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

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

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

Key words: lactosucrose; inflammation; colitis; Toll-like receptor 2; nuclear factor kappa B (NF-κB)
1. Introduction

Inflammatory bowel diseases (IBD), including Crohn’s disease (CD) and ulcerative colitis (UC), are chronically recurrent disorders of the intestine. IBD was involved in the dysregulation of the intestinal immune response. Immune and non-immune cells within the intestinal mucosa recognize conserved microbial structures through so-called pattern recognition receptors (PRRs), including toll-like receptors (TLRs). And TLR-mediated cellular responses can lead to chronic inflammation, which in turn contributes to the development of IBD. Polymorphisms in TLR1, TLR2, TLR4 and TLR9 loci have been associated with IBD in human.

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

Activation of NF-κB then upregulates the expression of numerous proinflammatory cytokines, such as interleukin-6 (IL-6) and IL-12, involved in intestinal inflammation. IL-6 is a pleiotropic cytokine. An elevation in serum IL-6 levels during remission in CD patients was found to be clinically relevant parameter for predicting inflammatory activity as well as for corresponding with a high frequency of disease relapse. TNBS-induced colitis can be abrogated with antibodies specific for IL-12, in which excessive production of IL-12 seems to be the crucial underlying genetic abnormality in susceptible mice. However, IL-10 is an anti-inflammatory cytokines that inhibit both antigen presentation and subsequent release of proinflammatory cytokines, thereby attenuating mucosal inflammation. IL-10 can inhibit the effector functions of activated macrophages and monocytes in vitro and downregulate IL-12
and inactivation of IL-10 in mice results in an increased production of IL-12. IL-10-/mice spontaneously develop colitis in a bacteria-containing environment but are healthy in germ-free conditions.

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. Fruit intake is negatively associated with the risk for IBD and can reduce the risk factors of colon cancer. 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. In addition, it is different for luminal and mucosa-associated microflora between IBD patients and healthy controls.

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. 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.

Lactosucrose (4G-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. Hino et al. reported that LS could stimulate overproduction of growth factor-β1 (TGF-β1) in peyers patches. LS was found to inhibit immunoglobulin E production and increase the secretive yield of IL-10 in ovalbumin/alum-immunized mice. In addition, LS has been shown to promote the growth of Bifidobacteria in patients with chronic inflammatory bowel disease or in healthy human. 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,
and promoted *Bifidobacterium* and *Lactobacillus*, decreased the number of *Escherichia* in TNBS-induced colonic inflammation. The main objective of present work is to investigate the effects of LS on TNBS-induced colitic rats and its effect on TLR-2, TLR-4 and nuclear NF-κB p65 expression in colitis.

2. Materials and Methods

2.1 Chemicals

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

2.2 Preparation of Lactosucrose

LS was catalytically synthesized from sucrose and lactose solution by β-fructofuranosidase. β-fructofuranosidase was derived from *Arthrobacter* sp.10138 in our laboratory. In this enzyme-catalyzed reaction (pH 7.0 and 30 °C), the solution contained 550 mmol/L sucrose, 550 mmol/L lactose, and β-fructofuranosidase (250 U/ml). After a 24h period, the whole solution was vacuum freeze-drying, and LS was obtained. And the concentration of saccharides were analyzed by high performance liquid chromatography (HPLC) 1200 from Agilent (USA) with evaporative light scattering detector (ELSD) model 300S (Softa, America) according to Ruan et al. The chromatographic column is a Kromasil 100-5NH₂ column (250 mm × 4.6mm). The mobile phase consisted of acetonitrile/water (75:25 V/V) flowed in the rate of 1.0 mL/min, and the temperature of column oven was 25 °C.
The drift tube and evaporator temperature was set at 70 °C and 30 °C, respectively.

2.3 Animals care

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

2.3.1 Introduction of colitis and experimental design

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

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

2.3.2 Evaluation of colitis severity

The parameters recorded in the experiments were the disease activity index (DAI), body weight, food intake and small intestinal length index. DAI ranging from 0-3 using the following parameters: weight loss, occult blood positivity, and stool consistency of the animal
2.4 Histological grading of colitis

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

2.5 Assay of blood clinical chemistry

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

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

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

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

2.7 Immunohistochemistry

Immunohistochemistry was performed as previously described\textsuperscript{37}. Samples from the colon were fixed in 4% paraformaldehyde in phosphate buffer solution and dehydrated with ethanol. Then those samples were embedded in paraffin. Sections (5\,\mu m thick) were prepared
and exposed to immunoperoxidase. Endogenous peroxidase was cleared by treatment with 3% H<sub>2</sub>O<sub>2</sub> phosphate buffered saline (PBS) for 20 min at room temperature. After washing with 0.05 mol/L PBS (pH 7.4), slides were blocked with 5% bovine serum albumin (BSA) in PBS for 20 min at room temperature to prevent non-specific protein binding. Then, the slides were sequentially exposed to the primary antibody TLR-2 (Santa Cruz Biotechnology, CA, USA, 1:200) and NF-κB p65 (Santa Cruz Biotechnology, CA, USA, 1:80) in PBS and incubated overnight at 4°C. After rinsing, the slides were incubated with a secondary antibody conjugated to biotinylated goat peroxidase and 0.1% (v/v) diaminobenzidine substrate. Slides processed without primary antibody served as the negative control. There are four slides every sample (rat) and there are four observations for a slide. The optical densities of TLR-2 and NF-κB p65 proteins in the colonic epithelial cells were determined by densitometric scanning using the Motic Med System 6.0 Software.

2.8 Western blot analysis of NF-κB p65 in nucleus

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

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

Real-time fluorescent quantitative polymerase chain reaction (PCR) analysis was used to measure the gene expression of TLR-2, TLR-4, and NF-κB. Colonic tissues from the normal, TNBS, LS, and SASP groups were immediately frozen in liquid nitrogen for RNA extraction. Total RNA was isolated from tissues using the Trizol reagent (Invitrogen, Amenrican) method as described by the manufacturer. cDNA synthesis was carried out using the Reverse
Transcription System Kit (Takara, Dalian, China), according to the manufacturer’s instructions. Fluorescent quantitative PCR was carried out using SYBR Premix Ex Taq (Takara, Japan) on an ABI 7900HT PCR instrument (Applied Biosystems, USA). The experimental conditions were: denaturation for 30s at 95 °C, annealing for 5s at 95 °C, and extension for 30 s at 60 °C, for 40 cycles. Rat primers were designed using Primer 3 and synthesized by Sangon Biotech Co., Ltd (Table 3) (Shanghai, China). The relative mRNA levels of the genes were calculated using the $2^{\Delta\Delta Ct}$ formula and the rat GAPDH as the housekeeping gene.

3.0 Statistical analysis

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

3. Results

3.1 Production and analysis of LS

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

3.2 LS improves colitis clinical symptoms and colon damage in rats

Rats exposed to TNBS developed symptoms of colitis as assessed by DAI. LS treatment for 14 days the DAI was significantly reduced compared to TNBS control rats. After SASP supplementation for 7 days, the DAI in SASP group was significantly decreased, as compared with TNBS control group. Compared to normal group, DAI in LS group or SASP group were no significantly difference after 14 days. There were no significant changes among all
treatment groups after 28 days (Fig 2 A).

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

3.3 Effects of LS supplementation on blood chemistry in colitis rats

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

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

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

IL-12 and IL-6 are known as a proinflammatory cytokine. IL-10 is a well known anti-inflammatory cytokine $^{41}$. In this study, TNBS-induced colitis was characterized by significantly increased the levels of IL-12 ($P < 0.01$) and IL-6 ($P < 0.05$), and a significantly
decreased in the level of IL-10 \((P < 0.05)\) compared to normal rats. Following administration of LS, the level of IL-12 was decreased \((P < 0.01)\) (Fig. 3A), and the level of IL-10 \((P < 0.05)\) was increased, compared to TNBS group rats (Fig. 3C). The level of IL-6 also tended to decrease in LS-treated rats \((P > 0.05)\), compared to the TNBS group (Fig. 3B). SASP supplementation decreased the level of IL-12 \((P < 0.01)\), and increased IL-10 level \((P < 0.05)\), as compared with TNBS group. When compared with normal rats, the content of IL-12, IL-10, and IL-6 in LS or SASP group had no significant difference. The effect of LS on cytokines modulation was similar with SASP. These results suggest that anti-inflammation of LS may be involved in the down-regulation of IL-12 level and up-regulation of IL-10 level.

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

Gut bacteria influence TLRs expression \(^{42}\). LS supplementation could modulate cecum and colonic bacteria in our previously study. Then, the mRNA levels for TLR-2, TLR-4 and NF-κB p65 were determined. Compared to normal rats, significant increases in mRNA abundances of these proteins in inflammatory TNBS group was observed after administration of TNBS (Fig. 4). The expression of TLR-2 and NF-κB p65 mRNA in the LS group was significantly down-regulated compared to the inflammatory TNBS group \((P < 0.05)\), and had no significant difference compared to normal group. TLR-4 mRNA was reduced by LS supplementation compared to colitis rat \((P > 0.05)\). SASP supplementation decreased the expression of TLR-2 \((P < 0.01)\) and TLR-4 \((P < 0.05)\) mRNA, as compared with colitis rats, and had no significant difference compared to normal rats (Fig. 4). The mRNA levels for TLR-2, TLR-4 and NF-κB p65 in LS group had no significant difference compared to SASP rats.

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

Next, the levels of TLR-2 and NF-κB p65 protein were determined by immunohistochemistry (Fig 5). The immunohistochemistry of TLR-2 in colon was showed in Fig. 5A-5D, and the NF-κB p65 was showed in Fig. 5F-5I. Arrows indicated the positive cells. The results showed that TNBS treatment caused a significantly increased in TLR-2 \((71.7±20.0\) to \(583.2±61.0)\) (Fig. 5E) and NF-κB p65 \((97.6±19.1\) to \(667.9±109.9)\) (Fig. 5J). TLR-2 and NF-κB p65 protein abundance in the LS group or SASP group was significantly
decreased compared to the TNBS group (inflammatory rats). Compared to normal group, the levels of TLR-2 and NF-κB p65 in LS or SASP group had no significant difference.

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

4. Discussion

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

In recent years, many studies have demonstrated that development of intestinal inflammation is partly attributed to TLRs activation. Among this family of receptors, TLR-2 and TLR-4 in colitis had been reported in numerous articles and paid much attention to the development of IBD. TLR-2/-, TLR-4/- and TLR-2/-4/- mice displayed reduced macroscopic signs of acute colitis and the amelioration of inflammation. In TNBS-induced murine colitis, the level of TLR-2 and TLR-4 were increased. In
conclusion, TLR-2 and TLR-4 play an important role in colitis. TLR-2 and TLR-4 are regulated by intestinal bacteria. TLR-2 recognizes lipoteichoic acid, peptidoglycan and related cell-wall glycopolymers in gram-positive bacteria and probiotics (including *Lactobacilli* and *Bifidobacteria*). TLR-4 recognizes LPS which is a bacteria cell wall component of gram-positive bacteria (including *Escherichia*). *Escherichia* stimulate TLR-4 activation \(^{57}\). Lee *et al.* \(^{58}\) reported *Lactobacillus* *suntoryeus* HY7801 inhibited TLR-4 linked NF-κB activation in TNBS-induced colitic mice. *Lactobacillus* protects intestinal epithelium from radiation injury in a TLR-2/cyclo-oxygenase-2-dependent manner \(^{59}\). Oral administration of *Bifidobacterium* bifidum activated TLR-2 in the intestinal epithelium necrotizing enterocolitis rat \(^{60}\). In our previous study, LS supplementation in TNBS-induced colonic inflammation increased the levels of *Bifidobacterium* and *Lactobacillus*, decreased the number of *Escherichia* \(^{32}\). Therefore, in this study, the expression of TLR-2 and TLR-4 were determined to explore the effect of LS on TNBS-induced rat colitis.

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

TLRs related signaling pathway might be influenced by microbe and short chain fatty acid. Intestinal microbe alone or combine with short chain fatty acid, such as butyric acid, impacted TLR-2 activation \(^{65-67}\). Caco-2 cells cultured with yeast increased the level of TLR-2 mRNA, while Caco-2 cells incubated with butyrate (10 mmol/L) and yeast decreased
the level of TLR-2 mRNA. Mirmonsef *et al.* reported the TLR-2 ligand could enhance IL-8 and TNF-α production in blood mononuclear cells and neutrophils pretreated with low levels of SCFA (0.02-2 mmol/L), while the TLR-4 ligand did not have such an effect. Kovarik *et al.* suggested that higher concentrations of n-butyrate (0.06-1 mmol/L) inhibited the release of IL-12/23p40 from blood mononuclear cells (obtained from IBD patients) after activation via TLR-2 agonists, but not TLR-4 engagement. According several reported papers with *vitro* experiments, SCFA play dual-directional regulation in TLR-2 signaling pathway. In our previously study, the numbers of *Lactobacilli* and *Bifidobacteria*, as well as the production of SCFA in LS-supplemented rats were increased, and the numbers of *Escherichia* were decreased, compared to the inflammatory group. In this study, after LS administration, TLR-2 level was decreased, the decreased of TLR-2 expression was related to the increase of *Lactobacilli* and *Bifidobacteria*, and short chain fatty acid need further research.

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

**5. Conclusion**

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

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


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Table 1. The composition and nutrient of experiment diet

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The vitamin-mineral premix provided (per kilogram feed): vitamin A, 14,000 IU; vitamin D₃, 1500 IU; vitamin E, 5 mg; vitamin K, 5 mg; thiamine, 13 mg; riboflavin, 12 mg; pyridoxine, 12 mg; vitamin B₁₂, 0.022 mg; niacin 60 mg; pantothenic acid, 24 mg; biotin, 0.2 mg; folic acid, 6 mg; choline chloride, 350 mg; Fe (as iron sulfate), 120 mg; Cu (as copper oxide), 10 mg; Mn (as manganous oxide), 75 mg; Zn (as zinc oxide), 30 mg; I (as ethylenediamine dihydroiodide), 0.5 mg; and Se (as sodium selenite), 0.2 mg.

Table 2. Disease activity index (DAI)

<table>
<thead>
<tr>
<th>Score</th>
<th>Weight loss (% of initial wt)</th>
<th>Blood in feces</th>
<th>Stool consistency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt;1</td>
<td>Negative</td>
<td>Normal pellets</td>
</tr>
<tr>
<td>1</td>
<td>1-4.99</td>
<td>Occult blood+</td>
<td>Slightly loose feces</td>
</tr>
<tr>
<td>2</td>
<td>5-10</td>
<td>Occult blood ++</td>
<td>Loose feces</td>
</tr>
<tr>
<td>3</td>
<td>&gt;10</td>
<td>Gross blood</td>
<td>Watery diarrhea</td>
</tr>
</tbody>
</table>

Table 3. Primers design for genes analysed by real-time quantitative PCR
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
<th>Product (bp)</th>
<th>Genebank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR-2</td>
<td>5’- AGAGGGAAATCGTGCGTGC-3’</td>
<td>5’- CCATACCCAAGGAAGGAG-3’</td>
<td>137</td>
<td>NM_198769</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>323</td>
<td></td>
<td></td>
<td>NM_019178</td>
</tr>
<tr>
<td>NF-κB</td>
<td>5’-GCACGTGTGAGGACGCTGATA-3’</td>
<td>5’-CGTGAGATTCCCAGGTTG-3’</td>
<td>96</td>
<td>AF 079314.1</td>
</tr>
<tr>
<td>p65</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>323</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-GGCAAGTTCAACGGCACAG-3’</td>
<td>5’-GCAGTAGACTCCACGACA-3’</td>
<td>142</td>
<td>NM_017008.3</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Normal</th>
<th>TNBS</th>
<th>LS</th>
<th>SASP</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/L)</td>
<td>31.8±3.1</td>
<td>42.2±6.4*</td>
<td>32.2±3.3*</td>
<td>38.7±3.2*</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>11.5±1.4</td>
<td>19.8±5.9*</td>
<td>15.0±2.8</td>
<td>16.3±3.5</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>62.6±21.8</td>
<td>41.0±15.4*</td>
<td>67.6±17.7*</td>
<td>73.7±15.0*</td>
</tr>
<tr>
<td>TP (g/L)</td>
<td>58.9±6.6</td>
<td>65.4±3.3*</td>
<td>67.6±4.9*</td>
<td>65.3±1.9*</td>
</tr>
<tr>
<td>ALB (g/L)</td>
<td>28.6±2.7</td>
<td>30.8±1.9</td>
<td>31.4±2.8*</td>
<td>31.4±1.1*</td>
</tr>
<tr>
<td>BUN (mmol/L)</td>
<td>5.90±0.88</td>
<td>6.48±0.44</td>
<td>5.99±1.10</td>
<td>6.78±1.18</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>0.36±0.09</td>
<td>0.43±0.07</td>
<td>0.46±0.09</td>
<td>0.48±0.06*</td>
</tr>
<tr>
<td>CHO (mmol/L)</td>
<td>0.97±0.17</td>
<td>1.25±0.17*</td>
<td>1.27±0.45*</td>
<td>1.02±0.35*</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D., n=6.* Mean values were significantly different compared with normal rats (\*P < 0.05); # Mean value were significantly different compared with TNBS rats (#P < 0.05).

AST, aspartate aminotransferase; ALT, alanine transaminase; ALP, alkaline phosphatase; TP, total protein; ALB, albumin; BUN, urea nitrogen; LDL, low-density lipoprotein; CHO, cholesterol.
Figures Legends

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

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.

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.

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
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.

**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 p65 positive 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.

**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$).
Lactosucrose, LS

Intestinal Inflammation

Pro-inflammatory cytokine (IL-12, IL-6)

Anti-inflammatory cytokine (IL-10)

40x25mm (600 x 600 DPI)
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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67x45mm (600 x 600 DPI)
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Structural formula of Lactosucrose
108x47mm (299 x 299 DPI)