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Original Research Article

Lactobacillus rhamnosus confers protection against colorectal cancer in rats

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Abstract

Purpose: *To investigate the protective effect and mechanism of action of Lactobacillus rhamnosus against colorectal cancer (CRC).*
Methods: *A total of 40 healthy female Sprague Dawley rats weighing 100 – 140 g (mean weight = 120 ± 20 g) were used for this study. The rats were randomly assigned to four groups of 10 rats each: normal control group, L. rhamnosus group; 1, 2-dimethylhydrazine (DMH) group and treatment group. Rats in L. rhamnosus group were inoculated with L. rhamnosus (1 x 10^8 CFU/mL) orally for 20 weeks, while rats in DMH group received 35 mg DMH/kg /week intraperitoneally for 10 weeks for induction of CRC. Treatment group rats received 35 mg DMH/kg bwt intraperitoneally for 10 weeks for induction of CRC, and were treated with L. rhamnosus (1 x 10^8 CFU/mL) orally for 20 weeks. After 20 weeks, the rats were euthanized using ether anesthesia. Expressions of inflammatory, angiogenesis and pro-apoptotic genes were determined using Western blotting and real-time quantitative polymerase chain reaction (qRT-PCR).*
Results: *Treatment with L. rhamnosus significantly reduced the incidence of CRC in the rats ($p < 0.05$). The incidence of multiple tumors in the treatment group was also significantly reduced, when compared to DMH group ($p < 0.05$). The protein expressions of inducible nitric oxide synthase (iNOS), tumor necrosis factor α (TNF-α), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), cyclooxygenase-2 (COX-2), bcl-2 and vascular endothelial growth factor α (VEGF-α) were significantly upregulated in DMH group, when compared with normal control group ($p < 0.05$). However, treatment with L. rhamnosus significantly down-regulated the expressions of these proteins ($p < 0.05$). DMH treatment also significantly upregulated the expressions of iNOS, TNF-α, VEGF-α, NF-kB, β-catenin and bax genes ($p < 0.05$). However, L. rhamnosus significantly reversed the effects of DMH on the expression levels of these genes ($p < 0.05$).*
Conclusion: *These results show that L. rhamnosus prevents CRC via suppression of expressions of inflammatory and angiogenesis genes, and upregulation of apoptotic gene expression.*

Keywords: *Colorectal cancer, Lactobacillus rhamnosus, Probiotics, Angiogenesis, Expression*

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of cancer-related deaths worldwide. In 2012 alone, it accounted for about 1.4 million new cases and 0.7 million deaths [1]. Colorectal cancer is expected to increase up to 60 % globally by 2030, with more than 2.2 million new cases and 1.1 million deaths [2]. In 2012, the incidence of CRC was put at 253,000, accounting for 139,000 deaths in China [3]. The disease is treated using chemotherapy and radiotherapy. However, these treatments do not prevent recurrence, nor do they prolong survival [4].

In recent times, the use of diets especially probiotics as therapy has received huge attention [5]. Probiotics are live, non-pathogenic microorganisms that can be consumed as dietary supplements. They confer beneficial effects on the host by improving intestinal microbial balance. Within the gastrointestinal tract (GIT), the overgrowth of pathogenic microorganisms stimulates intestinal immunity-producing components that may be associated with increased risk of cancer development [6,7]. These microorganisms (probiotics), through different processes promote reduction of cancer risk. Thus, the replacement of pathogenic bacteria by probiotics restricts microbial overgrowth in the GIT and modulates the severity of the disease.

At present, most of the available probiotics contain lactic acid bacteria including *Lactobacillus* and *Bifidobacterium* species. These probiotics help to maintain intestinal microbial balance, decrease pathogenic bacteria inside the gut flora, improve bowel movement and restore gut microbiota in antibiotic-associated diarrhea. The use of probiotics as dietary supplements in the prevention of CRC has been reported [5,8-10]. However, the exact mechanism of cancer prevention by probiotics remains unknown, although some mechanisms have been proposed. These include carcinogen binding and degradation, stimulation of protective enzymes, production of anti-mutagenic compounds, prevention of DNA damage, and activation of local immune response [5].

In the last five decades, *Lactobacillus rhamnosus* GG strain has been used for the treatment of various GIT abnormalities, including CRC. This bacterium decreases the risk of CRC through several mechanisms such as modulation of gut microbiota, modulation of human dendritic cells, inhibition of harmful enzymatic activities, and production of pro-inflammatory effects.

Colorectal cancer (CRC) is a complex process involving various events which lead to the formation of lesions at molecular, cellular and morphological levels. Inflammation/lesion is the hallmark of cancer and involves the interplay of various inflammatory genes. The nuclear factor kappa B (NF-$_k$B), an inflammatory gene was first detected in patients with leukemia, lymphoma, colon cancer, pancreatic cancer, oral cancer and breast cancer. The cyclooxygenase-2 (COX-2) gene has also been shown to be associated with the development and progression of CRC. Other inflammatory cytokines that may be involved in the pathogenesis of CRC include iNOS, TNF-α and VEGF-α. The oncogene, bcl-2 and tumor suppressor gene, p53 are associated with cell proliferation and apoptosis [11]. The present study investigated the protective effect of *Lactobacillus rhamnosus* against CRC, and the underlying mechanism(s).

EXPERIMENTAL

Materials

Peroxidase-labeled secondary antibody, cDNA synthesis kit, total RNA purification kit, and primers of iNOS, TNF-α, VEGF-α, NF-$_k$B, COX-2, bcl-2, and β-actin were products of Sigma-Aldrich (USA). Image J software was purchased from the National Institute of Health (USA). NanoDrop spectrophotometer was purchased from Thermo Fischer Scientific Co., Ltd. (USA). Flex RT-PCR System was obtained from Applied Biosystems.

Bacterial strain

Lactobacillus rhamnosus GG 1.2134 (LGG) was obtained from China General Microbiological Culture Center (CGMCC 1.2134). The strain was grown in de Man-Rogosa-Sharpe (MRS) broth, and cells in logarithmic growth phase were selected and used in this study. The cells were centrifuged at 5000 g for 10 min at 4 ^{o}C, and washed with sterile phosphate-buffered saline (PBS, pH 7.2). Then, the pelleted cells were diluted to a concentration of 10^8 CFU/mL.

Rats

A total of 40 healthy female Sprague Dawley rats weighing 100 – 140 g (mean weight = 120 ± 20 g) were obtained from The Eighth Affiliated Hospital, Sun Yat-Sen University, Shenzhen, Guangdong Province, China. The rats were maintained in polypropylene cages (2 - 3 rats per cage) in a well-ventilated room. Prior to the initiation of the study, the rats were acclimatized to the environment for one week under controlled

conditions at a temperature of 23 ± 2 ^{o}C, humidity of 55 – 65 %, and 12 h light/2h dark cycle. They were allowed access to standard rat feed and clean drinking water. The study protocol was approved by the Eighth Affiliated Hospital Animal Care and Use Committee (XY2017-15). The study lasted 20 weeks.

Experimental design

The rats were randomly assigned to four groups of 10 rats each: normal control group, *L. rhamnosus* group, DMH group and treatment group. Rats in *L. rhamnosus* group were inoculated with *L. rhamnosus* (1 x 10^8 CFU/mL) orally for 20 weeks, while DMH group received 35 mg DMH/kg bwt/week intraperitoneally for 10 weeks for induction of CRC. Rats in the treatment group received 35 mg DMH/kg bwt intraperitoneally for 10 weeks for CRC induction, and were treated with *L. rhamnosus* (1 x 10^8 CFU/mL) orally for 20 weeks. After 20 weeks, the rats were euthanized using ether anesthesia and colon tissues were excised for analysis.

Western blotting

About 100 mg of the colon tissue from each euthanized rat was homogenized in ice-cold Tris-mannitol buffer (pH 7.2) using mechanical homogenizer until total disruption was achieved. Then, the homogenate was centrifuged at 10,000 g for 10 min at 4 ^{o}C. The resultant supernatant was collected and kept at - 40 ^{o}C until when required. Protein concentration was determined using Bradford method [13]. A portion of total tissue protein (10 µg) from each sample was separated on a 12 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 ° C for 120 min. Subsequently, non-fat milk powder (5 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37 ^{o}C and incubated to block non-specific binding of the blot [14].

Incubation of the blots was performed overnight at 4 °C with primary antibodies of rabbit polyclonal anti- iNOS (1:500), TNF-α (1:2000), VEGF-α (1:2000), NF-$_k$B (1:500), COX-2 (1:2000), β-actin (1:5000) and bcl-2 (1:2000). Then, the membrane was washed thrice with TBS-T and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 3 h at room temperature. The blot was developed using an X-ray film. Then, diaminobenzidine (DAB, 10 mg/15 mL of PBS with 15 µL H_2O_2) was added to the membrane in the dark till the protein band developed to the required intensity. Immediately after the band reached the required intensity, the membrane was washed with Milli-Q water and air-dried. Using image J software, the bands obtained were visualized and analyzed. The density was expressed as gray values in densitrometric units. The respective protein expression levels were normalized to that of β-actin which was used as a standard.

cDNA synthesis and quantitative Real-Time PCR (qRT-PCR)

Colon tissue was extracted using GenElute™ total RNA purification kit to obtain mRNA. The concentration and purity of the extracted RNA was determined spectrophotometrically at 260/280 nm. The RNA was reverse-transcribed to cDNA using First Strand cDNA synthesis kit. The qRT-PCR was performed using QuantStudio™ 7 Flex Real-Time PCR System. The PCR cycling conditions were: initial denaturation at 95 ^{o}C for 30 sec, followed by 40 cycles of 95 ^{o}C for 30 sec, 55 ^{o}C for 30 sec and extension at 72 ^{o}C for 1 min. Dissociation melt-curve analysis was performed to determine non-specific amplification. The mean threshold value for each cycle was normalized to the expression of β-actin. The relative expression of RNA was calculated using $2^{-\Delta\Delta Ct}$ method. The primer sequences used are shown in Table 1.

Table 1: Primers sequences used for gene expression

Gene	Primer sequence 5'-3'
β-Catenin	5'- ACTGGCAGCAGCAATCTTAC-3' 5'- GAGGTGTCCACATCTTCTTC-3'
TNF-α	5'- CTTCTGTCTACTGAACTTCG-3' 5'- AAGATGATCTGAGTGTGAGG-3'
NF-$_k$b	5'-GCTTACGGTGGGATTGCATT-3' 5'-TTATGGTGCCATGGGTGATG-3'
iNOS	5'-TAAAGGGACAGCGTCAGCGA-3' 5'-TGGGGGAACACAGTAATGGC-3'
Bax	5'- GGCGAATTGGAGATGAACTG-3' 5'- CCCCAGTTGAAGTTGCCAT-3'
VEGF-α	5'-TATATCTTCAAGCCGTCCTGTG-3' 5'-TCTCCTATGTGCTGGCTTTG-3'
β-actin	5'-TGT TTG AGA CCT TCA ACA CC-3' 5'-TAG GAG CCA GGG CAG TAA TC-3'

Statistical analysis

Data are expressed as mean ± SEM, and the statistical analysis was performed using SPSS (13.0). Groups were compared using Student *t*-tests. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Outcome of CRC induction

Treatment with *L. rhamnosus* significantly reduced the incidence of CRC in the rats ($p < 0.05$). The incidence of multiple tumors in the treatment group was also significantly reduced, when compared with DMH group ($p < 0.05$). These results are shown in Table 2.

Table 2: Incidence of CRC among the groups

Group n	Number of rats with tumor	Number of rats with multiple tumors
Normal control 10	0 (0.00 %)	0 (0.00 %)
L. rhamnosus 10	0 (0.00 %)	0 (0.00 %)
DMH 10	10 (100 %)	7 (70 %)
Treatment 10	6 (60 %)*	2 (20 %)*

*$P < 0.05$, when compared with *DMH* group

Expressions of inflammation- and angiogenesis-related proteins

The protein expressions of iNOS, TNF-α, NF-$_k$B, COX-2, bcl-2 and VEGF-α were significantly upregulated in DMH group, when compared with normal control group ($p < 0.05$). However, treatment with *L. rhamnosus* significantly down-regulated the expression of these proteins ($p < 0.05$; Figure 1).

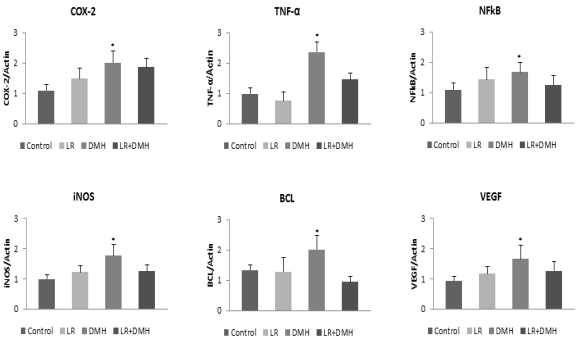

Figure 1: Protein expressions among the groups. *$P < 0.05$, when compared with normal control group

Levels of expression of inflammation- and angiogenesis-related genes

The expressions of iNOS, TNF-α, VEGF-α, NF-$_k$B and β-catenin genes were significantly upregulated by DMH ($p < 0.05$). In contrast, the expression of the pro-apoptotic gene, bax, was significantly down-regulated by DMH ($p < 0.05$). However, treatment with *L. rhamnosus* significantly reversed the effects of DMH on the expression levels of these genes ($p < 0.05$; Figure 2).

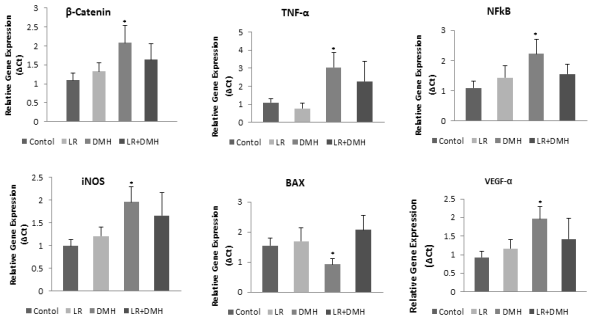

Figure 2: Gene expressions among the groups. *$P < 0.05$, when compared with normal control group

DISCUSSION

Colorectal cancer is one of the most dreadful tumors due to the fact that it is characterized by high morbidity and mortality. The use of probiotics has shown promising potential in the treatment of colorectal and other cancers. Dimethylhydrazine is a carcinogenic agent used to induce CRC with similar morphological and anatomical features as human colonic neoplasm [5-8]. Metabolites of DMH are highly specific, indirect carcinogens that produce CRC in a dose-dependent manner.

The doses of DMH and *L. rhamnosus* used in this study were obtained from literature [5,15]. The results of this study showed that treatment with *L. rhamnosus* significantly reduced the incidence of CRC in the rats. The incidence of multiple tumors in the treatment group was also significantly reduced, when compared with the DMH group. These results suggest that *L. rhamnosus* may be effective in reducing the incidence of tumor and its multiplicity, and are in agreement with those previously reported [5,16].

The mechanism by which probiotics prevent the development of cancer has not been fully elucidated. The NF-$_k$B is associated with pro-inflammatory response. This molecule mediates inflammatory response via induction of downstream cytokines such as iNOS, TNF-α and COX-2 [17]. Angiogenesis plays a vital role in the development of tumor and its progression.

In the present study, the expressions of NF-$_k$B protein and gene were significantly higher in DMH group than in the treatment group, and are in agreement with those previously reported [15]. The increased level of expression of NF-$_k$B was directly linked to increased expressions of iNOS, COX-2 and TNF-α in the DMH group. Increased

expression of COX-2 is associated with incidence of CRC, and altered COX-2 activity may reduce incidence of intestinal tumorigenesis.

The anti-inflammatory effect of *L. rhamnosus* is exerted via attenuation of the expressions of TNF-α and NF-$_k$B [18,19]. The reduced expressions of NF-$_k$B pathway proteins in the treatment group suggest that *L. rhamnosus* may be effective in preventing CRC. Therefore, a possible mechanism by which probiotics prevent development of CRC is by targeting the NF-$_k$B pathway. The enzyme COX-2 is activated during inflammation, and it promotes angiogenesis via stimulation of VEGF-α expression [20,21]. Angiogenesis, the formation of new blood vessels is an important process responsible for tumor growth and depends on TNF-α and VEGF-α. Another downstream cytokine of NF-$_k$B is VEGF-α.

In this study, the expression of VEGF-α was upregulated in the *DMH* group, when compared with normal control group. However, treatment with *L. rhamnosus* significantly suppressed the expression of NF-$_k$B and its downstream cytokines. Studies have shown that increased expression of NF-$_k$B is associated with increased levels of COX-2, VEGF and TNF-α [17]. These results suggest that NF-$_k$B downstream cytokines may be involved in the development of CRC, and are not in agreement with those reported in a previous study [22]. Apoptosis is a major anti-tumor process, and its deregulation is associated with cancer.

In this study, *L. rhamnosus* significantly increased the expression of the pro-apoptotic gene, bax, and significantly reduced its protein expression. Treatment with *L. rhamnosus* has been shown to induce apoptosis via activation of bax and suppression of bcl-2 [23]. Probiotics have been shown to upregulate bax protein expression and restore the normal expression of bcl-2 [5]. In the present study, the increased expression of bax and suppression of bcl-2 expression in the treatment group is an indication that the positive association between *L. rhamnosus* and epithelial cell apoptosis may be responsible for its anti-cancer effect. The inflammatory cytokine TNF-α induces the upregulation of bcl-2, an anti-apoptotic protein, and activates the survival pathway, thereby enabling cells to escape apoptosis [24].

The reduced expression of TNF-α in the treatment group suggests that the anticancer effect of *L. rhamnosus* may be exerted through induction of apoptosis and evasion of cell apoptosis. The cytokine TNF-α is associated with intra-mucosal and invasive CRC [25,26]. The tumor progression gene, *β*-catenin is involved in the pathogenesis of CRC. In this study, *β*-catenin gene expression was significantly higher in DMH group than in the treatment group, which is in agreement with those previously reported [15].

CONCLUSION

These results show that *L. rhamnosus* prevents the development of CRC via suppression of expressions of inflammatory and angiogenesis genes, and upregulation of apoptotic gene expression.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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