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Original Article

Long-term use of *Bifidobacterium longum* alleviates colorectal colitis in rats by regulating inflammatory cytokines and Treg cells

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Abstract: The therapeutic effects of *Bifidobacterium longum* (*B. longum*) in treating IBD patients still remain controversial, with the underlying mechanisms being unclear. *B. longum* was used to treat the rats *in vivo*, as well as rats' splenocytes *in vitro*. Histological damage of the colons was evaluated with standard methods. Inflammatory cytokines were measured in serum and cell culture medium. We found that treatment of *B. longum* reduced inflammation in TNBS-induced colitis more profoundly than salicylazosulfapyridine (SASP). *B. longum* increased the systemic anti-inflammatory effects in rats due to the up-regulated levels of IL-10, and the ratio of IL-10/IL-12, as well as the down-regulated levels of IL-12, IL-17, and IL-23 in the serum, more effectively than SASP treatment. And it seemed that pre-treatment of *B. longum* before TNBS enema combined with 3 weeks' treatment of *B. longum* afterwards exerted slightly stronger anti-inflammatory effects than 3 weeks' treatment of *B. longum* alone. *B. longum* also enhanced the proportion of the Treg cells significantly in the spleens of the TNBS-treated rats. Consequently this study showed that long-term treatment of *B. longum* subsp. *longum* exerted significant anti-inflammatory effects on chemical colitis in rats by regulating the proportion of Treg cells, and the expression of circulating inflammatory cytokines.

Keywords: *Bifidobacterium*, inflammatory bowel disease, T-regulatory cells, IL-10, IL-12

Introduction

Inflammatory bowel disease (IBD) is a chronic, refractory inflammatory disease consisting of two major forms, Crohn's disease (CD) and ulcerative colitis (UC) [1]. The exact pathogenesis of IBD is still obscure, even though biological agents, such as anti-tumor necrosis factor (anti-TNF), make mucosal healing possible in a small portion of these patients [2]. So far, it is considered that genetic disorders, immune aberration, environmental influence and intestinal microbial flora are main factors contributing to IBD's initiation and progression. There are one hundred trillion bacteria living in homeostasis with the intestinal immune system. Maintaining the homeostasis of intestinal microbial flora is critical to preserve human being's health [3]. It has been confirmed that there are defects of mucosal tolerance in IBD patients, causing the intestinal intolerance to microorganisms and food antigens.

Induction of a pro-inflammatory T-cell response in intestines by microorganisms can lead to development of either T-help-1 (Th1), Th2, or Th17 cells, and can be suppressed by the effect of regulatory T cells (Tregs). Treg cells, both the naturally occurred from thymus and the induced from peripheral tissues, typically expressed CD4, CD5 and the transcription factor Foxp3. Tregs limit the differentiation of Th1 and Th17, and suppress the secretion of the associated cytokines (interferon-γ, IL-12, IL-17 and IL-18) [4, 5]. It has been confirmed in animal models and in patients that lacking of Tregs or suppression of its function could result in severe colitis [6]. Both clinical trials and animal experiments have revealed that *Bifidobacterium*, used to balance the intestinal microflora, is effective in

treating the colitis by inducing Treg production and its function [7-9]. Other therapeutic mechanisms have been reported as regulating cytokines expression and improving the epithelial barrier function [10].

Even though there are encouraging results about the therapeutic effects of *Bifidobacterium* in treating IBD patients, negative results have also been reported. Silveira *et al* just reported that once the colitis had been established, *Bifidobacterium longum* did not provide any protection [11]. Srutkova *et al* reported that only some certain strains of *Bifidobacterium* had therapeutic effects [7]. One study carried out *in vitro* by three strains of *Bifidobacterium* indicated that only *Bifidobacterium infant* was effective in suppressing IL-17 function and enhancing IL-27 expression [12]. Another study showed that the proportion of *Bifidobacterium* in IBD patients increased compared with healthy controls [13]. Theoretically if the *Bifidobacterium* exhibited pronounced therapeutic effects, the wounded intestinal mucosa of the IBD patients should heal after the treatment of *Bifidobacterium*, which never happened actually. In our previous studies, we showed that *Bifidobacterium* could exert some therapeutic effects on IBD models but not as effective as *Faecalibacterium prausnitzii* [14, 15]. Simultaneously, we demonstrated that in these *Bifidobacterium*-treated rats the concentrations of certain related cytokines and the proportion of Treg cells did not change as much as those in the *Faecalibacterium prausnitzii*-treated rats.

For all of the above reasons, we put forward the following hypothesis, (1) not all strains of *Bifidobacterium* show therapeutic effects in IBD patients, (2) some strains might only possess preventive effects, and (3) the duration of *Bifidobacterium* treatment might influence the therapeutic effects. So we carried out this experiment to investigate the preventive and therapeutic effects of *Bifidobacterium longum* in animal models for 3 weeks, and explored how the expression and function of cytokines and Tregs were regulated.

Materials and methods

Experiment animals and induction of colitis

Sprague-Dawley rats (200-240 g) were obtained from Central Animal Laboratory of Nanjing Drum Towel Hospital (Nanjing, China). All rats were housed and handled under specific pathogen-free conditions in accordance with local animal welfare officer. The rats were allowed to adapt to our laboratory environment for 1 week before commencing of the experiment, during which period they had free access to standard rodent chow and tap water. Colitis was induced by trinitrobenzene sulphonic acid (TNBS, Sigma, St. Louis, MO, USA) consistent with our previous protocol [16, 17]. Briefly, rats were fasted for 12 hours before colitis induction and anesthetized with ether. TNBS (100 mg/kg, 10% TNBS in 50% ethanol; total volume 1.0 ml) was injected into the colon via a rubber catheter. The rats were thereafter maintained in a vertical position for 3 min. The control rats were treated with the same volume of 50% ethanol using the same technique. All rats were checked daily for behavior and body weights. The experiments were approved by the Animal Care and Use Committee of Nanjing Drum Tower Hospital. And all the experiments were performed in accordance with the relevant guidelines and regulations.

Animal treatment protocol

The rats were distributed into five groups randomly. (15 rats in each group) Details for the treatment were shown in **Figure 1**. The frozen *Bifidobacterium longum* subsp. *longum* (*B. longum*) and salicylazosulfapyridine (SASP) were gifts from Shanghai Sine Pharmaceutical Company. Eth group acted as the sham-operation group. TNBS group acted as the untreated group. SASP group (1.5 mg/kg/day) acted as the control group for treatment. The rats who accepted *B. longum* (2 g/rat/day) prophylactically for one week before TNBS enema, followed with 3 weeks' treatment of B. *longum* after TNBS enema, were defined as *B. long (P+T)* group. And the rats treated with *B. longum* for 3 weeks after TNBS enema were defined as *B. long (T)* group.

Macroscopic scores and histological analysis

After the animals were sacrificed, the colons were taken out and flushed with PBS, then cut open and photographed. The colonic specimens were scored by an observer who was unaware of the treatments according to the Morris' scoring system: 0, no damage; 1, localized hyperaemia, but no ulcers; 2, linear ulcers

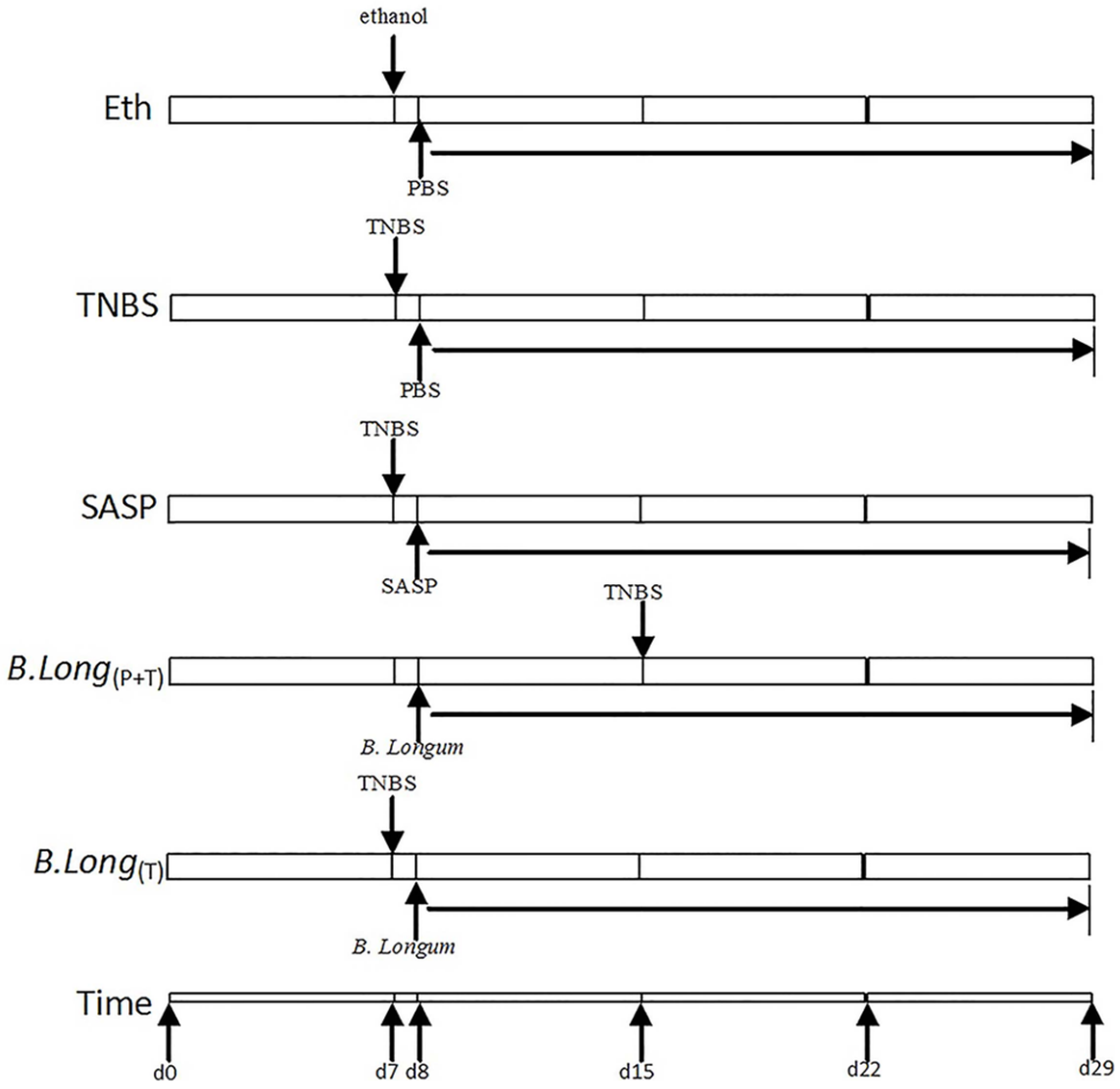

Figure 1. Diagram for the treatment protocol. Animals were housed in our local animal center to adapt to the environment from day 0 to day 7. Since day 8 the animals began to accept treatments according to the scheme. Eth group rats were given 50% ethanol enema on day 7, and from day 8 were given PBS by gavage once daily until sacrificed on day 29. TNBS group were given TNBS enema on day 7, and since day 8 were given PBS by gavage. SASP group received TNBS enema on day 7, and since day 8 received SASP (1.5 mg/kg) once daily by gavage. *B. Long (P+T)* group were given *B. Longum* (2 g/rat, dissolved in PBS) from day 8 to day 29 once daily, during which period TNBS was given on day 15 by enema. *B. Longum (T)* group were given TNBS enema on day 7, and since day 8 were given *B. Longum* (2 g/rat, dissolved in PBS) once daily by gavage.

with no significant inflammation; 3, linear ulcer with inflammation at one site; 4, two or more sites of ulceration and/or inflammation; 5, two or more major sites of inflammation and ulceration or one major site of inflammation and ulceration extending more than 1 cm along the colon [18]. The tissues were fixed in 10% buffered paraformaldehyde, dehydrated, and embedded in paraffin. The sections were dried, cleaned, hydrated, and then stained with hematoxylin and eosin (HE). Histological damage of the colons was evaluated by standard methods.

Splenocyte culture

Ten untreated rats were sacrificed and the spleens were obtained. The spleen cells were harvested and prepared as single cell suspension in plates (10^7 cells). Then they were incu-

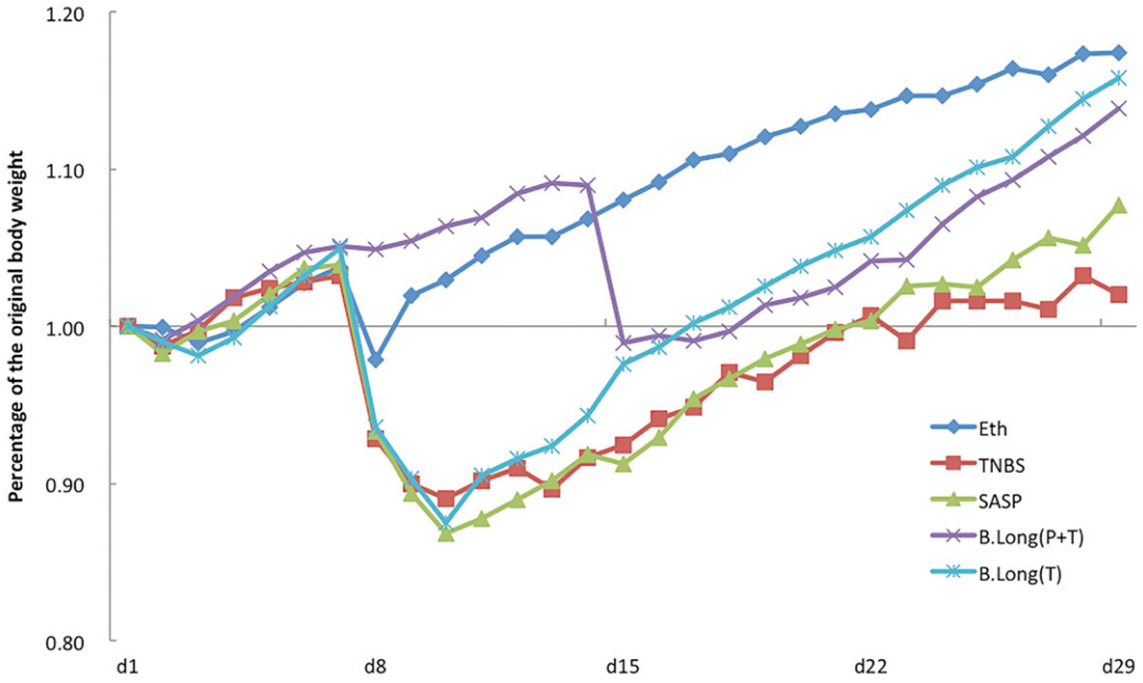

Figure 2. Body weight changes in different groups.

bated at 37°C for 48 h in 1 ml complete medium (control group), with recombinant TGF-β (2 ng/ml) plus IL-6 (20 ng/ml) or recombinant TGF-β plus IL-6 plus *B. longum* (10^7 cells). Culture supernatants were collected and stored at -80°C to be prepared for the cytokines detection.

Enzyme-link immunosorbent analysis (ELISA)

About 1.5 mL blood was collected from each rat just before it was sacrificed. We centrifuged the blood samples to remove the blood cells and obtain the serum. Concentrations of cytokines IL-10, IL-12, IL-17, and IL-23 in serum, as well as IL-10, IL-12, and IL-17 in supernatants from splenocyte culture, were detected by commercially available ELISA kits (R&D) according to the manufacturer's instructions. All samples were checked in duplicates. The proportions of IL-10/IL-12 were then calculated.

Splenocyte isolation and flow cytometry

The isolation of splenocytes was performed in accordance with the previous methods [17]. Briefly, the spleens were excised just after the rats were killed, and placed in RPMI-1640 medium. Single cell suspensions were then prepared. The tissues passed through a 70-μm-pore mesh and were washed again. Erythrocytes were lysed by ammonium chloride (BD Bioscience, San Diego, CA, USA). Cells were resuspended in supplemented RPMI-1640 and counted. The cells were labeled with FITC anti-rat CD4 (eBioscience), APC anti-rat CD25 (eBioscience) and PE anti-rat Foxp3 (eBioscience). The labeled cells were checked by flow cytometry (Becton Dickinson) and analyzed via the Cell Quest software (BD Bioscience) (2×10^5 cells per sample).

Statistics

All data were expressed as mean ± standard error of the mean (SEM). The statistical significance was evaluated by one-way *ANOVA* followed by least significant difference (LSD) or Dunnett's test. A *P*-value less than 0.05 was considered statistically significant.

Results

Bifidobacterium longum accelerated body weight recovery and improved survival rate

Body weight decreased remarkably in all the rats receiving TNBS enema. For the ethanol group, the rats' body weight decreased slightly and recovered soon after the normal diet. For

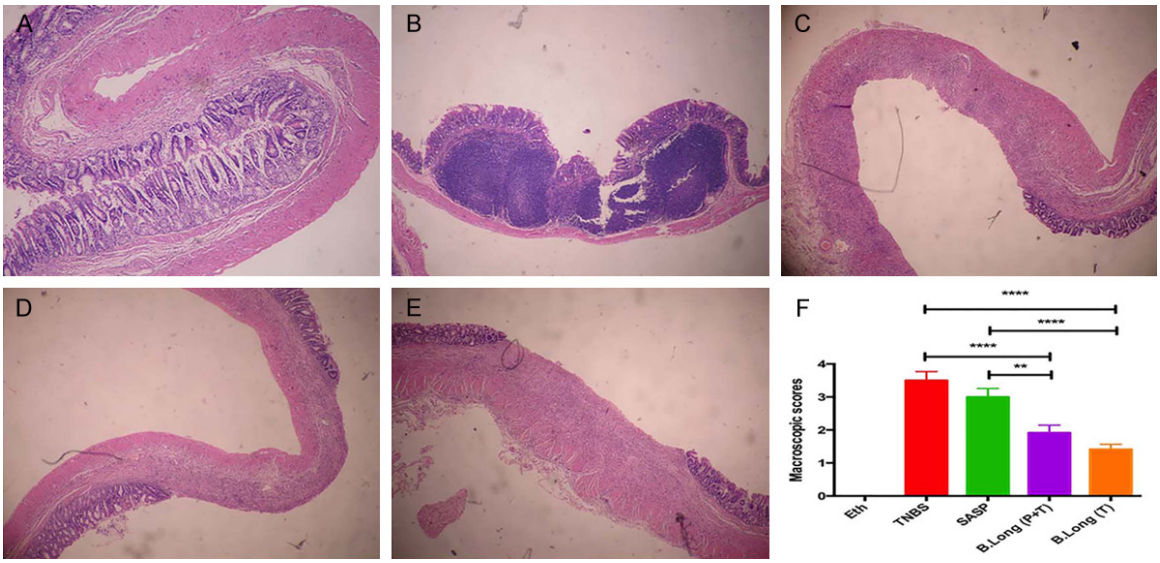

Figure 3. The macroscopic view scores and histological images of different treatment group (Original magnification 40 ×). A. Ethanol group: normal histological image; B. TNBS group: necrosis of epithelium and large amount of inflammatory cells infiltrating into mucosa and sub-mucosa layers; C. SASP group: no necrosis epithelium, small amount of inflammatory cells infiltrating into newly granulation tissues; D, E. *B. long (P+T)* and *B. long (T)* groups: superficial damage of the epithelium and no inflammatory cells, newly glands and granulation tissues could be founded; F. The macroscopic scores of different groups. After 3 weeks of recovery there was no damage in ethanol group, thus in this group the macroscopic score is zero.

other groups, after TNBS enema all the rats' body weights decreased sharply. Body weight recovery to initial level in *B. long (P+T)* group lasted 12 days and 14 days in *B. long (T)* group. However, the recovery time for SASP group and TNBS group were 17 days and 21 days, respectively. Changes of body weight were shown in **Figure 2**. One rat died accidently because of over-anesthetization during the modeling procedure in SASP group. At the end of the experiment, there were 15, 10, 10, 12 and 12 rats in ethanol, TNBS, SASP, *B. long (P+T)* and *B. long (T)* group respectively. As a result B. *longum* treatment decreased the time of body weight recovery to initial level after TNBS enema treatment, and slightly increased the survival rate of the rats.

Bifidobacterium longum reduced inflammation in TNBS induced colitis

After three weeks' recovery, the colonic mucosa became normal in the ethanol group, while shorter length, stiff colonic wall, bleeding, edema and ulcers of the colonic mucosa could be observed in the rats from TNBS group. In some rats the ulcers even caused chronic perforation, abdominal adhesions, and resulted in incomplete ileus in this group. Compared to TNBS group, the colons in SASP group were much longer, and the ulcers were smaller and more superficial. No chronic perforation or abdominal adhesion was detected. Besides, although edema and inflammation could be detected in *B. long (P+T)* group, they were not detected in *B. long (T)* group. And there were linear ulcers in 3 rats in *B. long (P+T)* group.

The histological view of the colons from ethanol group was normal. In TNBS group, we observed large amounts of destroyed glands, necrosis of the epithelium, and plenty of inflammatory cells infiltrating into mucosa and sub-mucosa layers. These damages were much alleviated in SASP group, in which the granulation tissues and fibrosis could be identified in some colons. Although superficial damage and swollen glands could be detected in a small portion of the rats in *B. long (P+T)* group, the granulation tissues and new glands could be found in most rats in *B. long (P+T)* and *B. long (T)* groups. The macroscopic view scores and histological images were shown in **Figure 3**. The scores were significantly down-regulated in *B. long (P+T)* and *B. long (T)* groups compared with TNBS group, as well as SASP group, demonstrating that *B. longum* reduced inflammation in TNBS induced colitis. However no statistical significance was observed between *B. long (P+T)* group and *B. long (T)* group.

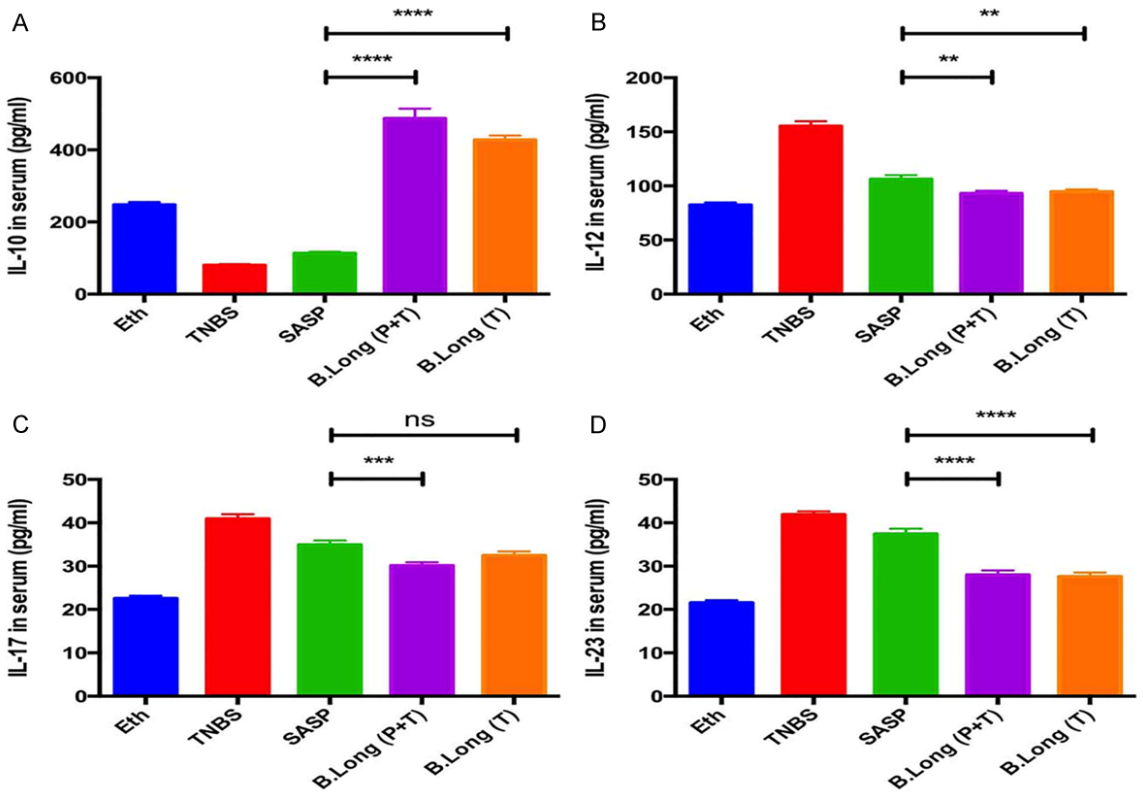

Figure 4. Concentrations of cytokines in rats' serum in different groups: A. IL-10 expression; B. IL-12 expression; C. IL-17 expression; D. IL-23 expression.

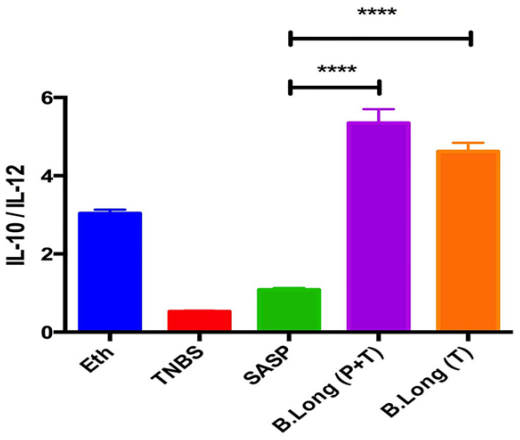

Figure 5. The ratio of IL-10/IL-12 in rats' serum in different groups.

Bifidobacterium longum altered the expression of cytokines

The levels of IL-10 and IL-10/IL-12 ratio decreased dramatically in the serum from TNBS group compared with ethanol group. Simultaneously, the levels of IL-12, IL-17 and IL-23 increased to different levels in TNBS group. After SASP treatment, levels of IL-10 concentration and IL-10/IL-12 ratio in the serum increased, which were enhanced to significantly higher levels after *B. longum* treatment. And this effect was slightly more obvious in *B. long (P+T)* group than in *B. long (T)* group (**Figures 4A** and **5**). While *B. longum* treatment induced significantly lower levels of IL-12, and IL-23 concentrations in the serum compared with SASP treatment. Interestingly IL-17 concentration was significantly lower only in *B. long (P+T)* group, but not in *B. long (T)* group, than SASP group (**Figure 4B-D**).

The concentrations of IL-10 in cultured splenocytes increased slightly after the co-culture of TGF-β plus IL-6, which increased further after the adding of *B. longum*. On the contrary, the concentration of IL-12 decreased sharply after the co-culture of TGF-β plus IL-6, which increased to the pre-stimulated level after the further adding of *B. longum.* The concentration of IL-17 and the ratio of IL-10/IL-12 increased apparently after the stimulation of the two cyto-

Table 1. Concentrations of cytokines in splenocyte culture supernatants

Cytokines	Control	TGF-β+IL-6	TGF-β+IL-6+B. Long
IL-10 (pg/ml)	145.60±27.85	177.70±56.26	279.70±53.60
IL-12 (pg/ml)	16.20±3.41	8.60±2.06	16.10±2.94
IL-17 (pg/ml)	104.80±20.92	396.25±79.09	220.95±48.17
IL-10/IL-12	9.39±2.71	22.89±2.71	18.34±11.14

The isolated splenocytes were cultured in medium with or without TGF+IL-6/TGF+IL-6+B. Long. Concentrations of IL-10, IL-12, and IL-17 in culture supernatants were detected by ELISA. There were ten samples in each group, and each sample was detected in duplicates. Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed by Dunnett's *t* test. P<0.05 was considered statistically significant.

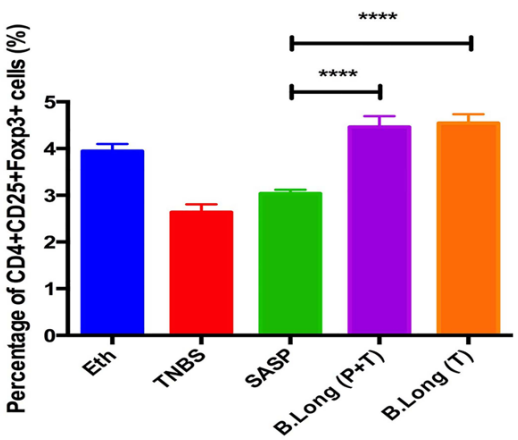

Figure 6. Percentage of CD4+CD25+Foxp3+ Treg cells in rats' spleens in different groups.

kines, and decreased after the further adding of *B. longum* (**Table 1**).

Bifidobacterium longum enhanced the proportion of Treg cells in spleens

The proportion of Treg cells in spleens decreased after the treatment of TNBS. SASP treatment increased the proportion of Treg cells, which was not as effective as *B. longum* treatment in TNBS induced colitis (**Figure 6**).

Discussion

The incidence of IBD has been raising dramatically over the past few decades all over the world [19]. Reduced microbial diversity has been associated with IBD and probiotic bacteria have been proposed for its prevention and/or treatment. Since IBD results from complex host-microbiota interactions, preventive strategies targeting the aberrant composition of the intestinal microbiota may provide new potentials to tackle the disease. *Bifidobacterium longum* is considered a key member of the human gut microbiota with many beneficial effects on the immune system [20]. It has been reported that *B. longum* prevented acute DSS-induced colitis in mice in a strictly strain-specific manner [7]. In our previous research, we also demonstrated that 7-day treating of *B. longum* exerted some protective effects on TNBS-induced colitis in rats by giving a significantly higher IL-10/IL-12 ratio and attenuating the TNBS-induced inflammation, but not as effective as *Faecalibacterium prausnitzii* [15]. However, more studies have shown negative results about the therapeutic effects of *B. longum* on IBD patients, probably because of the short duration of treatment or use of inappropriate strains. Here in this study we treated the TNBS induced colitis with a specific strain, *Bifidobacterium longum* subsp. *longum* which was a gift from Shanghai Sine Pharmaceutical Company, for a longer duration of 3 weeks, and found that *B. longum* could attenuate the rats' chemical colitis by regulating the inflammatory and tolerogenic T cell subsets differentiation, and excretion of the relevant cytokines. Besides we observed that *B. longum* facilitated the augmentation of Tregs in the rats' splenocytes.

B. longum has been proved to play a protective role against colitis by effectively inducing anti-inflammatory cytokines (e.g., TGF-β, IL-4, and IL-10), as well as suppressing the pro-inflammatory cytokines (e.g., IL-12) production when used to treat IBD patients or chemical colitis in animals [8, 21-25]. So the ratio of IL-10/IL-12 can be used to assess the anti-inflammatory effects *in vitro* and *in vivo* [26]. IL-23 acts as the upstream regulator of Th17 cells, which is very important in maintaining the stability and activation of Th17 cells [27]. Previously we showed that circulating IL-17 and IL-23 were both significantly increased by TNBS treatment in rats, suggesting their involvement in the onset of IBD. In the current study, *B. longum* increased the systemic anti-inflammatory effects in rats due to up-regulated levels of IL-10 and the ratio of IL-10/IL-12, as well as the down-regulated levels of IL-12, IL-17, and IL-23 in the serum, more profoundly than SASP treatment, sugges-

ting the therapeutic potential of *B. longum* for IBD. And it seems that pre-treatment of *B. longum* before TNBS enema combined with 3 weeks' treatment of *B. longum* afterwards exerted slightly stronger anti-inflammatory effects than 3 weeks' treatment of *B. longum* alone, reflected by the ratio of IL-10/IL-12 and levels of these cytokines, indicating that long-term use of *B. longum* might be effective in protecting the chemical colitis in the TNBS-treated rats.

However in the cultured splenocytes treated with TGF-β plus IL-6, we failed to observe the increase of IL-10/IL-12 ratio after the further adding of *B. longum*. We hypothesized that although Th17 cells induced by TGF-β plus IL-6 treatment functioned to down-regulate the IL-12 levels, which had to be kept above a certain threshold. As a result the further adding of *B. longum* was not able to decrease the IL-12 levels additionally. The hypothesis needs to be investigated in the future.

It is commonly accepted that IBD could be suppressed by the Treg cells [28, 29]. Previous studies have shown that Treg cells are important regulators to prevent and protect colitis, with the evidence that transfer of Treg into the colitis rodents can significantly attenuate the intestinal inflammation [30, 31]. In our study long-term treatment of *B. longum* increased the proportion of the Treg cells obviously in the spleens from the TNBS-treated rats, demonstrating the critical role of Treg cells in regulating the recovery of the chemical colitis induced by *B. longum*.

In summary, this study showed that long-term treatment of *B. longum* subsp. *longum* exerted significant anti-inflammatory effects on chemical colitis in rats by regulating the proportion of Treg cells, increasing the expression of circulating anti-inflammatory cytokines such as IL-10, and decreasing the expression of circulating pro-inflammatory cytokines such as IL-12. The therapeutic effects of *B. longum* in IBD patients and the underlying molecular mechanisms need to be investigated further. Considering our previous findings that *F. prausnitzii* also had potential protective effects in colitis, which was stronger than short-term use of *B. longum*, we want to explore whether long-term combinational use of *B. longum* and *F. prausnitzii* have even greater anti-inflammatory effects in treating IBD patients.

Acknowledgements

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Disclosure of conflict of interest

None.

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