Gut *Lactobacillus* protects against the progression of renal damage by modulating the gut environment in rats

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**ABSTRACT**

**Background.** The role of gut microbiota in the progression of chronic kidney disease (CKD) has not been fully elucidated.

**Methods.** Renal failure was induced in 6-week-old spontaneously hypertensive rats by 5/6 nephrectomy (Nx). We analyzed the gut microbiota population to identify the relevant species potentially involved in inducing renal damage. Human colon Caco-2 cells were used to delineate the mechanism involved in the molecular changes in the gut of Nx rats.

**Results.** Nx rats showed an increase in *Bacteroides* (Bact) and a decrease in *Lactobacillus* (Lact) species compared with sham-operated rats. Lact, but not Bact, populations were significantly associated with urinary protein excretion. Treatment of Nx rats with $1 \times 10^{10}$ CFU/kg/day Lact ameliorated increased urinary protein excretion and higher serum levels of the uremic toxins, indoxyl sulfate and p-cresyl sulfate, and serum urea nitrogen levels. Lact also attenuated systemic inflammation in Nx rats, as evaluated by serum lipopolysaccharide, interleukin-6 and C-reactive protein levels. Histologically, renal sclerosis in Nx rats was restored by Lact treatment. A reduction in the expression of tight junction proteins and the Toll-like receptor 2 (TLR2), a putative Lact receptor, in the colons of Nx rats were mitigated by Lact. Treatment of Caco-2 cells with indole downregulated tight junction protein expression, which was abolished by exposure to Lact. The effects of Lact were reversed by treatment with OxPAPC, a TLR inhibitor. Similarly, the increase in the permeability of the Caco-2 cell monolayer was reversed by the administration of Lact. Lact upregulated TLR2 expression in Caco-2 cells. Lact also attenuated the increase in serum indoxyl sulfate and urea levels and urinary protein excretion in Nx rats even in the pseudogerm-free environment.

**Conclusions.** Lact supplementation mitigated the systemic inflammation and proteinuria associated with renal failure, suggesting that in the gut microbiota, Lact plays a protective role against the progression of CKD.

**Keywords:** chronic kidney disease, gut microbiota, protein bound uremic retention solutes, tight junction, Toll-like receptor

**INTRODUCTION**

Chronic kidney disease (CKD) is a worldwide health problem that can lead to cardiovascular disease and end-stage kidney disease that requires renal replacement therapy. Although its pathogenesis and pathophysiology have not been fully elucidated, the progression of CKD is accompanied by the accumulation of uremic substances that have deleterious effects. For example, the protein-bound uremic solutes indoxyl sulfate (IS) and p-cresyl sulfate (PCS), which are normally excreted into the urine by the kidneys, accumulate in patients with CKD. These substances have been shown to cause damage to renal tubular cells or endothelial cells, as well as to other cells, by increasing cellular oxidative stress [1]. In an *in vivo* study where these substances were administered exogenously, they induced damage to renal or cardiovascular tissues by enhancing oxidative stress, inflammatory responses or fibrotic changes [2–4]. A recent study also demonstrated that another uremic solute, indole acetic acid (IAA), caused endothelial cell damage. In addition, IAA serum levels correlated with cardiovascular events in CKD patients, suggesting that it may be a cause of cardiovascular complications in CKD [5]. Since the precursors of these substances, indoles or cresols, are produced by bacteria in the intestine from the amino acid tryptophan or tyrosine, respectively, and are subsequently absorbed from the colon, the changes in the influx of these uremic solutes from the intestine may affect renal function [6]. This kind of interrelationship between the gut and kidney is called ‘the intestinal-renal syndrome’ [7], and the modulation of this axis, including regulation of the colon environment or colon barrier, may be a plausible strategy to slow the progression of CKD. A decreased expression of tight junction proteins in the colon has been observed...
in CKD, and stabilization of the intestinal barrier by changing the colon environment, using a charcoal absorbent for indole, was found to improve the associated endotoxemia, oxidative stress and inflammatory response [8].

Another factor that affects the intestinal environment is changes in the intestinal microbiota, which have been shown to correlate with kidney diseases [9, 10]. Manipulation of microbiota, such as with the use of probiotics, reduced blood urea nitrogen (BUN) levels and enhanced longevity in CKD rats [11, 12]. Changes in the distribution of intestinal microbiota and reductions in uremic toxins caused by treatment with probiotics have been observed in hemodialysis patients [13], although the clinical relevance of these alterations has not been determined [14].

This study assessed the role of the gut environment in CKD by using 5/6 nephrectomized (Nx) spontaneous hypertensive rats (SHRs), in which we previously observed severe renal damage and inflammation [15]. We found that a decrease in the population of Lactobacillus (Lact) in Nx rats, along with disruption of the intestinal barrier, contributed to the progression of CKD. We also showed that Lact supplementation ameliorated renal damage and intestinal changes through activation of a Toll-like receptor (TLR) that recognizes Lact. These findings provide compelling evidence for the clinical use of Lact in CKD patients to reverse the inflammatory response and protect against the progression of renal damage.

**Materials and Methods**

**Animal experiments**

Renal failure was induced in 6-week-old male SHRs (Charles River, Wilmington, MA, USA) via the Nx model, as described previously [15]. In the first experiment, rats were randomly assigned to two experimental groups, sham-operated SHR (Sham, n = 8) and Nx rats (n = 8). In the second experiment, rats were randomly assigned to three experimental groups: sham-operated SHR (Sham, n = 8), Nx (n = 8) and Nx rats treated with 1 × 1010 CFU/kg/day Lactobacillus acidophilus NT (Lact) (Nx+Lact, n = 8). Lact was kindly provided by Nitto Pharmaceutical Industries (Kyoto, Japan). Twelve weeks after nephrectomy, body weight and systolic blood pressure (SBP) were measured by the tail-cuff method (KN-210, Natsume, Tokyo, Japan). Twenty-four-hour urinary protein excretion and serum concentrations of BUN, serum creatinine and IS were measured as described previously [15, 16]. Serum PCS and IAA levels were measured by high-performance liquid chromatography [17]. Serum lipopolysaccharide (LPS) levels were measured by turbidimetric-kinetic assay using a toxinometer (Sham, n = 8). In the second experiment, Nx (n = 8) and Nx rats treated with 1 × 1010 CFU/kg/day Lactobacillus acidophilus NT (Lact) (Nx+Lact, n = 8). Lact was kindly provided by Nitto Pharmaceutical Industries (Kyoto, Japan).

**Table 1. The primers used for bacterial gene analysis**

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>Lactobacilli</td>
<td>GAGCTTAGGGGAATCTTCCA</td>
<td>CACCCGTACACATGGAG</td>
</tr>
<tr>
<td>Bacteroides</td>
<td>GAAGGCTCCCCACACTTG</td>
<td>CAAATCGGTCTTCTGAG</td>
</tr>
<tr>
<td>Prevotella</td>
<td>CACCAAGGCAGCACTCA</td>
<td>GGAATACCTGGATGACCT</td>
</tr>
<tr>
<td>Universal primers</td>
<td>TCTACCGGGAGGCAGCAT</td>
<td>GACATTACGGGTATCTAATCCTGTT</td>
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**Analysis of gut bacteria**

The gut microbiota population was examined as described previously [20]. Briefly, DNA was extracted from the bead-treated suspension of fecal samples. Amplification of fecal 16S rDNA was performed using 25 μM of the labeled primers 516f (5′ TGCCAGCAGCAGCGGTGA 3′) and 1510r (5′ GGTTA CCTTGTACGGACTT 3′). For terminal restriction fragment length polymorphism (T-RFLP) analysis of the microbiota, the resulting amplicons were digested with 2U of Bsl I (Cell Signaling, Danvers, MA, USA) for 1 h and the fragments were separated using an automated DNA sequencer (ABI PRISM 3130xl DNA Sequencer, Applied Biosystems, Carlsbad, CA, USA). The lengths and peak areas of the resulting electropherogram were determined using GeneMapper software (Applied Biosystems). The relative abundances of operational taxonomic units (OTUs) were calculated by dividing the peak area of each fragment by the sum of all peak areas. The T-RFLP patterns of each sample were compared using the dissimilarity index [21]. The amounts of bacteria in the fecal samples of each species were quantified by real-time quantitative PCR using the 7500 Fast Real-time PCR System (Applied Biosystems), as previously reported [22]. The primers for each species are shown in Table 1 [23]. Results with the specific Lactobacillus, Bacteroides and Prevotella primers were normalized relative to those for the universal primers.

**Cell culture**

Caco-2 sigmoid colon cancer cells (HTB-37; ATCC, LOT 60143947) were grown in non-fibrin-coated flasks in Dulbecco’s modified eagle’s medium (DMEM, Invitrogen, Auckland, NZ), containing glucose, L-glutamine, NaHCO3 and pyridoxine levels of interleukin-6 (IL-6) and C-reactive protein (CRP) were measured using commercial ELISA kits (Abcam Japan, Tokyo, Japan). At sacrifice, tissue samples of the kidney and ascending colon were obtained, snap frozen and used for subsequent experiments. All experiments were performed in accordance with the animal experiment guidelines of the Keio University School of Medicine.

**Morphological examination**

Kidneys were fixed in 10% formaldehyde and embedded in paraffin blocks. Glomerular sclerosis was assessed by periodic acid-Schiff (PAS) staining and renal fibrosis was assessed by Masson-trichrome staining. Glomerulosclerosis was evaluated by counting sclerotic glomeruli and calculating the glomerulosclerotic index [18, 19]. The proportion of the fibrotic area was measured using Image-Pro Plus 3.0 (Media Cybernetics, Silver Spring, MD, USA).
and claudin-1 (Invitrogen). Following incubation with a second-gels and transferred to nitrocellulose membranes, which were
Aliquots of supernatants were electrophoresed on SDS-PAGE
incubated in a lysis buffer and centrifuged at 15 000
pressed as the apparent permeability coef
for
X3). All permeability experiments were conducted in triplicate
experiments and found that 10
on this evidence, we tested the effects of indole in the preliminary
ranges from 250 to 1100
concentration used in the experiment was determined to be be-
be highly concentrated in the intestinal juice at least by 10- to
100-fold because of the adsorption of water [26]. Furthermore,
intestinal cells were covered with the mucous layer [26]. Based
this evidence, we tested the effects of indole in the preliminary
experiments and found that 10 μM of indole was toxic to Caco-2
cells. We further examined the effects of indole on the expression of
tight junction proteins at the concentrations ranging from 0.1
to 1 μM and observed that indole downregulated ZO-1 and
occcludin between the concentration of 0.1 and 1 nm, although claudin-1 was not altered even at 1 μM. Hence the appropriate
concentration used in the experiment was determined to be be-
tween 0.1 and 1 nm. To investigate the role of the Toll-like receptor
2 (TLR2), Caco-2 cells were pretreated with a nonselective
TLR blocker, oxidized 1-palmitoyl-2-arachidonyl-
phosphorylcholine (OxPAPC, InvivoGen, San Diego, CA, USA),
30 min prior to the administration of Lact or indole. The cells
were harvested 24 h after the treatment and the expressions of
tight junction proteins, including occludin, ZO-1 and claudin-1,
were examined by immunoblotting.

Permeability assay

Permeability was estimated by measuring the paracellular transport of FITC-labeled 4-kDa dextran (FD4) as described previously [27]. Briefly, sterilized FD4 was added into the apical well at 25 mg/mL with or without indole, Lact or a com-
bination of both for 6 h. The translocation of FD4 across the
cells was determined by sampling the basolateral solution.
FD4 fluorescence at 485/535 nm was measured using a Perkin
Elmer (Waltham, MS, USA) multimode plate reader (ARVO X3). All permeability experiments were conducted in triplicate
for five sets of cell preparations. FD4 permeability was ex-
pressed as the apparent permeability coefficient (Papp), calculated as described previously [27].

Immunoblotting

Ascending colon tissues and Caco-2 cells were lysed and so-
nicated in a lysis buffer and centrifuged at 15 000 g for 15 min.
Aliquots of supernatants were electrophoresed on SDS-PAGE
gels and transferred to nitrocellulose membranes, which were
then incubated with primary antibodies against occludin, ZO-1
and claudin-1 (Invitrogen). Following incubation with a second-
ary antibody (HRP-linked anti-rabbit IgG; GE Healthcare,
Backhamshire, UK), immunoreactive bands were detected
using an ECL detection kit (Amersham Biosciences, Uppsala,
Sweden).

Real-time reverse transcriptase polymerase chain reaction
(RT-PCR)

Total RNA was extracted from the ascending colon tissue
and Caco-2 cells using TRIzol reagent (Invitrogen). Equal
amounts (1 μg) of total RNA from each sample were converted
to cDNA using the Prime Script RT reagent kit with gDNA Era-
er (Takara, Ohtsu, Japan). Real-time RT-PCR was performed
using an ABI Step One Plus sequence detector (Applied Biosys-
tems). Primers used are shown in Table 2. Levels of mRNA were
normalized to those of β-actin.

Pseudogerm-free model experiment

A pseudogerm-free condition was created by administering
a broad-spectrum antibiotic cocktail consisting of ampicillin,
neomycin sulfate, vancomycin and metronidazole as described
previously [28]. Rats were randomly assigned to the following
groups: (1) sham-operated SHR (Sham, n = 6), (2) Nx
SHR (N x, n = 6), (3) Sham plus antibiotics (Sham+Anti,
n = 6), (4) Nx plus antibiotics (Nx+Anti, n = 6) and
(5) Nx+Anti treated with Lact (Nx+Anti+Lact, n = 6). Eight weeks after neph-
rectomy, 24-h urinary protein excretion levels and SBP were
measured and the rats were sacrificed. The concentration of
BUN and serum concentrations of creatinine and IS were mea-
sured as described [15].

Statistical analysis

Data are expressed as mean ± SEM and analyzed by one-way
analysis of variance, followed by the Bonferroni’s post hoc test.
A P-value <0.05 was considered statistically significant.

RESULTS

Decreased Lact population in nephrectomized rats

The microbiota between Nx SHRs, a model of severe ne-
phrosclerosis [15] and Sham-operated SHRs were compared.
Body weight was significantly lower (286.0 ± 38.2 g versus
366.8 ± 5.4 g, P < 0.05) and SBP was significantly higher
(Figure 1A) in Nx compared with Sham rats. In addition,
serum creatinine, IS and BUN levels, as well as urinary protein
excretion, were higher in Nx than in Sham rats (Figure 1B–E),
confirming that these rats were an appropriate model of severe
renal dysfunction. T-RFLP evaluation of intestinal microbiota
in feces from the terminal ileum showed that Bacteroides
(Bact) and Prevotella species were more abundant, whereas
Lact species were less abundant in the Nx rats compared with

Table 2. The primers used for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>TLR2 (rat)</td>
<td>GTACCAGTAGTGATGCTGAAGT</td>
<td>GGCCTGGTCATTGTCTTC</td>
</tr>
<tr>
<td>GAPDH (rat)</td>
<td>GTTACAGGGCTGCCTCTC</td>
<td>GCTGGTTCCCCGTATGACCC</td>
</tr>
<tr>
<td>TLR2 (human)</td>
<td>GGCAGCAGTTCAGATGTC</td>
<td>AGGCAATCCCGGTACTCTGAA</td>
</tr>
<tr>
<td>β-actin (human)</td>
<td>GTCAAGTTCACATCGGC</td>
<td>CATGATGACACAGATCCTC</td>
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Effects of Lactobacillus on CKD

403
the Sham rats (Figure 1F). Confirmatory real-time PCR using species-specific primers showed that Bact species were higher and Lact lower in number in Nx compared with Sham rats (Figure 1G), whereas Prevotella species were similar. The Lact population was significantly associated with urinary protein excretion levels (Figure 1H; $R^2 = 0.425$, $P < 0.01$), whereas the Bact population was not.

**Effects of L. acidophilus on rats with CKD**

Since decreased Lact in the intestine was associated with urinary protein excretion, we investigated the effects of Lact supplementation in our rat model of CKD by treating Nx rats with $1 \times 10^{10}$ CFU/kg/day Lact for 12 weeks (Nx+Lact). RT-PCR using DNA extracted from fecal samples revealed
that the population of \textit{Lact} was higher in Nx+\textit{Lact} than in Nx rats (Figure 2A). Daily chow intake was lower in Nx (24.3 ± 4.3 g/day) than in Sham (36.0 ± 2.7 g/day) rats, and this decrease was not improved by \textit{Lact} treatment (28.2 ± 3.1 g/day). Similarly, body weight was lower in Nx (296.4 ± 21.2 g) than in Sham (361.4 ± 9.4 g) rats, although this reduction improved in Nx+\textit{Lact} rats (322.0 ± 24.9 g). Systolic blood pressure was similar in Nx and Nx+\textit{Lact} rats (Figure 2B). Although the

![Figure 2](https://academic.oup.com/ndt/article-abstract/31/3/401/2460089)

**FIGURE 2**: Effects of \textit{Lactobacillus} on nephrectomized rats. Spontaneously hypertensive rats underwent sham nephrectomy (Sham) or 5/6 nephrectomy (Nx) or Nx followed by \textit{Lactobacillus} treatment (Nx+\textit{Lact}), as described. (A) Numbers of \textit{Lactobacillus} species in the colons, as determined by RT-PCR. Measurements were made of (B) SBP, (C) BUN, (D) serum creatinine level, (E) daily urinary protein excretion, (F) serum indoxyl sulfate (IS), (G) serum p-cresyl sulfate (PCS), (H) serum indole acetic acid (IAA), (I) serum lipopolysaccharide (LPS), (J) serum interleukin-6 (IL-6) and (K) serum C-reactive protein (CRP) levels. The evaluation of (L) glomerular sclerosis by PAS staining and (M) interstitial fibrosis by Masson-trichrome staining are shown. Lower panels represent the quantification of glomerular sclerosis and interstitial fibrosis. Scale bar, 100 μm. *P < 0.05, **P < 0.01 versus Sham; #P < 0.05 versus Nx (n = 8 per group).
increase in BUN levels caused by Nx improved following Lact treatment (Figure 2C), serum creatinine levels were not altered (Figure 2D), suggesting that supplementation with Lact was not sufficient for the improvement of renal dysfunction in Nx SHRs. The Lact-induced reduction of BUN may have been caused by decreased urea production in the intestine or decreased urea entry from the intestine. However, the increased urinary protein excretion in Nx rats was mitigated in Nx+Lact rats (Figure 2E), indicating that Lact had a renoprotective effect. Similarly, serum concentrations of IS (Figure 2F), PCS (Figure 2G), LPS (Figure 2I), IL-6 (Figure 2J) and CRP (Figure 2K), which were higher in Nx than in Sham rats, were mitigated in Nx+Lact rats. However, the increase in serum IAA levels (Figure 2H) in Nx rats was not altered in Nx+Lact rats. Nx rats showed a significantly higher degree of glomerulosclerosis, as evaluated by the glomerulosclerotic index (Figure 2L) [18, 19] and increased tissue fibrotic area on Masson-trichrome staining (Figure 2M) than Sham rats. Lact treatment improved the glomerulosclerotic but not the fibrotic index.

Molecular changes in the intestinal barrier induced by Lact

Disruption of the intestinal barrier has been shown to contribute to increased levels of serum cytokines during renal failure, as well as to the progression of renal impairment [8]. Colon epithelial tight junctions are involved in maintaining the intestinal barrier, wherein the proteins occludin, ZO-1 and claudin-1 are found to be key components of intestinal tight junctions. The expression of all three proteins was lower in the Nx model compared with Sham-operated rats. Although Lact supplementation restored the expression of occludin and ZO-1, it had no effect on claudin-1 expression (Figure 3A). It is possible that the concentrations of uremic toxins or their precursors affect the population of microbiota or the expression of tight junction proteins [29]. Although the fecal concentrations of indole, p-cresol and phenol increased in Nx, Lact treatment had no effect on any of these compounds (Figure 3B), indicating that the treatment with Lact had little effect on the production or degradation of these substances in the intestine. The expression of tight junction proteins has been reported to be regulated by the activation of pattern recognition receptors, TLRs [30, 31]. It has also been reported that Lact is recognized by TLR2 and activates TLR2 in various cells [32, 33]. We found that the expression of TLR2 was lower in the Nx than in the Sham group. This decrease was reversed in the Nx+Lact group (Figure 3C). These results suggested that the restoration of intestinal tight junction protein expression and the concomitant increase in TLR2 expression by Lact leads to the suppression of increased intestinal permeability and to the reduction in systemic IS levels as well as in LPS levels, thus mitigating renal damage and systemic inflammation in Nx rats [34, 35].

Indole- and Lact-modified tight junction molecules in Caco-2 cells

The mechanisms by which Nx and Lact induce molecular changes in the intestinal barrier were investigated using the Caco-2 colonic cell line. Indole, which is produced in the intestine by some microorganisms, dose-dependently downregulated the expression of occludin and ZO-1, while having no effect on claudin-1 expression (Figure 4A). These indole-induced reductions in occludin and ZO-1 were restored by the coadministration of Lact to Caco-2 cells. These effects of Lact were inhibited by the nonselective TLR blocker OxPAPC (Figure 4B). Although indole did not alter the expression of TLR2, it was increased by Lact treatment (Figure 4C). These data implied that, in Nx rats, the expression of tight junction proteins was disrupted by the reduction in the Lact population through the reduced activation of TLR2. Finally, the permeability of the cell layer was assayed using FD-4 dye as described in the Materials and Methods section. Indole, at a concentration of 1.0 nM, increased the permeability of the Caco-2 cell layer, and this increase was inhibited by treatment with Lact (Figure 4D). This result implied that the increased intestinal permeability in Nx rats was improved by Lact treatment. It is deduced that Lact contributes to maintenance of the intestinal environment.

Lact-mitigated proteinuria in rats with CKD under pseudogerm-free conditions

The probiotic effects of Lact on proteinuria in Nx rats were confirmed using pseudogerm-free conditions, where the effects of microbiota other than Lact are minimized. Treatment of Sham and Nx rats with an antibiotic cocktail consisting of ampicillin, neomycin sulfate, vancomycin and metronidazole yielded a pseudogerm-free condition in these animals, whereas some of these rats were supplemented with Lact, as confirmed by Gram staining of the feces of each experimental group (Figure 5A). Treatment of Sham and Nx rats with antibiotics had no effect on the levels of expression of serum creatinine, BUN, or IS, or on urinary protein excretion (Figure 5B–E). Treatment of Nx rats with Lact had no effect on serum creatinine levels (Figure 5B) but attenuated the increases in BUN and IS levels and urinary protein excretion even under pseudogerm-free conditions (Figure 5C–E). These findings confirmed the probiotic effects of Lact.

DISCUSSION

It has been strongly suggested that the gut microbiome is an important factor in the maintenance of a healthy condition [36, 37]. By using the recently developed T-RFLP technique followed by real-time PCR, this study analyzed the species present in the gut microbiota and found that the Lact population was reduced under CKD conditions. The number of Lact was found to be associated with urinary protein excretion levels, and supplementation with Lact mitigated proteinuria in rats with CKD. Furthermore, Lact helped to improve proteinuria by restoring the expression of intestinal barrier proteins and reducing systemic inflammation, even under pseudogerm-free conditions. Lact had a direct effect on intestinal cells by reversing the indole-induced downregulation of tight junction proteins through activation of the TLR2 pathway. Thus this study identified a specific species of microbiota that may be protective against renal damage and revealed the detailed mechanisms underlying these effects.
Colon microbiota are altered under uremic conditions. For example, the populations of certain microbes, such as Clostridiaceae, Enterobacteriacea and Verrucomicrobiacea, which produce indole or p-cresol, are higher, whereas the populations of microbial families with butyrate-producing enzymes, including Lact and Prevotellae, are lower in subjects with uremia compared with healthy subjects [38]. It was reported that in CKD patients, probiotics with the combination of L. acidophilus, Bifidobacterium longum and Streptococcus thermophilus produced a significant reduction of BUN and enhanced well-being [39]. Although several other studies also revealed that probiotics or prebiotics in CKD patients...
reduce BUN levels or serum IS levels, some papers have reported no beneficial effects of probiotics, prompting a controversy regarding probiotic therapy [6, 14, 40]. In this sense, it has been proposed that future studies are necessary to develop more efficient therapeutic strategies or to elucidate more precise mechanisms for dysbiosis in CKD [6, 14]. Our data provide relevant information for these future studies.

**FIGURE 4:** Expression of intestinal barrier proteins in Caco-2 cells. (A) Caco-2 cells were incubated with various concentrations of indole and the levels of expression of the tight junction proteins occludin (left panel), ZO-1 (middle panel) and claudin-1 (right panel) were assessed by immunoblotting. Each upper subpanel shows an immunoblot and each lower subpanel shows the densitometric analysis of that blot. *P < 0.05 versus unstimulated cells (n = 5). (B) Effects of Lactobacillus (Lact) and the nonselective TLR blocker OxPAPC (Ox) on the expression of occludin (upper panel), ZO-1 (middle panel) and β-actin (lower panel) in indole-treated (1.0 nmol/L) Caco-2 cells. Each upper subpanel shows an immunoblot and each lower subpanel shows the densitometric analysis of that blot. †P < 0.05 versus unstimulated cells, *P < 0.05 versus indole-stimulated cells treated with Lact (n = 5). (C) Effects of Lact and indole on the expression of TRL2 mRNA in indole-treated Caco-2 cells. *P < 0.05 versus control (n = 5). (D) Effects of Lact and indole on the permeability of the Caco-2 cell monolayer. *P < 0.05 versus unstimulated cells, †P < 0.05 versus indole-stimulated cells (n = 5).
In our experiment using the pseudogerm-free condition, although the number of Lact should be decreased by antibiotics, the antibiotic cocktail used in this study had no effects on proteinuria. These data imply that antibiotic therapy also reduces other microbiota with unfavorable effects on the kidney, such as Clostridium species or Bact species, which are the microbiota producing indole. Our analysis showed an increase in the population of Bact, and it was reported that ~20% of Bact species produce indole [41]. Consistently, fecal concentrations of indole in the intestine were higher in Nx compared with Sham rats. Because indole directly downregulates the expression of tight junction proteins that form a barrier of intestinal cells, and also because indole is a direct precursor of the uremic toxin IS, the increase in the population of Bact in the Nx model could lead to increased intestinal permeability and increased serum levels of IS as well as LPS. Our findings show that the gut contains various types of microbiota that may have an effect on renal damage, either positively or negatively. Both a decrease in renoprotective Lact and an increase in renal pathogenic Bact contributed to the progression or maintenance of renal damage in Nx rats. Collectively, it is suggested that the balance between these bacteria may have some role in CKD.

In the present study, Lact treatment salvaged the expression of tight junction proteins in the intestine of Nx rats. Our study provided the molecular mechanism and evidence for the direct action of Lact in intestinal cells. In the previous study, the downregulation of tight junction proteins by infection of Caco-2 cells with Escherichia coli or Salmonella was reversed by the addition of Lactobacillus amylophilus via modulation of the extracellular signal-regulated kinase (ERK) and c-Jun

**FIGURE 5:** The effects of Lactobacillus on nephrectomized rats in a pseudogerm-free environment. (A) Gram staining of stool samples of SHRs (Sham), 5/6 nephrectomized SHRs (Nx), Sham rats treated with antibiotics (Sham+Anti), Nx rats treated with antibiotics (Nx+Anti) and Nx rats treated with antibiotics and Lactobacillus (Nx+Anti+Lact). Measurements of (B) serum creatinine, (C) BUN (D) and serum IS levels and (E) daily urinary protein excretion in the five rat groups. *P < 0.05, **P < 0.01 versus Sham+Anti; #P < 0.05 versus Nx+Anti (n = 6 per group).
NH2-terminal kinase (JNK) pathways [42]. In addition, activation of the TLR2 pathway has been reported to lead to ERK or JNK activation [43, 44]. TLRs are mucosal pattern recognition receptors that recognize microbes, leading to the preservation of tight junction integrity [30, 31]. Taken together, our findings suggest that Lactobacillus regulates the expression of tight junction proteins through the direct activation of TLR2. Consistent with this hypothesis, our experiments using Caco-2 cells showed that the restoration of indole-induced downregulation of ZO-1 and occludin by Lactobacillus was inhibited by pretreatment with the TLR antagonist OxPAPC. In CKD, reduction in the Lactobacillus population and the subsequent downregulation of TLR2 also contributed to downregulation of tight junction proteins (Figure 6). Supplementation with Lactobacillus successfully reversed these changes. We also found that tight junction proteins were downregulated by the direct action of indole. Indole has been shown to activate arylhydroxycarbon (AhR) and the pregnane X receptors (PXR) expressed in cell nuclei [45, 46]. Our results suggest a possible cross-talk between these nuclear receptors and TLR signaling in the intestinal cells (Figure 6) [10, 47].

The intestine plays an important role in regulating systemic inflammation by serving as a barrier against the entry of various cytokines, endotoxins, uremic toxins and microorganisms [9, 32]. Tight junction proteins form an effective barrier against the influx of these noxious substances from the gastrointestinal lumen to the internal milieu. In a uremic state, intestinal barrier function is impaired, resulting in increased circulating endotoxin levels and enhanced systemic inflammation [48]. This systemic inflammation contributes to the progression of renal impairment [10]. In the previous report, Nx rats exhibited low-grade inflammation, leading to impairment of kidney structure and function [49]. In this study, Lactobacillus restored intestinal tight junction protein expression, which possibly reduced intestinal permeability as shown in the experiment described in Figure 4D. These effects stabilized intestinal barrier function, blocked the translocation of LPS and attenuated systemic inflammation, as indicated by the reduction in both serum IL-6 and CRP levels. These effects ultimately improved renal sclerotic changes and proteinuria in Nx rats.

Moreover, the increase in uremic solutes IS and PCS has been reported to induce renal inflammatory responses [4, 50, 51]. Our study implied that in Nx rats, besides an impaired clearance, the increase in intestinal indole and p-cresol production, presumably by an increase in the population of indole-

**Figure 6**: Scheme depicting the effects of CKD on microbiota populations and systemic inflammation. Intestinal indole downregulates the expression of tight junction proteins, presumably through binding to the arylhydroxycarbon receptor (AhR) or PXR. In contrast, Lactobacillus maintains protein expression through the activation of TLR2. In CKD, indole concentrations increase and the population of Lactobacillus decreases, which both result in the downregulation of expression of tight junction proteins. These mechanisms enhance intestinal permeability, thus allowing various uremic toxins such as indole and p-cresol to enter the systemic circulation, consequently elevating the systemic indoxylsulfate (IS) and p-cresylsulfate (PCS) levels. Increased permeability also allows bacterial lipopolysaccharide (LPS) to translocate to the systemic circulation, leading to systemic inflammation along with increases in serum cytokine levels such as IL-6 and CRP. These changes ultimately cause renal cell damage. Lactobacillus supplementation reverses these conditions and mitigates renal damage.
and p-cresol-producing microbiota, and the disruption of the intestinal barrier due to the increased levels of the indole itself or the reduction in the Lact population could be responsible for the observed increase in serum IS and PCS levels. Treatment of Nx rats with Lact stabilized barrier function and reduced the serum levels of IS and PCS, resulting in reductions in renal inflammation and in renal sclerosis. Because Lact treatment did not alter fecal concentrations of indole, as well as p-cresol, or renal excretory function, Lact-induced reductions in systemic IS and PCS were considered to be primarily due to blocking of indole and p-cresol entry from the intestine. These mechanisms may explain the renoprotective effects of Lact (Figure 6). This barrier-stabilizing effect may also explain why Lact reduced BUN levels without affecting renal function, as serum urea levels are also determined by urea production by intestinal microflora and by urea entry into the intestine [52]. Several other metabolic pathways, including uptake via the intestinal wall, and metabolic conjugation reactions, such as sulfotransferation of indole to IS or that of p-cresol to PCS by the intestinal wall, may be involved in uremic toxin generation [6]. The effects of Nx and Lact treatment on these pathways remain to be investigated.

In conclusion, Lact exhibited renal protective effects in CKD rats by modulating the gut environment and regulating systemic inflammation. These effects suggest a novel mechanism by which renoprotective Lact can improve systemic inflammation and slow the progression of renal damage.

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CONFLICT OF INTEREST STATEMENT

None declared.

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