Title: Anti-inflammatory properties of Bifidobacterium longum expressing human manganese superoxide dismutase using the TNBS–induced rats model of colitis

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Keywords: rhMn–SOD, Bifidobacterium longum, ulcerative colitis, Antiinflammatory
Anti-inflammatory properties of *Bifidobacterium longum* expressing human manganese superoxide dismutase using the TNBS-induced rats model of colitis

Yajie Xie¹, Qing Guo¹, Shiyu Li², Mengge Liu¹, Zhenrui Xu¹, Qian Zhang¹

Hanxiao Sun¹#

¹ Institute of Genomic Medicine, College of Pharmacy, Jinan University, Guangzhou, 510632, China.

² Institute of Genetic Engineering, Southern Medical University, Guangzhou, 510515, China.

# Corresponding author

E-mail address: sunhx718@126.com.

Telephone and Fax numbers: 020-38375022
Abstract

The chronic enteritis disease ulcerative colitis (UC) is a formidable opponent, and its etiology remains unclear. Current studies have shown that oxidative stress is closely related to the pathogenesis of inflammatory bowel disease. Antioxidant enzymes, such as manganese superoxide dismutase (MnSOD), have been deemed to exert an anti-inflammatory function. Normally, oral administration of MnSOD directly leads to an incapacitation because of poor penetration and stability. To address this problem, the probiotic *Bifidobacterium longum* (*B. longum*), which naturally occurs in the intestinal flora, was engineered to secrete a biologically active human MnSOD. Then this engineered bacterium was utilized against a rat model harboring UC induced through trinitrobenzene sulfonic acid. The data showed the engineered *B. longum* successfully secreted a penetratin-hMn-SOD fusion protein. The bioactivity assay demonstrated that this fusion protein was delivered into Caco-2 cells and significantly decreased TNF-α (P<0.01) and IL-6 (P<0.01) expression, as well as, ROS level (P<0.001) in LPS-induced Caco-2 cells. The rat UC model experiment indicated that the *B. longum* harboring rhMn-SOD (penetratin-hMn-SOD) successfully inhibited the release of cytokines like TNF-a, IL-6, IL-1β and IL-8, and reduce MPO activity and MDA levels. The histological analysis of the colon tissue section revealed that the engineered *B. longum* was efficient in attenuating UC damage. These results suggested that preventing UC by the use of *B. longum* harboring rhMn-SOD could be an alternative choice.

**Keywords:** rhMn-SOD, *Bifidobacterium longum*, ulcerative colitis, Antiinflammatory
Introduction

Inflammatory bowel disease (IBD), which has traditionally been divided into two subtypes, ulcerative colitis (UC) and Crohn’s disease (CD), is a chronic inflammatory disease of the intestinal tract [1]. Although its pathogens and etiology are not well known, IBD is associated with many factors, such as one’s immune system factor, genetic, and gut flora, leading to intestinal homeostasis damage, inflammation, and intestinal disorders [2-4].

Recently, extensive evidence has demonstrated that inflammatory responses to abnormal colitis are mainly characterized by enhanced formation of reactive oxygen species and nitrogen species, increased synthesis of pro-inflammatory cytokines, and activation of neutrophils [5-7]. Located in the mitochondria, manganese superoxide dismutase (MnSOD) is a major antioxidant enzyme that can effectively kill many harmful reactive oxygen species and lead to cell protection [8]. Previous studies have suggested that MnSOD could reduce oxidative stress and ROS generation, inhibit endothelial cell activation and regulate adhesion molecule expression and leukocyte-endothelial interactions, signifying a potentially and important tool for the treatment of IBD [7, 9, 10]. However, oral Mn-SOD results in a disabled pharmacological function because of poor penetration and stability in vivo, which limits its application [11]. Therefore, the development of a practical and efficient strategy to deliver Mn-SOD is a necessity.

With the rapid development of recombinant biotechnology, the hMn-SOD gene has been successfully expressed in *Escherichia coli*, yeast, insects, mammals, and other systems which provides a valuable reference for the study of its application and function. *Bifidobacterium* is a genus of non-pathogenic bacteria, and one of the most important groups of human intestinal flora, and this species has a fundamental
function within a human from birth to old age [12]. Many studies have shown that
*Bifidobacterium* has many health-promoting properties, such as fighting inflammation, microbial infections, and cancers [13, 14]. With these beneficial effects, some strains of *Bifidobacterium* have been used as probiotics, and they have also become popular in the medicine industry and in food science because their high security, elicits prominent abilities of fermentation, resists extreme intestinal environment and adapts to specific local communities [15, 16]. Further researches have suggested that *Bifidobacterium* has been successfully used as a safe and effective delivery system for genetic transformations [17, 18].

In this work, we constructed an expression system of *B. longum* secreting a biological active penetratin-hMn-SOD fusion protein and used its oral delivery to combat UC in a rat model induced by TNBS.

**Materials and methods**

**Construction of a secreted hMn-SOD Bifidobacterium**

The bacterial strains and plasmids used in this study are shown in Table 1. *E. coli* DH5α was used for general cloning purposes and grown at 37°C in Luria-Bertani broth with ampicillin continuous shaking culture. *B. longum* HB15 [17] were cultured at 37°C in MRS medium supplemented with 0.05 % (w/v) L-cysteine under anaerobic conditions. The pMB1 fragment containing a *Bifidobacterium* replicon, was obtained from the plasmid pDG7 (ShineGene Molecular Biotech, China) [19, 20]. The hup gene promoter and terminator as well as the amyB signal sequence, were amplified from *Bifidobacterium*. An Oligonucleotide consisting of a penetratin sequence, hMn-SOD gene, and a furin cleavage site between the penetratin and the hMn-SOD was synthesized using codon optimization for *Bifidobacteria* [21], and together with a promoter, a terminator, an amyB signal sequence, and pMB1, were inserted into
pBluescript II SK(-) (ShineGene Molecular Biotech, China) to yield a 5.9 kb *E. coli-Bifidobacterium* shuttle vector named pBsSOD (Fig. 1). The plasmid was transformed into *Bifidobacterium* cells through electroporation [22] to yield *B. longum*-rhMnSOD. The transformants were screened with agarized culture medium containing ampicillin (100 mg/L final concentration) in an anaerobic condition.

**Expression of hMn-SOD**

The culture solution was harvested and centrifuged at 3000 rpm for 10 minutes, collecting the supernatant and filtering it through 30 kD ultrafiltration membranes (Millipore Amicon, USA), precipitating with trichloroacetic acid. The collected fractions were dissolved in PBS and SDS-PAGE (12% gels) to separate the fusion protein. The mouse anti-SOD2 monoclonal antibody (TIANGEN, China) was used for a western blot to analyze the hMn-SOD per the manufacturer’s instructions. The hMn-SOD level in the culture supernatants were detected with an hMn-SOD ELISA kit (TIANGEN, China), per the manufacturer’s protocols, at 0, 5, 10, 15, 20, 25, and 30 hours after being sufficiently digested with furin.

**Cell culture**

Caco-2 cells (Bogoo, China) were grown in DMEM supplemented with 10% FBS, 1% NEAA, 1% L-glutamate, and antibiotic–antimycotic mixture (10000 U/ml penicillin G, 10000 μg/ml streptomycin sulfate and 25 μg/ml amphotericin B in 0.85% saline) at 37°C in culture flasks in a humidified air–5% CO2 atmosphere until the experiments were performed.

**Bioactivity assay**

Cells were seeded into 24-well plates at a density of 4×10^6 cells per well. *Bifidobacterial* culture supernatants were adjusted to an anticipated concentration (containing 60 μg/ml rhMn-SOD). After removing the growth medium, the cells were
incubated with *B. longum* or *B. longum*-rhMn-SOD culture supernatants diluted 1:1 (v/v) with fresh cell culture medium in *B. longum* and *B. longum*-rhMn-SOD groups. Cells in the normal and model groups should be treated with culture medium (for bifidobacterial) diluted 1:1 (v/v) with fresh cell culture medium. After one hour for incubation, cells were exposed to 5 μg/ml LPS (*E. coli*; Sigma). After another four hours, parts of the cells were harvested and measured with an ELISA kit (MultiSciences, China) to determine the amount of released TNF-α and IL-6 in the supernatants.

Other parts of the cells were used to check the ROS removal activity of rhMn-SOD. ROS level was assessed by using the oxidation-sensitive fluorescent probe, DCFH-DA. Cells were incubated with DCFH-DA (5 mM) for 30 minutes at 37°C in 5% CO2 and then washed with HBSS. The ROS formation level was analyzed via FACScan flow cytometry (Becton-Dickinson, USA).

**Transduction of rhMnSOD protein into Caco-2 cells**

Cells seeded into 24-well plates at a density of 4×10^6 cells per well were assigned to three groups: blank control, hMnSOD group, and penetratin-hMnSOD group. The hMnSOD group cells were treated with *B. longum* culture supernatants and control hMnSOD (60 μg/mL), and the penetratin-hMnSOD group cells were treated with *B. longum*-rhMnSOD culture supernatants (containing 60 μg/mL hMnSOD). After treatment for 90 minutes, cells were lysed in RIPA buffer, and the homogenates were cleared by centrifugation and analyzed for protein concentration by western blot. Our earlier research provided the control hMnSOD without penetratin [23].

**Animal assays**

These experiments were performed according to the Animal Care and Use Committee of Jinan University, China. Forty-eight non-pathogenic Wistar male rats,
weight 180±20g at 9-10 weeks old, were purchased from the Third Military Medical University (SYXK2002-032). They were housed in comfortable cages and kept under standardized conditions, with freely available food and water and a 12-hour light/dark cycle at a temperature of 22–25°C. Before the experiments were performed, the rats were allowed to acclimatize for three days. Rats were randomly assigned to four groups (n = 12/group): normal group, model group, B. longum group, and B. longum-rhMnSOD group. The rats in the model group, the B. longum group, and the B. longum-rhMnSOD group were induced colitis through the infusion of TNBS, as described previously by Wang et al [7]. The normal group rats received physiological saline. After the TNBS had induced colitis for 24 hours, the model group was orally administered daily with PBS; the B. longum group was orally administered daily with WT B. longum (2×10^9 c.f.u. per rat per day); and the B. longum-rhMnSOD group was orally administered daily with B. longum expressing rhMnSOD (2×10^9 c.f.u. per rat per day). The normal group was provided with tap water only. After treatment for seven days, all rats were sacrificed, and their colon tissues were harvested for MPO, MDA, TNF-a, IL-6, IL-1β, and IL-8 analysis.

**Histological evaluation**

On the seventh day after Bifidobacterium administration, samples from the middle colon were fixed in 4% buffered formaldehyde (pH 7.4), dehydrated, and subsequently embedded in paraffin. Sections were cut (7 μm) and then stained with hematoxylin and eosin (H&E) in accordance with standard procedures for the histological evaluation of colonic damage. Morphological changes in the colonic membrane were observed under a microscope. The histological damage was scored per the previously established criteria [24].

**MPO activity in colon tissues**
The tissue samples from the middle colon were obtained, weighed, and then homogenized in 0.5% (w/v) hexadecyltrimethylammonium bromide in 50 mM of potassium phosphate buffer (pH 6.0). The homogenates were then centrifuged for 15 minutes at 15,000 rpm at 4°C. Supernatants were collected to measure MPO activity by a microplate reader at 460 nm ($A_{460}$) as described by Wei [17]. One unit of MPO activity was defined as the degrading of 1 μmol of peroxidase per minute at 37°C.

**Measured MDA level in colon tissues**

Part of the harvested homogenate was used for measuring the MDA level. After centrifugation at 10,000 rpm at 4°C for 15 minutes, supernatants were collected for MDA and measured using assay kits (TIANGEN, China). The thiobarbituric acid (TBA) method determined the MDA level.

**Determination of cytokines production in colon tissues**

Appropriate colon samples were weighed and homogenized in PBS at pH 7.2 containing a protease cocktail, and then they were centrifuged at 12,000 rpm for 10 minutes at 4°C. The cytokine levels of TNF-α, IL-6, IL-1β, and IL-8 were quantified using ELISA in accordance with the manufacturer’s instructions (GenStar, China).

**Statistical analysis**

The data are presented as mean. Error bars represent the standard deviation of the means for at least three independent experiments. The data were evaluated using GraphPad Prism 5.0. Statistical analysis for significant differences was performed using one-way ANOVA, where appropriate. A p-value < 0.05 was considered statistically significant.

**RESULTS**

**Engineering a Bifidobacterium secreting penetratin-hMn-SOD**

The pBluescript II SK (-) is an *Escherichia coli* clone plasmid, and it was selected
as the object of reconstruction. To create an *E. coli-Bifidobacterium* shuttle vector, we
first inserted a fragment pMBI containing *Bifidobacterium* replicon into pBluescript II
SK (-). The constitutive and highly expressed hup promoter and terminator [17, 25]
were introduced to improve the expression. To make the penetratin-hMn-SOD secrete
from cells, the amyB signal peptide was introduced [26, 27]. The transporter peptide
penetratin (PEP-1: KETWWETWTEWSQPKKKRKV) was connected in the front
of hMn-SOD by a furin cleavage site, facilitating an increasing absorption and
delivering the hMn-SOD into cells [28-30]. As soon as penetratin-hMn-SOD entered
the body, an integral hMn-SOD would be obtained in the presence of a furin cleavage
in vivo. The pBsSOD vector is shown in Fig. 1.

**Determination of penetratin-hMn-SOD**

To confirm the transformants of *B. longum* secreting hMnSOD, supernatants from
the modified *Bifidobacterium* were analyzed with western blotting. As shown in Fig.
2A, and as expected in the modified *Bifidobacterium*, we found a prominent band of
about 22kD in the supernatants after furin digestion. This band matched the calculated
molecular weight of an intact hMn-SOD, while it weighed nothing in the WT *B.
longum*, allowing us to conclude that this band corresponded to the hMn-SOD protein.

Surprisingly, a band of about 25kD, matching the penetratin hMn-SOD fusion protein,
was observed in the modified *Bifidobacterium* with the non-furin treatment; this could
be explained by a cross reaction of the penetratin-hMn-SOD fusion with the
anti-SOD2 monoclonal antibody. Next, we determined the hMn-SOD level in the
culture supernatants after furin digestion over time. The data was revealed in Fig. 2B.
An increasing hMn-SOD concentration in the supernatants was seen along with rapid
cell multiplication; this appearance may be associated with cell growth periodicity.

**Anti-inflammatory activity of B. longum-rhMnSOD**
To determine the biological activity of rhMnSOD expressed by *B. longum*-rhMnSOD, we tested its anti-inflammatory activity with an LPS-induced inflammatory model of Caco-2 cells. We measured the expression levels of TNF-α and IL-6 after treatment with LPS and adjusted *bifidobacterial* culture supernatants. We found that *B. longum*-rhMnSOD supernatants containing 60 μg/ml rhMnSOD could significantly inhibit TNF-α and IL-6 expression compared to that of the model (p<0.01) and *B. longum* (p<0.05) group (Fig. 3A), indicating that rhMnSOD secreted by *B. longum*-rhMnSOD served as an excellent anti-inflammatory property.

**Antioxidant activity of *B. longum*-rhMnSOD**

To prove the correlation between ROS removal and anti-inflammatory properties, the ROS removal effect of rhMn-SOD was determined in the LPS-induced caco-2 cells using DCFH-DA fluorescent probe. The model group cells showed strong ROS production compared to the normal group cells (Fig. 3B). However, the level of ROS production decreased in the *B. longum* and *B. longum*-rhMn-SOD groups compared with the model group, particularly in *B. longum*-rhMn-SOD group, indicating that the rhMnSOD secreted by *B. longum*-rhMnSOD was complete and active (Fig. 3B).

**Observation of penetratin-hMn-SOD fusion protein into Caco-2 cells**

Researches have shown that Caco-2 cells are widely employed in determining drug absorption in the human small intestine and in studying drug transport mechanisms [31, 32]. In our study, western blot was used to analyze the hMnSOD concentration of the three groups, which indirectly reflected the intracellular delivery of rhMnSOD into the Caco-2 cells. As shown in Fig. 4, the protein concentration of hMnSOD in the penetratin-hMnSOD group was significantly higher than that of the other two groups (P<0.001), suggesting that the penetratin-hMnSOD accumulated in the cytosol and nucleus and could be transported into cells with the peptide penetratin.
Oral administration of *B. longum*-rhMnSOD to against TNBS-induced colitis

The given TNBS rats developed severe colitis that presented the typical features of diarrhea, poor coat quality, and reduced mobility. Following seven days of treatment with *Bifidobacterium*, these symptoms improved gradually in the *B. longum* group and the *B. longum*-rhMnSOD group; however, the *B. longum*-rhMnSOD group was more distinguished than the *B. longum* group.

Histological analyses of the colonic samples were performed after the application of oral cells for seven days. The colon samples were from normal rats that had histologically healthy mucosa (Fig. 5A). Those rats in the TNBS-induced colitis model group showed significant inflammatory damage with mucosa destruction, cell infiltration in the lamina propria, and goblet cell hyperplasia and destruction (Fig. 5B). After treating rats with *Bifidobacterium* supernatants, the *B. longum* group showed a reduction in inflammation; however, most tissues from this group still had severe inflammatory damage compared with the rat colons from normal rats (Fig. 5C). The *B. longum*-rhMn-SOD group displayed a relatively normal and integrated colonic histology (Fig. 5D). The histological scores for the model and treatment groups showed an increase in the degree of inflammation compared with normal rats (Fig. 5E). Treatment of rats with *B. longum*-rhMn-SOD supernatants significantly decreased damage scores in TNBS-induced colitis rats compared with the model and the *B. longum* group animals.

The MPO enzyme is a sign of neutrophil infiltration, which is closely related to IBD. MPO activity was significantly increased in the TNBS-induced colitis model group and the treatment groups compared to the normal group. However, the data reflected that the MPO activity on day seven was significantly reduced in the *B. longum*-rhMnSOD group compared with the model and the *B. longum* groups,
suggested reduced neutrophil infiltration in colon tissues (Fig. 6A).

The frequently-used oxidative damage indicator MDA was a marker for free radical-induced lipid peroxidation [33]. In model animal colon tissues, the MDA levels significantly increased compared with the normal rats (Fig. 6B). B. longum-rhMnSOD and B. longum treatment significantly reduced MDA levels compared with the model group (Fig. 6B).

Cytokines serve a critical role in regulating IBD. TNF-α, IL-6, IL-1β and IL-8 are some positive factors that promote IBD. The data showed that the levels of these cytokines were significantly attenuated in the B. longum-rhMn-SOD group compared with the model group and the B. longum group (Fig. 7).

DISCUSSION

IBD is a refractory immune-mediated disease that is characterized not only by immune disorders and various inflammatory factors but also by abnormalities of the intestinal microflora; its authentic pathogens and etiology remain unknown. Many drugs and measures were introduced to achieve IBD resistance. One of the effective strategies against UC is to recommend anti-inflammatory drugs to reduce the inflammatory effects. It has been well established that inflammatory mediators, such as proteases, cytokines, arachidonic acid metabolites, and ROS, are closely related to gut inflammation [34]. Increasing evidence has shown that ROS make a critical contribution to the pathogenesis of colitis [35, 36]. Therefore, controlling ROS in IBD became a significant concern. Work over the recent decades has produced amazing results, and researches in SOD and its mimetic could ameliorate colitis [7, 9]. Indubitably, these results are surprising; however, the existing problems and limitations remained unanswered. It is well known that ROS, such as superoxide anion, have a wide distribution and are not only located in blood but also in cytoplasm,
nucleus, intercellular substances, and mitochondria. Thus, the SOD would ideally journey to all these areas to eliminate harmful ROS. Conventional drug delivery systems, oral and injection, do not meet these requirements. Therefore, it is necessary to develop a method to eliminate this problem.

In our work, an optimistic transporter, penetratin, was introduced to deliver large proteins into mammalian cells. Penetratin is an antennapedia homeodomain-derived cationic peptide, and investigation showed that penetratin was the most promising cell-penetrating peptide for effectively translocating cell membranes while covalently or noncovalently conjugating to a larger cargo, such as polypeptides or oligonucleotides [37], or even delivering cargo molecules to the cytosol and nucleus [38] but not the mitochondria [39]. To deliver SOD to these areas, the MnSOD living in the mitochondria were chosen. Among the various penetratins, the PEP-1 peptide has been reported to successfully transfer SOD into cells [40-42]. PEP-1 peptide also has advantages in protein transduction, including high stability, no cytotoxicity, and low sensitivity to serum [43]. Thus far, many PEP-1 fusion proteins have been used to protect against numerous diseases, including atherosclerosis, neuronal disease, and skin inflammation [44-46]. In fact, our trans-membrane assay results showed that PEP-1 was capable of translocating hMn-SOD into Caco-2 cells, which vividly supported these reports (Fig. 4). Furthermore, we made SOD-conjugated penetratin via a linker of the furin cleavage. Therefore, SOD could travel to the blood, cytoplasm, and nucleus with the help of penetratin, and then it would obtain an integral hMnSOD after the furin cleavage in vivo and destroy ROS.

SOD is a typical protein, and administering it orally might result in an extremely low bioavailability because of its poor permeability [47] and rapid enzymatic degradation in the gastro-intestinal tract [48]. Therefore, we chose Bifidobacteria as a
delivery vehicle to improve the bioavailability of orally delivered SOD. *Bifidobacteria*, generally regarded as safe microorganisms that can regulate the balance of intestinal flora via the mucosal barrier effect, inhibits the growth of pathogenic bacteria and prevents gastrointestinal diseases, and it was engineered to secrete a biologically active penetratin hMn-SOD fusion protein. Oral administration of this modified *B. longum* would make SOD travel directly to the UC location, regardless of the terrible degradation in the upper gastrointestinal tract. Meanwhile, penetratin, as an absorption enhancer, readily increases SOD absorption in the colon to improve bioavailability. In this study, the use of *Bifidobacterium* as a delivery system makes rhMn-SOD feasible for oral administration and regulates the intestinal flora while controlling the inflammatory reaction to IBD treatment; this strategy may be more reasonable since IBD is tightly related to inflammatory factors and intestinal microflora disorder. The results presented that MPO, MDA, TNF-α, IL-6, IL-1β, and IL-8 were significantly reduced in the rats fed with *B. longum* secreting hMnSOD, and it also prevented colonic pathological damage in the colon, suggesting a statistically significant anti-inflammatory reaction on TNBS-induced colitis in the rat model. Furthermore, experimental and clinical studies have provided evidence of the beneficial effects of *Bifidobacterium* on inflammatory bowel disease [13, 49, 50]. Our experimental results also support these reports. When treated with *Bifidobacterium* supernatant alone, the *B. longum* group showed reduced inflammation, MPO activity, and MDA level compared with the model group. In summary, our work represents an alternative option to improve experimental IBD by *B. longum* expressing hMnSOD.

**ACKNOWLEDGMENTS**

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**Figure legends**

**Fig. 1** Schematic of the pBsSOD shuttle plasmid. The pMB1 represents the replication for *Bifidobacterium*. The pMB1, hup promoter, hup terminator and a synthesized nucleotide fragment containing amyB signal sequence, peptide penetratin (PEP-1), furin cleavage site and human MnSOD gene in turn were ligated into pBluescript II SK (-), yielding pBsSOD. Ampr, ampicillin resistance gene; ori, origin of replication of *E. coli*; lacZ’, lactose operon.

**Fig. 2** Determination of penetratin-hMn-SOD. A. Western blot analysis of hMnSOD expression in *B. longum*-rhMnSOD. A band of about 22kD (lane 2), matching hMnSOD, was observed in the supernatant of *B. longum*-rhMnSOD with furin digestion. A band of about 25kDa (lane 1), whose molecular weight corresponded to the penetratin-hMnSOD fusion protein was obtained in the sample not treated with furin. These bands were not detected in *B. longum* (lane 3, 4). B. Growth (measured as OD$_{600}$) and hMnSOD expression of *B. longum*-rhMnSOD were measured at the indicated time points (0, 5, 10, 15, 20, 25, and 30 hours).

**Fig. 3** Bioactivity assay of *B. longum*-rhMnSOD in LPS-induced Caco-2 cells. A. ELISA analysis of the effects of *B. longum*-rhMnSOD on TNF-α and IL-6 secretion. B. Intracellular ROS levels were measured by flow cytometry after staining with DCFH-DA. **P<0.01; *P<0.05.**
**Fig. 4** Western blot analysis of rhMnSOD was entered into Caco-2 cells: A. 1, blank control cells alone; 2, hMnSOD group; 3, penetratin-hMnSOD group. B. Relative gray value of three groups. ***P<0.001.

**Fig. 5** Histological analysis of colonic samples (original magnification, ×40) after oral administration of *B. longum* for seven days. A. Normal group, showing a normal structure. B. Model group showing strong inflammatory damage. C. *B. longum* group, showing a reduction of the extent and severity of histological cell damage. D. *B. longum*-rhMnSOD group, showing a relatively normal colonic histology. E. Microscopic colonic damage scores in TNBS-induced colitis in rats. *P<0.05; **P<0.01.

**Fig. 6** MPO activity and MDA level in colon tissues after *B. longum*-rhMn-SOD administration. *P<0.05; ***P<0.001; **P<0.01.

**Fig. 7** The levels of TNF-α, IL-6, IL-1β and IL-8 in TNBS-induced colitis in rats after *B. longum*-rhMnSOD administration. *P<0.05; **P<0.01.
Table 1 Bacterial strains, plasmids and primers used in this study.

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<td>pDG7</td>
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| pBluescript II SK(-) | Amp
|                    | *Escherichia coli* clone plasmid |                     |
| pBsSOD            | Amp
|                    | pBluescript II SK(-) derivative containing the pMBI replicon, a functional *hup* gene promoter, amyB signal sequence, penetratin, a furin-cleavable sequence, *hMnSOD* gene, *hup* gene terminator | This study |

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Fig. 2

A

B

- Growth curve
- hMnSOD concentration

OD<sub>600</sub>

Time (h)

HnSOD concentration (μg/mL)
Fig. 3

A

![Graph showing TNF-α and IL-6 concentrations with comparison between Normal, Model, B. longum, and B. longum-rhMnSOD groups]

B

![Graph showing DCFH-DA fluorescence and relative ROS level with comparison between Normal, Model, B. longum, and B. longum-rhMnSOD groups]
Fig. 4

A

1 2 3

hMn-SOD

GAPDH

B

hMn-SOD/GAPDH

1 2 3

** *** **
Fig. 5
Fig. 6

A MPO

B MDA

Normal  Model  B. longum  B. longum-hMn-SOD

0.0  0.5  1.0  1.5
MPO activity (u/g)

0.0  0.5  1.0  1.5
MDA level (nmol/mg prot)

*  **  ***

**  **

*  *  **

*
Fig. 7

The graph shows the cytokine levels (ng/g tissue) for different groups: Normal, Model, B. longum, and B. longum-Mn-SOD. The cytokines measured are IL-1β, TNF-α, IL-6, and IL-8. The bars indicate the mean levels with error bars representing standard deviation. Significant differences are indicated by asterisks: * for p < 0.05 and ** for p < 0.01.