



Basic nutritional investigation

Administration of probiotic mixture DM#1 ameliorated 5-fluorouracil-induced intestinal mucositis and dysbiosis in rats



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ABSTRACT

Objective: The use of probiotics to alleviate chemotherapy-induced intestinal mucositis is supported by clinical consensus. However, no studies to date, to our knowledge, have systematically analyzed the effects of a probiotic mixture on chemotherapy-induced mucositis or assessed changes in the intestinal microbiota after probiotic treatment. The aim of this study was to report the effects of a probiotic mixture, DM#1, on intestinal mucositis and dysbiosis of rats treated with 5-fluorouracil (5-FU).

Methods: Twenty-eight male Sprague Dawley rats weighing 180 to 220 g were randomly divided into four groups: control, 5-FU, probiotic high (PH), and probiotic low (PL). Except for the control group, all other groups received intraperitoneal injections of 5-FU for 5 d, and the PH and PL groups received DM#1 intragastrically (1×10^9 or 1×10^8 colony-forming units/kg, respectively) for 8 d. One day after the last administration, rats were sacrificed and the ilea were removed for histopathologic assessment and evaluation of permeability, myeloperoxidase activity, levels of cytokines (interleukin [IL]-4, IL-6, tumor necrosis factor [TNF]- α), and mRNA of toll-like receptors (TLR; TLR2, TLR4, and TLR9). Additionally, intestinal microbiota profiles were analyzed by polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis and quantitative real-time PCR.

Results: Treatment with DM#1 ameliorated 5-FU-induced intestinal mucosal injury in rats, possibly by reducing proinflammatory cytokine levels and neutrophil infiltration. The increased intestinal permeability caused by 5-FU was ameliorated. These results were closely associated with the reestablishment of intestinal microbial homeostasis and alteration of the TLR2/TLR4 signaling pathway.

Conclusions: Administration of the probiotic mixture DM#1 ameliorated 5-FU-induced intestinal mucositis and dysbiosis in rats.

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Introduction

More than 50% of patients receiving the chemotherapy drug 5-fluorouracil (5-FU) develop either oral or intestinal mucositis [1]. Injury of the intestinal mucosal barrier is one of the most

debilitating side effects of 5-FU treatment and is associated with pain, bacteremia, and malnutrition [2]. These complications result from reduced enterocyte proliferation and migration and increased cell apoptosis, which combine to disrupt normal intestinal barrier function [3]. Destruction of the intestinal mucosa leads to reduced nutrient absorption and increased vulnerability to infection [4].

In addition to inducing intestinal mucositis, chemotherapeutics also have detrimental effects on the composition of the intestinal microbiota. A shift in composition from predominantly gram-positive to predominantly gram-negative bacteria was observed following administration of 5-FU in Lewis rats [5]. A regimen of 5-FU and irinotecan elevated *Clostridium* cluster XI and *Enterobacteriaceae* [6]. Because commensal bacteria play

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pivotal roles in both the innate and adaptive immune systems of the host [7], intestinal dysbiosis contributes greatly to the development of chemotherapy-induced mucositis and diarrhea [8]. Therefore, normalization of intestinal homeostasis could be an appropriate strategy to improve the status of patients receiving chemotherapy.

In recent years, the use of probiotics to alleviate damage to intestinal mucosa has been supported by clinical consensus [9]. Both live probiotic strains and factors derived from probiotic culture supernatants have been tested in animal models to examine their effects on 5-FU-induced intestinal mucositis. *Streptococcus thermophilus* TH-4 improved the mitotic count, decreased crypt fission, and reduced histologic deficits caused by 5-FU [10]. Administration of probiotic factors derived from *Escherichia coli* Nissle 1917 and *Lactobacillus fermentum* BR11 partially protected the intestine from 5-FU-induced mucositis [11]. Furthermore, treatment with *Lactobacillus acidophilus* improved the inflammatory and functional aspects of 5-FU-induced intestinal mucositis [12]. In contrast, *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* BB12 did not alleviate mucositis caused by 5-FU [13]. However, to our knowledge, no studies have systematically analyzed the effects of a probiotic mixture on 5-FU-induced mucositis or assessed changes in the intestinal microbiota after probiotic intervention.

In the present study, we investigated the effects of a probiotic mixture, DM#1, on intestinal mucositis and dysbiosis of rats treated with 5-FU. DM#1 includes four previously isolated probiotic strains: *Bifidobacterium breve* DM8310, *L. acidophilus* DM8302, *Lactobacillus casei* DM8121, and *S. thermophilus* DM8309. We also investigated changes in intestinal barrier function after treatment with 5-FU and/or DM#1 and evaluated signaling pathways that may be involved in the regulation of intestinal barrier function. The results of this study will provide a valuable complementary reference for clinical treatment as well as theoretical support for the development of targeted probiotic therapy.

Materials and methods

Bacterial culture

The probiotic preparation is a probiotic mixture of four strains, namely *B. breve* DM8310, *L. acidophilus* DM8302, *L. casei* DM8121, and *S. thermophilus* DM8309. They were originally isolated from Chinese fermented food and were deposited in the Culture Collection of Dalian Medical University (DMCC), Dalian, China. To identify the strains, 16 S rDNA were amplified by polymerase chain reaction (PCR) and submitted to sequencing. The partial sequences of 16 S rDNA of the four strains were deposited in the National Center for Biotechnology Information (NCBI) GenBank under the accession number KP967559–KP967561.

To prepare the probiotic mixture, the probiotics were first inoculated into the corresponding culture medium—bismuth sulfite agar medium for *Bifidobacterium*, *Lactobacillus* selective agar medium for *Lactobacillus*, and *Streptococcus* selective agar medium for *Streptococcus*—and they were cultured under anaerobic condition, at 37°C for 24 h. After that, single colonies were picked up and inoculated in the corresponding liquid medium, and cultured under the same conditions for 24 h. The population of bacteria was calculated by optical detection at 600 nM. Different volumes of each bacterium were mixed and then centrifuged at 5000g, at 4°C for 2 min, and resuspended in 2 mL phosphate-buffered saline (PBS) and administered to each rat intragastrically 1 d at a time.

Animal experiments

Animal experiments were performed under the approval of Dalian Medical University Institutional Animal Care and Use Committee in accordance with the laboratory's animal ethics guidelines (SYXK [Liao] 2014-0002). Twenty-eight male SD rats weighting 180 to ~220 g (obtained from the SPF animal center of Dalian Medical University) were kept in a temperature-controlled room with ad libitum access to water and fasted for 24 h before all experiments. The rats were randomly divided into four experimental groups, the 5-FU group (n = 7) was intraperitoneally injected with 5-FU (30 mg/kg DBL®, Mayne Pharma Pty Ltd.,

Victoria, Australia) from day 1 to day 5; the probiotic low (PL) group (n = 7) and the probiotic high (PH) group (n = 7) were intraperitoneally injected with 5-FU (30 mg/kg in PBS) once a day for 5 d, and were intragastrically administered 1×10^9 colony-forming unit (CFU)/kg or 1×10^8 CFU/kg of probiotic consortium suspended in 2 mL PBS once a day for 8 d, respectively. The rats in the control and the 5-FU group were administered 2 mL of PBS daily. Mortality, food and water intake, body weight (BW), and fecal and urine output were recorded daily. At day 9, all rats were sacrificed via carbon dioxide overdose and cervical dislocation. The gastrointestinal tract was removed and emptied of contents. Separate 4-cm sections of distal ileum were collected in 10% buffered formalin. The blood and feces of rats were collected and frozen immediately at –80°C until detection.

Histologic analysis

The paraffin-embedded specimens of distal ileum were sectioned (5 µm) and stained with hematoxylin and eosin (H&E). Mean villus height and crypt depth measurements were obtained by evaluating 40 villi and crypts per section [14]. A qualitative histologic assessment of damage was taken from an earlier study [15]. Analysis of the distal ileum was conducted using seven parameters each individually scored from 0 (normal) to 3 (maximal damage), to achieve an overall damage severity score of ≤21. The parameters analyzed were villus fusion and stunting/villus: crypt ratio; enterocyte disruption; reduction in goblet cell numbers; crypt disruption; crypt cell disruption; thickening/edema of the submucosa and thickening of the muscularis externa. The median scores for each group were then compared.

Myeloperoxidase activity

Neutrophil infiltration in the ileum was determined by myeloperoxidase (MPO) assay. Forty-eight segments (4 cm; two samples from each rat were taken) of tissue were thawed and homogenized in 1 mL saline. Aliquots (200 µL) were then centrifuged (1300g, 10 min) and the pellet resuspended in hexadecyltrimethylammonium bromide buffer and vortexed for 2 min to release MPO from the tissue. Samples were then centrifuged (800g, 2 min) and 50 µL of SN added, in duplicate, to a 96-well plate. Hydrogen peroxide and O-dianisidine reaction mixture were then added to each well and absorbance of the reaction mixture measured at 450 nm (Sunrise Microplate Absorbance Reader, Tecan Austria GmbH, Grödig, Austria) at 1 min intervals for 15 min.

Assessment of gut permeability

Gut permeability was measured as described previously with minor modifications [16]. The segments of distal ileum were opened along the mesenteric border and mounted in the Ussing chamber with an aperture of 0.3 cm². The chamber was connected to a VCC MC6 amplifier, controlled and monitored using the Acquire & Analyze software (V2.3.177 Physiologic Instruments, San Diego, CA, USA). Experiments were carried out under current-clamp (open-circuit) conditions as described previously [17]. Segments were incubated in oxygenated (95% O₂; 5% CO₂) bicarbonate buffer at 37°C. Tissue was equilibrated for 15 min, thereafter a 3 mA current pulse was applied across the intestinal wall every 6 s for 30 min. The transepithelial potential was measured and recorded by the Acquire & Analyze software, and the change in potential induced by the current pulse was used to calculate transepithelial resistance (TER) according to Ohm's Law. Higher values of TER represent lower gut permeability.

Measurement of endotoxin and cytokines levels

The concentration of plasma endotoxin was determined using enzyme-linked immunosorbent assay (ELISA) using protocols supplied by the manufacturer (Uscn Life Science Inc., Wuhan). To detect cytokines levels, the ileum samples of rats were removed, flushed with PBS, and resuspended in tissue culture plates (Falcon 3046; Becton Dickinson Labware, Lincoln Park, NJ, USA) in RPMI 1640 medium supplemented with 50 mM 2-mercaptoethanol (2-ME), 10% fetal calf serum, streptomycin (100 U/mL), and penicillin (100 U/mL). Cultures were incubated at 37°C in 5% CO₂ for 24 h. Supernatants were harvested and stored at 70°C for cytokine-level quantification. Levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-4 in culture supernatants were measured by using ELISA kits (Uscn Life Science Inc., Wuhan) according to manufacturer's instructions. The results were expressed as pg/mL.

Denaturing gradient gel electrophoresis profiling

The meta-genomic DNA was extracted from the frozen cecal content of rats by the QIAamp DNA stool mini kit (Qiagen, Germany). PCR was conducted using universal primers F338+GC clamp and R518 targeting the hyper-variable V3 region of 16 S rRNA gene [18]. The resulting 16 S rDNA amplicons were analyzed using the DCode system (Bio-Rad, Hercules, California, USA) according to a previous description [18]. The digitized denaturing gradient gel electrophoresis

(DGGE) images were analyzed with Quantity One image analysis software (version 4.6.1, Bio-Rad). Phoretix 1 D (Phoretix International, UK) was used to analyze the abundance and relative intensity in the DGGE [19]. Similarities were displayed graphically as a dendrogram. The Shannon-Wiener index of diversity (H') [20] was used to determine the diversity of taxa present in fecal samples collected from the hyperthyroid and healthy group. This index was calculated by $H' = -\sum(p_i)(\ln p_i)$, where p_i was the proportion of the bands in the track and was calculated as follows: $P_i = n_i/\sum n_i$, where n_i was the average density of peak i in the densitometric curve. The evenness (E) that reflected uniformity of bacterial species distribution also was computed. This index was calculated by $E = H'/\ln S$, where S was the number of bands.

Sequence analysis

To identify some separated and strong bands, bands were excised under ultraviolet transilluminator. Gel slices were washed then kept in sterile water overnight at 4°C for diffusion. Four μ L of the solution was taken as the DNA template for reamplifying by PCR using 341 F and 518 R. The PCR products were purified from the gel, then cloned into the PMD18-T Easy vector (TakaRa, Japan), and transformed into *Escherichia coli* Nova blue cell. Positive clones that met the DGGE criteria were selected, and their cultures were subjected to sequencing (TakaRa, Dalian). Finally, the sequences were compared directly with those in GenBank by Blast search (NCBI).

Quantitative real-time PCR evaluation of intestinal microbial groups

Specific primers used to characterize the population of different bacterial groups by quantitative real-time PCR (qPCR) are listed in Supplementary Table 1. PCR amplification and detection were performed with Thermal Cycler Dice™ Real Time System (TaKaRa, Dalian, China). Each reaction mixture (20 μ L) consisted of 10 μ L SYBR Premix Ex Taq (TaKaRa), 0.75 mL of each of the specific primers at a concentration of 10 mM, and 60 ng of meta-genomic DNA extracted from the cecal content. The fluorescent products were detected at the last step of each cycle. A melting curve analysis was made after amplification to distinguish the targeted PCR product from the nontargeted PCR product. Bacterial concentration from each sample was calculated by comparing the Ct values obtained from the standard curves. Standard curves were created using serial 10-fold dilution of pure cultures of DNA, corresponding to 10^2 to 10^9 cells from the culture collection. Bacterial quantity was expressed as log10 bacteria/g of fecal content.

qPCR detection of TLRs mRNA expressions in rat intestine

Ileum samples from rats were extracted and preserved in RNeasy lysis solution. RNA extractions were carried out with the RNeasy mini Kit (Qiagen, Hilden, Germany), and the complementary DNA (cDNA) was synthesized using the AffinityScript Multiple Temperature cDNA synthesis Kit (Stratagene, La Jolla, CA, USA) according to the supplier's protocol. cDNAs obtained by reverse transcription were used to determine mRNA expression levels of TLRs by the specific primers listed in Supplementary Table 1. Reactions were run using the StepOnePlus Real-Time PCR System (Life Technologies, Carlsband, CA, USA). The reaction mixture (10 μ L) comprised 4.5 μ L FastStart Universal SYBR Green Master (Roche, Mannheim, Germany), 0.5 μ L of each primer 30 μ M (Table 1), 2.5 μ L of sterile distilled water, and 2 μ L of DNA template (100 ng/ μ L). Each sample was run in triplicate, and the mean Ct was determined from the three runs. Relative mRNA expression was expressed as Δ Ct = Ct TLR – Ct calibrator. GAPDH house-keeping gene expression was used as calibrator after verification of its stability under our experimental conditions. Then, relative mRNA expression was calculated as $\Delta\Delta$ Ct = Δ Ct treatment – Δ Ct control. Finally, the relative gene expression levels were converted and expressed as fold difference ($= 2^{-\Delta\Delta$ Ct}).

Statistical analysis

All values were expressed as mean \pm SEM. Group comparisons were statistically analyzed using analysis of variance followed by Bonferroni's test with the

Table 1
Microbial diversity index analysis among different experimental groups

Diversity index	Control	5-FU	5-FU + PL	5-FU + PH
Number of bands	41.67 \pm 2.51	28.67 \pm 4.93*	35.33 \pm 0.58†	38.33 \pm 4.04†
H'	3.57 \pm 0.06	3.16 \pm 0.23*	3.38 \pm 0.05†	3.51 \pm 0.12†
H'_{\max}	3.73 \pm 0.06	3.34 \pm 0.18*	3.57 \pm 0.01†	3.64 \pm 0.11†
E	0.96 \pm 0.01	0.94 \pm 0.02	0.95 \pm 0.01	0.97 \pm 0.01

5-FU, 5-fluorouracil; PH, probiotic high; PL, probiotic low

Values are means \pm SD

* $P < 0.05$ compared with control group.

† $P < 0.05$ compared with 5-FU group.

assistance of GraphPad Prism Program (Version 5.01, GraphPad Software Inc., LaJolla, CA, USA). Values were considered significant at $P < 0.05$.

Results

Effects of DM#1 on mortality, body weight, and metabolism parameters of 5-FU-treated rats

The experiment design is shown in Figure 1A, during the 8-d experiment, two rats died after 5-FU treatment in the model group (5-FU group) on days 3 and 4, respectively (Fig. 1B). In the low-dose (5-FU + PL) and high-dose (5-FU + PH) probiotics-treated groups, one rat from each group died on day 4. No control group rats died.

The average BW of rats in the control group increased from 202.29 \pm 12.88 g to 239.14 \pm 15.65 g throughout the study period, the changing of body weight (Δ BW) increased by 36.86 \pm 7.03 g (Fig. 1C). In contrast, weight in the 5-FU group decreased considerably (58.43 \pm 4.08 g) with the lowest weight registered on the last day after 5-FU administration. The BWs of rats in the 5-FU + PL and 5-FU + PH groups decreased by 42.29 \pm 7.61 g and 45.43 \pm 10.39 g, respectively, which were significantly less than the loss of average BW of 5-FU group ($P = 0.0141$, $P = 0.0214$ compared with 5-FU).

The metabolism data, including water and feed consumption, and urine and fecal output were recorded from days 0 to 8, although no significant differences between groups were observed (all $P > 0.05$). No significant differences were observed among treatment groups on individual days compared with 5-FU group (all $P > 0.05$, data not shown). Furthermore, in the period after 5-FU or probiotic administration, no differences were observed among groups for water consumption, urine and fecal output, and feed consumption (all $P > 0.05$, data not shown).

Histologic analysis and severity scoring

Segments of rat ileum were fixed and stained with H&E. Measurements of villus heights and crypt depths were performed via microscopy. Forty intact and well-oriented villi and crypts were measured and averaged for each sample. The microscopy analysis was double-blinded. Rats treated with 5-FU presented the following histopathologic changes in the ileum (Fig. 2A): mucosa with significantly shortened villi, goblet cell depletion, and intense inflammatory infiltrate compared with the control rats. The probiotic-treated groups experienced a significant improvement of histopathologic changes.

Treatment with 5-FU significantly increased the histologically determined severity score in the ileum for all treatment groups compared with healthy controls (all $P < 0.05$; Fig. 2B). Importantly, both the PL- and PH-treated rats displayed significantly lower severity scores compared to 5-FU-treated rats ($P = 0.0058$, $P = 0.0001$).

The villus height, crypt, and mucus depth in the ileum were significantly reduced in the 5-FU-treated rats (all $P < 0.05$; Fig. 2C–E), on the contrary, the crypt depth of rats receiving the probiotic mixture was not affected by 5-FU treatment, and the reduction of villus height and mucus depth was less pronounced in these rats, suggesting a protective effect. However, no significant differences were detected between the PH and PL groups (all $P > 0.05$).

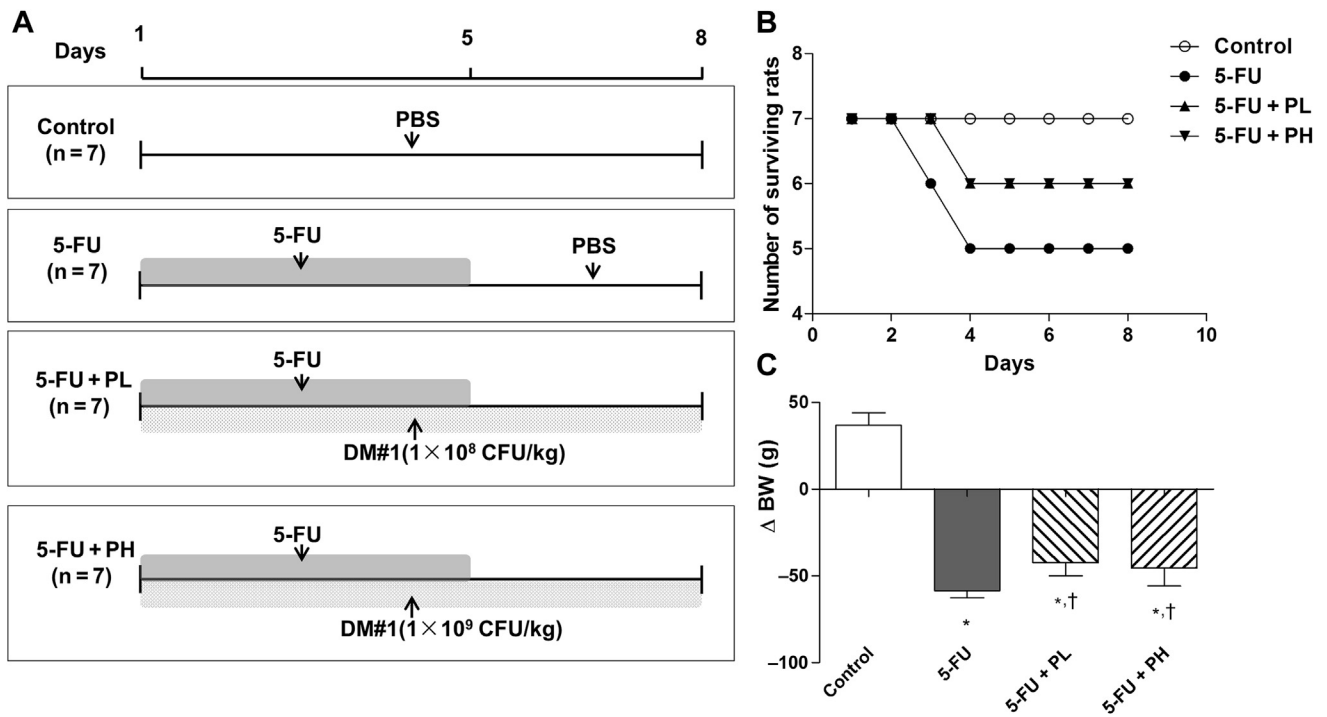


Fig. 1. The experiment design, mortality, and changing of rats' body weight. (A) Experiment design. The 5-FU group ($n = 7$) was intraperitoneally injected with 5-FU (30 mg/kg) from day 1 to day 5; the 5-FU + PL group ($n = 7$) and the 5-FU + PH group ($n = 7$) were intraperitoneally injected with 5-FU (30 mg/kg in saline) once a day for 5 d, and were intragastrically administrated 1×10^9 CFU/kg or 1×10^8 CFU/kg of probiotic mixture suspended in 2 mL PBS once a day for 8 d, respectively. The rats in control group and 5-FU group were administrated 2 mL of PBS every day. (B) Number of surviving rats in each group during experimental process. (C) Changing of rats' mean body weight in each experimental group. * $P < 0.05$ compared with control group; † $P < 0.05$ compared with 5-FU group. 5-FU, 5-fluorouracil; CFU, colony-forming unit; PBS, phosphate-buffered saline; PH, probiotic high; PL, probiotic low.

MPO activity of 5-FU-treated rats was ameliorated by DM#1

After treatment with 5-FU, the animals experienced a significant increase in neutrophil infiltration in the ileum (8.51 ± 1.02 UMPO/mg) compared with the control group (2.36 ± 0.66 UMPO/mg; $P = 0.0001$). Treatment with the probiotic mixture significantly reduced neutrophil infiltration in the ileum (5-FU + PL: 3.00 ± 0.19 UMPO/mg, 5-FU + PH: 2.09 ± 0.39 UMPO/mg) compared with those of the 5-FU group ($P = 0.0001$, $P = 0.0001$; Fig. 3A).

Levels of intestinal proinflammatory cytokines were reduced by DM#1

Figure 3B–E show that the rats treated with 5-FU present significantly increased levels of IL-4, IL-6, and TNF- α of the ileum (155.5%, 163.9%, and 159.8%, respectively) compared with those of the controls ($P = 0.0081$, $P = 0.0045$, $P = 0.0009$, respectively). This suggested a severe pattern of intestinal mucositis in rat. On contrast, the increases in IL-4, IL-6, and TNF- α concentration were significantly ($P = 0.0001$, $P = 0.0062$, $P = 0.0144$, compared with 5-FU group) reversed by treatment with the probiotic mixture. Compared with PL treatment, the PH mixture did not show superior effects in ameliorating the secretion of proinflammatory cytokines (all $P > 0.05$).

DM#1 affected intestinal permeability and plasma endotoxin level

The TER of the distal ileum in 5-FU-treated rats was reduced by 50.72% compared with the control rats (30.8 ± 5.5 versus

62.5 ± 6.5 Ohm cm^2 , $P = 0.0381$; Fig. 3E), suggesting a dramatic increase of intestinal permeability. Administration of the probiotic mixture resulted in elevation of TER values to 42.6 ± 4.3 (5-FU + PL group), and 45.8 ± 6.0 (5-FU + PH group) Ohm cm^2 , respectively, which were significantly higher than the 5-FU group ($P = 0.0453$, $P = 0.0349$), suggesting reduction of intestinal permeability. No significant difference was detected between the 5-FU + PL group and the 5-FU + PH group ($P > 0.05$).

The plasma endotoxin of rats was detected by ELISA. The results are presented in Figure 3F. In control rats, the endotoxin level was 0.08 ± 0.01 EU/mL. A significant increase (0.85 ± 0.21 EU/mL, $P = 0.0001$) in endotoxin level was observed in 5-FU-treated rats. In the probiotic-treated groups, the endotoxin levels of rats were much lower than that of 5-FU group ($P = 0.0001$, $P = 0.0001$) but still dramatically higher than the control group ($P = 0.0010$, $P = 0.0017$). No significant difference was detected between the 5-FU + PL group and the 5-FU + PH group ($P > 0.05$).

Intestinal microbial composition of 5-FU-treated rats was modified by DM#1

The intestinal microbiota of rats in different experimental groups was analyzed by PCR-DGGE using universal primers targeting the V3 region of 16 S rRNA (Fig. 4A). The DGGE fingerprint indicated that the complexity of microbiota in the 5-FU group differed significantly from that of the control group (Table 1), as the mean number of bands was 28.67 ± 4.93 in 5-FU group and 41.67 ± 2.51 in control group, respectively ($P = 0.0017$). The complexity of microbiota in the two probiotic-treated groups showed some amelioration, as the mean number of bands was

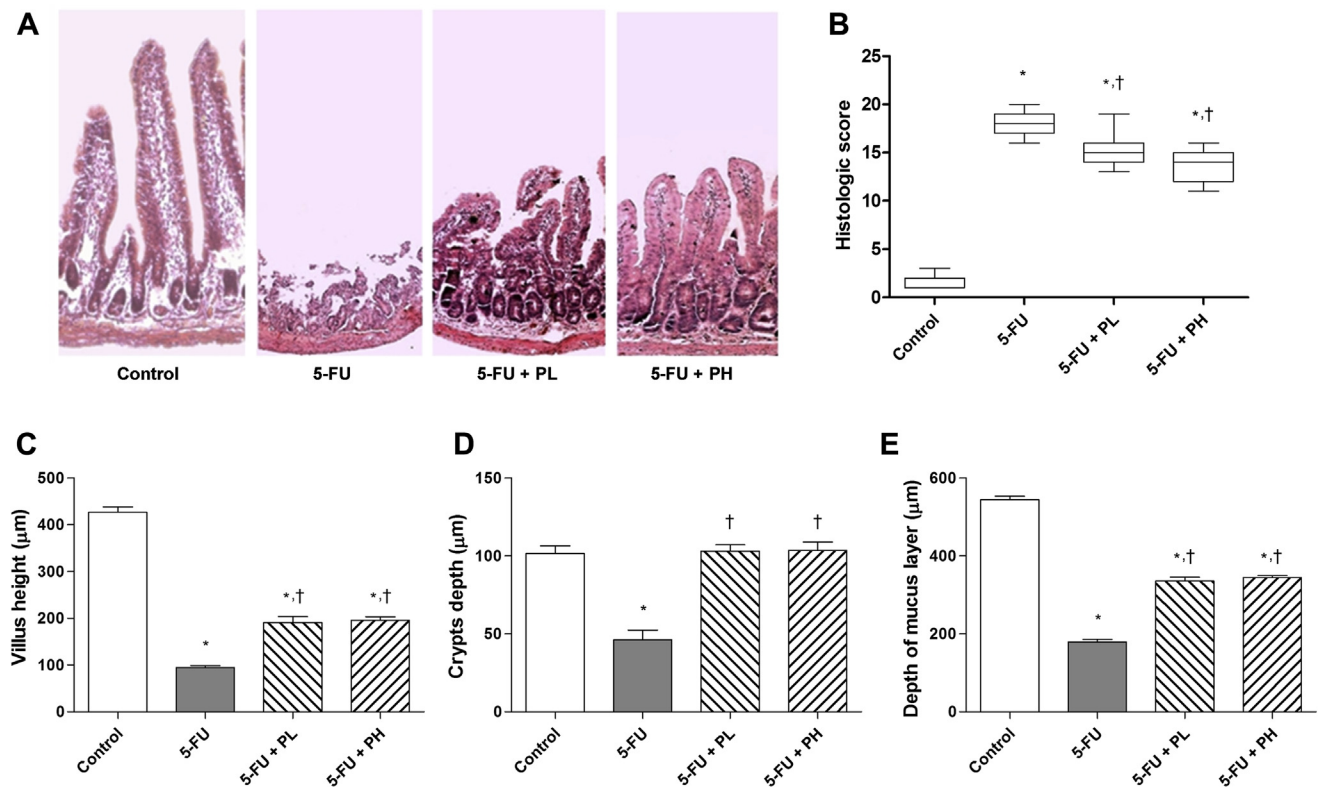


Fig. 2. Administration of DM#1 reduced intestinal damage in rats caused by 5-FU. (A) Photomicrographs (×200) of the ileum of rats treated with PBS (control), 5-FU, and probiotic mixture (5-FU + PL, 1×10^8 CFU/kg; 5-FU + PH, 1×10^9 CFU/kg). Note that treatment with DM#1 reversed 5-FU-induced shortening of the villi. Segments of the ileum were processed to measure the histologic score (B), the height of the villi (C), crypt depth (D), and the depth of the mucus layer (E). Values are expressed as the mean \pm SEM. The data were submitted to analysis of variance followed by Bonferroni's test. * $P < 0.05$ compared with the control group (PBS only). † $P < 0.05$ compared with the 5-FU group (5-FU + PBS). 5-FU, 5-fluorouracil; CFU, colony-forming unit; PBS, phosphate-buffered saline; PH, probiotic high; PL, probiotic low.

35.33 ± 0.58 in 5-FU + PL group and 38.33 ± 4.04 in 5-FU + PH group, which are much closer to that of control group, and significantly higher than 5-FU group ($P = 0.0450$, $P = 0.0089$). The dendrogram constructed based on analysis of the DGGE profile supported that they joined in different clusters. Figure 4B displays that there were two main clusters in the dendrogram. Lanes 1, 2, 3 from the control group, 7, 8, 9 from the 5-FU + PL group, and 10, 11, 12 from the 5-FU + PH group joined in one cluster, whereas 4, 5, and 6 from the 5-FU group formed the other cluster.

In Figure 4A, five bands that were obviously different between the control and 5-FU group were selected and excised for sequencing analysis. To verify the resolution capability of DGGE, bands in the same position but in different lanes were cut and sequenced. Results from sequencing suggested they belong to same bacterial groups, and are summarized in Supplementary Table 2. Surprisingly, all of the selected bacteria belonged to the phylum of *Firmicutes*. Bands a and b showed 98% and 94% similarity to a *Lactobacillus* strain and an uncultured bacterial clone, respectively. Bands c and d were identified as bacteria that belong to the genus of *Clostridium* (cluster XIVa and III, respectively). Bands e and f showed high similarity to *Lachnospiraceae* bacterium ACC2 (97%) and *Enterococcus faecium* Aus0004 (99%).

qPCR results (Fig. 4C) revealed that the abundance of total bacteria was significantly reduced by 5-FU treatment ($P = 0.0020$), the abundance of *Lactobacillus* group, the genus of *Enterococcus*, the family of *Lachnospiraceae*, and groups of *Clostridium* cluster III and XIVa in 5-FU-treated rats were

significantly altered compared with the control rats ($P = 0.0001$, $P = 0.0422$, $P = 0.0389$, $P = 0.0000$, $P = 0.0000$, respectively). Rats of the two probiotic-treated groups showed increased populations of *Lactobacillus* group, *Clostridium* cluster III and XIVa compared with the 5-FU-treated rats (5-FU + PL versus 5-FU, $P = 0.001$, $P = 0.0084$, $P = 0.0001$; 5-FU + PH versus 5-FU, $P = 0.0001$, $P = 0.0056$, $P = 0.0006$) and decreased abundance of *Enterococcus* spp., and *Lachnospiraceae* (5-FU + PL versus 5-FU, $P = 0.0421$, $P = 0.0424$; 5-FU + PH versus 5-FU, $P = 0.0201$, $P = 0.0456$). However, the differences between them are not statistically significant (all $P > 0.05$).

Ileac TLRs expression pattern in 5-FU-treated rats were reversed by DM#1

Figure 5 shows different expression patterns of TLR2, TLR4, and TLR9 mRNA in the ileum of rats. 5-FU treatment significantly increased the mRNA expression of TLR2 (by 2.04 ± 0.68 folds, $P = 0.0251$), and it slightly affected the mRNA expression of TLR4 (increased by 0.35 ± 0.08 folds, $P = 0.0480$) and TLR9 (decreased by 0.15 ± 0.05 folds, $P = 0.0475$) in the ileum of rats. Administration of the probiotic mixture reduced the expression levels of TLR2 and TLR4, the high-dose probiotic treatment even reversed the expression to the levels that are comparable to control (all $P > 0.05$ compared with control). However, no significant difference was detected between the 5-FU + PL group and the 5-FU + PH group (all $P > 0.05$).

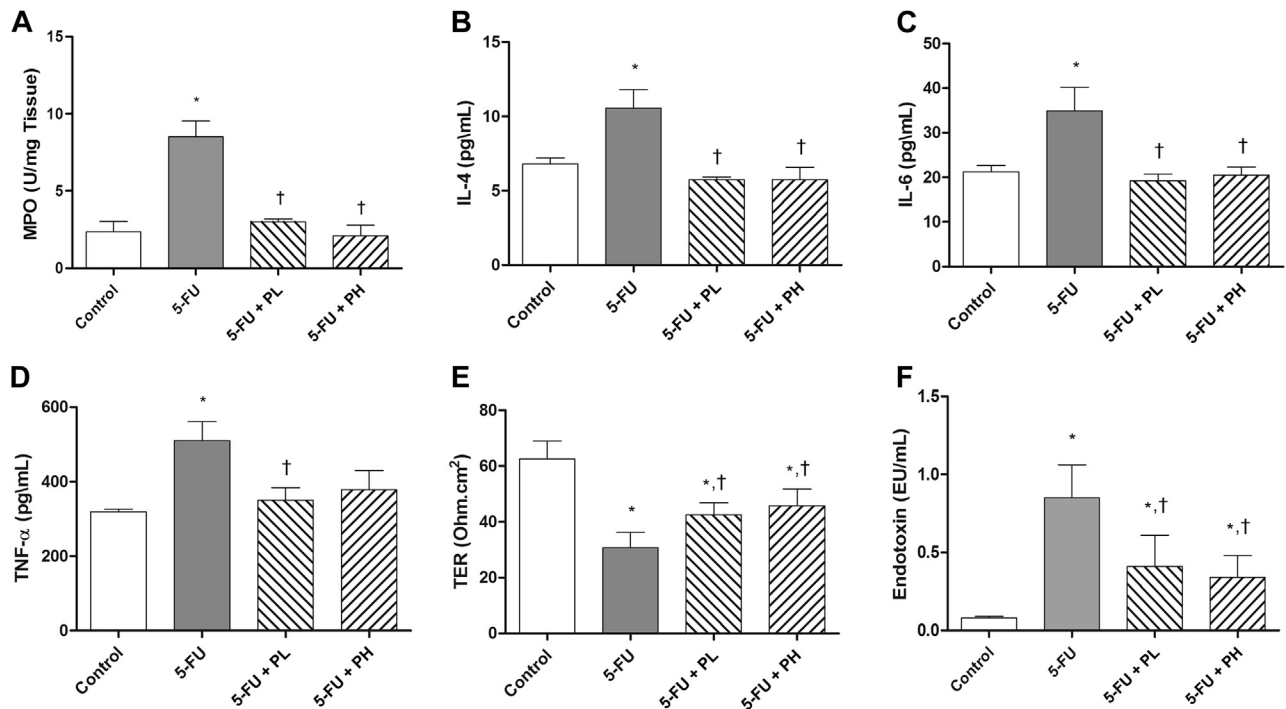


Fig. 3. Administration of DM#1 improved inflammatory parameters and permeability of rats with 5-FU-induced intestinal mucositis. The levels of MPO activity (A), the proinflammatory cytokines IL-4 (B), IL-6 (C), TNF- α (D), the ileum permeability (E), and plasma endotoxin (F) in each experimental group were detected. The results are presented as the mean \pm SEM. The data were submitted to analysis of variance followed by Bonferroni's test. * $P < 0.05$ compared with the control group (PBS only). † $P < 0.05$ compared with the 5-FU group (5-FU + PBS). 5-FU, 5-fluorouracil; CFU, colony-forming unit; IL, interleukin; MPO, myeloperoxidase; PBS, phosphate-buffered saline; TNF, tumor necrosis factor.

Discussion

The normal microbiota dwelling in the human intestinal tract antagonizes the growth of pathogens, promotes immune function, affects nutritional status, plays a role in detoxification, and has antiaging effects [21]. Therefore, the intestinal microbiota is important to the health of the host. Patients receiving cytotoxic therapy exhibit marked changes in the structure and composition of the intestinal microbiota. These changes most frequently include decreases in *Bifidobacterium* spp., *Clostridium* cluster XIVa, and *Faecalibacterium prausnitzii* and increases in *Enterobacteriaceae* and *Bacteroides* spp. [22]. Several animal studies have shown a drastic shift from commensal bacteria (i.e., *Bifidobacterium* and *Lactobacillus* spp.) to *Escherichia*, *Clostridium*, and *Enterococcus* spp. following a single intraperitoneal dose of 5-FU. The present study also indicated a dramatic change in the intestinal microbiota: Both the total abundance of bacteria and the populations of individual microbial groups (such as *Lactobacillus* spp. and *Clostridium* cluster XIVa) were significantly different between 5-FU-treated rats and control rats. However, in contrast to findings from previous studies, the intestinal population of *Clostridium* cluster III was reduced by 5-FU treatment, whereas the abundance of *Lachnospiraceae* increased markedly. These groups have been reported to play important pathologic roles in inflammation of the intestinal mucosa [23].

An altered intestinal ecology is closely associated with the pathology of mucositis after administration of chemotherapy [9]; therefore, normalization of intestinal homeostasis could be an appropriate strategy to improve the status of patients receiving such therapy. Several studies have reported different effects of

probiotics or factors derived from probiotic culture supernatants on mucositis induced by 5-FU [11]. However, to our knowledge, this is the first study to evaluate the effects of a probiotic mixture on 5-FU-induced intestinal dysbiosis and mucositis. Mixtures of probiotic strains from different genera have been successfully applied in the treatment of disease. The multistrain probiotic product VSL#3 was reported to reduce chronic inflammation in a mouse model of colitis [24], and it exhibited anti-inflammatory activity via the PI3 K/Akt and nuclear factor κ B (NF- κ B) pathways in a rat model of dextran sulfate sodium-induced colitis [25]. Furthermore, a mixture of three *Lactobacillus* strains enhanced the integrity of the gut mucosa in germ-free mice [26]. Ingestion of a mixture of *Lactobacillus gasseri* KS-13, *Bifidobacterium bifidum* G9-1, and *Bifidobacterium longum* MM-2 induced a less inflammatory cytokine profile and a potentially beneficial shift in the gut microbiota in older adults [27]. A mixture of *C. butyricum* and *Bifidobacterium infantis* restored the fecal microbiota and attenuated systemic inflammation in mice with antibiotic-associated diarrhea [28]. One study compared the effects of individual probiotic strains—*L. acidophilus* HB56003, *B. longum* HB55020, and *Streptococcus faecalis* HB62001—and a mixture of the three strains on postinfectious irritable bowel syndrome and showed that the mixture was superior in restoring intestinal barrier function and reducing inflammation [29].

Given the intestinal dysbiosis induced by 5-FU, we decided to use a mixture of four probiotic strains to treat rats with 5-FU-induced mucositis. The strains were originally isolated from traditional Chinese fermented foods. Preliminary safety evaluations conducted in mice showed that the strains had no effects

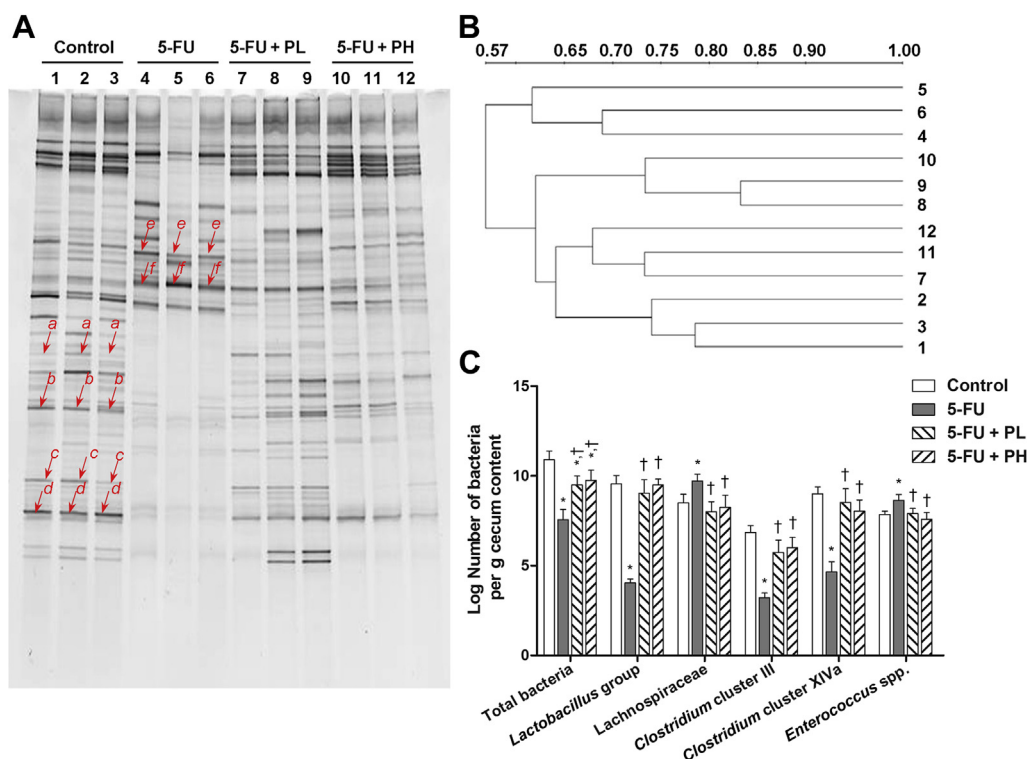


Fig. 4. The intestinal profile of rats in different experimental groups. (A) The V3 region of the 16S rRNA gene profile in rats of the control group (lanes 1, 2, 3), 5-FU group (lanes 4, 5, 6), 5-FU + PL group (lanes 7, 8, 9), and 5-FU + PH group (lanes 10, 11, 12). Bands 1A, 2A, 1B, 2B, 3B, 1C, 2C, 3C, 1D, 2D, 3D, 4D, 4E, 5E, 6E, 4F, 5F, and 6F were cut for sequencing. (B) Dendrogram of DGGE profiles analyzed by the unweighted pair group method with arithmetic mean method. (C) The population of selected bacterial groups in fecal contents of rats in different groups detected by qPCR. Data are presented as the average estimate of logarithms of bacterial copy numbers in 1 g of fecal contents. * $P < 0.05$ compared with the control group (PBS only). † $P < 0.05$ compared with the 5-FU group (5-FU + PBS). 5-FU, 5-fluorouracil; CFU, colony-forming unit; DGGE, denaturing gradient gel electrophoresis; PBS, phosphate-buffered saline; PH, probiotic high; PL, probiotic low; qPCR, quantitative real-time polymerase chain reaction.

on mortality, BW, or bacterial translocation and had no plasmids. The strains also had no effects on serum maleic dialdehyde (MAD) and liver glutathione levels (Supplementary Tables; Supplementary Fig. 1). Results of the present study demonstrate that the mixture has a remarkable ability to reestablish the intestinal homeostasis of 5-FU-treated rats and is especially effective in restoring *Lactobacillus* spp. and *Clostridium* clusters III and XIVa.

Normalization of the intestinal microbiota in probiotic-treated rats may contribute greatly to the improvement of

intestinal barrier function. Our study shows that DM#1 can reduce levels of proinflammatory cytokines and neutrophil infiltration in the intestine, as well as gut permeability and plasma endotoxin concentrations, in 5-FU-treated rats. Proinflammatory cytokines such as TNF- α and IL-6 contribute to the severity and maintenance of injury in intestinal mucositis [30], and IL-4 was found to participate as a proinflammatory cytokine in a model of 5-FU-induced intestinal damage [31]. Thus, the reduction of these cytokines suggests that the probiotic mixture has strong anti-inflammatory activity. An increase in intestinal

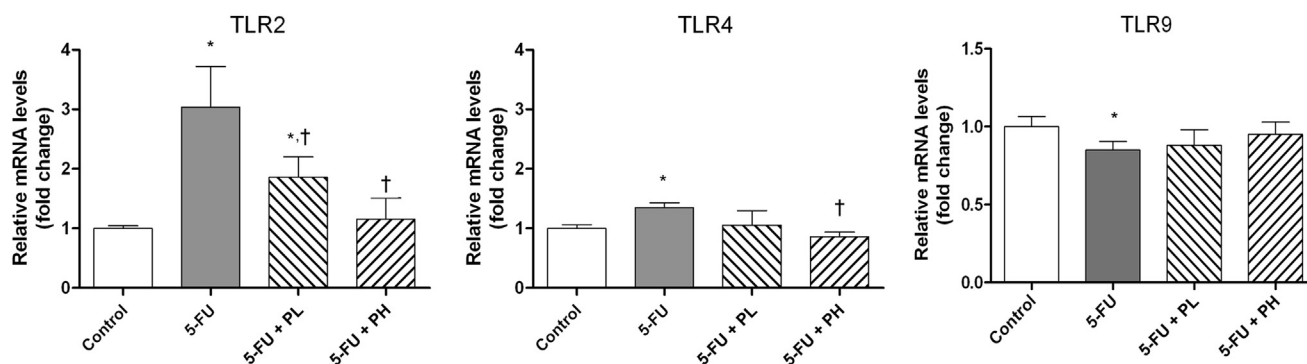


Fig. 5. qPCR analysis of TLR2, TLR4, and TLR9 mRNA expression levels (fold change) in the ileum of rats. The results are the mean values, and the vertical bars indicate SEM of five rats per group. * $P < 0.05$ compared with the control group (PBS only). † $P < 0.05$ compared with the 5-FU group (5-FU + PBS). 5-FU, 5-fluorouracil; PBS, phosphate-buffered saline; qPCR, quantitative real-time polymerase chain reaction; TLR, toll-like receptor.

permeability is one of the hallmarks of the third and fourth phases of chemotherapy-induced mucositis [32]. Both villous atrophy and the intestinal microbiota can influence gut permeability [33,34]. Indeed, several commensal bacteria have been shown to improve epithelial barrier function both in vitro and in vivo [35]. Likewise, we observed that treatment with the probiotic mixture greatly reduced intestinal permeability and serum endotoxin levels in 5-FU-treated rats. The rapid recovery of the intestinal *Lactobacillus* and *Clostridium* clusters III and XIVA groups may have contributed to these effects, since studies have shown that *Lactobacillus* group of bacteria can protect the intestinal epithelial barrier by improving the integrity of epithelial cells [36], they can also reduce paracellular permeability by modulating the expression of tight junction proteins [37]; depletion of *Clostridium* groups leads to decreased production of short-chain fatty acid (e.g., butyrate, acetate), which may impair the capability of the host to downregulate aberrant intestinal immune response [23].

The precise mechanisms by which microbes influence the immune and mechanical barriers of the gut are largely unknown. However, the interaction between gut microbes and the host is mediated through TLRs. These receptors recognize molecules of microbial origin and play a key role in innate immune responses [36]. Binding of bacterial products to TLRs on epithelial cells results in the activation of NF- κ B signaling, which triggers the production of proinflammatory cytokines and results in the development of an inflammatory response [37,38]. Conversely, probiotics such as *Bacteroides thetaiotaomicron* and *B. infantis* decrease NF- κ B activation [39,40], leading to a decrease in plasma levels of endotoxin and proinflammatory cytokines [41]. The *Clostridium* XIVA group may attenuate intestinal inflammation by exerting an effect on polyamine secretion, which in turn regulates the expression of TLR2 [42]. Additionally, TLR2 ligands have been found to stimulate the phosphorylation of protein kinase C, leading to a decrease in intestinal permeability [43]. To investigate whether changes in the intestinal microbiota induced by 5-FU affected the TLR expression pattern in the ileal mucosa, and whether administration of DM#1 attenuated such affects, we detected levels of TLR2, TLR4, and TLR9 mRNA by quantitative PCR. Changes in TLR expression have been associated with intestinal inflammatory responses caused by antibiotic-induced depletion of murine microbiota [44]. Our results showed that treatment with 5-FU induced a dramatic increase in the expression of TLR2 and TLR4 and slightly affected the expression level of TLR9. The increased expression of TLR2 and TLR4 was efficiently reduced by administration of a high dose of the probiotic mixture. We found that 5-FU-induced dysbiosis was characterized by significant reductions of *Lactobacillus* spp. and *Clostridium* cluster XIVA; therefore, decreased abundance of these microbial groups may be responsible for the alteration of the TLR2 signaling pathway. Administration of DM#1 to 5-FU-treated rats reestablished the intestinal ecosystem, particularly the populations of *Lactobacillus* spp. and *Clostridium* cluster XIVA, which in turn attenuated the increase in TLR2 mRNA expression and thus reduced the levels of proinflammatory cytokines and intestinal permeability.

Conclusions

Results from the present study indicated that, for treatment with 5-FU induced dysbiosis in the rat intestine, both microbial diversity and the populations of individual microbial groups were affected. Furthermore, this shifting of the intestinal microbiota may contribute greatly to the destruction of the

mucosal barrier in rats by influencing the TLR2 signaling pathway. Treatment with DM#1 ameliorated 5-FU-induced intestinal mucosal injury in rats, possibly by reducing inflammatory factors such as proinflammatory cytokines and neutrophil infiltration. Additionally, the increased intestinal permeability caused by 5-FU was normalized by treatment with DM#1. These results are closely associated with reestablishment of intestinal microbial homeostasis and alteration of the TLR2 signaling pathway. This probiotic mixture could increase patients' quality of life, positively influence treatment intensity, and decrease morbidity and mortality. However, some studies have shown that probiotics can cause invasive infections in patients [45]; therefore, further systematic evaluation of the safety of this probiotic preparation is recommended.

Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.nut.2016.05.003>.

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