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Lactobacillus rhamnosus induced epithelial cell apoptosis, ameliorates inflammation and prevents colon cancer development in an animal model

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ABSTRACT

Background/Aim: Probiotics have been suggested as prophylactic measure in colon carcinogenesis. This study aimed at determining the potential prophylactic activity of *Lactobacillus rhamnosus* GG CGMCC 1.2134 (LGG) strain on colorectal carcinogenesis via measuring its effect on Nuclear factor kappa B (NFκB) inflammatory pathway and apoptosis.

Materials and methods: 64 Sprague Dawley rats were grouped into four as follows; Group 1 (Healthy control), Group 2 (LGG), Group 3 (cancer control Dimethyl hydrazine (DMH)) and Group 4 (LGG + DMH). LGG was administered orally to LGG and LGG + DMH groups. Colon carcinogenesis was chemically induced in LGG + DMH and DMH groups by weekly injection of 40 mg/kg DMH. Animals were sacrificed after 25 weeks of experiment and tumor characteristics assessed. The change in expression of NFκB-p65, COX-2, TNFα, Bcl-2, Bax, iNOS, VEGFα, β-catenin, Casp3 and p53 were evaluated by western blotting and qRT-PCR.

Results: LGG treatment significantly reduced tumor incidence, multiplicity and volume in LGG + DMH treatment group compared to DMH cancer control group. Also, LGG treatment reduced the expression of β-catenin and the inflammatory proteins NFκB-p65, COX-2 and TNFα; the anti-apoptotic protein Bcl-2, but increased the expression of the pro-apoptotic proteins Bax, casp3 and p53 compared with DMH group.

Conclusion: LGG have a potential protection effect against colon carcinogenesis; inducing apoptosis and ameliorating inflammation, and may hold a promise as bio-therapeutic dietary agent.

1. Introduction

Colorectal cancer is the third most common cause of cancer related deaths in developed countries with more than a million new cases each year [1]. A recent study by Chen et al. [2] indicates that China alone registered 2.8 million deaths with 4.3 million new cases of cancer in 2015. While systems are working around the clock to find a lasting solution to cancer, the challenge still lies in the inability to identify new treatment strategies beyond chemotherapy to inhibit tumor progression.

Probiotics, which include lactic acid bacteria, have health benefits when applied in proper dose and have been proven to possess prophylactic functions such as improved nutrition, microbial balance and immuno-enhancement of the gastrointestinal tract (GIT) [3–5]. Probiotics have been used for the prevention and treatment of a wide range of disorders from gastroenteritis to intestinal neoplasia. The well-known benefits of probiotics' use in the management of antibiotic associated diarrhea lies in restoration of the normal microbiota [6]. However, the mechanisms of probiotics efficacy in many disorders are not well established. Probiotic-therapy has been explored in non-gastrointestinal diseases like the treatment and prevention of atopic eczema [7]. Nevertheless, the evidence to date suggests that the major clinical effects of probiotics are seen in GIT disorders. Animal model experiments have shown that the intestinal microbiota is important in stimulating normal immune system development,

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especially the gut-associated lymphoid tissue, as well as the development and persistence of oral tolerance to food antigens which may play a role in the prevention of GIT diseases [8–10].

Of the many probiotic strains available, *Lactobacillus rhamnosus* GG is a well characterized strain and has been used in clinical practice for more than 50 years [11]. Administration of *Lactobacillus rhamnosus* is shown to lower the risk of Colon cancer; through the inhibition of harmful enzymatic activities and the beneficial modulation of the bowel microbiota [8]. Furthermore, LGG is known to modulate human dendritic cells and exhibits strong pro-inflammatory effects [11].

Inflammation is a hallmark of cancer. The inflammatory gene nuclear factor kappa B (NFκB) have been identified in cancer tissues from patients including lymphoma, leukemia, breast, oral, pancreas and colon cancers [12–14]. Cyclooxygenase-2 (COX-2) is involved in inflammation and reports associates it with colorectal tumor development and progression [15]. Oncogenes and tumor suppressor genes such as Bcl-2 and p53 are involved in regulating cell behavior like apoptosis and proliferation [16]. Alteration or deregulation of the Bcl-2 and p53 genes occurs during development and progression of CRC. Additionally, lack of Bcl-2 expression correlates with local tumor invasion, metastasis, and recurrence [17].

This work focused on the preventive effect(s) of LGG on colorectal cancer through the identification and monitoring of biomolecular markers of inflammation and apoptosis.

2. Materials and methods

2.1. Animals

Four weeks old Female Sprague Dawley rats (n = 64) were obtained from SPF (Specific Pathogenic Free animals Centre) of Dalian Medical University (Liaoning, China) and housed in an environmentally controlled room at 22 °C with a 12 h light–dark cycle. Food and water were provided ad libitum.

This work followed the ethical guidelines and was approved by the Dalian Medical University Animal Care and Use Committee.

2.2. Experimental design and animals follow up

Following one week of acclimatization, the animals were divided into 4 groups of 16 animals each and were treated as follows:

- Group 1 (healthy control): animals never received any treatment and were considered as healthy controls.
- Group 2 (*L. rhamnosus* GG CGMCC 1.2134 (LGG)): animals received oral daily dose of LGG 1×10^9 CFU lactobacilli/1 ml.
- Group 3 (DMH): animals received a weekly single dose of 40 mg/kg body weight of DMH intraperitoneally for 10 consecutive weeks.
- Group 4 (LGG + DMH): animals received a weekly single dose of 40 mg/kg body weight of DMH intraperitoneally for 10 weeks and daily oral dose of LGG 1×10^9 CFU lactobacilli/1 ml for 25 weeks.

2.3. Preparation of probiotic strains

Lactobacillus rhamnosus GG 1.2134 (LGG) was procured from China General Microbiological Culture Center (CGMCC 1.2134). LGG was grown aerobically at 37 °C in MRS broth and maintained on MRS agar by regular sub-culturing at 7 days interval. For experimental inoculation, 18 h old cultures were centrifuged at 4000 x *g* for 10 min, washed, and suspended in phosphate buffered saline pH 7.2 to contain 1×10^9 CFU lactobacilli/1 ml.

2.4. Induction of colon carcinogenesis

DMH (Aladdin, Shanghai, China, CAS: 306-37-6) was freshly prepared in 1 mM EDTA and pH adjusted to 7.0 with 1 mM NaOH. Weekly single dose of DMH (40 mg/kg body weight) was given intraperitoneally (i.p.) to LGG + DMH and DMH groups for 10 consecutive weeks [18]. Animals were sacrificed after 25 weeks of DMH treatment under anesthesia using 10% urethane.

2.5. Western blotting analysis

Western blotting analysis was performed according to standard protocol. Briefly, 100 mg of colon tissue was homogenized with Radio Immuno-preciptation Assay (RIPA) lyses buffer (Beyotime, China, Cat No: P0013C) with phenylmethylsulphonyl fluoride (PMSF) and Cocktail protease inhibitor (Thermo Scientific, USA, Lot # PA196152), centrifuged at 15000 rpm for 20 min and supernatant collected and kept in aliquots. Protein concentration was determined using BCA Kit (Thermo Scientific, USA, Cat No: 23227) using Bovine serum albumin (BSA) as protein standard. Samples were stored at −80 °C for later use. For gel loading, protein samples were boiled with loading buffer for 5 min at 95 °C and separated in SDS PAGE. Proteins were then transferred to polyvinylidene fluoride membrane (PVDF), blocked with 5% skimmed milk and incubated with primary antibody overnight at 4 °C. The primary antibodies used are as follows; NFκB-p65 (1:500), Casp3 (1:500) (Wuhan Boster Ltd, Cat No BA0610, BA3968 Wuhan, China), and COX-2 (1:2000), BAX, p53 (1:2000), and β actin(1:5000) (ProteinTech, USA Cat No; 12375-1-AP, 50599-2-Ig, 10442-1-AP and 20536-1-AP respectively). The immunoprecipitated protein bands were detected with infrared scanner using a secondary goat anti-rabbit IgG-IR 680 antibody (1:10000) (Licor, Odessey, USA).

2.6. Tissue RNA extraction and quantitative RT-PCR

The procedure described in the TRIzol® Reagent Kit (life technology, USA Cat No: 15596-026) was followed. Briefly, rats' colon tissue was homogenized using liquid nitrogen and TRIzol® (Trizol) in a ratio of 50–100 mg tissue/ml of trizol reagent followed by centrifugation. The supernatant was removed and mixed with chloroform 200 μl/ml of trizol, incubated at room temperature for 5 min and centrifuged at 12000 rpm. The top upper layer was taken and mixed with isopropanol 500 μl/ml trizol and 75% of ethanol added. The mixture was vortexed, centrifuged and the supernatant removed and air dried to obtain RNA pellet. Finally RNA was eluted in 20 μl RNase-free water. The amount of RNA isolated was quantified by Nano drop UV absorbance at 260/280 nm (Nano drops Thermo scientific 2000 spectrometer).

2.7. cDNA synthesis and qRT PCR

cDNA was synthesized from total RNA as per the manufacturer's manual (*TransScript*® All-in-One First-Strand cDNA Synthesis SuperMix, Cat No: AT-341-01). The resulting cDNA was used for qRT PCR. The set of primers used were synthesized from Invitrogen, USA as presented in Table 1. qRT PCR was performed using SYBR® Premix Ex Taq II (Tli RNaseH Plus) (TAKARA Bio Inc, Japan, Cat No: RR820A). The reaction conditions consist of 40–45 cycles of initial denaturation at 95 °C for 30 s and elongation at 95 °C for 5 s, 55 °C for 30 s and 72 °C for 60 s followed by dissociation melt curve using Strategene MX3005p, Germany platform. The data generated was analyzed for relative gene expression using the comparative 2Ct method with β-actin as

Table 1
List of primer sequences used for qRT PCR.

Gene	Forward Primer	Reverse primer
TNFα	CTT CTG TCT ACT GAA CTT CG	AAG ATGATC TGA GTG TGA GG
β-Catenin	ACTGGCAGCAGCAATCTTAC	GAGGTGTCCACATCTTCTTC
β Actin	AGGATGCAGAAGGAGATTACTGCC	AAAACGCAGCTCAGTAACAGTGC
Bcl-2	GATGACTGAGTACCTGAACCG	CAGAGACAGCCAGGAGAAATC
BAX	GGCGAATTGGAGATGAACTG	CCCCAGTTGAAGTTGCCAT
iNOS	GGAGCAGGTTGAGGATTACTTC	TCAGAGTCTTGTGCCTTTGG
VEGFα	TATATCTTCAAGCCGTCCTGTG	TCTCCTATGTGCTGGCTTTG

endogenous control and healthy control group as internal calibrator.

2.8. Statistical analysis

All data were analyzed using GraphPad Prism Version 6.04, USA. Analysis of Variance (ANOVA) followed by Turkeys test when significant was employed for comparison of significance between the groups. Results were considered significant at $p < 0.05$.

3. Results

3.1. Treatment with LGG decreased tumor incidence, multiplicity and volume

The effect of probiotic LGG on CRC development was studied in DMH induced animal model (Fig. 1). At the end of the experiment, tumor incidence in DMH cancer group was 100% but reduced significantly by LGG + DMH treated group to 62.5% ($p < 0.05$). Also, the tumor multiplicity and volume was significantly reduced by LGG treatment in LGG + DMH group compared with DMH cancer control as indicated in (Table 2). However, no animal in healthy control and LGG group developed cancer. Probiotic LGG treatment prevented cancer by reducing tumor incidence, multiplicity and volume.

3.2. Probiotic LGG treatment inhibits inflammation and angiogenesis involved in tumor development

The effect of LGG administration on the inflammatory cytokines NFκB-p65, iNOS, TNFα and COX-2 and VEGFα for angiogenesis was investigated.

We observed an increased expression of NFκB-p65, TNFα and iNOS in DMH cancer group but decreased in LGG + DMH treated group (Fig. 2A, B). VEGFα expression increased in DMH Cancer control but decreased by LGG + DMH treated group. Hence, LGG suppresses inflammation and angiogenesis associated with cancer prevention.

3.3. LGG treatment induced colonic cells apoptosis and prevents tumor progression

The activity of LGG on apoptosis was studied to ascertain its anti-tumor effect. The pro- and anti-apoptotic proteins Caspase-3, Bax, Bcl-2 and p53 were analyzed (Fig. 3A). LGG treatment in LGG + DMH group is associated with increased pro-apoptotic proteins Casp3, Bax and p53, but decreased anti-apoptotic protein Bcl-2 (Fig. 3B) compared with DMH cancer control group as shown in Figs. 3. Also, the effect of LGG on the tumor marker β-catenin for tumor progression was also analyzed. β-catenin is observed significantly increased in DMH cancer control group but decreased in LGG + DMH group (Fig. 3B). Therefore, treatment with Probiotic LGG increased apoptosis of colonic cells; associated with anti-tumor activity, and prevents tumor advancement in colon carcinogenesis.

4. Discussion

In our study, we investigated the effect of probiotic LGG on CRC development using DMH induced CRC animal model due to its phenotypic and genotypic features similar to human sporadic CRC [19].

Oral administration of LGG effectively reduced the tumor incidence, multiplicity and volume in DMH induced CRC animal

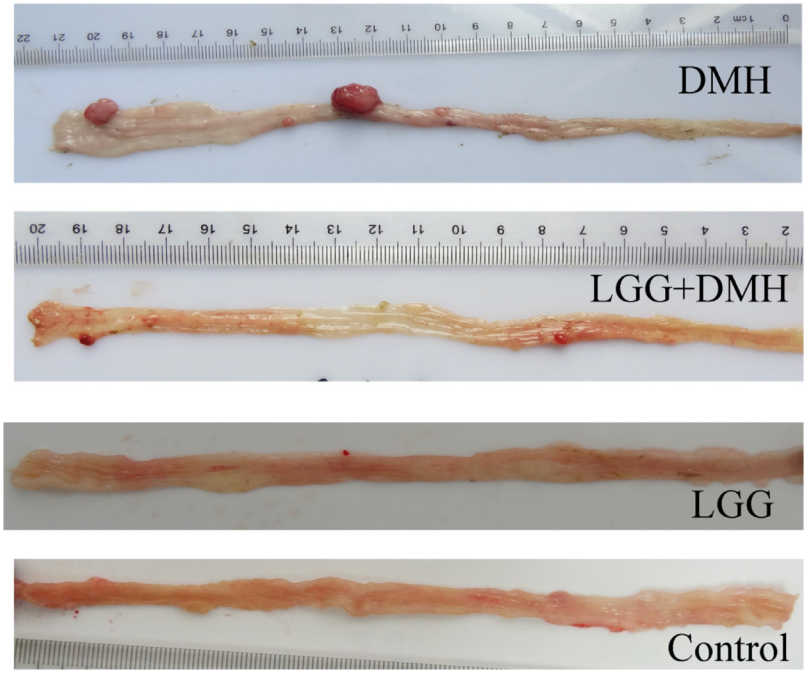

Fig. 1. LGG treatment reduces tumor incidence, multiplicity and volume in DMH Induced Colon cancer in SD rats. DMH group had more tumors both in size and numbers than the LGG + DMH treated animals. No tumors were observed in LGG or Control group animals at 25 weeks.

Table 2
Assessment of LGG-treatment on tumor Incidence multiplicity and volume.

Groups	No. of rats	No. of tumor bearing rats	Tumor incidence (%)	Tumor multiplicity (No. of tumor/tumor bearing rats)	Mean tumor volume (cm^3)
Group 1 Healthy control	16	0	0	0	0
Group2 LGG	16	0	0	0	0
Group 3 DMH	16	16/16	100[a]	5.75[b]	0.5940[c] ± 1.229
Group 4 LGG + DMH	16	10/16	62.5[a]	2.25[b]	0.07793[c] ± 0.1618

Administration of LGG significantly reduced tumor incidence, multiplicity and volume in LGG + DMH group compared to the DMH cancer group ($p < 0.05$). Values are represented as mean ± SD. Values of a common superscript letter (a–c) are significant at $p < 0.05$.

model. The change in incidence, multiplicity and size depicts a potential protective activity and chemoprevention effect of probiotic LGG on colon carcinogenesis development and progression. Le Leu et al. [20] reported the anticancer effect of lactic acid bacteria reducing tumor incidence, multiplicity and volume corroborating with findings in this study.

To address the mechanism whereby probiotic LGG overts tumor incidence, multiplicity and volume, we investigated the role of inflammation; a hallmark in cancer development and progression [16]. NFκB activation is involved in proinflammatory response and angiogenesis with role in tumor development and progression [21–23]. In normal condition NFκB is inactive. However, on activation following response to signals, NFκB translocates to the nucleus and sustains inflammation promoting proliferation, angiogenesis as well as preventing the elimination of cancer cells [24]. The results from this study observed an increase in NFκB in DMH group but decreased in DMH + LGG treated group. Femia et al. [25] studied the gene expression profile of DMH induced colon carcinogenesis and showed significant up-regulation of NFκB. The inflammatory effect of NFκB is mediated via the induction of downstream cytokines including COX-2, iNOS and TNFα [26]. The increase in NFκB in DMH group was observed associated with the direct increase in COX-2, iNOS and TNFα in this study but suppressed in LGG + DMH group. Studies by Iyer et al. [27] and Lee et al. [9] reported LGG's anti-inflammatory activity through attenuation effect on NFκB and TNF-α. Also, Wang et al. [28] reviewed the involvement of NFκB signaling in colon carcinogenesis as affirmed by the results in this study.

Cyclooxygenase-2 is reported increased in CRC [29,30]. In addition, it is activated during inflammation and it is known to promote angiogenesis; via induced expression of VEGF, and colon carcinogenesis [29,31]. The induced expression of COX-2 is also linked to the expression of iNOS involved in inflammation [31]. On the other hand, the formation of new blood vessels, hence angiogenesis, is essential for tumor growth and is reported dependent on VEGF and TNFα. VEGF and TNFα are downstream

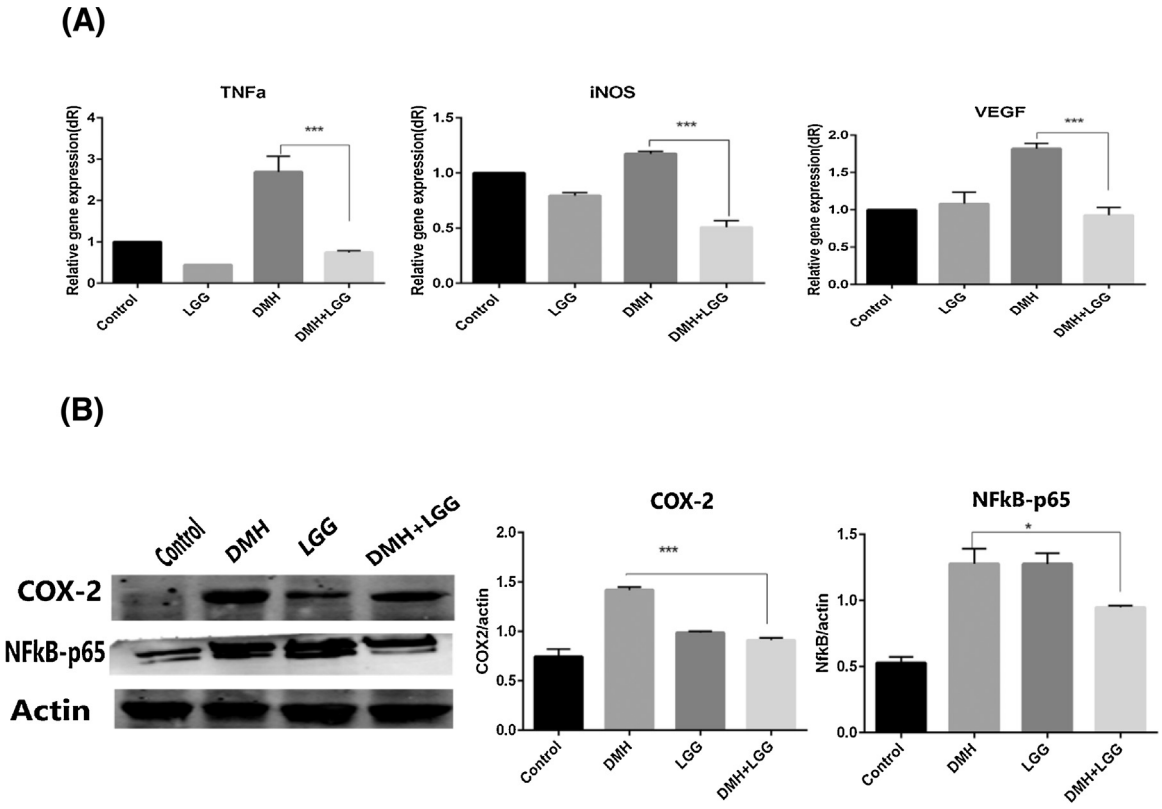

Fig. 2. LGG attenuate pro-inflammatory cytokines in DMH induced colon cancer in SD rats (A) quantitative RT-PCR gene expression of NFκB-p65, TNFa, iNOS and VEGFα relative to β-actin as endogenous gene control and Control group as calibrator. The values are presented as relative gene expression (dR) to the calibrator. (B) Western Blot analysis of COX-2 and NFκB-p65. Level of significance represents at * ($p < 0.05$), ** ($p < 0.001$), *** ($p < 0.0001$).

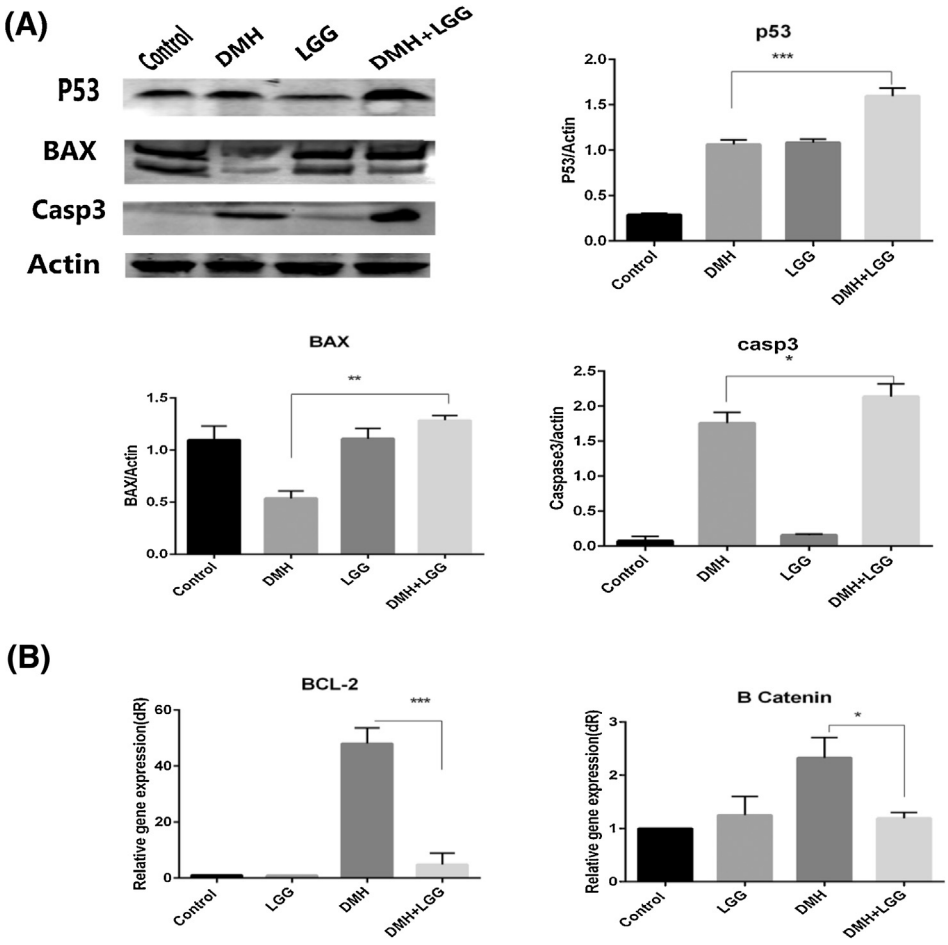

Fig. 3. LGG induce apoptosis and attenuates inflammation in DMH induced colon cancer in SD rats (A) Western blot analysis of p53, BAX and Casp3 from colon tissue samples (B) quantitative RT-PCR gene expression of BCL-2 and β-catenin relative to β-actin as endogenous gene control and Control group as calibrator. Results are presented as relative gene expression (dR) to the calibrator. Level of significance represents at * ($p < 0.05$), ** ($p < 0.001$), *** ($p < 0.0001$).

cytokines of NFκB. In this study, the increased expression of NFκB-p65 was associated with increased TNFα, COX-2 and VEGF as also reported by Huang et al. [32]; indicating the involvement of NFκB activation of these downstream cytokines involved in colon carcinogenesis but abated following probiotic LGG treatment in LGG + DMH group. In lung sepsis animal model, probiotic LGG attenuating effect on COX-2 and TNFα is been reported [33] and could similarly impact on colon carcinogenesis prevention via COX-2 and TNFα suppression in this study.

The induction of apoptosis is one of the major anti-tumor therapeutic direction of new treatments to evaluate their effectiveness on cell homeostasis [34,35]. In view of this, active apoptotic cells could be disabling various cellular target proteins and breaking down structural component of the cells [36]. Furthermore, deregulation of apoptosis is linked to several pathophysiological diseases including cancer [34]. The results of our studies show the induction of apoptosis of epithelial cells in LGG + DMH group but suppressed in DMH group. Not all, the observed increase in epithelial cell apoptosis in LGG + DMH group is associated with suppression of COX-2 contrary to the elevated COX-2 expression in DMH group associated with depressed apoptosis. High expression of COX-2, as observed in DMH group, is reported to prevent intestinal epithelial cells from apoptosis [37,38]. On the contrary, Caffrey et al. [39] reported that COX-2 inhibition induces apoptosis as observed in LGG + DMH treatment group. Inhibition of COX-2 is also known to activate downstream target p53 which has tumor suppression activity and a known apoptotic inducer [40]. In addition, Wu et al. [41] reported that LGG induces apoptosis through activation of p53 and Bax as well as suppression of anti-apoptotic protein Bcl-2. These reports confirms the findings in our study of p53 enhanced expression in LGG + DMH group associated with epithelial cell apoptosis but suppressed in DMH group linked with reduced epithelial cell apoptosis. Furthermore, the increase of TNF-α by cancer tumor cells is reported to activate survival pathways enabling cells to escape apoptosis by the upregulation of anti-apoptotic BcL-2 via NFκB activation [23]. LGG treatment reduced TNF-α and could be involved in its anticancer role via induction of apoptosis in LGG + DMH group.

β-catenin signaling has been shown to be involved in colorectal carcinogenesis and tumor progression [42] associated with intramucosal and invasive colorectal cancers [43]. High expression of β-catenin and low p53 expression in CRC is associated with tumor progression [44]. LGG is a potential antitumor agent as it significantly down-regulated β-catenin expression and increased p53 level in the DMH + LGG group than DMH group, associated with the prevention and progression of colon cancer in this study.

5. Conclusions

Treatment with probiotic *Lactobacillus rhamnosus* LGG reduces colon tumor incidence, volume and multiplicity.

Our data further suggests LGG anti-cancer effect associated with induction of epithelial cell apoptosis, and suppression of the NFκB signaling pathway associated with inflammation and holds a promise in the field of prevention of CRC as a bio-therapeutic agent.

Competing interests

The authors declare that they have no competing interests.

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