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

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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

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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

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51 function within a human from birth to old age [12]. Many studies have shown that
52 *Bifidobacterium* has many health-promoting properties, such as fighting inflammation,
53 microbial infections, and cancers [13, 14]. With these beneficial effects, some strains
54 of *Bifidobacterium* have been used as probiotics, and they have also become popular
55 in the medicine industry and in food science because their high security, elicits
56 prominent abilities of fermentation, resists extreme intestinal environment and adapts
57 to specific local communities[15, 16]. Further researches have suggested that
58 *Bifidobacterium* has been successfully used as a safe and effective delivery system for
59 genetic transformations[17, 18].

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

63 **Materials and methods**

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

65 The bacterial strains and plasmids used in this study are shown in Table 1. *E. coli*
66 DH5a was used for general cloning purposes and grown at 37°C in Luria-Bertani
67 broth with ampicillin continuous shaking culture. *B. longum* HB15 [17] were cultured
68 at 37°C in MRS medium supplemented with 0.05 % (w/v) L-cysteine under anaerobic
69 conditions. The pMB1 fragment containing a *Bifidobacterium* replicon, was obtained
70 from the plasmid pDG7 (ShineGene Molecular Biotech, China) [19, 20]. The *hup*
71 gene promoter and terminator as well as the amyB signal sequence, were amplified
72 from *Bifidobacterium*. An Oligonucleotide consisting of a penetratin sequence,
73 hMn-SOD gene, and a furin cleavage site between the penetratin and the hMn-SOD
74 was synthesized using codon optimization for *Bifidobacteria* [21], and together with a
75 promoter, a terminator, an amyB signal sequence, and pMB1, were inserted into

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76 pBluescript II SK(-) (ShineGene Molecular Biotech, China) to yield a 5.9 kb *E.*
77 *coli-Bifidobacterium* shuttle vector named pBsSOD (Fig. 1). The plasmid was
78 transformed into *Bifidobacterium* cells through electroporation [22] to yield *B.*
79 *longum*-rhMnSOD. The transformants were screened with agarized culture medium
80 containing ampicillin (100 mg/L final concentration) in an anaerobic condition.

81 **Expression of hMn-SOD**

82 The culture solution was harvested and centrifuged at 3000 rpm for 10 minutes,
83 collecting the supernatant and filtering it through 30 kD ultrafiltration membranes
84 (Millipore Amicon, USA), precipitating with trichloroacetic acid. The collected
85 fractions were dissolved in PBS and SDS-PAGE (12% gels) to separate the fusion
86 protein. The mouse anti-SOD2 monoclonal antibody (TIANGEN, China) was used for
87 a western blot to analyze the hMn-SOD per the manufacturer's instructions. The
88 hMn-SOD level in the culture supernatants were detected with an hMn-SOD ELISA
89 kit (TIANGEN, China), per the manufacturer's protocols, at 0, 5, 10, 15, 20, 25, and
90 30 hours after being sufficiently digested with furin.

91 **Cell culture**

92 Caco-2 cells (Bogoo, China) were grown in DMEM supplemented with 10% FBS,
93 1% NEAA, 1% L-glutamate, and antibiotic–antimycotic mixture (10000 U/ml
94 penicillin G, 10000 μg/ml streptomycin sulfate and 25 μg/ml amphotericin B in 0.85%
95 saline) at 37°C in culture flasks in a humidified air–5% CO2 atmosphere until the
96 experiments were performed.

97 **Bioactivity assay**

98 Cells were seeded into 24-well plates at a density of 4×10^6 cells per well.
99 *Bifidobacterial* culture supernatants were adjusted to an anticipated concentration
100 (containing 60 μg/ml rhMn-SOD). After removing the growth medium, the cells were

101 incubated with *B. longum* or *B. longum*-rhMn-SOD culture supernatants diluted 1:1
102 (v/v) with fresh cell culture medium in *B. longum* and *B. longum*-rhMn-SOD groups.
103 Cells in the normal and model groups should be treated with culture medium (for
104 bifidobacterial) diluted 1:1 (v/v) with fresh cell culture medium. After one hour for
105 incubation, cells were exposed to 5 μg/ml LPS (*E. coli*; Sigma). After another four
106 hours, parts of the cells were harvested and measured with an ELISA kit
107 (MultiSciences, China) to determine the amount of released TNF-α and IL-6 in the
108 supernatants.

109 Other parts of the cells were used to check the ROS removal activity of rhMn-SOD.
110 ROS level was assessed by using the oxidation-sensitive fluorescent probe,
111 DCFH-DA. Cells were incubated with DCFH-DA (5 mM) for 30 minutes at 37°C in
112 5% CO2 and then washed with HBSS. The ROS formation level was analyzed via
113 FACScan flow cytometry (Becton-Dickinson, USA).

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

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

123 **Animal assays**

124 These experiments were performed according to the Animal Care and Use
125 Committee of Jinan University, China. Forty-eight non-pathogenic Wistar male rats,

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

142 **Histological evaluation**

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

150 **MPO activity in colon tissues**

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

157 **Measured MDA level in colon tissues**

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

162 **Determination of cytokines production in colon tissues**

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

167 **Statistical analysis**

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

173 **RESULTS**

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

175 The pBluescript II SK (-) is an *Escherichia coli* clone plasmid, and it was selected

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

186 **Dtermination of penetratin-hMn-SOD**

187 To confirm the transformants of *B. longum* secreting hMnSOD, supernatants from
188 the modified *Bifidobacterium* were analyzed with western blotting. As shown in Fig.
189 2A, and as expected in the modified *Bifidobacterium*, we found a prominent band of
190 about 22kD in the supernatants after furin digestion. This band matched the calculated
191 molecular weight of an intact hMn-SOD, while it weighed nothing in the WT *B.*
192 *longum*, allowing us to conclude that this band corresponded to the hMn-SOD protein.
193 Surprisingly, a band of about 25kD, matching the penetratin hMn-SOD fusion protein,
194 was observed in the modified *Bifidobacterium* with the non-furin treatment; this could
195 be explained by a cross reaction of the penetratin-hMn-SOD fusion with the
196 anti-SOD2 monoclonal antibody. Next, we determined the hMn-SOD level in the
197 culture supernatants after furin digestion over time. The data was revealed in Fig. 2B.
198 An increasing hMn-SOD concentration in the supernatants was seen along with rapid
199 cell multiplication; this appearance may be associated with cell growth periodicity.

200 **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.

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226 **Oral administration of *B. longum*- rhMnSOD to against TNBS-induced colitis**

227 The given TNBS rats developed severe colitis that presented the typical features of 228 diarrhea, poor coat quality, and reduced mobility. Following seven days of treatment 229 with *Bifidobacterium*, these symptoms improved gradually in the *B. longum* group 230 and the *B. longum*-rhMnSOD group; however, the *B. longum*-rhMnSOD group was 231 more distinguished than the *B. longum* group.

232 Histological analyses of the colonic samples were performed after the application 233 of oral cells for seven days. The colon samples were from normal rats that had 234 histologically healthy mucosa (Fig. 5A). Those rats in the TNBS-induced colitis 235 model group showed significant inflammatory damage with mucosa destruction, cell 236 infiltration in the lamina propria, and goblet cell hyperplasia and destruction (Fig. 5B). 237 After treating rats with *Bifidobacterium* supernatants, the *B. longum* group showed a 238 reduction in inflammation; however, most tissues from this group still had severe 239 inflammatory damage compared with the rat colons from normal rats (Fig. 5C). The *B.* 240 *longum*-rhMn-SOD group displayed a relatively normal and integrated colonic 241 histology (Fig. 5D). The histological scores for the model and treatment groups 242 showed an increase in the degree of inflammation compared with normal rats (Fig. 243 5E). Treatment of rats with *B. longum*-rhMn-SOD supernatants significantly 244 decreased damage scores in TNBS-induced colitis rats compared with the model and 245 the *B. longum* group animals.

246 The MPO enzyme is a sign of neutrophil infiltration, which is closely related to 247 IBD. MPO activity was significantly increased in the TNBS-induced colitis model 248 group and the treatment groups compared to the normal group. However, the data 249 reflected that the MPO activity on day seven was significantly reduced in the *B.* 250 *longum*-rhMnSOD group compared with the model and the *B. longum* groups,

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251 suggesting reduced neutrophil infiltration in colon tissues (Fig. 6A).

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

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

261 **DISCUSSION**

262 IBD is a refractory immune-mediated disease that is characterized not only by
263 immune disorders and various inflammatory factors but also by abnormalities of the
264 intestinal microflora; its authentic pathogens and etiology remain unknown. Many
265 drugs and measures were introduced to achieve IBD resistance. One of the effective
266 strategies against UC is to recommend anti-inflammatory drugs to reduce the
267 inflammatory effects. It has been well established that inflammatory mediators, such
268 as proteases, cytokines, arachidonic acid metabolites, and ROS, are closely related to
269 gut inflammation [34]. Increasing evidence has shown that ROS make a critical
270 contribution to the pathogenesis of colitis [35, 36]. Therefore, controlling ROS in IBD
271 became a significant concern. Work over the recent decades has produced amazing
272 results, and researches in SOD and its mimetic could ameliorate colitis [7, 9].
273 Indubitably, these results are surprising; however, the existing problems and
274 limitations remained unanswered. It is well known that ROS, such as superoxide
275 anion, have a wide distribution and are not only located in blood but also in cytoplasm,

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276 nucleus, intercellular substances, and mitochondria. Thus, the SOD would ideally
277 journey to all these areas to eliminate harmful ROS. Conventional drug delivery
278 systems, oral and injection, do not meet these requirements. Therefore, it is necessary
279 to develop a method to eliminate this problem.

280 In our work, an optimistic transporter, penetratin, was introduced to deliver large
281 proteins into mammalian cells. Penetratin is an antennapedia homeodomain-derived
282 cationic peptide, and investigation showed that penetratin was the most promising
283 cell-penetrating peptide for effectively translocating cell membranes while covalently
284 or noncovalently conjugating to a larger cargo, such as polypeptides or
285 oligonucleotides [37], or even delivering cargo molecules to the cytosol and nucleus
286 [38] but not the mitochondria [39]. To deliver SOD to these areas, the MnSOD living
287 in the mitochondria were chosen. Among the various penetratins, the PEP-1 peptide
288 has been reported to successfully transfer SOD into cells [40-42]. PEP-1 peptide also
289 has advantages in protein transduction, including high stability, no cytotoxicity, and
290 low sensitivity to serum [43]. Thus far, many PEP-1 fusion proteins have been used to
291 protect against numerous diseases, including atherosclerosis, neuronal disease, and
292 skin inflammation [44-46]. In fact, our trans-membrane assay results showed that
293 PEP-1 was capable of translocating hMn-SOD into Caco-2 cells, which vividly
294 supported these reports (Fig. 4). Furthermore, we made SOD-conjugated penetratin
295 via a linker of the furin cleavage. Therefore, SOD could travel to the blood, cytoplasm,
296 and nucleus with the help of penetratin, and then it would obtain an integral hMnSOD
297 after the furin cleavage in vivo and destroy ROS.

298 SOD is a typical protein, and administering it orally might result in an extremely
299 low bioavailability because of its poor permeability [47] and rapid enzymatic
300 degradation in the gastro-intestinal tract [48]. Therefore, we chose *Bifidobacteria* as a

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

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REFERENCES

1. Eksteen B, Liaskou E and Adams DH. 2008. Lymphocyte homing and its role in the pathogenesis of IBD. Inflammatory Bowel Diseases. 14:1298-312.

2. Xavier RJ and Podolsky DK. 2007. Unravelling the pathogenesis of inflammatory bowel disease. Nature. 448:427-34.

3. Strober W, Fuss I and Mannon P. 2007. The fundamental basis of inflammatory bowel disease. Journal of Clinical Investigation. 117:514-21.

4. Abraham C and Cho JH. 2011. Inflammatory bowel disease. Practitioner 235:256-61.

5. Zhu H, Jia Z, Misra H and Y. Robert LI. 2012. Oxidative stress and redox signaling mechanisms of alcoholic liver disease: updated experimental and clinical evidence. Journal of Digestive Diseases. 13:133-42.

6. Korzenik JR and Podolsky DK. 2006. Evolving knowledge and therapy of inflammatory bowel disease. Nature Reviews Drug Discovery. 5:197-209.

7. Wang YH, Dong J, Zhang JX, Zhai J and Ge B. 2016. Effects of mimic of manganese superoxide dismutase on 2,4,6-trinitrobenzene sulfonic acid-induced colitis in rats. Archives of Pharmacal Research. 39:1-11.

8. Van-De-Wetering C, Coleman M, Dr, Smith B and Knudson C. 2008. Manganese superoxide dismutase gene dosage affects chromosomal instability and tumor onset in a mouse model of T cell lymphoma. Free Radical Biology & Medicine. 44:1677-86.

9. Seguí J, Gironella M, Sans M, Granell S, Gil F, Gimeno M, Coronel P, Piqué JM and Panés J. 2004. Superoxide dismutase ameliorates TNBS-induced colitis by reducing oxidative stress, adhesion molecule expression, and leukocyte recruitment

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351 into the inflamed intestine. Journal of leukocyte biology. 76:537-44.
352 10. Carroll IM, Andrus JM, Bruno-Bárcena JM, Klaenhammer TR, Hassan HM and
353 Threadgill DS. 2007. Anti-inflammatory properties of Lactobacillus gasseri
354 expressing manganese superoxide dismutase using the interleukin 10-deficient mouse
355 model of colitis. American Journal of Physiology Gastrointestinal & Liver Physiology.
356 293:G729-38.
357 11. Matés JM, Pérez-Gómez C and Núñez dCI. 1999. Antioxidant enzymes and
358 human diseases. Clinical Biochemistry. 32:595-603.
359 12. Martinez FA, Balciunas EM, Converti A, Cotter PD and Rp DSO. 2013.
360 Bacteriocin production by Bifidobacterium spp. A review. Biotechnology Advances.
361 31:482-488.
362 13. Srutkova D, Schwarzer M, Hudcovic T, Zakostelska Z, Drab V, Spanova A,
363 Rittich B, Kozakova H and Schabussova I. 2015. Bifidobacterium longum CCM 7952
364 Promotes Epithelial Barrier Function and Prevents Acute DSS-Induced Colitis in
365 Strictly Strain-Specific Manner. Plos One. 10.
366 14. Fu GF, Li X, Hou YY, Fan YR, Liu WH and Xu GX. 2005. Bifidobacterium
367 longum as an oral delivery system of endostatin for gene therapy on solid liver cancer.
368 Cancer Gene Therapy. 12:133-140.
369 15. Thierry A. 2008. Safety assessment of dairy microorganisms: Propionibacterium
370 and Bifidobacterium. International Journal of Food Microbiology. 126:316-20.
371 16. Turroni F, Van SD and Ventura M. 2010. Genomics and ecological overview of
372 the genus Bifidobacterium. International Journal of Food Microbiology. 149:37-44.
373 17. Wei P, Yang Y, Ding Q, Li X, Sun H, Liu Z, Huang J and Gong Y. 2015. Oral
374 delivery of Bifidobacterium longum expressing alpha-melanocyte-stimulating
375 hormone to combat ulcerative colitis. Journal of Medical Microbiology. 65:160.

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376 18. Wei P, Yang Y, Li T, Ding Q and Sun H. 2015. A engineered Bifidobacterium
377 longum secreting a bioative penetratin-Glucagon-like peptide 1 fusion protein
378 enhances Glucagon-like peptide 1 absorption in the intestine. Journal of Microbiology
379 & Biotechnology.
380 19. Rossi M, Brigidi P, Gonzalez VyRA and Matteuzzi D. 1996. Characterization of
381 the plasmid pMB1 from Bifidobacterium longum and its use for shuttle vector
382 construction. Research in Microbiology. 147:133-43.
383 20. Liu DW, Sun ZK, Guo YH and Huang LY. 2010. Establishment of an Efficient
384 Transformation System and Expression of GFP in Bifidobacterium Longum
385 NCC2705. Letters in Biotechnology.
386 21. Escogido MLR, Rodríguez ADL and Rosa APBDL. 2007. A novel binary
387 expression vector for production of human IL-10 in Escherichia coli and
388 Bifidobacterium longum. Biotechnology Letters. 29:1249-1253.
389 22. Turroni F, Duranti S, Bottacini F, Guglielmetti S, Van SD and Ventura M. 2014.
390 Bifidobacterium bifidum as an example of a specialized human gut commensal.
391 Frontiers in Microbiology. 5:437-437.
392 23. Feng W, Mei S, Wenjie Y and Luyuan H. 2011. High-level soluble expression of
393 recombinant human manganese superoxide dismutase in Escherichia coli , and its
394 effects on proliferation of the leukemia cell. Protein Expression & Purification.
395 77:46-52.
396 24. Elson CO, Beagley KW, Sharmanov AT, Fujihashi K, Kiyono H, Tennyson GS,
397 Cong Y, Black CA, Ridwan BW and Mcghee JR. 1996. Hapten-induced model of
398 murine inflammatory bowel disease: mucosa immune responses and protection by
399 tolerance. Journal of Immunology. 157:2174.
400 25. Shkoporov AN, Efimov BA, Khokhlova EV and Kafarskaia LI. 2008. Production

401 of human basic fibroblast growth factor (FGF-2) in Bifidobacterium breve using a
402 series of novel expression/secretion vectors. Biotechnology Letters. 30:1983-1988.
403 26. Wei P, Yang Y, Ding Q, Li X, Sun H, Liu Z, Huang J and Gong Y. 2015. Oral
404 delivery of Bifidobacterium longum expressing alpha-melanocyte-stimulating
405 hormone to combat ulcerative colitis. Journal of Medical Microbiology. 65.
406 27. Tian P, Xu B, An G, Sun H, Shi W and Li Z. 2014. A new therapies for intestinal
407 flora imbalance used ampicillin resistant probiotics. Journal of Microbiology &
408 Biotechnology.
409 28. Henriques ST and Castanho MA. 2005. Environmental factors that enhance the
410 action of the cell penetrating peptide pep-1 A spectroscopic study using lipidic
411 vesicles. Biochimica Et Biophysica Acta. 1669:75-86.
412 29. Deng SH, Ke XZ, Shi XY, Cai ZZ, Yang JN, Huang YZ and Chen P. 2012.
413 Construction of pET15b-VP3 Gene Prokaryotic Expression Vector by LP Recco PCR
414 Cloning. Journal of Chengdu Medical College.
415 30. Choi HS, An JJ, Kim SY, Lee SH, Kim DW, Yoo KY, Won MH, Kang TC, Kwon
416 HJ and Kang JH. 2006. PEP-1-SOD fusion protein efficiently protects against
417 paraquat-induced dopaminergic neuron damage in a Parkinson disease mouse model.
418 Free Radical Biology & Medicine. 41:1058-1068.
419 31. Liao S and Xie JW. 2005. Application of Caco-2 cell model in drug in vitro
420 research. Chinese New Drugs Journal.
421 32. Leung PH, Zhang L, Zuo Z and Lin G. 2005. Intestinal absorption of Stemona
422 alkaloids in a Caco-2 cell model. Planta Medica. 72:211-6.
423 33. Draper HH and Hadley M. 1989. Malondialdehyde determination as index of
424 lipid peroxidation. Methods in Enzymology. 186:421-431.
425 34. Seguí J, Gil F, Gironella M, Alvarez M, Gimeno M, Coronel P, Closa D, Piqué

ACCEPTED

426 JM and Panés J. 2005. Down-regulation of endothelial adhesion molecules and
427 leukocyte adhesion by treatment with superoxide dismutase is beneficial in chronic
428 immune experimental colitis. Inflammatory Bowel Diseases. 11:872-882.
429 35. Kruidenier L and Verspaget HW. 2002. Review article: Oxidative stress as a
430 pathogenic factor in inflammatory bowel disease—Radicals or ridiculous? Alimentary
431 Pharmacology & Therapeutics. 16:1997–2015.
432 36. JayPravda. 2005. Radical induction theory of ulcerative colitis. World Journal of
433 Gastroenterology. 11:2371-2384.
434 37. Dupont E, Prochiantz A and Joliot A. 2011. Penetratin Story: An Overview.
435 Methods in Molecular Biology. 683:21-29.
436 38. Muratovska A, Lightowlers RN, Taylor RW, Wilce JA and Murphy MP. 2001.
437 Targeting large molecules to mitochondria. Advanced Drug Delivery Reviews.
438 49:189-198.
439 39. Ross MF, Gait MJ and Murphy MP. 2004. Cell-penetrating peptides do not cross
440 mitochondrial membranes even when conjugated to a lipophilic cation: evidence
441 against direct passage through phospholipid bilayers. 383:457-468.
442 40. Huang GQ, Wang JN, Tang JM, Zhang L, Zheng F, Yang JY, Guo LY, Kong X,
443 Huang YZ and Liu Y. 2011. The combined transduction of copper, zinc-superoxide
444 dismutase and catalase mediated by cell-penetrating peptide, PEP-1, to protect
445 myocardium from ischemia-reperfusion injury. Journal of Translational Medicine.
446 9:73.
447 41. Wang JN, Ding P, Huang YZ, Luo LN, Guo LY, Kong X and Shao F. 2007. [The
448 protective effect of PEP-1-SOD1 preconditioning on hypoxia/reoxygenation injury in
449 cultured human umbilical vein endothelial cells]. Chinese Journal of Cardiology.
450 35:750-756.

ACCEPTED

451 42. Yang B, Tan LG, Zhang L, Zheng F, Kong X, Guo LY, Yang JY and Wang JN.
452 2013. Inhibitory effect of PEP-1-SOD1 fusion protein on synthesis of collagen Ⅰ
453 induced by angiotensin Ⅱ in rat cardiac fibroblasts. Journal of Xinxiang Medical
454 University.
455 43. Morris MC, Depollier J, Mery J, Heitz F and Divita G. 2001. A peptide carrier for
456 the delivery of biologically active proteins into mammalian cells. Nature
457 Biotechnology. 19:1173-6.
458 44. Kim DW, Kim DS, Kim MJ, Kwon SW, Ahn EH, Jeong HJ, Sohn EJ, Dutta S,
459 Lim SS and Cho SW. 2011. Imipramine enhances neuroprotective effect of
460 PEP-1-Catalase against ischemic neuronal damage. Bmb Reports. 44:647-652.
461 45. Wu Y, Xie G, Xu Y, Ma L, Tong C, Fan D, Du F and Yu H. 2015. PEP-1-MsrA
462 ameliorates inflammation and reduces atherosclerosis in apolipoprotein E deficient
463 mice. Journal of Translational Medicine. 13:316.
464 46. Kim MJ, Kim DW, Park JH, Kim SJ, Lee CH, Yong JI, Ryu EJ, Cho SB, Yeo HJ
465 and Hyeon J. 2013. PEP-1-SIRT2 inhibits inflammatory response and oxidative
466 stress-induced cell death via expression of antioxidant enzymes in murine
467 macrophages. Free Radical Biology & Medicine. 63:432-445.
468 47. Camenisch G, Alsenz J, Van dWH and Folkers G. 1998. Estimation of
469 permeability by passive diffusion through Caco-2 cell monolayers using the drugs'
470 lipophilicity and molecular weight. European Journal of Pharmaceutical Sciences.
471 6:317-24.
472 48. Woodley JF. 1994. Enzymatic barriers for GI peptide and protein delivery.
473 Critical Reviews in Therapeutic Drug Carrier Systems. 11:61-95.
474 49. Javed NH, Alsahly MB and Khubchandani J. 2016. Oral Feeding of Probiotic
475 Bifidobacterium infantis: Colonic Morphological Changes in Rat Model of

476 TNBS-Induced Colitis. Scientifica. 2016:9572596.
477 50. Philippe D, Heupel E, Blumsperisen S and Riedel CU. 2011. Treatment with
478 Bifidobacterium bifidum 17 partially protects mice from Th1-driven inflammation in
479 a chemically induced model of colitis. International Journal of Food Microbiology.
480 149:45-9.
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483 **Figure legends**

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

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

498 **Fig. 3** Bioactivity assay of *B. longum*-rhMnSOD in LPS-induced Caco-2 cells. A.
499 ELISA analysis of the effects of *B. longum*-rhMnSOD on TNF-α and IL-6 secretion.
500 B. Intracellular ROS levels were measured by flow cytometry after staining with
501 DCFH-DA. **P<0.01; *P<0.05.

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502 **Fig. 4** Western blot analysis of rhMnSOD was entered into Caco-2 cells: A. 1, blank
503 control cells alone; 2, hMnSOD group; 3, penetratin-hMnSOD group. B. Relative
504 gray value of three groups. ***P<0.001.
505 **Fig. 5** Histological analysis of colonic samples (original magnification, ×40) after oral
506 administration of *B. longum* for seven days. A. Normal group, showing a normal
507 structure. B. Model group showing strong inflammatory damage. **C.** *B. longum* group,
508 showing a reduction of the extent and severity of histological cell damage. D. *B.*
509 *longum*-rhMnSOD group, showing a relatively normal colonic histology. E.
510 Microscopic colonic damage scores in TNBS-induced colitis in rats. *P<0.05;
511 **P<0.01.
512 **Fig. 6** MPO activity and MDA level in colon tissues after *B. longum*-rhMn-SOD
513 administration. *P<0.05; ***P<0.001; **P<0.01.
514 **Fig. 7** The levels of TNF-a, IL-6, IL-1β and IL-8 in TNBS-induced colitis in rats after
515 *B. longum*-rhMnSOD administration. *P<0.05; **P<0.01.
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Table. 1 Bacterial strains, plasmids and primers used in this study.

	Relevant characteristics	Further informat
Bacterial strain		
B. longum HB15	Source of *hup* gene promoter and hup and transformation host	This study
E. coli DH5a	Cloning host	
Plasmids		
pDG7	Source of the pMBl replicon	
pBluescript II SK(-)	Amp^r; *Escherichia coli* clone plasmid	
pBsSOD	Amp^r; pBluescript II SK(-) derivative containing the pMBl replicon, a functional *hup* gene promoter, amyBsignal sequence, penetratin, a furin-cleavable sequence, *hMnSOD* gene, *hup* gene terminator	This study
Primer		
Phup-F	5’-GGACTAGTCCATACCCCCTTCGGGGAA-3’	SpeI
Phup-R	5’-GCTCTAGAGCAAAGCATCCTTCTTGGG-3’	XbaI
Thup-F	5’-TTGCGGCCGCAACCTTCTGCTCGTAGCG-3’	NotI
Thup-R	5’-GCCGCGGCCGCTGAACTAGTCCGG-3’	SacII

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Fig. 1

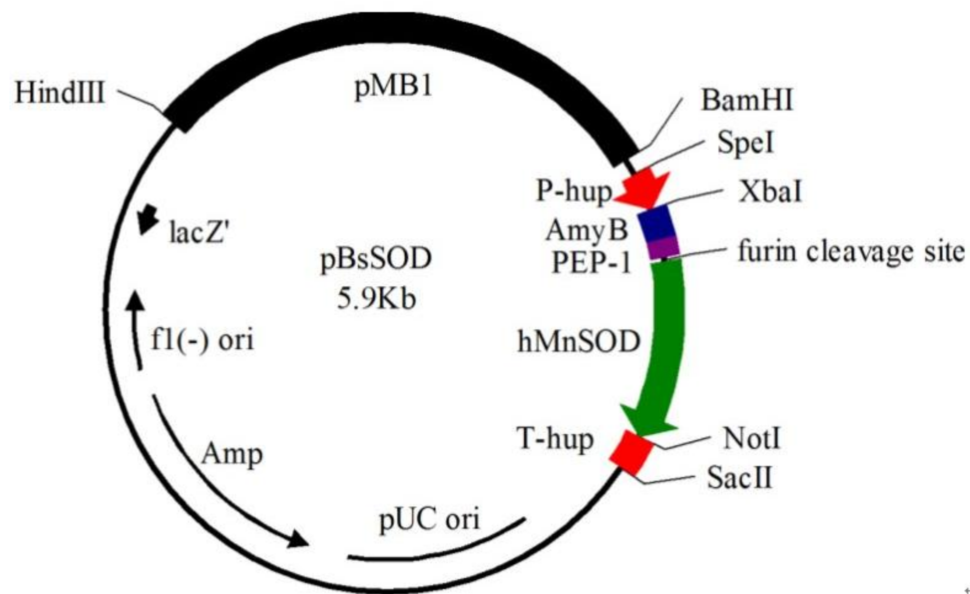

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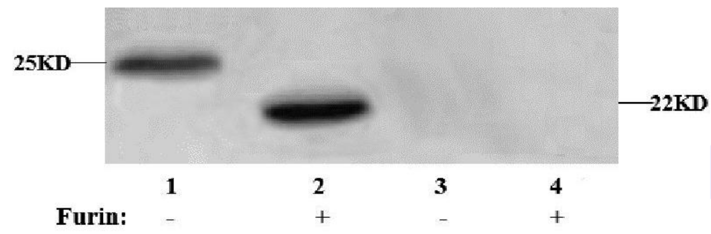
Fig. 2

A



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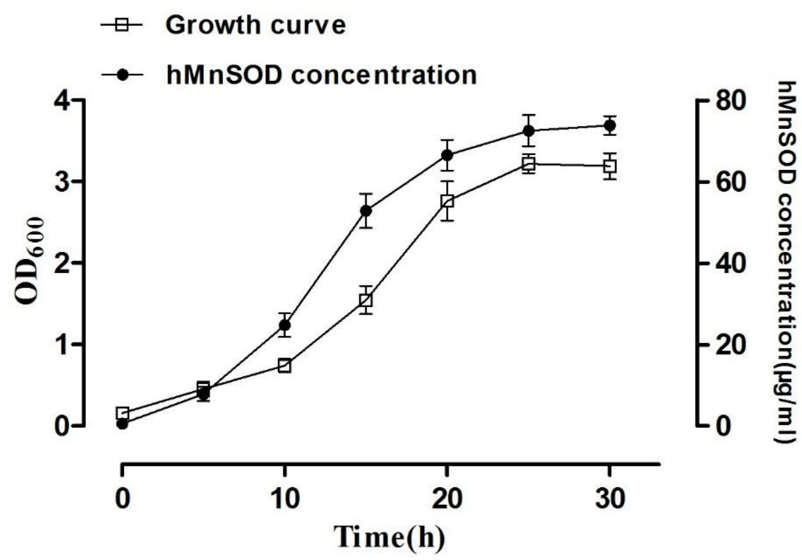

Fig. 3

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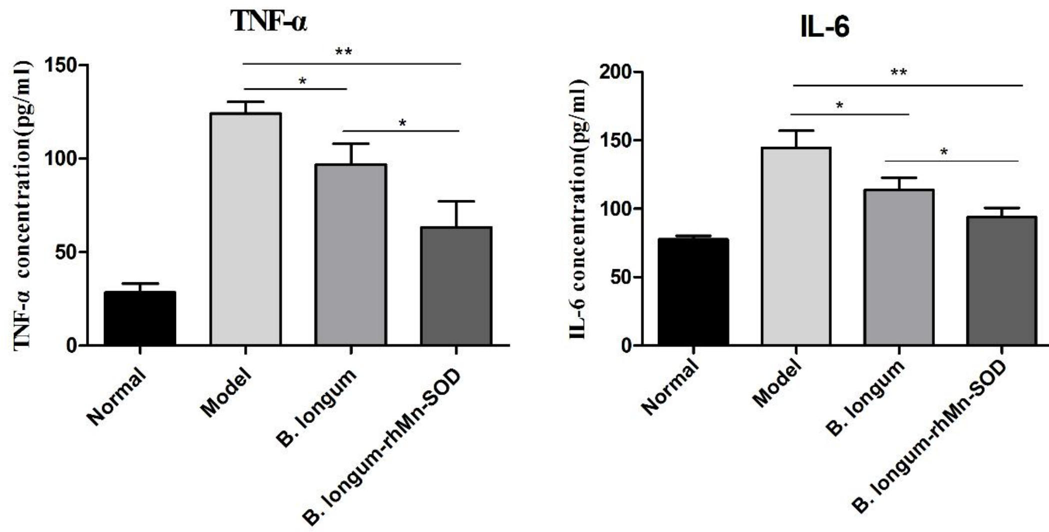

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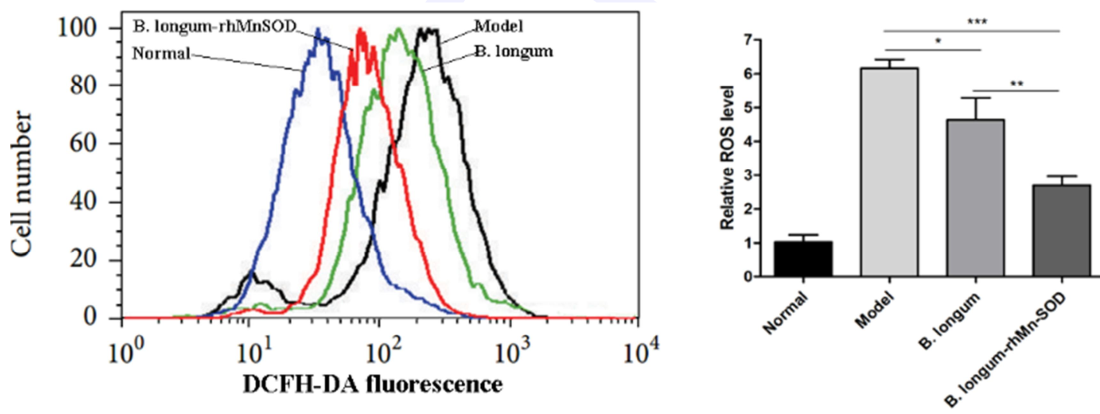
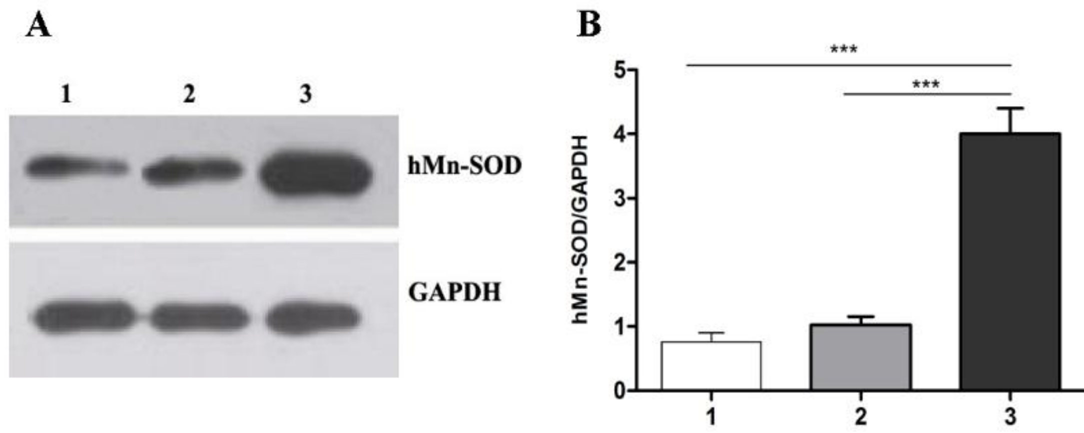


Fig. 4



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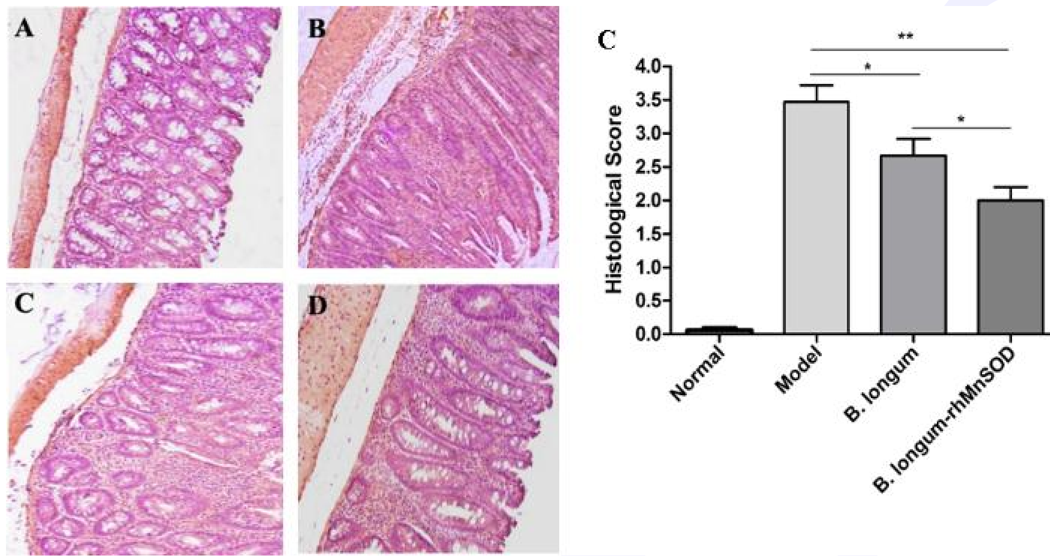

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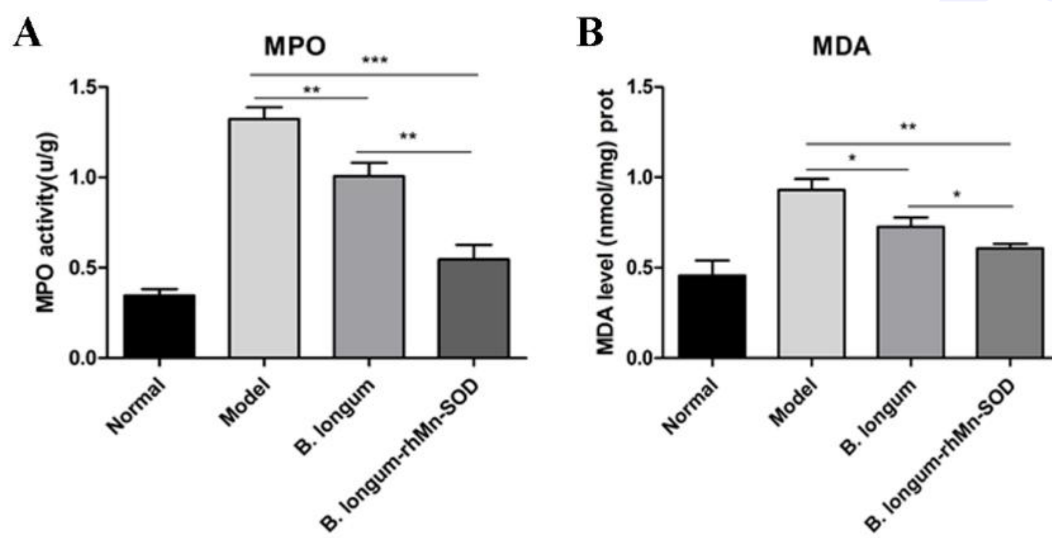
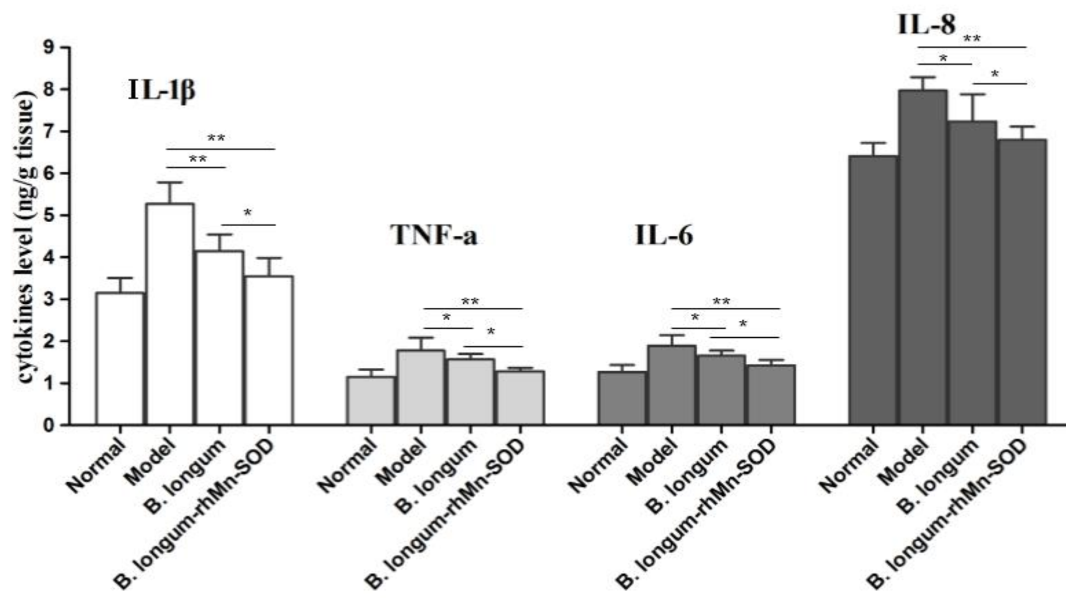


Fig.7



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