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1 **Diet with lactosucrose supplementation ameliorates trinitrobenzene**
2 **sulfonic acid-induced colitis in rats**

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16 **Author Contributions**
17 Z. Ruan was in charge of the whole project and involved in the designing of the study and
18 revised the paper. Y. Zhou conducted the animal trial and wrote the part of paper. H. Li, L.
19 Wang, S. Liu assisted with tissue collection, chemical analyses; X. Zhou, and C. Zhang,
20 assisted with RT-PCR; X. Huang assisted with H&E staining and immunohistochemistry. Z.
21 Deng, G. Wu, and Y. Yin assisted with discussion.
22

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23 **Abstract:**

24 Chronic intestinal inflammation contributes to an increased risk of colon cancer.
25 Lactosucrose (LS), a kind of functional trisaccharide, can modulate immunity and promote
26 microbe growth. The aim of this study was to investigate the effects of LS on
27 2,4,6-trinitrobenzenosulfonic acid (TNBS) induced colitic in rats. Rats had been randomly
28 into four treatments: Normal group, TNBS group, LS group, and salicylazosulfapyridine
29 (SASP) group for five weeks. LS supplementation ameliorated TNBS-induced colitis. LS
30 supplementation increased IL-10 production and suppressed the secretion of IL-12 in the
31 colon, as compared to TNBS group. LS decreased the production of TLR-2 protein and
32 nuclear NF-κB p65 protein, as well as mRNA levels, as compared with colitic rats. These
33 results indicated that chronic feeding of LS inhibited TNBS-induced chronic inflammation.
34 LS has potential nutraceutical intervention to combat colitis.

35

36 **Key words:** lactosucrose; inflammation; colitis; Toll-like receptor 2; nuclear factor kappa B
37 (NF-κB)

38

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39 **1. Introduction**

40 Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative
41 colitis (UC), are chronically recurrent disorders of the intestine [1]. IBD was involved in the
42 dysregulation of the intestinal immune response. Immune and non-immune cells within the
43 intestinal mucosa recognize conserved microbial structures through so-called pattern
44 recognition receptors (PRRs), including toll-like receptors (TLRs) [2]. And TLR-mediated
45 cellular responses can lead to chronic inflammation, which in turn contributes to the
46 development of IBD [3]. Polymorphisms in TLR-1,-2, -4 and -9 loci have been associated with
47 IBD in human [4-6].

48 Nuclear factor-kappa B (NF-κB) may serve as the main mediator of TLRs signaling in
49 IBD [7]. It is a key regulator of the inducible expression of numerous genes involved in
50 immune and inflammatory responses in the gut [8, 9]. Increased NF-κB activation has been
51 detected in the intestinal lamina propria of patients with CD or murine TNBS colitis models
52 [10, 11]. Colonic mucosal biopsies as well as lamina propria mononuclear cells from IBD
53 patients display increased presence of NF-κB p65 in the nucleus in comparison to mucosa
54 samples from healthy individuals [12]. Neurath et al. were able to prevent and even to abrogate
55 established TNBS-induced colitis in mice by administrating antisense oligonucleotides
56 directed against NF-κB p65 [13]. Growing evidences in the association of NF-κB activation and
57 IBD suggest that modulation of NF-κB signaling pathway is the main target for the
58 anti-inflammatory treatment of IBD.

59 Activation of NF-κB then upregulates the expression of numerous proinflammatory
60 cytokines, such as interleukin-6 (IL-6) and IL-12, involved in intestinal inflammation. IL-6 is
61 a pleiotropic cytokine. An elevation in serum IL-6 levels during remission in CD patients was
62 found to be clinically relevant parameter for predicting inflammatory activity [14] as well as for
63 corresponding with a high frequency of disease relapse [15]. TNBS-induced colitis can be
64 abrogated with antibodies specific for IL-12 [16], in which excessive production of IL-12 seems
65 to be the crucial underlying genetic abnormality in susceptible mice [17]. However, IL-10 is an
66 anti-inflammatory cytokines that inhibit both antigen presentation and subsequent release of
67 proinflammatory cytokines, thereby attenuating mucosal inflammation. IL-10 can inhibit the
68 effector functions of activated macrophages and monocytes in vitro and downregualte IL-12

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[18], and inactivation of IL-10 in mice results in an increased production of IL-12 [19]. IL-10-/-mice spontaneously develop colitis in a bacteria-containing environment but are healthy in germ-free conditions [20].

Although the etiology of IBD is not well understood, the promoting effects of functional oligosaccharides on intestinal health have been attracted more and more attention. Fructo-oligosaccharide attenuates the production of pro-inflammatory cytokines [21]. Fruit intake is negatively associated with the risk for IBD and can reduce the risk factors of colon cancer [22]. So far, a number of experimental findings have suggested that intestinal metabolites (short-chain fatty acids) and beneficial bacterium in gut lumen contribute to the remission of IBD. For example, butyric acid attenuates intestinal inflammation in murine colitis [23]. In addition, it is different for luminal and mucosa-associated microflora between IBD patients and healthy controls [24].

Several animal models have been developed to investigate the pathology of IBD. One of these models is 2,4,6-trinitrobenzene sulfonic acid (TNBS) induced colitis in which rodents receiving TNBS develop acute and chronic transmural colitis resembling colitis [25]. The TNBS/ethanol induced colitis model has been very useful in studying many important aspects of gut inflammation, including cytokine secretion patterns, cell adhesion, and immunotherapy [26].

Lactosucrose (4^G-β-D-galactosylsucrose, LS) is an indigestible trisaccharide, which is composed of galactose, glucose, and fructose. It was approved as commercial prebiotics by Japan Health Department before 20 years and is prevalent until now. Several studies have shown that LS confers anti-inflammatory and anti-allergic effects [27-30]. Hino *et al*. reported that LS could stimulate overproduction of growth factor-β1 (TGF-β1) in peyers patches [28]. LS was found to inhibit immunoglobulin E production and increase the secretive yield of IL-10 in ovalbumin/alum-immunized mice [29]. In addition, LS has been shown to promote the growth of *Bifidobacteria* in patients with chronic inflammatory bowel disease or in healthy human [30, 31]. Although a number of studies have investigated the effects of LS on inflammation, the anti-inflammatory effect and mechanisms of LS have not been studied thoroughly.

In our previous study, we found LS increased short chain fatty acid (SCFA) production,

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99 and promoted *Bifidobacterium* and *Lactobacillus*, decreased the number of *Escherichia* in
100 TNBS-induced colonic inflammation [32]. The main objective of present work is to investigate
101 the effects of LS on TNBS-induced colitic rats and its effect on TLR-2, TLR-4 and nuclear
102 NF-κB p65 expression in colitis.
103

104 **2. Materials and Methods**

105 2.1 Chemicals

106 TNBS (0.5% w/v) was purchased from Sigma-Aldrich Chemical Co. (MO, USA).
107 Rabbit polyclonal NF-κB p65, TLR-2, and Lamin B1 antibodies were purchased from Santa
108 Cruz Biotechnology (CA, USA). NE-PER nuclear and cytoplasmic extraction reagents and
109 enhanced chemiluminescence were purchased from Thermo Scientific (MA, USA).
110 Peroxidase conjugated anti-rabbit immunoglobulin G, IL-12, IL-10, and IL-6 ELISA kits
111 were purchased from Boster Biological Technology (Wuhan, China). Alanine transaminase
112 (ALT) and aspirate transaminase (AST) were obtained Nanjing Jiancheng Bioengineering
113 Institute (Nanjing, China). Blood clinical chemistry reagents for measure of alkaline
114 phosphatase (ALP), total protein (TP), albumin (ALB), urea nitrogen (BUN), low-density
115 lipoprotein (LDL) and cholesterol (CHO) were form Leadman Biochemistry (Beijing, China).
116 Trizol was purchased from Invitrogen (NY, USA). Reverse Transcription System Kit from
117 (Beijing, China).

118 2.2 Preparation of Lactosucrose

119 LS was catalytically synthesized from sucrose and lactose solution by
120 β-fructofuranosidase. β-fructofuranosidase was derived from *Arthrobacter* sp.10138 in our
121 laboratory. In this enzyme-catalyzed reaction (pH 7.0 and 30 ^{o}C), the solution contained 550
122 mmol/L sucrose, 550 mmol/L lactose, and β-fructofuranosidase (250 U/ml). After a 24h
123 period, the whole solution was vacuum freeze-drying, and LS was obtained. And the
124 concentration of saccharides were analyzed by high performance liquid chromatography
125 (HPLC) 1200 from Agilent (USA) with evaporative light scattering detector (ELSD) model
126 300S (Softa, America) according to Ruan *et al* [33]. The chromatographic column is a Kromasil
127 100-5NH_2 column (250 mm × 4.6mm). The mobile phase consisted of acetonitrile/water
128 (75:25 V/V) flowed in the rate of 1.0 mL/min, and the temperature of column oven was 25 ºC.

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129 The drift tube and evaporator temperature was set at 70 ºC and 30 ºC, respectively.

130 2.3 Animals care

131 Female Sprague-Dawley rats weighing 200 ± 20 *g* were obtained from SIPPR-BK
132 Experimental Animal (Shanghai, China). All animals were housed in a specific pathogen-free
133 condition, temperature-controlled atmosphere (25 ± 1 °C at 50% relative humidity) under a
134 12 h light/12 h dark cycle. The rats had free access to standard diet (GB14924.1-2001, Table
135 1) and sterile drinking water at all times. This study was carried out in Jiangxi Province
136 Center for Disease Control and Prevention (Nanchang, China), and performed in accordance
137 with the Chinese guidelines for the Laboratory Animals Care.

138 2.3.1 Introduction of colitis and experimental design

139 An ulcerative colitis model was induced by previously described methods [34]. Rats were
140 deprived of food but not water for 24 h before induction of colitis. Fasted animals were
141 lightly anesthetized with 1% pentobarbital sodium, and a plastic catheter was inserted rectally
142 into the colon. Then TNBS was located 8 cm proximal to the anus. One milliliter of TNBS
143 dissolved in 50% (v/v) ethanol was introduced into the lumen of the colon at a dose of
144 100 mg/kg body weight through the plastic catheter. Following administration of TNBS, the
145 animals were kept in a head-down position for 60 s to allow the samples to move through the
146 gastrointestinal tract.

147 The rats with intestinal inflammation induced by TNBS were assigned randomly into
148 one of the three treatments: the TNBS group (receiving physiological saline), the LS group
149 (intragastric administration, 250 mg/kg body weight every day), and the SASP group
150 (intragastric administration receiving salicylazosulfapyridine (SASP), 250 mg/kg body
151 weight every day) as positive control. SASP is commonly used in clinical therapeutics for
152 IBD patients. Normal rats that had not been exposed to TNBS were given physiological
153 saline (Normal group). There were 6 rats in each group. All rats in each group were treated
154 for 35 days, and then sacrificed with ether anesthesia to obtain tissue samples.

155 2.3.2 Evaluation of colitis severity

156 The parameters recorded in the experiments were the disease activity index (DAI) [35],
157 body weight, food intake and small intestinal length index. DAI ranging from 0-3 using the
158 following parameters: weight loss, occult blood positivity, and stool consistency of the animal

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159 (Table 2).

160 2.4 Histological grading of colitis

161 Histological examination was performed as previously described [36]. The colon tissues
162 were fixed in 10% (v/v) buffered formalin phosphate, embedded in paraffin, and cut into 5
163 μm section. Then the sections were stained with hematoxylin and eosin, and then assessed
164 under light microscopy. The degree of inflammation was graded semi-quantitatively from 0 to
165 4 (0, no signs of inflammation; 1, very low level of inflammation; 2, low level of leukocyte
166 infiltration; 3, high level of leukocyte infiltration, high vascular density, and thickening of the
167 colon wall; and 4, transmural infiltration, loss of goblet cells, high vascular density, and
168 thickening of the colon wall). Grading was performed in a blinded fashion.

169 2.5 Assay of blood clinical chemistry

170 When rats were sacrificed, fresh blood samples were obtained and collected in tubes
171 containing heparin. The blood samples were centrifuged at 3500 r/min and the supernatant
172 fluid (plasma) was obtained. All samples were stored at -20 °C until analysis.

173 Hematological parameters ALP, BUN, LDL, TP, CHO, and ALB were determined using
174 a biochemistry analyzer (Beckman, USA). ALT and AST were determined by
175 spectrophotometry using commercial kits.

176 2.6 Assay of secretory production of IL-12, IL-6 and IL-10 in the colon

177 The production of IL-12, IL-6 and IL-10 in the colon was measured using
178 enzyme-linked immunosorbent assay (ELISA). The sections of the proximal colon (100 mg)
179 were taken from inflammed areas and homogenized in physiological saline (10 ml). The
180 solution was centrifuged at 3500 r/min for 15 min. The supernatant was obtained for the
181 analysis of cytokines. These cytokines were measured with ELISA kits (Boster Biological
182 Technology, Wuhan, China) according to the manufacturer's protocol. The ELISA microplate
183 was read using an ELISA reader (Dynatech Laboratories, USA) with an absorbance
184 maximum at 450 nm.

185 2.7 Immunohistochemistry

186 Immunohistochemistry was performed as previously described [37]. Samples from the
187 colon were fixed in 4% paraformaldehyde in phosphate buffer solution and dehydrated with
188 ethanol. Then those samples were embedded in paraffin. Sections (5 μm thick) were prepared

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189 and exposed to immunoperoxidase. Endogenous peroxidase was cleared by treatment with
190 3% H_2O_2 phosphate buffered saline (PBS) for 20 min at room temperature. After washing
191 with 0.05 mol/L PBS (pH 7.4), slides were blocked with 5% bovine serum albumin (BSA) in
192 PBS for 20 min at room temperature to prevent non-specific protein binding. Then, the slides
193 were sequentially exposed to the primary antibody TLR-2 (Santa Cruz Biotechnology, CA,
194 USA, 1:200) and NF-κB p65 (Santa Cruz Biotechnology, CA, USA, 1:80) in PBS and
195 incubated overnight at 4°C. After rinsing, the slides were incubated with a secondary
196 antibody conjugated to biotinylated goat peroxidase and 0.1% (v/v) diaminobenzidine
197 substrate. Slides processed without primary antibody served as the negative control. There are
198 four slides every sample (rat) and there are four observations for a slide. The optical densities
199 of TLR-2 and NF-κB p65 proteins in the colonic epithelial cells were determined by
200 densitometric scanning using the Motic Med System 6.0 Software.

201 2.8 Western blot analysis of NF-κB p65 in nucleus

202 The activation of NF-κB in colon was assessed by western blot. Nuclear protein in colon
203 tissue was extracted by commercial kit (Thermo Scientific, MA, USA) according to the
204 manufacturer. The protein content was estimated by coomassie brilliant blue method. Proteins
205 (100 μg) were separated with 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis
206 and then transferred to a polyvinylidene difluoride (PVDF) membrane (2 h, 200 mA). The
207 membranes were blocked in 5% BSA for 1 h at room temperature. The different proteins
208 were visualized using NF-κB p65 (1:1000) and Lamin B1 (1:500) primary anti-bodies and
209 developed using enhanced chemiluminescence (Thermo Scientific, MA, USA). The image of
210 the blot was acquired with Molecular Imager Gel Doc XR (Bio Rad). The optical density of
211 bands was calculated and analyzed by means of Image J (NIH, Bethesda, MD, USA).

212 2.9 Quantitative analysis of gene expression with fluorescent quantitative polymerase chain
213 reaction

214 Real-time fluorescent quantitative polymerase chain reaction (PCR) analysis was used to
215 measure the gene expression of TLR-2, TLR-4, and NF-κB. Colonic tissues from the normal,
216 TNBS, LS, and SASP groups were immediately frozen in liquid nitrogen for RNA extraction.
217 Total RNA was isolated from tissues using the Trizol reagent (Invitrogen, Amenrican) method
218 as described by the manufacturer. cDNA synthesis was carried out using the Reverse

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219 Transcription System Kit (Takara, Dalian, China), according to the manufacturer's
220 instructions. Fluorescent quantitative PCR was carried out using SYBR Premix Ex Taq
221 (Takara, Japan) on an ABI 7900HT PCR instrument (Applied Biosystems, USA). The
222 experimental conditions were: denaturation for 30s at 95 °C, annealing for 5s at 95 °C, and
223 extension for 30 s at 60 °C, for 40 cycles. Rat primers were designed using Primer 3 and
224 synthesized by Sangon Biotech Co., Ltd (Table 3) (Shanghai, China). The relative mRNA
225 levels of the genes were calculated using the $2^{-\Delta\Delta Ct}$ formula [38] and the rat GAPDH as the
226 housekeeping gene.

227 3.0 Statistical analysis

228 All values in the figures and text are expressed as mean ± standard deviation (SD).
229 Differences between the means of individual groups were determined by one-way analysis of
230 variance with the Duncan multiple range test. *P* value <0.05 were considered statistically
231 significant. The statistical software package SPSS 11.5 (SPSS Institute, Illinois, USA) was
232 used for these analyses.

233

234 **3. Results**

235 3.1 Production and analysis of LS

236 In present work, LS production was carried out using reaction mixture of
237 β-fructofuranosidase, sucrose and lactose (1:1) in incubator at 30 °C for 24 h. In this reaction,
238 the fructosyl moiety of sucrose is transferred to lactose by fructofuranosidase, thus forming
239 LS. The concentration of oligosaccharides formation during course of the periods was
240 analyzed by HPLC (Fig.1). The maximum amount of LS formation was observed 55.04%
241 (w/w) in which the concentration of lactose was remarked 22.81% (w/w), sucrose 19.04%
242 (w/w), glucose 1.86% (w/w), and fructose 1.25% (w/w) respectively.

243 3.2 LS improves colitis clinical symptoms and colon damage in rats

244 Rats exposed to TNBS developed symptoms of colitis as assessed by DAI. LS treatment
245 for 14 days the DAI was significantly reduced compared to TNBS control rats. After SASP
246 supplementation for 7 days, the DAI in SASP group was significantly decreased, as compared
247 with TNBS control group. Compared to normal group, DAI in LS group or SASP group were
248 no significantly difference after 14 days. There were no significant changes among all

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249 treatment groups after 28days (Fig 2 A).

250 Histological analysis of colon (Fig. 2 B, C, D, and E) showed that there was different for
251 intestinal microstructure between 4 groups. In the TNBS group, colonic architecture such as
252 crypt and surface epithelial were changed as well as massive transluminal infiltration of
253 inflammatory cells. In contrast, infiltration of inflammatory cells was mild in submucosal
254 area in animals fed with the LS or SASP diets. Histologic scores of inflammatory group were
255 significantly increased after TNBS enema compared to normal animals (Fig. 2 F). Dietary LS
256 or SASP supplementation significantly mitigated intestinal injury compared to TNBS group,
257 and no significant difference was found between LS and SASP group. Hence, LS treatment
258 rat had a recovery effect for inflammation.

259 3.3 Effects of LS supplementation on blood chemistry in colitis rats

260 TNBS significantly increased the activities of AST ($P < 0.05$) and ALT ($P < 0.05$) as well
261 as concentrations of TP ($P < 0.05$) and CHO ($P < 0.05$) in serum, but decreased ALP ($P <$
262 0.05) activity, as compared with normal rats (Table 4). Compared to normal group, the
263 content of TP ($P < 0.05$), ALB ($P < 0.05$), and CHO ($P < 0.05$) in LS or SASP group were
264 increased. The activity of AST ($P < 0.05$) and LDL ($P < 0.05$) content in SASP group were
265 increased, as compared with normal rats.

266 ALP is a membrane-bound enzyme that can dephosphorylate multiplex phosphate
267 substrates [39]. ALP removes the phosphate group of lipopolysaccharide (LPS) to generate
268 monophage lipid A which is much less toxic than LPS [40]. AST activity was significantly
269 decreased ($P < 0.05$) and ALP activity was increased ($P < 0.05$) in LS group compared to
270 TNBS group. No significantly difference of AST activity and ALP activity were found in LS
271 group compared to normal group. In SASP group, the activity of ALP was increased
272 compared to TNBS group colitic rats ($P < 0.05$), and there was no significant difference
273 between SASP group and normal group (Table 4). The effect of LS and SASP showed similar
274 effect on blood chemistry.

275 3.4 Effect of LS supplementation on IL-12, IL-6 and IL-10 secretion

276 IL-12 and IL-6 are known as a proinflammatory cytokine. IL-10 is a well known
277 anti-inflammatory cytokine [41]. In this study, TNBS-induced colitis was characterized by
278 significantly increased the levels of IL-12 ($P < 0.01$) and IL-6 ($P < 0.05$), and a significantly

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279 decreased in the level of IL-10 ($P < 0.05$) compared to normal rats. Following administration
280 of LS, the level of IL-12 was decreased ($P < 0.01$) (Fig. 3A), and the level of IL-10 ($P < 0.05$)
281 was increased, compared to TNBS group rats (Fig. 3C). The level of IL-6 also tended to
282 decrease in LS-treated rats ($P > 0.05$), compared to the TNBS group (Fig. 3B). SASP
283 supplementation decreased the level of IL-12 ($P < 0.01$), and increased IL-10 level ($P < 0.05$),
284 as compared with TNBS group. When compared with normal rats, the content of IL-12, IL-10,
285 and IL-6 in LS or SASP group had no significant difference. The effect of LS on cytokines
286 modulation was similar with SASP. These results suggest that anti-inflammation of LS may
287 be involved in the down-regulation of IL-12 level and up-regulation of IL-10 level.

288 3.5 Modulatory effect of LS on the TLR-2, TLR-4 and NF-κB mRNA levels

289 Gut bacteria influence TLRs expression [42]. LS supplementation could modulate cecum
290 and colonic bacteria in our previously study. Then, the mRNA levels for TLR-2, TLR-4 and
291 NF-κB p65 were determined. Compared to normal rats, significant increases in mRNA
292 abundances of these proteins in inflammatory TNBS group was observed after administration
293 of TNBS (Fig. 4). The expression of TLR-2 and NF-κB p65 mRNA in the LS group was
294 significantly down-regulated compared to the inflammatory TNBS group ($P < 0.05$), and had
295 no significant difference compared to normal group. TLR-4 mRNA was reduced by LS
296 supplementation compared to colitis rat ($P > 0.05$). SASP supplementation decreased the
297 expression of TLR-2 ($P < 0.01$) and TLR-4 ($P < 0.05$) mRNA, as compared with colitis rats,
298 and had no significant difference compared to normal rats (Fig. 4). The mRNA levels for
299 TLR-2, TLR-4 and NF-κB p65 in LS group had no significant difference compared to SASP
300 rats.

301 3.6 Inhibitory effect of LS supplementation on TLR-2 and NF-κB p65 protein levels in
302 TNBS-induced colitis

303 Next, the levels of TLR-2 and NF-κB p65 protein were determined by
304 immunohistochemistry (Fig 5). The immunohistochemistry of TLR-2 in colon was showed in
305 Fig. 5A-5D, and the NF-κB p65 was showed in Fig. 5F-5I. Arrows indicated the positive cells.
306 The results showed that TNBS treatment caused a significantly increased in TLR-2
307 (71.7±20.0 to 583.2±61.0) (Fig. 5E) and NF-κB p65 (97.6±19.1 to 667.9±109.9) (Fig. 5J).
308 TLR-2 and NF-κB p65 protein abundance in the LS group or SASP group was significantly

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309 decreased compared to the TNBS group (inflammatory rats). Compared to normal group, the
310 levels of TLR-2 and NF-κB p65 in LS or SASP group had no significant difference.

311 The expression of NF-κB p65 in nucleus was determined by western blot. NF-κB was an
312 important downstream molecular of TLRs activation. When NF-κB was activated, it was
313 transported into nucleus and stimulated various pro-cytokines production. In this study, the
314 expression NF-κB p65 in the nucleus of intestinal cells in TNBS group rats was increased (P
315 < 0.01), as compared with normal group. Colitis rats supplemented with LS ($P < 0.01$) or
316 SASP ($P < 0.01$) decreased the expression of nuclear NF-κB, as compared with colitis rats,
317 and had no significant difference compared to normal rats (Fig. 6). The anti-inflammatory
318 effect of LS or SASP may correlate with the inhibition of NF-κB activation, and the effects of
319 LS on the inhibition of NF-κB activation is equivalent with SASP.

320

321 **4. Discussion**

322 The incidence of IBD is on the rise not only in developed countries but also in
323 developing countries, such as China [43]. Thus, it is imperative to increase the repertoire of
324 methods available for inhibition of intestinal inflammation. Recent studies have shown that
325 dietary compounds remission IBD by its anti-inflammatory effect and modification of
326 microbiota composition [44-46]. Prebiotics, including inulin and fructooligosaccharide, may play
327 an important role in controlling inflammatory disease [47, 48]. Oligosaccharides have not been
328 extensively studied, but they may offer ideal treatment or co-treatment options due to their
329 capacity to reduce intestinal inflammation. Here, we demonstrated that LS supplementation
330 successfully ameliorates colitis. The mitigatory effect of LS on colitis was found to be similar
331 to that of SASP. Thus, functional oligosaccharides, including LS and fructooligosaccharide,
332 may serve as novel alternative options for the treatment or remission in IBD.

333 In recent years, many studies have demonstrated that development of intestinal
334 inflammation is partly attributed to TLRs activation [3, 49]. Among this family of receptors,
335 TLR-2 and TLR-4 in colitis had been reported in numerous articles and paid much attention
336 to the development of IBD [5, 50-53]. TLR-2-/-, TLR-4-/- and TLR-2/-4-/- mice displayed
337 reduced macroscopic signs of acute colitis and the amelioration of inflammation [54]. In
338 TNBS-induced murine colitis, the level of TLR-2 and TLR-4 were increased [55, 56]. In

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339 conclusion, TLR-2 and TLR-4 play an important role in colitis.

340 TLR-2 and TLR-4 are regulated by intestinal bacteria. TLR-2 recognizes lipoteichoic
341 acid, peptidoglycan and related cell-wall glycopolymers in gram-positive bacteria and
342 probiotics (including *Lactobacilli* and *Bifidobacteria*). TLR-4 recognizes LPS which is a
343 bacteria cell wall component of gram-positive bacteria (including *Escherichia*). *Escherichia*
344 stimulate TLR-4 activation [57]. Lee *et al.* [58] reported *Lactobacillus suntoryeus* HY7801
345 inhibited TLR-4 linked NF-κB activation in TNBS-induced colitic mice. *Lactobacillus*
346 protects intestinal epithelium from radiation injury in a TLR-2/cyclo-oxygenase-2-dependent
347 manner [59]. Oral administration of *Bifidobacterium* bifidum activated TLR-2 in the intestinal
348 epithelium necrotizing enterocolitis rat [60]. In our previous study, LS supplementation in
349 TNBS-induced colonic inflammation increased the levels of *Bifidobacterium* and
350 *Lactobacillus*, decreased the number of *Escherichia* [32]. Therefore, in this study, the
351 expression of TLR-2 and TLR-4 were determined to explore the effect of LS on
352 TNBS-induced rat colitis.

353 In the present study, LS supplementation to colitis rats significantly decreased the level
354 of TLR-2 mRNA and protein compared to colitis rats ($P < 0.05$). The expression of TLR-4
355 mRNA was decreased compared to inflammatory rats ($P > 0.05$). These results indicated that
356 LS alleviated colitis associated with down-regulated TLR-2 expression rather than TLR-4.
357 When TLR-2 was activated by its ligands, the production of pro-inflammatory cytokines
358 (such as IL-12) was increased and production of anti-inflammatory cytokines (such as IL-10)
359 was decreased, and suggesting inducing inflammation [61, 62]. It was reported that TLR-2
360 polymorphisms and protein was up-regulated in IBD patients and animal models with colitis
361 [51, 52, 55]. Inhibited or down-regulated TLR-2 expression may alleviate colonic inflammation.
362 TLR-2 monoclonal antibody supplementation in dextran sulfate sodium-induced colitis mice
363 decreased colonic inflammation [63]. Following granulocyte and monocyte adsorption apheresis
364 treatment, TLR-2 cells in the colorectal mucosa was decreased [64].

365 TLRs related signaling pathway might be influenced by microbe and short chain fatty
366 acid. Intestinal microbe alone or combine with short chain fatty acid, such as butyric acid,
367 impacted TLR-2 activation [65-67]. Caco-2 cells cultured with yeast increased the level of
368 TLR-2 mRNA, while Caco-2 cells incubated with butyrate (10 mmol/L) and yeast decreased

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369 the level of TLR-2 mRNA [65]. Mirmonsef *et al.* [66] reported the TLR-2 ligand could enhance
370 IL-8 and TNF-α production in blood mononuclear cells and neutrophils pretreated with low
371 levels of SCFA (0.02-2 mmol/L), while the TLR-4 ligand did not have such an effect.
372 Kovarik *et al.* [67] suggested that higher concentrations of n-butyrate (0.06-1 mmol/L) inhibited
373 the release of IL-12/23p40 from blood mononuclear cells (obtained from IBD patients) after
374 activation via TLR-2 agonists, but not TLR-4 engagement. According several reported papers
375 with vitro experiments, SCFA play dual-directional regulation in TLR-2 signaling pathway. In
376 our previously study, the numbers of *Lactobacilli* and *Bifidobacteria,* as well as the
377 production of SCFA in LS-supplemented rats were increased, and the numbers of *Escherichia*
378 were decreased, compared to the inflammatory group [32]. In this study, after LS administration,
379 TLR-2 level was decreased, the decreased of TLR-2 expression was related to the increase of
380 *Lactobacilli* and *Bifidobacteria*, and short chain fatty acid need further research.

381 LS possesses anti-inflammatory effect by up-regulating IL-10 level, down-regulating
382 IL-12 and NF-κB levels in colon. IL-10 is an important anti-inflammatory cytokines for
383 preventing intestinal inflammation. It is evidenced by the spontaneous colitis in IL-10-/- mice
384 [68]. Taniguchi *et al.* [29] reported LS increased the production of IL-10 in
385 ovalbumin/alum-immunized mice. NF-κB can be activated by TLRs, such as TLR-2 and
386 TLR-4, and and then stimulate the production of various pro-inflammation cytokines (IL-12
387 and IL-6 et al.) [69]. Inhibiting of NF-κB was a possible way for combating against
388 inflammation. *B. longum* HY8004 and curcumin, have been reported to attenuate colitis via
389 inhibition of NF-κB activation [57, 70]. In our studies, Dietary supplemented with LS or SASP in
390 colitis rats inhibited IL-12 production and nuclear NF-κB p65 expression, and increased
391 IL-10 production in the colon.

392

393 **5. Conclusion**

394 LS has an inhibitory effect on intestinal inflammation induced by TNBS. The LS exerts
395 similar effects on ameliorating colitis that is shown by SASP widely used in the treatment of
396 IBD. LS can increase the production of anti-inflammatory cytokine IL-10 and decrease the
397 production of IL-12, meanwhile inhibite TLR-2 and NF-κB expression at mRNA and protein
398 level, and inhibite NF-κB activation. LS may be used as a functional food for IBD patients.

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399

400 **Acknowledgments**

401 This research was financially supported by National Natural Science Foundation of
402 China (Grant No. 31001014) and the Research Foundation (SKLF-TS-201108 and
403 SKLF-TS-200817) and the Open Project Program (SKLF-KF-201005 and SKLF-KF-201216)
404 of State Key Laboratory of Food Science and Technology, Nanchang University.

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534 **Table 1.** The composition and nutrient of experiment diet

Ingredients	Content (%)	Chemical composition	Content
Wheat	14	Digestible energy (Mal/kg)	3.40
Corn	43	Crude protein (%)	21.0
Soybean meal	24	Crude Fat (%)	4.5
Full fat soybean extruded	8	Calcium (%)	1.0
Soybean oil	1.4	Total phosphate (%)	0.7
Whey powder	3		
Fish meal	3.2	Sodium (%)	0.3
Limestone	1.3	Met + Cys (%)	0.78
Dicalcium phosphate	1.1	Lys (%)	1.35
Vitamin-mineral premix[#]	1.0	Thr (%)	0.88

535 The vitamin-mineral premix provided (per kilogram feed): vitamin A, 14,000 IU; vitamin D_3,
536 1500 IU; vitamin E, 5 mg; vitamin K, 5 mg; thiamine, 13 mg; riboflavin, 12 mg; pyridoxine,
537 12 mg; vitamin B_{12}, 0.022 mg; niacin 60 mg; pantothenic acid, 24 mg; biotin, 0.2 mg; folic
538 acid, 6 mg; choline chloride, 350 mg; Fe (as iron sulfate), 120 mg; Cu (as copper oxide), 10
539 mg; Mn (as manganous oxide), 75 mg; Zn (as zinc oxide), 30 mg; I (as ethylenediamine
540 dihydroiodide), 0.5 mg; and Se (as sodium selenite), 0.2 mg.

541

542 **Table 2.** Disease activity index (DAI)

Score	Weight loss (% of initial wt)	Blood in feces	Stool consistency
0	<1	Negative	Normal pelletets
1	1-4.99	Occult blood+	Slightly loose feces
2	5-10	Occult blood ++	Loose feces
3	>10	Gross blood	Watery diarrhea

543

544 **Table 3.** Primers design for genes analysed by real-time quantitive PCR

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		Primers	Product (bp)	Genebank accession no.
TLR-2	forward primer	5'- AGAGGGAAATCGTGCGTGAC -3'	137	NM_198769.2
	reverse primer	5'-CCATACCCAGGAAGGAAGGCT -3'		
TLR-4	forward primer	5'-AGAATGAGGACTGGGTGA-3'	323	NM_019178
	reverse primer	5'-GCTAAGAAGGCGAGTACAACCT-3'		
NF-κB p65	forward primer	5'-GCACTGTGAGGACGGCATA-3'	96	AF 079314.1
	reverse primer	5'-CGTGAAGTATTCCCAGGTTTG -3'		
GAPDH	forward primer	5′-GGCAAGTTCAACGGCACAG-3′	142	NM_017008.3
	reverse primer	5′-CGCCAGTAGACTCCACGACAT-3′		

545

546 **Table 4.** Effect of lactosucrose on blood profile in TNBS-induced colitis rats

Group	Normal	TNBS	LS	SASP
AST (U/L)	31.8±3.1	42.2±6.4*	32.2±3.3#	38.7±3.2*
ALT (U/L)	11.5±1.4	19.8±5.9*	15.0±2.8	16.3±3.5
ALP (U/L)	62.6±21.8	41.0±15.4*	67.6±17.7#	73.7±15.0#
TP (g/L)	58.9±6.6	65.4±3.3*	67.6±4.9*	65.3±1.9*
ALB (g/L)	28.6±2.7	30.8±1.9	31.4±2.8*	31.4±1.1*
BUN (mmol/L)	5.90±0.88	6.48±0.44	5.99±1.10	6.78±1.18
LDL (mmol/L)	0.36±0.09	0.43±0.07	0.46±0.09	0.48±0.06*

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CHO (mmol/L)	0.97±0.17	1.25±0.17*	1.27±0.45*	1.02±0.35*

547 Values are expressed as mean ± S.D., n=6.* Mean values were significantly different
548 compared with normal rats (* $P < 0.05$); # Mean value were significantly different compared
549 with TNBS rats (# $P < 0.05$).
550 AST, aspartate aminotransferase; ALT, alanine transarninase; ALP, alkaline phosphatase; TP,
551 total protein; ALB, albumin; BUN, urea nitrogen; LDL, low-density lipoprotein; CHO,
552 cholesterol.
553

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554 **Figures Legends**

555 **Fig. 1.** HPLC chromatogram of LS formation

556 HPLC chromatogram of LS: (1) fructose; (2) glucose; (3) sucrose; (4) lactose; (5) LS.

557

558 **Fig. 2.** Administration of LS attenuates the development of TNBS induced colitis in rat.

559 (A) The disease activity index (DAI) indicated the grade of intestinal inflammation.
560 Histologic findings of colon samples stained with hematoxylin and eosin. (B) Normal control
561 rat. Micrograph showed normal mucosa (NM), submucosa (SM), epithelium (Ep) and crypt
562 (Cr). (C) TNBS induced colitis rat. Micrograph showed surface epithelial (arrow-heads) and
563 crypt (hollow arrow-heads) loss , severe infiltration of inflammatory cells in NM (arrows)
564 and SM area (hollow arrows). (D) LS-treated and (E) SASP-treated rat with colitis induced
565 by TNBS, micrograph showed that integrated surface epithelium (arrow-heads) and normal
566 crypt (hollow arrow-heads), the infiltration of inflammatory cells was mild in NM (arrows)
567 and SM area (hollow arrows). (F) The values mean a grading scale for histological
568 assessment of inflammation in colitis. Values are expressed as mean ± S.D., n=6.* Mean
569 values were significantly different compared with normal rats ($^{*}P < 0.05$; $^{**}P < 0.01$); #
570 Mean value were significantly different compared with TNBS rats ($^{\#}P < 0.05$; $^{\#\#}P < 0.01$),
571 n=6.

572

573 **Fig. 3.** Effects of LS on inflammatory cytokines IL-12 (A), IL-6 (B), and IL-10 (C) in colon
574 of colitis rats.

575 The contents of IL-12 (A), IL-6 (B), and IL-10 (C) were measured using ELISA. Values
576 are expressed as mean ± S.D., n=6. * Mean values were significantly different compared with
577 normal rats ($^{*}P < 0.05$; $^{**}P < 0.01$); $^{\#}$ Mean value were significantly different compared with
578 TNBS rats ($^{\#}P < 0.05$; $^{\#\#}P < 0.01$), n=6.

579

580 **Fig. 4.** Effects of LS on mRNA expression of TLR and NF-κB.

581 Total RNA was extracted from colon tissues for quantification by real-time fluorescent
582 quantitative PCR. The relative mRNA expression of the target genes was calculated using the

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583 $2^{-\Delta\Delta Ct}$ formula and were normalized using GAPDH as the housekeeping gene. Values are
584 expressed as mean ± S.D., n=6.* Mean values were significantly different compared with
585 normal rats ($^{*}P < 0.05$; $^{**}P < 0.01$); # Mean value were significantly different compared with
586 TNBS rats ($^{\#}P < 0.05$; $^{\#\#}P < 0.01$), n=6.
587
588 **Fig. 5.** Representative images for the effect of LS on TLR-2 and NF-κB p65 protein in the
589 colon.
590 TLR-2 and NF-κB p65 protein in colonic tissue were measured by immunohistochemistry
591 methods. Immunohistochemistry of TLR-2 expression in colon: (A) Normal control rat, (B)
592 TNBS induced colitis rat, (C) LS-treated rat with colitis induced by TNBS, (D) SASP-treated
593 rat with colitis induced by TNBS. (E) Integrated optical density of TLR-2.
594 Immunohistochemistry of NF-κB p65 expression in colon: (F) Normal control rat, (G) TNBS
595 induced colitis rat, (H) LS-treated rat with colitis induced by TNBS, (I) SASP-treated rat with
596 colitis induced by TNBS. (J) Integrated optical density of NF-κB p65. TLR-2 and NF-κB
597 p65positive cells (arrows) were stained in brown. Values are expressed as mean ± S.D., n=6.
598 * Mean values were significantly different compared with normal rats ($^{*}P <0.05$; $^{**}P < 0.01$);
599 # Mean value were significantly different compared with TNBS rats ($^{\#}P < 0.05$; $^{\#\#}P < 0.01$),
600 n=6.
601
602 **Fig. 6.** Effect of LS on the expression of nuclear NF-κB p65 in colon of colitis rats.
603 (A) Western analysis of NF-κB p65 and Lamin B1 expression in nucleus of colon. (B)
604 Relative density of NF-κB p65, the density of NF-κB p65 band was normalized to that of
605 Lamin B1.
606 Values were expressed as the mean ± S.D.; n = 3 per group. * Mean values were significantly
607 different compared to normal rats ($^{**}P < 0.01$); # Mean value were significantly different
608 compared with TNBS rats ($^{\#\#}P < 0.01$).
609

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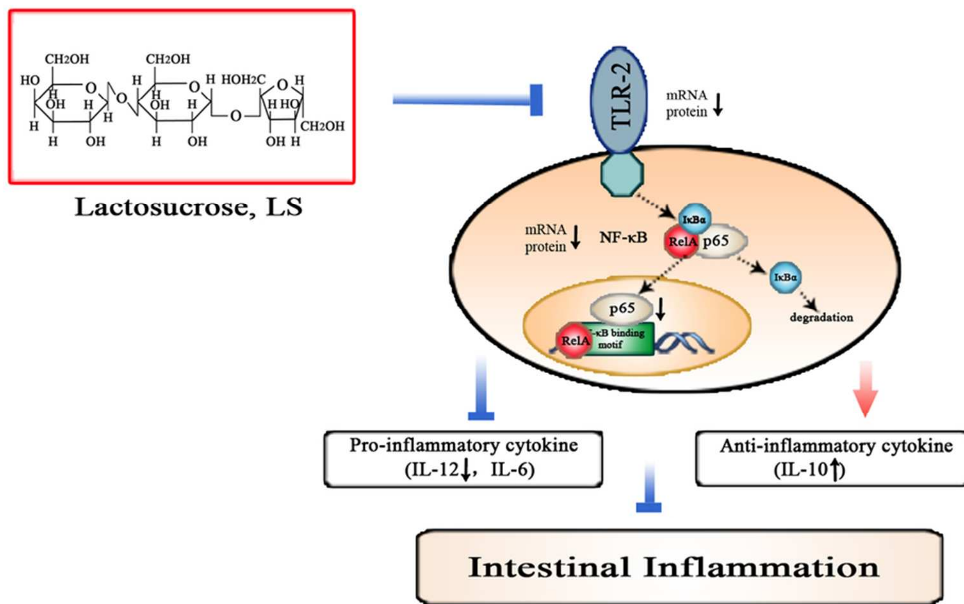

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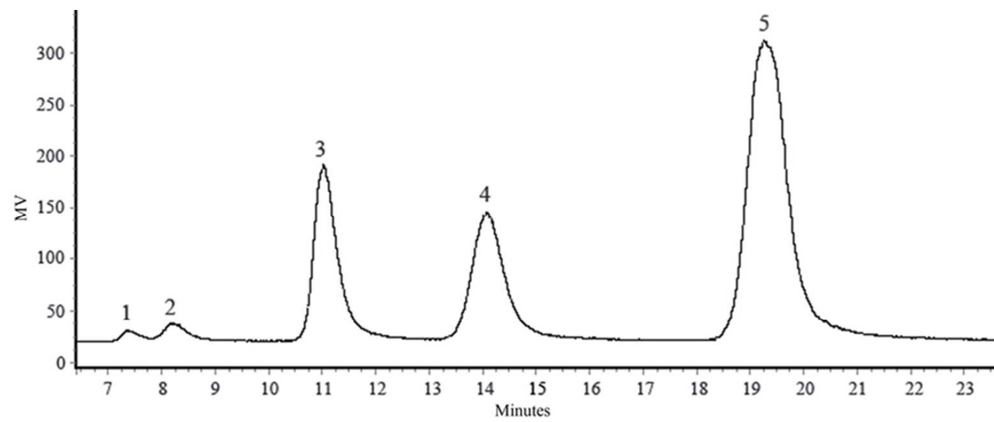

Fig. 1. HPLC chromatogram of LS formation
HPLC chromatogram of LS: (1) fructose; (2) glucose; (3) sucrose; (4) lactose; (5) LS.

35x14mm (600 x 600 DPI)

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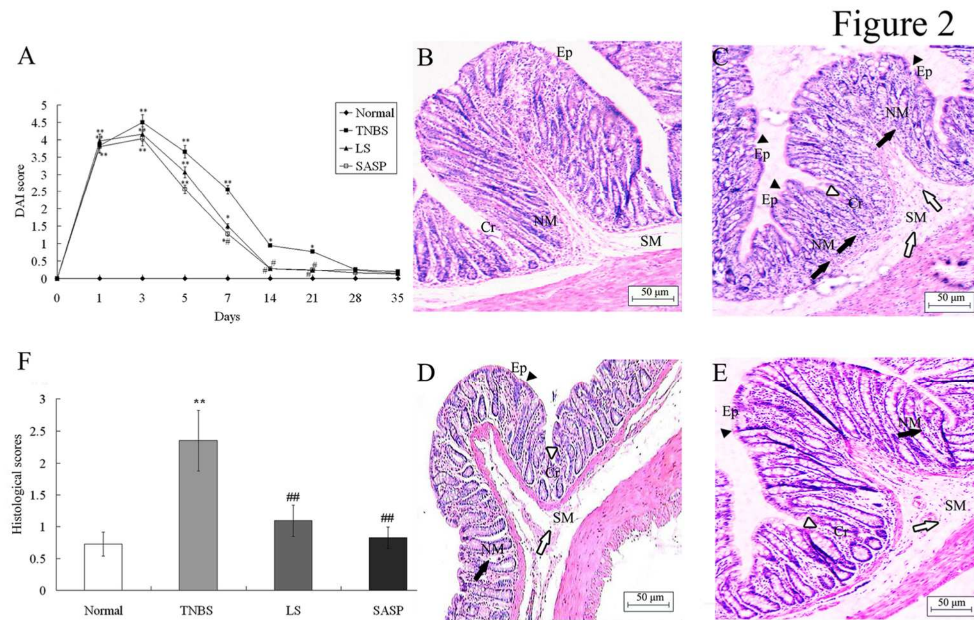

Fig. 2. Administration of LS attenuates the development of TNBS induced colitis in rat.
(A) The disease activity index (DAI) indicated the grade of intestinal inflammation. Histologic findings of colon samples stained with hematoxylin and eosin. (B) Normal control rat. Micrograph showed normal mucosa (NM), submucosa (SM), epithelium (Ep) and crypt (Cr). (C) TNBS induced colitis rat. Micrograph showed surface epithelial (arrow-heads) and crypt (hollow arrow-heads) loss , severe infiltration of inflammatory cells in NM (arrows) and SM area (hollow arrows). (D) LS-treated and (E) SASP-treated rat with colitis induced by TNBS, micrograph showed that integrated surface epithelium (arrow-heads) and normal crypt (hollow arrow-heads), the infiltration of inflammatory cells was mild in NM (arrows) and SM area (hollow arrows). (F) The values mean a grading scale for histological assessment of inflammation in colitis. Values are expressed as mean ± S.D., n=6.* Mean values were significantly different compared with normal rats (* $P < 0.05$; ** $P < 0.01$); # Mean value were significantly different compared with TNBS rats (# $P < 0.05$; ## $P < 0.01$), n=6.

67x45mm (600 x 600 DPI)

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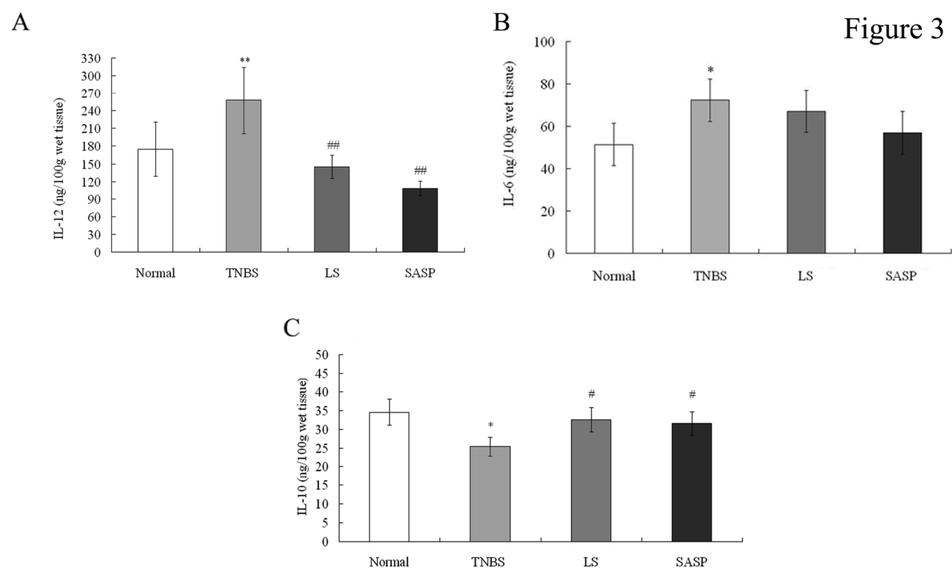

Fig. 3. Effects of LS on inflammatory cytokines IL-12 (A), IL-6 (B), and IL-10 (C) in colon of colitis rats. The contents of IL-12 (A), IL-6 (B), and IL-10 (C) were measured using ELISA. Values are expressed as mean ± S.D., n=6. * Mean values were significantly different compared with normal rats (*P < 0.05; **P < 0.01); # Mean value were significantly different compared with TNBS rats (#P < 0.05; ##P < 0.01), n=6.

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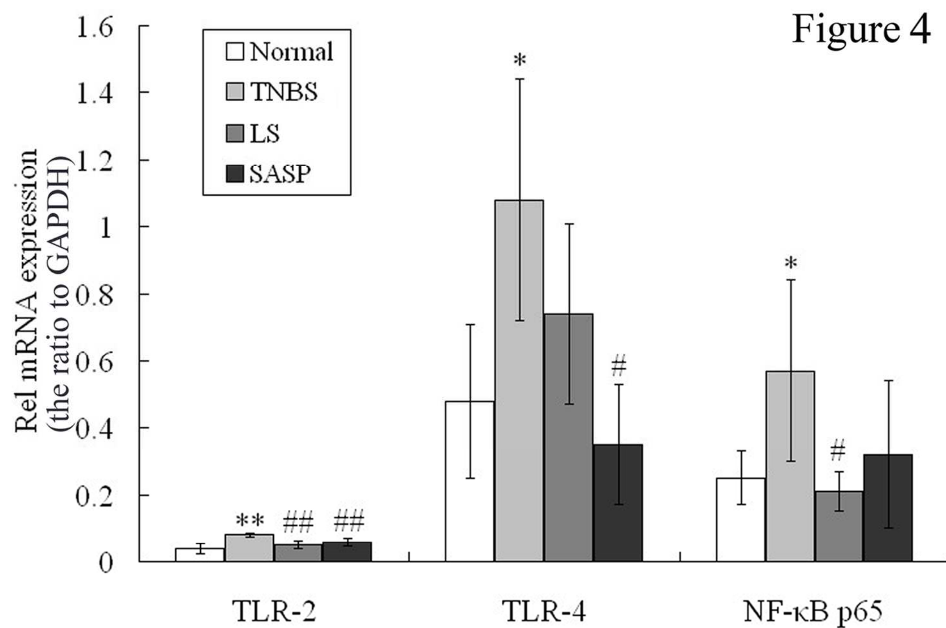

Fig. 4. Effects of LS on mRNA expression of TLR and NF-κB.
Total RNA was extracted from colon tissues for quantification by real-time fluorescent quantitative PCR. The relative mRNA expression of the target genes was calculated using the 2-ΔΔCt formula and were normalized using GAPDH as the housekeeping gene. Values are expressed as mean ± S.D., n=6.* Mean values were significantly different compared with normal rats (* $P < 0.05$; ** $P < 0.01$); # Mean value were significantly different compared with TNBS rats (# $P < 0.05$; ## $P < 0.01$), n=6.

99x65mm (600 x 600 DPI)

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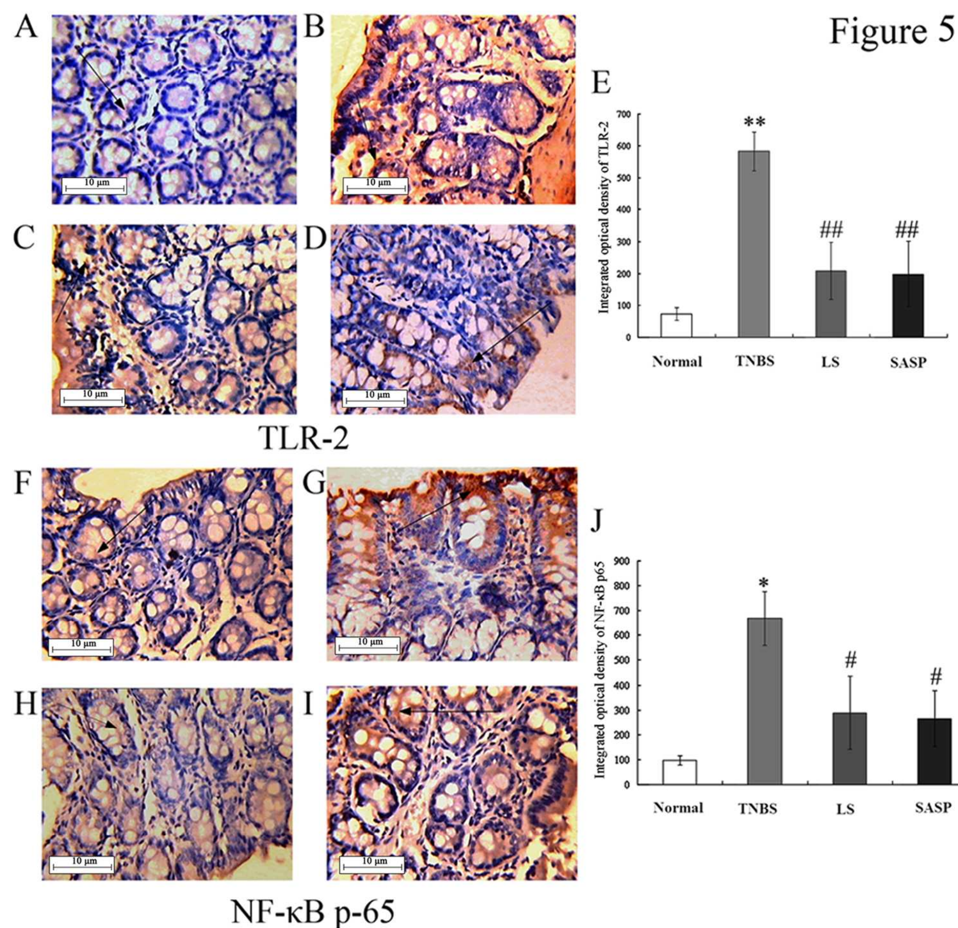

Fig. 5. Representative images for the effect of LS on TLR-2 and NF-κB p65 protein in the colon. TLR-2 and NF-κB p65 protein in colonic tissue were measured by immunohistochemistry methods. Immunohistochemistry of TLR-2 expression in colon: (A) Normal control rat, (B) TNBS induced colitis rat, (C) LS-treated rat with colitis induced by TNBS, (D) SASP-treated rat with colitis induced by TNBS. (E) Integrated optical density of TLR-2. Immunohistochemistry of NF-κB p65 expression in colon: (F) Normal control rat, (G) TNBS induced colitis rat, (H) LS-treated rat with colitis induced by TNBS, (I) SASP-treated rat with colitis induced by TNBS. (J) Integrated optical density of NF-κB p65. TLR-2 and NF-κB p65positive cells (arrows) were stained in brown. Values are expressed as mean ± S.D., n=6. * Mean values were significantly different compared with normal rats (* P <0.05; ** P < 0.01); # Mean value were significantly different compared with TNBS rats (# P < 0.05; ## P < 0.01), n=6.

64x62mm (600 x 600 DPI)

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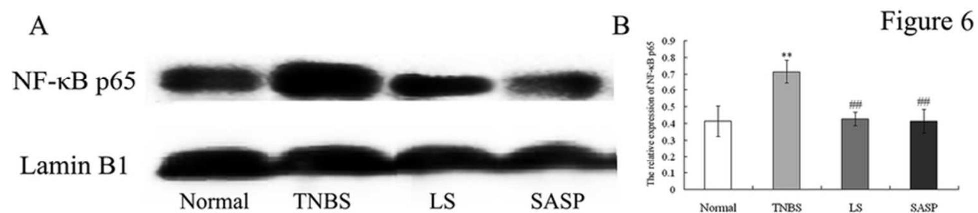

Fig. 6. Effect of LS on the expression of nuclear NF-κB p65 in colon of colitis rats. (A) Western analysis of NF-κB p65 and Lamin B1 expression in nucleus of colon. (B) Relative density of NF-κB p65, the density of NF-κB p65 band was normalized to that of Lamin B1. Values were expressed as the mean ± S.D.; n = 3 per group. * Mean values were significantly different compared to normal rats (** $P < 0.01$); # Mean value were significantly different compared with TNBS rats (## $P < 0.01$).

38x9mm (600 x 600 DPI)

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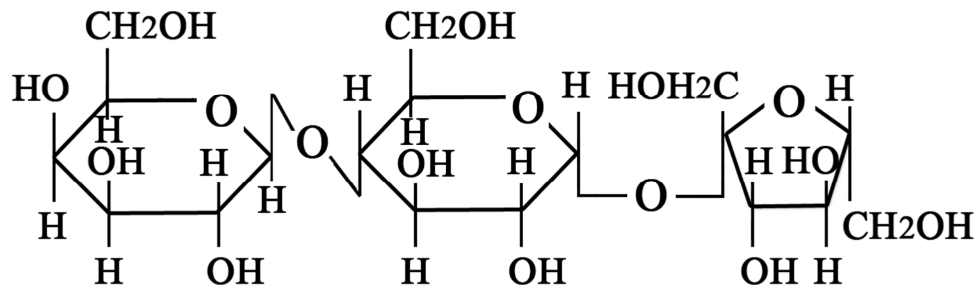

Structural formula of Lactosucose
108x47mm (299 x 299 DPI)

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