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Angela Verma [a] & Geeta Shukla [a]
[a] Department of Microbiology, Panjab University, Chandigarh, India
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Probiotics *Lactobacillus rhamnosus GG, Lactobacillus acidophilus* Suppresses DMH-Induced Procarcinogenic Fecal Enzymes and Preneoplastic Aberrant Crypt Foci in Early Colon Carcinogenesis in Sprague Dawley Rats

Angela Verma and Geeta Shukla
Department of Microbiology, Panjab University, Chandigarh, India

Diet makes an important contribution to colorectal cancer (CRC) risk implying risks for CRC are potentially reducible. Therefore, the probiotics have been suggested as the prophylactic measure in colon cancer. In this study, different probiotics were used to compare their protective potential against 1,2 dimethylhydrazine dihydrochloride (DMH)-induced chemical colon carcinogenesis in Sprague Dawley rats. Animals belonging to different probiotic groups were fed orally with 1×10^9 lactobacilli daily for 1 week, and then a weekly injection of DMH was given intraperitoneally for 6 wks with daily administration of probiotic. *Lactobacillus GG* and *L.acidophilus* + DMH-treated animals had maximum percent reduction in ACF counts. A significant decrease ($P < 0.05$) in fecal nitroreductase activity was observed in *L.casei* + DMH and *L.plantarum* + DMH-treated rats whereas β-glucuronidase activity decreased in *L.GG* + DMH and *L.acidophilus* + DMH-treated rats. Animals treated with *Bifidobacterium bifidum* + DMH had significant decreased β-glucosidase activity. However, not much difference was observed in the colon morphology of animals belonging to various probiotic + DMH-treated rats compared with DMH-treated alone. The results indicated that probiotics, *L.GG*, and *L.acidophilus* can be used as the better prophylactic agents for experimental colon carcinogenesis.

INTRODUCTION

Colon cancer is the third most common cancer worldwide and is one of the major causes of mortality (1,2). Colon carcinogenesis is a multistep process in which preneoplastic lesions accumulate in mucosal cells due to genetic changes involving more than 20 genes (3). The highest incidence rates of colon cancer are in Australia, New Zealand, Europe, and North America, and the lowest in Africa and Southcentral Asia due to differences in genetic makeup and dietary and environmental factors (1).

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Address correspondence to Geeta Shukla, Department of Microbiology, Panjab University, Chandigarh 160014. Tel: +91 (172) 2541770. Fax: +91 (172) 2541770. E-mail: geeta_shukla@pu.ac.in

Experimental studies on colon cancer provide evidence that the colonic microflora and microbial enzymes β-glucuronidase, nitroreductase, and azoreductase play an important role in chemical carcinogenesis (4,5). The enzymes, mainly β-glucuronidase and β-glucosidase, convert carcinogen, 1,2 dimethylhydrazine dihydrochloride (DMH) into metabolites that hydrolyze glucuronide conjugates into carcinogenic aglycones that might be an important activation pathway to induce colon cancer (6). Nitroreductase too causes the formation of reactive nitroso-intermediates, N-hydroxy-intermediates, and aromatic amines well-known carcinogens (7-,8). The excessive activities of these enzymes may be a primary factor in the etiology of colon cancer.

The initiation phase in colorectal cancer (CRC) can be recognized by the development of reliable biomarkers; the aberrant crypt foci (ACF) lesions, which persist, grow in the distal colon, and can develop into cancerous tissue (9–13). Therefore, in the present study, the number and growth features of ACF have been used as to identify the initiators and modulators of colon carcinogenesis (12).

Because prognosis for advanced CRC is poor, the prophylactic measure is required to control the incidence and occurrence of the disease (14). Moreover, the endogenous microflora and diet are important in the development of colon cancer thus, the probiotics have been driven for improving the gut microbial function and reducing the occurrence of colon cancer (15–17). Although the effect of probiotics on colon cancer have been shown, a comparative study to find the efficacy of different probiotics as the prophylactic agent under similar experimental conditions on chemical colon carcinogenesis in early stage of cancer needs to be further investigated. Therefore, it is pertinent to assess the effect of different probiotics on initial and early promotional phase of colon carcinogenesis in Sprague Dawley rats.

MATERIALS AND METHODS

Chemicals

DMH and phenolphthalein-β-D-glucuronide were obtained from Sigma Chemical Company (St. Louis, MO).

M-nitrobenzoic acid and nitrophenyl-β-D-glucoside were obtained from HiMedia (Mumbai, India).

Animals

Sprague Dawley (SD) rats (100–150 g) were procured from the Central Animal House, Panjab University, Chandigarh, India. These were housed in polypropylene cages in the animal house and were acclimatized for 7–10 days before being used. Water and standard pellet diet (Hindustan Lever Products, Kolkata, India) were given ad libitum.

Induction of Colon Carcinogenesis

DMH was prepared in 1 mM EDTA and the pH was adjusted to 7.0 with 1 mM NaOH. In a week, single dose of DMH (20 mg/kg body weight) was given intraperitoneally (i.p.) to animals and the treatment was continued for 6 wk.

Probiotic Strains

Lactobacillus rhamnosus GG MTCC #1408*, Lactobacillus casei* MTCC #1423*, Lactobacillus plantarum* MTCC #1407 were procured form Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. *Lactobacillus acidophilus* NCDC #15 and *Bifidobacterim bifidum* NCDC #234 were procured from National Dairy Research Institute, Karnal, India.

Preparation of Probiotic Dose

Probiotic strains were grown in De Mann Rogosa Sharpe broth and maintained on De Mann Rogosa Sharpe agar slants by regular subculturing at an interval of 15 days by incubating at 37°C for 24 h. For experimental inoculation, 18-h old culture was cold centrifuged at 3500 g for 10 min, washed, and suspended in phosphate buffered saline (PBS, pH 7.2) to contain 1×10^9 lactobacilli/0.1 ml.

Experimental Design

Animals were divided into 12 groups. Each group comprised of 6 animals and was treated as follows:

- Group I (control): Animals received single dose of 1 mM EDTA saline (pH 7.0) weekly for 6 wk.
- Group II (DMH): Animals received a single dose of DMH intraperitoneally weekly for 6 wk.
- Group III (*L.rhamnosus GG*), Group IV (*L.rhamnosus, L.GG* + DMH), Group V (*L.casei*), Group VI (*L.casei* + DMH), Group VII (*L.acidophilus*), Group VIII (*L.acidophilus* + DMH), Group IX (*L.plantarum*), Group X (*L.plantarum* + DMH) and Group XI (*B.bifidum*), Group XII (*B. bifidum* + DMH): Animals belonging to Groups IV, VI, VIII, X, and XII were fed orally with 1×10^9 lactobacilli/0.1 ml daily for a week. Thereafter, a single dose of DMH was given i.p. and continued for 6 wk. During this period, the probiotic was fed daily to animals. Animals belonging to Groups III, V, VII, IX, and XI were fed orally with 1×10^9 lactobacilli/0.1 ml daily for 6 wk (18).

Followup of Animals

The protocol involved feeding the probiotic for 1 wk, followed by dosing with the carcinogen weekly and continued administration of probiotic for 6 wk covering the initiation and early promotion stages of carcinogenesis. During this period, body mass was monitored weekly. After respective treatment, in different groups feces were collected a day before sacrificing the animals for enzymes analysis. Animals were sacrificed after 6 wk of DMH treatment under an overdose of ether anesthesia and cervical dislocation for histopathological studies and aberrant crypt foci count.

Estimation of Body Mass

The body mass of rats belonging to all groups was recorded weekly on ordinary balance (SD-300, S.D. Fine Chemicals Ltd, Chandigarh, India).

Enumeration of ACF

Aberrant crypts are defined as easily recognizable mucosal alterations that are characteristically larger and more elongated than normal crypts, with a thicker lining of epithelial cells and a larger pericryptal zone. The entire colon was removed, and distal colon was cut into small sections (2×5 cm). The section was stained with 0.2% methylene blue and ACF were counted using light microscope. The total number of ACF/rat was calculated as the sum of the small, medium, and large ACF (19).

Enzyme Assays

A day before sacrifice, fresh fecal samples were collected and processed immediately. Briefly, for β-glucuronidase and β-glucosidase assay fecal samples were suspended in cold prereduced 0.1 M potassium phosphate buffer (pH 7.0) and for nitroreductase assay in 0.2 M Tris-HCl buffer (pH 7.8). The fecal suspension was homogenized and disrupted with sonication for 3 min at 4°C. The samples were cold centrifuged at 500 g for 15 min and from the supernatant the enzymes were assayed immediately. The fecal protein concentrations in the supernatant were determined by Lowry's method (20).

Nitroreductase Assay

The specific activity of fecal nitroreductase was determined with the modified method of Goldin and Gorbach (21). The enzyme reaction was run at 30°C for 1 h at pH 7.8. The total volume of the reaction mixture was 200 μl, containing a final concentration of 0.08 M Tris-HCl buffer, 0.35 mM m-nitrobenzoic acid, 0.5 mM NADPH and 80 μl of the sample. The reaction was stopped by addition of 300 μl of 1.2 N HCl. The amount of m-aminobenzoic acid produced was then measured using diazotization reaction and readings were taken at 540 nm. The amount of m-aminobenzoic acid produced was calculated comparing with standard m-aminobenzoic acid. The nitroreductase activity was expressed as microgram of m-aminobenzoic acid formed per hour per milligram of fecal protein.

β-Glucosidase Assay

The specific activity of fecal β-glucosidase assay was determined as the modified method of Goldin and Gorbach, 1976 (21). The enzyme reaction was run at 37°C for 1 h at pH 7.4. The total volume of the reaction mixture was 1 ml containing a final concentration of 0.1 M potassium phosphate buffer, 1 mM nitrophenyl-β-D-glucoside, and 0.2 ml of sample. The reaction was stopped by addition of 5 ml of 0.01 M sodium hydroxide. Readings were taken at 420 nm. The amount of nitrophenol released was determined by comparison with a standard nitrophenol curve. The β-glucosidase activity was expressed as microgram of nitrophenol formed per minute per milligram of fecal protein.

β-Glucuronidase Assay

The specific activity of fecal β-glucuronidase assay was determined as the modified method of Goldin and Gorbach (21). The enzyme reaction was run at 37°C for 15 min at pH 6.8. The total volume of the reaction mixture was 1 ml containing a final concentration of 0.02 M potassium phosphate buffer, 0.1 M EDTA, 1 mM phenolphthalein-β-glucuronide, and 0.1 ml of sample. The reaction was stopped by addition of 5 ml 0.2 M glycine buffer (pH 10.4) containing 0.2 M NaCl. Readings were taken at 540 nm. The amount of phenolphthalein released was determined by comparison with a standard phenolphthalein curve. The β-glucuronidase activity was expressed as microgram of phenolphthalein formed per minute per milligram of fecal protein.

Histopathological Study

The formalin fixed colonic tissue was dehydrated in different grades of alcohol. The tissue was dipped in molten paraffin wax and was cooled quickly to prevent crystallization. Thin sections of tissue were cut, and embedded tissue sections were kept in a water bath at 50°C to remove the wax. Sections were mounted on separate clean glass microscope slides and were stained with hematoxylin and eosin (H & E) stain and were examined by light microscopy.

Statistical Analysis

Results were expressed as mean ± standard deviation. The intergroup variation was assessed by 1-way analysis of variance. Statistical significance of the results was calculated at $P < 0.05$.

RESULTS

Body Mass

The change in body mass of animals treated with probiotics alone and probiotics + DMH were significantly more ($P < 0.05$) compared with DMH-treated animals. Among the various probiotic and DMH-treated animals, *L.GG* + DMH treated rats had maximum change in their body mass (i.e., their body mass increased in spite of DMH treatment) (Fig. 1).

ACF Count

Animals belonging to probiotic + DMH treated groups had significant decrease ($P < 0.05$) in ACF/colon compared with DMH-treated rats. The percentage reduction of ACF in animals with *L.GG* + DMH, *L.casei* + DMH, *L.acidophilus* + DMH, *L.plantarum* + DMH and *B.bifidum* + DMH was 98%, 45%, 96%, 89%, and 74%, respectively, compared with DMH treated rats. Among the different probiotics + DMH treated groups, animals belonging to *L.GG* + DMH and *L.acidophilus* + DMH

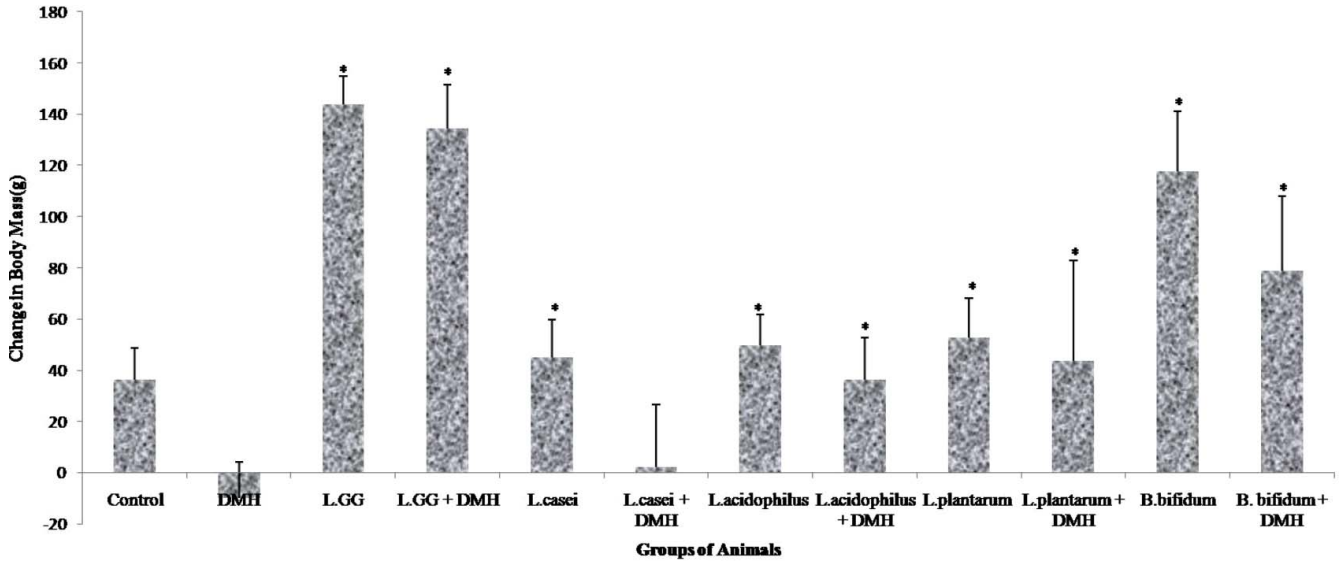

FIG. 1. Change in body mass. Values are mean ± standard deviation. *$P < 0.05$ v/s 1,2 dimethylhydrazine dihydrochloride (DMH). LGG = *Lactobacillus GG* (Color figure available online).

TABLE 1
Aberrant crypt foci (ACF) counts in various groups of animals

Groups of animals	Control	DMH	*L.GG* + DMH	*L.casei* + DMH	*L.acidophilus* + DMH	*L.plantarum* + DMH	*B.bifidum* + DMH
ACF count	0	37.75 ± 2.21	0.5 ± 1*	20.75 ± 3.09*	1.5 ± 1.2*	4.25 ± 1.2*	9.5 ± 2.08*

Values are mean ± SD. LGG = *Lactobacillus GG*.
*$P < 0.05$ v/s 1,2 dimethylhydrazine dihydrochloride (DMH).

treated groups had significantly less ($P < 0.05$) ACF counts (Table 1, Fig. 2).

Enzyme Assay

Both *L.casei* + DMH and *L.plantarum* + DMH treated animals had significantly decreased ($P < 0.05$) nitroreductase activity compared with DMH-treated and other probiotic + DMH-treated groups (Fig. 3). Animals treated with probiotics alone showed significantly less ($P < 0.05$) nitroreductase levels as compared with DMH-treated group. Interestingly, it was also observed that among various probiotics given alone, animals administered with *L.GG* and *B.bifidum* had significantly decreased ($P < 0.05$) β-glucosidase activity compared with DMH-treated animals. Similar results were observed when probiotic *B.bifidum* was given in combination with DMH (Fig. 4). The glucuronidase activity was found to be significantly less ($P < 0.05$) in animals receiving only probiotics compared with DMH. Similarly, animals belonging to *L.GG* + DMH and *L.acidophilus* + DMH groups had significantly decreased ($P < 0.05$) β-glucuronidase activity compared with other probiotic + DMH-treated groups (Fig. 5).

Histopathological Studies

The colon segments of control animal showed closely packed normal mucus glands with fewer lymphocytic clusters compared with glandular dilation, increased lymphocytic clusters, and edema in submucosa of DMH-treated rats (Fig. 6a and 6b). The animals belonging to various probiotic control groups showed no difference in the colon histology compared with control (Fig. 6c). Animals belonging to *L.casei* + DMH and *L.plantarum* + DMH groups had expanded mucosal folds with accumulation of inflammatory lymphocytes indicating colitis (Fig. 6e and 6g), whereas *L.acidophilus* + DMH-treated animals had moderate infiltration of lymphocytes with edema in submucosa and mucosa (Fig. 6f), whereas *L.GG* + DMH and *B. bifidum* + DMH-treated animals had closely packed glands with few lymphocytes only indicating normal morphology of colon (Fig. 6d and 6h).

DISCUSSION

Evidence from epidemiological and experimental studies implies that diet and intestinal microflora are important in the etiology of CRC (22). It has also been found that probiotics can change the colonic microbiota that might prevent diseases (23,24). Therefore, the present study was designed with the aim to find the effective prophylactic probiotic for colon carcinogenesis.

In the present study, the body mass of rats increased with supplementation of probiotics even with DMH treatment and

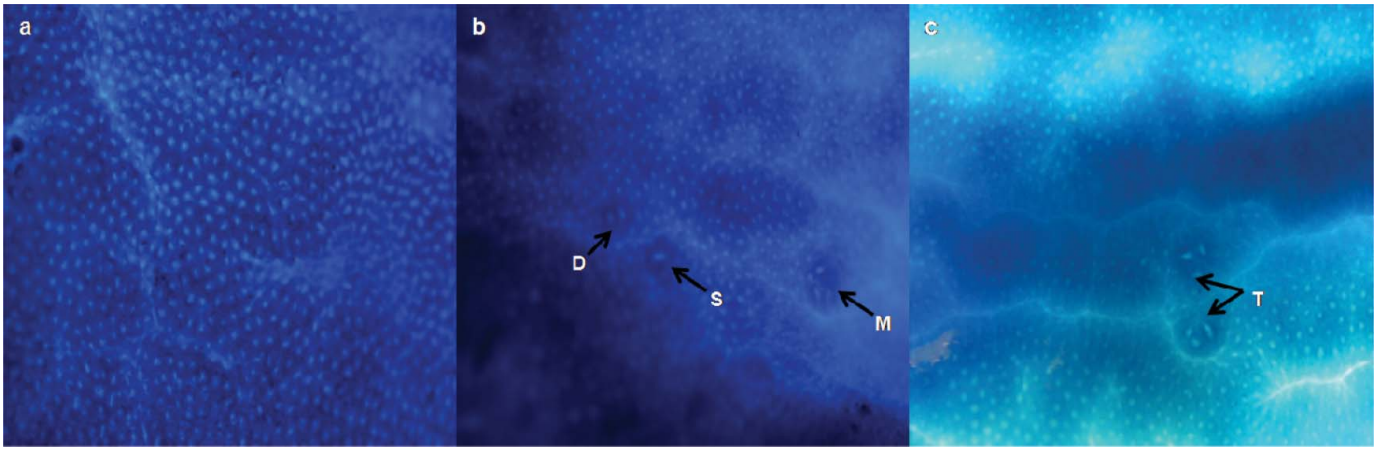

FIG. 2. Topographic view showing aberrant crypt foci stained with methylene blue. a:Normal (b and c) 1,2 dimethylhydrazine dihydrochloride (DMH) treated. Arrows indicate small aberrant crypt foci (ACF): singlet (S), doublet (D), triplet (T), and medium ACF (M; 40×) (Color figure available online).

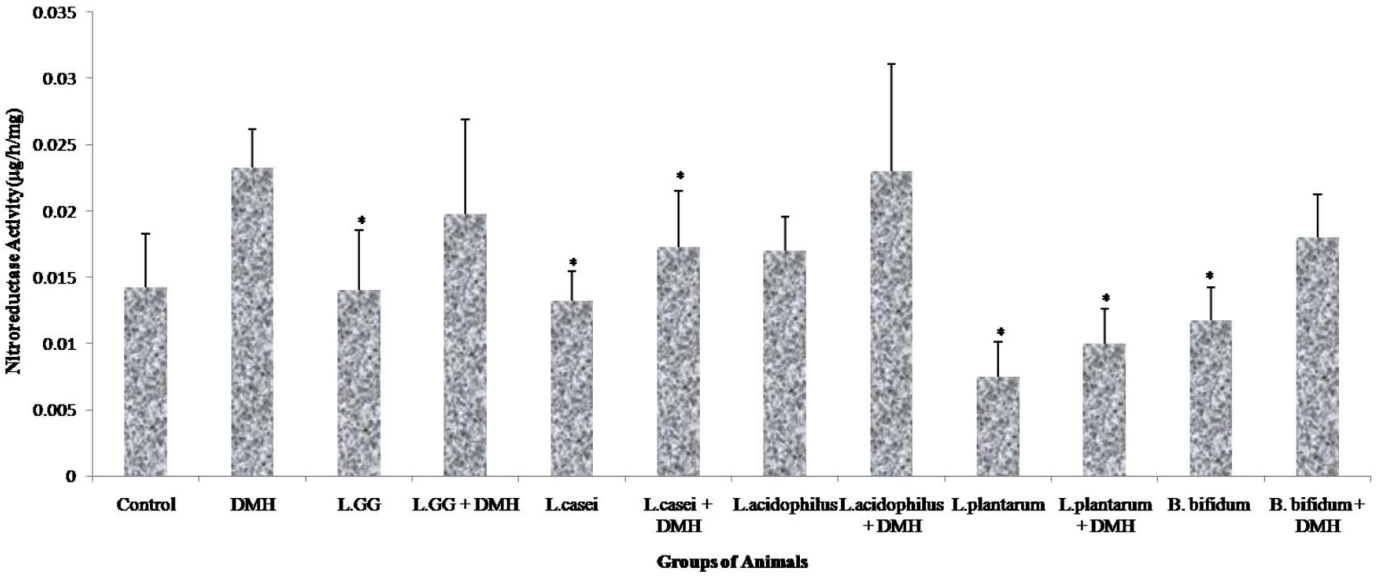

FIG. 3. Nitroreductase activity (μg/h/mg). Values are mean $\pm$ standard deviation. $^*P < 0.05$ v/s 1,2 dimethylhydrazine dihydrochloride (DMH). LGG = *Lactobacillus GG* (Color figure available online).

corroborates with the earlier study. Lee and Lee have also documented the increased body mass with supplementation of lactic acid bacteria in azoxymethane (AOM) treated rats (25).

The total number of ACF is considered to be the valid reliable biomarkers at early stage of carcinogenesis. The present observation of significantly reduced percentage of colonic preneoplastic ACF lesions in rats administered with probiotic for 1 wk before the DMH treatment is comparable with the observation of Goldin et al. (4). They have found that 3 wk prior administration of *L.GG* in DMH-treated rats resulted in significant decrease in the incidence of colon tumors indicating probiotics interference with the initiation or early promotional stages of DMH-induced intestinal tumorigenesis. Though, we have also found that supplementation of lactic cultures significantly inhibited colonic preneoplastic lesions (ACF) yet different probiotics had different percentage of ACF inhibition. More specifically, *L.GG*-, *L.acidophilus*-, and *L. plantarum*-treated animals had maximum percent inhibition of ACF, with no significant difference amongst them. Previous studies have also reported that probiotics *B.longum* and *L.acidophilus* reduced the total ACF in AOM-treated rats and is in accordance with our study (26,27). The possible mechanism of reduced ACF in probiotics and DMH-treated animals can be due to the prior administration of lactic cultures which may result in their

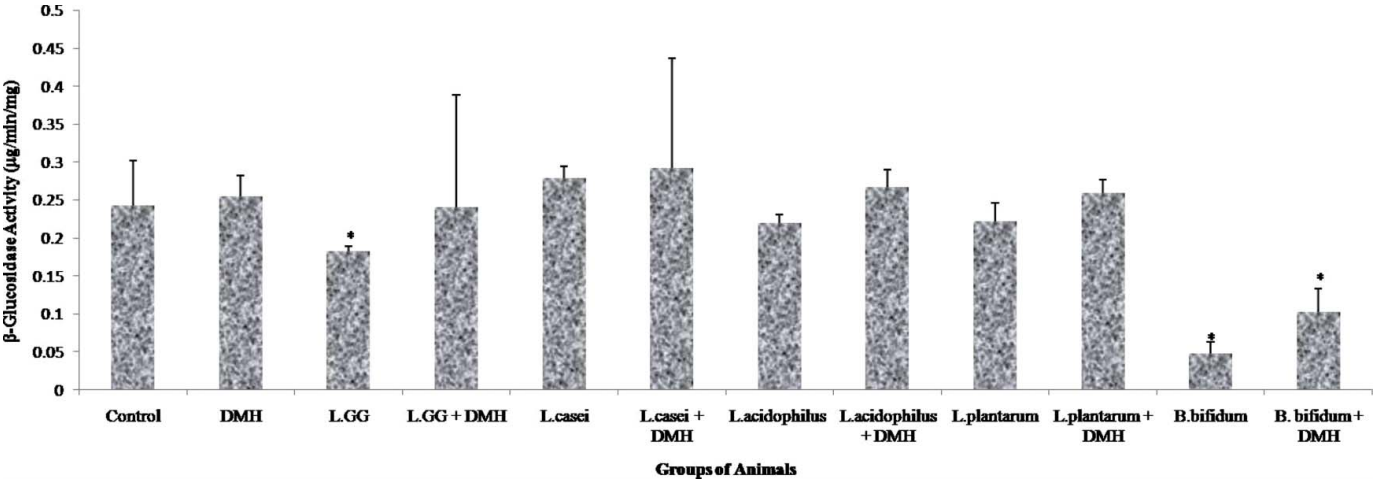

FIG. 4. β-glucosidase activity (μg/min/mg). Values are mean $\pm$ standard deviation. $^*P < 0.05$ v/s 1,2 dimethylhydrazine dihydrochloride (DMH). LGG = *Lactobacillus GG* (Color figure available online).

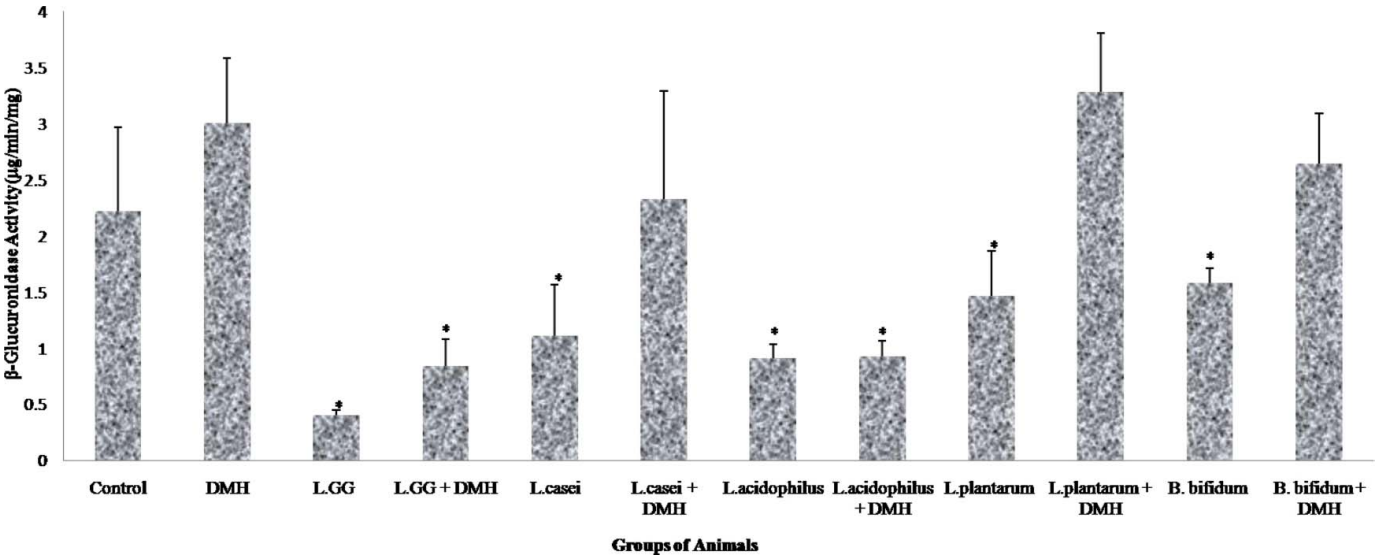

FIG. 5. β-glucuronidase activity (μg/min/mg). Values are mean $\pm$ standard deviation. $^*P < 0.05$ v/s 1,2 dimethylhydrazine dihydrochloride (DMH). LGG = *Lactobacillus GG* (Color figure available online).

direct interaction with DMH metabolites thus preventing DNA damage in the colon.

In the present study, we have observed that 1-wk supplementation of probiotic to DMH treatment led to variable decrease in nitroreductase and β-glucuronidase activity in different probiotic and DMH-treated animals. Probiotic treatment of *L.casei* and *L.plantarum* in DMH-treated animals led to decrease nitroreductase activity whereas *L.GG* and *L.acidophilus* decreased

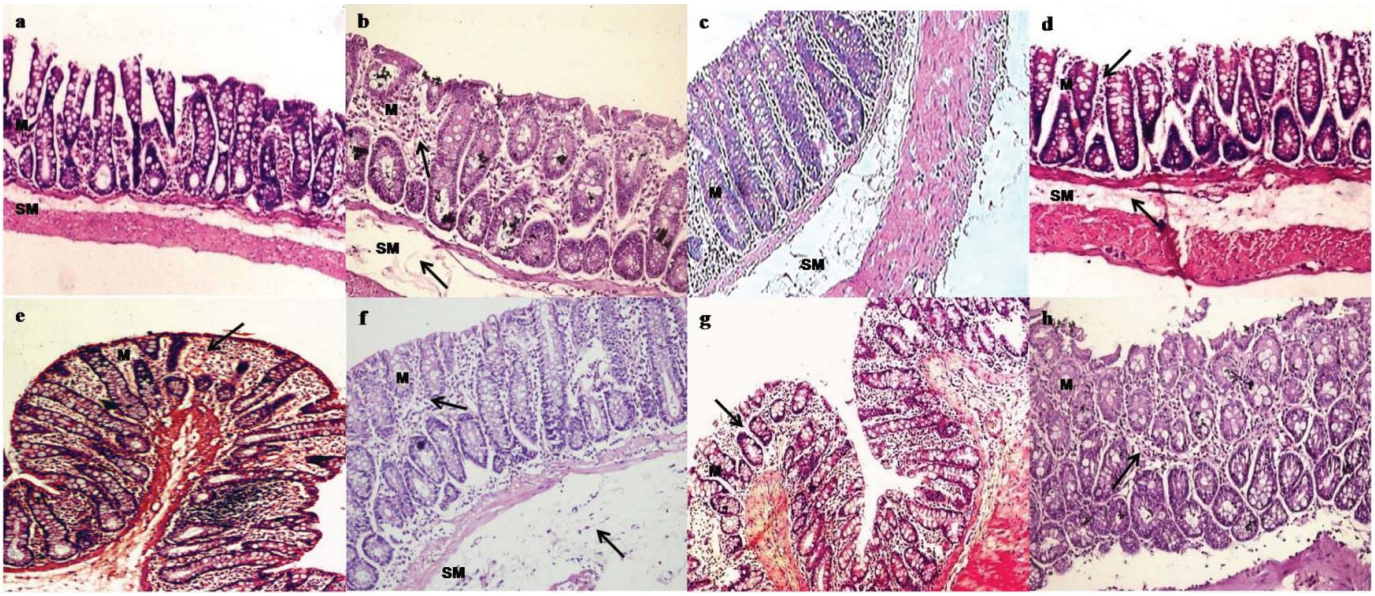

FIG. 6. Photomicrograph of colon showing (a) normal closely packed mucus glands with fewer lymphocytic clusters in control animals (Group I); (b) glandular dilation, clusters of lymphocytes in spaces between the glands and also edema in submucosa in DMH-treated animals (Group II); (c) normal closely packed mucus glands in probiotic alone treated animals (Group III, V, VII, IX, XI); (d) closely packed glands with few lymphocytes in *L.GG* + DMH treated animals (Group IV); (e) inflammatory cells (lymphocytes) in the expanded mucosal folds indicating colitis in *L.casei* + DMH animals (Group VI); (f) moderate amount of lymphocytes with edema in submucosa and mucosa in *L. acidophilus* + DMH-treated animals (Group VIII); (g) inflammatory cells (lymphocytes) in the expanded mucosal folds in *L.plantarum* + DMH-treated animals (Group X); (h) closely packed glands in *B.bifidum* + DMH-treated animals (Group XII). Arrows indicates lymphocytic infiltration in mucosa (M) and oedema in submucosa (SM) (H & E 100×) (Color figure available online).

β-glucuronidase activity. The present observation of decreased fecal enzymes is in accordance with the earlier studies where the reduction in specific activities of fecal microbial enzymes with the lactic acid bacteria treatment in human volunteers has been documented (28,29). Moreover, it has also been observed that diet influences both the microflora and the microbial enzymes activities in the intestinal tract that can produce substances with genotoxic, carcinogenic, and tumor producing activity from dietary components (30,31). Recently, Stein et al.'s in vitro study have also shown wheat aleurone, a prebiotic, to possibly have a cancer preventive potential that could be partially favored by addition of probiotics, *L.GG/B. animalis subsp. lactis* 12 supporting the present observation of reduced fecal enzymes in SD rats (32). Further, they have also documented that probiotic*s L.GG* and Bb12 in HT 29 cells decrease the tumor promoter deoxycholic acid (DCA, secondary bile acid), which may be due to the reduced activities of bacterial enzymes important for DCA formation (32). More specifically, the normal microflora *E. coli* and *Clostridium* have the highest level of β-glucuronidase activity, whereas *Bacteriodes fragilis* has high nitroreductase activity (33,34). Therefore, the change in the intestinal flora with lactobacilli and *Bifidobacterium* supplementation may have led to the decreased fecal enzymes activity as these cultures have low levels of these enzymes (35). Animals supplemented with *L.GG* and *L.acidophilus* along with DMH treatment had reduced β-glucuronidase activity, suggesting their role in preventing chemical carcinogenesis as β-glucuronidase is responsible for converting DMH to its ultimate carcinogen, methylazoxymethanol.

Animals supplemented with lactic cultures in DMH-treated animals showed decreased levels of nitroreductase and β-glucuronidase activity but they had high levels of β-glucosidase activity, whereas *B.bifidum* + DMH-treated animals had decreased levels of β-glucosidase activity. This may be due to the higher β-glucosidase activity in lactobacilli compared with the bifidobacteria that has less β-glucosidase activity (36). Marteau et al. have also shown that ingestion of a fermented dairy product containing *L. acidophilus, B. bifidum, Streptococcus lactis,* and *S. cremoris* increased β-glucosidase activity (37). The decreased enzyme activity (nitroreductase and β-glucuronidase) in probiotic and DMH-treated animals is also supported by the normal architecture of colon as observed by histopathological study where *L.GG, L.acidophilus,* and *B.bifidum* showed less lymphocytic infiltration and normal mucus glands compared with DMH-treated rats.

Taken together, it can be stated that probiotic *L. rhamnosus GG* and *L.acidophilus* are better prophylactic agent in DMH-induced early colon chemical carcinogenesis mainly because of their better interaction with the DMH metabolites resulting in reduced ACF and ability to reduce fecal enzymes resulting in decreased conversion of procarcinogens into carcinogens. However, a detailed study pertaining to *L.GG* and *L.acidophilus* as the effective prophylactic agent in combination with prebiotic inulin in chemically induced colon carcinogenesis in SD rats is underway. Thus, it can be concluded in anticipation that probiotic therapy holds promise as a useful prophylactic measure and can contribute to an overall better state of health for the consumers.

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