Attenuation of chronic kidney disease (CKD) and uremia induced gut-leakage by *Lactobacillus rhamnosus L34*, a probiotic derived from Thai population, in 5/6 nephrectomy model mice; an experimental study

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การใช้โพรไบโอติกชนิด แลคโตบาซิลลัส แรมโนสุส แอล 34 ที่แยกได้จากประชากรชาวไทยเพื่อใช้ในการลดความรุนแรงของภาวะไตวายเรื้อรังผ่านการลดการรั่วของเยื่อบุทางเดินอาหารจากภาวะยูรีเมียในหนูที่ถูกตัดไต 5/6 ส่วน

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สมกัญญา ตั้งสง่า : การใช้โพรไบโอติกชนิด แลคโตบาซิลลัส แรมโนซุส แอล 34 ที่แยกได้จากประชากรชาวไทยเพื่อใช้ในการลดความรุนแรงของภาวะไตวายเรื้อรังผ่านการลดการรั่วของเยื่อบุทางเดินอาหารจากภาวะยูรีเมียในหนูที่ถูกตัดไต 5/6 ส่วน. (Attenuation of chronic kidney disease (CKD) and uremia induced gut-leakage by *Lactobacillus rhamnosus L34*, a probiotic derived from Thai population, in 5/6 nephrectomy model mice; an experimental study) อ.ที่ปรึกษาหลัก : รศ. นพ.พิสุทธิ์ กตเวทิน, อ.ที่ปรึกษาร่วม : รศ. ดร. อัษฎาศ์ ลีฬหวนิชกุล

ที่มาของงานวิจัย โรคไตเรื้อรังมักทำให้เกิดการเปลี่ยนแปลงของเชื้อจุลชีพในลำไส้ ส่งผลให้เกิดการรั่วของเยื่อบุลำไส้ ทำให้สารพิษยูรีมิกที่สร้างภายในลำไส้เคลื่อนผ่านผนังเยื่อบุลำไส้เข้าสู่กระแสเลือดมากขึ้น เกิดภาวะยูรีเมีย ซึ่งอาจทำให้โรคไตเรื้อรังมีการดำเนินโรคแย่ลงได้ การใช้โพรไบโอติกชนิด แลคโตบาซิลลัส แรมโนซุส แอล 34 (แอล 34) อาจช่วยลดการรั่วของเยื่อบุทางเดินอาหารและลดภาวะแทรกซ้อนดังกล่าวได้ จึงทำการศึกษาผลของแอล 34 ต่อการเกิดพังผืดในไตและปริมาณสารพิษยูรีมิกในหนูที่ถูกตัดไต 5/6 ส่วน

ระเบียบวิธีการวิจัย สำหรับการทดลองในหนู หนูทดลองที่ถูกตัดไต 5/6 ส่วนถูกแบ่งออกเป็น 2 กลุ่ม ได้แก่ กลุ่มที่ได้โพรไบโอติกชนิด แอล 34 ปริมาณ $1x10^6$ หน่วยก่อรูปเป็นโคโลนี และกลุ่มควบคุมที่ได้สารละลายฟอสเฟตบัฟเฟอร์ร่วมกับมีหนูทดลองกลุ่มควบคุมอีก 1 กลุ่มที่ได้รับการผ่าตัดหลอกและได้สารละลายฟอสเฟตบัฟเฟอร์ หนูทดลองจะได้รับโพรไบโอติกหรือสารละลายฟอสเฟตบัฟเฟอร์ เริ่มที่ 6 สัปดาห์หลังผ่าตัด เป็นเวลานาน 14 สัปดาห์ และดูผลการทดลองที่ 20 สัปดาห์หลังผ่าตัด นอกจากนี้ยังมีการทดสอบผลของโพรไบโอติกชนิด แอล 34 ในภาวะยูรีเมียจากสารอินดอกซิลซัลเฟต ต่อการกระตุ้นการอักเสบ การเกิดการรั่วของเซลล์เยื่อบุลำไส้ใหญ่ (Caco-2 enterocyes) และการกระตุ้นการสร้างเยื่อพังผืดในเซลล์เยื่อบุผนังท่อไต (HK2 renal tubular cells)

ผลการศึกษา หนูทดลองที่ถูกตัดไต 5/6 ส่วนที่ได้โพรไบโอติกชนิด แอล 34 มีการบาดเจ็บของไต (ปริมาณเนื้อเยื่อพังผืดจากชิ้นเนื้อไต ระดับครีอะตินินในเลือด และปริมาณโปรตีนในปัสสาวะ) ระดับสารพิษยูรีมิกที่สร้างภายในลำไส้ (ไตรเมทิลามีนเอ็นออกไซด์ และอินดอกซิลซัลเฟต) ระดับเอนโดทอกซินในเลือด ระดับทูเมอร์เนคโครซิสแฟคเตอร์-แอลฟาในเลือด และมีการเปลี่ยนแปลงของสมดุลย์เชื้อจุลชีพ ที่ 20สัปดาห์หลังผ่าตัด น้อยกว่าหนูทดลองที่ถูกตัดไต 5/6 ส่วนที่ได้สารละลายฟอสเฟตบัฟเฟอร์ สำหรับการทดลองในเซลล์พบว่า ภาวะยูรีเมียทำให้เซลล์เยื่อบุลำไส้ใหญ่เกิดการแสดงออกของยีนควบคุมสารกระตุ้นการอักเสบ (อินเตอร์ลูคิน 8 และนิวเคลียร์แฟคเตอร์แคปปาบี) และเกิดการเกิดการรั่วของเซลล์โดยการวัดค่าทรานส์อิพิเทเลียลอิเลคทริคัลรีซิสแทนท์ นอกจากนี้ยังทำให้เซลล์เยื่อบุผนังท่อไตเกิดการแสดงออกของยีนควบคุมสารกระตุ้นการเกิดพังผืด (ทูเมอร์เนคโครซิสแฟคเตอร์-แอลฟา อินเตอร์ลูคิน 8 คอลลาเจนชนิดที่ 3 และ 4 อย่างไรก็ตามพบว่าโพรไบโอติกชนิด แอล 34ช่วยลดผลดังกล่าวได้ทั้งในเซลล์เยื่อบุลำไส้ใหญ่และเซลล์เยื่อบุผนังท่อไต

สรุป โพรไบโอติกชนิด แอล 34 ช่วยชะลอการเสื่อมของไตในภาวะไตเรื้อรังได้ ผ่านการลดภาวะยูรีเมีย ลดกระบวนการอักเสบจากภาวะยูรีเมีย และลดการรั่วของเยื่อบุลำไส้

สาขาวิชา อายุรศาสตร์ ลายมือชื่อนิสิต .....
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Somkanya Tungsanga : Attenuation of chronic kidney disease (CKD) and uremia induced gut-leakage by *Lactobacillus rhamnosus L34*, a probiotic derived from Thai population, in 5/6 nephrectomy model mice; an experimental study. Advisor: Assoc. Prof. Pisut Katavetin Co-advisor: Assoc. Prof. ASADA LEELAHAVANICHKUL, Ph.D.

Background: Although pathogenic gut microbiota causes gut leakage, increases translocation of uremic toxins into circulation, and accelerates CKD progression, the local strain of Lactobacillus rhamnosus L34 (L34) might attenuate gut leakage. We explored the effects of L34 on kidney fibrosis and levels of gut-derived uremic toxins (GDUTs) in 5/6-nephrectomy (5/6 Nx) mice.

Methods: At 6 weeks post-5/6 Nx in mice, either L34 ($1x10^6$ CFU) or phosphate buffer solution (as 5/6 Nx control) were daily fed for 14 weeks. *In vitro*, the effects of L34-conditioned media with or without indoxyl sulfate (a representative GDUT) on inflammation and cell integrity (transepithelial electrical resistance; TEER) were assessed in Caco-2 (enterocytes). In parallel, the effects as such on pro-inflammatory cytokines and collagen expression were assessed in HK2 proximal tubular cells.

Results: At 20-weeks post-5/6 Nx, L34-treated mice showed significantly lesser renal injuries, as evaluated by i) kidney fibrosis area ($p<0.01$) with lower serum creatinine and proteinuria, ii) GDUT including trimethylamine-N-oxide (TMAO) ($p=0.02$) and indoxyl sulfate ($p<0.01$), and iii) endotoxin ($p=0.03$) and serum TNF-α ($p=0.01$), than 5/6 Nx-controls. Fecal-microbiome analysis revealed an increased proportion of Bacteroidetes in 5/6 Nx-controls. After incubation with indoxyl sulfate, Caco-2 enterocytes had higher *IL-8*, *NF-κB* expression, and lower TEER value, and HK2 cells demonstrated higher gene expression of *TNF-α*, *IL-6*, and *collagen* (type III and type IV). These indoxyl sulfate-activated parameters were attenuated with L34-conditioned media indicating the protective role of L34 on enterocyte integrity and renal fibrogenesis.

Conclusion: Lactobacillus rhamnosus L34 attenuated uremia-induced systemic inflammation by reducing GDUTs and gut-leakage that provided reno-protective effects in CKD.

Field of Study:	Medicine	Student's Signature .....
Academic Year:	2021	Advisor's Signature .....
		Co-advisor's Signature .....

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Somkanya Tungsanga

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CHAPTER I

BACKGROUND AND RATIONALE

Background

The global burdens of chronic kidney disease (CKD) are substantial and have been rising during the past decades,[1] making retarding CKD progression the ultimate goal in the management of CKD. CKD leads to accumulation of metabolic substances, so-called “uremic toxins”, resulting in cardiovascular complications and CKD progression.[2] Most uremic toxins are derived from dietary compounds. However, some are produced from gastrointestinal tract, so-called gut-derived uremic toxins (GDUTs),[3] including trimethylamine-N-oxide (TMAO), indoxyl sulfate (IS), p-cresol sulfate, hippuric acid, and phenylacetic acid.[4, 5]

The human intestine harbors thousands of bacterial species that differ in individuals but tend to be similar among those who live in a close environment and consume similar diets.[6] Under physiological settings, the intestinal barrier limits the translocation of microbial products into the systemic circulation. Due to the defect of toxin elimination through kidneys in advanced CKD, some accumulated toxins are compensatory excreted through intestinal tract, which may promote overgrowth of pathogenic bacteria in the intestinal lumen, so-called gut dysbiosis.[7] Gut microbiota plays a significant role in regulating the production of GDUTs; therefore gut dysbiosis

could enhance the production of GDUTs.[8] Both gut dysbiosis and GDUTs can impair intestinal tight junctions, which lead to translocation of organic molecules and toxins into blood circulation, so-called gut leakage.[9] The gut leakage might be caused by either direct uremic toxin cytotoxicity or uremia-enhanced gut dysbiosis.[10]

Uremia-induced gut leakage increases endotoxin and GDUTs in the blood circulation,[8, 11] facilitates inflammatory reactions[10] and, consequently, accelerates CKD progression. Thus, there is a vicious cycle in that CKD causes uremic toxin accumulation and gut dysbiosis, and the latter two factors further induce gut leakage, leading to worsening of CKD, so-called gut-kidney axis.[12-14] This implies a new therapeutic possibility. Inflammation due to gut translocation of endotoxin, the main component of Gram-negative bacteria, which are the most abundant gut organisms, worsens GDUT-induced inflammatory condition[10] similar to adverse effects from other causes of inflammation toward CKD.[15] Because both GDUTs and endotoxemia might worsen CKD progression,[9, 16, 17] probiotics could prevent gut dysbiosis and could help to delay CKD progression.[18, 19] Recent studies showed the benefits of probiotics on reduction of GDUTs and inflammatory cytokines.[20, 21] However, there are only a few small studies which illustrate an effect of probiotics on delaying kidney fibrosis by histopathology. [22, 23] Accordingly, *Lactobacillus rhamnosus* L34 (L34), a strain of intestinal flora isolated from the Asian population,[24] improve intestinal integrity in several animal models of acute illnesses.[25, 26] Thus, this probiotic strain might also help delay CKD progression.

Circulating GDUTs induce cell damage, including enterocytes and renal tubular cells.[26-28] It is possible that probiotics produce renal protective molecules that could be delivered through the leaky gut.[10, 29] Because i) systemic inflammation worsens kidney fibrosis and facilitates CKD progression,[30] ii) uremia causes gut leakage that possibly enhances systemic inflammation,[10] and iii) the anti-inflammatory property of probiotics is documented,[25, 31, 32] we hypothesized that attenuation of CKD progression by L34 might be partly through an anti-inflammatory effect.

In this study, we explored the effects of *Lactobacillus rhamnosus* L34 on renal histopathology, CKD progression, inflammatory markers, and gut leakage in the 5/6 nephrectomy (5/6 Nx) mouse model. To understand the mechanisms on the gut-kidney axis, we also conducted the *in vitro* experiments in enterocytes (Caco-2 cells) and renal tubular cells (HK2 cells) to examine the effects of L34-conditioned media (LCM), that potentially contain protective molecules derived from probiotics, on cellular injuries caused by IS, a representative GDUT.

Research question

1. Primary research question

Could *Lactobacillus rhamnosus L34* attenuate CKD progression in 5/6 nephrectomized mice?

2. Secondary research questions

- Could *Lactobacillus rhamnosus L34* reduce gut leakage in 5/6 nephrectomized mice?
- Could *Lactobacillus rhamnosus L34* reduce levels of gut-derived uremic toxin in 5/6 nephrectomized mice?
- Could *Lactobacillus rhamnosus L34* reduce inflammation in 5/6 nephrectomized mice?

Objectives

1. Primary objective

- To study the efficacy of *Lactobacillus rhamnosus L34* in attenuation of kidney fibrosis by histopathology

2. Secondary Objectives

- To study the effect of *Lactobacillus rhamnosus L34* on gut leakage in 5/6 nephrectomized mice

- To study the effect of *Lactobacillus rhamnosus L34* on levels of gut-derived uremic toxins in 5/6 nephrectomized mice
- To study the effect of *Lactobacillus rhamnosus L34* on renal progression in 5/6 nephrectomized mice
- To study the effect of *Lactobacillus rhamnosus L34* on levels of inflammatory markers in 5/6 nephrectomized mice
- To explore the potential mechanistic effects of *Lactobacillus rhamnosus L34* on enterocyte injuries.
- To explore the potential mechanistic effects of *Lactobacillus rhamnosus L34* on renal tubular cell injuries.

Hypothesis

- 5/6 nephrectomy mice, which have been treated with *Lactobacillus rhamnosus L34,* have lower fibrosis by kidney histopathology than those which have not been treated.
- 5/6 nephrectomy mice, which have been treated with *Lactobacillus rhamnosus L34,* have lower gut leakage than those which have not been treated.
- 5/6 nephrectomy mice, which have been treated with *Lactobacillus rhamnosus L34,* have lower levels of gut-derived uremic toxin than those which have not been treated.

- 5/6 nephrectomy mice, which have been treated with *Lactobacillus rhamnosus L34,* have lower levels of serum creatinine and proteinuria than those which have not been treated.
- 5/6 nephrectomy mice, which have been treated with *Lactobacillus rhamnosus L34,* have lower levels of inflammatory markers than those which have not been treated.

Research design

This study is an experimental study including mouse model.

Conceptual framework

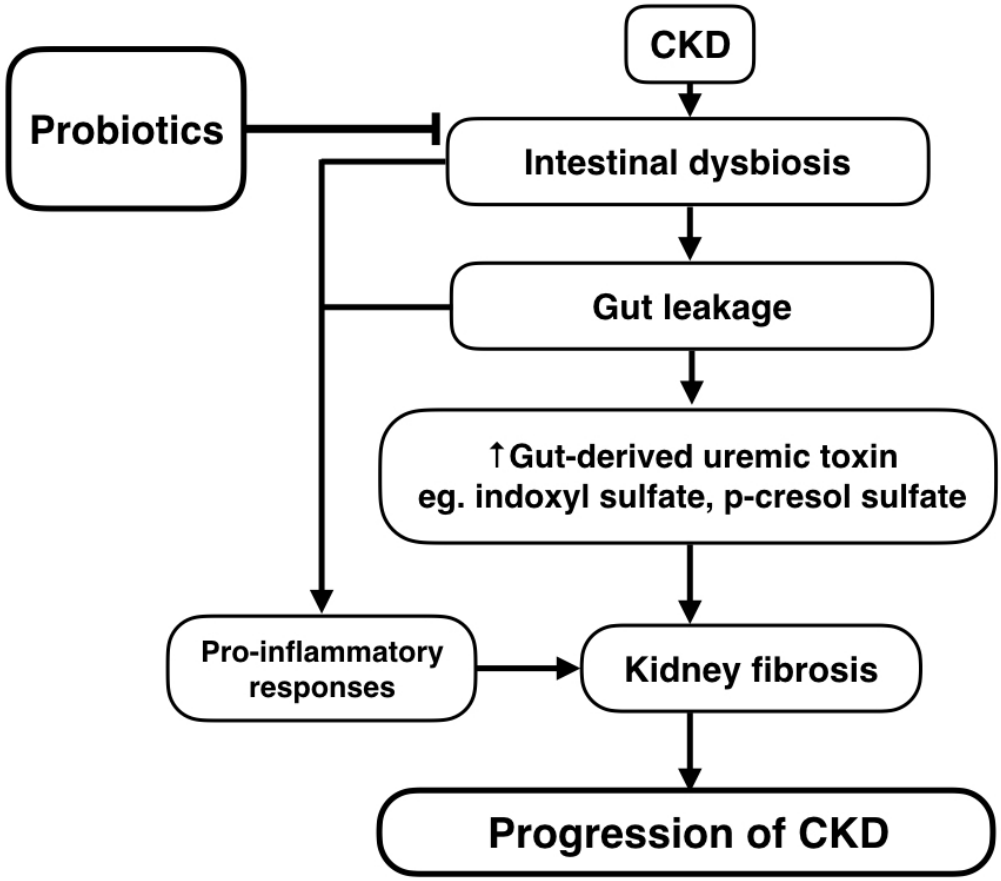

Figure 1 Conceptual framework of factors affecting progression of chronic kidney disease

CHAPTER II

REVIEW OF LITERATURE

Chronic kidney disease

Chronic kidney disease (CKD) is defined as the presence of abnormalities of either kidney function or structure for more than 3 months. CKD with decreased glomerular filtration rate (GFR) is defined as having a GFR of less than 60 ml/min/1.73 m2, equally to GFR categories G3a-G5). Other functional or structural abnormalities than decreased GFR include;[33]

- Albuminuria or microalbuminuria
- Urinary sediment abnormalities (microscopic hematuria, WBC casts, RBC casts, oval fat bodies, granular casts)
- Renal tubular disorders (renal tubular acidosis, nephrogenic diabetes insipidus, electrolyte abnormalities from tubular dysfunction, Fanconi syndrome, non-albumin proteinuria, cystinuria)
- Pathological abnormalities detected by histology or inferred
- Structural abnormalities (polycystic kidneys, dysplastic kidneys, horseshoe kidney, hydronephrosis, cortical scarring, renal masses, renal artery stenosis, small and hyperechoic kidneys)
- History of kidney transplantation

CKD is classified by causes, GFR, and albuminuria. The severity and prognosis of CKD is categorized by the levels of GFR and albuminuria, as shown in figure 2.[33]

Prognosis of CKD by GFR and Albuminuria Categories: KDIGO 2012

GFR categories (ml/min/ 1.73m²) Description and range				Persistent albuminuria categories Description and range		
				A1	A2	A3
				Normal to mildly increased	Moderately increased	Severely increased
				<30 mg/g <3 mg/mmol	30-300 mg/g 3-30 mg/mmol	>300 mg/g >30 mg/mmol
	G1	Normal or high	≥90			
	G2	Mildly decreased	60-89			
	G3a	Mildly to moderately decreased	45-59			
	G3b	Moderately to severely decreased	30-44			
	G4	Severely decreased	15-29			
	G5	Kidney failure	<15			

Figure 2 Summary of categories of CKD by GFR and albuminuria and prognosis of CKD progression. (Green, low risk; Yellow, moderate risk; Orange, high risk; Red, very high risk)[33]

Most causes of CKD are chronic and irreversible, leading to a life-long course of eGFR decline, so-called CKD progression.[33] Eventually, CKD may progress to the end stage requiring renal replacement therapy (RRT), so-called end stage kidney disease (ESKD). The ten most common etiologies of CKD in Thailand include:[34-36]

1. Diabetic nephropathy
2. Hypertensive nephropathy
3. Chronic tubule-interstitial nephritis
4. Chronic urinary tract obstruction
5. Presumed glomerulonephritis
6. Lupus nephritis
7. Polycystic kidney diseases
8. Chronic urate nephropathy
9. Biopsy-proven glomerulonephritis
10. Analgesic-induced nephropathy

Burden of chronic kidney disease

CKD is an important non-communicable disease that causes a major health burden worldwide. In 2017, the global prevalence of all-staged CKD in the all-age population was 9.1%, 697.5 million cases.[1] Among these CKD patients, 1.2 million cases died from renal causes while 1.4 million cases died from cardiovascular causes attributable to impaired kidney function, making CKD the 12th leading cause of death.[1, 37] CKD also contributed to 35.8 million disability-adjusted life-years (DALYs) in 2017, more commonly in the low to middle-income countries, especially in the three lowest quintiles of the Socio-demographic Index (SDI).[1] In Thailand, the estimated

prevalence of CKD was 17.5% in the overall population. CKD stage G3 and G2 are the two most common.[34]

During the past 20 years, the prevalence of renal replacement therapy in Thailand tended to increase every year, from 99 to 667 and 2274 cases per million population in 2000, 2010, and 2019, respectively. This brings about the consumption of resources and an estimated budget of over 7 million Thai Bahts per case per year, along with the burden for patients and caregivers.[36]

Gut microbiota

A large number of microbial cells co-evolved with the human hosts. This symbiotic ecosystem provides the largest reservoir of the microbiome in man with the highest diversity. The predominant bacteria include *Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, Fusobacteria,* and *Verrucomicrobia.*[38] Gut microbial diversity is influenced by environmental exposure, especially dietary habits.[6] Under normal physiology, the microbiota played an important role in the metabolic activities of hosts,[39] immune regulation,[40] certain vitamins and amino acid synthesis,[41] and bile acid metabolism.[42]

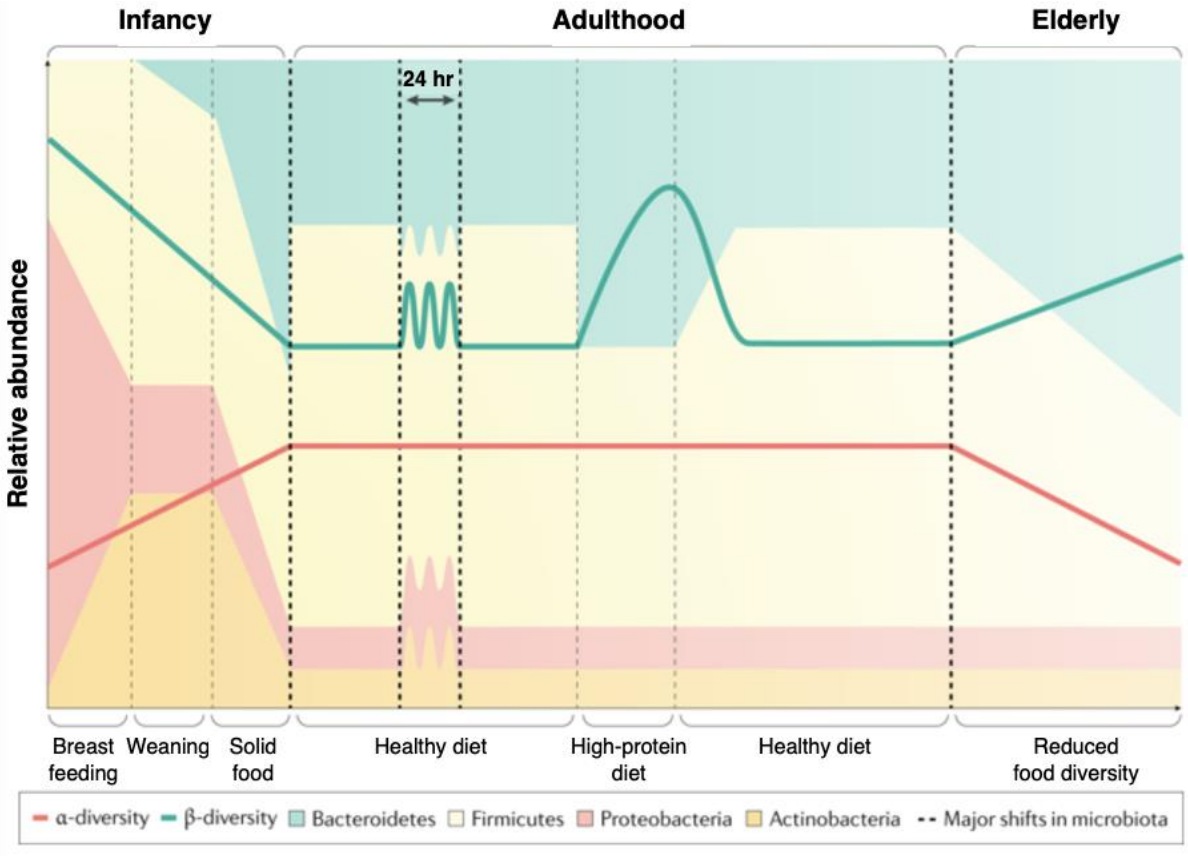

Figure 3 Temporal dietary modulation of the gut microbiota[43]

Gut-Kidney axis

In CKD patients, uremia was found to affect gut microbiota in several ways. First, it altered the composition of the gut microbiome. *Varizi et al*[44] showed the association of uremia and increase in intestinal pathobionts, so-called dysbiosis. Second, an increase in bile acid metabolism and increase in protein and carbohydrate fermentation would generate toxic metabolites, including uremic toxins. Third, dysbiosis would induce the production of lipopolysaccharide (LPS), which could activate macrophages and induce proinflammatory responses.[45] Finally,

the released cytokines and uremia-induced dysbiosis, led to local inflammation and impaired intestinal barrier.[46]

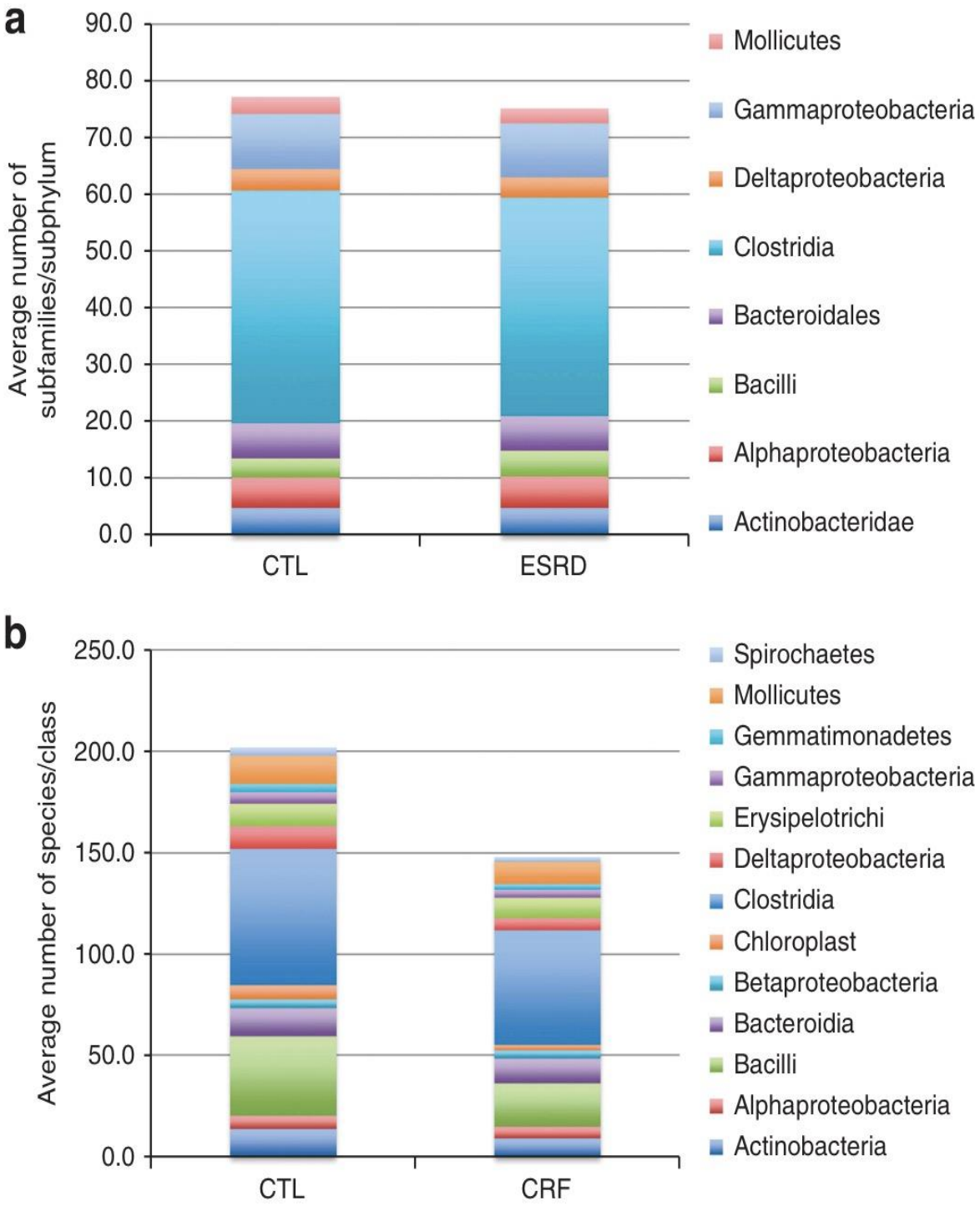

Figure 4 Altered gut microbiome in CKD animal model[47]

Gut-derived uremic toxins

Gut microbiota induced the production of uremic solutes in CKD and could lead to neurologic disorders, protein-energy wasting, cardiovascular disease, and progression of CKD. Moreover, the retention of uremic solutes could increase the generation of the dysbiotic microbiome. [48] These uremic toxins as such are indoxyl sulfate (IS), p-cresyl sulfate (PCS), trimethylamine-N-oxide (TMAO), and short-chain fatty acids (SCFF).

1. Indoxyl sulfate (IS)

As a result of tryptophan metabolism, indoles are absorbed from the gut and conjugated to IS in the liver. When IS accumulates in renal proximal tubules, it will activate nuclear factor-kappa B (NF-*k*B) and plasminogen activator inhibitor type 1 expression, the pro-inflammatory mediators. IS also increases the expression of tissue inhibitors of metalloproteinases and transforming growth factor-β (TGF- β 1), which are the mediators of tubulointerstitial fibrosis.[49]

2. P-Cresyl sulfate (PCS)

Several colonic bacteria generate phenols and its alkylated derivative, P-cresol, from the partial breakdown of tyrosine and phenylalanine. P-Cresol, mainly excreted in the urine, is progressively accumulated in CKD patients. PCS could activate the intrarenal renin-angiotensin system and epithelial-mesenchymal transformation, leading to kidney fibrosis.[50] Phenylacetylglutamine is another metabolite derived from β-phenylethylamine formed in the process of bacterial

proteolysis. It impairs immune regulation and produces oxidative stress that could worsen kidney fibrosis.[51]

3. Trimethylamine-N-Oxide (TMAO)

TMAO is generated during the bacterial metabolism of dietary lipid phosphatidylcholine (lecithin). An increase in TMAO levels is associated with an increased risk of major adverse cardiovascular events (MACE), including death, myocardial infarction, or stroke.[52] In animal models, TMAO was found to increase tubulointerstitial fibrosis and collagen deposition.[53]

4. Short-chain fatty acids (SCFF)

SCFF is a metabolite produced by gut microbiota during the fermentation of unabsorbed food components, especially fibers. SCFF supports multiple host functions, including energy metabolism, immune regulation, gut motility, and blood pressure regulation.[54, 55]

Table 1 Sources of Gut bacteria-associated uremic toxins and adverse reactions[48]

Uremic toxins	MW (Da)	Related organism	Source	Adverse reactions
Low-MW molecules (<0.5 kDa)				
TMAO[2]	75	*Faecalibacterium prausnitzii, Bifidobacterium spp.*	Dietary lipid phosphatidyl-choline	Progression of tubulointerstitial fibrosis, Smad3 phosphorylation of Smad3 (regulator of profibrotic TGF/Smad3 signaling), increase in mortality
Ammonia[56]	17	*Clostridium spp. Escherichia coli Enterococcus Shigella*	Glutamine, glycine, serine, threonine	Uremic enterocolitis, systemic inflammation, carcinogenesis
1-Methyl guanidine[57]	73	*Pseudomonas stutzeri*	Creatinine	Increase in mortality
Homocysteine[58]	135	*Bifidobacterium spp.*	Gut bacteria regulates production of homocysteine by folate production.	Increase in CV events and mortality, oxidative stress, inhibition of transmethylation pathways
D-Lactic acid[59]	90	*Enterococcus, Streptococcus spp.*	Bacterial production	Neurotoxicity, encephalopathy

Uremic toxins	MW (Da)	Related organism	Source	Adverse reactions
Oxalate[60]	90	*Oxalobacter formigenes, Bifidobacterium lactis, Enterococcus faecalis, Eubacterium spp.*	Gut bacteria degrades oxalate.	Urolithiasis, atherosclerosis
Protein-bound molecules				
p-Cresyl sulfate[17]	188	*Clostidium difficile, Bifidobacterium, Subdoligranulum, Lactobacillus spp, F prausnitzii,*	Tyrosine, phenylalanine	Progression of CKD, CV events and mortality, endothelial permeability, endothelial adhesion-molecule expression
Indoxyl sulfate[61]	213	*Clostridium sporogenes, Escherichia coli*	Tryptophan	Vascular stiffness and calcification, CV mortality, vascular smooth muscle proliferation, tubulointerstitial fibrosis
Indole-3-acetic acid[62]	175	*Clostridium sporogenes, Clostridium bartlettii, Escherichia coli*	Tryptophan	Glomerulo-sclerosis and interstitial fibrosis, predictors of mortality and CV events in CKD, oxidative stress, systemic inflammation

Uremic toxins	MW (Da)	Related organism	Source	Adverse reactions
Phenylacetic acid[63]	136	*Clostridium, Bacteroides spp.*	Tryptophan	GI irritation, convulsion, oxidative stress, osteoclast dysfunction. Immune dysregulation
Hippuric acid[64]	179	*Clostridia spp*	Aromatic compounds, polyphenols	Anion gap metabolic acidosis, impaired erythropoiesis and platelet COX activity, glucose intolerance

Manipulation of microbiome composition and its effects

As the dysbiosis of the gut microbiome causes various adverse consequences, several therapeutic trials were conducted in order to re-generate symbiosis, including pro- and prebiotics. Probiotics are the diet or supplements that contain beneficial organisms (symbionts), while prebiotics contain food for normal flora. Both probiotics and prebiotics target on normalization of the gut flora compositions.[65]

Prebiotics

The Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO) defined prebiotics as “a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota.”[66] Prebiotics are mainly the non-digestible high fiber-containing diet, the

so-called non-digestible oligosaccharides (NDOs), such as galacto-oligosaccharides, fructo-oligosaccharides, polyphenols (flavonols and quercetin), polydextrose, zinc, conjugated linoleic acid, L-carnitine, choline, sphingomyelin, and ellagitannins. Foods with high contents of prebiotics include wheat, onion, and garlic. The main effect of prebiotics is to promote the growth of the beneficial *Lactobacilli* and *Bifidobacteria spp.*[67] The first available prebiotics for human are the human milk oligosaccharides (HMO), which is associated with the development and maturation of the immune system, gastrointestinal tract, and gut microbiota diversity in newborns.[68] NDOs regulate the immune responses to vaccines, [69] allergic reactions, and the pro-inflammatory responses against systemic infection by stimulating the immune development and functions.[70] Galacto-oligosaccharides can reduce the diarrhea manifestation in chronic inflammatory bowel disease, especially irritable bowel syndrome.[71] Recent meta-analysis of Wilson et al[72] showed the increase in total abundance of *Bifidobacteria spp.* after treatment of prebiotics in patients with irritable bowel syndrome or functional bowel disorders. However, the effects on the quality of life or gastrointestinal symptoms are comparable to the placebo. Fructo-oligosaccharides are shown to induce satiety by increasing the secretion of satiety-inducible peptide YY and decreasing appetite-induced ghrelin, and subsequently control calory intake and induce weight loss in obese patients.[73] Long-chain fructo-oligosaccharides supplementation enhances gastrointestinal calcium absorption and was shown to improve bone mineralization in adolescents.[74] Moreover, prebiotics

reduced the markers of colonic cancer in rats[75] but was failed to show benefits on overall risk for colorectal cancer in human.[76]

Probiotics

In 2001, The Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO) defined prebiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host."[66] The available probiotics include the major bacterial species, *Bifidobacterium spp.* (*B. adolescentis, B. animalis, B. bifidum, B. breve,* and *B. longum*), *Lactobacillus spp.* (*L. acidophilus, L. casei, L. fermentum, L. gasseri, L. johnsonii, L. paracasei, L. plantarum, L. rhamnosus, L. salivarius*), and *Saccharomyces spp.* (*S. boulardii, S. thermophilus*) with an adequate amount of $1x10^9$ colony-forming units (CFU) per serving.[77]

Administration of probiotics has proven benefits on chronic inflammatory gastrointestinal diseases, such as *Clostridium difficile* diarrhea, inflammatory bowel disease, and irritable bowel syndrome.[78] The meta-analysis of Goldenberg, et al.[79] proved the efficacy of probiotics in the prevention and the additional benefits on the treatment outcomes in combination with standard antibiotics in *Clostridium difficile* diarrhea patients without severe deteriorated features or immunocompromise. In combination with gluten-free diets, probiotics can improve the severity of Celiac disease through the restoration of symbiosis and suppression of autoimmunity.[80, 81]

Probiotics also were effective in reducing the severity and diarrhea symptoms of irritable bowel syndrome.[82] *L. rhamnosus GG* and *L. reuteri* were effective in treating pediatric patients with irritable bowel syndrome.[83] According to the Australian and New Zealand Neonatal Network (ANZNN) data, probiotics could prevent the incidence of severe necrotizing enterocolitis and the occurrence of late-onset sepsis in the high-risk preterm newborns.[84] A systematic review of Oak, et al. showed the overall benefits of the probiotics on lactose intolerance and metabolic syndrome.[85] Multiple large-scale studies and meta-analyses demonstrated the effects of probiotics on chronic skin allergy, food allergy, and atopic diseases.[86-88]

Gut microbiota can alter the levels of circulating tryptophan, serotonin, and short-chain fatty acids, which are the gut-derived toxins that affect brain glial cells and blood-brain barrier, so-called the gut-brain axis.[89] Recently, probiotics showed the potential therapeutic effects on the Alzheimer's disease,[90] neurodegenerative diseases,[91] anxiety,[92] autism spectrum disorder,[93] Parkinson's disease,[94] major depressive disorder,[95] and other behavioral and neuropsychiatric disorders.[96, 97]

Effects of probiotics and prebiotics on CKD

Uremia-induced gut leakage increases endotoxin and GDUTs in the blood circulation,[8, 11] facilitates inflammatory reactions[10] and, consequently, accelerates CKD progression. Thus, there is a vicious cycle in that CKD causes uremic toxin accumulation and gut dysbiosis, and the latter two factors further induce gut leakage,

leading to worsening of CKD, the so-called gut-kidney axis (figure 5, 6).[12-14] The use of probiotics was reported to have beneficial effects on CKD hosts when administered in proper quantities in clinical and experimental studies (table 2, 3). Several studies showed the benefits of probiotics on the reduction of GDUTs and inflammatory cytokines.[20, 21] However, there are only a few small studies that illustrate an effects of probiotics on delaying kidney fibrosis by histopathology.[22, 23] The recent meta-analysis and systematic review demonstrated the effects of probiotics on significant reduction of blood urea levels in non-dialysis CKD patients. However, the benefits on clinical outcomes or reduction of uremic toxins in end-stage kidney disease could not be proved.[98]

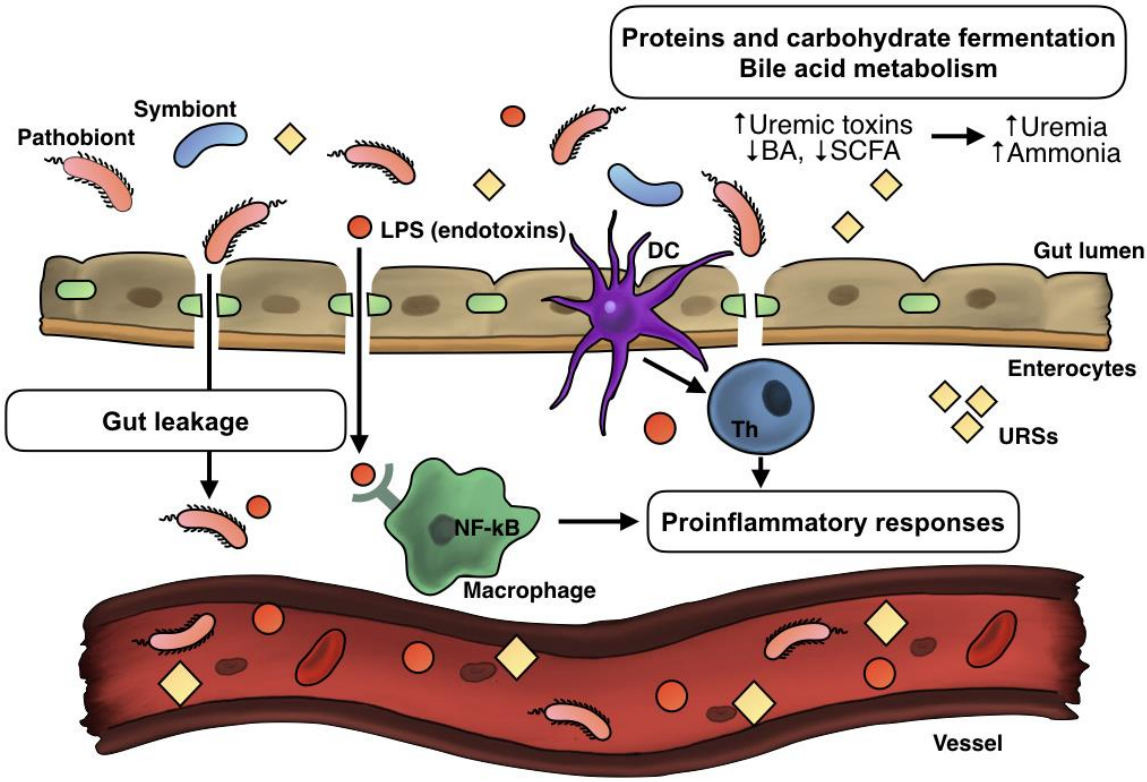

Figure 5 Effects of CKD on gut microbiota [48]

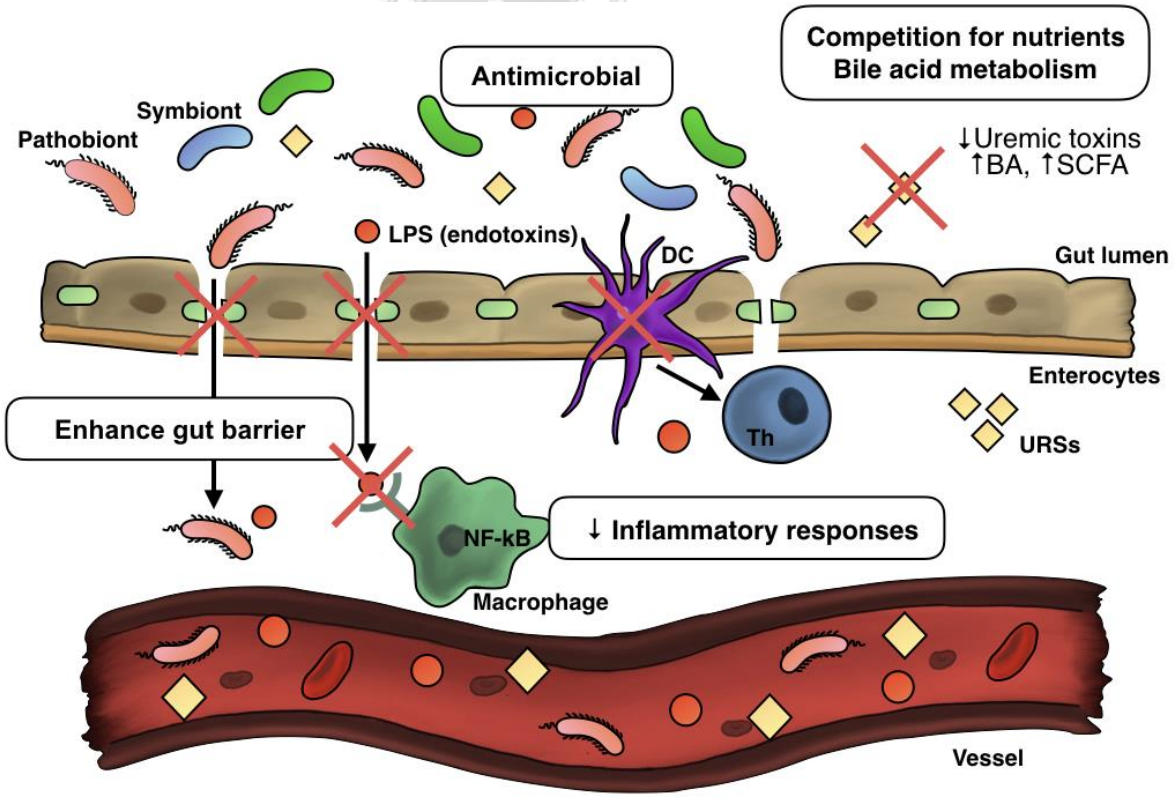

Figure 6 Effects of probiotics on gut microbiota and gut barrier [48]

Table 2 Previous studies of gut microbiota in CKD patients

Studies	Probiotics	N	Methods	Results
Rossi et al.[20] (CJASN 2016)	*Lactobacillus, Bifidobacteria, S. genera +prebiotics*	37	Single-center RCT, cross-over CKD G4-5 6 weeks, washout 4 weeks	↓p-cresol, not IS Modified stool microbiomes
Viramontes-Homer D et al.[99] (J Ren Nutr 2015)	*L. acidophilus B. lactis + prebiotics*	42	Multicenter RCT 2 months	↓GI symptoms, trend to ↓CRP
Wang et al.[19] (Benef Microbes 2015)	*B. bifidum, B. catenulatum, B. longum, L plantarum*	39	Single-center RCT PD 6 months	↓TNF-a, IL5, IL6, LPS preserve RRF
Cruz-Mora J et al.[100] (J Ren Nutr 2014)	*L. acidophilus B. lactis + prebiotics*	18	Double-blinded, placebo-controlled HD	↑Bifidobacteria in feces ↓Lactobacilli in feces ↓GI symptoms
Pavan et al.[23] (Minerva Urol Nefrol 2014)	*Prebiotic+Probiotic*	24	Single-center RCT CKD G3-4 12 months	↓CKD progression
Guida et al.[21] (Nutr Metab Cardiovasc Dis 2014)	*Lactobacillus, Bifidobacteria + prebiotics*	30	Single-center RCT, cross-over CKD G3-4 4 weeks	↓p-cresol

Studies	Probiotics	N	Methods	Results
Natarajan et al.[101] (BioMed Res Int 2014)	*S. thermophilus, L acidophilus, B. longum*	22	Single-center RCT, cross-over HD 8 weeks	↑QOL, trend to ↓IG&CRP
Miranda Alatriste et al.[102] (Nutr Hosp 2014)	*L. casei shirota*	30	Single-center RCT, cross-over CKD G3-4 8 weeks	↓urea by 11%
Nakabayashi et al.[103] (Nephrol Dial Transplant 2010)	*L. casei shirota, B. breve +prebiotics*	9	Single-center observational HD 4 weeks	↓ p-cresol normalize bowel habit
Ranganathan et al.[104] (Adv Ther 2010)	*S. thermophilus, L acidophilus, B. longum*	46	Multicenter RCT, cross-over CKD G3-4 6 months	↑QOL, ↓BUN
Ranganathan et al. [105] (Curr Med Res Opin 2009)	*S. thermophilus, L acidophilus, B. longum*	16	Single-center RCT, cross-over CKD G3-4 6 months	↑QOL, ↓BUN, ↓uric level
Taki et al.[106] (J Ren Nutr 2005)	*B. longum*	27	Single-center controlled trial HD 12 weeks	↓Hemocysteine, IS, TG

Studies	Probiotics	N	Methods	Results
Takayama et al.[107] (AJKD 2003)	*B. longum*	22	Single-center controlled trial HD 5 weeks	↓IS
Ando et al.[108] (Nihon Jinzo Gakkai Shi 2003)	*B. longum*	27	Single-center observational All staged CKD 6 months	↓CKD progression
Hida et al.[109] (Nephron 1996)	*B. infantis, L. acidophilus, E. faecalis*	25	Single-center observational HD 4 weeks	↓Indican in feces&serum ↓ p-cresol in feces
Simenhoff et al.[110] (Miner Electrolyte Metab 1996)	*L. acidophilus*	8	Single-center observational HD 1 course	↓Dimethylamine, ↓Nitrosodimethylamine

Table 3 Previous studies of gut microbiota in CKD animal model

Studies	Probiotics	Methods	Results
Prakash et al.[111] (Nat Med 1996)	*Genetically engineered E. coli with urease*	Uremic rats (5/6 nephrectomy) 35 days	↓Plasma urea
Ranganathan et al.[112] (Curr Med Res Opin 2005)	*Various combinations*	Uremic rats (5/6 nephrectomy) 16 weeks	↑lifespan, ↓BUN
Ranganathan et al.[113] (ASAIO J 2006)	*Sporosacina pasteurii*	Uremic rats (5/6 nephrectomy) 16 weeks	↑lifespan, ↓BUN
Andrade-Oliveira et al.[114] (JASN 2015)	*B. adolescentis, B. longum*	Bilateral IR-injury 2 weeks	↑Acetate production Protect from IR injury

Assessment of kidney fibrosis by histopathology

The significant histopathologic findings in CKD are tubulointerstitial fibrosis, tubular atrophy, global glomerulosclerosis, and capillary loss. Among these, the severity of interstitial fibrosis has the most association with a progressive decline of kidney functions and prediction of long-term outcomes.[115, 116] The gold standard in assessing kidney fibrosis is to measure the extension of a fibrotic area in kidney histopathology. The composition in the kidney fibrotic scar consists of the accumulation of extracellular matrix (ECM), which includes glycosaminoglycans, type I, type III, and type IV collagen, and fibronectin.[117] Fibroblasts, fibrocytes, pericytes, dendritic cells, and mast cells are known to aggravate tubulointerstitial fibrosis.[118]

In addition to conventional histologic stains (hematoxylin and eosin; H&E, Periodic acid–Schiff; PAS, Jone's Silver Methenamine), the visual measurement using the Masson-trichrome staining is currently the standard stain to detect the area fibrosis, where the collagen and fibrins are dyed blue.[119] Masson-trichrome staining has the highest correlation with eGFR decline with the most reproducibility. However, it has some limitations in detecting milder fibrosis.[120] Sirius red (or Picrosirius red) staining can also dye the collagen type I and III in red, but with some discrepancies between under light and polarized microscopes. Therefore, it is not commonly used.[121] Collagen type III immunohistochemistry is developed to assess fibrosis.

However, it lacks availability and clinical validation. It also cannot detect other types of collagen or extracellular matrix substances.[122]

Expression of some biomarkers, including Kidney Injury Molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), SRY-box 9, WAP four-disulfide core domain 2, and NK6 homeobox 2, in the kidney histopathology is also associated with the severity of tubulointerstitial fibrosis and chronic renal injuries.[123] However, such biomarkers can also be elevated in the context of acute kidney injury.

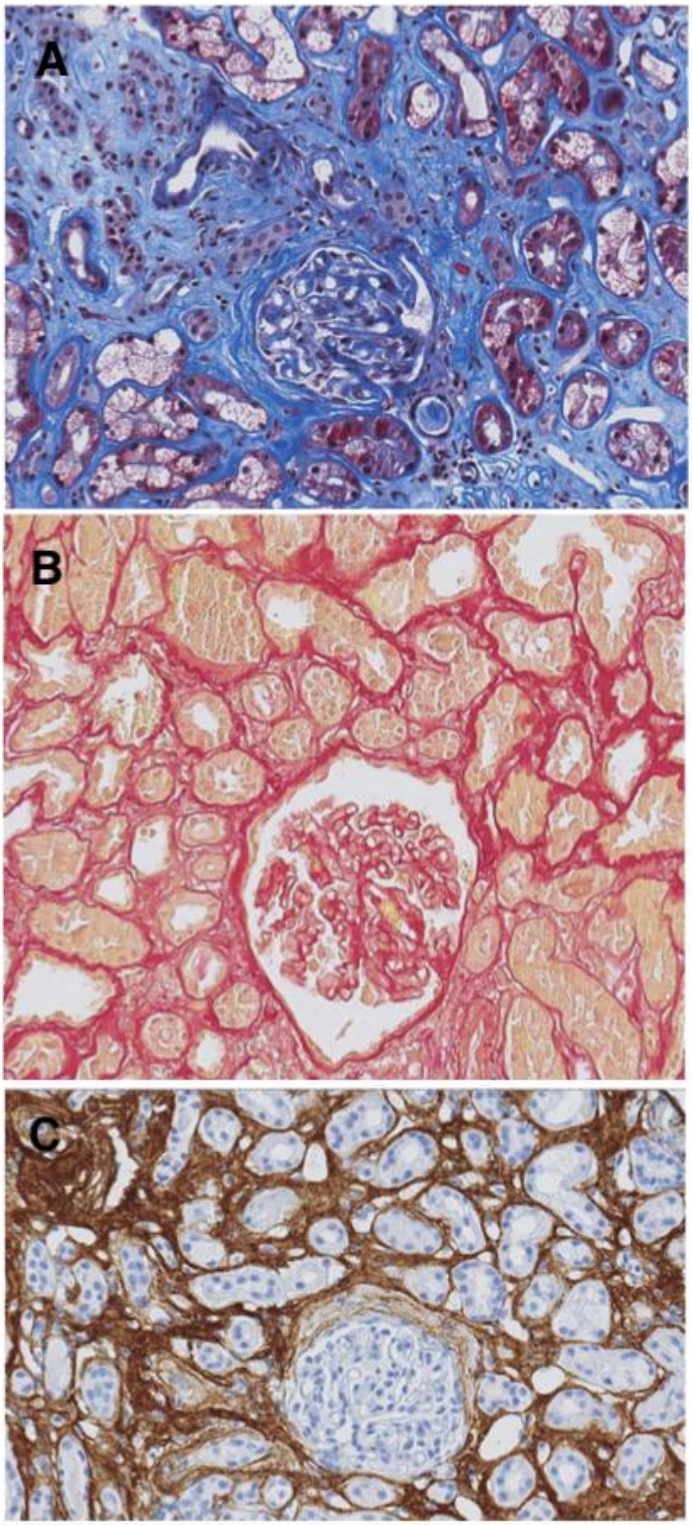

Figure 7 The histopathologic staining for kidney fibrosis, including Masson-trichrome (A), Sirius red (B), and Collagen type III immunohistochemistry (C)

CHAPTER III

MATERIALS AND METHODS

Animal study

Animals and animal model

Animal care and experiments were performed according to the National Research Council Guide for the Care and Use of Laboratory Animals.[124] The study protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (CU-ACUP No. 018/2562). Eight-week-old male C57BL/6 mice were purchased from Nomura Siam, Pathumwan, Bangkok, Thailand. The 5/6 Nx was performed via flank approach under isoflurane anesthesia.[125] Briefly, the upper and lower poles of the left kidney were removed. One week thereafter, a right nephrectomy was performed. Renal capsules were peeled off before the nephrectomy to avoid injury to the adrenal gland. To ascertain the optimal resected kidney mass for the CKD development model, only mice with a weight of resected left kidney in a ratio of 0.55-0.72 to removed right kidney were selected.[125] Then, 5/6 Nx mice were divided into 2 groups by block of four randomizations with allocation concealment, the probiotics gavage (5/6 Nx-L34) group and the phosphate buffer solution (PBS) gavage (5/6 Nx-PBS) control. Another group of mice were undergone sham surgery (Sham group). Study personnel involving clinical assessment, data collection, and data analysis were blinded to the treatment. At 6 weeks post-surgery, 50 µL of blood was collected through tail vein nicking, and

urine was collected in metabolic cages (Hatteras Instruments, NC, USA). At 20 weeks post-surgery, urine and feces were collected in metabolic cages (Hatteras Instruments, NC, USA). Blood samples were collected via cardiac puncture from all mice under isoflurane anesthesia. The animals were sacrificed thereafter.

Sample size calculation

$$n = 1+2C\,(s/d)^2$$

Tail	= One
α err prob	= 0.05
Power (1-β err prob)	= 0.90
C	= 10.51
sd	= 1.05×10^3
difference	= 2×10^3

(Ref. Tan RZ, et al. *Ren Fail*. 2019; 41(1):555-66.)

then $n = 1+21\,(1.05 \times 10^3/2 \times 10^3)^2$

$= 7$

To compare the treatment effects in between the 5/6 Nx-L34 and 5/6 Nx-control group, a minimum number of mice needed is 7 mice per group to obtain statistical significance of $p < 0.05$. With dropout rate of 30%, 10 mice per group were needed to maintain the power of this study. In this study, 20 mice were enrolled. They were divided into 2 groups: the 5/6 Nx-L34 group (n=10) and the 5/6 Nx-PBS

control group (n=10). Another group was enrolled and served as the sham control (n=5).

Animal Care

All animal procedures will be performed according to the US National Research Council (NRC) Guide for the Care and Use of Laboratory Animals (revision 2011).

1. Husbandry

- Housing: stainless-steel shoebox-shaped cage, solid bottom, open top size 19 x 28.5 x 13 cm with 4 animals / cage
- Bedding: wood shavings with weekly replacement
- Temperature: 23 ± 2 ˚C
- Relative humidity: 60-65 %
- Light: standard fluorescent
- Light intensity: 130-250 lux
- Diurnal cycle: light/dark = 12/12 hours
- Noise frequency: less than 85 decibels

2. Feeding

- *Ad libitum* food feeding with commercial diets (average of 5-7 grams per day)

- *Ad libitum* water feeding with all-day provide of uncontaminated drinking water in bottles with drinking tube (8-10 ml per day)

Probiotic administration

L34 from the stock was cultured on deMan-Rogosa-Sharpe (MRS) agar (Oxoid™, Hampshire, UK) under anaerobic conditions (10% CO_2, 10% H_2, and 80% N_2) using gas generation sachets (AnaeroPack®-Anaero; Mitsubishi Gas Chemical, Tokyo, Japan) at 37°C for 24- 48 hours before use. Commencing at 6 weeks after the right nephrectomy or sham operation, L34 at $1x10^6$ colony-forming unit (CFU) in 0.25 mL PBS were gavaged 3 times a week for 14 consecutive weeks until 1 day before sacrifice (figure 8).

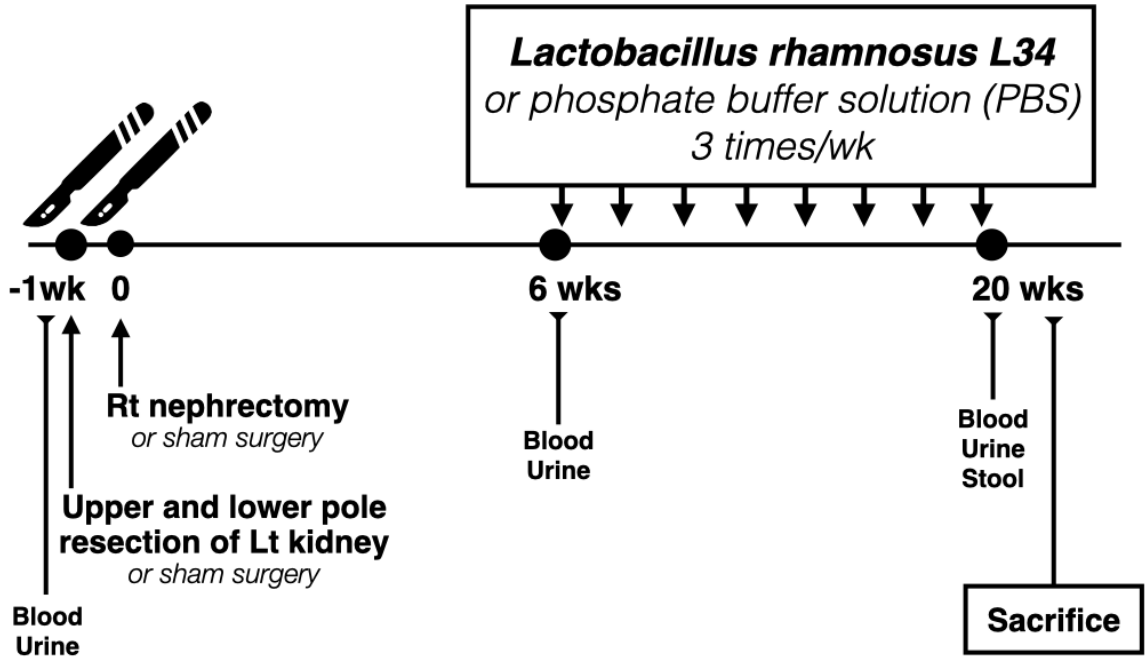

Figure 8 Schema of the mouse experiments

Observational measurements

1. Independent variable

- Probiotic administration (*L34* or vehicle).

2. Dependent variables

- Kidney injuries and uremic characteristics: area of kidney fibrosis by histopathology, plasma levels of gut derived uremic toxins (indoxyl sulfate, p-cresol sulfate, and TMAO), serum creatinine, 24-hour urine volume, 24-hour urine protein, anemia, and body weight gain.
- Gut microbiome analysis.

- Gut permeability: plasma levels of endotoxin, fluorescein isothiocyanate dextran (FITC)-dextran assay, detection of tight junction molecules in colonic pathology.
- Serum level of TNF-α

Mouse sample analysis

Hematocrit was measured by the microhematocrit method with the Coulter Counter (Hitachi 917; Boehringer Mannheim GmbH, Mannheim, Germany). Serum creatinine and albuminuria were measured using the colorimetric method (QuantiChrom™ Creatinine Assay Kit, BioAssay System, Hayward, CA, USA) and enzyme-linked immunosorbent assay (ELISA) (Albuwell M, Exocell™, Philadelphia, PA, USA), respectively. Serum TMAO was determined by liquid chromatography-mass spectrometry (LC-MS/MS) using silica column (Luna® silica; 00G-4274-E0, Phenomenex®, Torrance, CA, USA).[126] Various concentrations of non-isotopically labelled TMAO standards were spiked into control serum to construct calibration curves. Internal standard d9-TMAO was used for quantification and calculating the recovery rate of TMAO. Serum IS was determined by high-performance liquid chromatography (HPLC Alliance® 2695; Waters, Zellik, Belgium).[127] Serum TNF-α were evaluated by ELISA (PeproTech™, Cranbury, NJ, USA).[128]

Renal histopathologic studies

After sacrifice, the remaining kidneys were fixed in 10% formalin, paraffin-embedded, and stained with Masson's trichrome method.[30] Area of kidney fibrosis was determined by computerized image analysis software (ImageJ©software, Bethesda, MD, USA) in a 200x magnification field with 10 fields per sample.

Gut permeability determination and immunofluorescent

Three different methods proven to indicate gut leakage were determined: i) FITC-dextran assay, ii) spontaneous endotoxemia, and iii) detection of tight junction molecule in intestinal tissues. The detection of FITC-dextran (intestinal non-absorbable molecule) in serum after oral administration or spontaneous serum elevation of endotoxin without systemic inflammation indicate gut leakage.[31, 129-131] In the FITC-dextran assay, 12.5 mg of FITC-dextran (4.4 kDa) (FD4; Sigma-Aldrich®, St. Louis, MO, USA) was gavaged. At 3 hours thereafter, serum FITC-dextran was measured by Fluorospectrometer (NanoDrop™ 3300; Thermo Fisher Scientific™, Wilmington, DE, USA). Serum lipopolysaccharide (LPS or endotoxin) was evaluated by HEK-Blue LPS Detection Kit 2 (InvivoGen™, San Diego, CA, USA). Due to the lower limit of the standard curve of the test, the value <0.01 EU/ mL was recorded as 0. To examine intestinal tight junction, the section of cecum and colon obtained post-sacrifice were embedded in Cryogel (Leica Biosystems, Richmond, IL, USA). The 5 μm thick frozen sections were fixed in acetone, blocked by blocking buffer, stained with

fluorescent antibody against Occludin-1, an intestinal tight junction molecule, and labelled with secondary antibody in green (Alexa Fluor® 488; Life Technologies, Carlsbad, CA, USA). The detection of the green fluorescent color area of the tight junction at 630x from histological images were performed by the software of ZEISS® LSM 800 (Carl Zeiss AG, Jena, Germany) using 10 different fields per slide and presented as the percentage of tight junction area.

Fecal microbiome analysis

Feces (0.25 g per mouse) were collected from the mice in different cages per group to avoid the influence of allocoprophagy. Fecal microbiome analysis was performed.[29] Metagenomic DNA was extracted from prepared samples using DNAeasy Kit (Qiagen, Hilden, Germany) with DNA quality assessment using Nanodrop spectrophotometry. Universal prokaryotic primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGT WTCTAAT-3') with appended 50 Illumina adapter and 30 Golay barcode sequences were used for 16S rRNA gene V4 library construction. The abundance of total Gram-negative bacteria in feces was determined by calculating the bacterial abundance in the microbiome analysis on the phylum level.

In vitro study

1. The *in vitro* experiments of *Lactobacillus rhamnosus* L34-conditioned media

To examine the possible direct effect of probiotics on the intestine and kidney, IS, a water-soluble form of indoxyl, a molecule converted from Tryptophan amino acid by gut bacteria,[132] was used as a representative GDUT. The Caco-2 (ATCC HTB-37), and HK2 renal proximal tubular cells (ATCC CRL-2190) (American Type Culture Collection, Manassas, VA, USA), were used as representative of intestinal and kidney cells, respectively. The LCM, a representative of secreted molecules from the probiotics, was prepared.[25] Briefly, L34 at OD600 of 0.1 were incubated anaerobically for 48 hours before centrifugation for cell-free supernatants. Then, the supernatants were concentrated by speed vacuum drying at 40°C for 3 hours (Savant Instruments). The cell-free concentrated pellets were resuspended in an equal volume of DMEM, stored at -20°C until use. Caco-2 (at $2x10^6$ cells/well) or HK2 cells (at $5x10^4$ cells/well) maintained in Dulbecco's modified Eagle medium (DMEM) were incubated with IS (Sigma-Aldrich) at 500 μM/well (200 μL),[28] with or without 5% v/v LCM under 5% CO_2 at 37°C for 24 hours before determination of supernatant cytokines and the expression of several genes. To investigate the size of the potentially protective molecules in LCM, 3 fractions of LCM separated by 3 kDa filter (the finest commercially available filter) (Minisart®; Sartorius Stedim Biotech GmbH, Göttingen, Germany), including <3kDa, >3kDa and non-filtered LCM were used in the Caco-2 cell experiment.

Intestinal tight junction defect in uremic gut allows translocation of toxins with lower molecular weight (MW) into the systemic circulation, including endotoxin. To explore the effect of LCM with lower MW exclusively on renal tubular cells, LCM was filtered through a 3kDa membrane filter (Minisart®; Sartorius Stedim Biotech GmbH, Göttingen, Germany) in the HK2 cell experiment. After incubation with DMEM with LCM or DMEM alone (see details above), the supernatant was determined for cytokine (IL-8) and expression of several genes with ELISA method (Quantikine Immunoassay; R&D Systems, Minneapolis, MN, USA), and expression of selected genes related to fibrosis with quantitative reverse transcription-polymerase chain reaction (qRT-PCR).[133] The qRT-PCR of several genes in relative to *β-actin* (a house-keeping gene) with the $2^{-\Delta\Delta_{CT}}$ method was determined using cDNA (SuperScript™Vilo™ cDNA synthesis assay, Invitrogen™, Waltham, MA, USA) prepared from 50 ng of TRIzol-extracted total RNA (invitrogen™, Waltham, MA, USA) by a qPCR machine (LightCycler® 2.0, Roche Diagnostics, Indianapolis, IN, USA) with several primers, including *TNF-α*, *IL-6*, *IL-8*, Nuclear Factor kappa B (*NF-κB*), *Collagen* (type I, III, IV), *Fibronectin I,* and Hypoxia-inducible factor (*HIF-1α*) (table 4).[133]

The effect of GDUTs on intestinal integrity was determined by Transepithelial electrical resistance (TEER).[134] To establish confluent monolayer, Caco-2 cells at $5x10^4$ cells per well were seeded onto the upper compartment of 24-well Boyden chamber trans-well, using DMEM-high glucose supplemented with 20% Fetal Bovine

Serum (FBS), 1% N-hydroxyethylpiperazine-N-ethanesulfonate (HEPES), 1% sodium pyruvate, and 1.3% Penicillin/Streptomycin for 15 days. TEER, in ohm (Ω) xcm^2, was measured with epithelial volt-ohm meter (EVOM2™, World precision instruments, Sarasota, FL, USA) by placing electrodes in the supernatant at basolateral and in apical chambers. TEER values in media culture without Caco-2 cells was used as a blank and was subtracted from other measurements (figure 9).

Table 4 Primer sequences used for qRT-PCR in the *in vitro* experiments

Name	Forward primer	Reverse primer
Tumor necrosis factor-α (*TNF-α*)	5'-CTCTTCTGCCTGCTGCACTTTG -3'	5'- ATGGGCTACAGGCTTGTCACTC -3'
Interleukin-6 (*IL-6*)	5'- ATGAACTCCTTCTCCACAAGC -3'	5'- GTTTTCTGCCAGTGCCTCTTTG -3'
Interleukin-8 (*IL-8*)	5'- TGGCTCTCTTGGCAGCCTTC -3'	5'- TGCACCCAGTTTTCCTTGGG -3'
Nuclear Factor kappa B (*NF-κB*)	5'- CTTCCTCAGCCATGGTACCTCT -3'	5'- CAAGTCTTCATCAGCATCAAACTG -3'
Collagen type I	5'-CGATGGATTCCAGTTCGAGT-3'	5'-TTTTGAGGGGGTTCAGTTTG-3'
Collagen type III	5'-GTCCTATTGGTCCTCCTGGC-3'	5'-ACCAGGGAAACCAGCAGG-3'
Collagen type IV	5'-ATGGGGCCCCGGCTCAGC-3'	5'-ATCCTCTTTCACCTTTCAATAGC-3'
Fibronectin I	5'-CCGTGGGCAACTCTGTC-3'	5'-TGCGGCAGTTGTCACAG-3'
Hypoxia-inducible factor (HIF-1α)	5'-TTCACCTGAGCCTAATAGTCC-3'	5'-CAAGTCTAAATCTGTGTCCTG-3'
β-actin	5'-CCTGGCACCCAGCACAAT-3'	5'-GCCGATCCACACGGAGTACT-3'

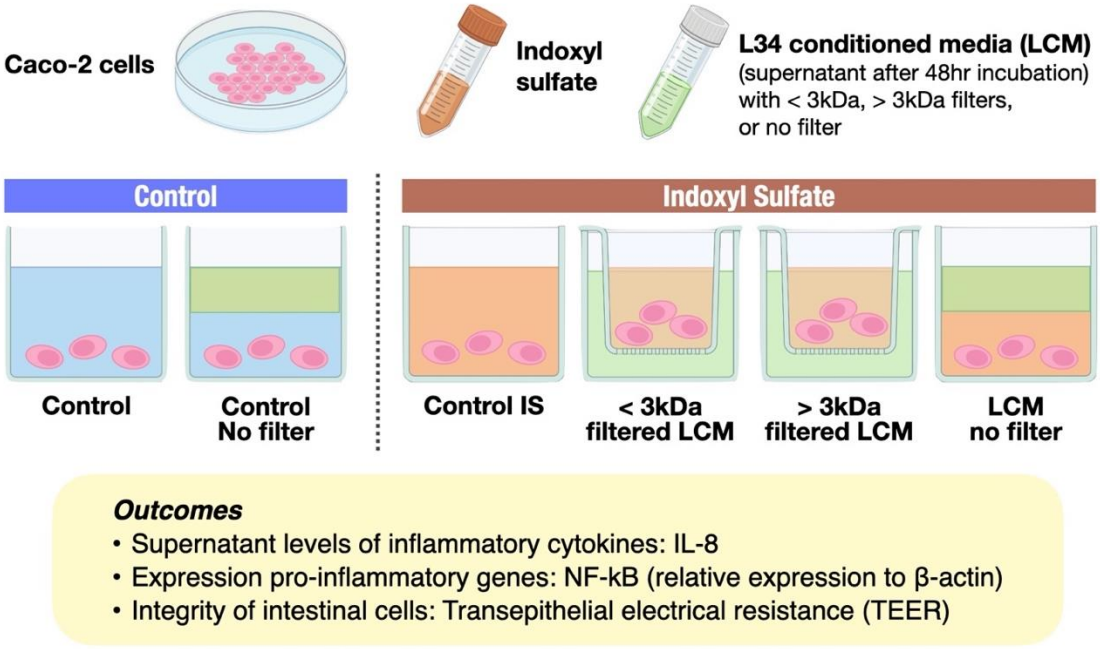

Figure 9 Schema of *in vitro* experiments on enterocytes (Caco-2 cells)

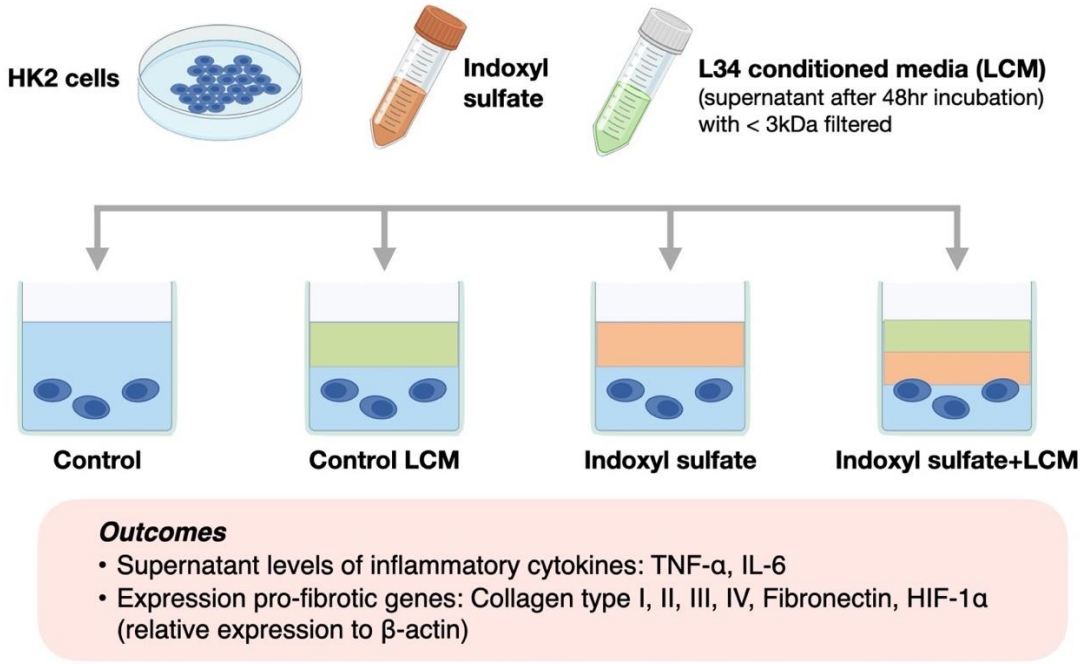

Figure 10 Schema of *in vitro* experiments on renal tubular cells (HK2 cells)

2. Characterization of the active anti-inflammatory molecules of LCM

The preliminary identification of active substances from LCM (<3kDa fraction) on anti-inflammation of HK2 cells[25] was performed (Figure 10). As such, the neutralization of LCM (<3kDa fraction) anti-inflammatory properties (attenuation in uremic toxin-induced TNF-α production by HK2 cells) after processing (enzyme inactivation and heat) before utilization indicated the biochemical properties of LCM. Accordingly, the enzyme sensitivity of LCM was tested by incubation with one of the various enzymes (Sigma-Aldrich), including α-amylase, lipase, proteinase K, and lysozyme. At a concentration of 1 mg/ml of LCM, each enzyme was incubated at 37°C (25°C for amylase and lysozyme) for 6 h and heated in a 100°C water bath for 10 min for enzyme inactivation. For thermal stability, the LCM was exposed to a 100°C water bath for 0.25, 0.5, 1, or 2 h before use. Then, processed LCM was tested for the suppressive activity on TNF-α production using HK2 cells (ATCC CRL-2190) as described above.

Statistical analysis

Analyzed data are presented as mean ± standard error (SE) using GraphPad Prism version 9.0 (La Jolla, CA, USA). Comparisons among groups were determined by one-way analysis of variance (ANOVA) after proving the three assumptions; normal distribution using Shapiro-Wilk test, homogeneity of variance using Bartlett's test, and

independence, followed by Tukey's analysis for multiple pairwise comparisons. Tukey's analysis would prevent the inflation of type I error and control the family-wise error rate (FWER) at specified level of 0.05 across such multiple pairwise comparisons. Any pair with t-score above the critical value was considered as having the Honestly Significant Difference (HSD). Comparison of resected kidney mass between the two 5/6 Nx groups was determined by an unpaired t-test. A *p*-value of <0.05 was considered statistically significant.

CHAPTER IV

RESULTS

Lactobacillus rhamnosus L34 attenuated kidney injury and kidney fibrosis in CKD mice

All mice were survived at 20 weeks of the observation. The weight of resected kidneys performed 6 weeks before initiation of the treatment, between the 5/6Nx-L34 group and the 5/6Nx-control group, did not differ significantly (figure 11). The uremic characteristics of 5/6 Nx mice were demonstrated by retardation of weight gain, anemia, increased serum creatinine and proteinuria at 6 and 20 weeks after surgery compared with sham controls (figure 12A-D). At 20 weeks after surgery, the percentage of weight gain and hematocrit (Hct) reduction in sham control and 5/6Nx mice were 43.85±11% versus 10.7±12%, and 3.74±11% and 19±12%, respectively (figure 12E-F). The changes in serum creatinine and proteinuria from baseline in sham control and 5/6 Nx mice were -0.04±0.07 versus 0.56±0.3 mg/dL, and 2.5±2.83 versus 31.9±9 mg/day, respectively (figure 12G, H). The renal histopathology showed more prominent fibrosis in the 5/6 Nx mice (figure 13A, B). These characteristics supported the CKD progression, the major feature of CKD in our 5/6 Nx model.[125]

After L34 administration for 14 weeks, the 5/6 Nx-L34 mice demonstrated less serum creatinine (0.60±0.15 versus 0.94±0.41 mg/dL, p=0.033) and proteinuria

(26.91±8.29 versus 36.9±8.38 mg/day, p=0.015) (figure 12C, D). L34 administration also attenuated area of kidney fibrosis (9.29±2.67 versus 16.01±6.23 %, p=0.004) (figure 13A, B) and percentage of global glomerulosclerosis (15.56±18.22 versus 41.67±11.11 %, p=0.013), when compared with 5/6 Nx-PBS mice.

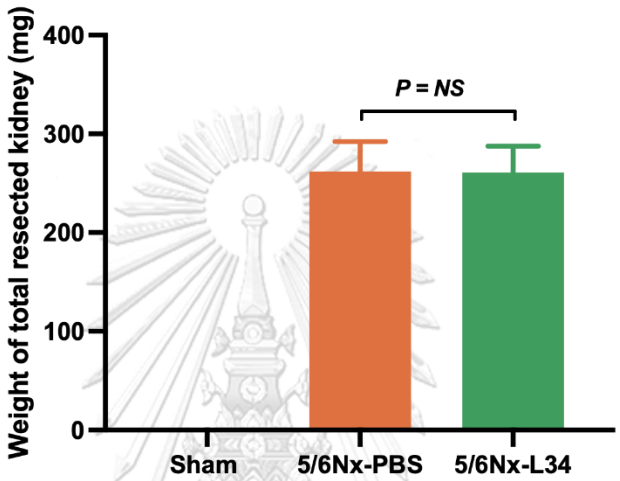

Figure 11 The weight of total resected kidney in each group

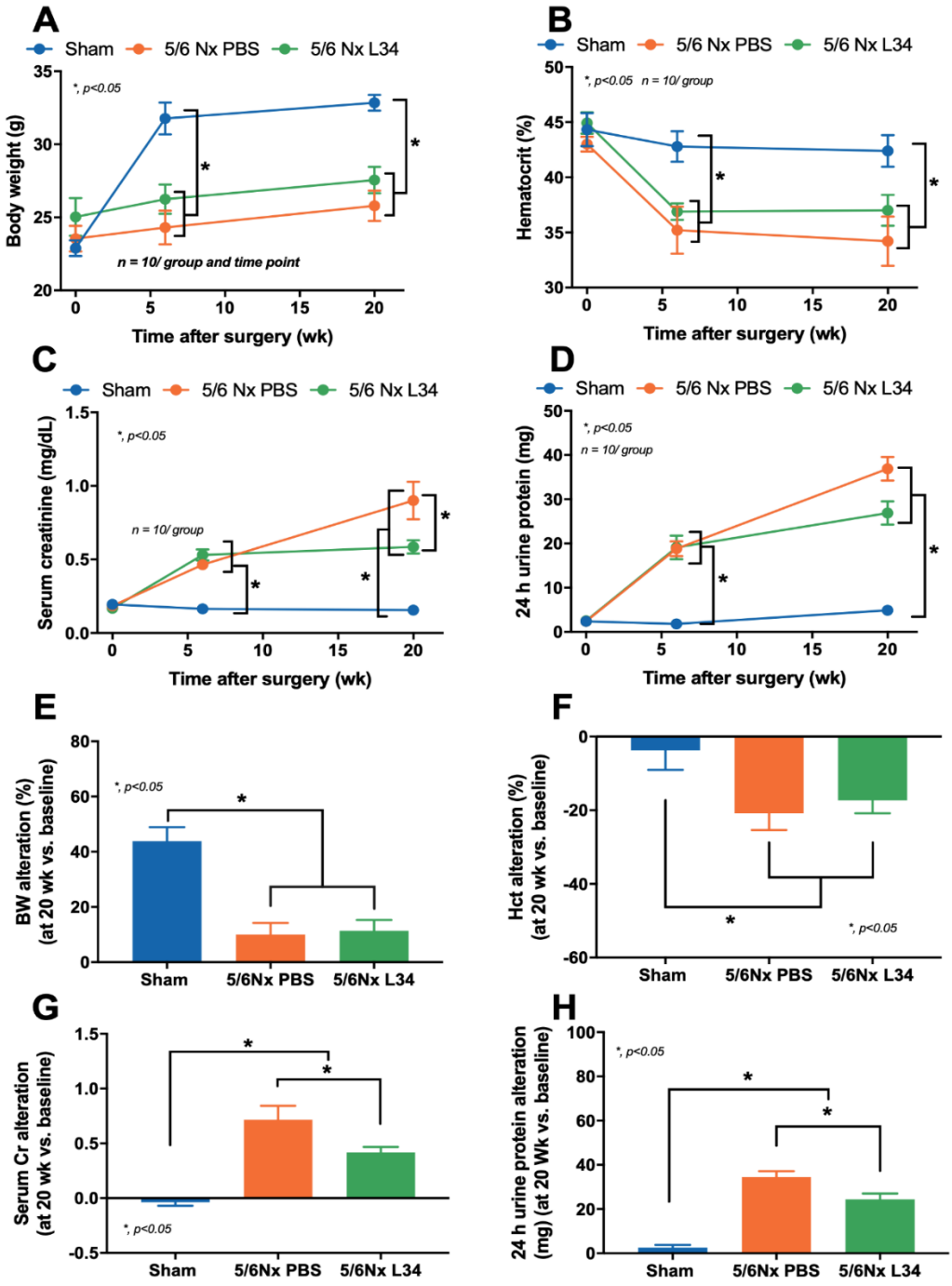

Figure 12 The characteristics of Sham control, 5/6 nephrectomy mice with phosphate buffer solution (5/6 Nx+PBS) or with Lactobacillus rhamnosus L34 (5/6 Nx+L34) as indicated by the time-point of body weight (A), hematocrit (B), serum creatinine (C), and 24-hour urine protein (D) (n=5 in Sham control, n=10 in 5/6 Nx+PBS and 5/6+L34 groups).

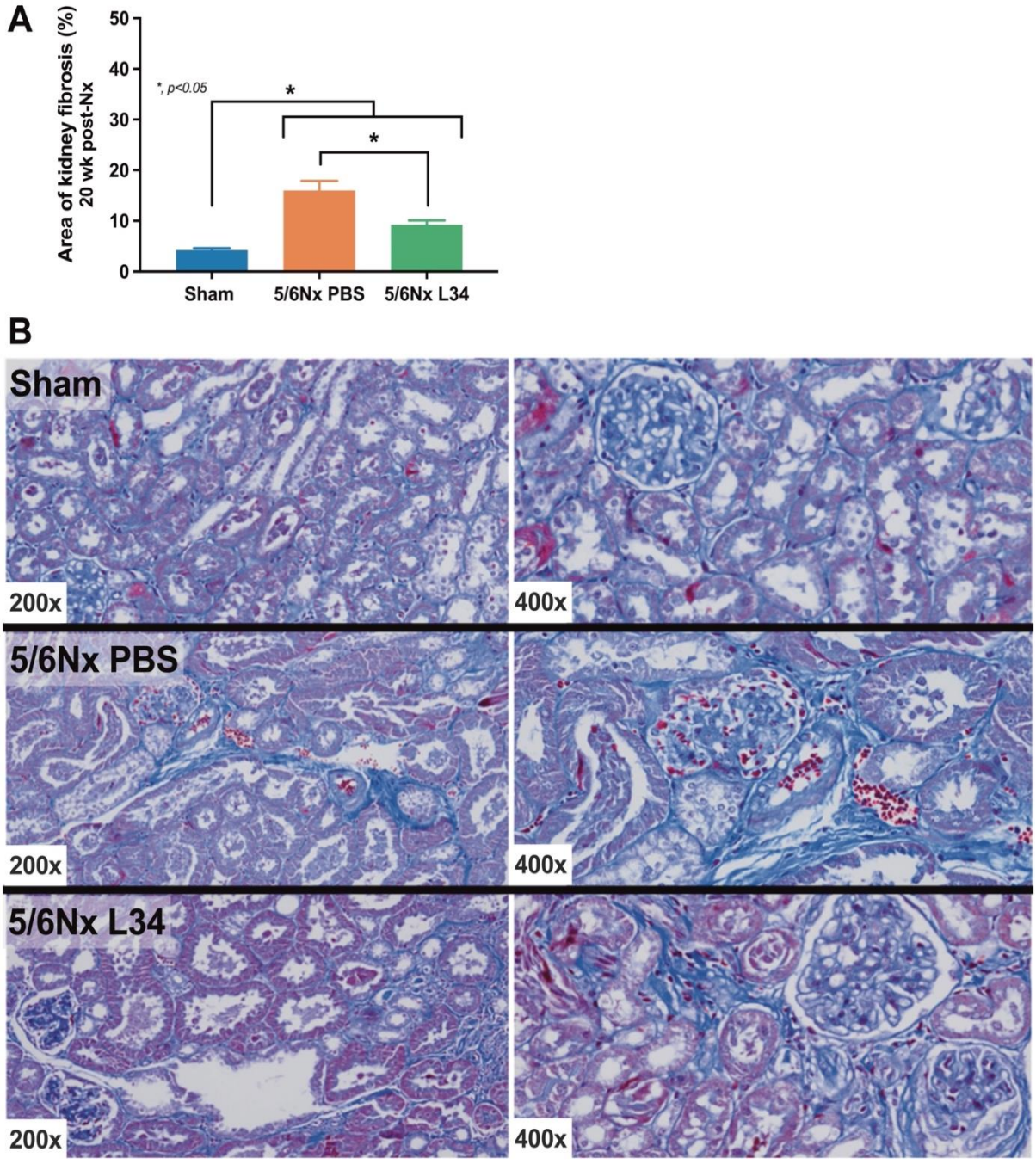

Figure 13 The characteristics of Sham control, 5/6 nephrectomy mice with phosphate buffer solution (5/6 Nx+PBS) or with Lactobacillus rhamnosus L34 (5/6 Nx+L34) as indicated by the area of kidney fibrosis with the representative Masson's Trichrome stained kidney histopathology (original magnification 200x, 400x) (A, B) (n=5 in Sham control, n=10 in 5/6 Nx+PBS and 5/6+L34 groups).

Lactobacillus rhamnosus L34 attenuated gut dysbiosis, gut leakage, and systemic inflammation in CKD mice

At 20 weeks post 5/6 Nx, there were i) gut leakage, as indicated by endotoxemia, FITC-dextran assay, and the reduction of Occludin, in the uremic cecum and colon (figure 14A-D), despite the normal intestinal histology (represented by caecum) (figure 14E) and ii) systemic inflammation (serum TNF-**α**) (figure 14F). L34 attenuated such gut leakage and systemic inflammation and decreased TMAO and IS (figure 14G-H).

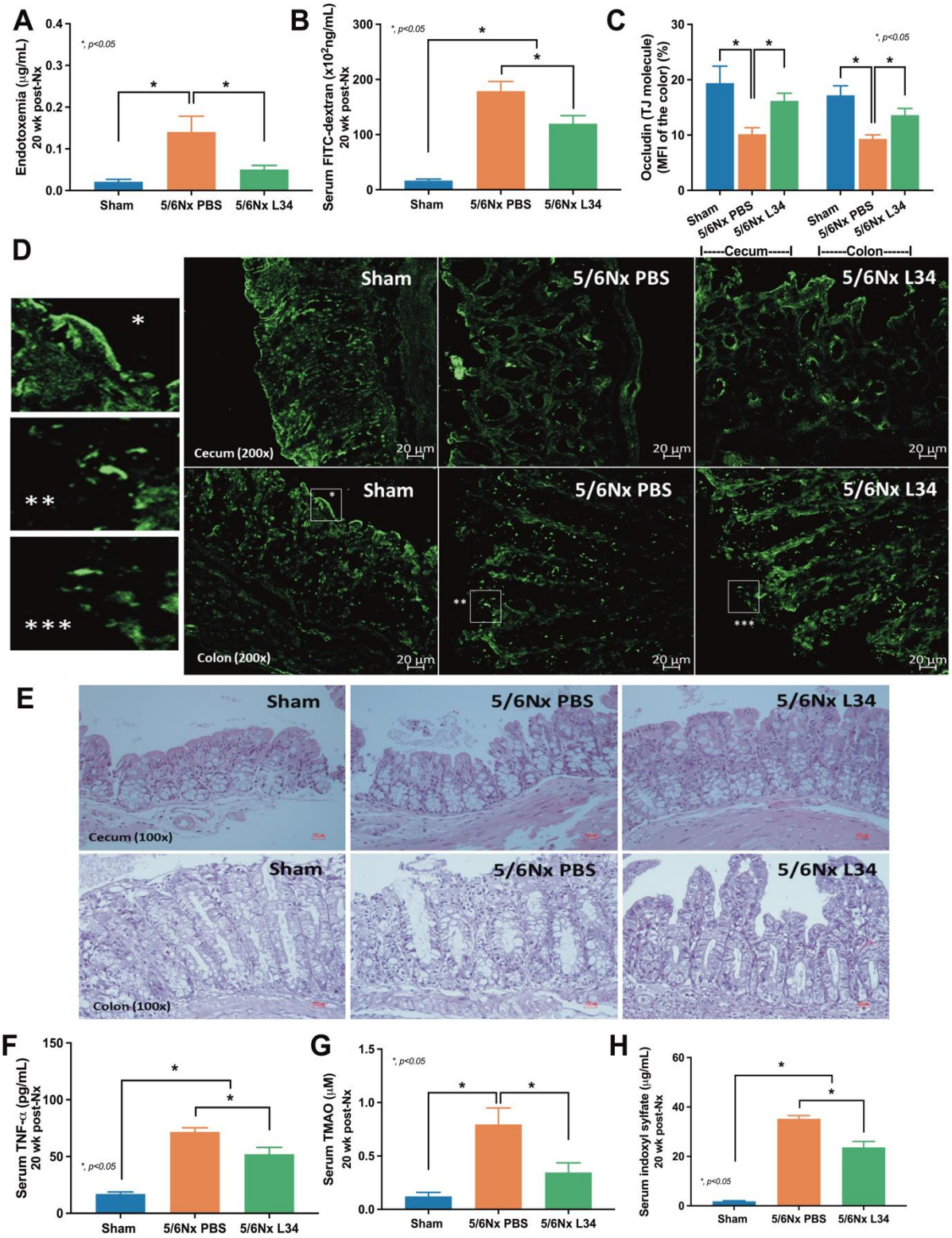
A
Endotoxemia (μg/mL) 20 wk post-Nx
*, p<0.05
Sham
5/6Nx PBS
5/6Nx L34
B
Serum FITC-dextran (x10²ng/mL) 20 wk post-Nx
C
Occludin (TJ molecule) (MFI of the color) (%)
I-----Cecum-----I
I-----Colon-----I
D
Sham
5/6Nx PBS
5/6Nx L34
Cecum (200x)
Colon (200x)
20 μm
E
Cecum (100x)
Colon (100x)
F
Serum TNF-α (pg/mL) 20 wk post-Nx
G
Serum TMAO (μM) 20 wk post-Nx
H
Serum indoxyl sylfate (μg/mL) 20 wk post-Nx

Figure 14 The characteristics of Sham control, 5/6 nephrectomy mice with phosphate buffer solution (5/6 Nx+PBS) or with Lactobacillus rhamnosus L34 (5/6 Nx+L34) as indicated by gut permeability defect (endotoxemia, FITC-dextran assay and area of Occludin tight junction molecule) with the representative fluorescent stained histology from cecum and colon (original magnification at 200x) (A-D), are demonstrated. The inset picture focuses on an apical area of the colon, showing the linear and granular fluorescent staining on Sham and 5/6 Nx mice, respectively. Additionally, the representative Hematoxylin & Eosin staining pictures (E), systemic inflammation (serum TNF-α) (F) and gut-derive uremic toxins; Trimethylamine N-oxide (TMAO) and indoxyl sulfate (G, H) (n=5 in Sham control, n=10 in 5/6 Nx+PBS and 5/6+L34 groups).

Fecal microbiome analysis in 5/6 Nx mice showed an increase in *Bacteroides* species, the most abundant Gram-negative anaerobes with potential pathogenicity,[31] with a decrease in *Firmicutes* species, the highest Gram-positive anaerobes in a healthy gut,[135] without alteration in *Proteobacteria* species (pathogenic bacteria) (figure 15A-E), and total Gram-negative bacteria (data not shown) when compared with sham controls. L34 administration attenuated uremia-induced gut dysbiosis as indicated by increased *Firmicutes* and reduced *Bacteroides* and total abundance of Gram-negative bacteria without affecting Proteobacteria (figure 15A-E). Notably, the alpha diversity and observed taxonomy units (OTUs) were not different among groups (figure 15F).

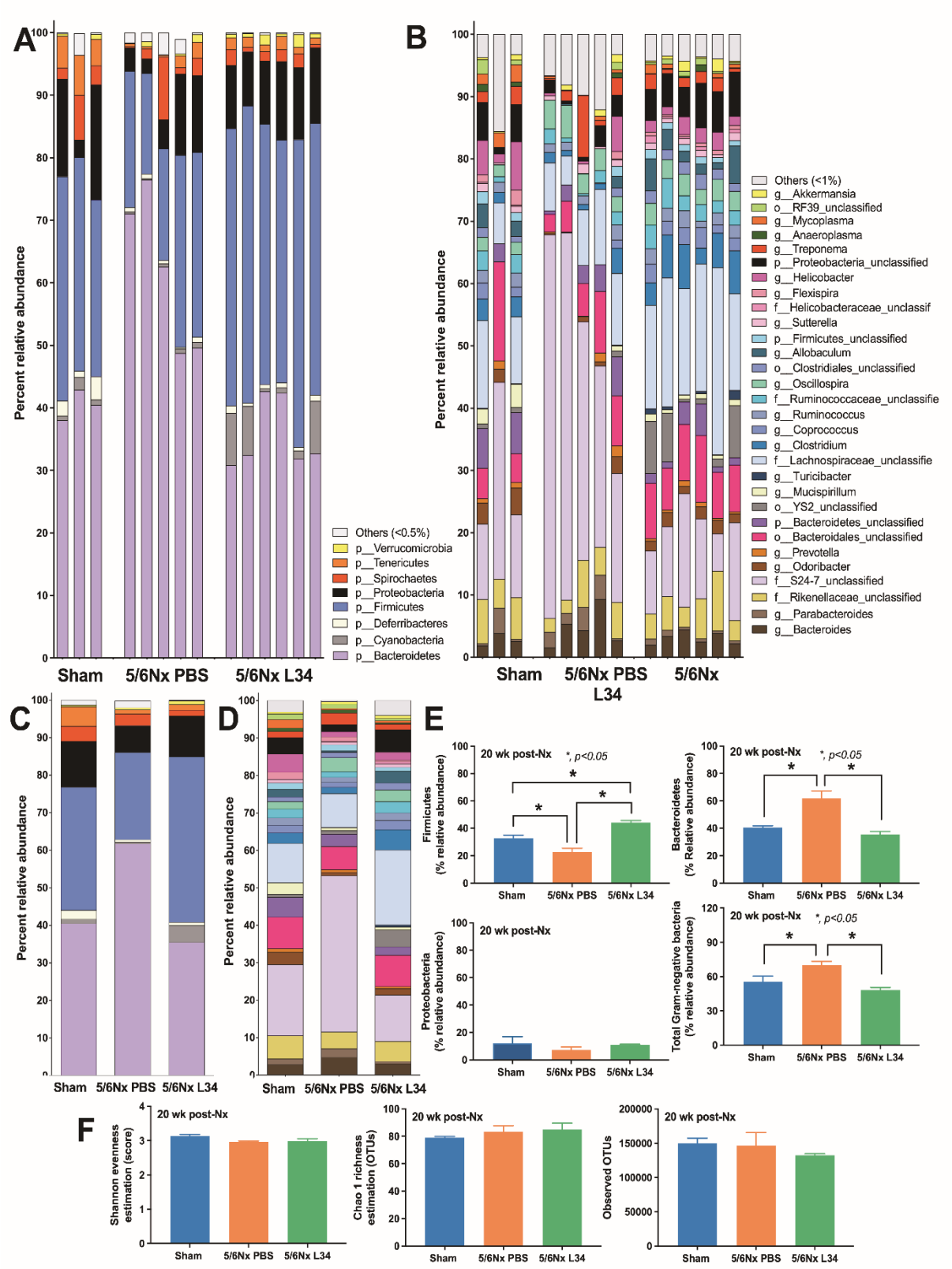
A
B
C
D
E
F
Percent relative abundance
Sham
5/6Nx PBS
5/6Nx L34
Others (<0.5%)
p__Verrucomicrobia
p__Tenericutes
p__Spirochaetes
p__Proteobacteria
p__Firmicutes
p__Deferribacteres
p__Cyanobacteria
p__Bacteroidetes
Others (<1%)
g__Akkermansia
o__RF39_unclassified
g__Mycoplasma
g__Anaeroplasma
g__Treponema
p__Proteobacteria_unclassified
g__Helicobacter
g__Flexispira
f__Helicobacteraceae_unclassif
g__Sutterella
p__Firmicutes_unclassified
g__Allobaculum
o__Clostridiales_unclassified
g__Oscillospira
f__Ruminococcaceae_unclassifie
g__Ruminococcus
g__Coprococcus
g__Clostridium
f__Lachnospiraceae_unclassifie
g__Turicibacter
g__Mucispirillum
o__YS2_unclassified
p__Bacteroidetes_unclassified
o__Bacteroidales_unclassified
g__Prevotella
g__Odoribacter
f__S24-7_unclassified
f__Rikenellaceae_unclassified
g__Parabacteroides
g__Bacteroides
20 wk post-Nx
*, p<0.05
Firmicutes (% relative abundance)
Bacteroidetes (% Relative abundance)
Proteobacteria (% relative abundance)
Total Gram-negative bacteria (% relative abundance)
Shannon evenness estimation (score)
Chao 1 richness estimation (OTUs)
Observed OTUs

Figure 15 The fecal microbiome analysis of Sham control, 5/6 nephrectomy mice with phosphate buffer solution (5/6 Nx+PBS) or with Lactobacillus rhamnosus L34 (5/6 Nx+L34) as indicated by the relative abundance in phylum (A) and in genus (B), the average relative abundance in phylum (C) and in genus (D), and the graph presentation of relative abundance in phylum levels (E) and the alpha diversity (Shannon and Chao estimation) with total observed taxonomy units (OTUs) (F)

Lactobacillus rhamnosus L34 attenuated uremia-induced injury on enterocytes and renal proximal tubular cells

After incubation of IS in Caco-2 cells, there were increased inflammatory markers (supernatant IL-8 and expression of *IL-8* and *NF-kB*) and decreased cell integrity (TEER) (figure 16A-D). It is possible that L34 produce both low MW molecules that might pass through uremic gut to kidneys and higher MW molecules that might affect enterocytes. Thus, LCM with or without filtration by 3kDa cut-off filter was used for *in vitro* experiments. Accordingly, all fractions of LCM (<3kDa, >3kDa, and non-filtered LCMs) attenuated GDUT-induced effects on Caco-2 enterocytes (pro-inflammation and TEER) (figure 16A-D). Because of the possible delivery of low MW molecules from LCM during gut leakage, only <3kDa-filtered LCM was tested in the HK2 cell experiment. The addition of IS to HK2 cells caused increased inflammatory responses (supernatant TNF-α and IL-6) (figure 17A, B), and upregulated expression of pro-inflammatory genes and pro-fibrotic genes, including *TNF-α*, *IL-6*, *Collagen type III* and *type IV*, *fibronectin,* and

Hypoxia-inducible factor (*HIF-1α*), but not *Collagen type I* (figure 17C-I). Interestingly, incubation of <3kDa-filtered LCM attenuated such IS-induced HK2-cell injuries (figure 17A-I). To explore the chemical nature of the active molecule from <3kDa-filtered LCM on IS-induced HK2 cells, the anti-inflammatory properties of LCM after several treatments were determined. Interestingly, the structure of active molecules against IS in <3kDa-filtered LCM were heat-labile polysaccharides and protein (possibly the glycoproteins) because the anti-inflammatory property was neutralized by either amylase and proteinase enzymes or heat exposure (figure 17J, K).

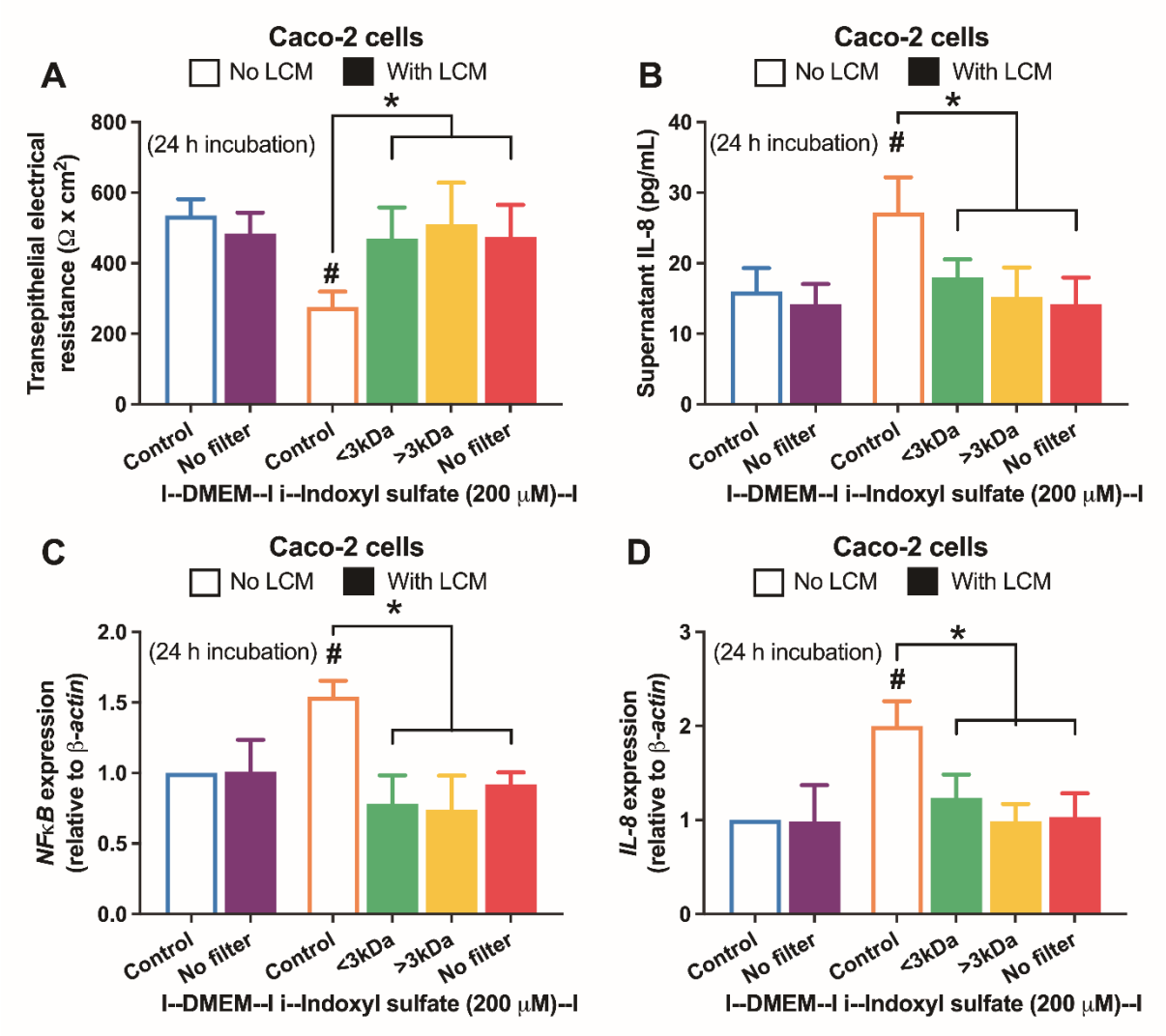

Figure 16 The characteristics of enterocytes (Caco-2 cells) after 24 h incubation by the culture media control (using DMEM; Control) or Lactobacillus condition media (LCM), after treated by 3 kDa filtered (<3kDa LCM and > 3kDa LCM) or no filter, with control (DMEM) or with indoxyl sulfate uremic toxin activation (200 μM) as indicated by transepithelial electrical resistance (TEER), supernatant IL-8, gene expression of *NF-**KB*** and *IL-8* (A-D)

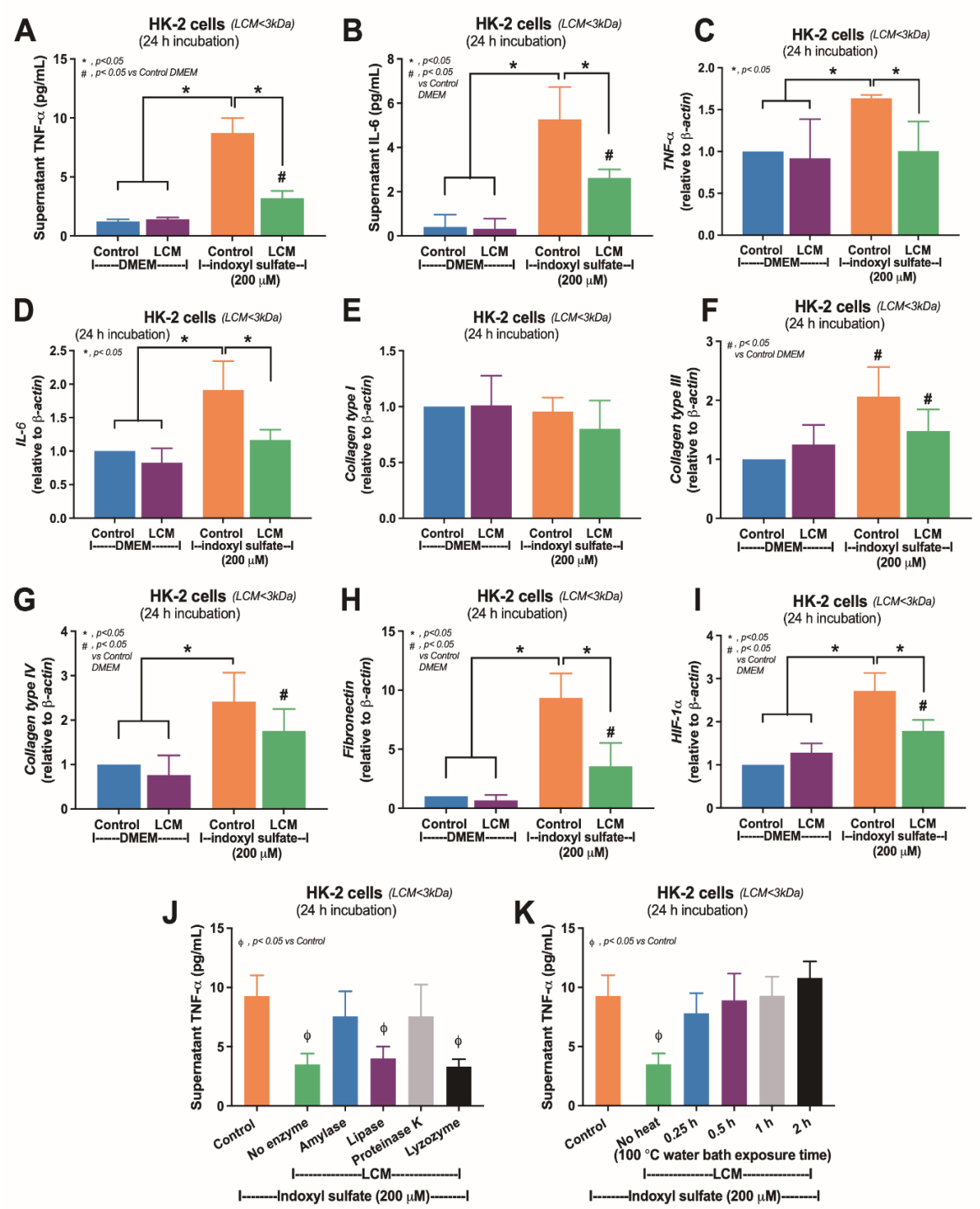

Figure 17 The characteristics of renal tubular cells (HK2 cells) after 24 h incubation by the culture media (Control) or indoxyl sulfate uremic toxin (200 μM) with 3 kDa filtered Lactobacillus condition media (LCM) or control as indicated by supernatant cytokines (TNF-**α** and IL-6) (A, B), and the expression of several genes,

including *TNF-α, IL-6, collagen type III* and *type IV, fibronectin*, and *hypoxia-inducible factor 1-α (HIF-1α)*, (C-H)

CHAPTER V

DISCUSSION

Gut dysbiosis, gut leakage, systemic inflammation, and CKD progression in 5/6 Nx mice

After 5/6 Nx, mice developed retardation of weight gain, anemia, azotemia, proteinuria, and significant renal fibrosis that supported advanced stage of CKD.[125] Alteration in gut microbiome in these mice, including decreased bacterial flora and increased pathogenic bacteria, supported CKD-induced gut dysbiosis.[46] Moreover, 5/6 Nx mice demonstrated loss of Occludin and increased FITC-dextran uptake. There was an increase in circulating GDUTs and endotoxin through impaired renal clearance and uremia-induced gut leakage (gut translocation of large MW toxins), respectively. Increased serum TNF-α level also indicated enhanced systemic inflammatory responses. Although our results support CKD induced gut dysbiosis and gut leakage, gut bacterial translocation, using 16S ribosomal DNA (16S rDNA) in blood, and the association between GDUTs and CKD staging could not be demonstrated in patients.[136, 137] Further studies on the gut-kidney axis are still in need.

Lactobacillus rhamnosus L34 attenuated uremia-induced gut dysbiosis and CKD progression in 5/6 Nx mice

Administration of L34 in 5/6Mx mice attenuated i) uremia-induced gut dysbiosis and gut leakage, ii) systemic inflammation (GDUTs in serum, endotoxemia, and inflammatory markers), and iii) CKD progression (azotemia, proteinuria, and kidney fibrosis). These data supported the benefits of probiotics in patients with CKD, as previously published.[138, 139] In addition, *in vitro* adverse effects of IS on Caco-2 and HK2 cells were minimized in LCM. Although our findings were similar to previous observations that advanced CKD induces gut dysbiosis,[7] gut leakage,[8, 9] circulating GDUTs, endotoxemia,[8, 11] and inflammatory markers[10] resulting in the accelerated CKD progression,[12] an effect of L34 on improved renal histology implied a prominent effect of L34 on CKD, which were different from other studies. Increased intestinal excretion of accumulated uremic toxins in CKD could induce gut dysbiosis by promoting the growth of pathogenic bacteria. Increased GDUTs and enhanced systemic inflammation exert injuries to vascular endothelium[140] and renal parenchymal cells.[50] These effects cause a vicious cycle of uremic toxin-induced gut dysbiosis, and dysbiosis further enhances GDUTs, worsening gut leakage, systemic inflammation, and CKD progression.

Although probiotic has been proven to reduce certain GDUTs in animal experiments,[112, 113] clinical studies,[20, 21, 104, 105, 141, 142] and in meta-analysis,[20, 98] only a few studies[21] demonstrated capacity of delaying CKD progression. Interestingly, the

beneficial effects of L34 were strong enough to delay CKD progression in 5/6 Nx mice, as indicated by decreased severity in renal histopathology. Although mechanisms of L34 in attenuation of CKD progression and kidney fibrosis is unclear, it is possible to be associated with the anti-inflammatory effect of L34 on gut dysbiosis and direct effects on intestinal and renal parenchymal cells. With probiotics-attenuated gut dysbiosis, reduction in GDUT production and subsequent decrease in circulating GDUTs were observed (figure 14G, H). Attenuation of endotoxemia (figure 14A) without alteration in total fecal Gram-negative bacteria after probiotic administration suggested the limited gut translocation of endotoxin (MW 50-100 kDa), but not the reduced endotoxin in gut contents. Likewise, L34 attenuated gut leakage as indicated by reduced intestinal absorption of FITC-dextran, improved tight junction molecule (figure 14B-D), and strengthened enterocyte barriers as indicated by TEER (figure 16A). These findings support the concept of probiotic-attenuating gut leakage in CKD.[46] L34 also reduce the level of some toxins, which are small enough to pass through normal intestinal tight junction (<0.6 kDa).[12]

Anti-inflammatory effect of *Lactobacillus rhamnosus* L34 against uremia-induced cell injury in enterocytes and renal tubular cells

Several uremic toxins induce systemic inflammation, uremic enteropathy, and CKD progression.[4, 11] IS reduced Caco-2 cell integrity (TEER) through pro-inflammatory

responses (supernatant IL-8) from up-regulated *NF-**K**B* transcriptional factor that might be responsible for gut leakage in CKD mice. Although IS activation on Caco-2 cells may differ from IS impact in mice because IS is converted from indole by the liver and distributed to enterocytes through blood circulation, possibly only at the basolateral side of the cells,[9, 16] our findings provide proof of concept on the uremia-induced intestinal injury. In parallel, IS activated inflammation, including *NF-**K**B* and *HIF-1**α*** expression, and facilitated collagen production in HK2 cells as demonstrated by up-regulation of several pro-fibrotic genes as a previous publication.[143] While ischemia up-regulated *HIF-1**α*** (a key molecule for cell survival after injury) in proximal tubular cells,[144] IS also accelerates *HIF-1**α*** levels here. On the other hand, an elevation of *HIF-2* in peritubular interstitial fibroblast-like cells facilitated Erythropoietin (EPO) production that maintains Hct[145] and IS dysregulates oxygen metabolism (and possibly HIF-2 production) that might lead to renal anemia.[146, 147] Moreover, IS also influence other molecules, including aryl hydrocarbon receptor (Ahr) and EPO production,[146, 148] which might be affected by LCM. Hence, more exploration on the effect of LCM on IS-induced injury in other aspects (such as Ahr and anemia) in the proper cells will be interesting.

Additionally, IS promotes the production of reactive oxygen species that directly induce cell damage and apoptosis. Reduction in supernatant pro-inflammatory cytokines in Caco-2 cells and decrease in cytokine production and pro-

fibrotic genes in HK2 cell experiment after addition of LCM indicated the therapeutic effect of L34 on IS-induced cytotoxicity to intestinal and renal epithelial cells. All fractions of LCM (<3 kDa, >3k Da, or non-filtered fractions) attenuated IS-induced inflammatory responses in Caco-2 cells, implying the production of anti-inflammatory substances in varying MW.[25] Although gut translocation of probiotics-derived substances with MW >3 kDa (endotoxin) is possible in 5/6 Nx mice, the majority of molecules that enter systemic circulation (and kidneys) would preferentially be <3 kDa. Despite a technical limitation in verifying the molecular sizes of the filtrated-LCM, LCM with the <3kDa fraction reduced IS-induced HK2 cell damage (up-regulation of cytokines and pro-fibrotic genes), suggesting proof of concept for the potential benefits of small molecules produced from L34, which might be the molecules with polysaccharide and protein (or glycoproteins) structure. Because the anti-inflammatory molecules for LPS-activated enterocytes from non-filtered LCM were carbohydrate molecules[149] and benefits of heat-killed probiotics are also mentioned,[150, 151] the effective molecules of probiotics might depend on the insults and the specific fractions of probiotics. Hence, the extraction and utilization of the beneficial molecules from the proper fraction of probiotics are interesting for further exploration as the new chemical drugs which are easier and less expensive for clinical usage than the viable probiotics.

Hence, increased systemic inflammation and kidney fibrosis through uremia-induced gut leakage in 5/6 Nx mice and attenuation by L34 supported a causal

relationship between gut dysbiosis (and gut leakage) with inflammation-induced kidney fibrosis. Our working hypothesis of the beneficial effect of probiotics on CKD (figure 17) consists of the effects on attenuation of uremia-induced gut leakage and GDUTs,[10] and the possible delivery of probiotics-producing anti-fibrotic substances to the kidney through gut leakage. Although gut leakage initiates gut translocation of several harmful pathogen molecules, taking advantage of gut leakage as an additional delivery method of some beneficial molecules or by some other interventions might be a captivating strategy. Also, some other available probiotics might also directly produce anti-fibrotic substances, the combination of such probiotics or extraction of anti-fibrotic molecules is interesting.

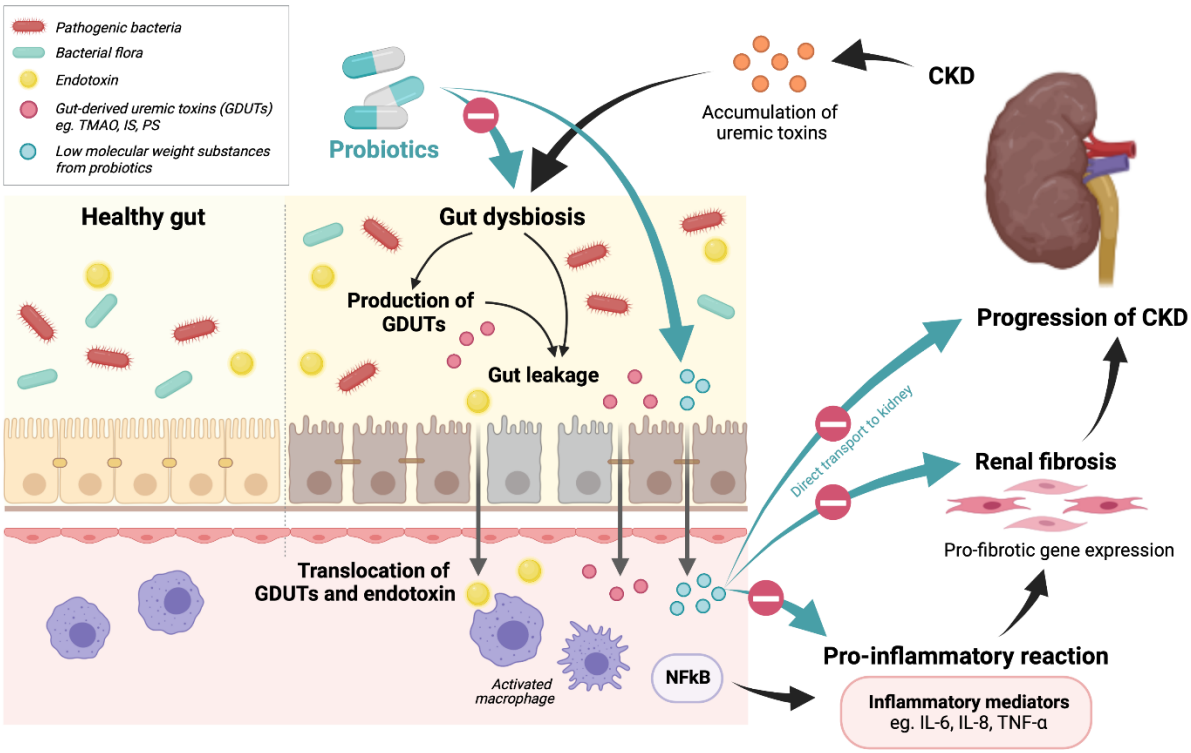

Figure 18 The proposed mechanism of the gut-kidney axis.

Uremic toxin accumulation in CKD leads to an increase in pathogenic bacteria and a decrease in bacterial flora, so-called gut dysbiosis. The pathogenic bacteria induce the production of gut-derived uremic toxins (GDUTs). Both gut dysbiosis and gut-derived uremic toxins impair intestinal tight junction integrity, so-called gut leakage, which leads to translocation of GDUTs and endotoxin into the systemic circulation. Circulating GDUTs and endotoxin stimulate macrophages to activate *NF-kB* transcription factor, which would induce pro-inflammatory reactions. Subsequently, the expression of pro-fibrotic genes was upregulated. The worsening of renal parenchymal fibrosis is characterized as the progression of CKD, continuing the vicious cycle. Administration of probiotics reduce gut dysbiosis and release low molecular weight substances (LMWS), which inhibit pro-inflammatory reaction and expression of pro-fibrotic genes, leading to disruption of the vicious cycle before proceeding to the progression of CKD. IS, indoxyl sulfate; PS, p-cresol

In CKD mice, the population is uniform, and all the confounders that would affect the progression of kidney fibrosis are considered and controlled. However, there are many more contributable factors and variations among the human population, such as the etiologies of CKD, lifestyle, adherence to the treatments, and environmental factors. Finding the proper prescription, which consists of the appropriate dose and the adequate duration of therapy, would yield further benefits after maximizing the necessary treatments with low adverse effects and low costs. Thus, despite the benefits of L34 in vivo and in vitro from the current data, clinical studies for a potential application in CKD and further mechanistic studies to explore other reno-protective mechanisms of probiotics-derived molecules are warranted.

Conclusion

Lactobacillus rhamnosus L34, derived from the Asian population, attenuated GDUTs, gut leakage, and systemic inflammation that provided the reno-protective effects in the CKD model. For the L34 benefits on renal tubular cells, L34 seems to have both direct renal impact (anti-inflammation on HK2 cells with <3kDa LCM fraction) and indirect renal influence through endotoxemia attenuation, partly through improved dysbiosis and reduced endotoxin in fecal contents (decrease in total Gram-negative bacteria). Hence, probiotics would have an important therapeutic role in retarding CKD progression in the future.

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PUBLICATION	1. Tungsanga S, Katavetin P, Panpetch W, Visitchanakun P, Saisorn W, Praditpornsilpa K, Eiam-Ong S, Tungsanga K, Tumwasorn S, Leelahavanichkul A. Lactobacillus rhamnosus L34 Derived from Thai Population Reduced Gut-derived Uremic Toxins and Attenuate Chronic Kidney Disease Progression in 5/6 Nephrectomy Mouse Model. Nephrol Dial Transplant. 2022;gfac032. 2. Tungsanga S, Panpetch W, Bhunyakarnjanarat T, Udompornpitak K, Katavetin P, Chancharoenthana W, Chatthanathon P, Somboonna N, Tungsanga K, Tumwasorn S, Leelahavanichkul A. Uremia-induced gut barrier defect in 5/6 nephrectomized mice is worsen by Candida administration through a synergy of uremic toxin, lipopolysaccharide, and (13)-β-D-glucan, but is attenuated by Lacticaseibacillus rhamnosus L34. Int J Mol Sci. 2022;23:2511. 3. Kulvichit W, Sarnvanichpitak K, Peerapornratana S, Tungsanga S, Lumlertgul N, Praditpornsilpa K, Tungsanga K, Eiam-Ong S, Kellum JA, Srisawat N. In-hospital Mortality of Critically Ill Patients with Interactions of Acute Kidney Injury and Acute Respiratory Failure in the Resource-limited Settings: Results from SEA-AKI Study. J Crit Care.

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Samransamruajkit R, Tungsanga S, Tiankanon K, Lumlertgul N, Leelahavanichkul A, Sriphojanart T , Tantawichien T, Thisyakorn U, Chirathaworn C, Praditpornsilpa K, Tungsanga K, Eiam-Ong S, Sitprija V, Kellum JA, Srisawat N. Endotoxemia and circulating bacteriome in severe COVID-19 patients. Intensive Care Med. 2020;8(1):72.

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AWARD RECEIVED

- Young Investigator Award, The 19th Asia Pacific Congress of Nephrology 2021, Asia Pacific Society of Nephrology.
- Excellent Award in the category of MSc/PhD students, Graduate Research Competition 2020, Graduate Affairs, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.
- Chief Fellow, Division of Nephrology, Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.
- Oral presentation: session of acute kidney injury ASN Kidney Week 2019, San Diego, American Society of Nephrology.
- Best oral presentation of nephrology session, Royal College Physicians of Thailand 2018.
- Best research oral presentation of residents, Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand 2018.
- Very good award in the category of Residents, Graduate Research Competition 2018, Graduate Affairs, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.
- Chief Resident, Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.
- Chief Intern, Sawanpracharak hospital, Nakhon Sawan, Thailand