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Synbiotic mulberry ameliorates kidney injury in dextran sodium sulfate–induced colitis rats

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ABSTRACT

Objective: To investigate the effect of synbiotic mulberry on kidney injury in a dextran sodium sulfate (DSS)-induced colitis model.**Methods:** Male Wistar rats were given drinking water containing 4% DSS for 7 days. Subsequently, the rats were treated by oral gavage with synbiotic mulberry at 250, 500, and 1000 mg/kg, sulfasalazine at 100 mg/kg, or synbiotic at 1000 mg/kg for an additional 7 days while receiving 0.4% DSS in drinking water. The severity of colitis was evaluated based on the disease activity index score. On day 14, plasma was collected, and the kidneys were harvested to evaluate kidney injury parameters and histological changes. In addition, the expression of genes associated with kidney injury was determined by quantitative RT-PCR.**Results:** Treatment with all doses of synbiotic mulberry significantly lowered the disease activity index score, accompanied by reductions in kidney histopathological changes, malondialdehyde concentration, and plasma cystatin C levels. Kidney fibrosis was also ameliorated by 500 and 1000 mg/kg of synbiotic mulberry. Treatment with 250 and 500 mg/kg of synbiotic mulberry downregulated *IL-18* mRNA expression, while *KIM-1* mRNA expression was reduced and plasma lipopolysaccharide-binding protein level was restored by 1000 mg/kg of synbiotic mulberry.**Conclusions:** Synbiotic mulberry ameliorates kidney injury in rats with DSS-induced colitis. It may be further explored as a treatment of kidney injury under colitis conditions.**KEYWORDS:** Colitis; Mulberry; Synbiotic; Dextran sodium sulfate; Kidney injury

1. Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gastrointestinal tract, and its incidence is rising globally. The two most common forms, ulcerative colitis, and Crohn's disease share similar symptoms that significantly reduce the patient's quality of life, including abdominal pain, severe diarrhea, rectal bleeding, weight loss, and fever[1]. Although the exact

Summary

Question: How does synbiotic mulberry alleviate kidney injury in colitis conditions?**Findings:** In a rat model of dextran sodium sulfate-induced colitis, synbiotic mulberry treatment could alleviate colitis symptoms and restore plasma lipopolysaccharide-binding protein levels. It also reduced plasma cystatin C levels, kidney histopathological damage, fibrosis, malondialdehyde concentration, and the expression of *KIM-1* and *IL-18* mRNA.**Meaning:** Synbiotic mulberry demonstrates beneficial effects in ameliorating kidney injury under colitis conditions.

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mechanism leading to IBD remains unknown, environmental factors, genetic predisposition, and gut microbiota are underlying causes[2]. The pathogenesis of the disease involves the destruction of the gut epithelial barrier, which leads to bacterial invasion into the mucosal layer, triggering an inflammatory immune response and eventually promoting systemic inflammation[3,4]. Clinical and epidemiological data suggest that IBD is a systemic disease, capable of affecting various organ systems, including the musculoskeletal system, skin, liver, lungs, and kidneys[5]. The effect of IBD on organs beyond the gastrointestinal tract is referred to as extraintestinal manifestations. Kidney involvement can occur in 4% to 23% of individuals with IBD[6,7]. Evidence has shown that renal diseases, such as glomerulonephritis, tubulointerstitial nephritis, and nephrolithiasis, are found in IBD patients[8].

In experimental animal models, dextran sodium sulfate (DSS) is commonly used to induce colitis to observe the pathological characteristics of IBD, such as dysregulation of gut mucosal permeability and inflammation. This acute intestinal injury induced by DSS leads to systemic inflammation-associated tissue injury. The study reported that DSS can induce systemic inflammation-associated acute kidney injury by regulating macrophage pro- and anti-inflammatory mediators[9]. Additionally, the DSS-induced colitis mouse model has been shown to damage the kidney's glomerular structure, leading to collagen fiber accumulation and reduced expression of glomerular filtration barrier-related proteins[10]. These findings highlight the renal manifestations of IBD-associated kidney injury.

Growing evidence indicates that gut dysbiosis is commonly observed in patients with IBD[11–13]. Modifying gut microbiota composition can effectively manage IBD and reduce the development of extraintestinal manifestations in these patients. Several studies suggest that probiotic and prebiotic supplementation can help modulate microbiota diversity and maintain gut health. Probiotics are beneficial microorganisms that support and promote gut health, while prebiotics are non-digestible food components that serve as selective probiotic substrates. A recent study demonstrated that treatment with multi-strain probiotics successfully alleviated gut inflammation and improved beneficial gut microbiota in rats with DSS-induced colitis[14]. Additionally, the prebiotic galactooligosaccharide showed anti-inflammatory effects in an *in vitro* intestinal cell exposed to DSS[15]. These findings suggest that a synbiotic, a combination of probiotics and prebiotics, could be an effective strategy for modifying gut microbiota profiles, promoting gut health, and reducing the severity and progression of IBD.

Mulberry is a well-known edible fruit that belongs to the genus *Morus* of the Moraceae family. It has been widely used as a fruit and medicinal herb due to its numerous health benefits. The most common species of mulberry consists of black mulberry (*Morus nigra* L.), white mulberry (*Morus alba* L.), and red mulberry (*Morus rubra* L.)[16]. Studies have demonstrated that mulberries exhibit

several positive biological effects, including antioxidant, anti-inflammatory, anti-tumor, and hypoglycemic activities[17]. A study in a mouse model of DSS-induced ulcerative colitis demonstrated that mulberry leaf flavonoids could reduce colitis symptoms and inhibit inflammatory pathways[18]. Furthermore, mulberries were shown to improve gut microbiota composition in a mouse model of DSS-induced colitis, leading to enhanced gut epithelial barrier integrity and a reduction in the severity of the disease[19]. This positive effect on gut microbiota suggests that mulberry exhibits prebiotic characteristics.

In the present study, we aimed to evaluate the therapeutic effects of synbiotic mulberry (SM), a combination of mulberry fruit powder with selective probiotic *Lactobacilli* and prebiotic inulin, in colitis-related kidney injury. Using DSS to mimic the inflammatory state of the gastrointestinal tract in rat models, we hypothesized that SM would be beneficial in alleviating kidney injury under colitis conditions.

2. Materials and methods

2.1. Preparation of SM

SM was formulated by combining mulberry fruit powder, probiotic biomass, and inulin. Mulberry (*Morus alba* L.) fruits were rinsed, dried in an oven at 50 °C, and ground into a fine powder. The probiotic strains, *Lactobacillus salivarius* and *Lactobacillus paracasei* were cultivated at 37 °C in MRS broth for 24 h. The probiotic biomass was then harvested by washing with NaCl solution (0.85% w/v) and centrifugation. The mulberry powder (100 g) was mixed with 2.5 g of inulin, 10^9 CFU of *Lactobacillus salivarius*, and 10^9 CFU of *Lactobacillus paracasei*. The mixture was freeze-dried and stored in a sealed aluminum foil bag at room temperature until used. The analysis of SM components and their subchronic oral toxicity was recently reported, with findings indicating no observed adverse effect of SM in the rat model[20].

2.2. Animals and ethical statement

Six-week-old male Wistar rats weighing 170–220 g were purchased from Nomura Siam International Co., Ltd., Bangkok, Thailand. The rats were acclimatized for one week and housed in the animal facility at the Animal Center, University of Phayao, Phayao, Thailand. All rats were maintained in a ventilated room at a controlled temperature of $(25 \pm 1)^\circ\text{C}$ under a 12-hour light-dark cycle. A standard rat diet and water were provided *ad libitum*. All animal study procedures followed the guidelines for laboratory animal care and use and were approved by the Animal Ethics Committee, University of Phayao, Phayao, Thailand (Permit number: 1-004-65; May 2022).

2.3. Experimental protocol and induction of colitis

All rats were randomly divided into eight groups ($n=5$ per group). The experimental periods lasted 14 days, and the protocol was as follows (Figure 1):

Group 1: The normal control group;

Group 2: The SM control group received 1000 mg/kg of SM for 14 d;

Group 3: The colitis control group received 4% (*w/v*) DSS for 7 d, followed by 0.4% (*w/v*) DSS for 7 d;

Group 4, 5, and 6: The colitis groups received 4% (*w/v*) DSS for 7 d, followed by 0.4% (*w/v*) DSS and 250, 500, or 1000 mg/kg of SM for 7 d, respectively;

Group 7: The colitis group received 4% (*w/v*) DSS for 7 d, followed by 0.4% (*w/v*) DSS and 100 mg/kg of sulfasalazine for 7 d;

Group 8: The colitis group received 4% (*w/v*) DSS for 7 d, followed by 0.4% (*w/v*) DSS and 1000 mg/kg of synbiotic for 7 d.

DSS was given in drinking water, and SM, sulfasalazine, and synbiotic were administered by oral gavage in a single daily dose. For group 8, the used synbiotic contained 7% (5×10^{10} CFU) of mixed probiotic strains (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium infantis*, *Bifidobacterium bifidum*, *Bifidobacterium longum*, *Lactococcus lactis*) and 3.7% oligosaccharides (Probac7, Interpharma, Bangkok, Thailand).

2.4. Disease activity index (DAI)

To determine the severity of DSS-induced colitis, DAI was assessed based on body weight loss, stool consistency, and rectal bleeding, as previously reported[21]. Each parameter was scored from 0 to 4, as shown in Supplementary Table. The severity score for each

parameter was averaged for each group, and the DAI was calculated as the sum of all parameter scores divided by 3.

2.5. Blood collection and kidney harvesting

On day 14, all rats were sacrificed. The rats were anesthetized by an intraperitoneal injection of 50 mg/kg of thiopentone sodium. While the rats were unconscious, blood samples were collected *via* cardiac puncture. The blood samples were centrifuged at 1500 rpm for 15 min at 4°C to obtain plasma. The plasma samples were stored at -80°C until used for biochemical analysis. Following the blood collection, the heart was harvested to ensure euthanasia. The kidneys were removed, decapsulated, and cleaned with a phosphate-buffered saline solution. One kidney was immediately stored at -80°C for further analysis, and the other was fixed in 4% paraformaldehyde for histological study.

2.6. Biochemical analysis

For the biochemical analysis of kidney injury, plasma cystatin C level was measured using a specific ELISA kit (E-EL-R0304, Elabscience Biotechnology Co., Houston, TX, USA) according to the manufacturer's instructions. Plasma lipopolysaccharide-binding protein (LBP) level was measured to assess systemic inflammation using a specific ELISA kit (E-EL-R0589, Elabscience Biotechnology Co., Houston, TX, USA), following the manufacturer's protocol.

2.7. Kidney histopathological study

The kidney samples were sliced, processed, and embedded into

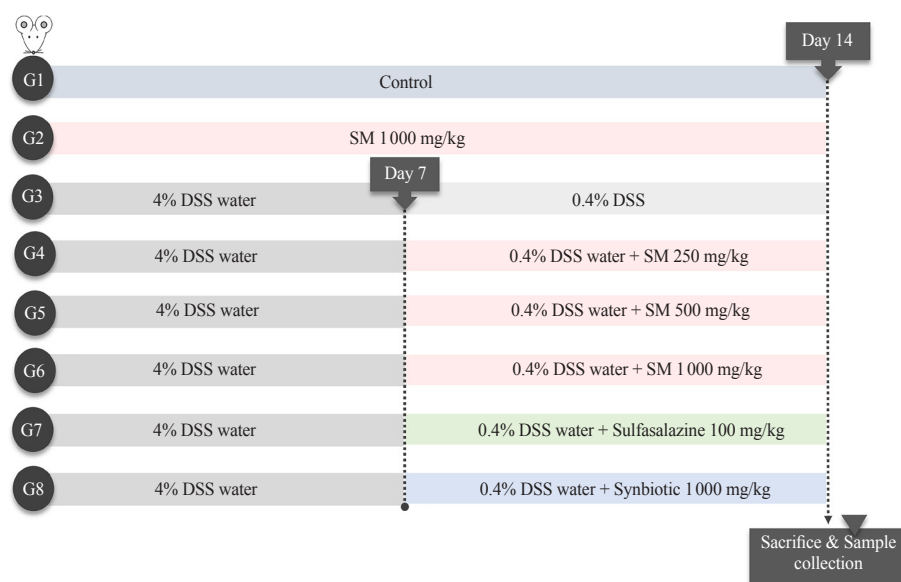


Figure 1. Experimental protocol. Dextran sodium sulfate (DSS) was administered in drinking water for 7 days. A single daily dose of synbiotic mulberry (SM), sulfasalazine, or synbiotic (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium infantis*, *Bifidobacterium bifidum*, *Bifidobacterium longum*, *Lactococcus lactis*) was administered by oral gavage for an additional 7 days. G: group.

paraffin blocks for histopathological examination. Tissue blocks were sectioned into 4 μ m thick slices using a microtome and stained with hematoxylin and eosin (H&E). The stained sections were sorted, and the group assignments were blinded. Five non-overlapping images per tissue section from each rat ($n=3$) were analyzed. Kidney damage parameters were assessed and graded using a kidney injury score, as described in the previous study[22]: grade 0 = no injury; grade 1 = 0%-5% injury; grade 2 = 5%-25% injury; grade 3 = 25%-75% injury; and grade 4 = >75% injury.

Collagenous connective tissue fibers deposited in the kidney tissues were examined using Masson's trichrome stain. Deparaffinized kidney tissue sections were stained with a trichrome stain kit (ab150686, Abcam, USA) according to the manufacturer's instructions. Masson's trichrome staining images were sorted, and the analysis was blinded. Five non-overlapping images per rat in each group were analyzed. The collagen-positive blue areas were assessed to determine the fibrosis score, as previously described[23,24]. The fibrosis score was determined on a scale from 0 to 4 based on the percentage of collagen deposition: grade 0 = no damage/staining; grade 1 = <25% area; grade 2 = 25%-50% area; grade 3 = 50%-75% area; and grade 4 = >75% area.

All images were captured using a Zeiss Axioscan Z1 slide scanner with Zeiss Zen Software (Carl Zeiss MicroImaging, Jena, Germany) and a 10 $\times$ objective lens. The images were exported in Tagged Information File Format (TIFF) for analysis.

2.8. Measurement of kidney oxidative stress

Lipid peroxidation measurement was performed to assess oxidative stress in kidney tissue by measuring the concentration of malondialdehyde (MDA). Kidney cortical tissues were removed and homogenized with cell lysis solution (C3228, CellLyticTM MT Mammalian Tissue Lysis/Extraction Reagent, Sigma-Aldrich, Missouri, USA) containing a 1% protease inhibitor cocktail (05892791001, Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. The homogenized tissue samples were centrifuged at 1600 $\times g$ at 4 $^{\circ}$ C for 10 min. The level of MDA in the supernatant was measured using a thiobarbituric acid assay kit (10009055, Cayman Chemical, MI, USA) according to the manufacturer's protocol.

2.9. Quantitative reverse transcription polymerase chain reaction (RT-PCR)

According to the manufacturer's instructions, total RNA was isolated from kidney tissue samples using an RNA isolation kit (R6834-01, E.Z.N.A. Total RNA Kit I, Omega Bio-tek, Inc., Norcross, Georgia). The concentration of total RNA was measured spectrophotometrically at 260 nm, and RNA quality was assessed by the absorbance ratio at 260 and 280 nm. One microgram of purified RNA was reverse-transcribed into single-stranded complementary DNA (cDNA) using the iScriptTM Reverse Transcription Supermix for RT-qPCR Kit (1708841, Bio-Rad Laboratories, Inc., California, USA). Quantitative RT-PCR was performed using the Bio-Rad CFX96 Sequence Detection System in 20- μ L reactions containing 2 $\times$ iTaqTM Universal SYBR[®] Green supermix (1725121, Bio-Rad Laboratories, Inc., California, USA), 4 μ L of cDNA, and 1 μ L of primers. The primer sequences of *KIM-1*, *IL-18*, *renin*, and *GAPDH* mRNA are shown in Table 1. The amplification thermal cycling protocol included an initial enzyme activation step at 95 $^{\circ}$ C for 30 s, followed by 40 cycles of 95 $^{\circ}$ C for 5 s, and 55 $^{\circ}$ C or 60 $^{\circ}$ C for 30 s. Melting curve analysis of the PCR product was conducted to verify identity and specificity, with the analysis performed at 65 $^{\circ}$ C to 95 $^{\circ}$ C in 0.05-second increments. The mRNA levels of *KIM-1*, *IL-18*, and *renin* in different groups were analyzed using the 2^{- $\Delta\Delta C_t$} method, with *GAPDH* used as the housekeeping gene and reference control.

2.10. Statistical analysis

Data were expressed as mean $\pm$ standard error of the mean (SEM). Differences among the eight groups were evaluated using a one-way analysis of variance (ANOVA) followed by Fisher's least significant difference test, facilitated by IBM SPSS Statistics 25. Statistical significance was set at P -values less than 0.05.

3. Results

3.1. Effect of SM on the severity of DSS-induced colitis

As shown in Figure 2A, rats in the DSS group exhibited significant increases in DAI scores compared to the control group (1.40 ± 0.12

Table 1. Primers used for quantitative RT-PCR.

Gene	Sequence	Annealing temperature
<i>KIM-1</i>	Forward: 5'-AACGCAGCGATTGTGCATCC-3'	60 $^{\circ}$ C
	Reverse: 5'-GTACACTCACCATGGTAACC-3'	
<i>IL-18</i>	Forward: 5'-GACAAAAGAAACCCGCTG-3'	59 $^{\circ}$ C
	Reverse: 5'-ACATCCTTCCATCCTTCACAG-3'	
<i>Renin</i>	Forward: 5'-AGACACAGCCAGCTTTGGAC-3'	60 $^{\circ}$ C
	Reverse: 5'-GAATTCACCCCATTCAGCAC-3'	
<i>GAPDH</i>	Forward: 5'-CCTGGAGAAACCTGCCAAGTAT-3'	60 $^{\circ}$ C
	Reverse: 5'-AGCCCAGGATGCCCTTTAGT-3'	

vs. 0.13 ± 0.08 , $P < 0.001$). The rise in DAI score of DSS-induced colitis rats was significantly reduced by treatment with 250, 500, and 1000 mg/kg of SM compared to the DSS group (0.07 ± 0.07 , 0.13 ± 0.08 , and 0.13 ± 0.08 vs. 1.40 ± 0.12 , respectively, $P < 0.001$). Treatment with sulfasalazine and synbiotic also led to a significant decrease in DAI scores compared to the DSS group (0.07 ± 0.07 and 0.13 ± 0.08 vs. 1.40 ± 0.12 , respectively, $P < 0.001$).

3.2. Effect of SM on plasma LBP

As shown in Figure 2B, the DSS-induced colitis group exhibited significantly reduced plasma LBP levels compared to the control group [$(1.39 \pm 0.08) \mu\text{g/mL}$ vs. $(1.81 \pm 0.14) \mu\text{g/mL}$, $P < 0.05$]. SM treatment at 1000 mg/kg significantly increased plasma LBP levels compared to the DSS group [$(1.75 \pm 0.05) \mu\text{g/mL}$ vs. $(1.39 \pm 0.08) \mu\text{g/mL}$, $P < 0.05$], while 250 and 500 mg/kg of SM did not improve the plasma LBP levels. Interestingly, treatment with sulfasalazine and synbiotic significantly increased plasma LBP levels compared to the DSS group [$(1.83 \pm 0.11) \mu\text{g/mL}$ and $(2.08 \pm 0.08) \mu\text{g/mL}$ vs. $(1.39 \pm 0.08) \mu\text{g/mL}$, $P < 0.05$ and $P < 0.001$, respectively].

3.3. Effect of SM on plasma cystatin C level

The plasma cystatin C level is shown in Figure 3A. Compared to the control group, DSS caused a significant increase in plasma cystatin C [$(230.90 \pm 8.69) \text{ ng/mL}$ vs. $(136.06 \pm 5.52) \text{ ng/mL}$, $P < 0.001$]. Treatment with SM at doses of 250, 500, and 1000 mg/kg significantly reduced DSS-induced increase in serum cystatin C compared to the DSS group [$(189.87 \pm 6.13) \text{ ng/mL}$, $(178.10 \pm 4.00) \text{ ng/mL}$, and $(171.09 \pm 2.39) \text{ ng/mL}$ vs. $(230.90 \pm 8.69) \text{ ng/mL}$, respectively, $P < 0.001$]. Similarly, significant reductions in plasma cystatin C were observed in the sulfasalazine and synbiotic treatment groups compared to the DSS group [$(152.34 \pm 5.23) \text{ ng/mL}$ and $(169.23 \pm 2.64) \text{ ng/mL}$ vs. $(230.90 \pm 8.69) \text{ ng/mL}$, respectively, $P < 0.001$].

3.4. Effect of SM on kidney oxidative stress

As shown in Figure 3B, MDA concentrations were significantly increased in the DSS group compared to the control group [$(3.44 \pm 0.53) \text{ nmol/mg}$ vs. $(2.12 \pm 0.18) \text{ nmol/mg}$, $P < 0.05$]. Treatment with

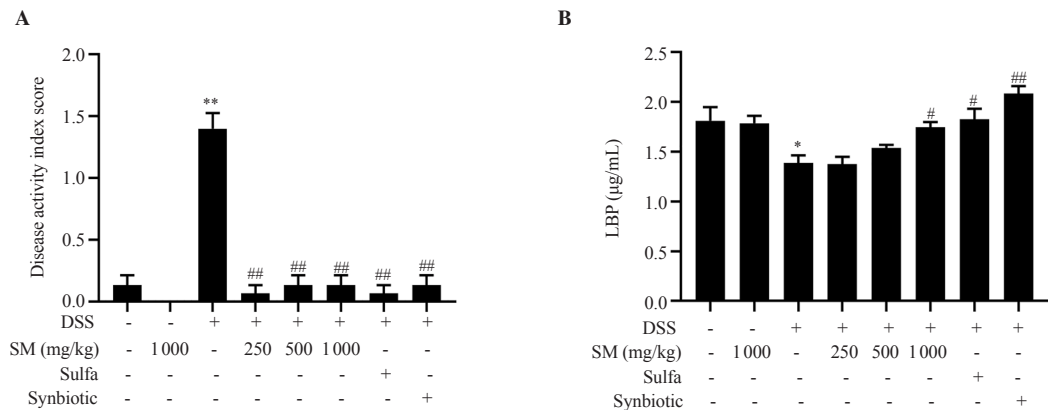


Figure 2. Effects of SM on disease activity index score (A) and plasma lipopolysaccharide-binding protein (LBP) level (B) in rats with DSS-induced colitis. Data are expressed as mean $\pm$ SEM ($n=5$). * $P < 0.05$ and ** $P < 0.001$ versus the control group; # $P < 0.05$ and ## $P < 0.001$ versus the DSS group. Sulfa: sulfasalazine.

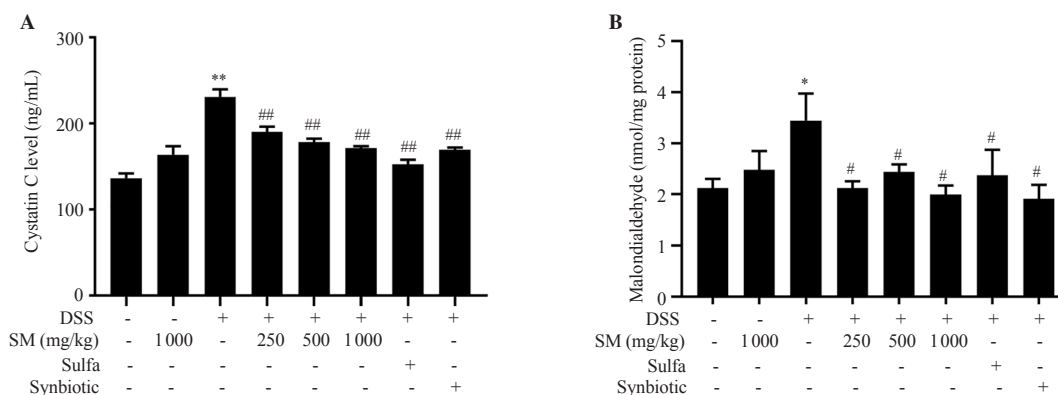


Figure 3. Effects of SM on plasma cystatin C level (A) and kidney malondialdehyde concentration (B) in rats with DSS-induced colitis. Data are expressed as mean $\pm$ SEM ($n=5$). * $P < 0.05$ and ** $P < 0.001$ versus the control group; # $P < 0.05$ and ## $P < 0.001$ versus the DSS group.

250, 500, and 1000 mg/kg of SM significantly reduced MDA levels compared to the DSS group [(2.12 ± 0.13) nmol/mg, (2.44 ± 0.15) nmol/mg, and (2.00 ± 0.18) nmol/mg *vs.* (3.44 ± 0.53) nmol/mg, $P < 0.05$]. Treatment with sulfasalazine and synbiotic demonstrated an anti-oxidative stress effect by significantly decreasing MDA compared to the DSS group [(2.37 ± 0.50) nmol/mg and (1.91 ± 0.27) nmol/mg *vs.* (3.44 ± 0.53) nmol/mg, $P < 0.05$].

3.5. Effect of SM on kidney histopathological changes

The kidney histopathology is shown in Figure 4A. The normal control and 1000 mg/kg SM alone groups exhibited no histopathological lesions in the kidneys. The glomeruli were round and displayed a normal ratio of cells to capillaries. The renal tubule epithelium was intact, with a clear lumen. DSS administration induced kidney histopathological changes, including sloughing of cells into the tubular lumen and mild glomerulomegaly. All treatments mitigated these histological changes induced by DSS. Notably, the DSS group treated with 500 mg/kg of SM demonstrated minimal damage and significantly improved glomerulomegaly. The kidney histological structures of the DSS group treated with sulfasalazine or synbiotic showed microscopic features similar to those observed in the DSS group given 500 mg/kg of SM.

The kidney histopathological changes were estimated using the kidney injury score, as shown in Figure 4B. DSS caused a significant increase in the kidney injury score compared with the control group

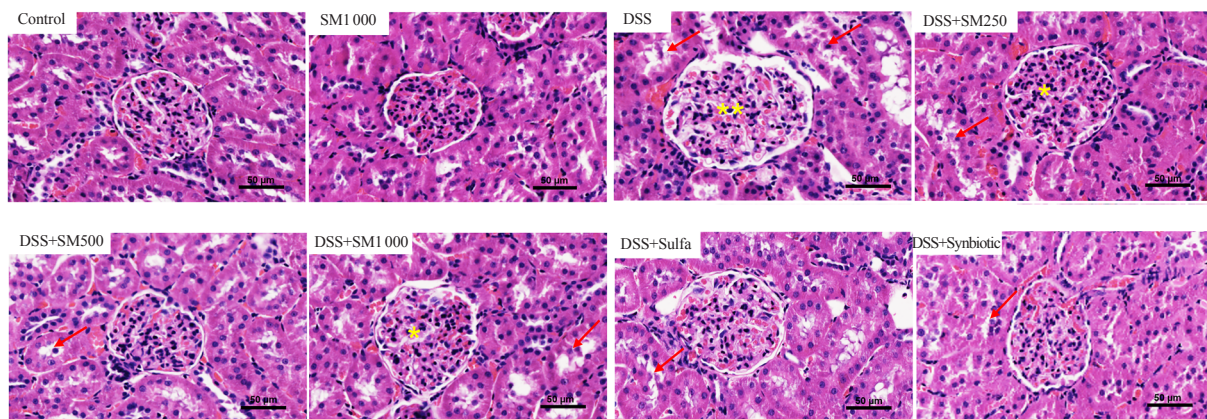
$(0.79 \pm 0.09$ *vs.* 0.31 ± 0.12 , $P < 0.001$). Treatment with 250, 500, and 1000 mg/kg of SM showed significantly decreased kidney injury scores compared to the DSS group (0.40 ± 0.08 , 0.35 ± 0.01 , and 0.44 ± 0.10 *vs.* 0.79 ± 0.09 , respectively, $P < 0.05$). Additionally, sulfasalazine and synbiotic treatments significantly reduced the kidney injury score compared to the DSS group (0.32 ± 0.06 and 0.42 ± 0.08 *vs.* 0.79 ± 0.09 , respectively, $P < 0.05$).

3.6. Effect of SM on kidney fibrosis

Masson's trichrome staining used for observing kidney fibrosis is shown in Figure 5A. The blue-stained areas indicate collagen fiber deposition. Minimal collagen was observed in Bowman's capsule and renal tubules in the normal control and 1000 mg/kg SM alone groups, as well as the DSS groups treated with 500 and 1000 mg/kg SM or synbiotic. In contrast, the DSS group and the DSS group treated with 250 mg/kg exhibited a high degree of collagen accumulation. Notably, extensive collagen fiber was observed in the DSS group receiving sulfasalazine treatment.

The representation of the collagen deposition area corresponded with the kidney fibrosis score as shown in Figure 5B. A significant increase in the fibrosis score was observed in the DSS group (1.00 ± 0.12), the DSS group treated with 250 mg/kg of SM (0.87 ± 0.29), and the DSS group treated with sulfasalazine (1.47 ± 0.37) compared to the control group (0.27 ± 0.18) ($P < 0.05$). Treatment with SM at 500 and 1000 mg/kg, as well as synbiotic significantly reduced the

A



B

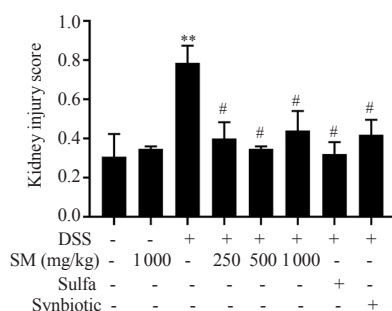


Figure 4. Histopathological examination of kidney tissues. (A) Hematoxylin and eosin staining of kidney tissues (scale bar=50 μm); (B) kidney injury score. Red arrow: sloughing of cells into the tubular lumen; yellow star: glomerulomegaly with minimal damage (*) and mild damage (**). Data are expressed as mean ± SEM ($n=3$). ** $P < 0.001$ versus the control group; # $P < 0.05$ versus the DSS group. SM250: synbiotic mulberry 250 mg/kg; SM500: synbiotic mulberry 500 mg/kg; SM1000: synbiotic mulberry 1000 mg/kg.

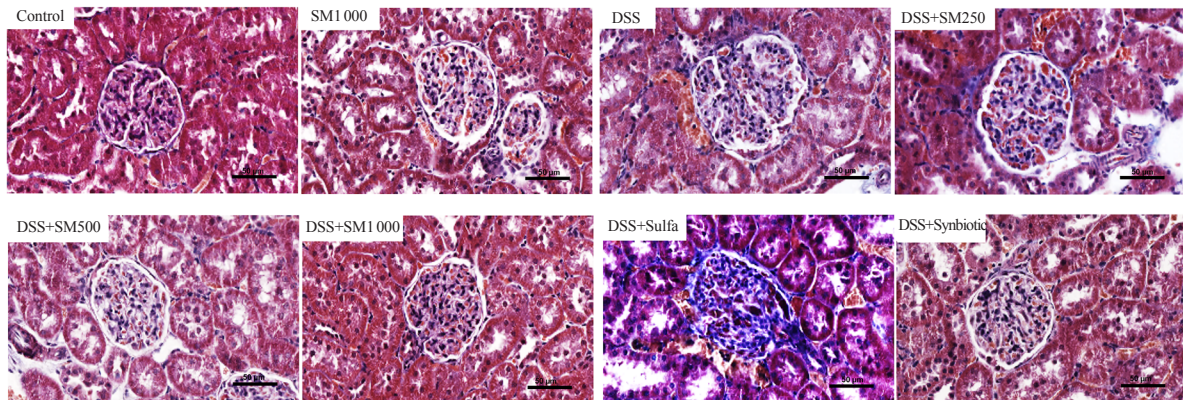
fibrosis score compared to the DSS group (0.20 ± 0.12 , 0.20 ± 0.12 , and 0.33 ± 0.07 vs. 1.00 ± 0.12 , respectively, $P < 0.05$).

3.7. Effect of SM on the expression of genes associated with kidney injury

As shown in Figure 6A and 6B, the expression levels of *KIM-1* and *IL-18* mRNA were significantly elevated in the DSS-induced colitis group compared to the control group (1.49 ± 0.17 and 1.28 ± 0.14 vs. 1.00 ± 0.00 , respectively, $P < 0.05$). Treatment with 250 and 500 mg/kg of SM significantly reduced the expression levels of *IL-18* mRNA compared to the DSS group (0.98 ± 0.12 and 0.90 ± 0.12 vs.

1.28 ± 0.14 , $P < 0.05$). *KIM-1* mRNA expression was significantly decreased by 1000 mg/kg of SM treatment (0.98 ± 0.19 vs. 1.49 ± 0.17 , $P < 0.05$). A significant reduction in *KIM-1* and *IL-18* mRNA expression was observed in the synbiotic-treated group compared to the DSS group (0.98 ± 0.23 vs. 1.49 ± 0.17 , $P < 0.05$; 0.49 ± 0.07 vs. 1.28 ± 0.14 , $P < 0.001$). Additionally, sulfasalazine treatment significantly decreased *IL-18* mRNA expression (0.48 ± 0.02 vs. 1.28 ± 0.14 , $P < 0.001$), with no significant effect on *KIM-1* mRNA expression (1.06 ± 0.05 vs. 1.49 ± 0.17 , $P > 0.05$). There were no significant changes in *renin* mRNA expression among the DSS, SM-treated, and synbiotic groups (Figure 6C). However, sulfasalazine treatment significantly decreased *renin* mRNA expression compared

A



B

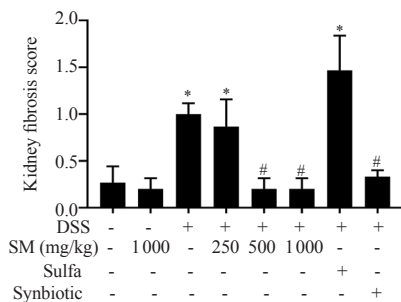
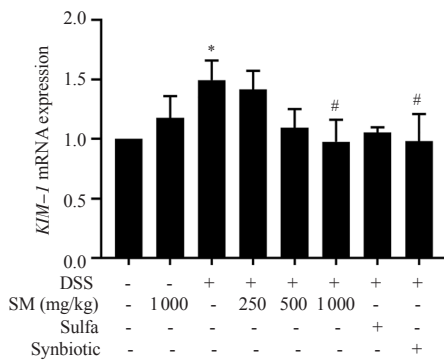
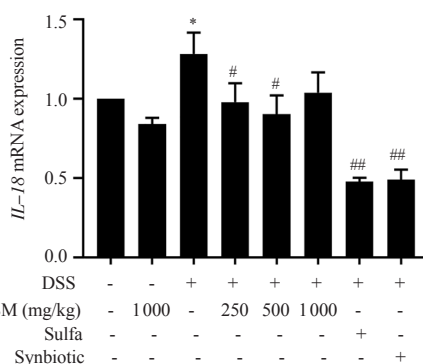


Figure 5. Effects of SM on collagen fiber deposition of the kidneys. (A) Masson's trichrome staining of kidney tissues (scale bar = 50 μm); (B) kidney fibrosis score. Data are expressed as mean ± SEM ($n=3$). * $P < 0.05$ versus the control group; # $P < 0.05$ versus the DSS group.

A



B



C

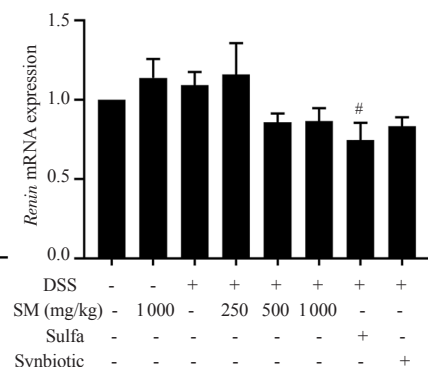


Figure 6. Effects of SM on the expression level of (A) *KIM-1*, (B) *IL-18*, and (C) *renin* genes associated with kidney injury. Data are expressed as mean ± SEM ($n=5$). * $P < 0.05$ versus the control group; # $P < 0.05$ and ## $P < 0.001$ versus the DSS group.

to the DSS group (0.75 ± 0.11 vs. 1.09 ± 0.08 , $P < 0.05$).

4. Discussion

IBDs are multifactorial disorders that are included in inflammatory diseases and show renal manifestations and complications. The present study provided data showing that SM had therapeutic effects on kidney injury in a DSS-induced colitis rat model.

In this study, 4% of DSS was used to induce colitis for 7 d. The results confirmed symptoms of colitis, demonstrated by increased DAI score, including body weight decrease, stool consistency, and rectal bleeding. We noticed that kidney destruction was found in the rat model with colitis. DSS influenced kidney histopathological changes including sloughing of cells into the tubular lumen and mild glomerulomegaly. In line with these results, glomerulopathy and acute tubular injury are renal manifestations of IBD[8]. However, there was a study that showed no significant histopathological changes in the kidney tissue observed in rats exposed to DSS (5%) for 7 d[25]. Thus, additional studies of kidney histopathological damage in colitis should be supplied.

To clarify the injury of kidneys in colitis, the expression of markers involved in kidney injury was examined. We demonstrated that *KIM-1* and *IL-18* mRNA were overexpressed in the kidney tissues of colitis rats. *KIM-1* is a biomarker for acute kidney injury found in renal tubular epithelial cells[26]. A previous study reported that experimental colitis rats induced by 2,4,6-trinitrobenzene sulfonic acid showed increased mRNA expression of *KIM-1* in the kidney tissues[27]. *IL-18*, a pro-inflammatory cytokine, is expressed in renal tubular epithelial cells and associated with renal inflammation and fibrosis[28]. It was also shown that the overproduction of *IL-18* by renal tubular epithelial cells promotes renal inflammation and fibrosis in hypertensive mice[29]. DSS (2%)-induced colonic inflammation in mice causes intestinal fibrosis and the kidney becomes fibrotic in this model[30]. In agreement with these results, the present findings showed an accumulation of collagen in the kidney tissues of colitis rats. Taking together, increased *IL-18* mRNA expression is suggested to be associated with cytokine-mediated kidney fibrosis in this model. Moreover, the bioinformatic study revealed the changes in fibrosis-related gene expression of IBD similar to kidney fibrosis[31]. Thus, it could be suggested that fibrosis might be the common pathway of extraintestinal manifestations in IBD. The results also showed an increased level of plasma cystatin C in the colitis rats. Cystatin C has been identified as a biomarker for the early detection of both acute and chronic changes in kidney function[32,33]. The present study confirms that kidney injury and kidney dysfunction were promoted in the DSS-induced colitis rats.

The overproduction of kidney MDA suggests that oxidative stress was present in this colitis rat model. In contrast to the present work, the study conducted by da Paz Martins *et al.*[34] did not find any changes in kidney oxidative stress profile in colitis mice (5% DSS *v/v*; for 7 d). However, a recent study has demonstrated that

gut bacteria modification significantly promotes the initiation and progression of colitis associated with kidney injury. The potential mechanism of this extraintestinal manifestation is involved in the lipopolysaccharide (LPS)-induced increase of cellular oxidative stress[35]. Damage to intestinal integrity and dysregulation of microbiota composition in colitis lead to the release of LPS into the bloodstream, causing systemic inflammation. Remarkably, our results demonstrated that plasma LBP levels were decreased in colitis rats. LBP is a circulating protein that promotes the host immune response to LPS by transferring it into the cytoplasm. At low concentrations of LBP, it enhances LPS-mediated activation of cytokines released by mononuclear cells[36]. Therefore, it can be proposed that kidney oxidative stress observed in colitis rats might be caused by DSS-induced LPS release, suggesting a possible mechanism for kidney injury in the present study.

The results demonstrate the therapeutic effects of SM on intestinal and kidney injury. All dosages of SM used in the present study reduced the DAI score, which was accompanied by decreases in the kidney injury score, plasma cystatin C levels, and kidney MDA concentration. Additionally, SM at doses of 500 and 1 000 mg/kg reversed kidney fibrosis. However, the dosage of SM to alleviate *KIM-1* mRNA expression is presented at 1 000 mg/kg whereas this dosage did not significantly lower *IL-18* mRNA expression. This result suggests the optimum dosage of SM for these two biomarkers in the kidney. No changes in *renin* mRNA expression were found with the SM treatment. Although the renin-angiotensin system is known to be altered in acute kidney injury, further studies are needed to explore the relationship of this system with colitis. Interestingly, sulfasalazine, an anti-inflammatory drug widely used for treating IBD, was found to enhance kidney fibrosis significantly in the present study. Sulfasalazine-induced renal injury has been previously reported[37]. Although the current results showed this drug could protect against DSS-induced kidney oxidative stress, alleviate histopathological changes, and lower *IL-18* mRNA expression, and plasma cystatin C level, we suggest that the potential adverse effects of sulfasalazine on kidney fibrosis should be considered.

As mentioned above, DSS-induced LPS release may contribute to kidney oxidative stress. This study reveals that SM exhibits an anti-oxidative stress effect on the kidney and improves plasma LBP levels in colitis rats, suggesting that SM may reduce kidney oxidative stress by the reduction of systemic inflammation-induced kidney injury. Mulberry also demonstrates its antioxidant effect. The phenolic compounds are the primary source of mulberry's antioxidant ability. The major contents of polyphenols in this fruit were anthocyanins and cyanidin-3-*O*-glucoside[17]. Previous studies have demonstrated that mulberry anthocyanins decreased DSS-induced colitis by improving intestinal barrier integrity, regulating gut microbiota composition, and alleviating intestinal inflammation and oxidative stress[38]. The study in kidney cell cytotoxicity has shown that a bioactive compound, phytosterols, from mulberry fruit has a protective effect against kidney epithelial cell apoptosis induced by cisplatin[39]. Additionally, the probiotics used in this study were

Lactobacillus salivarius and *Lactobacillus paracasei*. In a previous study on DSS-induced colitis mice, *Lactobacillus rhamnosus* GG exhibited anti-inflammatory activity[40]. The probiotic *Lactobacillus* could reduce kidney inflammation and necrosis in obesity-induced kidney disease in BALB/c mice[41].

Some limitations should be considered in this study. First, gut microbiota profiles and gut integrity were not investigated, both of which are essential for evaluating the role of SM in intestinal health. Second, the long-term effects of SM were not assessed, which is important for determining its safety as a treatment. Lastly, further research should explore kidney injury parameters related to signaling pathways to provide stronger evidence for the therapeutic efficacy of SM.

In conclusion, this study demonstrates the effect of SM on kidney injury in a DSS-induced colitis rat model. SM treatment alleviated colitis symptoms and restored plasma LBP levels. It lowered plasma cystatin C levels, suggesting improved kidney function. SM also attenuated kidney injury by reducing histopathological damage, fibrosis, MDA concentration, and expression of *KIM-1* and *IL-18* mRNA. These findings highlight the potential role of SM in mitigating kidney manifestations associated with colitis. However, further studies are needed to validate and confirm its protective effects on the extraintestinal manifestations of IBD.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding author upon request.

Authors' contributions

KW, AO, PW, and DA developed the conception and experimental design. KW, AO, and KS performed data collection, data analysis, and interpretation. KW contributed to drafting the article, critical revision of the article, final approval of the version to be published, and supervised the project.

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Supplementary Table 1. Disease activity index (DAI) score assessment.

Score	Body weight loss (%)	Stool consistency	Rectal bleeding
0	<1	Normal	Normal
1	1-5	Loose stools	Gross bleeding
2	5-10		
3	10-20		
4	>20	Diarrhea	Gross bleeding