Original Paper

Bifidobacterium Infantis Ameliorates Chemotherapy-Induced Intestinal Mucositis Via Regulating T Cell Immunity in Colorectal Cancer Rats

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Key Words

Bifidobacterium infantis • Intestinal mucositis • 5-fluorouracil • Oxaliplatin • CD4⁺CD25⁺Foxp3⁺ Tregs • CD4⁺CD17A⁺ cells • Colorectal cancer

Abstract

Background/Aims: Intestinal mucositis (IM) is a commonly encountered side effect in cancer patients receiving chemotherapy. This study aimed to investigate the effect of Bifidobacterium infantis (B. infantis) in attenuating the severity of chemotherapy-induced intestinal mucositis by regulating the T cell subsets in rats with colorectal cancer (CRC). **Methods:** Thirty male Sprague-Dawley (SD) rats were injected dimethyl hydrazine (DMH) subcutaneously for 10 weeks, and then injected SW480 cells in rectal mucosa to create a CRC model, and the rats were randomly divided into three groups: Control group (saline + saline), Chemotherapy group (saline + 5-FU+Oxaliplatin), B. infantis group (B. infantis + 5-FU+Oxaliplatin). IM was evaluated based on diarrhea severity, intestinal villus height, crypt depth, pro-inflammatory cytokines (IL-6, IL-1 β , TNF- α), T cell subsets (CD4⁺ IL17A⁺ cells and CD4⁺ CD25⁺ Foxp3⁺ Tregs) and related cytokine profiles. **Results:** The results showed that the B. infantis group demonstrated a higher body weight (BW) and intestinal villus height and a deeper crypt depth compared to the Chemotherapy group. The level of IL-6, IL-1 β and TNF- α which increased by chemotherapy, was lowered by B. infantis administration. Real time reverse transcription- polymerase chain reaction (RT-PCR) showed B. infantis reduced relative expression of Th17 and Th1 cells related cytokines, and increased relative expression of CD4⁺ CD25⁺ Foxp3⁺ Tregs related cytokines. Furthermore, Flow cytometry analysis showed B. infantis reduced CD4⁺ IL17A⁺ cells and increased CD4⁺ CD25⁺ Foxp3⁺ Tregs in mesenteric lymph nodes (MLNs) compared to the Chemotherapy group. **Conclusion:** B. infantis effectively attenuates chemotherapy-induced intestinal mucositis by decreasing Th1 and Th17 response and increasing CD4⁺ CD25⁺ Foxp3⁺ Tregs response.

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Introduction

Chemotherapy is an effective and widely used treatment in colorectal cancer (CRC), also diffusely adapted as postoperative adjuvant treatment. Oxaliplatin is frequently used as part of a chemotherapeutic regimen with 5-fluorouracil in the treatment of CRC [1]. Chemotherapy-induced mucositis caused by the breakdown of the mucosal barrier is a common and often severe side effect for CRC patients during their treatment [2, 3]. Some studies suggested that the mucosa-associated microbiota was dynamically associated with CRC [4]. The study suggested that chemotherapy-induced mucositis could aggravate dysbacteriosis [5].

Probiotics are live microorganisms that could benefit health when supplied in adequate amounts [6]. Certain probiotics have been associated with immunoregulatory responses on T-helper (TH) cells and T regulatory cells (Tregs) [7]. Among them, Bifidobacterium infantis is a commensal microbe isolated from the human gastrointestinal mucosa and has shown beneficial effects on gastrointestinal disease by modulating the immune function [8] and has shown efficient in treating inflammatory bowel disease [9]. B. infantis also can ameliorate trinitro-benzene-sulfonic acid (TNBS)-induced colitis [10] and chemotherapy-induced intestinal mucositis [4, 11] in a normal mouse model.

The mechanisms behind the amelioration of intestinal inflammation by B. infantis are still largely unknown. B. infantis feeding increased the proportion of CD4⁺ CD25⁺ Foxp3⁺ Tregs (Foxp3⁺ Tregs) and reduced the numbers of TH1 and TH17 cells within the lamina propria (LP) in dextran sulfate sodium (DSS)-induced colitis [12]. Moreover, B. infantis induced the expression of IL-10 and inhibited the expression of Th17 related IL-17 in DSS-induced colitis mice [13] and decreased Th1 pro-inflammatory cytokines (IFN- γ , IL-12 and TNF- α) and maintained the level of TNF- α in IL-10 knockout mice [14].

However, the effects of single B. infantis feeding on chemotherapy-induced intestinal mucositis and the underlying immune mechanism were still poorly understood. We hypothesized that B. infantis may alleviate 5-FU+oxaliplatin induced IM in CRC rats by adjusting the differentiation of CD4⁺ T subsets and related cytokines expression.

Materials and Methods

Cell Lines and culture conditions

SW480 (ATCC, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) supplemented with 10% fetal bovine serum (HyClone, USA), 1mM glutamine and 100U/ml each of penicillin and streptomycin. The cells were incubated in a humidified incubator at 37°C with 95% O₂ and 5% CO₂.

Animals

Animal experiments were approved by the Committee on Ethics of Animal Experiments of Dalian Medical University. Male Sprague-Dawley (SD) rats (160-170g) were obtained from the Experiment Animal Center of Dalian Medical University and acclimatized in appropriate cages under controlled conditions and fed with commercial solid food. Rats received food and water ad libitum but were fasted for 24h before sacrificed.

Experimental Design and Animal Model

The rats were injected with dimethyl hydrazine (DMH) (J & K Chemical Technology, Beijing) (30 mg/kg body weight) subcutaneously weekly for 10 weeks, and then injected with SW480 cells in rectal mucosa to create a CRC model. Rats were randomly divided into 3 groups (n=10): Control group (saline + saline), Chemotherapy group (saline + 5-FU+Oxaliplatin), and B. infantis group (B. infantis + 5-FU+Oxaliplatin). After 2 weeks, the rats were orally administrated B. infantis (1×10⁹ cfu/d) (B. infantis group) or suspension saline (Control group and Chemotherapy group) daily for 11 days. On the 8th day, 5-fluorouracil (Haipu, China) (75 mg/kg body weight) and Oxaliplatin (Hengrui, China) (8 mg/kg body weight) (Chemotherapy group and B. infantis group) or saline (Control group) was injected intraperitoneally (i.p.) for 3 days. Frozen stocks of B. infantis (TaKaRa, Japan) were stored at -80 °C prior to use. The probiotic was adjusted to 1×10⁹ cfu/ml in sterile saline and stored at 4 °C.

Diarrhea assessment

Stool passages of all animals were recorded daily. Diarrhea severity was assessed by using Bowen's score system [15] and was graded as follows: 0, normal stool; 1, slightly wet and soft stool indicating mild diarrhea; 2, wet and unformed stool indicating moderate diarrhea; and 3, watery stool indicating severe diarrhea. All diarrhea assessments were conducted in a blinded fashion by two investigators.

Histological Analysis

A 1-2 cm wide ring from the proximal jejunum was dissected and flushed with chilled isotonic saline to remove contents. The segments were fixed in 10% buffered neutral formalin for 24 hours, dehydrated in an ascending series of ethanol concentrations, cleared in xylol, and embedded in paraffin wax. Sections of 4 µm thickness were cut and mounted on glass slides then. Sections were routinely stained with haematoxylin and eosin (HE). HE stained goblet cells were expressed as the number of goblet cells per 10 villus-crypt units as described in the literature. Specimens were viewed under a TissueFAXS automatic scanning system, captured by a digital camera and analyzed by Histo-Quest software (Tissue Gnostics, Vienna, Austria). Measurements of villus height and crypt depth of the small intestine were determined for whole well orientated villi and crypts per small intestinal tissue section per mouse and the values were averaged.

Pro-inflammatory Cytokines Analysis

Blood was collected from the hearts immediately after those rats were sacrificed. Blood samples were centrifuged to yield serum. Serum levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) were assessed by enzyme-linked immune sorbent assay (ELISA) Kit (Elabscience, China). All assays were performed according to the manufacturer's instructions.

Flow cytometry analysis

Single cell suspension was prepared from MLNs of each rat. In order to identify Tregs in MLNs, cells were first surface labeled with fluorescein isothiocyanate (FITC) labeled anti-mouse CD4 (eBioscience, USA) and phycoerythrin (PE) labeled anti-mouse CD25 antibodies (eBioscience, USA). After that, cells were fixed, permeated and intracellularly stained with PE-Cyanine5 labeled anti-Foxp3 antibody (eBioscience, USA). To measure Th17 cells, cells were pre-stimulated for 4 h with PMA (50 ng/mL, Sigma) and ionomycin (500 ng/mL, Sigma) in the presence of Brefeldin A (1 mg/mL, eBioscience, USA) at 37°C and 5% CO₂. Then, cells were washed in phosphate buffer solution (PBS) and surface labeled with CD3-FITC and CD4-PE-Cy5. For intracellular labeling of IL-17, these cells were permeabilized with IL-17 fixation/permeabilization buffer (eBioscience, USA) and stained with anti-IL-17-PE (eBioscience, USA). Cells were incubated with affinity purified anti-mouse CD16/32 to block non-specific staining. IgG isotypes (BD pharmingen) were used as a control in all FACS experiments. Data were acquired on a FACS Calibur flow cytometer with CELLQuest software (version 5.1, BD BioScience).

RNA extraction and real-time RT-PCR

Total RNA was extracted from MLNs using TRIzol reagent (TaKaRa, Japan) according to the manufacturer's instructions. Total RNA (2 µg) was transcribed to cDNA using a reverse-transcription kit (TaKaRa, Japan). Primer sequences were as follows: (Table. 1). Real time RT-PCR was performed with the ABI 7300 system (Applied Biosystems; USA) according to the manufacturer's instructions. All the PCR experiments were performed under the same condition as follows: 95°C for 10 min, 95°C for 15 s and 60°C for 1 min, up to 40 cycles. GAPDH was used as an endogenous control.

Table 1. Primers used in the present study

Primer-Name (mRNA)	Forward	Reverse
T-bet	5'-ACGAGCCATTACAGGATTTTGTG-3'	5'-TCCAGTGGGCTCCAGTGTGTG-3'
IL-2	5'-GCAGCGTGTGTTGGATTTGAC-3'	5'-TGCTGGCTCATCATCGAATTG-3'
IL-10	5'-CAGACCCACATGCTCCGAGA-3'	5'-CAAGGCTTGGCAACCCAAAGTA-3'
TGF-β	5'-CATTGCTGTCCGTGCAGA-3'	5'-AGGTAACGCCAGGAATTGTTGCTA-3'
IL-12	5'-AATGGAATTGGTCCACCGAGA-3'	5'-ACCAGCCATGAGCAGGTGAA-3'
IFN-γ	5'-GAAACCATGTTTGGTGACGGAAATA-3'	5'-ACAGTAATTGGAATTCAGCGTGGAC-3'
IL-23	5'-TCCCGGAATCTCTGCACACT-3'	5'-CAGACCTTGGCGGATCCTT-3'
IL-21	5'-TCCTGAACCTTCTAACAGCTCCACAA-3'	5'-CCTCAGCTGTGCCAACAAAGTC-3'
IL-17	5'-GGGAAGTTGGACCAACACA-3'	5'-CCCACAGCATCTTCTCCA-3'
RORγt	5'-TCTGCAAGACTCATCGACAAGG-3'	5'-CACATGTTGGCTGCACAGG-3'
Foxp3	5'-TGAGCTGGCTGCAATTCTGG-3'	5'-ATCTAGCTGCTCTGCATGAGGTGA-3'
GAPDH	5'-GGCACAGTCAAGGCTGAGAATG-3'	5'-ATGGTGGTGAAGACGCCAGTA-3'

Fig. 1. (A) Daily body weight change in the rats. The rats were weighted daily and the results of all groups were compared with each other. Control group: the rats were administered with saline +saline; Chemotherapy group: the rats were administered with saline +(5-FU+Oxaliplatin); *B. infantis* group: the rats were administered with *B. infantis* +(5-FU+Oxaliplatin). Those rats in Chemotherapy group had higher body weight loss than those in Control group (Control group vs. Chemotherapy group #P <0.01 Fig. 1A). *B. infantis* ameliorated chemotherapy-induced weight loss. (Chemotherapy group vs. *B. infantis* group, *P <0.05 Fig. 1A). Fig. 1B Diarrhea score after administrating *B. infantis* (*B. infantis* group) or saline (Chemotherapy group and Control group) with/without 5-FU+Oxaliplatin treatment. Since the 8th day, 5-FU+Oxaliplatin (Chemotherapy group and *B. infantis* group) or saline (control group) was injected i. p. for 3 days. The rats in Chemotherapy group had more serious diarrhea than those in Control group (Control group vs. Chemotherapy group #P <0.01 Fig. 1B). However, the diarrhea score was clearly decreased in those mice treated with *B. infantis*. (Chemotherapy group vs. *B. infantis* group *P <0.05).

Statistical analysis

All experiment results were analyzed with SPSS software (version 21.0). Data of rats were analyzed by Wilcoxon rank sum test, Kruskal-Wallis H test and Nemenyi test. P values less than 0.05 were considered statistically significant.

Results

Effect on body weight

After completion of the experiment, all animals tolerated well. No mortality was noted. The rats were weighted daily starting from the day when *B. infantis* was administrated and the results of all groups were compared. There were no significant differences in initial body weight between the 3 groups prior to 5-FU+Oxaliplatin or saline injection. After chemotherapy treatment, those rats in Chemotherapy group had higher BW loss than those in Control group (#P <0.01). However, the BW loss was partially prevented in rats receiving *B. infantis* (*B. infantis* group vs. Chemotherapy group, *P <0.05) (Fig. 1A).

Effect on diarrhea

Diarrhea score of the rats was recorded daily starting from the day when *B. infantis* or saline were administrated and the results of all groups were compared. There were no significant differences in initial diarrhea score. However, marked diarrhea developed in the Chemotherapy group and *B. infantis* group after 5-FU and Oxaliplatin administrations. Rats in Chemotherapy group had more serious diarrhea than those in Control group (Control group vs. Chemotherapy group #P <0.01). However, the severity of diarrhea was clearly attenuated in those rats treated with *B. infantis* (Chemotherapy group vs. *B. infantis* group *P <0.05) (Fig. 1B).

Effect on the intestinal mucosal and the tumor

All rats in our experiment were found colorectal cancer by the anatomy of intestine. The gross specimens of colorectal cancer were collected from the rats in the Control

Fig. 2. The gross specimen of colorectal cancer were collected from the rats in the Control group (A), Chemotherapy group (B), and *B. infantis* group (C). The tumor diameter of the rats was measured, and the results were compared among the 3 groups (D). However, the difference of the tumor size among the 3 groups did not reach the significance ($P > 0.05$).

Fig. 3. The microscope specimen of intestinal tissue that were collected from the rats. Hematoxylin and eosin-stained sections (magnification 100×) of intestinal tissue of (A) saline +saline-treated, (B) saline+5-FU+Oxaliplatin-treated and (C) *B. infantis* +5-FU+Oxaliplatin-treated rats. The microscope specimen (D) (Hematoxylin and eosin-stained) (magnification 400×) of colorectal cancer was collected from the rats in this experiment. The histological examination of Villus height (E) and Crypt depth (F) measurements per rat in Control group (saline +saline), Chemotherapy group (saline +5-FU+Oxaliplatin) and *B. infantis* group (*B. infantis* +5-FU+Oxaliplatin). E: Villus height was significantly reduced in all rats receiving chemotherapy compared to the Control group ($^{\#}P < 0.05$). However, *B. infantis*-treated rats showed a higher villus height compared to Chemotherapy group ($^*P < 0.05$). F: The length of crypt depth in Chemotherapy group longer significantly ($^{\#}P < 0.05$) compared with the Control group. The crypt depth in *B. infantis* group was significantly restored by *B. infantis* treatment (Chemotherapy group vs. *B. infantis* group $^*P < 0.05$).

group (Fig. 2A), Chemotherapy group (Fig. 2B) and *B. infantis* group (Fig. 2C). The tumor diameter of the rats was measured, and the results were compared among the 3 groups (Fig. 2D). However, the difference of the tumor size among the 3 groups did not reach the significance ($P > 0.05$). The microscope specimen (Fig. 3D) of colorectal cancer were collected from the rats in this experiment. The histological examination of villus height,

Fig. 4. The level of IL-6 (A), IL-1 β (B) and TNF- α (C) determined by ELISA per rat of Control group (saline +saline), Chemotherapy group (saline +5-FU+Oxaliplatin) and *B. infantis* group (*B. infantis* +5-FU+Oxaliplatin). Chemotherapy increased the level of IL-6, IL-1 β and TNF- α in plasma compared with Control group. However, the level of IL-6, IL-1 β and TNF- α were decreased in the *B. infantis* group compared to the Chemotherapy group. A: IL-6 (Control group vs. Chemotherapy group, $^{\#}P<0.05$; Chemotherapy group vs. *B. infantis* group, $^{\#}P<0.05$); B: IL-1 β (Control group vs. Chemotherapy group, $^{\#}P<0.05$; Chemotherapy group vs. *B. infantis* group, $^{\#}P<0.05$); C: TNF- α (Control group vs. Chemotherapy group, $^{\#}P<0.05$). However, no significantly difference in the level of TNF- α after *B. infantis* feeding.

crypt depth and goblet cells measurements revealed that chemotherapy caused substantial damage intestinal mucosal layer including villus atrophy, crypt derangement and intense inflammatory cell infiltration (Fig. 3 A, B, C). In contrast, the intestines of *B. infantis*-treated rats only exhibited mild morphological damage. Villus height was significantly reduced in all rats receiving chemotherapy compared to the Control group ($^{\#}P < 0.05$). However, *B. infantis* -treated rats showed a higher villus height compared to Chemotherapy group ($^{\#}P < 0.05$) (Fig. 3E). Besides, chemotherapy significantly lengthened crypt depth of the intestine compared with the Control group. On the contrary, the crypt depth was significantly restored by *B. infantis* treatment (Fig. 3F).

Effect on levels of pro-inflammatory factors

ELISA analyses revealed that increased plasma IL-6, IL-1 β and TNF- α levels were detected in all rats in the Chemotherapy group compared to Control group ($^{\#}P < 0.05$). However, the level of IL-6, IL-1 β and TNF- α were decreased in the *B. infantis* group compared to the Chemotherapy group. However, no significantly difference in the level of TNF- α after *B. infantis* feeding. (Fig. 4)

B. infantis reversed the increased level of Th17 cells and decreased level of Foxp3⁺Tregs in the MLNs of chemotherapy-induced intestinal mucositis in CRC rats

We measured the numbers of CD4⁺CD17A⁺T cells (Fig. 5A) and CD4⁺CD25⁺Foxp3⁺ cells (Fig. 5B) in the MLNs in all rats by flow cytometry to determine if they were altered after interventions. Flow cytometric analyses revealed that chemotherapy increased the percentage of CD4⁺CD17A⁺T cells (Fig. 5C) and decreased the percentage of CD4⁺CD25⁺Foxp3⁺ cells (Fig. 5D) in MLNs compared with saline control. *B. infantis* feeding decreased the percentage of CD4⁺CD17A⁺T cells (Fig. 5C) and increased the percentage of CD4⁺CD25⁺Foxp3⁺ cells (Fig. 5D) in MLNs compared with Chemotherapy group.

B. infantis reversed the upregulation of Th1- and Th17-responses and downregulation of Foxp3⁺Treg responses in chemotherapy-induced intestinal mucositis in CRC rats

We measured the relative mRNA expression of different T cell cytokines by real-time RT-PCR. Compared with the Control group the relative mRNA expression of Th1 cytokines (IL-2

Fig. 5. Flow plots of CD4⁺ IL17A⁺ cells (A) and CD4⁺ CD25⁺ Foxp3⁺ cells (B) in MLNs from CRC rats. Level of CD4⁺CD17A⁺T cells (C) and CD4⁺CD25⁺Foxp3⁺ cells (D) determined by flow cytometry analysis per rat of Control group (saline +saline), Chemotherapy group (saline +5-FU+Oxaliplatin) and *B. infantis* group (*B. infantis* +5-FU+Oxaliplatin). Chemotherapy increased the percentage of CD4⁺CD17A⁺T cells ($P < 0.05$, Fig. 5C) and decreased the percentage of CD4⁺CD25⁺Foxp3⁺ cells ($P < 0.05$, Fig. 5D) compared to saline control. *B. infantis* feeding decreased the percentage of CD4⁺CD17A⁺T cells ($P < 0.05$, Fig. 5C) and increased the percentage of CD4⁺CD25⁺Foxp3⁺ ($P < 0.05$, Fig. 5D) cells in MLNs compared with Chemotherapy group.

mRNA, IL-12 mRNA, IFN- γ mRNA and T-bet mRNA) and Th17 cytokines (ROR γ t mRNA, IL-17 mRNA, IL-21 mRNA, IL-23 mRNA) increased in Chemotherapy group. However, the relative mRNA expression of Foxp3⁺ Tregs cytokines (IL-10 mRNA, Foxp3 mRNA, TGF- β mRNA) decreased in Chemotherapy group compared with the control group. Administration of *B. infantis* reversed upregulation of Th1- and Th17-responses and downregulation of Foxp3⁺Treg responses in chemotherapy-induced IM in rats (Fig. 6). However, the relative mRNA expression of IL-21 in rats of *B. infantis* group also decreased, but did not reach the significance in comparison with the Chemotherapy group. The difference of the relative mRNA expression of Foxp3 between the Chemotherapy group and *B. infantis* group did not reach the significance either. Furthermore, the difference of the relative mRNA expression of TGF- β between the Control group and Chemotherapy group had no significance (Fig. 6).

Discussion

Recently, the incidence of colorectal cancer had increased rapidly, which seriously threatened the health of human beings. Some studies demonstrated that CRC was often accompanied the disorder of intestinal and body immune system seriously. For CRC patients, chemotherapy was inevitable treatment, and which aggravated the disorder of immunological function. Intestinal mucositis was one of the most frequent and deleterious side effects in cancer patients undergoing combined chemotherapy, and had no effective preventive and control measures currently. CRC patients with chemotherapy-induced intestinal mucositis experienced changes in their chemotherapy treatment, including delays in therapy, dose reductions, reduction in dose intensity, and even discontinuation of therapy [16, 17]. *B. infantis* had shown beneficial effects on the mucosal barrier of intestinal, and effective for gastrointestinal disease, such as inflammatory bowel disease [9]. However, prior to our experiment, it had not been tested whether *B. infantis* administration could prevent the development of chemotherapy-induced intestinal mucositis in CRC.

It was reported that probiotics could ameliorate chemotherapy-induced intestinal mucositis [4, 11] in a normal mouse model. However, they used the animal model without

Fig. 6. Level of relative mRNA expression of T cell-related cytokines from MLNs determined by real-time RT-PCR analysis per rat from Control group (saline+ saline), Chemotherapy group (saline+ 5-FU+Oxaliplatin) and *B. infantis* group (*B. infantis*+ 5-FU+Oxaliplatin). Chemotherapy increased the relative mRNA expression of Th1 cytokines (IL-2 mRNA, IL-12 mRNA, IFN-γ mRNA and T-bet mRNA) and Th17 cytokines (RORγt mRNA, IL-17 mRNA, IL-21 mRNA and IL-23 mRNA) and decreased the relative mRNA expression of Foxp3⁺Treg cytokines (IL-10 mRNA, Foxp3 mRNA and TGF-β mRNA) compared with the Control group. *B. infantis* administration reversed upregulation of Th1- and Th17-responses and downregulation of Foxp3⁺Treg responses in chemotherapy-induced IM in rats. However, the relative mRNA expression of IL-21 in rats of *B. infantis* group also decreased, but did not reach the significance in comparison with the Chemotherapy group. The difference of the relative mRNA expression of Foxp3 between the Chemotherapy group and *B. infantis* group did not reach the significance either. Furthermore, the difference of the relative mRNA expression of TGF-β between the Control group and Chemotherapy group had no significance. A: IL-2 (Control group vs. Chemotherapy group, $P < 0.05$; Chemotherapy group vs. *B. infantis* group, $P < 0.05$); B: IL-12 (Control group vs. Chemotherapy group, $P < 0.05$; Chemotherapy group vs. *B. infantis* group, $P < 0.05$); C: IFN-γ (Control group vs. Chemotherapy group, $P < 0.05$; Chemotherapy group vs. *B. infantis* group, $P < 0.05$); D: T-bet (Control group vs. Chemotherapy group, $P < 0.05$; Chemotherapy group vs. *B. infantis* group, $P < 0.05$); E: IL-17 (Control group vs. Chemotherapy group, $P < 0.05$; Chemotherapy group vs. *B. infantis* group, $P < 0.05$); F: IL-21 (Control group vs. Chemotherapy group, $P < 0.05$); G: IL-23 (Control group vs. Chemotherapy group, $P < 0.05$; Chemotherapy group vs. *B. infantis* group, $P < 0.05$); H: RORγt (Control group vs. Chemotherapy group, $P < 0.05$; Chemotherapy group vs. *B. infantis* group, $P < 0.05$); I: Foxp3 (Control group vs. Chemotherapy group, $P < 0.05$); J: IL-10 (Control group vs. Chemotherapy group, $P < 0.05$; Chemotherapy group vs. *B. infantis* group, $P < 0.05$); K: TGF-β (Chemotherapy group vs. *B. infantis* group, $P < 0.05$).

CRC, which could not be direct and realistic basis for the treatment of clinical CRC patients. In our study, we used the CRC rat model to explore whether *B. infantis* could ameliorate the development of severe 5-FU combined with Oxaliplatin induced intestinal mucositis and to explore the mechanism. About the animal model, there were several methods to establish an animal with CRC, for instance, carcinogen DMH treatment, transgenic animal technology, orthotopic transplantation tumor and so on. DMH was one of most common

chemical carcinogens of CRC, the experimental model induced by DMH had similar features to human CRC [18], but was more time-consuming. Another common method to establish CRC model was using SW480 colon adenocarcinoma cells, and the tumor growth was faster. However, the tumor formation rate was lower. In our preliminary experiment, 30 male SD rats were injected DMH subcutaneously for 10 weeks, and then injected SW480 cells in rectal mucosa to create a CRC model. After a week, the growth of tumors in colorectal mucosa were detected on 18 rats, and after two weeks, tumors were measured on 29 rats. The tumor formation rate of CRC model reached 96.67%. In addition, the preparatory experiments showed that the mucosal damage were detected obviously in 48-72 hours after 5-FU and Oxaliplatin injection. Evidence from preliminary experiments showed that experimental colorectal tumors created by DMH and SW480 cells are similar histology in epithelial, morphology and anatomy to human colorectal neoplasm. This animal model is of great importance for the research on CRC.

We used this animal model to verify the assumption that *B. infantis* might have beneficial effects on intestinal mucositis induced by chemotherapy and to make a primary exploration of the mechanisms. Firstly, we should have a basic understanding of the mechanisms behind the chemotherapy-induced intestinal mucositis. The ruling points of view on chemotherapy-induced IM might include the dysbiosis of the intestinal microbiota or the activation of intestinal mucosal immune system [18-20]. The intestinal tumor was one of the reasons which cause the derangement in the intestinal mucosa barrier function, therefore, chemotherapy treatment also aggravated the dysfunction of intestinal barrier that present as the infiltration of inflammation cells, damage of intestinal mucous, and then appearing diarrhea and body weight loss [15]. However, chemotherapy was inevitable for CRC, we had to demonstrate whether the supplement of probiotics could alleviate chemotherapy induced intestinal mucositis in CRC rats. The intestinal microflora balance had great importance on intestinal mucosa barrier function. *B. infantis* as a key member of probiotics might encourage the balance of intestinal microflora and promote the healthy function of intestinal mucosa barrier. It was reported that 5-FU treatment led to the body weight loss and serious diarrhea on normal rats [21], those clinical symptoms could be alleviated by the supplement of probiotics [15, 21, 22]. In our study, we observed that *B. infantis* administration diminished the severity of intestinal damage caused by 5-FU and Oxaliplatin, prevented the loss in body weight and the decrease in villus height, and reduced the occurrence of diarrhea. We had proposed that *B. infantis* could have beneficial effects on intestinal mucositis induced by chemotherapy in CRC rats.

The pathogenesis of intestinal inflammation induced by chemotherapy is complex. It had been proposed that chemotherapy drugs cause DNA and non-DNA damage to the epithelium cells, generating reactive oxygen species, and then following with the pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6) being upregulated rapidly [23]. TNF- α and IL-1 β play an important role in the aggravation of intestinal mucositis [24]. Our results demonstrated that the supplement of *B. infantis* decrease the high level of pro-inflammatory cytokines caused by chemotherapy in CRC rats.

The immune cell responses played a crucial role in the pathogenesis of chemotherapy-induced intestinal mucositis. Activated effector T-helper cells (Th1, Th2 and Th17) released enormous pro-inflammatory cytokines and initiated excessive inflammation, resulting in intestinal mucosal damage [25], while the uncontrolled Th cells responses could be avoided by the tolerance mechanisms of the immune system. Foxp3 Tregs could suppress Th cells and secrete anti-inflammatory cytokines IL-10 and TGF- β [26].

This study showed the levels of Th1 cells and its cytokines (IL-2, IL-12 and IFN- γ) were up-regulated in the chemotherapy-induced intestinal mucositis rats, which can be reversed by *B. infantis*. IFN- γ , an essential pro-inflammatory cytokine is responsible for macrophage activation [25]. It was identified IL-2 as a key molecule for IFN- γ induction by polymerized polyphenols. IL-12 also could induce naïve T cells to produce IFN- γ and plays an important role in the Th1 differentiation [27]. In our study, chemotherapy-induced intestinal mucositis rats showed down-regulated of IL-2, IL-12 and IFN- γ mRNA with supplement of *B. infantis*.

B. infantis also down-regulated the level of T-bet (Th transcription factor) in chemotherapy-induced intestinal mucositis rats. T-bet required for Th1 lineage commitment is a member of T-box family of transcription factors that appears to regulate lineage commitment in CD4 Th lymphocytes in part by activating IFN- γ [28]. These outcomes showed *B. infantis* could recede Th1 cell response by regulating its cytokines and differentiation-related factors expression in chemotherapy-induced intestinal mucositis rats.

We identified the levels of Th17 cells and its cytokines (IL-17, IL-21 and IL-23) were up-regulated in the chemotherapy-induced intestinal mucositis rats. IL-17 is a mediator of inflammatory responses; and T-cell derived IL-17 and IFN- γ synergistically increase chemokine IL-8 secretion by intestinal epithelial cells [29]. IL-21 and IL-23 were found to promote the differentiation of Th17 cells from naïve CD4 immunohomeostasis T cells and prevent the expression of Foxp3 by naïve CD4 immunohomeostasis T cells [30, 31]. Furthermore, IL-23 induced pro-inflammatory cytokines (IFN- γ , IL-6) in the colon [32]. Orphan nuclear receptor ROR γ (ROR γ t) is the pivotal transcription factor to Th17 differentiation [33] and mainly expressed by Th17 cells. ROR γ t could regulate the production of IL-17 to induce colitis [34]. Our animal experiment showed a decrease in ROR γ t mRNA in chemotherapy-induced intestinal mucositis rats fed with *B. infantis*. In summary, *B. infantis* suppressed Th17 responses by regulating its cytokines and differentiation-related factors expression in chemotherapy-induced colitis rats.

Foxp3⁺ Tregs are essential for normal immunohomeostasis by suppressing T-helper effector cells [26]. The importance of Tregs in the prevention of colitis has been confirmed by previous studies [35]. The immunosuppressive function of Tregs requires a high level of Foxp3 expression [36]. Decreased Foxp3 expression could lead to impaired Treg function and be causal for immune disorders. TGF- β drives the differentiation of naïve T cells to a Treg phenotype [37]. TGF- β is a potent regulatory cytokine that inhibits T-helper cell proliferation, differentiation and activation. IL-10 is a potent anti-inflammatory cytokine, and Treg-specific deletion of IL-10 resulted in intestinal mucositis [38, 39]. In our study, *B. infantis* promoted Foxp3⁺ Treg responses in association with increased levels of Foxp3, IL-10 and TGF- β mRNA in chemotherapy-induced intestinal mucositis rats.

Conclusion

The current study demonstrates that administration of *B. infantis* attenuated chemotherapy-induced intestinal mucositis via suppressing Th1 and Th17 responses as well as promoting Foxp3⁺ Treg responses in CRC rats. This suggests *B. infantis* may serve as an alternative therapeutic strategy for the prevention of chemotherapy-induced mucositis. However, we failed to discount the systemic bacterial translocation from the intestine. The limitation of this study was that the bacterial translocation and the change in gut microbiota during and after chemotherapy with and without administration of *B. infantis* were not examined. These might be relevant in the pathogenesis of chemotherapy-induced intestinal mucositis.

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Disclosure Statement

All authors declared no conflicts of interest.

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