloride (DMH). Colorectal carcinogenesis was induced by injecting DMH subcutaneously (40mg/kg body weight) twice a week for 2 weeks. Rats were divided into five groups, twenty four in each group and fed with the buffalo milk or probiotic supplements (20g) in addition to basal diet for 32 weeks. The rats were fed with buffalo milk or probiotic Dahi alone or in combination with PXC in addition to basal diet *ad libitum* and euthanized at 8th, 16th and 32nd weeks of the experiment and examined for development of aberrant crypt foci (ACF), mucin-depleted foci (MDF) and proliferating cell nuclear antigen (PCNA) labeling index. Administration of DMH in rats induced formation of preneoplastic lesions (ACF and MDF) and increased PCNA index in colorectal tissue. Probiotic Dahi alone or in combination with piroxicam showed a significant ( $P < 0.05$ ) protective effects by lowering the initiation and progression of DMH induced formation of preneoplastic lesions and PCNA labeling index. These observations suggest that probiotic LaLp Dahi alone or in combination with PXC may be used as a potential nutraceutical intervention in prophylaxis and treatment of colorectal cancer.

**Keywords:** Probiotics; DMH; Piroxicam; Aberrant crypt foci; Mucin depleted foci; Colorectal carcinogenesis**Peer Reviewers:** Daniela De Stefano, PhD, Institute of Immunology, IERFC Fondazione ONLUS HSR DIBIT1 Via Olgettina, Italy**Editor:** Sihua Peng, PhD, Department of Pathology, Zhejiang University School of Medicine, China**Received:** May 12, 2013; **Accepted:** August 15, 2013; **Published:** August 25, 2013**Competing Interests:** The authors have declared that no competing interests exist**Copyright:** 2013 Mohania D *et al.* This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited**\*Correspondence to:** Dheeraj Mohania, Department of Research, Sir Ganga Ram Hospital, Rajinder Nagar, New Delhi, India. Email: [dmohania@gmail.com](mailto:dmohania@gmail.com); **Present Address:** Department of Research, Sir Ganga Ram Hospital, Rajinder Nagar, Email: [dmohania@gmail.com](mailto:dmohania@gmail.com)

## Introduction

Colorectal cancer is the fourth most common cancer in men and the third most common cancer in women worldwide and is therefore a major health problem underlining the need for effective chemopreventive strategies [1]. Several studies have suggested that probiotics such as lactobacilli or bifidobacteria modulate the host resistance against intestinal infections and provide protective effects against colon cancer development [2, 3]. There exists a potential role for foods that contain probiotics to change the colonic microbiota in a way that might prevent diseases such as colorectal cancer. ACF are considered to be putative pre-neoplastic colon lesions since they are found in the colon of carcinogen-treated rodents [4, 5] and in patients at high risk for colon cancer development [6]. Biochemical, genetic and morphological studies have shown that ACF and colon tumors share similar alterations, further supporting the hypothesis that ACF are precursors of colorectal cancer. Mucin-depleted foci (MDF) have been identified as dysplastic lesions or crypts devoid of mucin that occur in the colon of carcinogen treated rats [6, 7] and also in humans at high risk of colon cancer [8]. MDF harbor mutations in genes affecting colon carcinogenesis and, like colonic tumours, show Wnt signaling activation, features suggesting that these lesions are precancerous [9, 10]. The expression of MUC2, the most abundant mucin in the colon, is dramatically reduced in MDF which leads to speculation that the focal loss of the protective mucous layer might activate local inflammation, which might drive carcinogenesis to more advanced stages [11]. MDF are dose-dependently induced by 1,2-dimethylhydrazine dihydrochloride (DMH)

and progressively increase in size after carcinogen administration. Recent investigations suggest that MDF can be also used as a promising biomarker for studying the effect of chemopreventive agents in colon carcinogenesis [8, 9,11]. Assessment of PCNA expression as an indicator of colonic crypt cell proliferation is another putative intermediate marker of colon cancer risk [12]. There are abundant reports on beneficial effects on probiotic Dahi on health and many investigators have suggested that the consumption of fermented dairy products, including probiotic Dahi, elicits anti-tumor effects [6, 7]. Dahi is fermented buffalo milk consumed in Indian sub-continent as a part of daily diet. It is prepared using mesophilic culture of lactococci, which are not probiotic in nature. However, Dahi can be a good medium for delivery of probiotic bacteria. We have prepared a buffalo milk based probiotic Dahi by co-culturing the selected strains of *Lactobacillus acidophilus* LaVK2 and *Lactobacillus plantarum*Lp9 and Dahi starter. Recent studies from our laboratory have shown that this product attenuates the diet induced hypercholesterolemia in rats [13], and improves macrophage activity and confers protection against enteric infection in mice [13]. It reverses age related dysregulation of tissue antioxidant activities and decline in expression of biomarkers of ageing (peroxisome proliferators activated receptors- $\alpha$ , senescence marker protein-30 and klotho) in hepatic and kidney tissues [14], and alleviates age induced decline in macrophage and lymphocyte functions [15]. Being a fermented milk product, Dahi can be an excellent medium for delivery of probiotic strains that can provide protection against colorectal cancer. In this context, the present study was carried out for the first time

to evaluate the chemopreventive role of probiotic Dahi and its intervention with PXC on the development of preneoplastic lesions such as ACF, MDF and PCNA labeling index in male Wistar rats. The present study shows that this probiotic Dahi prevents the initiation and progression of pre-neoplastic lesions in DMH treated rats, and also improves the efficacy of treatment with piroxicam.

## Material and Methods

### Bacterial strains

*L. acidophilus* LaVK2, *Lactococcus lactis* ssp. *cremoris* NCDC-86 and *L. lactis* ssp. *lactis biovar diacetylactis* NCDC-60 were obtained from National Collection of Dairy Cultures, National Dairy Research Institute (NDRI), Karnal, India. *Lactobacillus plantarum* Lp9 was a generous gift from Dr. V. K. Batish, Emeritus Scientist, Dairy Microbiology Division, NDRI, Karnal. The *lactobacilli* and *lactococci* were propagated and maintained in MRS-broth and M17 broth (Himedia Laboratories Pvt. Ltd., Mumbai, India) at 37 °C and 30 °C, respectively, and were stored at 4-8 °C between transfers. *L. plantarum* and *L. acidophilus* were propagated and cultured at 37 °C for 24 h and 37 °C for 48 h, respectively.

### Preparation of Dahi and probiotic Dahi (LaLp Dahi)

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 was standardized to 3.0% fat, heated to 90 °C for 15 min and was then cooled to 37 °C. Dahi was prepared by culturing standardized buffalo milk with Dahi starter culture (*Lactococcus lactis* ssp. *cremoris* and

*Lactococcus lactis* ssp. *Lactis biovar diacetylactis*, 1% each) for 8 h at 30 °C. Probiotic LaLp Dahi was prepared by inoculating buffalo milk with *L. acidophilus* LaVK2, *L. plantarum* Lp9 and Dahi starter (1.0%). The final product contained  $2 \times 10^9$  cfu/g of lactococci, *L. plantarum* and *L. acidophilus* each.

### Chemicals

1,2-dimethylhydrazine dihydrochloride (DMH), piroxicam, alcian blue stain, Harris' haematoxylin, monoclonal anti-PCNA antibody and 3,3-diaminobenzidine were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The source of mouse ABC immunostaining system was Santa Cruz Biotechnology (Santa Cruz, California, U.S.A), *N-N'*-dimethyl-*m*-phenylenediamine and *N-N'*-dimethyl-*p*-phenylenediamine were procured from National Chemicals Ltd., Bangalore, India. All other chemicals were obtained from S D Fine-Chem Limited, Mumbai, India or Hi-Media Lab. Ltd., Mumbai, India.

### Animals and Diet

Male Wistar rats (3 weeks of age) were obtained from Small Animal House of National Dairy Research Institute, Karnal, India. The animals were housed in stainless steel cages (three animals per cage) throughout the study, and room temperature was maintained at  $25 \pm 2$  °C;  $55 \pm 5\%$  humidity and at a 12-h light/12-h dark cycle. The animals were used and cared for in accordance with the principles and guidelines for human use and protocols were approved by the Institutional Ethics Committee. The composition of basal diet was starch, 63%; casein, 20%; soybean oil, 5.5%; cellulose, 5%; mineral mixture, 5%; vitamin mixture, 1%; D-L methionine, 0.2%; and choline chloride, 0.2%.

Mineral and vitamin mixture were prepared and mixed according to AOAC [16, 17].

### Experimental design

Animals were randomly divided into five groups, twenty four in each group and fed with the buffalo milk or probiotic supplements (20g) in addition to basal diet for 32 weeks. Rats of group I was fed with buffalo milk which served as control. Rats of group II (Milk-DMH) was administered DMH and served as the DMH control; rats of group III (Milk-DMH-PXC) was administered milk and PXC in DMH induced rats. Rats of group IV (LaLp Dahi-DMH) were accessible to probiotic Dahi and DMH, and rats of group V (LaLp Dahi-DMH-PXC) were offered both probiotic Dahi and PXC in DMH induced rats. Following 28 d feeding, DMH (40 mg/kg body weight) was administered subcutaneously (s.c.) to the rats of the corresponding groups at twice a week for 2 weeks. PXC(4 mg/rat or 200 mg/kg of supplements) was given daily orally along with the supplements (milk/probiotic Dahi) and its feeding started one week after the last dose of DMH and continued till termination of experiment. Rats were sacrificed by cervical dislocation at 8th, 16th, 32th weeks of experimental time and colorectal tissues were examined for aberrant crypt foci (ACF), mucin depleted foci (MDF) and proliferating cell nuclear antigen (PCNA) labeling index.

### Identification of aberrant cryptfoci (ACF)

Colorectal segments were removed, washed with saline, cut open longitudinally, fixed flat in 10% buffered formalin and stained with methylene blue [18]. The ACF were characterised by their enlarged crypts elevated crypts from the surrounding epithelium, thickened epithelial layer, increased pericryptal space and their round or elongated luminal

openings [19].

### Identification of mucin deficient foci (MDF)

After ACF determination, the colorectal segments were processed for high iron diaminealcian blue staining [15]. The MDF were characterized by having or very little mucin and fulfilled the criteria according to Caderniet *al.* [9]. The tissue were coded and scored independently by two observers.

### Immunostaining for proliferating cell nuclear antigen (PCNA)

Following MDF determination, the colorectal tissues were embedded in paraffin such that the crypts could be sectioned longitudinally. The proliferative activity in the mucosa was evaluated by determining proliferating cell nuclear antigen (PCNA) immunoreactivity [20]. Sections were cut from paraffin-embedded tissue and mounted on poly-L-lysine coated slides were de-paraffinized and re-hydrated. The endogenous peroxidase activity was blocked with 1.0 % hydrogen peroxide in absolute methanol. The sections washed with phosphate-buffered saline (PBS) were stained with mouse ABC immunostaining system. Non-specific background staining was blocked with normal blocking serum, the slides were incubated for 60 min at 37°C with the primary antibody (mouse monoclonal anti-PCNA antibody) diluted 1:100 in PBS. After washing (thrice with PBS) the slides were incubated for 30 min at 37°C with biotinylated goat anti-polyvalent secondary antibody. The slides were again washed (thrice with PBS) and then incubated for 30 min with avidin-biotinylated horseradish peroxidase reagent. Finally, the slides after rinsing thrice with PBS were treated for 5 min with 3, 3-diaminobenzidine. The slides were washed with deionized water for 5 min and counter stained with Harris'

haematoxylin. Proliferative activity was evaluated by counting PCNA-positive nuclei in at least 10 full longitudinal crypt sections (from the base to the bottom of the crypt) under the light microscope [20]. The dark brown stained nuclei were considered PCNA positive. The PCNA positive index was calculated as the proportion of positive stained nuclei expressed in percentage. The microscopic slides were coded and read independently by two observers.

### Statistical analysis

The results were expressed as the means  $\pm$  SD for each group (n=8) and analyzed by One-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

### Effect on feed intake and body weight

A significant decline in average feed intake as well as in body weight gain was observed in rats treated with DMH (Table 1). Treatment of DMH induced rats with either LaLp Dahi or PXC alone or LaLp Dahi adjuvant with PXC, restored feed intake to normal levels and increased the body weight significantly ( $P < 0.05$ ).

**Table 1** Effect of feeding probiotic Dahi and piroxicam (PXC) treatment on feed intake and body weight of dimethylhydrazine (DMH) treated rats.

Groups	Average feed intake (g/d/rat)	Body weight (g)		
		Initial	Final	Gain
Buffalo milk	17.8 <sup>a</sup> $\pm$ 1.9	22.2 <sup>a</sup> $\pm$ 0.3	325.2 <sup>a</sup> $\pm$ 1.0	303.0 <sup>a</sup> $\pm$ 1.0
Buffalo milk-DMH	11.4 <sup>b</sup> $\pm$ 2.0	22.8 <sup>a</sup> $\pm$ 0.4	237.0 <sup>b</sup> $\pm$ 3.5	214.3 <sup>b</sup> $\pm$ 3.6
Buffalo milk-DMH-PXC	16.8 <sup>a</sup> $\pm$ 2.0	23.2 <sup>a</sup> $\pm$ 0.4	304.5 <sup>c</sup> $\pm$ 3.1	281.3 <sup>c</sup> $\pm$ 3.2
LaLp Dahi-DMH	16.2 <sup>a</sup> $\pm$ 1.9	23.1 <sup>a</sup> $\pm$ 0.3	324.9 <sup>a</sup> $\pm$ 1.4	301.8 <sup>a</sup> $\pm$ 1.4
LaLp Dahi-DMH-PXC	17.0 <sup>a</sup> $\pm$ 2.0	23.1 <sup>a</sup> $\pm$ 0.4	343.6 <sup>d</sup> $\pm$ 6.1	320.5 <sup>d</sup> $\pm$ 5.9

Values are mean  $\pm$  SD for n=8. DMH, 1,2-dimethylhydrazine dihydrochloride; PXC, piroxicam. <sup>a,b,c,d</sup>Mean values within a column with unlike superscript letters were significantly different ( $P < 0.05$ ).

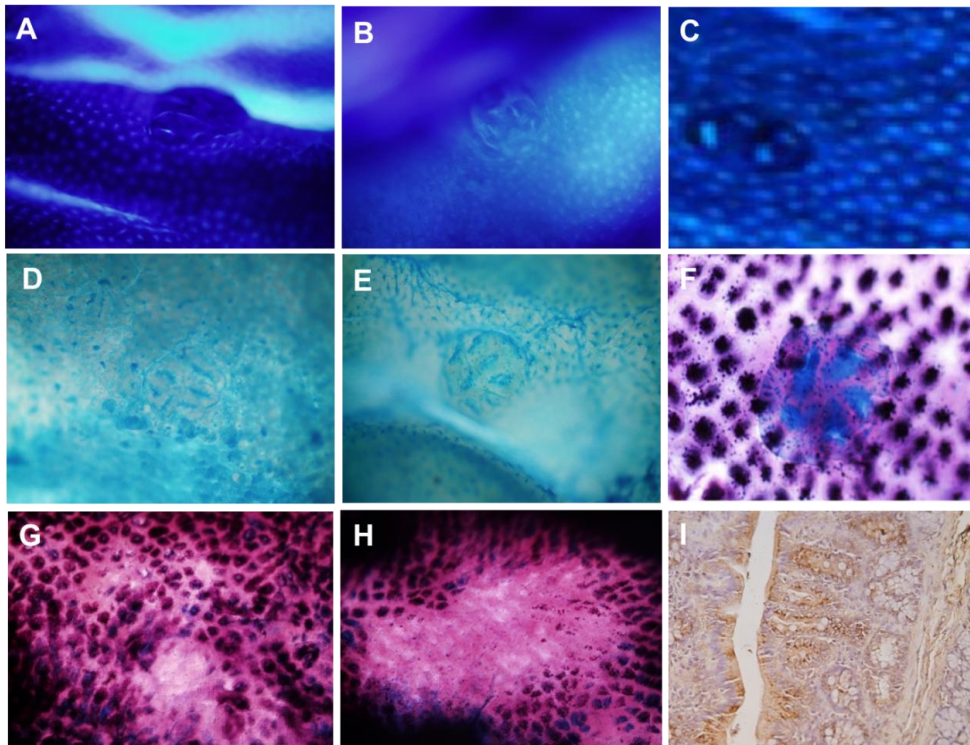
### Effect on aberrant crypts (AC) and aberrant crypt foci (ACF) formation

Figure 1A shows the lateral view of aberrant crypt foci (ACF) in methylene blue stained colonic mucosa. The ACF protrudes towards the lumen and consists of several crypts that differ from surrounding crypts by their increased size, and thicker and deeply stained epithelial lining. Figure 1B depicts ACF

consisting of 6 aberrant crypts. Figure 1C depicts ACF consisting of 4 aberrant crypts. Figure 1D and 1E depicts alcian blue-stained ACF having elliptical luminal opening. Figure 1F shows the topographic view of ACF in high iron diamine-alcian blue (HID-AB) stained colonic mucosa, wherein the ACF consisting of



five aberrant crypts was stained blue while normal crypts purplish-black.



**Figure 1** Topographic view and histological section of colonic mucosa. A, Lateral view of aberrant crypt foci (ACF) protruding towards the lumen, stained in methylene blue (magnification, 200x). B, ACF stained in methylene blue showing 6 aberrant crypts (AC) /ACF (magnification, 200x). C, ACF stained in methylene blue showing 5 aberrant crypts (AC) /ACF (magnification, 400x). D, ACF stained in alcian blue showing 6 AC/ACF (magnification, 200x). E, ACF stained in alcian blue showing 8 AC/ACF (magnification, 200x). F, ACF stained in high iron diamine-alcian blue showing 5AC/ACF (magnification, 400x). G, Mucin depleted foci (MDF) stained in HID-AB (magnification, 200x). H, Mucin depleted foci (MDF) stained in HID-AB (magnification, 400x). I, Section of colonic mucosa treated with immunohistochemistry technique showing proliferating cell nuclear antigen (PCNA) expression (magnification, 400x).

The development of aberrant crypt foci (ACF) at different time intervals in DMH injected rats has been shown in Figure 2 which clearly indicate the protective potential of probiotic LaLp Dahi alone or in combination with PXC against colorectal carcinogenesis. The ACF in DMH treated rats were present throughout the length of colorectal segment, with the incidence higher in mid and distal regions, and these regions accounted for approximately 90% of the total ACF. Further, the number of ACF increased with time in DMH treated rats. The DMH induced rats fed with LaLp Dahi or PXC or PXC and LaLp Dahi combined registered

significantly lower numbers of AC and ACF than in Milk-DMH group. The total number of ACF at 8 week decreased by 49.5, 61.6 and 72.0% in rats fed with piroxicam, LaLp Dahi, and LaLp Dahi and piroxicam combined, respectively, relative to Milk-DMH group. Although the total number of ACF at 16 week increased in each group, the proportion relative to Milk-DMH group remained almost same in rats fed piroxicam or LaLp Dahi or LaLp Dahi and piroxicam combined (i.e., 46.8, 53.5 and 72.0%, respectively). While the total number of ACF continued to rise beyond 16 week in Milk-DMH group, it almost plateaued in rats

fed with piroxicam or LaLp Dahi or piroxicam and LaLp Dahi combined. When expressed as percent, total number of ACF at 32 weeks decreased by 59.2, 65.8, and 82.2% in rats fed piroxicam, LaLp Dahi, and LaLp Dahi and piroxicam combined, respectively, relative to Milk-DMH group. The LaLp Dahi was more efficacious than PXC in reducing the number of ACF in colorectum. A further reduction in number of ACF in colorectum was observed when animals were treated with probiotic Dahi and PXC combined. Figure 3 show that the feeding LaLp Dahi or piroxicam treatment

significantly ( $P < 0.05$ ) reduced the multiplicity of aberrant crypts (AC) in ACF in colorectal segments. The proportion of ACF having more than three crypts at 16 weeks was 20.4, 24.7, and 23.1% in rats fed with piroxicam, LaLp Dahi, and LaLp Dahi and piroxicam combined, respectively, compared with 37.0% in Milk-DMH group. Furthermore, the proportion of ACF having more than 3 crypts at 32 weeks was 48.3% in Milk-DMH group, and it was reduced to 14.9, 21.0, and 12.0% in animals fed with piroxicam, LaLp Dahi, and LaLp Dahi and piroxicam combined, respectively.

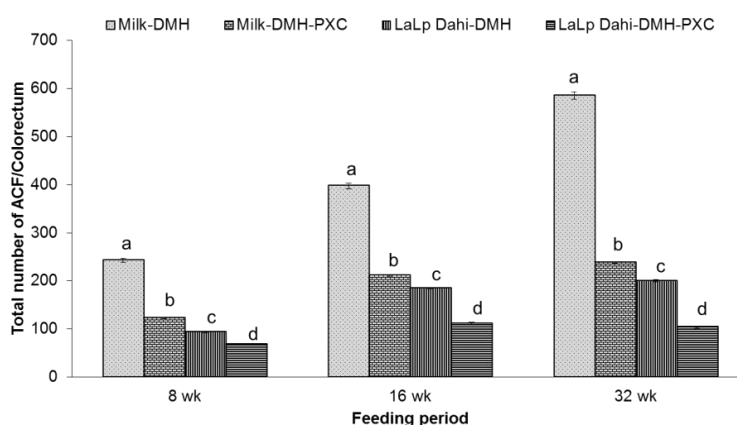


Figure 2. Effect of feeding probiotic Dahi and piroxicam (PXC) treatment on development of aberrant crypt foci (ACF) in colorectal tissue of dimethylhydrazine (DMH) treated rats. Values (mean  $\pm$  SD for n=8) with different superscript letters are significantly different ( $P < 0.05$ ).

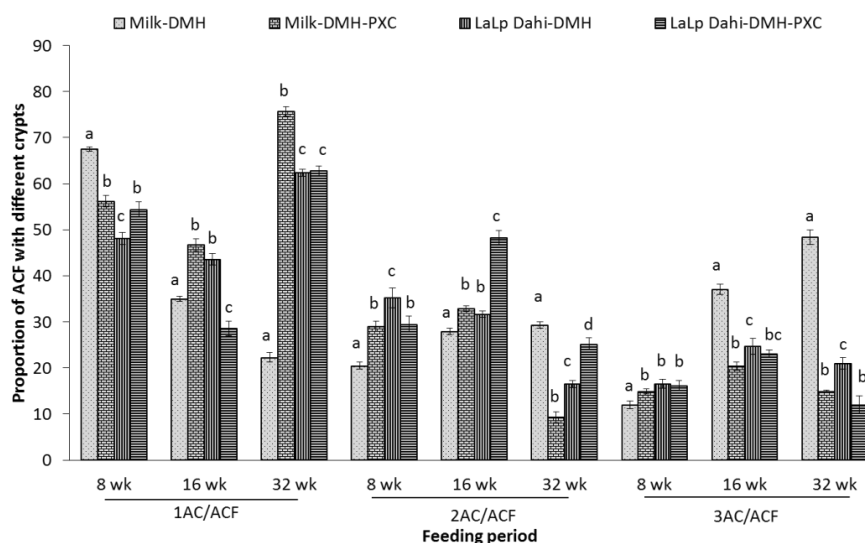
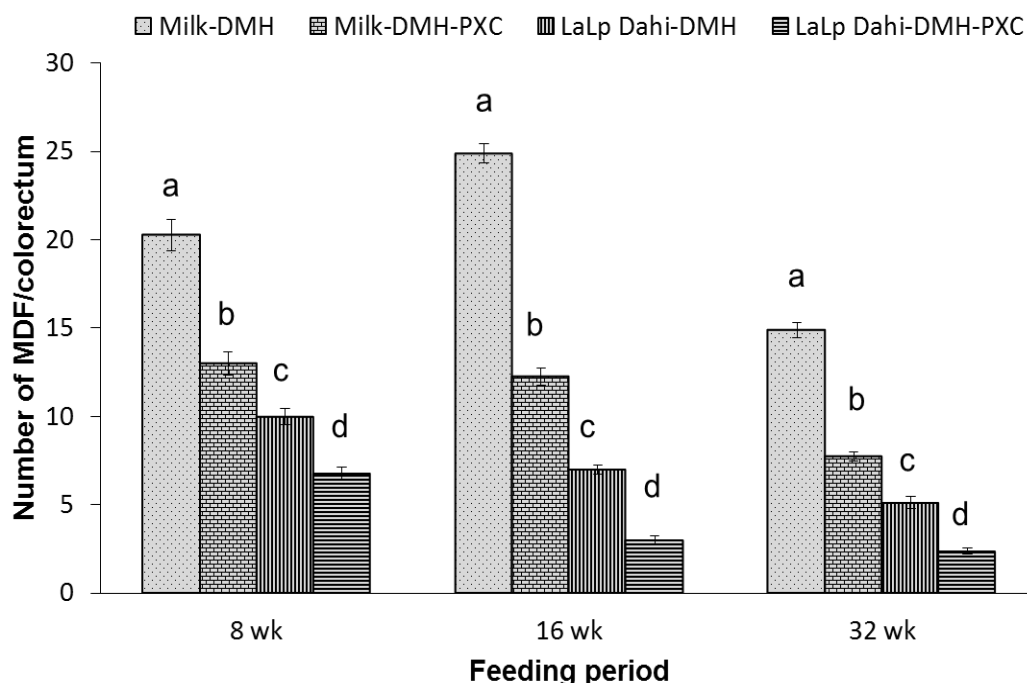


Figure 3 Effect of feeding probiotic Dahi and piroxicam (PXC) treatment on multiplicity of crypt per aberrant crypt foci (ACF) in colorectal tissue of dimethylhydrazine (DMH) treated rats. Values (mean  $\pm$  SD for n=8) with different superscript letters are significantly different ( $P < 0.05$ ).

### Effect on mucin depleted foci (MDF) formation

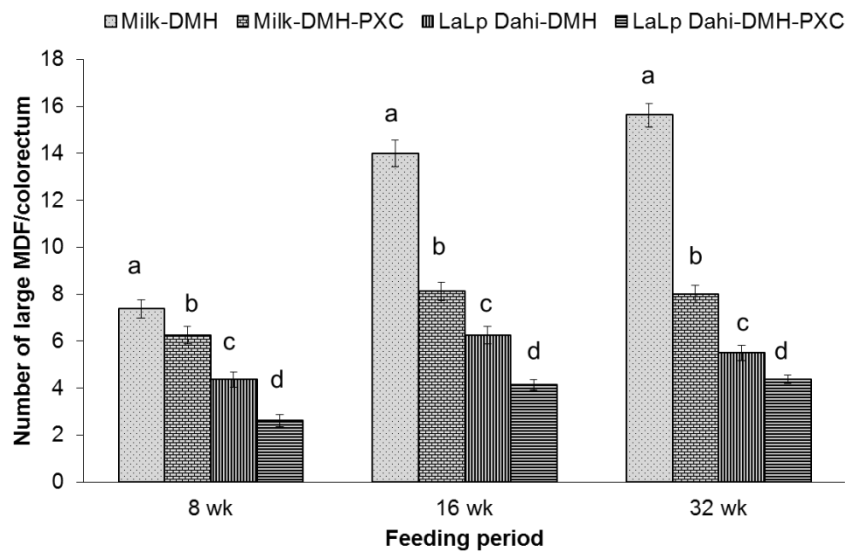
MDF are the preneoplastic lesions characterized by the absence or scant production of mucin, elevation of the lesion above the surface, nuclear stratification, crypt enlargement, nuclear crowding and severe dysplasia. Figure 1G and 1H shows the topographic view of mucin depleted foci (MDF) in HID-AB stained colonic mucosa. The administration of DMH in rats resulted in development of MDF in colorectum, which decreased in number after 16 weeks (Figure 4). The number of DMH induced MDF decreased significantly by treatment with piroxicam or LaLp Dahi. The number of large MDF (containing more than 4 AC) in Milk-DMH group increased with time throughout the experimental period (Figure 5). The treatment

with piroxicam or probiotic Dahi significantly decreased the number of total as well as large MDF in colorectal segment. Unlike, in Milk-DMH group, there was no increase in number of large MDF beyond 16 weeks in groups treated with piroxicam or probiotic Dahi or probiotic Dahi and piroxicam combined. The probiotic Dahi was more effective than piroxicam in reducing the progression in number as well as in size of MDF. Further, in animals treated with combination of piroxicam and probiotic Dahi the number as well as the size of MDF in colorectal segment were significantly lower than in groups treated with piroxicam or probiotic Dahi alone, suggesting that probiotic Dahi improves the efficacy of cancer treatment with piroxicam.

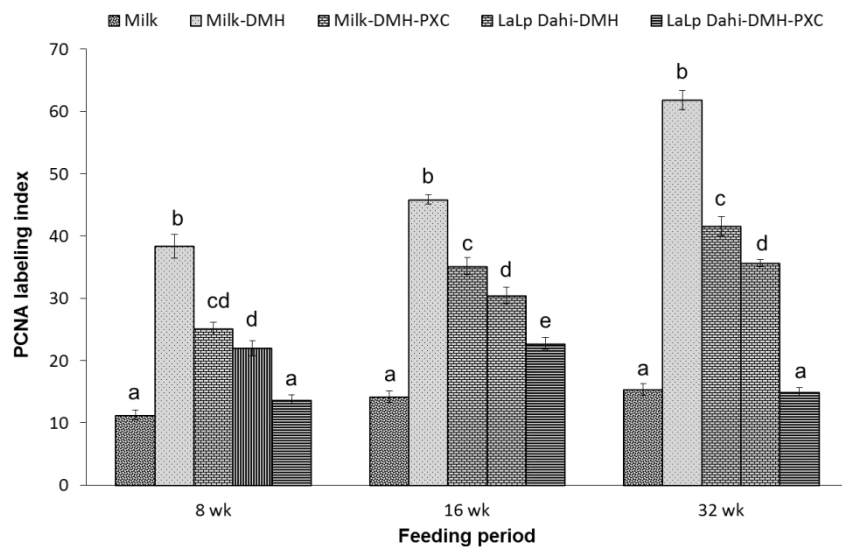


**Figure 4** Effect of feeding probiotic Dahi and piroxicam (PXC) treatment on development of mucin depleted foci (MDF) in colorectal tissue of dimethylhydrazine (DMH) treated rats. Values (mean ± SD for n=8) with different superscript letters are significantly different ( $P < 0.05$ ).





**Figure 5** Effect of feeding probiotic Dahi and piroxicam (PXC) treatment on development of large (containing  $\geq 4$  aberrant crypts) mucin depleted foci (MDF) in colorectal tissue of dimethylhydrazine (DMH) treated rats. Values (mean  $\pm$  SD for n=8) with different superscript letters are significantly different ( $P < 0.05$ ).



**Figure 6** Effect of feeding probiotic Dahi and piroxicam (PXC) treatment on proliferating cell nuclear antigen (PCNA) labeling index\* in colorectal tissue of dimethylhydrazine (DMH) treated rats. Values (mean  $\pm$  SD for n=8) with different superscript letters are significantly different ( $P < 0.05$ ). \*PCNA labeling index is the proportion of PCNA positive cells in a crypt.

At 16 weeks, the number of MDF were reduced by 50.6, 71.9 and 88.0 % in rats treated with piroxicam, LaLp Dahi, and LaLp Dahi and piroxicam combined, respectively, and the corresponding diminutions in large MDF were 42.1, 55.0 and 70.8%, respectively compared with Milk-DMH group. At 32 weeks, the total

number of MDF decreased by 47.6, 65.8 and 83.9% in rats fed piroxicam, LaLp Dahi, and LaLp Dahi and piroxicam combined, respectively, relative to Milk-DMH group (Figure 5). The corresponding decrease in large MDF was 48.7, 64.7, and 71.8%, respectively.

### Effect on proliferating cell nuclear antigen (PCNA) labeling index

Proliferating cell nuclear antigen (PCNA), a non-histone nuclear protein essential for DNA replication and repair, the content of which is increased during carcinogenesis, is used a prognostic marker in development of cancer. Figure 11 shows immunohistochemically stained longitudinal section of normal-looking crypt of buffalo milk fed rats showing expression of PCNA protein in cells of rat colonic epithelium. The content of PCNA is increased during carcinogenesis, is used a prognostic marker in development of cancer. The data represented in Figure 6 shows that content of PCNA in cells is increased with the age even in normal animals, reaching at 32 weeks 48% above 0 d level. In DMH-injected control rats the accumulation of PCNA at 32 weeks increased 6-fold of that at 0 d level. The treatment with piroxicam or probiotic Dahi decreased DMH induced accumulation of PCNA in epithelial cells of colorectal tissue. The reductions in DMH induced accumulation of PCNA in epithelial cells of colorectal tissue were more pronounced in animals treated with the combination of piroxicam and probiotic Dahi.

### Discussion

Aberrant crypt foci appear predominantly in the distal colon, are the precursors of colon cancer [6]. ACF growth is reported to occur through the mechanism of “crypt fission” [21] and therefore, the ACF with more number of crypts are characteristic of advanced cancer stage. Probiotic LaLp Dahi significantly ( $P<0.05$ ) decreased the DMH induced progression of aberrant crypt foci. Piroxicam treatment also decreased the numbers of ACF; and the effect of PXC and probiotic Dahi was additive. The numbers of ACF having multiple aberrant crypts were very few in rats fed probiotic Dahi or treated with PXC or with combination of

probiotic Dahi and PXC, while in DMH treated control these increased linearly with time.

Mucin depleted foci are also promising biomarker for studying the effects of chemopreventive agents on colon carcinogenesis [7, 22, 23]. The occurrence of MDF in high-risk patients provides evidence that these lesions have a counterpart in human pathology and, as observed in rodents, may represent the very early stages of colorectal cancer. In our study, feeding probiotic Dahi to DMH induced rats decreased significantly ( $P<0.05$ ) the induction and progression of MDF. The treatment with PXC also decreased the induction and progression of DMH induced MDF, and the probiotic Dahi improved its efficacy. The reduction in number of MDF at 32 weeks might be due to their transformation into tumors. The number of large MDF in DMH treated control group continued to rise throughout experimental period. The trend in appearance of neoplastic lesions in DMH treated control rats and the effect of piroxicam is similar to that reported earlier in the colon of F344 rats [7, 9]. The mechanism could possibly involve the interaction of mucin such as sialomucins or sulfomucins in surface and upper crypt goblet cells with the metabolites of lactic acid culture in colorectal mucosa [32].

It is well known that PCNA is used as an indicator of DNA synthesis and cellular proliferation and has been used as an intermediate biomarker in chemoprevention of colorectal cancer [24]. Using immunohistochemistry, we identified that there was a large amount of positive expression of PCNA in the colorectum of DMH-injected control rats was increased six-fold of that at 0 d level. The PCNA labeling index increased significantly ( $P<0.05$ ) in epithelial cells of rats injected with DMH, and the treatment with piroxicam or probiotic Dahi decreased this rise in PCNA index. The reductions in DMH induced accumulation of PCNA in epithelial cells of colorectal mucosa were more pronounced in animals treated with the

combination of piroxicam and probiotic Dahi. This indicates that the colorectal tissue were in an more active state of proliferation in DMH-injected control rats, which is a similar result to that reported in previous studies [25]. LaLp Dahi was equally or even more effective than piroxicam in reducing PCNA labeling index in colorectum of DMH treated rats. In addition, it is well established that increased PCNA- labeling index in colonic ACF, the known pre-neoplastic lesions, leads to higher risk for malignant progression [33]. It was concluded that suppression of cellular proliferation may represent one of the mechanisms through which probiotic Dahi alone or as an adjunct with PXC exert their chemopreventive effects against DMH-induced colorectal cancer in the initiation phase and that probiotic Dahi had the potential to inhibit/slow colon tumorigenesis and to obstruct the growth and progression of hyperplastic foci into frank invasive tumors in the post-initiation phase in this rat model. These effects of probiotic Dahi are mediated, at least in part, through its ability to suppress cellular proliferation. Whether Probiotic Dahi induces apoptosis in our animal model of DMH-induced colorectal carcinogenesis and the possible mechanisms underlying this apoptotic efficacy are being investigated in our laboratory and the primary results are encouraging.

The dietary treatment with probiotic Dahi was started four weeks before carcinogen administration showed protective effect was seen within two weeks. These results suggest that probiotics are active during initiation and early promotional phase of the carcinogenic process. The 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 mucosal structure [22, 23, 25]. The intestinal bacteria are capable of activating or deactivating proximal carcinogens, behaving as

promoters and some as anti-promoters in colon carcinogenesis [25]. Several recent studies from our laboratory have shown that dietary LaBb-Dahi down regulates carcinogen activating cytochrome P450 enzymes CYP1A1, CYP1A2 and CYP1B1 in liver, and up regulates carcinogen detoxifying  $\gamma$ -glutamyltranspeptidase, UDP-glucuronosyltransferase and quinone reductase activities in liver as well as in colon [26]. The potential of this product to improve macrophage and lymphocyte functions [14] and antioxidative status [15] has been also established. The effect of probiotic Dahi on biotransformation and/or disposition of DMH metabolites could influence the injury to the colonic mucosa and lower cell proliferation (PCNA labeling index) leading to reduced number of preneoplastic lesions. Recently, Mohania *et al.* [22] showed that this probiotic Dahi could also act as an antioxidant and can lower levels of TBARS, faecal  $\beta$ -glucuronidase and enhance the activity of glutathione-S-transferase (GST) in liver and colorectal tissues. However, future research may be needed to explore the molecular mechanism underlying the anticarcinogenic potential of probiotic Dahi alone or in combination of PXC to alleviate or reduce the colorectal carcinogenic effects of DMH.

## Conclusion

In conclusion, the present study demonstrated that probiotic LaLp Dahi, administered alone or in combination with PXC has a chemopreventive effect against DMH-induced colorectal carcinogenesis in rat. This study also provided an evidence that probiotic Dahi appreciably suppressed the progression of preneoplastic lesions and cell proliferation in the post-initiation phase, which might make probiotic Dahi a promising agent to combat the progression of benign polyps and other pre-neoplastic lesions into malignant metastatic tumors and to manage colorectal cancer. These

findings suggest that probiotics Dahi could have a therapeutic potential to decrease the risk of colorectal cancer and be used as a potential nutraceutical intervention in prophylaxis and treatment of colorectal cancer.

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## Abbreviations:

AC, Aberrant crypt;  
ACF, Aberrant crypt foci;  
MDF, Mucin-depleted foci;  
PCNA, Proliferating cell nuclear antigen;  
DMH, 1, 2-dimethylhydrazine dihydrochloride;  
PXC, Piroxicam;  
NDRI, National Dairy Research Institute

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