Original Article

Heat-treated and/or lysozyme-treated *Enterococcus faecalis* (FK-23) improves the progression of renal disease in a unilateral ischemia-reperfusion injury rat model

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(Received 16 February, 2024; Accepted 29 March, 2024; Released online in J-STAGE as advance publication 4 April, 2024)

The prevalence of chronic kidney disease (CKD) is increasing owing to the elderly population. Here, we investigated the effects of heat-treated *Enterococcus faecalis* (FK-23) and lysozyme-treated FK-23 (LFK) on the progression of CKD in rats. A CKD model was established using male Wistar rats by subjecting them to right nephrectomy (1K), followed by ischemia and reperfusion (IR). FK-23 or LFK was fed *ad libitum* as a mixed diet after right nephrectomy. Animals subjected to renal ischemia-reperfusion injury (IRI) showed increased plasma creatinine and blood urea nitrogen levels. Furthermore, in the kidneys, collagen accumulation and α-smooth muscle actin, indicative of fibroblast activation and fibrosis-related gene and protein expression, increased 3 weeks after IRI. FK-23 and LFK suppressed the increase in the mRNA levels of some of these genes. The increase in oxidative stress markers, 4-hydroxy-2-nonenal, endothelial nitric oxide synthase, and nitrotyrosine in the kidney, as well as increased plasma uremic toxins after IRI, were also ameliorated by FK-23 and LFK. Metagenomic analysis of fecal samples revealed that gut microbial alteration caused by IRI was also ameliorated by LFK treatment. These results suggest that *Enterococcus faecalis* ingredients may improve CKD progression by suppressing oxidative stress and correcting the balance of the intestinal microflora.

***Key Words*: chronic kidney disease (CKD), heat-treated *Enterococcus faecalis* (FK-23), lysozyme-treated FK-23 (LFK), intestinal microflora, renal fibrosis**

More than 850 million people worldwide suffer from kidney diseases, which is approximately twice the number of people with diabetes (422 million) and 20 times the prevalence of cancer (42 million) or AIDS/HIV (36.7 million) worldwide. In particular, the number of patients with chronic kidney disease (CKD) is increasing owing to population aging and the rise in the number of patients with diabetes. CKD is a progressive condition that affects >10% of the general population worldwide.[1] Acute kidney injury (AKI) significantly increases the risk of CKD development, which is closely associated with AKI severity. Between 5.3 and 10.5 million people require dialysis or transplantation, although many do not receive treatment due to the lack of resources or financial barriers. Therefore, controlling the progression of AKI to CKD is important. Furthermore, intestinal-kidney nephropathy has become an issue, as urea toxins derived from intestinal bacteria accumulate in the blood of patients with CKD, and bacteria in the intestinal tract are transferred to the blood and other organs owing to increased intestinal permeability caused by decreased barrier function of the intestinal epithelium.

The human intestinal flora consists of four major groups: *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria*, which account for 99% of all intestinal bacteria at the phylum level.[2] Previous studies have reported that dysbiosis, the quantitative and qualitative imbalance of the intestinal microbiota, is prevalent in patients with CKD. The proportion of so-called good bacteria, such as *Lactobacillus*, *Prevotella*, and *Bifidobacteria*, has been reported to decrease,[3] while that of *Brachybacterium*, *Catenibacterium*, and *Enterobacteriaceae* is reported to increase in the intestinal microbiota of patients with CKD.[3,4]

Based on this phenomenon of intestinal microbiota alteration associated with CKD, intestinal bacteria abundance is being studied for the development of diagnostics and new treatment methods for diabetic nephropathy at Tohoku University. Lactic acid-producing bacteria are used as probiotics. Among them, *Lactobacillus* sp., *Bifidobacterium bifidum*, and *Enterococcus* sp. are the most used, and, to date, no probiotic-associated adverse events have been reported. Furthermore, the effects of administering dead bacteria and their components are unknown.

A previous study showed that patients with CKD show significantly decreased free radical scavenging capacity in the plasma compared to healthy controls.[5] The percent values of scavenging capacity were significantly different for all assessed free-radical species, namely, hydroxyl radical, 73 ± 12% ($p = 0.001$); superoxide radical, 158 ± 50% ($p = 0.001$); alkoxyl radical, 121 ± 30% ($p = 0.005$); alkylperoxyl radical, 123 ± 32% ($p>0.1$); alkyl

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doi: 10.3164/jcbn.24-29

radical, 26 ± 14% ($p = 0.001$); and singlet oxygen, 57 ± 18%, except alkylperoxyl radical.[5]

FK-23 is a heat-treated strain of *Enterococcus faecalis* (*E. faecalis*), whereas LFK is a lysozyme-treated plus heat-treated strain of *E. faecalis*. The radical scavenging capacity for the six free-radical species was increased after 7 days of FK-23 intake (1.5 g, 900 billion cells/package/day) in the serum and saliva of healthy subjects. Therefore, in this study, we studied the effect of heat-treated *E. faecalis* (FK-23) and enzyme-treated FK-23 (LFK) on CKD progression in rats.

Prebiotics are substrates that are selectively utilized by host micro-organisms and are beneficial to health,[6] and biogenics are described as a food ingredient that works directly or via the intestinal flora for biological regulation, biological defense, disease prevention, recovery, and aging control; they have immunostimulatory, cholesterol-lowering, blood pressure-lowering, anti-tumor, anti-thrombotic, and hematopoietic effects. FK-23 and LFK, which are not viable, act as prebiotics, biogenics, and/or postbiotics.[4,7] Previous studies have shown that FK-23 suppresses fat accumulation in the liver,[8] as well as allergic reactions.[9] In this study, we investigated the effects of FK-23 and LFK, which are thought to improve intestinal flora, on CKD progression in rats with induced kidney damage.

Materials and Methods

Reagents. General reagents were purchased from Wako Pure Chemicals (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan).

Animals. Seven-week-old male Wistar rats (CLEA Japan, Tokyo, Japan), at least $n = 6$ per group, were used. Rats were treated with three mixed anesthetics: medetomidine hydrochloride (0.15 mg), midazolam (2 mg), and butorphanol tartrate (2.5 mg) in saline (1.45 ml) at 0.25 ml per 100 g body weight. The Sham group underwent a sham operation, and the other group underwent a right nephrectomy only (1K). In the 1K-IR group, two weeks after right nephrectomy, for simulating renal ischemia-reperfusion (IR) injury, the left renal arteriovenous system was occluded using a clamp for 45 min and then reperfused. The FK-23 and LFK were provided by Nichinichi Pharmaceutical Co., Ltd. (Mie, Japan). After nephrectomy, the FK-23 (3%) and LFK (0.3% or 3%) were fed *ad libitum* with mixed pellets of CE-2 (CLEA Japan). They were dissected under intraperitoneal administration of mixed anesthetic agents [medetomidine (0.15 mg/0.15 ml/kg), midazolam (2 mg/0.4 ml/kg), butorphanol (2.5 mg/0.5 ml/kg), and saline (1.45 ml/kg)] at 3 or 8 weeks after CKD treatment (12 and 17 weeks old at autopsy). Blood was collected from the inferior vena cava (IVC), and tissues were collected after perfusion with saline via the IVC. The samples (plasma, urine, tissues, and feces) were immediately stored at −80°C until further analyses. Partly, the kidneys, heart, and liver were fixed in a 10% neutral buffered formalin solution (Wako Pure Chemicals, Osaka, Japan). Animal experiment protocols were approved by the Use of Laboratory Animals Committee of Osaka Metropolitan University (approval number: 18053). The study of animal experiments was carried out in accordance with the ARRIVE guidelines and national animal experiment guidelines.

Biochemical analysis. Plasma blood urea nitrogen (BUN) and creatinine were measured and subcontracted to SRL Inc. (Tokyo, Japan). Plasma uremic substances were analyzed by an LC-MS system (Exion LC-AD/X500R, SCIEX, Tokyo, Japan). Standard reagents were purchased as described below. Phenylsulfate (PS) and *p*-cresyl sulfate (PCS) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Indoxylsulfate (IDS) and indoxylsulfate $^{13}C_6$ were purchased from Sigma-Aldrich. PCS-d_6 and creatinine (CRE) were purchased from Toronto Research Chemicals. CRE was purchased from Nacalai Tesque. The samples brought in were diluted 10-fold with an isotope-labeled internal standard aqueous solution. PS, PCS, IDS, and CRE were analyzed using an LC-MS system (Exion LC-AD/X500R). The LC column was an InertSustain AQ-C18 HP column (2.1 mm × 150 mm, 3 μm; GL Sciences, Tokyo, Japan). A gradient condition consisting of 2 mM ammonium bicarbonate in ultrapure water (A) and methanol (B) was adopted. The gradient, expressed as changes in mobile phase B, was as follows: 0 min, start at 5% B; 0–15 min, a linear increase from 5% to 50% B; 15–16 min, a linear increase from 50% to 100% B; 16–21 min, hold at 100% B; and 21–26 min, equilibration at 5% B. The flow rate of the mobile phase was 0.2 ml/min. The high-resolution multi-reaction monitoring (MRM^{HR}) mode was used for the quantitation. The *m/z* values are shown in Supplemental Table 1*. The working parameters for the ESI source were the following: The ion spray voltage was −4,500 V (negative ion). The source temperature was maintained at 300°C. The settings for the nebulizer and heater gases were 40 and 60 psi, respectively.

Histochemical analysis. The tissues collected were fixed with formaldehyde. They were then embedded in paraffin, cut into thin sections (4 μm), and mounted on silane-coated glass slides. After deparaffinization for immunohistochemistry, the tissue was immersed in Target Retrieval Solution 10X Concentrate (pH 6: S1699) (Dako, Tokyo, Japan) diluted 10-fold with dH_2O and autoclaved at 121°C for 5 min for antigen activation. After cooling, the samples were rinsed and then stained using the VECTASTAIN UNIVERSAL ABC-AP kit (VECTOR, Burlingame, CA). Then the slides were incubated overnight at 4°C using primary antibodies (α-SMA or HNE) diluted in Can Get Signal immunostain solution A (Toyobo, Osaka, Japan). After overnight incubation, the samples were incubated for 30 min with a biotin-labeled universal antibody for color development. An alkaline phosphatase substrate kit (Vector Laboratories, Inc., Newark, CA) was used, and nuclear staining was performed with hematoxylin. Azan staining or Masson trichrome staining was performed at the laboratory of Osaka Metropolitan University.

Preparation of the cytosol stock solution. Kidney homogenates (20%) were prepared using RIPA Buffer (pH 7.4) with protease inhibitor cocktail tablets (1 tablet/10 ml, Roche: Basel, Switzerland) on ice for 20 s at a speed of 30,000 rpm using a homogenizer (Microtech Nition, Chiba, Japan). The sample was then centrifuged (Kubota Shoji, Tokyo, Japan) at 4°C and 14,000 rpm for 30 min. The supernatant was collected and used as the cytosol stock solution. Protein concentrations were measured using the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA).

Western blot. Cytosol solution was prepared by adding ultrapure water and 6× SDS Buffer (0.35 M Tris-HCl, 10% SDS, 15% 2-mercaptoethanol, 30% glycerol, 0.06% bromophenol blue), and the sample was heated at 95°C for 5 min. SDS-PAGE was performed as previously described.[10] Primary antibodies (Supplemental Table 2*) were diluted in Can Get Signal Solution 1, and then their solutions were applied at 4°C overnight. After incubation with secondary antibody in Can Get Signal Solution 2 at room temperature for 1 h and extensive washing, blots were placed in Amersham ECL™ Prime Western Blotting Detection Reagent (Cytiva, Tokyo, Japan), allowed to react for 2 min, and then imaged with LuminoGraph1 WSE (ATTO, Tokyo, Japan). Blots were measured using a CS Analyzer (ver. 3.0, ATTO) to determine the emission intensity of each band. The band intensities of the same samples were normalized by β-actin.

RNA isolation from the kidney and quantitative reverse transcriptase PCR (RT-PCR). Total RNA was isolated from kidney samples using NucleoSpin® RNA (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. cDNA was synthesized using RT-PCR using a Rever Tra Ace qPCR RT kit (Toyobo). Quantitative PCR (qPCR) was performed

*See online. https://doi.org/10.3164/jcbn.24-29

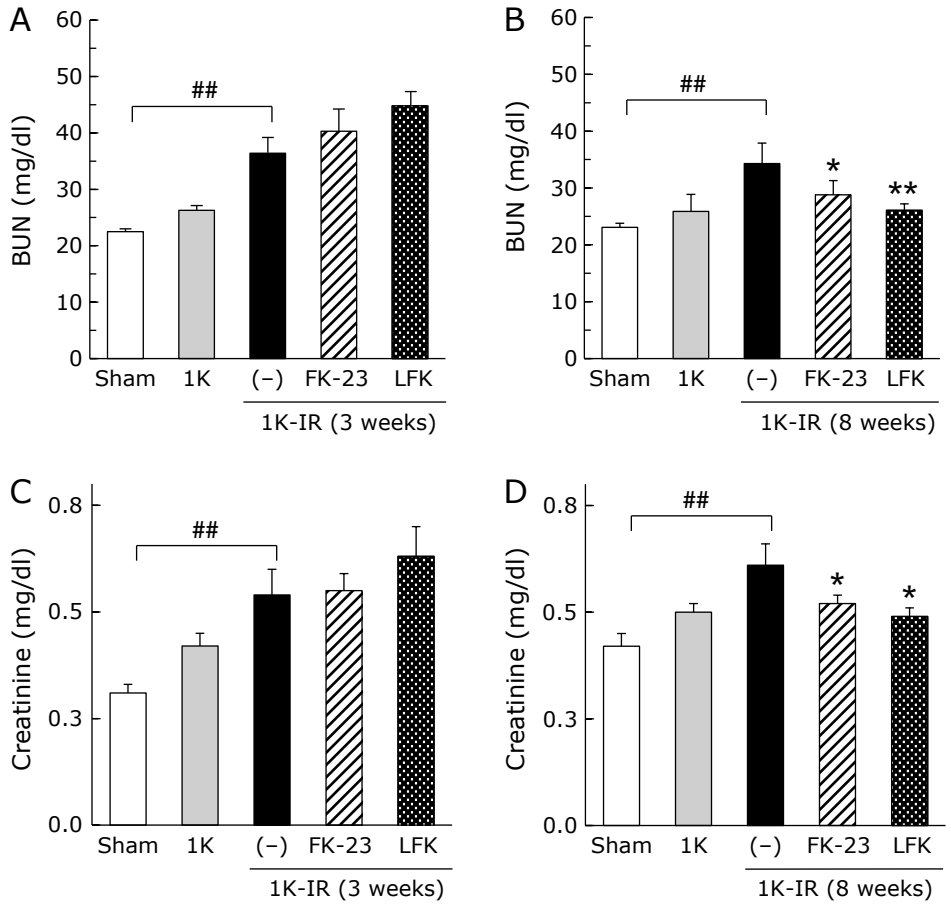

Fig. 1. Effects of FK-23 or LFK on blood urea nitrogen (BUN) and creatinine levels. Male Wistar rats ($n = 6$ per group) were treated as described in the Materials and Methods. The Sham group underwent a sham operation, and the 1K group underwent a right nephrectomy only. In the 1K-IR group, two weeks after right nephrectomy (1K), for simulating renal ischemia-reperfusion injury, the left renal arteriovenous system was occluded using a clamp for 45 min and then repercussed. LFK and FK-23 were fed as mixed diets (3%), and BUN and creatinine levels were measured after 3 and 8 weeks. (A) BUN (3 weeks); (B) BUN (8 weeks); (C) Creatinine (3 weeks) and (D) Creatinine (8 weeks). Values are expressed as the mean ± SE. $^{\#\#}p<0.01$ vs Sham, $^{*}p<0.05$, $^{**}p<0.01$ vs 1K-IR.

using gene-specific primers or TaqMan Gene Expression Assays (Thermo Fisher Scientific) (Supplemental Table 3*) and the THUNDERBIRD Probe qPCR Mix (Supplemental Table 4*, Toyobo). PCR was performed using 7500 Fast (Applied Biosystems, Foster City, CA) with the following cycling conditions: denaturation at 95°C for 15 s and 60°C for 60 s for 40 cycles. All qPCR targets were quantified based on standard curves run on the sample plate. The mRNA levels of specific genes were normalized to those of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase, in the same sample.

Metagenome analysis of rectal feces samples. Metagenomic analysis of the bacterial flora in feces was performed using 16S rRNA sequencing with SheepMedical (Tokyo, Japan).

Statistical analysis. Statistical analyses were performed using Statcel 4 (OMS Publications) or Excel statistics with multiple comparison tests. Statistical significance was determined using the Tukey–Kramer method, and differences were considered significant at $p<0.05$.

Data availability. The datasets collected and analyzed are available from the corresponding author on request.

Results

Changes in body weight, food intake, and kidney weight. In the 1K-IR group, two weeks after the 1K, the left renal arteriovenous system was occluded by the clamp for 45 min and then repercussed. A transient period of remarkably lower body weight was observed in the 1K-IR group at 9–10 weeks (1 week postoperatively) compared to the Sham group; however, after 8 weeks of IR, there was no weight difference between the groups (Supplemental Fig. 1A*). The amount of food consumed decreased during the week of performing renal IR in 1K-IR (Supplemental Fig. 1B*).

The kidney weight increased by approximately 1.3-fold in the 1K group and 1.6- to 2.5-fold in the 1K-IR group (Supplemental Table 5*). The administration of FK-23 or LFK did not significantly affect these changes.

Plasma BUN and creatinine levels. Three weeks after renal IR injury (IRI), BUN and creatinine levels increased in the 1K-IR group compared to those in the Sham group and were not suppressed by *E. faecalis* intervention (Fig. 1). Eight weeks after IRI, BUN and creatinine levels increased in the 1K-IR group compared to those in the Sham group and were suppressed by FK-23 and LFK administration (Fig. 1).

Kidney fibrosis. Collagen accumulation in the tubulointerstitial space, a common feature of kidney fibrosis, was observed at 3 weeks after IRI in the 1K-IR group (Supplemental Fig. 2*). At 8 weeks after IRI, marked collagen accumulation was observed in the 1K-IR group, indicating fibrosis of the renal tubular interstitium (Fig. 2). However, collagen accumulation in the 1K-IR group was significantly suppressed by FK-23 and LFK administration (Fig. 2).

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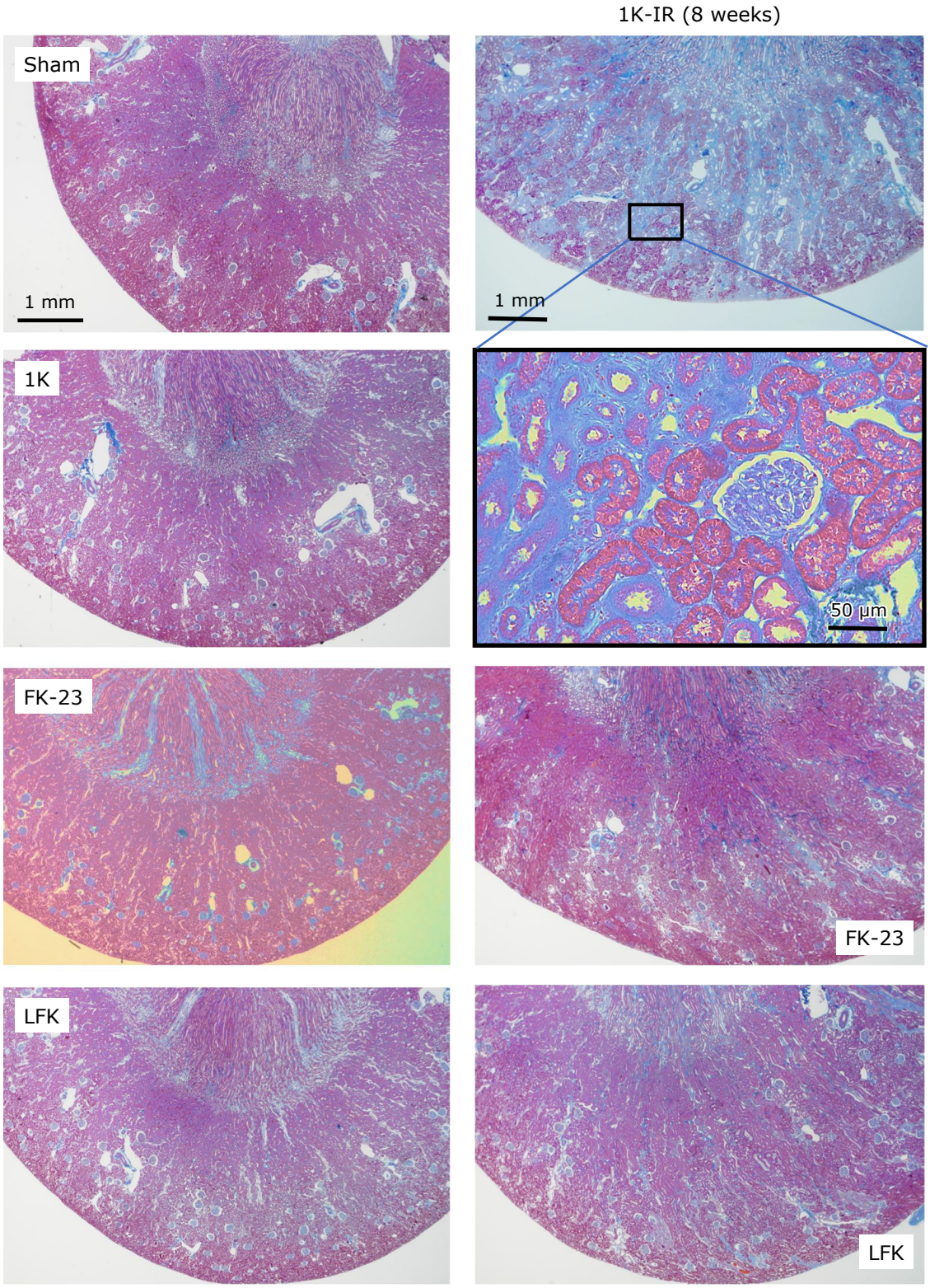

Fig. 2. Representative histology of renal fibrosis. Eight weeks after ischemia-reperfusion, the kidney was treated as described in the Materials and Methods section. The embedded paraffin sections were stained with Masson's trichrome. The left panels show no ischemia or reperfusion. The right panels show renal sections of the 1K-IR groups. The right second row shows the close-up section of the 1K-IR control.

Collagen production. The gene expression of α-SMA was significantly higher in the 1K-IR (3 weeks) group than in the Sham group (Fig. 3). The fold change of α-SMA expression was 1.79 ± 0.18 (data not shown) in the 1K-IR group (8 weeks). Gene expression was increased in 1K-IR (3 weeks) and was not affected by FK-23 or LFK (Fig. 3).

The levels of α-SMA protein at 8 weeks increased 1.5-fold in the 1K-IR group compared with those in the Sham group, and

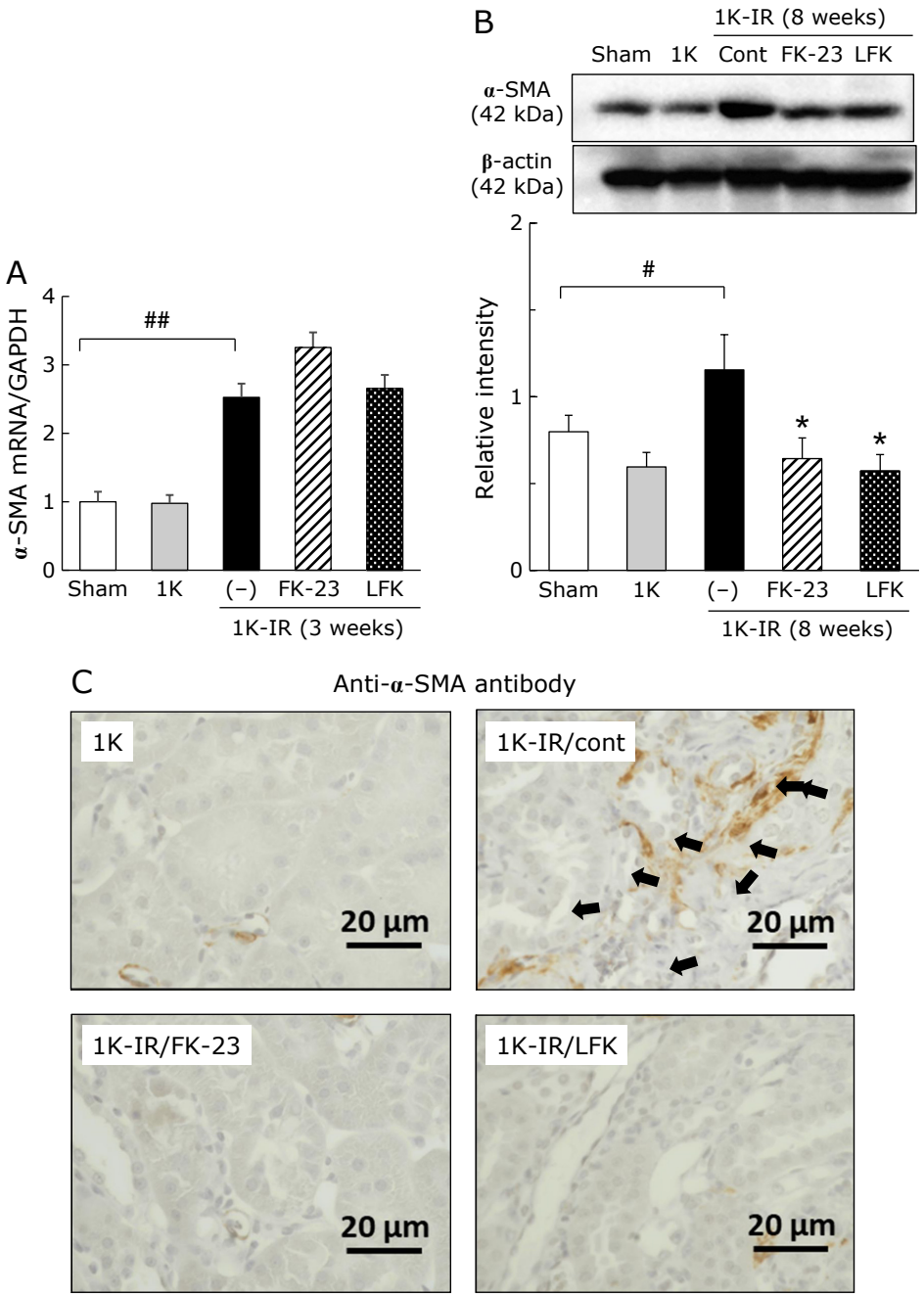

Fig. 3. Effects of FK-23 and LFK on α-smooth muscle actin (α-SMA) expression. Eight weeks after ischemia-reperfusion, rat kidneys (n = 4–11) were treated as described in the Materials and Methods section. (A) gene expression of α-SMA; (B) protein levels of α-SMA; and (C) representative immunohistochemistry. Arrows in (C) indicate that kidney fibroblasts are α-SMA-positive. Values are expressed as mean ± SE. $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs Sham, $^{*}p<0.05$ vs 1K-IR(–).

FK-23 or LFK decreased comparable to that in the Sham group (Fig. 3). Immunostaining of α-SMA showed that only vascular smooth muscle was stained in the Sham group, whereas fibroblasts were also stained in the 1K-IR group. FK-23 or LFK restored levels in the 1K-IR group comparable to those in the Sham group (Fig. 3).

The gene expression of Col1A1 in the 1K-IR group was significantly higher than that in the Sham group. LFK in the 1K-IR (8 weeks) group significantly decreased this increase (Fig. 4). HSP47, a collagen-specific chaperone protein, increased 2-fold in the 1K-IR group than in the Sham group. FK-23 or LFK significantly improved to the level of the Sham group (Fig. 4).

Effects of FK-23 and LFK on renal fibrinolysis. Figure 5A shows the expression of MMP-2, MMP-13, and TIMP-1 genes. The expression of all genes was significantly increased in the 1K-IR (3 weeks) group. FK-23 and LFK significantly reduced TIMP-1 mRNA expression, but not MMP-2 or MMP-13 expression. The protein levels of TIMP-1 and PAI-1 markedly increased in the 1K-IR (8 weeks) group (Fig. 5B). FK-23 and LFK significantly decreased these increases, that is, inhibition of fibrinolysis enzymes (Fig. 5B).

Effects of FK-23 and LFK on the gene expression of inflammatory cytokines. Figure 6 shows the gene expression of TNF-α, IL-6, and TGF-β after 3 and 8 weeks of IR. All genes

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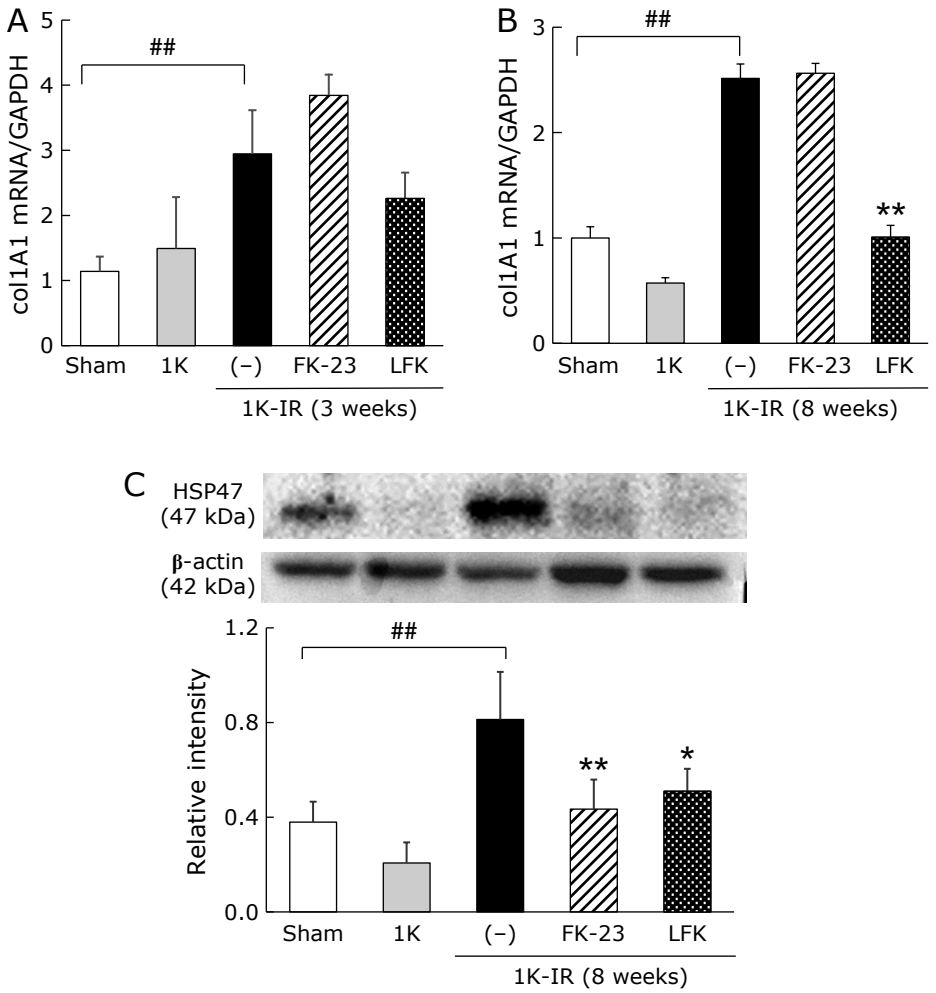

Fig. 4. Effects of FK-23 and LFK on col1A1 mRNA expression and the levels of collagen-specific chaperone protein and HSP47. Three or eight weeks after ischemia-reperfusion, rat kidneys (n = 4–12) were treated as described in the Materials and Methods section. (A) col1A1 mRNA (3 weeks); (B) col1A1 mRNA (8 weeks); and (C) HSP47 protein (8 weeks). Values are expressed as mean ± SE. $^{\#\#}p<0.01$ vs Sham, $^{*}p<0.05$, $^{**}p<0.01$ vs 1K-IR.

were significantly upregulated after IR. FK-23 and LFK significantly decreased the increase in TNF-α mRNA levels at 3 weeks onward and that in IL-6 mRNA levels at 8 weeks. The increase in the levels of TGF-β mRNA (a fibroblast activation factor) was decreased at 8 weeks by LFK.

Effects of FK-23 and LFK on oxidative and nitrosative stress. Lipid peroxidation in the kidney at 8 weeks after 1K-IR significantly increased, as evaluated by anti-HNE antibody staining (Fig. 7A). The staining-positive cells were observed in inflammatory cells such as infiltrated macrophages (Fig. 7B). FK-23 and LFK significantly improved this phenomenon (Fig. 7A and B). Furthermore, LFK reduced macrophage infiltration into the kidneys (Supplemental Fig. 3*). In addition, endothelial nitric oxide synthase (eNOS) protein (Fig. 7C) and nitrotyrosine (Fig. 7D) levels increased in the 1K-IR (8 weeks) group, which indicated a decrease in NO bioavailability due to increased oxidative stress in NO release and nitrosative stress. FK-23 and LFK treatment ameliorated this increase. Furthermore, the metabolomic analysis showed that plasma arginine and citrulline levels were increased in the 1K-IR (8 weeks) group, suggesting increased NO production (data not shown).

Effects of FK-23 and LFK on plasma indoxyl sulfate and PCS levels. Plasma indoxyl sulfate levels significantly increased in the 1K-IR group (3 weeks and 8 weeks). LFK (0.3% and 3%) restored indoxyl sulfate levels dose-dependently (Fig. 8A). PCS levels increased in the 1K-IR group (8 weeks), and FK-23 and LFK (3%) restored PCS to normal levels (Fig. 8B). There was a difference in phenyl sulfate levels between the groups (Supplemental Fig. 4*).

Effects of LFK on the gut microbial composition. *Prevotellaceae* and *Bacteroidaceae* (Bacteroidetes Phylum) were more abundant in the Sham group, but their abundance decreased in the 1K-IR (8 weeks) group (Fig. 9A). LFK (0.3% and 3%) restored the decrease of *Prevotellaceae* (Fig. 9A and B). The ratio of the *Ruminococcaceae* and *Lachnospiraceae* families (Firmicutes Phylum) to Bacteroidetes (F/B ratio), which has been associated with several pathological conditions, increased in the 1K-IR (8 weeks) group (Fig. 9C). LFK (0.3% and 3%) suppressed this increase. At 3 weeks, there was no difference in the intestinal flora of all the groups (data not shown).

Discussion

Our results showed that treatment with FK-23 or LFK ameliorated 1K-IR-induced renal fibrosis by attenuating oxidative stress and inflammation. This is the first report showing that FK-23 and LFK, that is, *E. faecalis* components of heating and heating plus lysozyme treatment, respectively, improved renal failure symptoms, such as uremic toxin accumulation.

Renal IRI is a complex syndrome that causes CKD after

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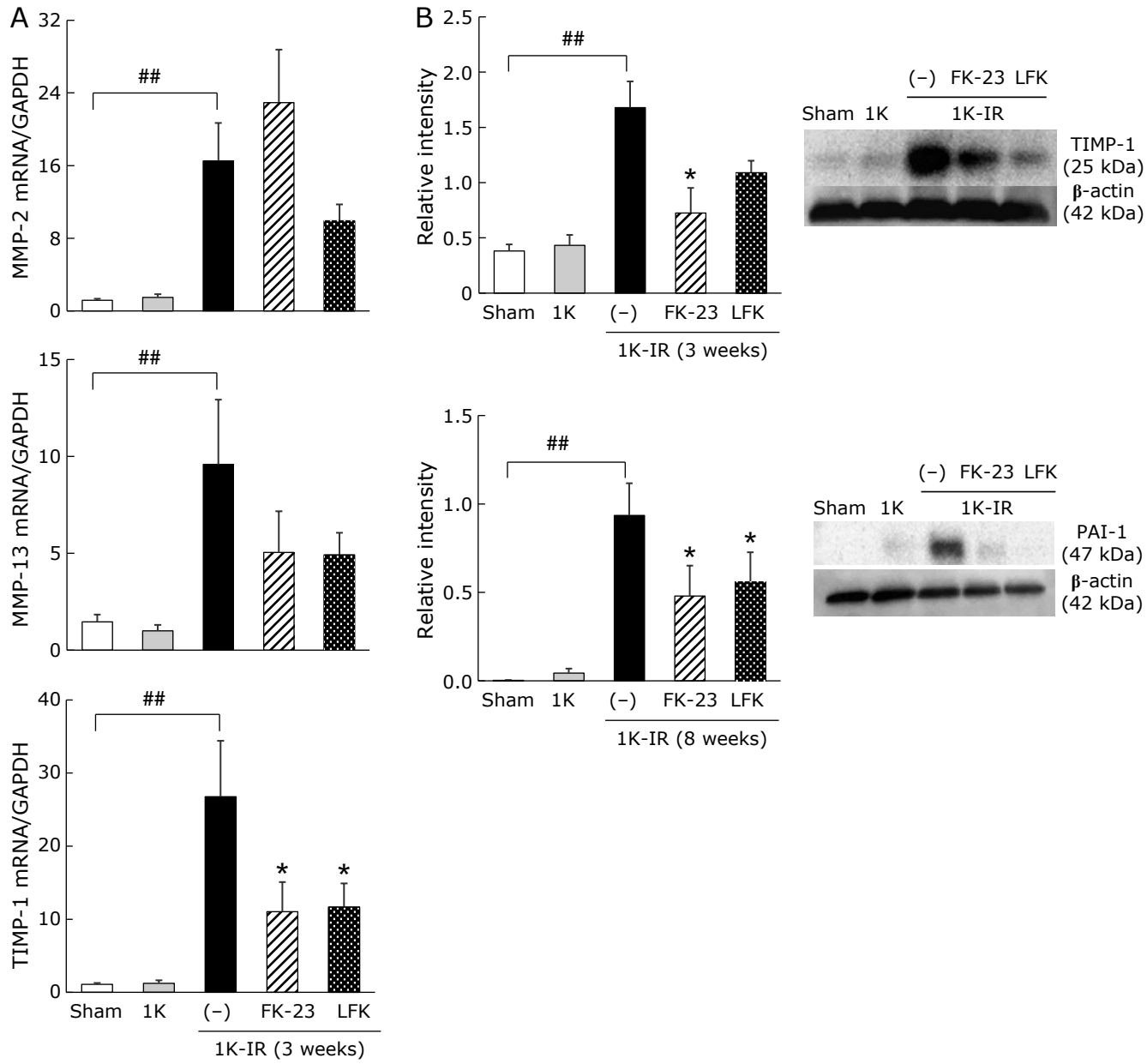

Fig. 5. Effects of FK-23 and LFK on the expression of fibrosis-related genes (A) and proteins (B). Three weeks after ischemia-reperfusion, rat kidneys (n = ~5–18) were treated as described in the Materials and Methods. (A) Gene expression and (B) TIMP-1 and PAI-1 levels. Values are expressed as mean ± SE. $^{\#\#}p<0.01$ vs Sham, $^{*}p<0.05$ vs 1K-IR.

recovery from IRI-mediated AKI. Therefore, no single therapy can effectively prevent renal injury following ischemia. In this study, ischemia-reperfusion injury was induced in one kidney in rats, which exhibited incomplete recovery from AKI and subsequent progression of CKD with an increase in plasma creatinine, worsening of proteinuria, and deleterious histopathological changes, including interstitial fibrosis. The BUN value of the 5/6 nephrectomy model used as a CKD model was approximately 40 mg/dl, which was close to the 36 mg/dl value of this model (Fig. 1).[11] These findings suggest that this model may transition from acute to chronic kidney injury. In addition, the hemoglobin (18.7 ± 0.2–16.7 ± 0.4 g/dl) and hematocrit (54.2 ± 0.6%–48.7 ± 1.1%) values at 3 weeks in this model significantly decreased ($p<0.05$), and FK-23 (3%) or LFK (3%) restored the levels (data not shown). It is suggested that this model is equivalent to stage 3 in CKD patients because anemia occurs slightly in this model.

FK-23 and LFK significantly improved renal fibrosis (Fig. 2) and suppressed the activation of renal fibroblasts (Fig. 3–5). Both FK-23 and LFK also suppressed the increases in the mRNA levels of inflammatory cytokines, such as TNF-α and IL-6 (Fig. 6). These mechanisms suggest that FK-23 and LFK reduced fibroblast accumulation mainly by suppressing the mRNA and protein expression of fibrinolysis inhibitory factor (TIMP-1) (Fig. 5). However, there were differences in gene expression levels of MMP2 and MMP13 between FK-23 and LFK (Fig. 5A). MMP2 has a higher affinity for non-fibrous type 4 collagen, while MMP13 has a higher affinity for fibrous type 2 collagen and specifically degrades it. Thus, LFK may better degrade interstitial collagen than FK-23.

This suggests that FK-23 and LFK improve CKD pathophysiology by suppressing inflammatory cytokines (Fig. 6) and oxidative stress caused by inflammatory macrophages (Fig. 7). LFK

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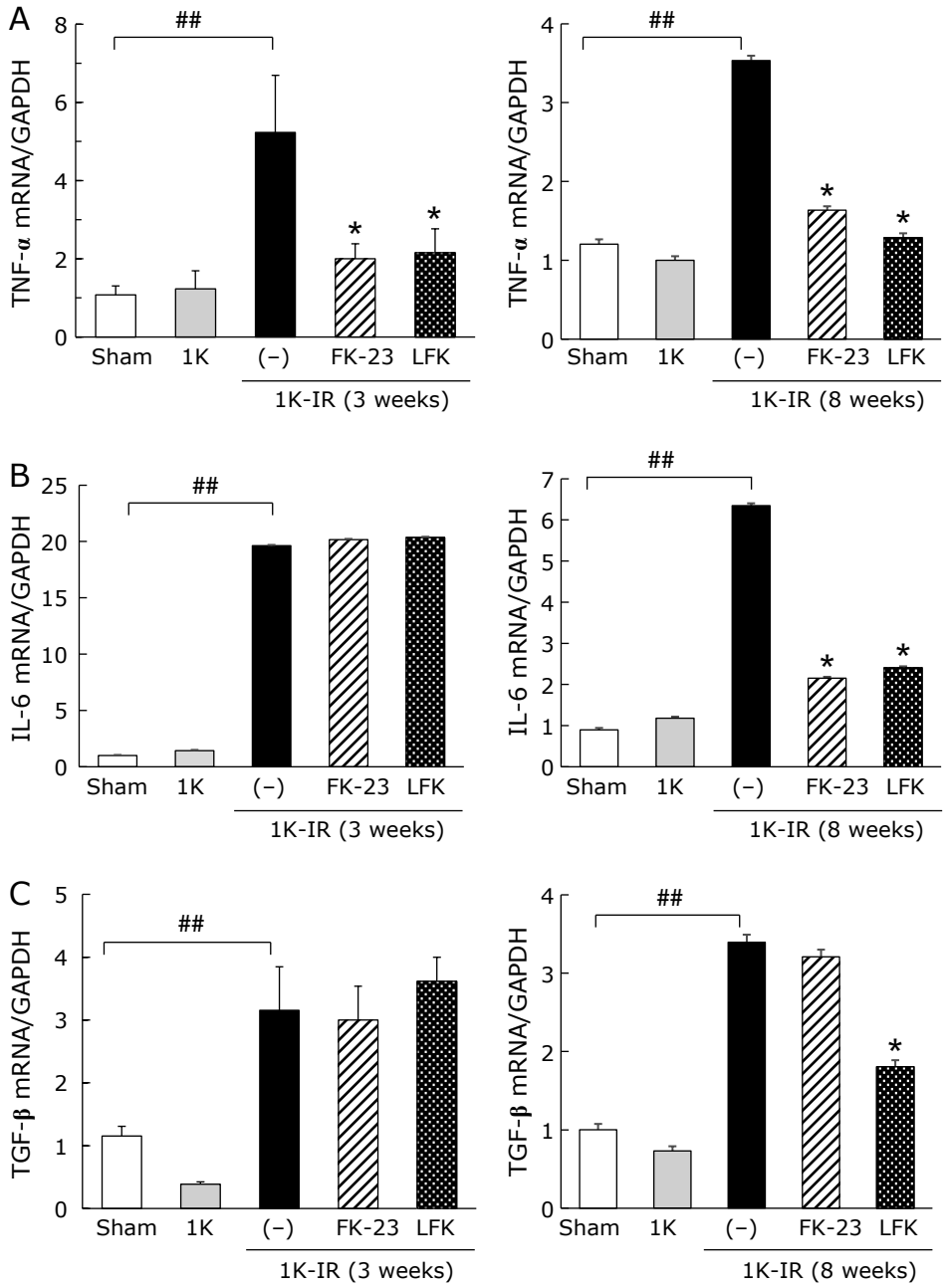

Fig. 6. Effects of FK-23 and LFK on the gene expression of inflammatory cytokines. Three or eight weeks after ischemia-reperfusion, rat kidneys (n = 5–18) were treated as described in the Materials and Methods section. (A) TNF-α mRNA; (B) IL-6 mRNA; and (C) TGF-α1 mRNA. Values are expressed as mean ± SE. $^{\#\#}p<0.01$ vs Sham, $^{*}p<0.05$ vs 1K-IR.

reduced the number of infiltrating macrophages (Supplemental Fig. 3*). LFK acts as a potent antioxidant. Furthermore, eNOS and nitrotyrosine levels in the 1K-IR group were markedly elevated, indicating decreased NO bioavailability (Fig. 7C and D).

In CKD, the production and accumulation of uremic toxins are affected by changes in intestinal function and bacterial flora, in addition to decreased urinary clearance. Among the dietary components, tryptophan, tyrosine, and carnitine are not fully absorbed in the small intestine but are converted to indole, *p*-cresol, and trimethylamine, respectively, by intestinal bacterial metabolism when they reach the large intestine. In our model, uremic toxins increased in the plasma (Fig. 8 and Supplemental Fig. 4*). The ratio of *Firmicutes/Bacteroidetes* (F/B) plays an important role in maintaining intestinal homeostasis,[12] and alteration of the F/B ratio is regarded as dysbiosis, which leads to pathological conditions.[13] While some members of both *Firmicutes* and *Bacteroidetes* are probiotics, an overall increase in *Firmicutes* coincides with obesity, and an increase in *Bacteroidetes* coincides with inflammatory bowel disease.[13] An increase in the F/B ratio reduces the abundance of acetate- and butyrate-producing bacteria.[14] Under CKD conditions, a high-fiber diet is beneficial as it increases microbial biodiversity and the abundance of Bacteroidetes, leading to a lower F/B ratio, accompanied by lower concentrations of indoxyl sulfate and *p*-cresol sulfate.[15] In this study, LFK decreased the F/B ratio and uremic toxin levels (Fig. 9C and Supplemental Fig. 4*), leading to improved kidney function. Furthermore, it has been reported that inhibition of the microbiota-dependent urinary toxin

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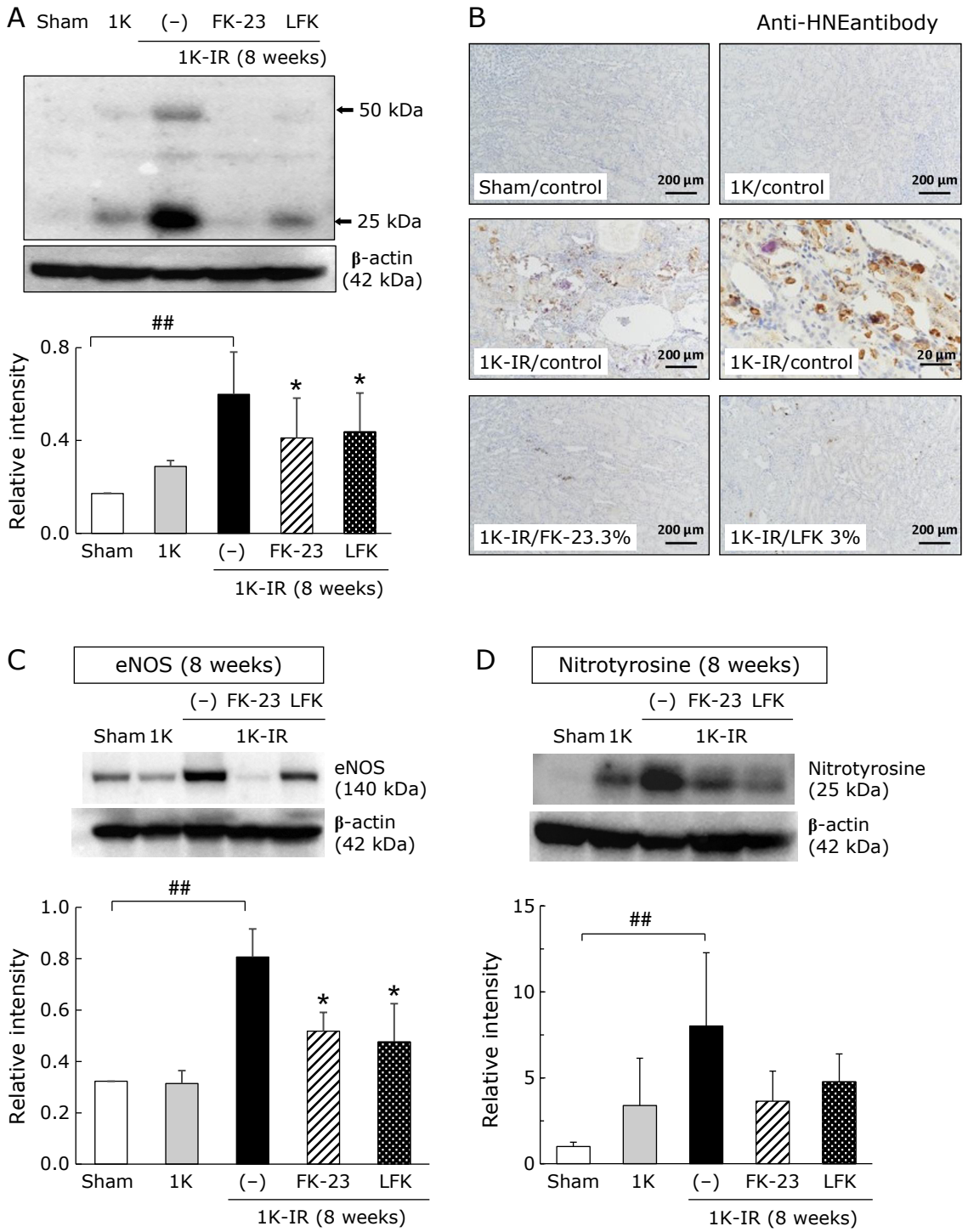

Fig. 7. Effects of FK-23 and LFK on 4-hydroxy-2-nonenal (4-HNE)-modified proteins NOS3 and nitrotyrosine in the kidneys. Eight weeks after ischemia-reperfusion, rat kidneys were treated as described in the Materials and Methods section. (A) 4-HNE-modified proteins. Values are expressed as mean ± SE (n = 5–11). (B) Immunohistochemistry for the anti-HNE antibody; (C) NOS3; and (D) nitrotyrosine. Values are expressed as mean ± SE (n = 4–11). $^{\#\#}p<0.01$ vs Sham, $^{*}p<0.05$ vs 1K-IR.

trimethylamine *N*-oxide attenuates pathophysiological conditions, such as renal fibrosis, in adenine-induced CKD model mice.[16] Therefore, the inhibition of urinary toxin production may also be important for improving CKD pathology. Thus, LFK is effective in improving the pathophysiology of CKD.

Probiotics and prebiotics protect the intestinal environment. Probiotics were proposed as viable additives that are beneficial to the host animal by improving the balance of intestinal microorganisms.[17] They have since been redefined as "live microorganisms that have a health benefit to the host, or foods containing them."[18] In a systematic review of probiotic treatment for patients with CKD, various sample sizes (18 and 101 patients), study durations (4–24 weeks), and dosages (2.0×10^{12}–1.6×10^{10} CFU and 15 g; with no specific standard dosage

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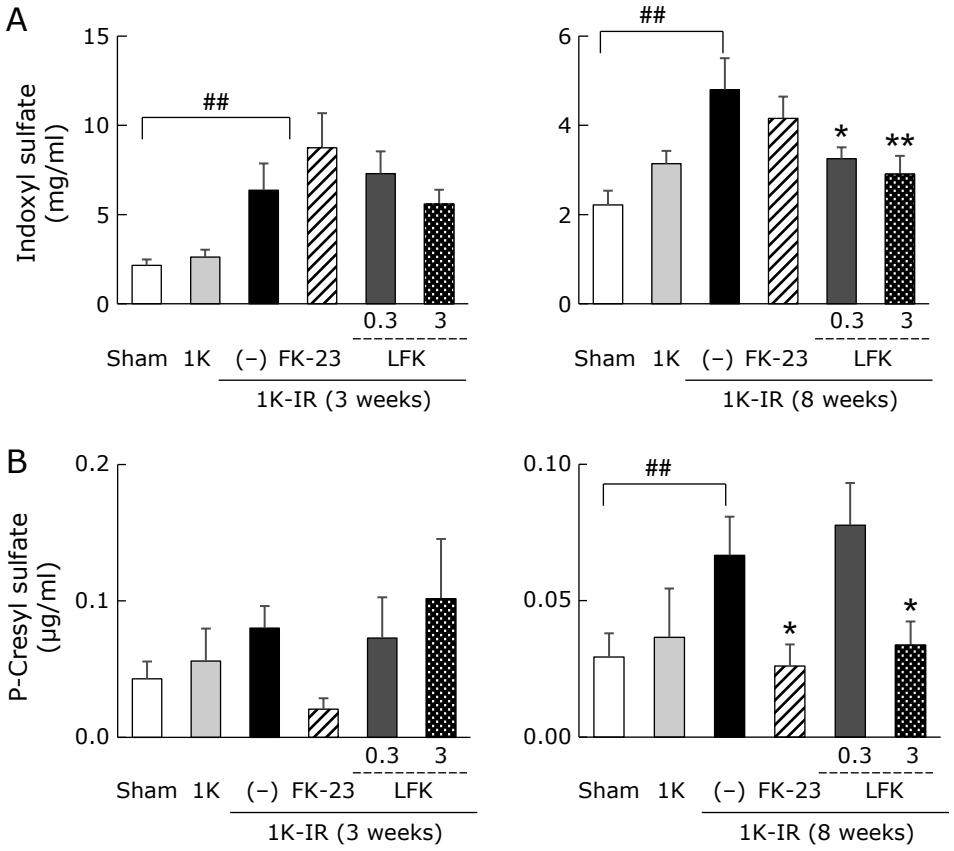

Fig. 8. Effects of FK-23 and LFK on indoxyl sulfate and p-cresyl sulfate levels in plasma and fecal microbiome analyses. Plasma was collected 3 or 8 weeks after ischemia-reperfusion, as described in the Materials and Methods section, and (A) indoxyl sulfate and (B) p-cresyl sulfate levels were determined (n = 5–14). Values are expressed as mean ± SE. $^{\#\#}p<0.01$ vs Sham, $^{*}p<0.05$, $^{**}p<0.01$ vs 1K-IR.

criteria) were studied. Probiotic agents were also prescribed in different forms in relation to diet and time of day, such as immediately after or during meals. Most studies reported positive effects on renal function and decreased levels of urea, BUN, ammonia, and plasma uremic toxins.[19]

Recently, scientists found that not only the live strains but also some inactivated strains, components, and their metabolites can also be beneficial for humans.[20] Therefore, the term "postbiotics" from the consensus statement issued by the International Scientific Association for Probiotics and Prebiotics in 2021 was put forward and has attracted great concern from the industry in recent years. Both FK-23 and LFK, which are true postbiotics and biogenic, have been found to increase plasma antioxidant activity in humans, as mentioned in the Introduction. Although FK-23 and LFK used in our rat study were dead bacteria, they were given at a dose of 1.2×10^{12} CFU/kg body weight per day. This dose showed similar antioxidant activity in rat plasma (data not shown).

FK-23 has been shown to suppress Th17 cell development and attenuate allergic airway responses in mice[21] as well as show good results in a double-blind test.[22] LFK reduced mortality[8] and inflammation[23] caused by the Influenza A virus in mice. Furthermore, FK-23 improved hepatitis C virus infection in humans[24] and opportunistic fungal infections in mice.[25] FK-23 inhibits fatty acid oxidation during hepatic steatosis in mice.[8] Therefore, the postbiotics FK-23 and LFK have several benefits. As shown in Supplemental Fig. 5*, plasma serotonin, circled by the dotted line in metabolic map #7, increased in the CKD model and improved with LFK administration. The bioactive substance serotonin has been implicated in inflammation in the kidney. The impaired renal function affects peripheral serotonin metabolism.[26] Inflammation is also involved in kidney fibrosis and may be involved in the pathogenesis of CKD, a chronic fibrotic process. Nevertheless, the active ingredients of FK-23 and LFK that provide the various health benefits remain unclear and must be identified in future studies.

To the best of our knowledge, this is the first study to demonstrate the ability of heat-killed *E. faecalis* (FK-23) and lysozyme-treated FK-23 (LFK), a postbiotic, and their components to improve CKD pathology. The number of patients with CKD is estimated to be 13.3 million, and CKD prevention is a major concern in Japan and worldwide. Our findings may offer a safe and effective alternative for CKD. Further studies should be conducted to elucidate the clinical impact of postbiotic use in CKD in humans as well as in other animals.

Author Contributions

ST and YM contributed to the conceptualization of the study. NI, AY, HI, KN, ST, and MO contributed to data analysis and interpretation. ST and YM contributed to drafting the manuscript. TY supervised the study. All authors have approved the submitted version of the manuscript and agreed to be accountable for any part of this work.

Acknowledgments

Western blotting and RT-qPCR were performed with the research support platform of the Graduate School of Medicine, Osaka Metropolitan University. We would like to thank Editage (www.editage.jp) for English language editing.

*See online. https://doi.org/10.3164/jcbn.24-29

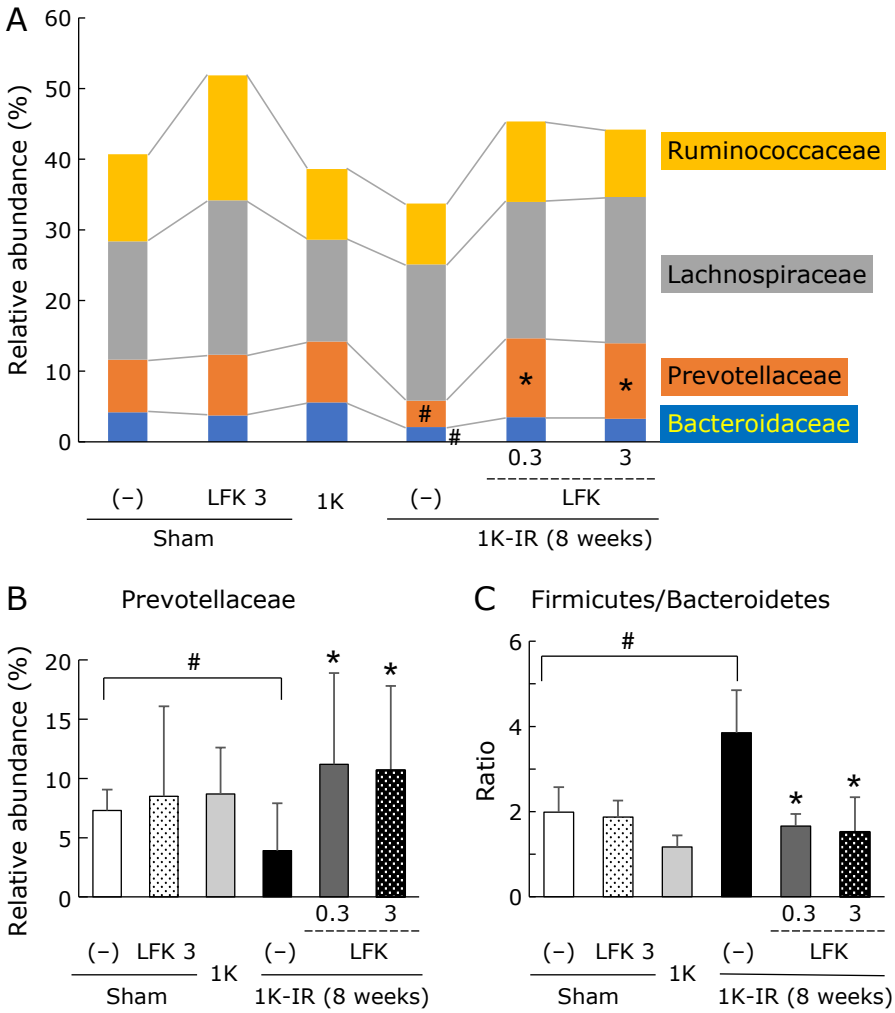

Fig. 9. Effects of FK-23 and LFK on fecal microbiome analysis. (A) Metagenomic analysis of fecal samples. Four bacterial species related to intestinal immunity were analyzed. (B) Changes in the relative abundance of the Prevotellaceae family. (C) The ratio of Firmicutes to Bacteroidetes (F/B ratio). Values are expressed as mean ± SD (n = 4–9). $^{\#}p<0.05$ vs Sham, $^{*}p<0.05$ vs 1K-IR.

Funding

This work was partially supported by grants-in-aid from Nichinichi Pharmaceutical Co., Ltd. (Mie, Japan).

Conflict of Interest

ST, YM, and TY received an unrestricted grant from Nichinichi Pharmaceutical Co., Ltd. (Mie, Japan). NI is supported by Nichinichi Pharmaceutical Co., Ltd. as an outside work in this study. The funders had no role in the data analysis and interpretation. KN is employed by Nichinichi Pharmaceutical Co., Ltd. The remaining authors declare no competing interests.

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