博士論文

論文題目　**Galacto-oligosaccharides modify gut microbiota and attenuate renal injury**

（ガラクトオリゴ糖は腸内細菌叢を変化させ，腎組織障害を改善する）

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ABSTRACTS

Tubulointerstitial injury is central to the progression of end-stage renal disease. Recent studies have reported that indoxyl sulfate (IS), one of the most investigated uremic toxins, causes tubulointerstitial injury through oxidative stress or endoplasmic reticulum (ER) stress. Because indole, the precursor of IS, is synthesized from dietary tryptophan by the gut microbiota, I hypothesized that the gut microbiota was altered in kidney disease, and the intervention targeting the gut microbiota with galacto-oligosaccharides (GOS) would attenuate the renal injury. After 2 weeks of GOS administration for 5/6 nephrectomized (Nx) or sham-operated (Sham) rats, the gut microbiota were analyzed with pyrosequencing methods, cecal indole and serum IS were measured, and renal injury was evaluated. Pyrosequencing analysis revealed that the bacterial family Clostiridiaceae was increased in the Nx rats compared with the Sham rats and decreased with GOS, and GOS increased three bacterial families and decreased five families in the Nx rats. Cecal indole and serum IS were decreased and renal injury was improved with decreased infiltrating macrophages in GOS-treated Nx rats. The expression levels of ER stress markers and apoptosis were significantly increased in the Nx rats and decreased

with GOS. Taken altogether, my data show that the gut microbiota was altered in kidney disease, and GOS modified the gut microbiota, decreased cecal indole and serum IS, and attenuated renal injury in the Nx rats. GOS could be a novel therapeutic agent to protect against renal injury.

INTRODUCTION

Chronic kidney disease, uremia, and uremic toxins.

Chronic kidney disease (CKD), whose concept was first proposed by the National Kidney Foundation in 2002, is a significant clinical issue on health care worldwide [1]. CKD is defined as kidney damage for more than 3 months (kidney damage: structural or functional abnormalities), or estimated glomerular filtration rate < 60 mL/min/1.73m^2 for more than 3 months [1]. More than ten million people in Japan are suffering from CKD [2]. Because CKD has been reported to be an independent risk factor for mortality and significantly associated with cardiovascular disease [1], [3], reducing CKD patients is of great importance.

In CKD, uremic toxins, organic waste products that are cleared by the kidneys and excreted to urine in healthy state, accumulate and induce characteristic symptoms of CKD [4]. Moreover, IS (Figure 1) is among the most investigated uremic toxins and has been reported to cause cell and tissue injury [5]–[9]. Niwa et al. indicated that IS

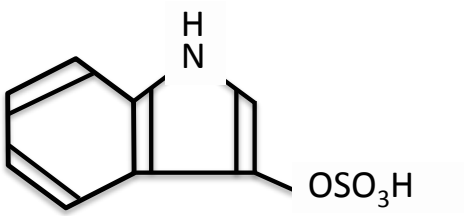

Figure 1. **Graphical formula of indoxyl sulfate.**

administration to 5/6 nephrectomized rats accelerated glomerular sclerosis [5], and they also showed that IS upregulated the expression of fibrosis related protein, transforming growth factor (TGF)-β1, tissue inhibitor of metalloproteinase (TIMP)-1, and proα1(I) collagen [6]. Palm et al. reported that IS increased oxygen consumption and caused cellular damage through oxidative stress in tubular cells [7]. Kawakami et al. indicated that IS caused tubular cell damage through endoplasmic reticulum (ER) stress [8]. Shimizu et al. showed tubular cell damage through IS-induced cellular senescence [9]. These evidences suggest that IS not only accumulates in the CKD condition but also causes *per se* tubulointerstitial injury. Therefore, decreasing serum IS is one of the most important clinical issues to solve among CKD patients.

Indole, the precursor of IS, is synthesized from dietary tryptophan by the gut microbiota and is metabolized into IS via indoxyl through sulfate conjugation in the liver [10]. IS enters the systemic circulation and causes cell and tissue injury (Figure 2).

Serum IS is produced almost by the gut microbiota, because serum IS of hemodialysis (HD) patients with total colectomy was reported almost to be under detectable level [11], and serum IS of germ free mice weren't almost detectable as well [10].

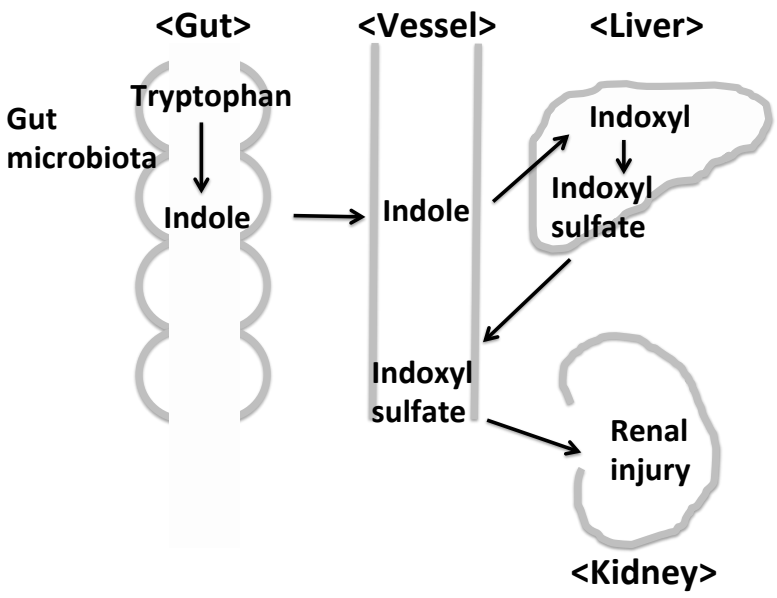

Figure 2. **Synthesis of indoxyl sulfate (IS) from tryptophan.**

Indole is synthesized from dietary tryptophan, and is metabolized into IS via indoxyl. IS enters the systemic circulation and cause cell and tissue injury.

Chronic kidney disease, the gut microbiota, and indoxyl sulfate.

The human gut is housed by hundreds kinds of species, hundreds of trillions of bacteria [12]. The gut microbiota are typically dominated (90% of all the bacteria) by bacteria belonging to the two phylums, Firmicutes (e.g., *Clostridium*, *Lactobacillus*) and Bacteroides (e.g., *Prevotella*). Bacteria belonging to the four phylums, Firmicutes, Bacteroides, Actinobacteria (e.g., *Bifidobacterium*), and Proteabacteria (e.g., *Enterobacteriaceae*) account for 99% of all the bacteria in the gut [12]–[14]. It also has been reported that human and rat gut microbiota are similar at phylum level [15], [16].

The gut microbiota have been reported to utilize dietary carbohydrates residues that cannot be digested by the host's digestive enzymes in the small intestine to synthesize short-chain fatty acids (SCFAs; e.g., succinic acid, acetic acid, lactic acid, formic acid, propionic acid, and butyric acid) [17]–[20]. These SCFAs are utilized as nutrient sources by colonic bacteria and colonic epithelial cells, are absorbed from colon with contributing to the metabolism of the host, and are used to decrease intestinal luminal pH [20]. The gut microbiota can also utilize amino acid residues that are not absorbed from the small intestine to synthesize ammonia or the corresponding amines.

CKD has been reported to enhance the protein putrefaction in the gut because of insufficient protein assimilation in the small intestine resulting in more protein entering the colon [21], [22], prolonged colon transit time [21], and increased luminal pH secondary to urea diffusion [23], all of which can effect colonic environment and microbial composition. Vaziri et al. reported that the diversity of the gut microbiota and the proportions of Lactobacillaceae and Prevotellaceae were decreased in the feces of 5/6 nephrectomized rats compared with control rats [23], and Hida et al. reported that Bifidobacteria were decreased and *Clostridium perfringens* was increased in the feces of HD patients [24]. If the gut microbiota is truly altered in kidney disease and contributes to uremic toxin production, an intervention targeting the gut microbiota could offer a potential therapeutic option for tissue injury related to uremic toxins.

Currently a number of agents are used to change the gut microbiota composition. Probiotics are live microbial feed supplements that beneficially affect the host by improving its intestinal balance [25], and prebiotics are non-digestible food ingredients which beneficially affect the host by stimulating some bacterial growth in the gut [26]. Galacto-oligosaccharides (GOS) are prebiotics; they are made from lactose by

β-galactosidase and are utilized by the limited colonic bacteria (Figure 3).

Administrations of probiotics and/or prebiotics to CKD patients or CKD model are reported in several papers [24], [27]–[29]. Hida et al. reported that lactic acid bacteria administration to the HD patients reduced serum IS and fecal putrefactive metabolites [24]. Takayama et al. reported that *Bifidobacterium* administration to the HD patients reduced serum IS [27]. Meijers et al. reported that administration of oligofuructose-enriched inulin to the HD patients reduced serum *p*-cresyl sulfate [28]. Nakabayashi et al. reported that administration of *Lactobacillus casei* strain Shirota, *Bifidobacterium breve* strain Yakult, and GOS to the HD patients reduced serum *p*-cresol [29]. Koppe et al. reported that administration of arabino-xylo-oligosaccharides to 5/6 nephrectomized mice decreased serum *p*-cresyl sulfate [30].

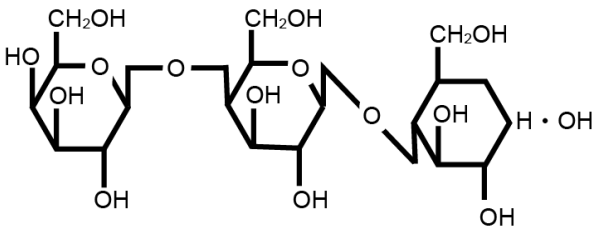

Figure 3. **Graphical formula of 4'-galactosyllactose, the major element of galacto-oligosaccharides.**

Analyzing gut microbiota.

To analyze the gut microbiota, cultural methods such as isolating, culturing, and gram staining, have been widely performed [31], but these methods can be applied only to the culturable bacteria. Recently, quantitative PCR has been widely used, and DNA extracted from the gut contents is amplified using bacteria-specific primers. The method has high quantitative performance, but it can be applied only to the bacteria whose specific primers are known. More recently, pyrosequencing gene analysis methods are getting more popular [32], [33]. 16s rDNA consists of the conservative regions, whose base sequences are shared by most bacteria, and the hypervariable regions [31]. Extracted DNA is amplified and sequenced with next-generation sequencing systems. Identification of bacteria is performed through analyzing the sequences corresponding to the hypervariable regions with the sequence database [34]. These methods can be also applied to the unculturable bacteria if the sequences of 16s rDNA are reported, and enable comprehensive analysis of the gut microbiota; indeed quantitative PCR is more sensitive for detection of specific bacteria than pyrosequencing methods.

Endoplasmic reticulum stress and apoptosis.

Because IS has been reported to cause renal injury through ER stress [8], I investigated the change of renal injury and ER stress caused by GOS.

ER regulates protein synthesis, folding, trafficking, modification, and degradation [35]. The ER functions are influenced by various conditions such as ischemia, glucose deprivation, oxidative stress, or genetic mutations, which can cause abnormal protein folding [36]–[38]. The accumulation of the unfolded or malfolded protein in the ER lumen causes ER dysfunctions, called ER stress. Cells with ER stress trigger intracellular signaling known as unfolded protein response (UPR), which consists of adaptive UPR and maladaptive UPR. Cells with ER stress, at first, trigger adaptive UPR to ameliorate ER functions. But cells with too severe ER stress or with irrecoverable ER functions by adaptive UPR trigger maladaptive and proapoptoic UPR, resulting in apoptosis [39], [40].

Adaptive UPR increase protein-folding capacity by upregulation of ER chaperones (e.g., glucose-regulated protein [GRP] 78) [41], and reduce new protein synthesis, with preventing further accumulating of unfolded proteins. In addition, misfolded protein

degradation system (ER-associated degradation: ERAD), and oxidant-detoxifying enzymes (e.g., hemoxygenase-1 [HO-1]) maintain ER function for cell survival [42].

GRP78, a central regulator of UPR, normally binds to N-termini of ER localized transmembrane protein (e.g., inositol-requiring enzyme 1 [IRE1], double-stranded RNA-activated protein kinase-like ER kinase [PERK], and activating transcription factor 6 [ATF6]). IRE1, PERK, and ATF6 are core UPR signal transducers. When unfolded protein accumulated in the ER lumen, GRP78 releases the ER membrane proteins and binds to unfolded proteins, resulting in adaptive UPR triggered [42].

Cells with too severe ER stress or with irrecoverable ER functions by adaptive UPR trigger proapoptotic UPR [36], [38]. ER stress-induced apoptosis is mainly mediated by CCAAT/enhancer-binding protein homologous protein (CHOP, also called growth arrest and DNA damage 153; GADD153). CHOP is a transcription factor that induces some proapoptoic factors, downregulates antiapoptoic Bcl-2, and leads to apoptosis [39]. The other maladaptive UPR pathways are mediated with c-Jun N-terminal kinase (JNK) or caspase-12 [43], [44].

Accumulation of the unfolded protein has been reported to be associated with various

diseases, such as neurodegenerative disease (e.g., Alzheimer's disease [45]), diabetes, atherosclerosis, and liver disease [37] . In addition, several research groups including us reported that ER stress was associated with kidney disease, especially tubulointerstitial injury [46].

Tubulointerstitial injury is central to the progression of end-stage renal disease [47]. Ohse et al. reported that proteinuria caused ER stress-induced apoptosis via caspase-12 pathway in the proximal tubular cells [48]. Kawakami et al. reported that CHOP expression was upregulated in tubular cells of 5/6 Nx nephrectomized rats and was significantly downregulated when serum IS was reduced by AST-120 [8]. This report indicates that IS causes ER stress and upregulates CHOP expression in tubular cells.

HYPOTHESES AND AIMS

I hypothesized that kidney disease altered the composition of the gut microbiota and the intervention targeting the gut microbiota could attenuate renal injury through the inhibition of indole and IS production. In detail, I investigated the gut microbiota of 5/6 nephrectomized rats with/without GOS using pyrosequencing methods, measured the gut indole and serum IS concentration, and evaluated the renal injury with ER stress and apoptosis in tubular cells**.**

MATERIALS AND METHODS

Animal Models.

All of the animal experiments were approved by the ethical committee of Graduate School of Medicine, the University of Tokyo (P11-038) and performed in accordance with the guidelines of the Committee on Ethical Animal Care and Use at the University of Tokyo.

Eight-week-old male Sprague-Dawley rats weighing 240-260 g were purchased from CLEA Japan (Tokyo, Japan) and were housed in an air-conditioned room under a 12-hour light/dark cycle with free access to food and water. Only one rat was housed in each cage, because co-housing the rats would have caused homogenization of the microbiota via coprophagy. Blood pressure (BP) and body weight (BW) were measured, and blood sampling from the tail vein was performed weekly.

The rats were divided into two groups: a 5/6 nephrectomized group (Nx, n = 13) and a sham-operated group (Sham, n = 7). In the Nx group, right nephrectomy was performed (-1 wk), and 1 week later, the posterior and one anterior branch of the left

main renal artery were ligated and infarction of approximately two-thirds of the left kidney was induced (0 wk). In the Sham group, only laparotomy was performed (-1 wk, 0 wk). Each operation was performed under pentobarbital anesthesia. Two weeks after the nephrectomy (2 wk), all of the rats were divided into a control-diet group (Con; Con Nx, n = 7; Con Sham, n = 4) or a GOS-diet group (GOS; GOS Nx, n = 6; GOS Sham, n = 3) so that the mean BPs and the mean concentrations of blood urea nitrogen (BUN) and serum creatinine (sCre) at 1 wk and 2 wk would be comparable between the control-diet group and GOS-diet group. After 2 weeks of GOS treatment, the rats were sacrificed under pentobarbital anesthesia (4 wk), and the kidneys were fixed with neutral-buffered formalin or methyl Carnoy's solution.

Galacto-oligosaccharides treatment regimen.

For GOS treatment, a GOS diet was prepared by replacing half of the sucrose in a control-diet, AIN-93G (Oriental Yeast Co., Tokyo, Japan) with GOS (Table 1), which was supplied by the Yakult Central Institute. The preliminary data showed that more GOS sometimes caused diarrhea due to high intestinal osmolarity and low food intake.

Table 1. *Ingredients composition of the diet (%)*

	Control-diet	GOS-diet
Casein	20.00	20.00
L-cystine	0.30	0.30
Corn starch	52.95	52.95
Sucrose	10.00	5.00
Soybean oil	7.00	7.00
Powdered cellulose	5.00	5.00
AIN-93G Mineral Mix	3.50	3.50
AIN-93G Vitamin mix	1.00	1.00
Choline Bitartrate	0.25	0.25
Galacto-oligosaccharide	-	5.00
Total	100.00	100.00

Physiological measurements.

BP was measured with Softron BP-98A Unit®, an occlusive tail-cuff plethysmograph attached to a pneumatic pulsetransducer (Softron, Tokyo, Japan). BW was measured with a compression balance. The mean food consumption in the 3 days immediately before sacrifice was recorded.

Pyrosequencing analysis.

In rodents, cecum is the major fermentation organ [49]. To examine the composition of the rats' gut microbiota, the cecal contents was analyzed using pyrosequencing

methods [32]. DNA was extracted from the cecal content of the rats. 16S rDNA genes of each sample were amplified with 66F-linker A forward primer and 338Rm-linker B reverse primer. The sequences of the primers were as follows: A primer, 5'-*CCATCTCATCCCTGCGTGTCTCCGAC* – TCAG – NNNNNNNNNN - GCYTAAYACATGCAAGTMGA-3'; B primer, 5'-*CCTATCCCCTGTGTGCCTTGGCAGTC* – TCAG – NNNNNNNNNN - GCTGCCWCCCGTAGGWGT-3'. The italicized sequences represent 454 Life Sciences linker A or linker B, respectively (Roche, Basel, Switzerland). 'TCAG' was inserted as a key sequence as recommended by the manufacture, and NNNN… represented a unique 10-base barcode to tag each PCR product. The underlined sequence was the broadly conserved bacterial primer 66F or 338Rm, respectively. PCR was performed with SYBR *Premix Ex Taq*® (TAKARA BIO, Shiga, Japan) under the following conditions: initial denaturing at 95°C for 5 min, followed by 13-17 cycles of 95°C for 30 s, 55 °C for 30 s, and 72°C for 1 min using ABI PRISM7500® (Applied Biosystems, Tokyo, Japan). Amplicons were sequenced using a Roche 454 GS Junior pyrosequencer® (Roche) with a GS FLX Titanium emPCR Kit® (Lib-L, Roche).

Sequences generated from the PCR amplicons of the pyrosequencing-barcoded 16S rDNA gene were analyzed using default settings in the open source software package QIIME (Quantitative Insights Into Microbial Ecology; http://qiime.org/) [50]. After filtering, the total number of remaining reads was 40,656 reads. The average sequence length of the remaining reads was 1626.2 ± 491.1 in each sample. These remaining reads were subjected to a data analysis pipeline implemented in QIIME. 16S rDNA gene sequences were assigned to operational taxonomic units using the QIIME implementation of cd-hit and a threshold of 97% pairwise identity.

Biochemical measurements.

The concentrations of six SCFAs (succinic acid, lactic acid, formic acid, acetic acid, propionic acid, and butyric acid) were also measured as representative markers of microbiota changes. The concentrations were determined with HPLC (Waters 432 Conductivity Detector®, Waters Corporation, Milford, MA, USA), as previously described [51]. The cecal indole and serum IS concentrations were also analyzed with HPLC (GL-7400 series HPLC®; GL Sciences, Tokyo, Japan) as previously described

[52]. The assays for the concentrations of BUN and sCre were performed with commercial kits (Wako Pure Chemical Industries, Osaka, Japan).

Histological evaluation.

Formalin-fixed, paraffin-embedded tissue was sectioned (3 μm thick) and the sections were dewaxed with Histoclear® (National Diagnostics, Atlanta, GA, USA) and rehydrated through graded ethanols. Periodic acid-Schiff (PAS) staining was performed for semiquantitative evaluation of tubulointerstitial injury. Tubulointerstitial injury scores were graded (0 to 5) as previously reported [53] on the basis of the percentage of tubular cellularity, basement membrane thickening, cell infiltration, dilation, atrophy, sloughing, or interstitial widening as follows: 0, no change; 1, <10% tubulointerstitial injury; 2, 10% to 25% injury; 3, 25% to 50% injury; 4, 50% to 75% injury; and 5, 75% to 100% injury. The injury was evaluated in randomly selected 30 fields, and all of the quantifications were performed in a blinded manner.

Immunohistochemistry.

To identify macrophages, CHOP, GRP78, and cleaved caspase-3, indirect immunoperoxidase staining was performed on methyl Carnoy's-fixed (macrophage) or formalin-fixd (CHOP, GRP78, and cleaved caspase-3) paraffin-embedded tissue sections (3 μm thick). After dewaxing and rehydration, antigen retrieval was performed with autoclaving in citrate buffer (pH 6.0) for CHOP or GRP78 or in EDTA buffer (pH 8.0) for cleaved caspase-3 [54]. Blocking of pseudoperoxidase with 3% hydrogen peroxide, nonspecific protein binding and endogenous biotin activity were then performed. The sections were incubated overnight at 4°C with mouse monoclonal anti-macrophage antibody (ED-1, 1:500 dilution; Chemicon, Temecula, CA, USA), rabbit polyclonal anti-CHOP antibody (1:100 dilution; Santa Cruz Biotechnology, Dallas, TX, USA), goat polyclonal anti-GRP78 antibody (1:100 dilution; Santa Cruz Biotechnology), or rabbit monoclonal anti-cleaved caspase-3 antibody (Asp175, 1:250 dilution; Cell Signaling Technology, Danvers, MA, USA). The sections were incubated for 40 min at room temperature with the secondary antibodies, biotinylated horse anti-mouse IgG antibody (1:1000 dilution; VECTOR, Burlingame, CA, USA), goat

anti-rabbit IgG antibody (1:1000 dilution; VECTOR), or rabbit anti-goat IgG antibody (1:1000 dilution; Dako, Glostrup, Denmark) appropriately. Color was developed by incubation with diaminobenzidine (DAB; Wako Pure Chemical Industries) and hydrogen peroxide.

Macrophages, CHOP- or cleaved caspase-3-positive cells, or the GRP78-positive area was evaluated in randomly selected 30 fields with ImageJ® software (National Institutes of Health). All of the quantifications were performed in a blinded manner.

Total RNA isolation and real-time quantitative PCR.

Mechanically homogenized tissue samples were subjected to RNA quantification. Total RNA was isolated with RNAiso® (TAKARA BIO, Shiga, Japan) and was reverse-transcribed with RT Master Mix® (TAKARA BIO) according to the manufacturer's protocols. cDNA was subjected to real-time quantitative PCR using THUNDERBIRD qPCR Mix® (Toyobo, Osaka, Japan) and an iCycler system® (Bio-Rad Laboratories, Hercules, CA, USA). The sequences of the primers used in the

present study were as follows: rat CHOP, forward 5’-CCAGCAGAGGTCACAAGCAC-3’, reverse 5’-CGCACTGACCACTCTGTTTC-3’; rat beta-actin, forward 5’-CTTTCTACAATCAGCTGCGTG-3’, reverse 5’-TCATGAGGTAGTCTGTCAGG-3’. Quantitative PCR was performed under the following conditions; 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. mRNA levels of CHOP were normalized with β actin as an internal control.

Western blot analysis.

Kidney tissue was homogenized and sonicated for 1 min in RIPA buffer (5 mM EDTA, 150 mM sodium chloride, 1% NP40, 1% Triton X-100, 50 mM Tris buffer [pH 7.4]) with a proteinase inhibitor cocktail (cOmplete Mini®, Roche) and was centrifuged at 15000 × *g* for 10 min at 4°C. The supernatant was denatured by incubation in 4× sample buffer (2% sodium dodecyl sulfate [SDS], 10% glycerol, 60 mM Tris [pH 6.8], 10 mM dithiothreitol, and 0.01% bromophenol blue) at 96°C for 5 min. Proteins were

separated with 10% SDS polyacrylamide gel electrophoresis, and were transferred onto a polyvinylidene fluoride membrane (Amersham Biosciences, Piscataway, NJ, USA). Blocking of non-specific protein binding was performed with 5% skim milk in Tris-buffered saline (pH 7.4) containing 0.5% Tween 20.

For the detection of GRP78 or cleaved caspase-3, goat polyclonal anti-GRP78 antibody (1:1000 dilution; Santa Cruz Biotechnology) or rabbit monoclonal anti-cleaved caspase-3 antibody (Asp175, 1:800 dilution; Cell Signaling Technology) was used as the primary antibody, and rabbit polyclonal anti-β actin antibody (1:2000 dilution, Sigma-Aldrich) was used as an internal control. The membranes were incubated with the primary antibodies at 4°C overnight and were incubated at room temperature for 45 min with HRP-conjugated donkey anti goat IgG antibody (1:10000 dilution, Santa Cruz Biotechnology) or goat anti rabbit IgG antibody (1:10000 dilution, Bio-Rad Laboratories) as the secondary antibody. The bands were detected with an enhanced chemiluminescence system (ECL Plus® and Image Quant LAS 4000®, GE Healthcare, Buckinghamshire, UK) and were subjected to quantitative densitometry with Image Quant TL® software (GE Healthcare).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay.

Formalin-fixed, paraffin-embedded sections (8 μm thick) were used for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. TUNEL-positive cells were identified using a TACS 2 TdT-Blue Label In Situ Apoptosis Detection Kit® (Trevigen, Gaithersburg, MD, USA) and were counted in randomly selected 30 fields.

Statistical analysis.

All of the values are expressed as the means ± SD. Data for two groups were analyzed using Student's two-tailed *t*-test, and data for more than two groups were compared using one-way ANOVA with Bonferroni's test as a post-hoc test. All of the analysis were performed with GraphPad Prism® software version 5.5 for Mac (GraphPad software, San Diego, CA, USA). With Student's *t*-test, differences with P values < 0.05 were considered statistically significant, and adjusted P values underwent Bonferroni's test.

RESLUTS

BP, BW, and food consumption.

The systolic BP of the Con Nx and GOS Nx rats were significantly elevated compared with the corresponding Sham groups, but no significant differences were found between the Con Nx and GOS Nx rats or between the Con Sham and GOS Sham rats. The BW and daily food consumption of GOS Nx rats showed no significant differences compared with Con Nx rats (Figure 4 and Table 2).

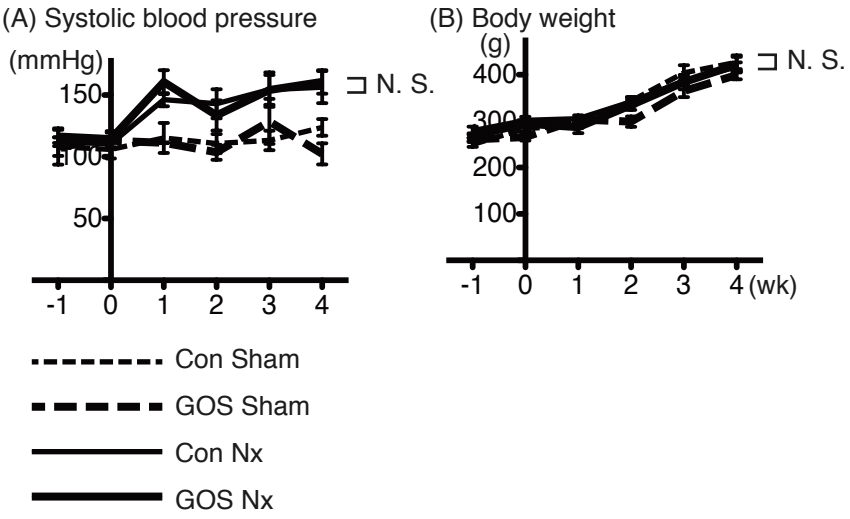

Figure 4. **Blood pressure and body weight.** The systolic blood pressures (A) were significantly higher in the Con Nx and GOS Nx rats than in the corresponding Sham groups, but no significant differences were found between the Con Nx and GOS Nx rats or between the Con Sham and GOS Sham rats. No significant changes in body weight (B) were found among the four groups. (Con Sham, n = 4; GOS Sham, n = 3; Con Nx, n = 7; GOS Nx, n = 6)

Table 2. *Daily food consumption at 4 wk*

	Con Sham	GOS Sham	Con Nx	GOS Nx
Daily food consumption, g/day	28.1 ± 3.2	28.8 ± 5.1	27.2 ± 2.9	24.4 ± 3.1

Values are means ± SD. The difference determined by ANOVA was significant in all parameters.

The four groups did not show significant difference in daily food consumption.

Gut microbiota and short-chain fatty acids production.

We analyzed total 40656 reads with a mean average of 1626 ± 491 sequences in each sample. A total of 38 bacterial families were detected, and the proportion of the bacterial family 'Clostridiaceae' in the cecal contents was significantly increased in Con Nx rats compared with Con Sham rats and significantly decreased in GOS Nx rats compared with Con Nx rats. By GOS administration, three of the families' proportions were significantly increased and five families' proportions were significantly decreased. The proportions of 'Bifidobacteriaceae', 'Clostridiales; Incertae Sedis XIV', and 'Porphyromonadaceae' were significantly increased in GOS Nx rats compared with Con Nx rats and the proportions of 'Ruminococcaceae', 'Peptostreptococcaceae', 'Streptococcaceae', 'Veillonellaceae', and 'Clostridiales; Incertae Sedis XIII' were significantly decreased in GOS Nx rats compared with Con Nx rats (Figure 5).

The cecal SCFAs concentrations were changed by GOS. The succinic acid concentration was significantly higher in GOS Nx rats compared with Con Nx rats. Although the acetic acid concentration of GOS Nx rats was higher, the difference did not reach statistical significance (Figure 6).

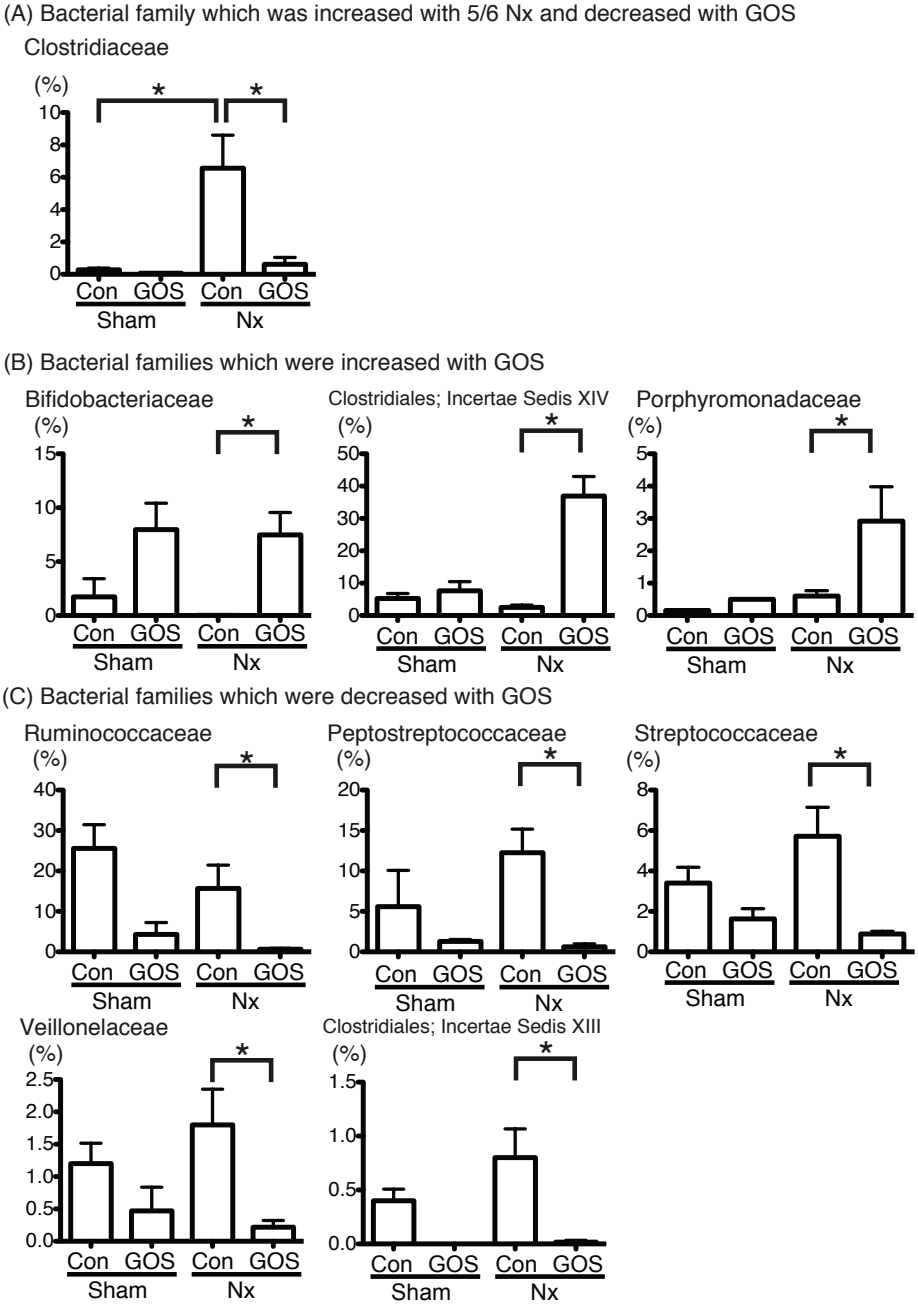

Figure 5. **Proportions (%) of the bacterial families in the cecum analyzed with pyrosequencing methods.** The proportion of 'Clostridiaceae' (A) was significantly increased in Con Nx rats compared with Con Sham rats and was significantly decreased in GOS Nx rats compared with Con Nx rats. The proportions of 'Bifidobacteriaceae', 'Clostridiales; Incertae Sedis XIV', and 'Porphyromonadaceae' (B) were significantly increased in GOS Nx rats compared with Con Nx rats. The proportions of 'Ruminococcaceae', 'Peptostreptococcaceae', 'Clostridiaceae', 'Streptococcaceae',

‘Veillonellaceae’, and ‘Clostridiales; Incertae Sedis XIII’ (C) were significantly decreased in GOS Nx rats compared with Con Nx rats. (Con Sham, n = 4; GOS Sham, n = 3; Con Nx, n = 7; GOS Nx, n = 6) *; Difference with adjusted *P* value < 0.05.

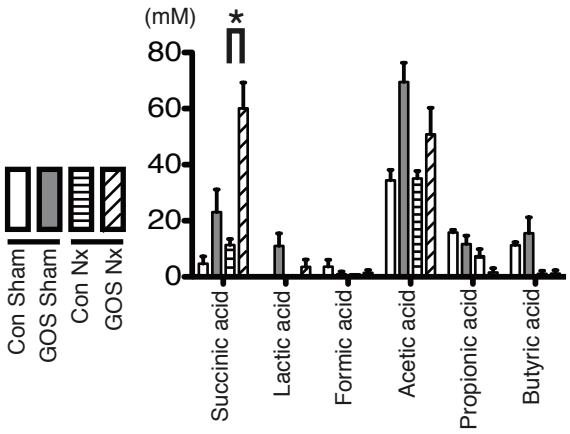

Figure 6. **Concentrations of short-chain fatty acids (SCFAs) in the cecum.** Six SCFA concentrations were examined (D), and succinic acid was significantly increased in GOS Nx rats compared with Con Nx rats. (Con Sham, n = 4; GOS Sham, n = 3; Con Nx, n = 7; GOS Nx, n = 6) *; Difference with adjusted *P* value < 0.05.

Decrease in the cecal indole and serum indoxyl sulfate with GOS.

The serum IS concentration was significantly increased in Con Nx rats compared with Con Sham rats, and was significantly decreased in GOS Nx rats (at 3 wk and 4wk) compared with Con Nx rats.

In contrast, although cecal indole concentrations were significantly lower in GOS Nx rats compared with Con Nx rats, a significant difference was not detected between the Con Sham and Con Nx rats (Figure 7).

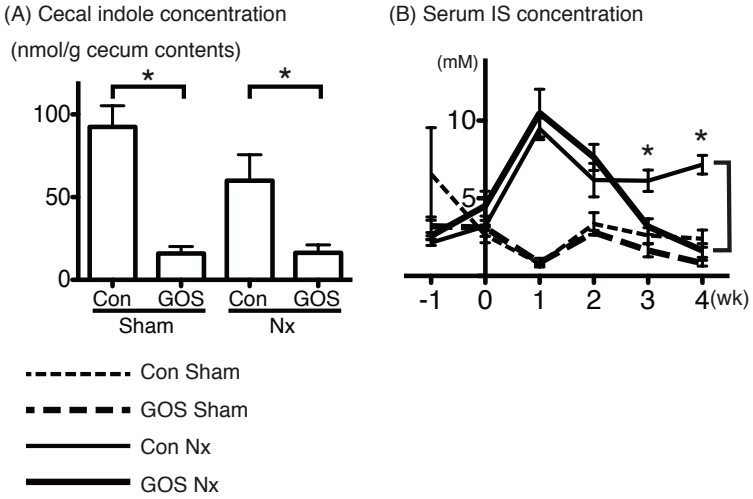

Figure 7. **Concentrations of cecal indole (A) and serum indoxyl sulfate (B).** A: GOS significantly decreased the cecal indole concentrations both in the Sham and Nx rats (amount per 1 g of the cecal contents). B: GOS significantly decreased the serum indoxyl sulfate concentrations in GOS Nx rats compared with Con Nx rats at 3 wk and 4 wk. (Con Sham, n = 4; GOS Sham, n = 3; Con Nx, n = 7; GOS Nx, n = 6) *; Difference with *P* value < 0.05.

Renal function with GOS.

The BUN and sCre concentrations were significantly increased in the Con Nx and GOS Nx rats compared with the corresponding Sham groups. Although BUN showed a lower tendency in GOS Nx rats compared with CON Nx rats, no significant differences in BUN or sCre were found between the Con Nx and GOS Nx rats (Figure 8).

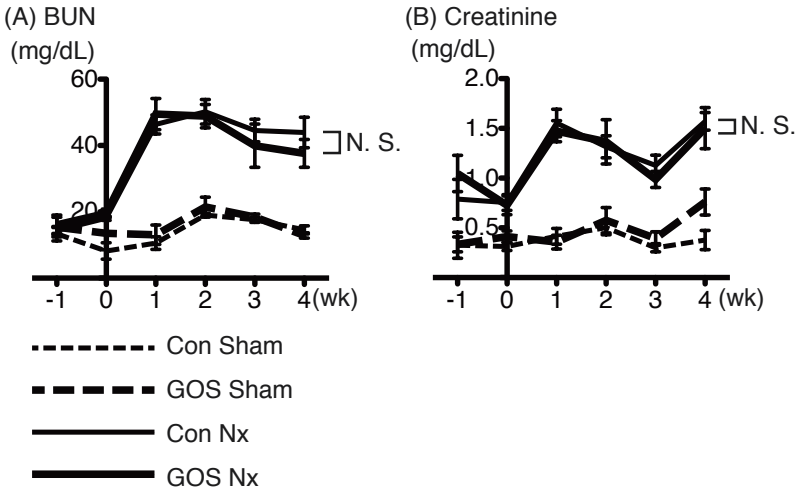

Figure 8. **Concentrations of blood urea nitrogen (BUN) and serum creatinine (sCre).** BUN (A) and sCre (B) were significantly higher in the Con Nx and GOS Nx rats than in the corresponding Sham groups, but no significant changes were found between the Con Nx and GOS Nx rats. (Con Sham, n = 4; GOS Sham, n = 3; Con Nx, n = 7; GOS Nx, n = 6)

Tubulointerstitial injury with GOS.

Semiquantitative analysis of tubulointerstitial injury showed that Con Nx rats demonstrated significantly more severe injury than Con Sham rats and GOS Nx rats demonstrated the significantly less severe injury than Con Nx rats (Figure 9).

Immunohistochemistry of macrophages was also performed to evaluate tubulointerstitial injury. The number of infiltrating macrophages was significantly increased in Con Nx rats compared with Con Sham rats and significantly decreased in GOS Nx rats compared with Con Nx rats (Figure 10).

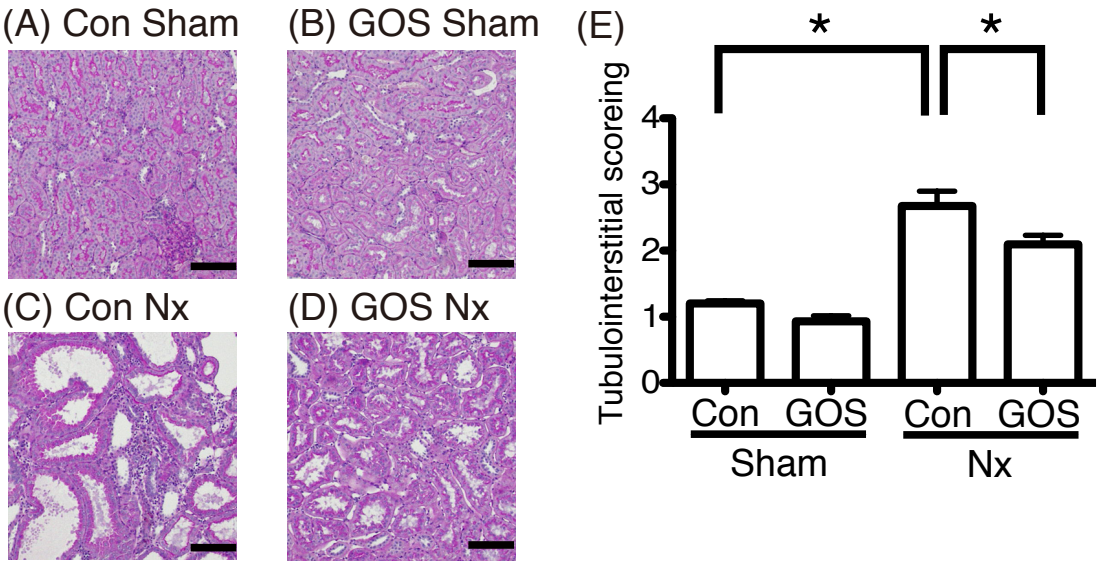

Figure 9. **Semiquantitative analysis of tubulointerstitial injury.** The injury worsened in Con Nx rats (C) compared with Con Sham rats (A) and ameliorated in GOS Nx rats (D) compared with Con Nx rats. Semiquantitative analysis of tubulointerstitial injury (E) revealed the significant deterioration in Con Nx than Con Sham and the significant amelioration in GOS Nx rats compared with CON Nx rats. (Con Sham, n = 4; GOS Sham, n = 3; Con Nx, n = 7; GOS Nx, n = 6) Original magnification: × 200; Scale bars: 50μm. *; Difference with adjusted *P* value < 0.05.

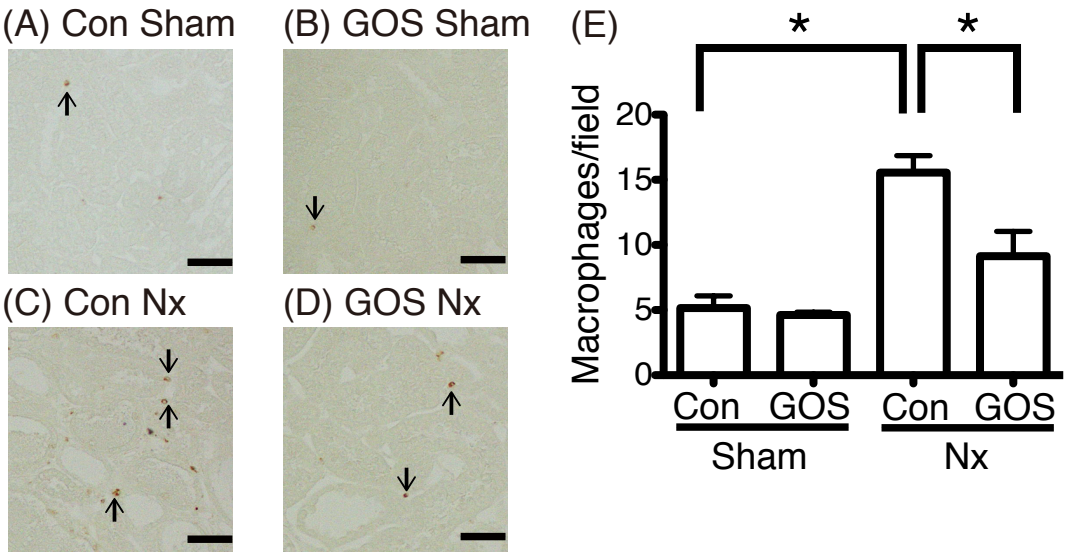

Figure 10. **Immunohistochemistry of macrophages (ED-1).** Infiltrating macrophages were increased in Con Nx rats (C) compared with Con Sham rats (A) and decreased in GOS Nx rats (D) compared with Con Nx rats. Quantitative analysis of infiltrating macrophages (E) revealed a significant increase in Con Nx rats compared with Con Sham rats and a significant decrease in GOS Nx rats compared with Con Nx rats. (Con Sham, n = 4; GOS Sham, n = 3; Con Nx, n = 7; GOS Nx, n = 6) Original magnification: × 200; Scale bars: 50μm. *; Difference with adjusted *P* value < 0.05.

Endoplasmic reticulum stress and apoptosis with GOS.

Quantitative PCR revealed that CHOP expression was significantly upregulated in Con Nx rats compared with Con Sham rats and significantly downregulated in GOS Nx rats compared with Con Nx rats.

The protein expression of CHOP was tested with immunohistochemistry. CHOP-positive cells were significantly increased in Con Nx rats compared with Con Sham rats and significantly decreased in GOS Nx rats compared with Con Nx rats (Figure 11).

The expression of GRP78 was tested with immunohistochemistry and Western blotting. Immunohistochemistry showed that the GRP78-positive area was significantly increased in Con Nx rats compared with Con Sham rats and significantly decreased in GOS Nx rats compared with Con Nx rats. Western blotting of GRP78 revealed that the expression was significantly upregulated in Con Nx rats compared with Con Sham rats and significantly downregulated in GOS Nx rats compared with Con Nx rats (Figure 12).

Because ER stress has been reported to cause apoptosis through CHOP expression [39], tubular cell apoptosis was examined with TUNEL assay and cleaved caspase-3 expression. The assay revealed that TUNEL-positive cells were also significantly increased in Con Nx rats compared with Con Sham rats and significantly decreased in GOS Nx rats compared with Con Nx rats (Figure 13). Immunohistochemistry showed that cleaved caspase-3-positive cells were significantly increased in Con Nx rats compared with Con Sham rats and significantly decreased in GOS Nx rats compared with Con Nx rats. Western blotting of cleaved capsase-3 revealed that the expression was significantly upregulated in Con Nx rats compared with Con Sham rats and significantly downregulated in GOS Nx rats compared with Con Nx rats (Figure 14).

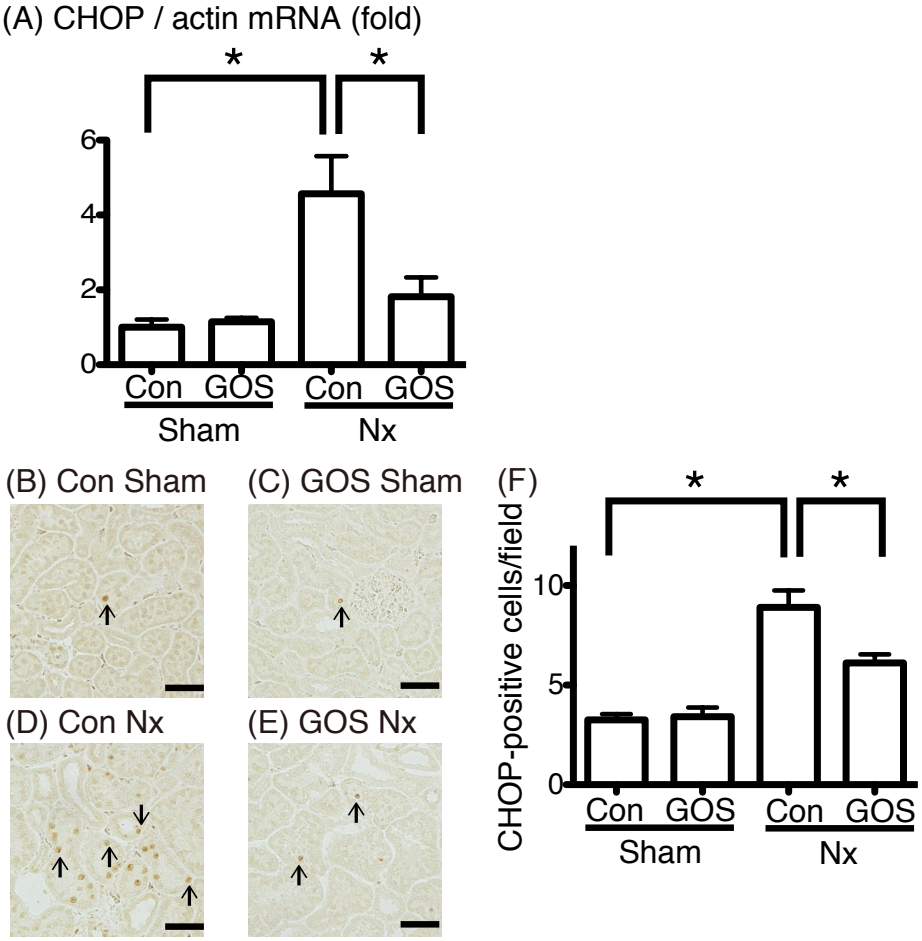

Figure 11. **Expressions of CCAAT/enhancer-binding protein homologous protein (CHOP).** CHOP mRNA expression (A) was examined with quantitative PCR. The expression was significantly upregulated in Con Nx rats compared with Con Sham rats and significantly downregulated in GOS Nx rats compared with Con Nx rats. Immunohistochemistry revealed that CHOP-positive cells were increased in Con Nx rats (D) compared with Con Sham rats (B) and decreased in GOS Nx rats (E) compared with Con Nx rats. Quantitative analysis of CHOP-positive cells (F) revealed a significant increase in Con Nx rats compared with Con Sham rats and a significant decrease in GOS Nx rats compared with Con Nx rats. (Con Sham, n = 4; GOS Sham, n = 3; Con Nx, n = 7; GOS Nx, n = 6) Original magnification: × 200; Scale bars: 50μm. *; Difference with adjusted *P* value < 0.05.

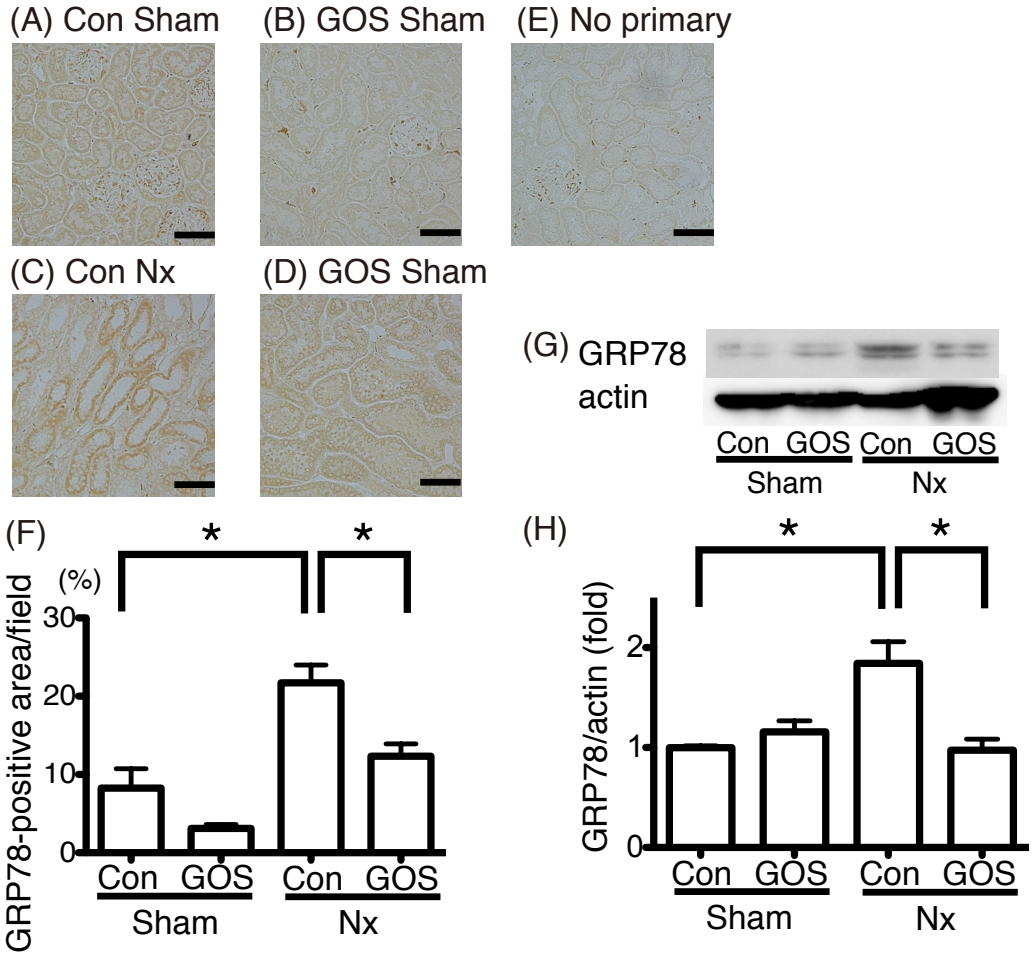

Figure 12. **Expressions of glucose-regulated protein (GRP) 78.** Immunohistochemistry revealed that the GRP78-positive area was increased in Con Nx rats (C) compared with Con Sham rats (A) and decreased in GOS Nx rats (D) compared with Con Nx rats. Quantitative analysis of the GRP78-positive area (F) revealed a significant increase in Con Nx rats compared with Con Sham rats and a significant decrease in GOS Nx rats compared with Con Nx rats. Western blotting of GRP78 (G, H) showed the same statistically significant changes. (Con Sham, n = 4; GOS Sham, n = 3; Con Nx, n = 7; GOS Nx, n = 6) Original magnification: × 200; Scale bars: 50μm (G-J). *; Difference with adjusted *P* value < 0.05.

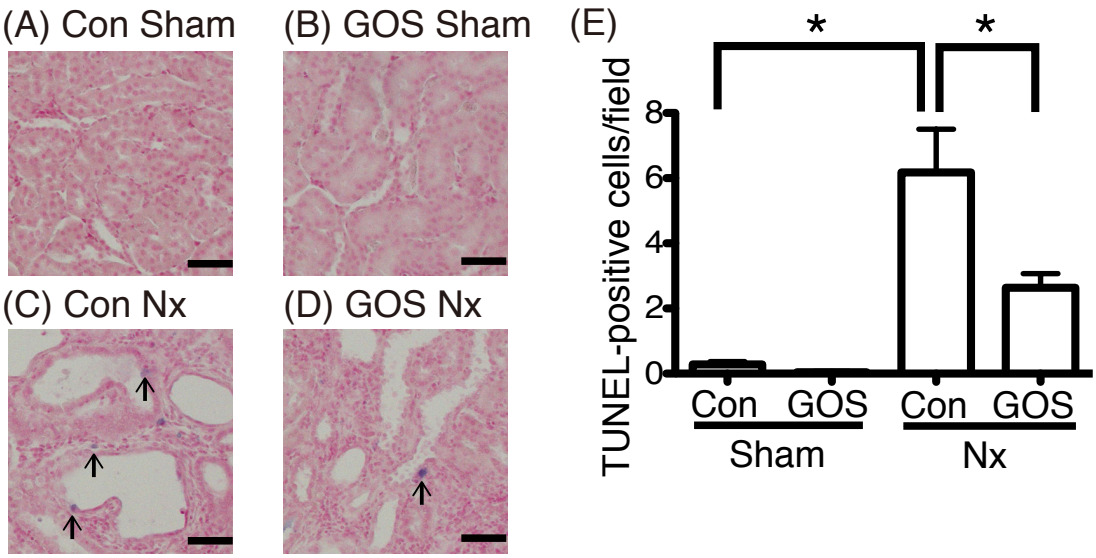

Figure 13. **Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay.** TUNEL-positive cells were increased in Con Nx rats (C) compared with Con Sham rats (A) and decreased in GOS Nx rats (D) compared with Con Nx rats. Quantitative analysis of TUNEL-positive cells (E) revealed a significant increase in Con Nx rats compared with Con Sham rats and a significant decrease in GOS Nx rats compared with Con Nx. The counter staining was performed with Nuclear Fast Red. (Con Sham, n = 4; GOS Sham, n = 3; Con Nx, n = 7; GOS Nx, n = 6) Original magnification: × 400; Scale bars: 30μm. *; Difference with adjusted *P* value < 0.05.

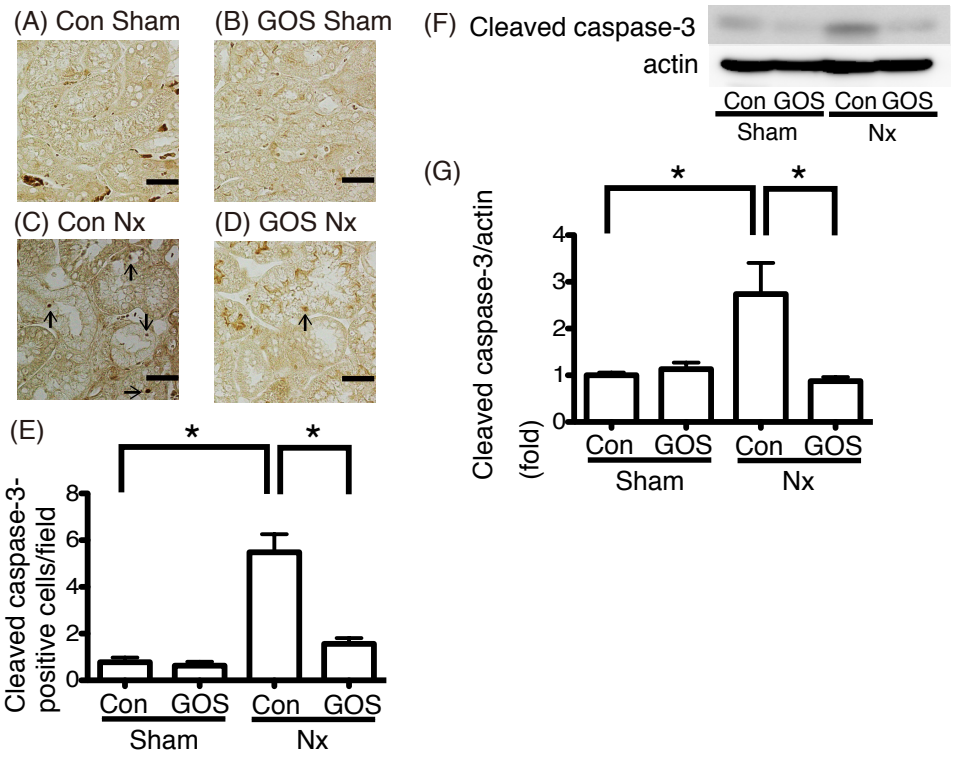

Figure 14. **Expression of cleaved caspase-3 (Asp175).** Immunohistochemistry showed that cleaved caspase-3-positive cells were increased in Con Nx rats (C) compared with Con Sham rats (A) and decreased in GOS Nx rats (D) compared with Con Nx rats. Quantitative analysis of cleaved caspase-3-positive cells (E) revealed a significant increase in Con Nx rats compared with Con Sham rats and a significant decrease in GOS Nx rats compared with Con Nx rats. Western blotting of cleaved caspase-3 (F, G) showed the same statistically significant changes. (Con Sham, n = 4; GOS Sham, n = 3; Con Nx, n = 7; GOS Nx, n = 6) Original magnification: × 400; Scale bars: 30μm. *; Difference with adjusted *P* value < 0.05.

DISCUSSION

I have demonstrated that the gut microbiota was altered in Nx rats and GOS administration in Nx rats modified the gut microbiota and attenuated the renal injury with the decrease of cecal indole and serum IS. Furthermore, decreased IS ameliorated ER stress and apoptosis in the renal injury.

The cecal proportion of 'Clostridiaceae' was significantly increased in Con Nx rats compared with Con Sham rats. Recently, the associations of the gut microbiota with diseases have been reported, such as obesity, type 2 diabetes, allergy, inflammatory bowel disease, and kidney disease [12], [14], [23], [55]–[57], and interventional studies with probiotics and/or prebiotics have been widely attempted [56], [58]–[60].

In the present study, to analyze the changes in microbiota composition, I employed novel pyrosequencing methods, which were able to analyze the gut microbiota in families or a more detailed level, and I decided to use GOS, which has been reported to be utilized only by the limited bacterial families and to change the gut environment including an increase in *Bifidobacterium*, an indole-negative bacterium [61].

I successfully found a significant increase of the proportion of ‘Clostridiaceae’ in Con Nx rats. The results that the gut microbiota were altered in kidney disease are in agreement with previous reports [23], [24]. Taking it into consideration that ‘Clostridiaceae’ was increased after 5/6 nephrectomy and decreased with GOS, ‘Clostridiaceae’ was indicated to play an important role in the gut in kidney disease. It has been reported that ‘Clostridiaceae’ include indole-positive bacteria, indole-negative bacteria, and indole-degradative bacteria [62]–[64]. The role of ‘Clostridiaceae’ in the gut in kidney disease should be further investigated.

In the Nx group, GOS significantly increased the cecal proportions of three families, ‘Bifidobacteriaceae’, ‘Clostridiales; Incertae Sedis XIV’, and ‘Porphyromonadaceae’ and significantly decreased the proportions of six families, ‘Ruminococcaceae’, ‘Peptostreptococcaceae’, ‘Streptococcaceae’, ‘Veillonellaceae’, ‘Clostridiales; Incertae Sedis XIII’, and ‘Clostridiaceae’. In addition, the concentrations of cecal acetic acid and succinic acid were increased in GOS Nx rats. The changes in SCFAs caused by GOS supported the actual changes in the composition of the microbiota, also indicating that the gut environment changed.

In GOS Nx rats, the decrease in cecal indole can be explained partly by the increase in indole-negative bacteria (e.g., most species of Bifidobacteriaceae [65]), and the decrease in the indole-positive bacteria (e.g., some species of Clostridiaceae [63] and Peptostreptococcaceae [66]), and the decrease in serum IS can be explained partly by the decrease in cecal indole.

Despite the significant increases of IS in Con Nx rats compared with Con Sham rats, no significant difference of the cecal indole concentration was detected between the Con Nx and Con Sham rats. Vaziri et al. reported intestinal hyperpermeability in renal dysfunction [67]. High permeability might have caused high indole absorption from the guts in Con Nx rats, but the exact mechanisms should be further investigated.

The renal injury was significantly ameliorated in GOS Nx rats compared with Con Nx rats. Because IS has been previously reported to induce tubular ER stress [8], and CHOP has been reported to mediate ER stress-induced apoptosis [39], I examined the effects of GOS on tubular ER stress and apoptosis. GOS Nx rats showed significantly lower expression of CHOP, GRP78, and cleaved capsase-3 and significantly fewer TUNEL-positive cells compared with Con Nx rats. Collectively, GOS ameliorated ER

stress and apoptosis, which can be considered among the mechanisms for renal injury. These results are in agreement with those from a previous study [8].

The present study has limitations. The study did not investigate the causal relationships among the gut microbiota, changes of indole and IS concentrations, and renal injury. The details of the indole production of each bacterium remain unknown. The mechanism of indole absorption from the gut is poorly understood. Moreover, the direct effects of ‘Clostridiaceae’ on indole synthesis and renal injury are unknown. Colonic sterilization with antibiotics should be performed to investigate the effect of the gut microbiota. Further investigations must be performed in the future.

In conclusion, GOS administration ameliorated renal injury by decreasing cecal indole and serum IS concentrations, ER stress, and apoptosis, and kidney disease altered the gut microbiota (Figure 15). To the best of our knowledge, the present study is the first report to examine the effects of GOS on the gut microbiota of 5/6 nephrectomized rats using pyrosequencing methods and analysis of SCFAs production. GOS could be a novel therapeutic agent to protect against renal injury.

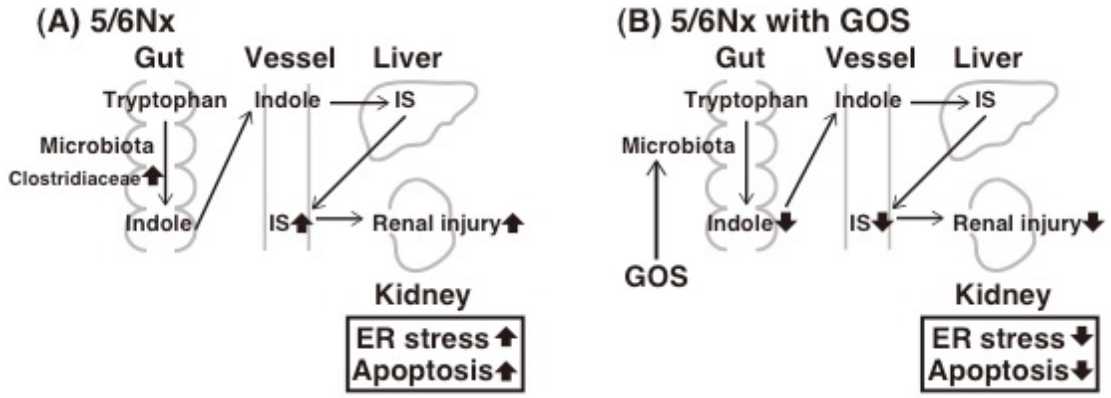

Figure 15. **The alterations in the gut microbiota and the amelioration of renal injury.** In 5/6 Nx rats without GOS (A), Clostridiaceae was increased, and serum IS was elevated, resulting in more severe renal injury with more severe ER stress and apoptosis. In 5/6 Nx rats with GOS (B), the microbiota were altered, and serum IS was decreased with lower indole concentration, resulting in less mild renal injury with less ER stress and apoptosis.

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