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MODULATION OF APOPTOSIS AND IMMUNE RESPONSE BY SYNBIOTIC IN EXPERIMENTAL COLORECTAL CANCER

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ABSTRACT

The study was designed with an aim to delineate the apoptotic and immunomodulating potentials of synbiotic (*L.rhamnosus* + *L.acidophilus* + inulin) in experimental colon carcinogenesis. Ten groups of Sprague Dawley rats received either probiotic, prebiotic or synbiotic for 18 weeks along with the inducement of tumors by DMH. It was found that synbiotic administration to DMH -treated animals promoted apoptosis in colonic tumors by down regulating Bcl-2, K-ras expression and enhancing the expression of wild type P53 compared with the tumors of DMH-only-treated animals. Immunologically, the colonic tissue of synbiotic +DMH -treated animals had significantly increased levels of TNF-α, IFN-γ and IL-10 ($p<0.05$) while IL-6 level decreased compared with DMH-only-treated animals. The synbiotic administration promoted apoptosis in the colonic tissues by modulating the gene expression and cytokine levels, suggesting the prophylactic potentials of synbiotic against colon cancer.

KEYWORDS: Synbiotic, probiotic, prebiotic, apoptosis, colon

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common fatal malignancies worldwide and is described as the paradigm between inflammation and cancer etiology. CRC is the third most common cancer in men (663,000 cases, 10.0% of the total) and the second in women (570,000 cases, 9.4% of the total) worldwide [1,2]. Pathophysiology of CRC clearly indicates that apoptosis plays an important role in maintaining the normal architecture of organs/tissue/cells and gets altered in various stress conditions [3,4]. It has also been observed that the transformation of colorectal epithelium to carcinoma is associated with a progressive inhibition of apoptosis that usually proceeds through a series of pathological progressions to produce colorectal polyps, adenomas and adenocarcinomas [5,6]. Studies have documented the role of probiotics in the maintaining cell proliferation and apoptosis, which are the key mechanisms for the prevention of CRC[7]. It has been observed that *Lactobacillus reuteri* suppresses TNF induced NF-κB activation in a dose and time-dependent manner thus inducing apoptosis in human myeloid leukemia-derived cells [8]. Scientists have also reported regulatory and anti-inflammatory potentials of the probiotic mixture, VSL#3 which decreased COX-2 expression in Colo320 and SW480 intestinal epithelial cells. In dextran sulfate sodium induced colitis in rats, the VSL#3 decreased the colonic expression of COX-2, NF-kB, TNF-α, IL-6 and inducible nitric oxide synthase, while increased IL-10 expression [9,10]. Further, the anti-inflammatory efficacy of probiotic *L.plantarum* Lp91 has been observed in the colon tissues of mice with TNBS-induced colitis where it significantly reduced COX-2 and TNF-α, while upregulating IL-10 levels and MUC2 gene [11]. Probiotics have also shown to exhibit their immunomodulatory potential via binding to pattern recognition receptors (PRRs) of dendritic cells, macrophages, monocytes and intestinal epithelial cells [12,13]. Gut functionality is affected by the fermentation of probiotics, as preclinical studies have documented that prebiotic product butyrate may have chemopreventive effect in carcinogenesis, while propionate may have anti-inflammatory effects on colon cancer cells [14,15,16]. Recently, the pro-apoptotic effect of synbiotic (combination of resistant starch and *Bifidobacterium lacti*s) has been documented in experimentally induced colon cancer [17,18]. More specifically, apoptosis as a regulatory factor has great implication on the expansion of tumors and on the activities of several genes known in cancer progression [19]. Though, studies have demonstrated the role of probiotics in the regulation of cell proliferation and apoptosis but their role in combination with prebiotics as the synbiotic have not been studied and warrants further investigation. Thus, the study was designed to elucidate the apoptotic and immunomodulatory mechanism of synbiotic in experimentally induced colon cancer.

MATERIALS AND METHODS

1. Chemicals: 1,2-Dimethylhydrazine dihydrochloride (DMH), dithiothreitol and TRIzol reagent were obtained from Sigma Chemical Company, St. Louis, MO, USA. The prebiotic inulin (fructooligosaccharide) was procured from Hi-Media, Mumbai, India. All the primers used were purchased from Genex Life Sciences Pvt. Ltd, Bengaluru, India.

2. Animals: Sprague Dawley (SD) rats weighing 100-200g were housed in polypropylene cages in the animal house after their procurement from the Central Animal House, Panjab University, Chandigarh, India. These were acclimatized for 7–10 days before being used and were provided with water and pellet diet (Hindustan Lever Products, Limited, Kolkata, India) *ad libitum*. Care, use and disposal of animals were done in accordance with the guidelines of Panjab University Animal Ethical Committee (IAEC), Chandigarh and approved by the Committee for the Purpose of Control and Supervision on Experiments on Animals (IAEC/156; 25/08/2011).

3. Induction of colon carcinogenesis: DMH was prepared in EDTA (1mM) and pH was adjusted to 7.0 with NaOH (1 mM). A single dose of DMH (20 mg/kg body weight) was given intraperitoneally (i.p) per week for eighteen weeks to animals [20].

4. Preparation of probiotic dose: Lactobacilli strains (*Lactobacillus rhamnosus GG* MTCC # 1408 and *Lactobacillus acidophilus* NCDC # 15) were grown in de Mann Rogosa Sharpe (MRS) broth and maintained on MRS agar slants by regular sub-culturing at an interval of 15 days by incubating at 37˚C for 24 hours. For experimental inoculation, 18 hour old bacterial culture was cold centrifuged at 3500 g for 10 minutes, washed and suspended in phosphate buffered saline (PBS, pH 7.2) to contain 1 x 10^9 lactobacilli/0.1ml which was fed orally with orogastric gavage [20].

5. Prebiotic: Animals were administered orally with prebiotic inulin (5 mg/0.1ml) by orogastric gavage [21].

6. Synbiotic: *L. rhamnosus GG* together with *L. acidophilus* was the probiotic component and in combination with the prebiotic inulin formed the synbiotic. Animals were administered synbiotic orally (1 x 10^9 lactobacilli/0.1ml + 5 mg/0.1 ml inulin) with orogastric gavage [22].

7. Experimental Design: Eighty animals were divided into ten groups. Group I (Control): Animals received a single dose of EDTA (1mM) saline intraperitoneally per week for eighteen weeks. Group II (DMH-only-treated): Animals received a single dose of DMH intraperitoneally per week for eighteen weeks. Group III (*L. rhamnosus GG*), Group IV (*L. rhamnosus, L.GG* + DMH), Group V (*L. acidophilus*), Group VI (*L. acidophilus* + DMH): Animals belonging to group III and V were fed orally with probiotic (1 x 10^9 lactobacilli/0.1ml) daily for eighteen weeks. Animals belonging to group IV and VI were fed orally only with probiotic (1 x 10^9 lactobacilli/0.1ml) daily for a week and thereafter, a single dose of DMH was administered intraperitoneally (i.p) weekly for eighteen weeks along with daily oral administration of probiotic. Group VII (Inulin): Animals belonging to these group were fed orally daily with inulin for eighteen weeks. Group VIII (Inulin + DMH): Animals belonging to this group were fed with inulin orally daily for a week. Thereafter, from 2nd week onwards, animals were administered a single dose of DMH intraperitoneally per week for eighteen weeks. However, oral feeding of inulin was continued daily till the end of 18th week. Group IX (Synbiotic) Animals were fed orally with *L. rhamnosus GG* and *L. acidophilus* and inulin together daily for eighteen weeks. Group X (Synbiotic+DMH): Animals were fed orally with synbiotic for a week and thereafter, a single dose of DMH intraperitoneally per week was given for eighteen weeks along with daily administration of synbiotic.

8. Follow up of the animals: At the end of treatment, blood was collected from the animals by cardiac puncture, serum was prepared and stored at -60°C till further use. Thereafter, animals were sacrificed under an overdose of ether anesthesia by cervical dislocation and colon was removed. The whole colonic tissue, tumors and the adjacent mucosa to the tumors were processed for isolation of colonocytes, RNA, DNA and cytokines estimation.

9. Isolation of colonocytes: The colonocytes were isolated by Sanders method [23]. The Sander's method for isolation of colonocytes is designed to remove intact crypts and surface cells leaving behind the lamina propria. After sacrificing the rats, entire colon was removed, flushed with Ca^{2+} and Mg^{2+} free-PBS and was cut longitudinally to expose the lumen. The cut colon was placed in Ca^{2+} and Mg^{2+} free-Hank's buffered salt solution (HBSS), 30 mM EDTA, 5 mM dithiothreitol (DTT), 0.1% bovine serum albumin (BSA), incubated at 37˚C on shaker for 15 minutes. The mucosal side was gently scraped, isolated cells were cold centrifuged at 600g and washed twice with HBSS containing Ca^{2+} and Mg^{2+} and 0.1% BSA. The final volume was made up to 2 ml, cells were counted in a hemocytometer and were adjusted to contain 1 x 10^5 cells/ml. The cell viability was measured by the trypan blue exclusion method.

10. Ethidium bromide/acridine orange staining for determination of apoptotic cells: The colonocytes were observed under a fluorescence microscope after staining with ethidium bromide/acridine orange stain. The apoptotic cells were counted manually and were expressed as percentage in comparison to 100% of control.

11. Total RNA Extraction and Reverse Transcription–Polymerase Chain Reaction (RT-PCR): Total RNA was extracted from both colonic mucosa and colon tumors using TRIzol reagent, a mixture of guanidine thiocyanate and phenol in a monophase solution (Sigma Aldrich,

USA) following manufacturer's protocol. The RNA was suspended in nuclease free water and stored at -80°C. The purity of RNA was monitored in 1.5% agarose ethidium bromide gel and quantitated using Nano-Drop 1000 spectrophotometer (Thermo Fisher Scientific Inc., UK). Equal amount of RNA (2 µg) was used for synthesis of complementary DNA (cDNA) using commercially available kit (Fermentas Life Sciences, Canada). The cDNA was stored at -20°C for further use.

12. Expression of Bax and Bcl-2, the apoptotic markers: From the cDNA, PCR was performed using the following sets of primers (Genex Life Sciences Pvt. Ltd, Bangalore, India, [24]): Bax (forward primer; 5/-GTTTCATCCAGGATCGAGCAG-3/, reverse primer, 5/-CATCTTCTTCCAGATGGT-3/), Bcl-2 (forward primer; 5/-CCTGTGGATGACTGAGTACC-3/, reverse primer; 5/-GAGACAGCCAGGAGAAATCA-3/), β-actin, as a control (forward primer; 5/-ATGGAATCCTGTGGCATCCA-3/, reverse primer, 5/-TCCACACAGAGTACTTGCGCTC-3/). PCR was performed using the following PCR programme: 94°C for 2 minutes for initial denaturation; then 35 cycles of denaturation at 94°C for 1 minute; annealing at 55°C for 1 minute; extension at 72°C for 1.5 minute and a final elongation at 72°C for 7 minutes. The amplified DNA was resolved in 1.5% agarose ethidium bromide gel and analysed by Gel Doc EZ Imager (Bio-Rad).

13. Expression of protooncogene K-ras and tumor suppressor gene P53: From the cDNA, PCR was performed using the following set of primers (Genex Life Sciences Pvt. Ltd, Bangaluru, India, [25]): The primers which amplify wild-type K-ras were utilized: forward (5/-ACTTGTGGTAGTTGGCCCT-3/) and reverse (5/-TCCCCAGTTCTCATGTACTG-3/). PCR was performed using following programme: 95°C for 3 minutes for initial denaturation; then 35 cycles of denaturation at 93°C for 30s; annealing at 57°C for 45s; extension at 74°C for 45 s and a final elongation at 72°C for 7 minutes. The primers which amplify wild-type P53 [26], (5/-GGCTCCTCCCCAACATCTTATC-3/) and the downstream (5/-TCTCCCAGGACAGGCACAAAC- 3/) were used. PCR was performed using following PCR programme: 95°C for 3 minutes for initial denaturation; then 35 cycles of denaturation at 94°C for 30s; annealing at 58°C for 30s; extension at 72°C for 30s and a final elongation at 72°C for 5 minutes. The amplified DNA was resolved in 1.5% agarose ethidium bromide gel and analysed by Gel Doc EZ Imager (Bio-Rad).

14. Estimation of cytokines in colonic tissue and serum: Each colonic tissue was homogenized in 2 ml of 0.25 mM Tris-Cl (pH 7.8) and resulting homogenates were cold centrifuged at 1,600 g for 20 minutes [27]. The supernatants and serum were subjected to the quantification of cytokines (TNF-α, IL-6, IFN-γ, IL 10) by ELISA based kits (Ray-Biotech, India), in triplicate as per the manufacturer's instructions. The results were expressed as the concentration of cytokines (pg/ng) per milliliter of the sample using standard cytokines provided in the kit.

15. Histopathological study: After the animals were sacrificed, part of the colon was removed, fixed in 10% buffered formalin, processed, and stained with hematoxylin and eosin (H&E stain). The slides were blot-dried, mounted with distyrene plasticizer xylene, and examined microscopically.

16 Statistical analysis: Results were expressed as mean ± standard deviation (SD). The inter group variation was assessed by one way analysis of variance (ANOVA) followed by Post Hoc LSD Tests. Statistical significance of the results was calculated at $p<0.05$.

RESULTS

1. Quantification of apoptosis

The colonocytes isolated from animals belonging to synbiotic+DMH, *L.rhamnosus*+DMH and inulin+DMH showed significant increased percentage ($p<0.01$) of the apoptotic cells and was 115.37%, 113.93% and 154.02% respectively compared with DMH-only-treated animals (Fig 1A, 1B).

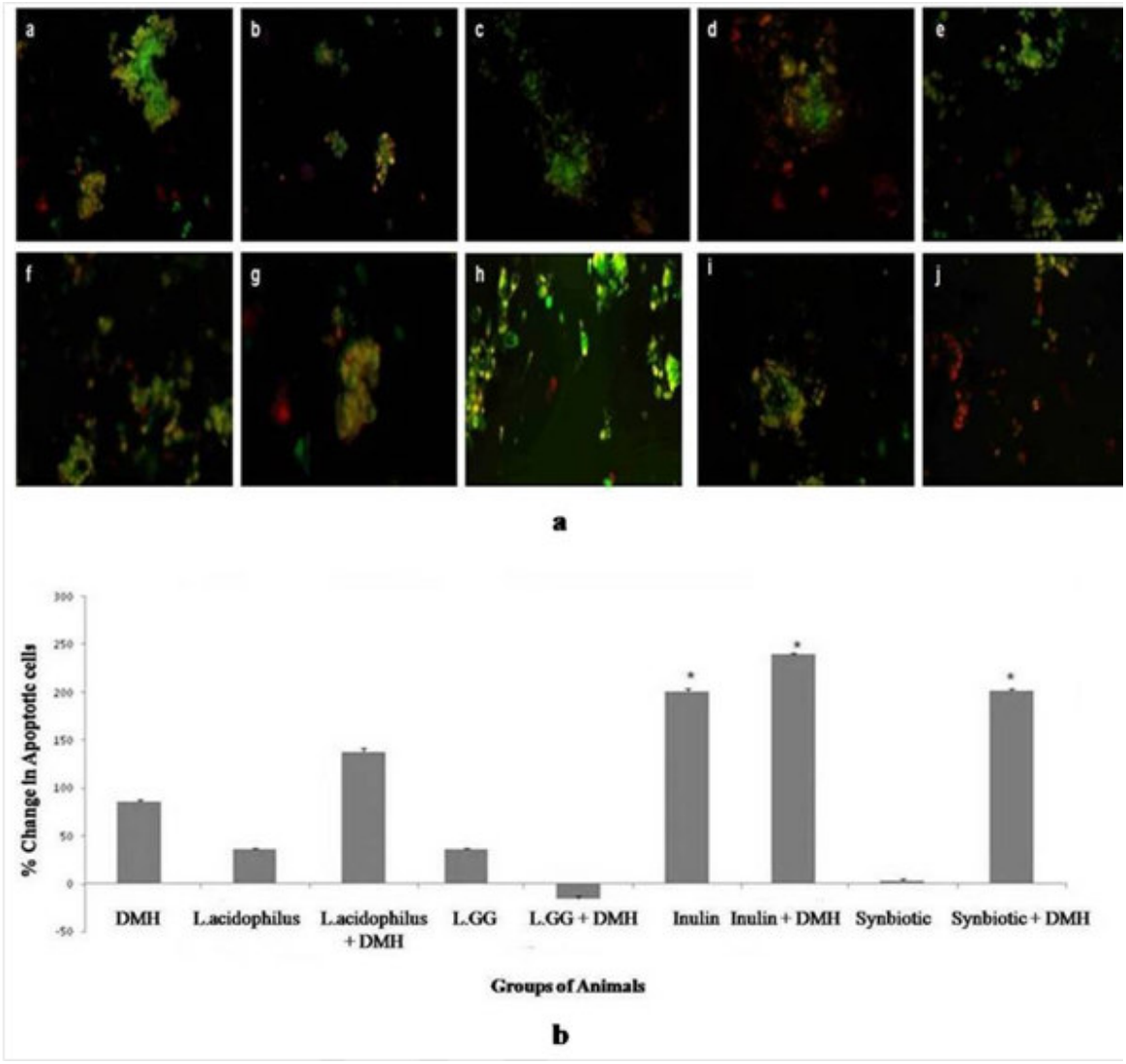

Figure 1

(a) EtBr/AO stained colonocytes in different groups of animals: (a) Control; (b) DMH-treated; (c) L.acidophilus; (d) L.acidophilus+DMH; (e) L.GG; (f) L.GG+DMH (g) Inulin (h) Inulin+DMH (i) Synbiotic (j) Synbiotic + DMH (400X). (b) Percentage of apoptotic cells in different groups of animals. Values are mean± SD. *p<0.05 DMH-treated.

2. Expression of apoptotic markers Bax and Bcl-2

There was no significant difference in the Bax and Bcl-2 expression of groups control, L.GG, *L.acidophilus*, inulin and synbiotic therefore only the expression in control group was used for comparison to other treated groups. The colonic tumors of animals belonging to DMH, L.GG+DMH, inulin+DMH and synbiotic+DMH-treated groups showed no significant difference in the Bax expression (Fig 2A, C). However, a significant downregulation of Bcl-2 expression ($p<0.05$) was observed in the colonic tumors of synbiotic+DMH, *L.acidophilus*+DMH, inulin+DMH and LGG+DMH-treated animals compared with the tumors of DMH-only-treated animals (Fig 2B, C). It was interesting to note that Bcl-2 expression was also downregulated ($p<0.05$) in the mucosal areas of animals belonging to synbiotic+DMH, *L.acidophilus*+DMH and LGG+DMH-treated compared with colonic tumors of these groups of animals (Fig 2B, C). While, the mucosal areas of animals belonging to synbiotic+DMH and L.GG + DMH showed significant increased ($p<0.05$) expression of Bax, the *L.acidophilus*+DMH group had significant decreased ($p<0.05$) expression compared with their respective colonic tumors (Fig 2A, C).

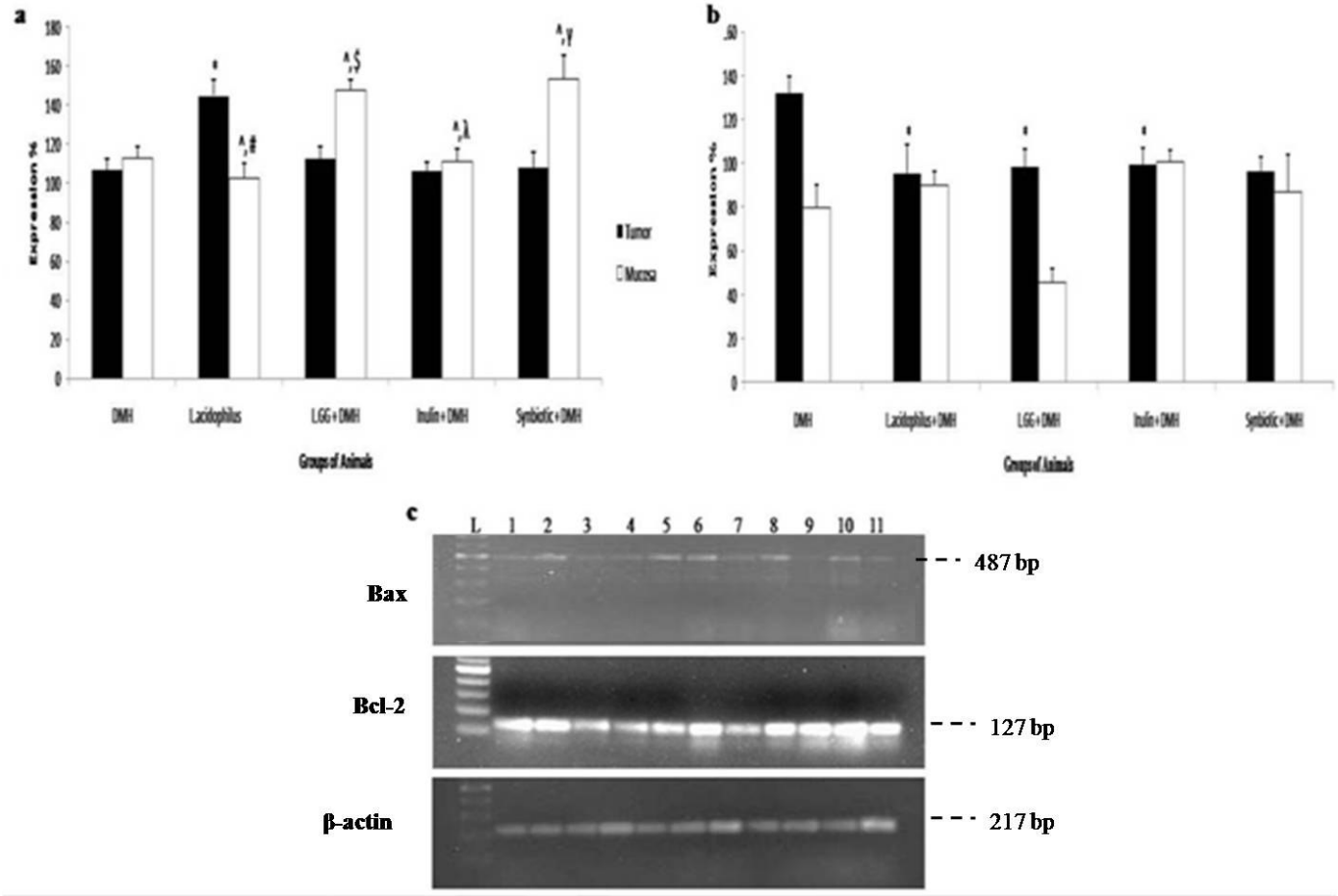

Figure 2

Densitometric analysis of Bax (a) and Bcl-2 (b) and gel electophoresis of the RT-PCR products (c) of colonic tumors (T) and adjacent mucosa (M) of animals belonging to different groups. The bar diagram represents the densitometric analysis of Bax and Bcl-2 expression (percent relative to control where expression in control was considered to be 100%. Lanes- L: DNA ladder, 1: Control, 2: DMH-treated (T), 3:DMH-treated (M), 4: L.acidophilus + DMH (T), 5: L.acidophilus + DMH (M), 6: L.GG +DMH (T), 7: L.GG +DMH (M), 8: Inulin + DMH (T), 9: Inulin + DMH(M), 10: Synbiotic + DMH (T), 11: Synbiotic + DMH (M). Values are mean ± SD. *p<0.05 v/s DMH-treated (T), ^p<0.05 v/s DMH-treated (M), $^{\#}$p<0.05 v/s L.acidophilus + DMH (T), $^{\$}$p<0.05 L.GG + DMH (T), ^{Y}p<0.05 v/s Synbiotic + DMH (T).

3. Expression of proto-oncogene K-ras and tumor suppressor gene P53.

As there was no significant difference in the K-ras and P53 expression of control, L.GG, *L.acidophilus*, inulin and synbiotic groups, therefore the RT-PCR expression result of the control group was used for comparison of expression with different treated groups. K-ras expression decreased significantly ($p<0.01$) in the colonic tumors of animals belonging to synbiotic+DMH and LGG+DMH -treated compared with DMH-only-treated animals. However, no significant difference was observed in the expression of K-ras of the colonic tumors and mucosal areas of animals belonging to LGG+DMH, inulin +DMH and synbiotic+DMH-treated, but *L.acidophilus* +DMH-treated animals had reduced K-ras expression in the mucosal area than colonic tumors (Fig 3).

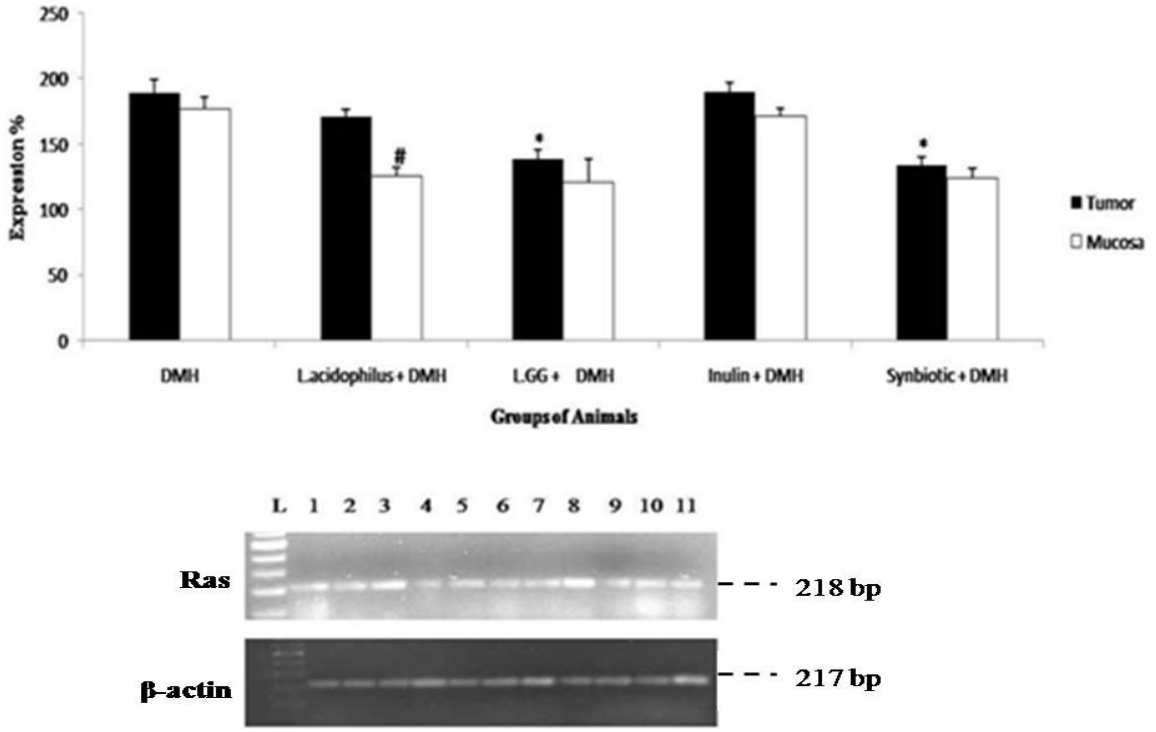

Figure 3

K-ras expression densitometric analysis and gel electrophoresis of the RT-PCR product of colonic tumors (T) and adjacent mucosa (M). The bar diagram represents the densitometric analysis of K-ras expression (percent relative to control where expression in control was considered to be 100%Lanes- L: DNA ladder, 1: Control, 2: DMH-treated (T), 3:DMH-treated (M), 4: L.acidophilus + DMH (T), 5: L.acidophilus + DMH (M), 6: L.GG +DMH(T), 7: L.GG +DMH(M), 8: Inulin + DMH(T), 9: Inulin + DMH(M), 10: Synbiotic + DMH (T), 11: Synbiotic + DMH (M). Values are mean ± SD. *p<0.05 v/s DMH-treated, #p<0.05 v/s L.acidophilus + DMH (T).

Further, it was observed that synbiotic+DMH-treated animals had significant ($p<0.05$) upregulation of wild type P53 expression in both the colonic tumor and mucosal areas compared with DMH-only-treated animals (Fig 4). Animals belonging to *L.acidophilus*+DMH-treated group had significantly higher ($p<0.05$) expression of P53 in mucosal areas than its corresponding colonic tumors while the mucosa of L.GG+DMH had lower ($p<0.05$) P53 expression compared with its tumors. However, inulin+DMH treated animals did not show significant difference in the expression of P53 in both colonic tumors and mucosal regions compared with DMH-only-treated animals (Fig 4).

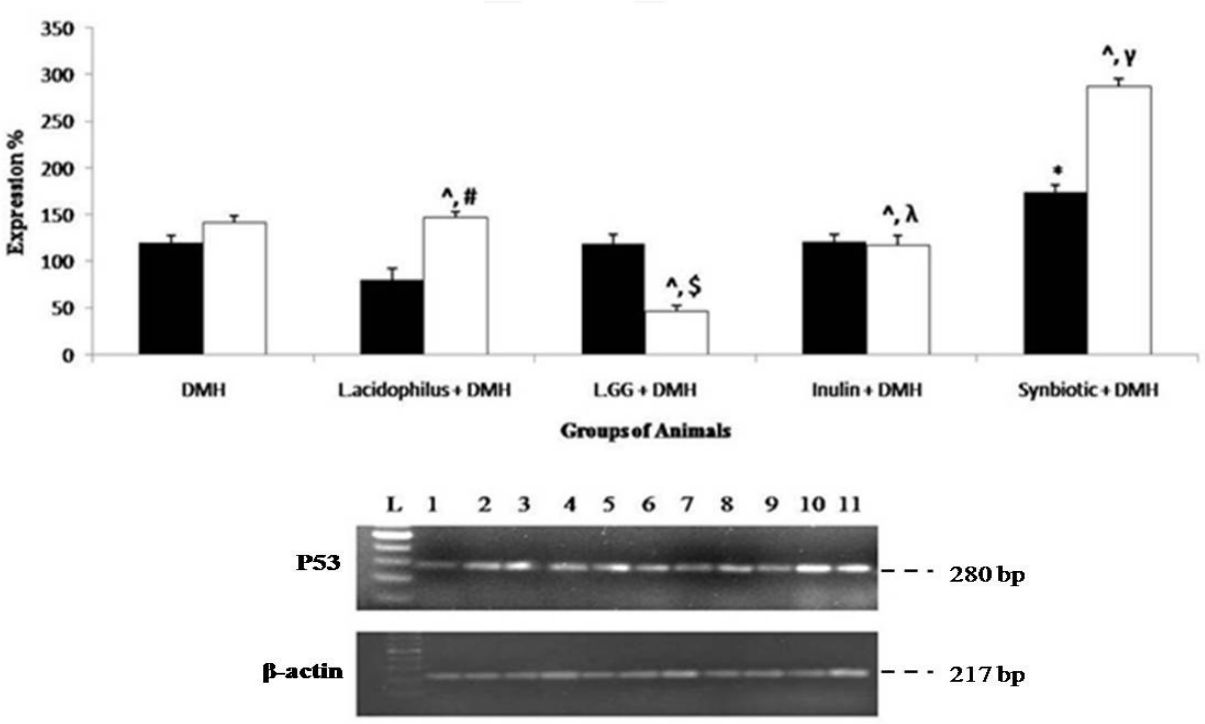

Figure 4

P53 expression densitometric analysis and gel electrophoresis of the RT-PCR product of colonic tumors (T) and adjacent mucosa (M). Lanes- L: DNA ladder, 1: Control, 2: DMH-treated (T), 3:DMH-treated (M), 4: L.acidophilus + DMH(T), 5: L.acidophilus + DMH (M), 6: L.GG+DMH (T), 7: L.GG +DMH (M), 8: Inulin + DMH (T), 9: Inulin + DMH (M), 10: Synbiotic + DMH (T), 11: Synbiotic + DMH (M). Values are mean ± SD. *p<0.05 v/s DMH-treated (T), ^p<0.05 v/s DMH-treated (M), $^{\#}$p<0.05 v/s L.acidophilus + DMH (T), $^{\$}$p<0.05 L.GG + DMH(T), $^{\lambda}$p<0.05 v/s Inulin + DMH (T), $^{\gamma}$p<0.05 v/s Synbiotic + DMH (T).

4. Cytokines levels in tissue and serum

TNF-α levels in serum decreased significantly ($p<0.05$) in synbiotic+DMH, LGG+DMH, *L.acidophilus*+DMH, and inulin +DMH -treated animals compared with DMH-only-treated animals. However, levels of TNF-α in colonic tissue were found to be significantly higher ($p<0.05$) in synbiotic+DMH, LGG+DMH, and inulin +DMH-treated animals compared with DMH-only-treated animals (Fig 5A). We also found that levels of IL-6 cytokines decreased significantly ($p<0.05$) in the colonic tissue but were similar in LGG+DMH, *L.acidophilus*+DMH and synbiotic+DMH-treated animals compared with DMH-only-treated animals (Fig 5B). However, IFN-γ levels in the colonic tissues increased significantly ($p<0.05$) in synbiotic+DMH-treated animals compared with DMH-only-treated animals but no significant difference was observed in the serum of LGG+DMH, *L.acidophilus*+DMH, inulin +DMH and synbiotic+DMH-treated animals (Fig 5C). The levels of anti-inflammatory cytokine, IL-10 increased significantly ($p<0.05$) in the colonic tumors of animals belonging to synbiotic+DMH, LGG+DMH, and inulin +DMH -treated groups compared with DMH-only-treated animals while no significant difference was observed in the serum of these animals (Fig 5D).

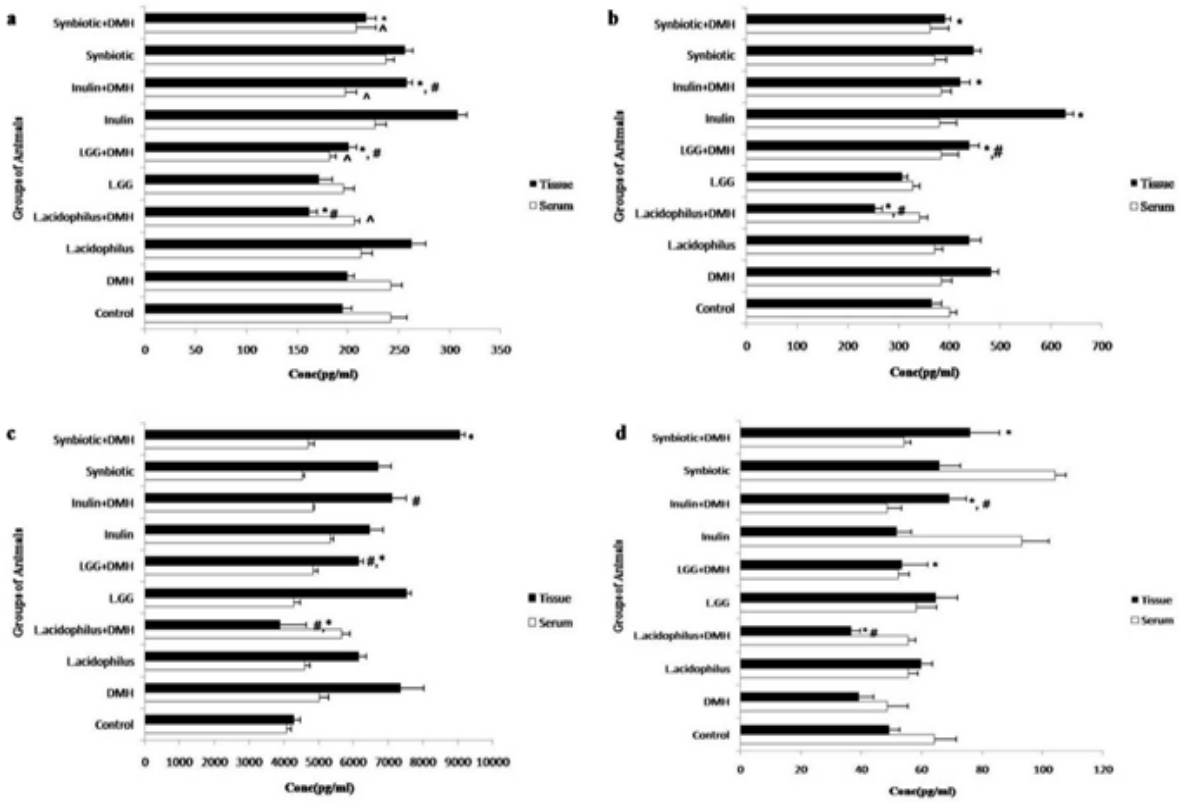

Figure 5

Cytokine levels in serum and colonic tissues: (a) TNF-α, (b) IL-6, (c) IFN-γ, (d) IL -10. Values are mean ± SD. *p<0.05 v/s DMH-treated (tissue), ^ p<0.05 v/s DMH-treated (Serum), #p <0.05 v/s Synbiotic+DMH (Tissue).

5. Histopathological studies

Macroscopically, it was found that in all DMH treated groups the tumors were sessile which rested on the mucosa without a pedicle. However, the site of tumor occurrence was not specific (6A, 6B).

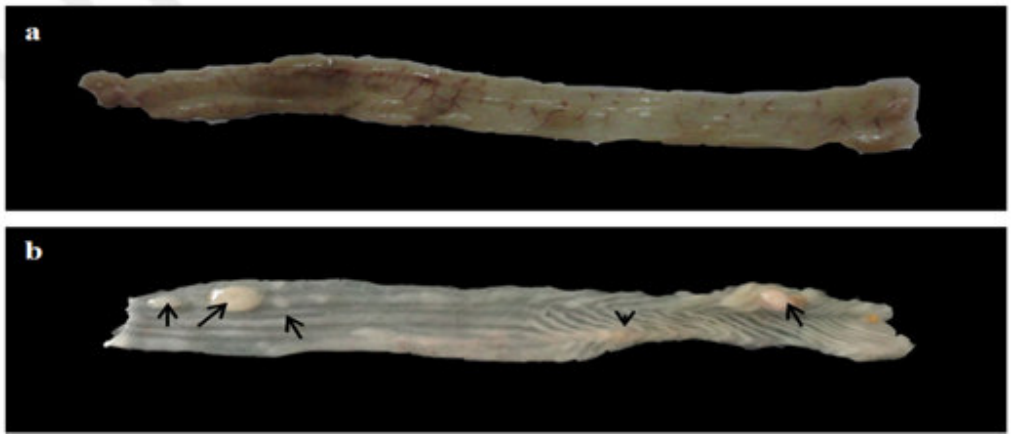

Figure 6
Photograph showing the colon of (a) Control rats (b) DMH treated rats with arrows pointing to the tumors.

Histopathologically, as compared to the normal straight tubular crypt pattern of the colonic tissue in the normal control groups the tumors of the DMH treated groups had completely lost the normal glandular pattern of tubular crypts and also had eosinophilic amorphous necrotic material referred to as 'dirty' necrosis admixed with hematoxylin staining nuclear debris (→N) and inflammatory cell nuclei(→L) (Fig 7A, 7B).

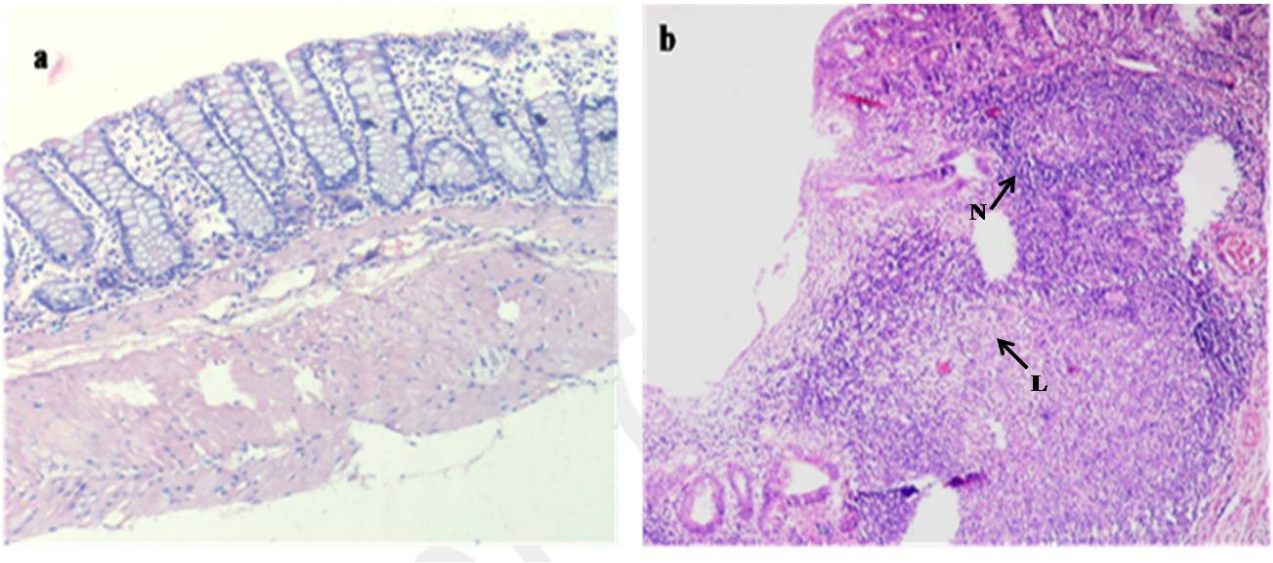

Figure 7
Photomicrograph of the rat colon in different groups showing (a) Normal colon (b) adenocarcinoma in the DMH treated groups of animals.

DISCUSSION

The present study is an extension of our previous study, where we demonstrated that prior supplementation of probiotics (L.GG, *L.acidophilus*) or prebiotic (inulin) reduced the activities of procarcinogenic enzymes and ACF lesions in DMH induced colon cancer in rats [20, 21]. Further, the combination of probiotic and prebiotic as synbiotic was used in long term

study where DMH was induced for 18 weeks and it was observed that synbiotic showed antitumorigenic and antioxidative effect [22]. To further understand the molecular and immonological roles of synbiotic in experimental colorectal cancer, the present study was designed to study the effect of synbiotic on tumor progression with respect to apoptosis, expression of K-ras and P53 along with cytokines level. Apoptosis is an innate cellular defense against oncogenesis that acts by removal of cells with genomic instability or mutation which might otherwise progress to malignancy [28]. In the present study, we found increased apoptosis in the colonic tissue of DMH-treated animals administered either with *L.acidophilus,* L.GG, inulin or synbiotic suggesting their ability to upregulate apoptosis. Further, it was interesting to note that prior administration of synbiotic to DMH-induced colon carcinogenesis led to decreased expression of Bcl-2, a protooncogene and this observation is consistent with the earlier studies wherein scientists have demonstrated that milk fermented with *Lactobacillus helveticus* reduced Bcl-2 expression and enhanced apoptosis in a murine cancer model [29]. Also *L. casei* supplementation has been reported to suppress Bcl-2 gene expression in mucosal T lymphocytes of patients with Crohn's disease [30]. Most notably, prior administration of synbiotic to DMH-treated animals led to significant decreased expression of K-ras and increased P53 expression. This may be attributed to probiotic potential of activating tumor suppressor genes resulting in reduced cell proliferation. It has been reported in the earlier studies that rats fed with probiotic *Bifidobacterium longum* rich diet exerted strong anti-tumor activity on the colonic mucosa by reducing the expression ras-p21 and cell proliferation [31,32]. Similarly, in another study milk fermented with two probiotic lactic acid bacteria, *L. rhamnosus* GG (LGG) and *L. casei* strain Shirota (Lcs), alone or with chlorophyllin adjunct reduced the expression of c-myc, bcl-2, cyclin D1 and ras-p21 genes in the hepatic cancer [33]. Further, Pool-Zobel et al., have also reported that oral administration of LAB was effective in reducing DNA damage, induced by chemical carcinogens, in the gastric and colonic mucosa of rats [34]. It is known that decreased Bcl-2 expression, activates p53-induced apoptosis in cancer cells and in our present study, we also observed decreased Bcl-2 expression which may have increased the expression of wild type P53 in synbiotic+DMH-treated animals leading to programmed cell death or apoptosis in colonic tumors [35]. Synbiotic+DMH- treated animals had increased levels of TNF-α, IFN-γ, IL-10 and decreased IL-6 in colonic tissues indicating the immunomodulatory effect of synbiotic. These effects of immunomodualtion corresponds with the results of our previous study, that showed reduced tumor incidence, burden and multiplicity in the synbiotic+DMH treated animals as compared to the DMH-only treated rats[22]. Not only there was reduction in the number of tumors, the histopathology also showed the mucosa of the synbiotic+DMH treated groups with fewer dysplastic changes in the crypts with increased number of goblet cells as compared to the high grade dysplasia and complex structures in the DMH-only treated group. Earlier studies have also reported that in both animal and human studies, probiotic supplementation (L.GG, VSL#3) induced proinflammatory cytokines IFN-γ as well as regulatory cytokines, IL-10 [36,37]. Further, Shida et al, [38] have also shown that, *L. casei* strain Shirota supplementation stimulated human peripheral blood mononuclear cells to secrete IL-12, IFN-γ, TNF-α, and IL-10. In addition to these, metabolic teichoic acids from LAB have also been found to elicit proinflammatory cytokine TNF-α [39]. More specifically, decreased levels of IL-6 in synbiotic+DMH-treated animals may be responsible for less tumor growth in colon and is in accordance with earlier studies where in murine experimental breast cancer model, decreased level of IL-6 was observed in mice fed with *L.helveticus* fermented milk or kefir clearly indicating inhibition of tumor growth [31,40]. The interesting observation in the present study was that the mucosal region of synbiotic+DMH-treated animals had significantly decreased Bcl-2 and increased Bax and P53 expression with no significant difference in K-ras expression compared with

the colonic tumors. Thus, it can be stated that prior administration of synbiotic to DMH-treated animal activates genes in favor of apoptosis and tumor suppression in the colonic tumors. In our earlier study, we have found that animals fed either with probiotic or prebiotic had great implication in reduction of CRC as prior administration of either probiotics or prebiotics for six weeks in DMH-induced colon cancer improved the gut microbiome and other in vivo factors like pH, substrate availability. Also, decreased activity of procarcinogenic enzymes (nitroreducatse, β-glucuronidase, β-glucosidase) and reduced preneoplastic lesions (aberrant crypt foci) was observed [20,21]. Further, we have observed that prior administration of synbiotic (*L.rhamnosus*, *L.acidophilus* and inulin) attenuated the oxidative stress by quenching the toxic radicals being generated by DMH, that otherwise might have damaged DNA leading to either mutations in proto-oncogenes and/or inactivation of tumor suppressor genes [22]. Expression of wild type tumor suppressor gene P53 was also found to be enhanced that in turn might have downregulated the Bcl-2 and upregulated Bax expression resulting into increased apoptosis. The observed enhanced apoptosis may have also been modulated by the proinflammatory cytokines IL-6 and TNF-α. IL-6 is a multifactorial cytokine having growth promoting and antiapoptotic activity as it activates IL-6-Jak-Stat3 signaling pathway that affects the K-ras activation [41]. More specifically, administration of synbiotic to DMH-treated animals led to decreased IL-6 levels and down regulation of K- ras expression leading to lesser cellular proliferation and decreased tumorigenesis which may be due to interaction and incorporation of orally administered probiotic into M cells, via Toll-like receptor (TLR) 2 mediated transcytosis and transport of LAB to peyer's patches (PPs), leading to stimulation of mucosal immune cells, thereby ameliorating the mucosal immunity [42, 43].

CONCLUSION

Taken together, it can be concluded that synbiotic (L.GG, *L.acidophilus* and inulin) administration in experimental colon carcinogenesis attenuates colonic tumors by modifying cytokine production which in turn promotes apoptosis by downregulation of K-ras expression and upregulation of p53. Thus, it can be concluded that synbiotic presents an attractive option as prophylactic intervention against the development of CRC, however further detailed clinical study needs to be carried out.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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