Dietary Fiber Protects against Diabetic Nephropathy through Short-Chain Fatty Acid–Mediated Activation of G Protein–Coupled Receptors GPR43 and GPR109A

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ABSTRACT

Background Studies have reported "dysbiotic" changes to gut microbiota, such as depletion of gut bacteria that produce short-chain fatty acids (SCFAs) through gut fermentation of fiber, in CKD and diabetes. Dietary fiber is associated with decreased inflammation and mortality in CKD, and SCFAs have been proposed to mediate this effect.

Methods To explore dietary fiber's effect on development of experimental diabetic nephropathy, we used streptozotocin to induce diabetes in wild-type C57BL/6 and knockout mice lacking the genes encoding G protein–coupled receptors GPR43 or GPR109A. Diabetic mice were randomized to high-fiber, normal chow, or zero-fiber diets, or SCFAs in drinking water. We used proton nuclear magnetic resonance spectroscopy for metabolic profiling and 16S ribosomal RNA sequencing to assess the gut microbiome.

Results Diabetic mice fed a high-fiber diet were significantly less likely to develop diabetic nephropathy, exhibiting less albuminuria, glomerular hypertrophy, podocyte injury, and interstitial fibrosis compared with diabetic controls fed normal chow or a zero-fiber diet. Fiber beneficially reshaped gut microbial ecology and improved dysbiosis, promoting expansion of SCFA-producing bacteria of the genera *Prevotella* and *Bifidobacterium*, which increased fecal and systemic SCFA concentrations. Fiber reduced expression of genes encoding inflammatory cytokines, chemokines, and fibrosis-promoting proteins in diabetic kidneys. SCFA-treated diabetic mice were protected from nephropathy, but not in the absence of GPR43 or GPR109A. *In vitro*, SCFAs modulated inflammation in renal tubular cells and podocytes under hyperglycemic conditions.

Conclusions Dietary fiber protects against diabetic nephropathy through modulation of the gut microbiota, enrichment of SCFA-producing bacteria, and increased SCFA production. GPR43 and GPR109A are critical to SCFA-mediated protection against this condition. Interventions targeting the gut microbiota warrant further investigation as a novel renoprotective therapy in diabetic nephropathy.

Diabetic nephropathy (DN) is the leading cause of CKD globally and continues to grow in incidence and prevalence. Although hyperglycemia has historically been considered the driving force behind diabetic complications, increasing evidence points to chronic inflammation as having a central role.[1–4] Intensive glycemic control, blockade of the renin-angiotensin-aldosterone system, and use of SGLT2 inhibitors can delay disease progression in diabetic

nephropathy.[5,6] However, these regimes provide only partial therapeutic effect, highlighting the need for further insights into the pathogenesis of diabetic nephropathy to mitigate the residual risk of progression to ESKD.

The gut microbiota evolves and exists in a symbiotic relationship with host, contributing to a plethora of physiologic functions including regulation of the gut barrier, development and maintenance of immune responses and energy metabolism.[7] Disturbance of the normal gut microbiota (dysbiosis) has been implicated in the pathogenesis of obesity, diabetes, autoimmunity, inflammatory bowel disease, and more recently, kidney disease.[8] Multiple studies have consistently demonstrated quantitative and qualitative "dysbiotic" changes to the gut microbiota in CKD and diabetes, which disturb the symbiotic relationship. The hallmark of this dysbiosis is depletion of short-chain fatty acid (SCFA)–producing bacteria, and in the case of CKD, an increase in pathobionts contributing to uremic toxicity.[9] Production of uremic toxins, translocation across an impaired intestinal barrier and subsequent activation of the innate immune system with systemic inflammation, are some of the proposed mechanisms by which the gut microbiota may potentiate kidney disease.[10] We have previously demonstrated the integral role innate immunity and inflammation play in progression of diabetic nephropathy through TLR2/4, and the potential for blockade of ligand-TLR interactions to prevent diabetic kidney injury and inflammation.[3,4,11]

Diet remains the largest exogenous determinant of gut microbiota composition[12] and has garnered interest as a therapeutic avenue to re-establish symbiosis. Epidemiologic studies have demonstrated a link between higher dietary fiber intake and lower risk of inflammation and mortality, particularly in those with CKD.[13] In clinical and experimental studies, the gut microbiome has been shown to determine clinical responses to immune-based anticancer therapies.[14–16] Susceptibility to develop experimental type 1 diabetes[17] was also shown to be microbiome dependent and manipulation of the microbiome was shown to alter susceptibility. SCFAs, the predominant metabolites produced by bacterial fermentation of dietary fiber, are proposed to play a key role in microbiota–host crosstalk. SCFAs have been demonstrated to reduce inflammatory responses in kidney ischemia reperfusion injury[18] and modulate inflammation *in vitro*.[19,20] Pathways regulated by the microbiome in these studies are known to be critically important in the pathogenesis of diabetic nephropathy and CKD; however, the role of the microbiome in diabetic nephropathy has not been elucidated, and evidence that manipulation of the microbiome may retard nephropathy is currently lacking.

In this study, we tested the hypothesis that gut dysbiosis contributes to the pathogenesis of diabetic nephropathy. Using a murine model, we sought to determine whether dietary modification or supplementation, both designed to increase SCFAs, would attenuate renal inflammation and retard the development of diabetic nephropathy. Here, we show for the first time that beneficial reshaping of the gut microbiota

Significance Statement

The gut microbiota and its metabolites, in particular short-chain fatty acids derived from gut microbes' fermentation of fiber, are emerging therapeutic targets for systemic inflammatory and metabolic diseases, including diabetic nephropathy. The authors report that high-fiber diets or supplementation with short-chain fatty acids (acetate, butyrate, or propionate) afforded protection against development of kidney disease in diabetic mice. Dietary fiber restored gut microbial ecology, corrected "dysbiotic" changes, and increased production of short-chain fatty acids. Mice deficient in the metabolite-sensing G protein–coupled receptors GPR43 or GPR109A were not protected by short-chain fatty acids, suggesting that protection was mediated by downstream binding to these receptors. Tapping into the metabolic potential of the gut microbiota through diet may offer a novel approach to address diabetic nephropathy.

prevents nephropathy through the activation of SCFA-sensing receptors.

METHODS

Animals

Wild-type (WT) C57BL/6 mice were obtained from the Animal Resource Centre (Perth, Western Australia). $Gpr43^{-/-}$ and $Gpr109A^{-/-}$ mice on a C57BL/6 background were bred and maintained in our facility. Mice were housed in a specific pathogen-free facility at the University of Sydney. Male mice aged 7–9 weeks were used in experiments. Animal care and experimental protocols were approved by the University of Sydney Animal Ethics Committee.

Induction of Diabetes

Diabetes was induced with intraperitoneal injections of streptozotocin (STZ), 55 mg/kg daily for five consecutive days. Age-matched littermates serving as nondiabetic controls received volume- and pH-matched citrate buffer. Mice with a blood glucose level >20 mmol/L were used to assess diabetic kidney injury. Blood glucose level was measured with an Accu-Check glucometer (Roche Diagnostics) using tail vein blood. All mice were euthanized 12 weeks after injection.

Diets and SCFA Treatment

All custom fiber-adjusted diets were purchased from Specialty Feeds, Australia, and on the basis of modifications to normal chow (NC) diet (AIN93G). Resistant starch (RS) diet (SF11–025) is enriched with 63.6% RS, High-fiber (HF) diet (SF11–029) is enriched in guar gum and cellulose (35% crude fiber), and zero-fiber (ZF) diet (SF09–128) is completely devoid of fiber (Supplemental Table 1). Mice received NC upon arrival at our facility, followed by randomization to specific diets. Sodium acetate (SA) 100 mM, sodium butyrate (SB) 50 mM, and sodium propionate (SP) 100 mM (Sigma-Aldrich) were dissolved and administered *ad libitum* in drinking water. Control mice received pH- and sodium-matched water. Diets and drinking solutions were refreshed three times per week.

Study Design

Diet Experiment

To assess the influence of dietary fiber supplementation, 3 weeks after STZ injection diabetic WT mice were randomized to four specific diet groups (with five mice per nondiabetic control group): (1) DN+RS, $n=5$; (2) DN+HF, $n=10$; (3) DN+NC, $n=9$; and (4) DN+ZF, $n=9$.

SCFA Experiment

WT diabetic mice were maintained on the control diet and randomized to receive acetate or butyrate in drinking water from 3 weeks after STZ injection, in the following groups (with five mice per nondiabetic control group): (1) DN+SA, $n=10$; (2) DN+SB, $n=10$; (3) DN+SP, $n=10$; and (4) DN+ water $n=10$.

Mechanistic Experiments

Diabetic mice deficient in G protein–coupled receptors (GPRs) GPR43 and GPR109A were randomized to RS diet or SCFA treatment at 3 weeks after STZ injection in the following groups: (1) $Gpr43^{-/-}$+SA, $n=10$; (2) $Gpr43^{-/-}$+ water, $n=10$; (3) $Gpr109A^{-/-}$+SB, $n=11$; (4) $Gpr109A^{-/-}$+ water, $n=10$; (5) WT B6+ water, $n=10$; (6) $Gpr43^{-/-}$+RS, $n=10$; (7) $Gpr43^{-/-}$+NC, $n=10$; (8) WT B6+RS, $n=10$; and (9) WT B6+NC, $n=10$.

Sample Collection

Blood, urine, and kidney tissue were harvested at 12 weeks as previously described.[11] Feces were collected under sterile conditions, frozen on dry ice immediately after collection, and stored at $-80°C$.

Quantification of Urinary Albumin Excretion

Urinary albumin excretion was quantified using the Murine Albumin ELISA Quantitation Set (Bethyl Laboratories) as described previously.[11] Urine creatinine was measured enzymatically by the Biochemistry Department of Royal Prince Alfred Hospital, Australia.

Histology

Periodic acid–Schiff (PAS) and picrosirius red staining were performed on 3 and 5 μm formalin-fixed kidney sections, respectively. Glomerular tuft area (A_G) was measured in 20 glomerular profiles per mouse, using DP2-BSW software v2.2 (OLYMPUS). Glomerular volume (V_G) was calculated using the following formula: $V_G=(\beta/\kappa) \times (A_G)^{3/2}$, where $\beta=1.38$ (shape coefficient for spheres) and $\kappa=1.1$ (size distribution coefficient).[21] Glomerular extracellular matrix was defined as the PAS-positive area quantified by image analysis software (ImagePro Premier 9). Interstitial collagen was assessed in a blinded fashion by point counting using an ocular grid at $\times 400$ magnification, in 20 consecutive fields.[22]

Immunohistochemistry

Staining for CD68$^+$ and Wilms Tumor 1 (WT-1) was performed on 7 μm acetone-fixed frozen sections after application of a biotin blocker system (DAKO). Staining for type 1 collagen (Col-1) was performed on formalin-fixed paraffin sections (5 μm) after deparaffinization and antigen retrieval by boiling in 10 mM sodium citrate buffer (pH 6). After blocking with 20% normal horse serum, primary antibodies rat anti-mouse CD68 (ABD Serotec Inc), rabbit anti-WT1 (Abcam), or rabbit anti–Col-1 (Abcam) were applied and incubated for 60 minutes. For visualization of bound primary antibodies, sections were incubated with secondary antibodies biotinylated anti-rat IgG or anti-rabbit IgG (BD Pharmingen). Vector stain ABC kit (Vector Laboratories Inc) was applied, followed by 3,3'-diaminobenzidine solution (DAKO), and counterstaining with Harris hematoxylin.

Quantification of Immunostaining

Macrophage (CD68$^+$) staining was assessed in a blinded manner by analysis of 20 consecutive high-power fields ($\times 400$ magnification) of renal cortex in each section. CD68-positive cells were counted using an ocular grid and expressed as cells per field. WT-1–positive cells were counted in 20 glomerular cross-sections (high-power fields, $\times 400$ magnification). Results were expressed as podocytes per glomerulus using the equation $N_{podglom}=N_{Vpodglom}\times V_{glom}$.[23]

Real-Time PCR

RNA was extracted from kidney tissue using TRIzol (Invitrogen). Complementary DNA was synthesized using oligo d(T) primers (Applied Biosystems) and the SuperScript III reverse transcription kit (Invitrogen). Complementary DNA was amplified in Universal Master Mix (Applied Biosystems) with gene-specific primers and probes, using the Roche Lightcycler 480 (Roche Applied Science). Specific TaqMan primers and probes used for TLR2, TLR4, IL6, IFNγ, TNFα, CCL2, CXCL10, TGFβ1, fibronectin, and GAPDH were obtained from Applied Biosystems. Results were normalized to GAPDH expression.

Bacterial DNA Sequencing

Bacterial genomic DNA was extracted from feces and purified using QIAamp DNA stool mini kit (QIAGEN), according to the manufacturer's protocol. DNA was amplified using tagged amplicons spanning the V4 region of bacterial 16S ribosomal RNA gene (515f/806r) on the Illumina MiSeq Platform (2×250 bp), at the Ramaciotti Centre for Genomics (University of New South Wales, Sydney, Australia). Data were deposited in the European Nucleotide Archive under accession number PRJEB36826.

Bioinformatics Analysis

Bioinformatics analysis was performed using the QIIME v1.9.1 pipeline. Paired-end reads were assembled, chimeric sequences were detected and removed using Chimeraslayer, and operational taxonomic units were picked at 97% sequence identity using the uclust algorithm. Taxonomies were assigned with BLAST against the Greengenes database. Taxa present at

<0.01% were filtered. Rarefaction analysis was used to compare the adequacy of sequencing depth (Supplemental Figure 1). Data were log2-transformed to account for nonnormal distribution of taxonomic count data. α Diversity was measured using Shannon diversity and evenness. Bacterial community profiles were compared using weighted UniFrac clustering of OTU abundances. A Pearson correlation-based network showing relationships between albuminuria and bacterial taxa was visualized in Calypso (http://cgenome.net/calypso/).

SCFA Measurements

Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was used for metabolic profiling of feces and serum. Fecal pellets were homogenized in cold deuterated water (D_2O) by bead beating at 5 m/s for 40 seconds. The fecal slurry was centrifuged at $16,000 \times g$ for 5 minutes at 4°C. Aliquots of serum or fecal supernatant were passed through Amicon ultra cellulose filters with a cut-off molecular mass of 3 kDa (Millipore), mixed with buffers (200 mM Na_3PO_4/D_2O and 5.0 mM DSS/D_2O, pH 7.0), before being transferred into 3-mm NMR tubes for analysis. Spectra were recorded at 298K on a Bruker 600 MHz AVANCE III spectrometer. The spectra were processed and analyzed using Chenomx NMR Suite v8.4 (Chenomx Inc). Phase and baseline corrections were carried out, with the known concentration of DSS used to determine the concentrations of metabolites.

Primary Culture and Stimulation of Mouse Tubular Epithelial Cells and Podocytes

Mouse kidney tubular epithelial cells (TECs) and podocytes were isolated from C57BL/6 mice, then cultured as described previously.[4] Cultured podocytes and TECs at 80% confluence were rinsed and incubated with serum-free DMEM/F12 medium for 48 hours. The cells were then exposed to 30 mM D-glucose (Invitrogen) or mannitol (5.5 mM glucose plus 24.5 mM mannitol) in the presence and/or absence of acetate (25 mM), propionate (12 mM), or butyrate (3.2 mM) for 12 hours.[18] After stimulation, cells were harvested for RNA extraction and assessment of mRNA expression by RT-PCR.

Statistical Analyses

All results are expressed as mean±SD or mean±SEM. Data were analyzed using two-tailed t tests, or one- or two-way ANOVA with *post hoc* Bonferroni correction (GraphPad, San Diego, CA) where appropriate. A P value of <0.05 was considered statistically significant.

RESULTS

Dietary Fiber Provides Protection against Albuminuria in Diabetic Nephropathy

To assess the effect of dietary fiber on diabetic nephropathy, we fed WT diabetic mice diets containing standard (NC), deprived (ZF), or enriched amounts of fiber (RS), which are known to alter SCFA levels.[24] After 12 weeks, all mice displayed a similar profile in the progression of hyperglycemia (Figure 1A), weight change (Figure 1B), and food intake (Figure 1C) over a 12-week period after STZ injection. Importantly, feeding with RS or ZF did not significantly alter the glucose profile relative to diabetic controls on NC.

Albuminuria is the earliest sign of diabetic nephropathy, reflecting damage to the glomerular filtration barrier, perpetuating disease progression through downstream inflammatory and fibrogenic pathways. NC- and ZF-fed diabetic mice developed progressive albuminuria, with significant elevation at 12 weeks after STZ injection compared with nondiabetic controls (U/ACR 138.7±28 and 149.88±44 versus 86.56±29 mg/mmol; $P<0.01$). Despite equivalent degrees of hyperglycemia, RS-fed diabetic mice were protected from the development of albuminuria with a significantly lower U/ACR at 12 weeks compared with NC- or ZF-fed diabetic mice (74.4±24 versus 138.7±28 and 149.88±4 mg/mmol; $P<0.01$) with no difference compared with nondiabetic controls (Figure 1D).

Dietary Fiber Attenuated Kidney Hypertrophy and Glomerular Injury

Diabetes-induced kidney hypertrophy was evident in NC- and ZF-fed mice, with an increase in kidney-to-body weight ratio compared with nondiabetic WT controls. RS feeding prevented kidney hypertrophy, with no significant increase in kidney-to-body weight ratio compared with nondiabetic controls at 12 weeks (Figure 2A). Histologic examination by light microscopy revealed attenuation of diabetic changes in the kidneys of RS-fed mice. Computerized morphometric analysis of PAS-stained sections demonstrated significant glomerular hypertrophy, hypercellularity, and increased mesangial matrix in NC- and ZF-fed diabetic mice, whereas these parameters were significantly reduced in RS-fed diabetic mice (Figure 2, B–E).

Progression of diabetic nephropathy is also characterized by loss of podocyte density, which typically parallels albuminuria. This was quantified by WT-1 expressed as a ratio to mean V_G. Mice fed NC showed a marked reduction in the number of podocytes per glomerulus compared with nondiabetic WT controls at 12 weeks. Podocyte number was preserved in diabetic mice fed RS and was similar to nondiabetic WT mice of similar age (Figure 2, F and G).

Dietary Fiber Decreased Renal Fibrosis and Interstitial Macrophage Recruitment

Accumulation of extracellular matrix components, particularly collagen, is a prognostic marker for progressive diabetic nephropathy. Col-1 is minimally expressed in healthy kidneys but increases with disease processes causing fibrosis. To quantify collagen deposition, sections were stained using picrosirius red and immunostained for Col-1. Collagen deposition was significantly increased in NC-fed diabetic mice,
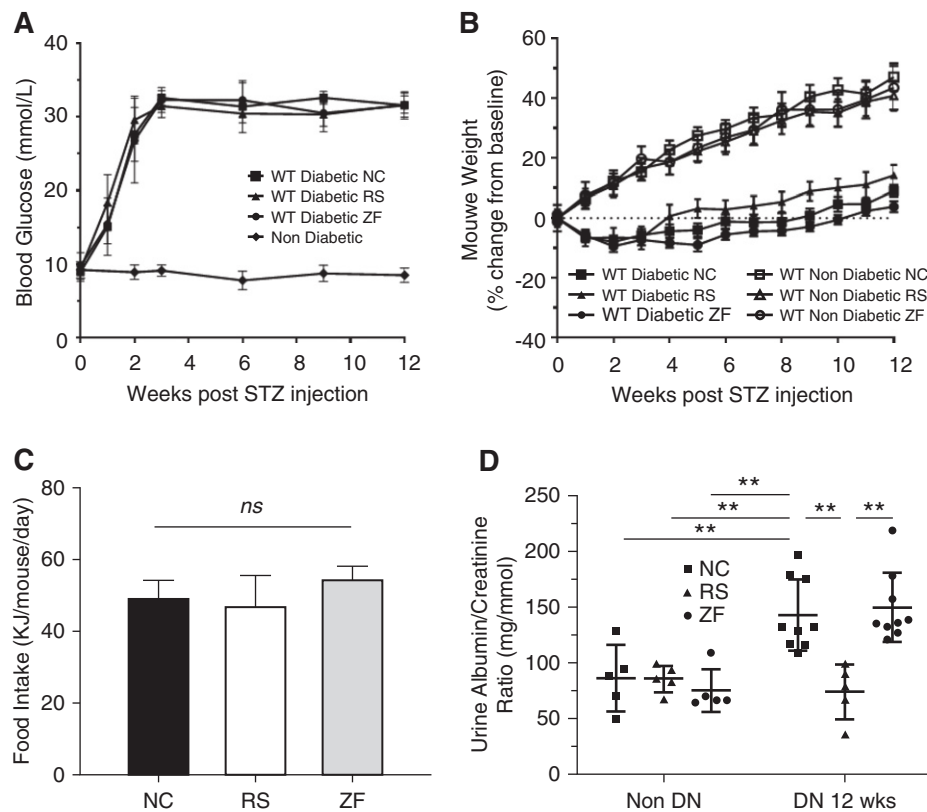
Figure 1. RS feeding provided protection against albuminuria in DN. STZ treatment induced diabetes in WT mice with similar severity, as indicated by (A) blood glucose, (B) body weight, and (C) food intake profiles. Progressive albuminuria is seen in diabetic ZF- and NC-fed mice compared with controls. (D) However, diabetic mice fed RS developed significantly less albuminuria, with no difference compared with nondiabetic controls at week 12. Data are shown as means±SD; **$P<0.01$.

predominantly localized to the tubulointerstitial space, whereas these changes were attenuated with RS feeding (Figure 2, H–J).

Macrophages are the predominant immune cell infiltrating diabetic kidneys and contribute to progression of renal injury. Immunostaining for the pan-macrophage marker CD68 revealed a dramatically increased number of interstitial macrophages at week 12 in the kidneys of diabetic NC- or ZF-fed mice. This accumulation of CD68-positive cells was significantly reduced in mice fed RS (Figure 2, K and L).

Collectively, our results support a protective role for dietary fiber against progression of diabetic nephropathy. This is reinforced by the finding that diabetic mice fed a second type of HF diet enriched in guar gum were also protected against nephropathy (Supplemental Figure 2).

Dietary Fiber Reduced the Expression of Inflammatory and Fibrotic Genes in Diabetic Kidneys

We next examined the expression of inflammatory molecules in diabetic kidneys. mRNA expression of innate immune receptors TLR2 and TLR4 was significantly increased in diabetic mice at 12 weeks; however, this upregulation was not observed in the kidneys of RS-fed mice. Downstream inflammatory cytokines and chemokines (IL6, TNFα, CCL2 and CXCL10)

and fibrosis-related genes (TGFβ and fibronectin) were also upregulated in diabetic kidneys, and abrogated with RS feeding (Figure 3).

Dietary Fiber Altered Gut Microbiota Composition and Improved Dysbiosis in Diabetic Mice

With the observation that dietary fiber could attenuate the development of diabetic nephropathy, we next investigated whether the observed renoprotective effects were attributable to diet-induced alterations in gut microbial ecology. Feces were collected before and after STZ injection, and after maintenance on treatment diet. Microbiota composition was assessed by analyses of amplicons generated across the V4 region of the bacterial 16S ribosomal RNA genes.

We first assessed gut microbiota α diversity and composition before and after STZ injection with mice on NC. Although most commonly used in research as a diabetogenic drug for its cytotoxic effect on pancreatic islet β cells, STZ, derived from *Streptomyces achromogenes*, also has antibiotic activity.[25] Expectedly, STZ caused a reduction in α diversity and disruption of the fecal bacterial community shortly after injection (Supplemental Figure 3). However, this change was relatively short-lived, with mice that remained on NC demonstrating spontaneous recovery of α diversity by 3 weeks after
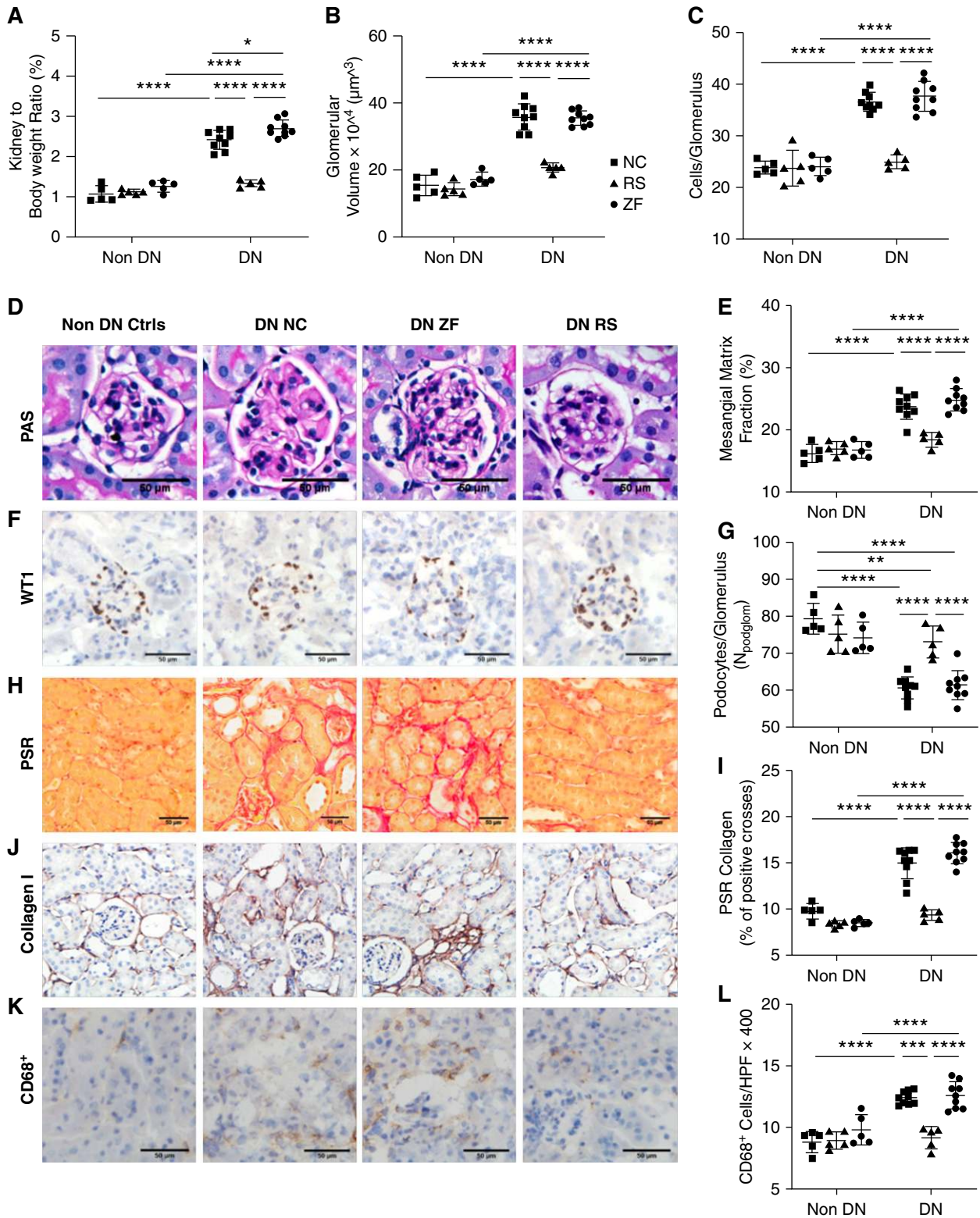
Figure 2. RS feeding reduced glomerular and interstitial injury in DN. Diabetic mice fed RS are relatively protected from diabetes-induced glomerular and interstitial injury compared with NC- and ZF-fed mice, as indicated by (A) decreased kidney-to-body weight ratio, (B and D) V_G, (C) glomerular hypercellularity, and (E) mesangial expansion. (D) Representative sections of glomeruli from NC-, ZF-, and RS-fed diabetic and NC-fed nondiabetic mice at 12 weeks (PAS-stained, ×400 magnification). (F and G) Representative sections of glomeruli stained for WT-1 at 12 weeks (×400 magnification), demonstrating podocyte density in nondiabetic mice, which is reduced in
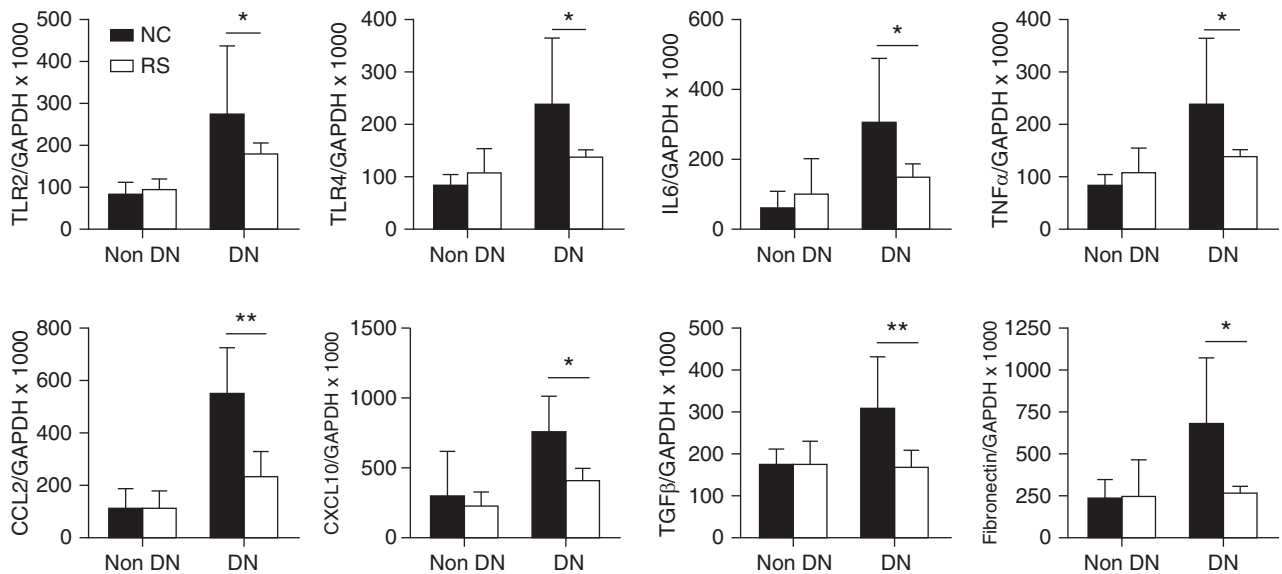
Figure 3. RS feeding reduced inflammatory cytokine and chemokine mRNA expression in diabetic kidneys. mRNA expression of innate immune receptors (TLR2, TLR4), proinflammatory cytokines (IL6, TNFα), chemokines (CCL2, CXCL10), and fibrosis related genes (TGFβ, fibronectin) were significantly reduced in RS-fed mice compared with NC-fed diabetic controls. Data are shown as means±SD; *$P<0.05$; **$P<0.01$.

injection and before diet randomization. After randomization, the microbial composition in NC-fed, fiber-supplemented, and depleted groups all polarized according to diet type, highlighting the profound ability of diet to influence the gut microbiota.

Diabetes induced a disease-related dysbiosis, evidenced by reduced α diversity as expressed by Shannon index ($P=0.007$) at 12 weeks compared with nondiabetic mice on an equivalent diet (Figure 4A). Relative abundance of bacteria at a phylum level was altered in diabetic mice, exhibiting increased *Firmicutes* and *Actinobacteria* but reduced *Bacteroidetes* compared with nondiabetic mice (Figure 4B). At the genus level, this correlated with relative loss of SCFA producers *Bacteroides* (species *Bacteroides acidifaciens*), *Ruminococcus*, and *Rikenella* (Figure 4C). Diabetes was also associated with a reduction in the genus *Akkermansia* (species *Akkermansia muciniphila*), a mucin-degrading bacteria integral to maintenance of gut integrity.

To assess the effect of dietary fiber on dysbiosis, we analyzed the gut microbial composition associated with our four diets in diabetic mice: NC, which has an equivalent amount of fiber to that recommended for humans; ZF; and the two different HF diets (RS and guar gum amylose). Principal coordinate analysis of UniFrac distances showed clear cluster separation

of diabetic mice according to diet type (analysis of similarities $P<0.001$; $R=0.93$) (Figure 4E). The relative abundance of bacterial phyla in each group of mice is shown in Figure 4F. RS and HF feeding ameliorated diabetes-related dysbiosis, with increased relative abundance of phylum *Bacteroidetes* at the expense of *Firmicutes*. Expansion of the SCFA producing genera *Prevotella* and *Bifidobacterium* with relative reduction of *Bilophila* (containing the known pathobiont *Bilophila wadsworthia*) was also seen in fiber-fed diabetic mice compared with NC-fed controls. HF diet, but not RS diet, increased the relative abundance of *A. muciniphila* in diabetic mice (Figure 4G).

To identify bacteria associated with severity of albuminuria, an early hallmark of diabetic nephropathy, we used Pearson correlation analyses shown as a heatmap in Figure 4D. At a family level, *Lactobacillaceae, Enterobacteriaceae,* unclassified *Clostridiales*, and *Streptococcaceae* were most strongly associated with increased albuminuria, all of which were most abundant in NC- and ZF-fed diabetic mice. In contrast, "protective bacteria" associated with lower levels of albuminuria were found predominantly in diabetic mice fed RS or HF diets.

Dietary Fiber Promotes Increased SCFA Production
As dietary fiber is known to be fermented by the gut microbiota to release SCFA, we next measured SCFA levels in feces

NC- and ZF-fed mice, with relative preservation in with RS feeding. Representative sections of kidney from diabetic and nondiabetic mice at 12 weeks demonstrating the increased interstitial collagen deposition being attenuated by RS feeding using (H and I) picrosirius red staining and immunostaining for (J) Col-I. (K) Representative sections of kidney from diabetic and nondiabetic mice stained for CD68. (L) Increased interstitial CD68^{+} macrophage accumulation is evident in diabetic NC- and ZF-fed, but not RS-fed mice. Data are shown as means±SD; *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$.
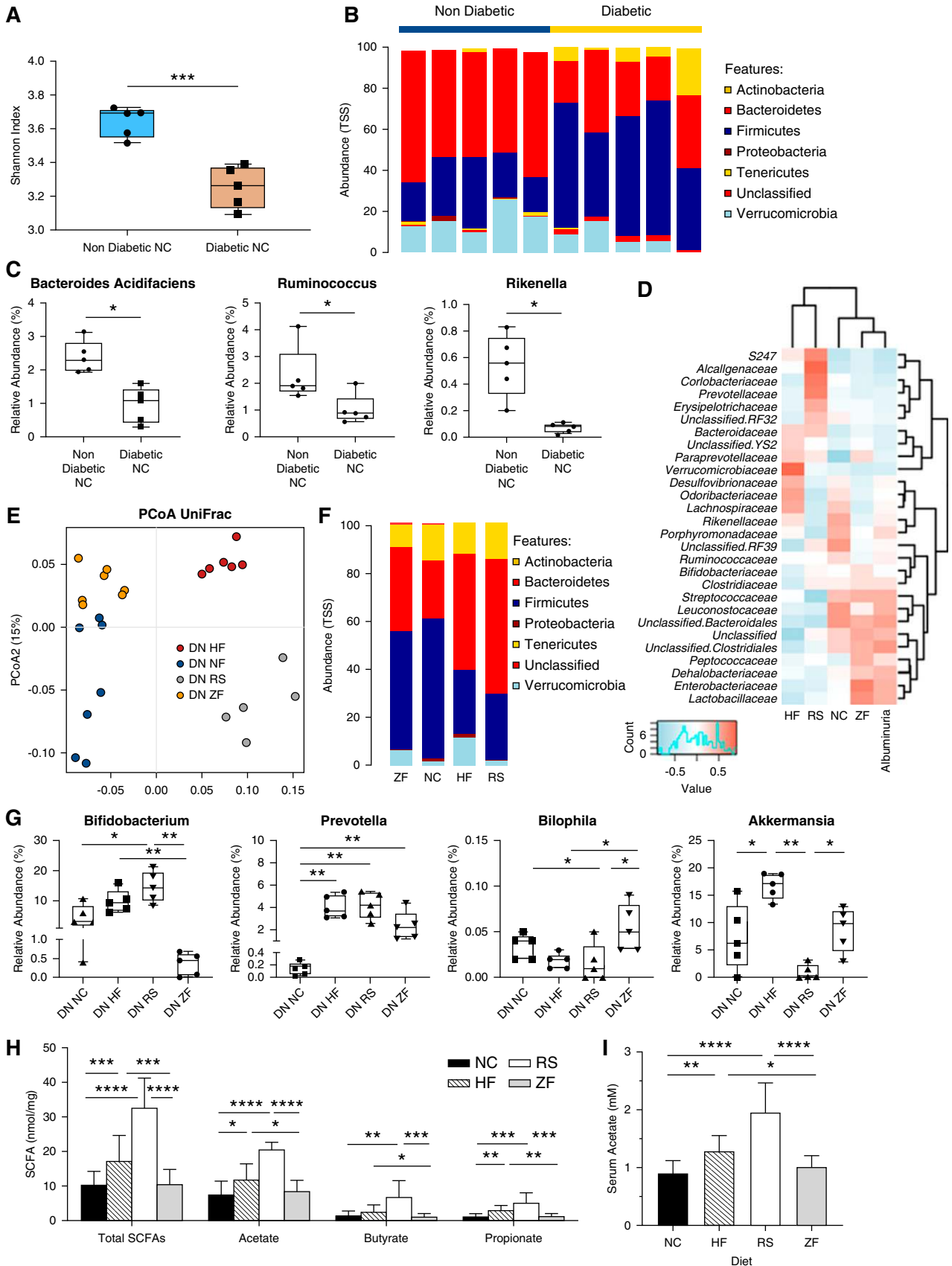
Figure 4. Diet-induced alterations in microbial ecology contribute to protection against DN through enrichment of SCFA-producing species. (A) Diabetes influences microbiota composition, with OTU-based α diversity metrics showing loss of diversity in diabetic versus

and serum of diabetic mice using ^{1}H NMR spectroscopy. In accordance with the changes in gut microbial ecology, diabetic mice fed RS and HF diets had significantly greater levels of serum acetate (Figure 4I) and fecal SCFAs (Figure 4H) compared with those on NC. However, SCFA levels in ZF-fed mice remained similar to NC-fed controls.

SCFA Are a Key Mediator of the Renoprotective Effects of Dietary Fiber

To determine whether changes in SCFA levels could directly mediate the renoprotective effects seen with dietary fiber, we treated diabetic mice with drinking water containing 100 mM acetate, 50 mM butyrate, or 100 mM propionate from week 3 postinjection while maintained on control diet. Mice treated with acetate in drinking water achieved similar peripheral concentrations of acetate to fiber-fed mice, with no changes to systemic acid-base balance (data not shown). Protection against progression of diabetic nephropathy was found in SCFA-treated mice, with reductions in albuminuria, glomerular hypertrophy, mesangial expansion, and interstitial fibrosis compared with controls (Figure 5). Similar to the effect seen with HF feeding, protection against diabetic nephropathy was accompanied by preservation of podocyte number (Figure 5, E and F) and a reduction in interstitial macrophage infiltration (Figure 5, I and J). We have previously demonstrated that SCFA supplementation in drinking water does not alter gut microbiota composition,[26] supporting a direct effect of SCFA in mediating protection against diabetic nephropathy.

Acetate Treatment Suppressed Expression of Inflammatory and Fibrotic Genes in Diabetic Kidneys

To further explore the molecular mechanisms underlying the effect of SCFA, real-time PCR was used to evaluate gene expression of inflammatory cytokines, chemokines, and fibrosis-related genes in diabetic kidneys. Expression of IL6 and IFNγ mRNAs were upregulated in WT diabetic kidneys compared with nondiabetic WT controls; however, this upregulation was not observed in the kidneys of acetate supplemented mice. Gene expression of chemokines CCL2 and CXCL10 were also substantially increased in WT diabetic

kidneys, but not in acetate supplemented mice. Expression of the fibrosis-related genes TGFβ and fibronectin were also suppressed with acetate supplementation (Figure 6).

GPR43 and GPR109A, GPRs for Acetate and Butyrate, Are Necessary for Fiber and SCFA Mediated Protection against Diabetic Nephropathy

The molecular actions of SCFA on cell function are mediated through diverse pathways, including signaling *via* "metabolite-sensing" GPRs.[27] To determine whether the beneficial effects of SCFA on diabetic nephropathy were mediated *via* GPRs, we treated groups of diabetic $Gpr43^{-/-}$ and $Gpr109A^{-/-}$ mice on a C57BL/6 background with acetate- and butyrate-supplemented drinking water, respectively. $Gpr43^{-/-}$ and $Gpr109A^{-/-}$ mice were equally susceptible to STZ-induced diabetes, with similar progression of hyperglycemia to WT mice (Figure 7A).

Acetate is known to be a selective and strong agonist for GPR43. WT B6 $Gpr43^{+/+}$ mice supplemented with acetate were largely protected from the development of diabetic nephropathy, whereas $Gpr43^{-/-}$ mice were not protected and albuminuria was exacerbated (Figure 7C). We next tested GPR109A, the primary receptor for butyrate. Butyrate supplementation in $Gpr109A^{-/-}$ mice provided partial protection from diabetic nephropathy, with a small reduction in albuminuria compared with diabetic WT controls (Figure 7D). Thus although acetate-mediated protection appears dependent upon GPR43, butyrate facilitates its beneficial effects *via* both GPR109A and additional pathways.

To determine whether the beneficial effects of dietary fiber on DN were mediated *via* these same GPRs, we fed diabetic WT C57BL/6 $Gpr43^{+/+}$ and $Gpr43^{-/-}$ mice RS, the diet that produced our highest SCFA levels. RS-fed $Gpr43^{-/-}$ mice showed some improvement in albuminuria ($P<0.01$) compared with WT controls, but absence of GPR43 did not account for the full protective effects of a HF diet (Figure 7B).

SCFA Treatment Inhibits Proinflammatory Responses in TECs and Podocytes Exposed to High Glucose

To determine the effects of SCFA on kidney cells under hyperglycemic conditions, primary TEC and podocyte cultures were

nondiabetic mice (Shannon index). (B) Relative abundance of bacteria presented at the phylum level, depicting changes in microbiota composition in diabetic versus nondiabetic mice. (C) At the genus and species levels, diabetic mice had lower levels of known SCFA producing *Ruminococcus*, *Rikenella* genera, and bacteria from the *Bacteroides acidifaciens* species than nondiabetic controls. (E) Fecal microbiota composition was analyzed by 16S ribosomal RNA sequencing from feces of diabetic mice at 12 weeks. The composition of gut microbiota in diabetes is influenced by diet. Principal coordinates analysis using weighted UniFrac distances depicts significant differences in the composition of the gut microbiota between HF-, RS-, NC-, and ZF-fed diabetic mice (analysis of similarities $P<0.001$). (F) Distribution of OTUs (key) in feces from diabetic mice fed different diets (horizontal axis); each OTU is presented as relative abundance at the phylum level. (G) RS and HF feeding ameliorates disease-related dysbiosis, resulting in expansion of the SCFA-producing *Prevotella* and *Bifidobacterium* genera, with relative reduction in pathobiont *Bilophila*. RS and HF diets had contrasting effects on relative abundance of *Akkermansia*. (D) Pearson correlation-based heatmap at a family level, identifying the bacteria associated with severity of albuminuria, with cool colors representing negative correlation and hot colors representing positive correlations. (H and I) Quantification of SCFA levels in feces and serum from diabetic mice fed fiber supplemented (HF, RS), standard (NC), or deplete (ZF) diets by ^{1}H NMR spectroscopy. Data are shown as means±SD with a minimum of five mice per group; *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$.
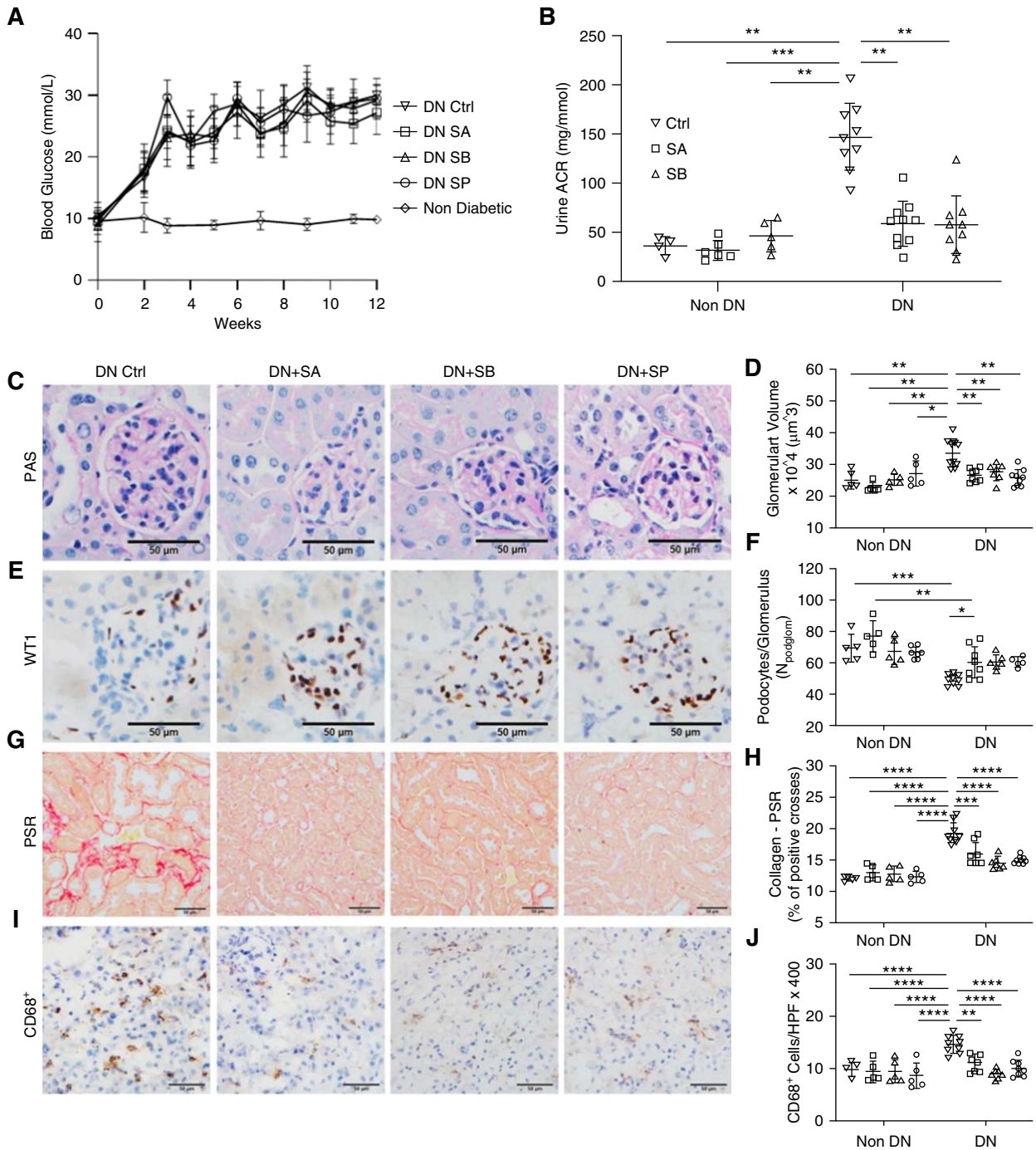
Figure 5. Treatment with SCFA acetate, butyrate, and propionate protects against DN. (A) Diabetic mice remained similarly hyperglycemic regardless of whether they received acetate, butyrate, propionate, or pH- and sodium-matched water. (B) Acetate and butyrate treatment reduced albuminuria at 12 weeks compared with control diabetic mice. Diabetic glomerular and interstitial changes including (C and D) V_G, (E and F) podocyte injury, and (G and H) interstitial macrophage accumulation (I and J) were diminished with SCFA treatment versus control diabetic mice. Data are shown as means±SD; *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$.

treated with acetate, butyrate, or propionate under high and normal glucose conditions. mRNA expression of proinflammatory cytokine (IL6), chemokines (CCL2, CXCL10), and profibrotic genes (TGFβ, fibronectin) were increased two- to seven-fold in TECs exposed to high glucose relative to osmotic controls. Upregulation of IL6, fibronectin, and TGFβ was significantly attenuated by all three SCFAs. CCL2 and CXCL10 expression was diminished by only acetate (Figure 8A).

In podocytes, upregulation of IL6, TGFβ, and fibronectin was similarly attenuated by all three SCFAs. In contrast to
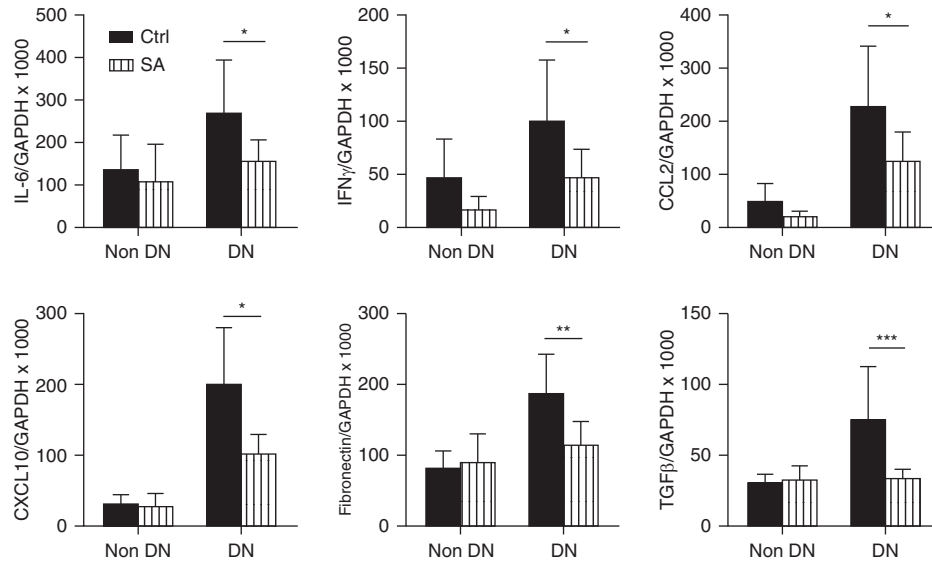
Figure 6. Acetate treatment reduced inflammatory and fibrotic mRNA gene expression in diabetic kidneys at 12 weeks. RT-PCR demonstrated substantial upregulation of mRNA expression of IL-6, IFNγ, CCL2, CXCL10, fibronectin, and TGFβ in WT diabetic kidneys, all of which were markedly diminished by acetate treatment. Data are shown as means±SD; *P<0.05; **P<0.01; ***P<0.001.

TECs, mRNA expression of TNFα in podocytes was exacerbated by exposure to glucose, and diminished by butyrate and propionate. No significant difference was seen in CCL2 expression (Figure 8B).

DISCUSSION

The gut microbiota and its metabolites play an integral role in the pathogenesis and development of CKD and other types of inflammatory and metabolic diseases.[28,29] Higher fiber intake has been associated with decreased inflammation and mortality in CKD,[13] yet the mechanisms of the gut–kidney axis are yet to be fully explored. Using a murine model of STZ induced diabetes, our study provides the first insights into the interplay between diet, the gut microbiome and development of pathologic inflammatory responses in diabetic nephropathy. In this study, we observed that dietary fiber is integral to a healthy gut microbiota, the maintenance of which protected mice against the clinical and histologic manifestations of diabetic nephropathy without altering glycemia. Supplementation with microbial-derived metabolites acetate and butyrate afforded similar protection, achieved principally through binding to metabolite-sensing receptors GPR43 and GPR109A. Our results reveal a role for dietary manipulation of the gut microbiome in attempting to delay progression of diabetic nephropathy, with SCFA being the key mediators of the protection afforded by fiber consumption.

Diet is the dominant exogenous factor known to influence the gut microbiome, and a key determinant of its composition and diversity.[12] In our study, changes in fiber consumption led to clear changes in the microbiota, evidenced by distinct cluster separation of zero-, normal-, and high fiber-fed diabetic mice using principal coordinate analysis. A high fiber diet ameliorated diabetes-related dysbiosis, and supported expansion of SCFA producers, in contrast to reduction of these groups in NC-fed diabetic controls. We also detected increased numbers of *Bilophila wadsworthia* in diabetic mice, a known urease producing pathobiont linked to increased gut permeability and systemic inflammation in mice.[30,31] These results parallel findings in human studies of a "dysbiotic" microbiota in CKD, with expansion of urease producing and contraction of SCFA-producing bacteria being predominant features.[9,31] The expansion of SCFA-producing bacteria seen in our diabetic fiber-fed mice highlights the capacity of diet to influence gut microbial composition. Despite this, treatment with pre-, pro-, or synbiotics in humans with CKD have produced conflicting results,[32] emphasizing the need for greater mechanistic insights to guide future translational studies.

Our two high fiber diets both resulted in expansion of the SCFA-producing *Bifidobacterium* and *Prevotella* genera, but had contrasting effects on *Akkermansia*. *A. muciniphila* has attracted interest as a probiotic for its beneficial effects in murine studies on metabolic syndrome, inflammation, and gut permeability, with a growing body of human translational work targeting obesity.[33,34] The HF diet promoted a three-fold expansion in contrast to relatively low abundance in RS-fed mice, yet both yielded similar degrees of protection against diabetic nephropathy. This finding highlights the complexity of the microbial ecosystem and provides support for the concept that no single species is responsible, rather that diet can induce net microbial changes, which tip the balance away from dysbiosis.

Dietary fibers are fermented by commensal colonic bacteria, resulting in the release of SCFAs locally and *via* the portal
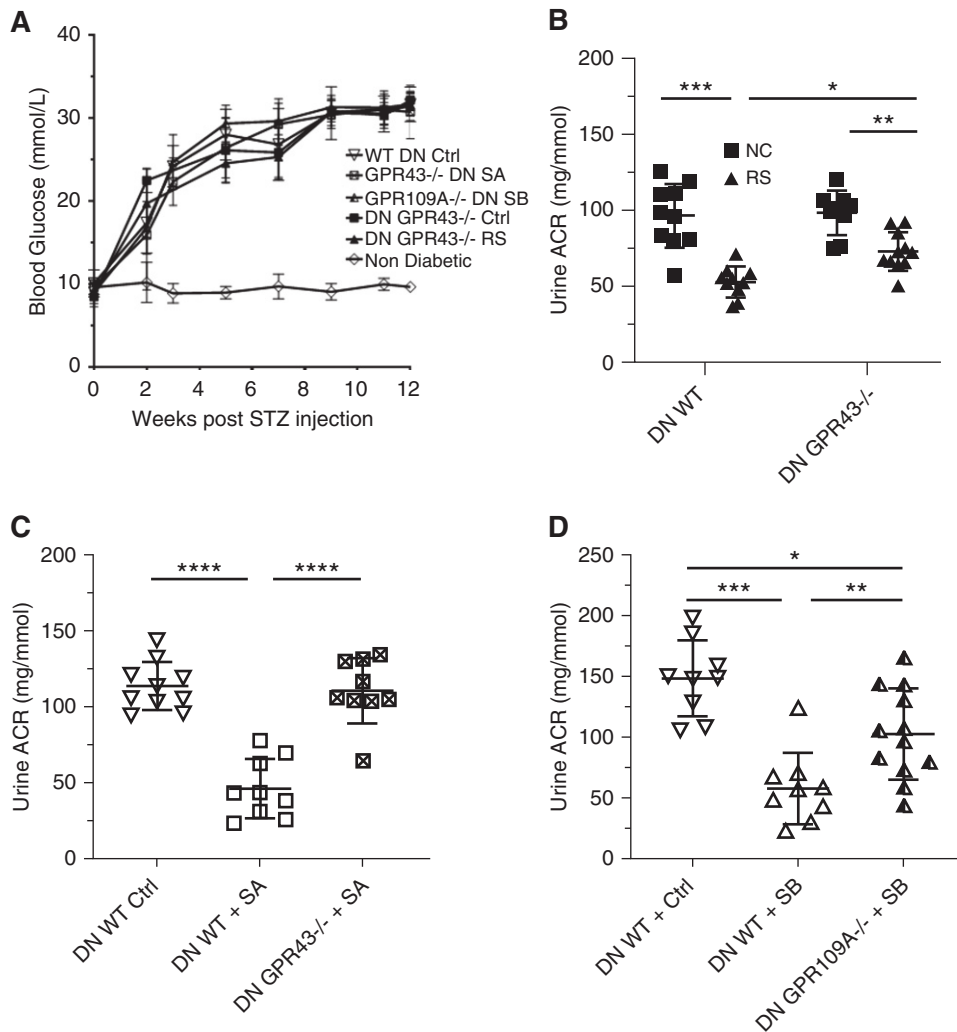
Figure 7. GPR43 and GPR109A are required for SCFA- and fiber-mediated protection against DN. (A) *Gpr43*$^{-/-}$ and *Gpr109A*$^{-/-}$ mice displayed a similar progression of hyperglycemia to WT counterparts. Blood glucose profiles were not significantly altered with SCFA treatment or RS diet. (B) Albuminuria in *Gpr43*$^{-/-}$ mice fed a HF diet, demonstrating partial reduction at 12 weeks. (C and D) *Gpr43*$^{-/-}$ and *Gpr109A*$^{-/-}$ mice were treated with oral acetate and butyrate, respectively, after induction of diabetes. Albuminuria measured at 12 weeks in (C) *Gpr43*$^{-/-}$ mice and (D) *Gpr109A*$^{-/-}$ mice. Data are shown as means±SD of at least *n*=5 mice per group; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

circulation to mediate extraintestinal effects.[35,36] Our HF diets induced changes in microbial composition that correlated with increased fecal and circulating SCFA levels. Systemic SCFA levels have been shown to respond in a dose-dependent manner to dietary fiber, confirming gut microbial fermentation of fiber as the primary factor influencing circulating levels.[37] In keeping with this, our RS diet, which contained the highest proportion of nondigestible fiber suitable for bacterial fermentation, produced the highest SCFA levels, followed by HF and then NC.

SCFA supplementation reproduced the beneficial effects of HF diets on diabetic nephropathy, pointing to these as key mediators of gut microbiota–host crosstalk. Although previous studies have demonstrated a role for SCFA in improving glucose tolerance through AMPK-, PPY-, and GLP-1–dependent

mechanisms,[38–40] we found no difference in glucose profiles between SCFA-treated diabetic WT, *Gpr43*$^{-/-}$, or *Gpr109A*$^{-/-}$ mice. This likely reflects these studies being performed in high-fat feeding models of type 2 diabetes. The improvements found highlight the effect of SCFA on obesity and insulin resistance,[41,42] with these effects yielding no glucose difference in our lean diabetic mice.

At the molecular level, SCFA mediate cellular effects through multiple mechanisms, including GPRs, effects on cell proliferation, apoptosis, and histone acetylation.[27] GPR43 and GPR109A are expressed on gut epithelial cells and innate immune cells that respond to SCFA produced in the colon, indicating a broad role in inflammatory and immune responses.[43,44] We and others have previously demonstrated the SCFA-GPR signaling pathway as critical to
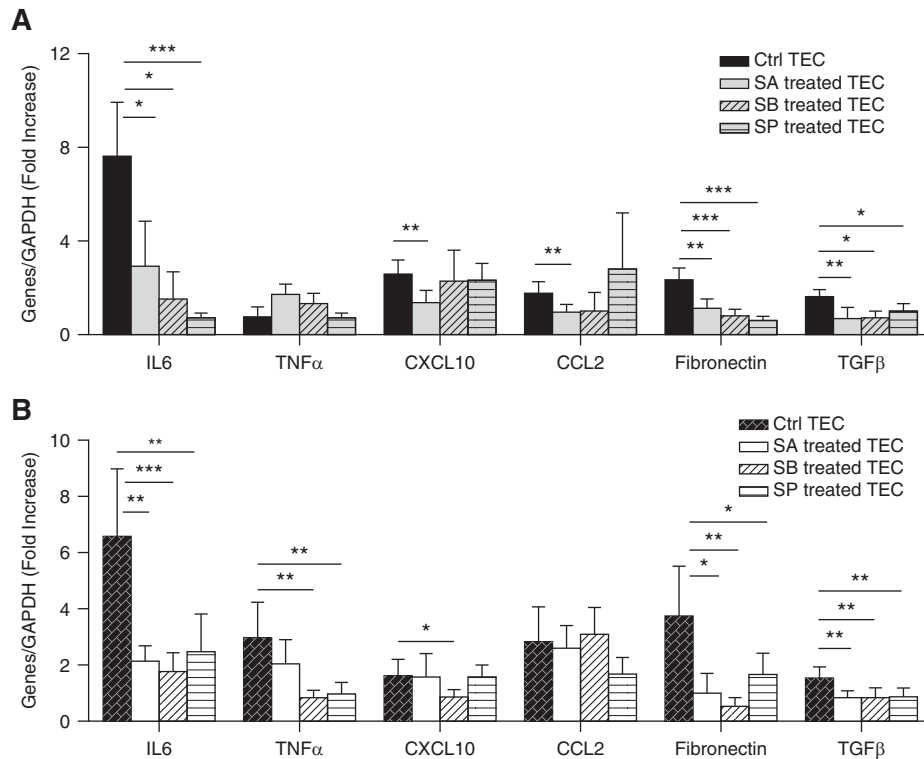
Figure 8. SCFA treatment reduced proinflammatory gene expression in glucose stimulated TECs and podocytes. Primary TECs and podocytes from WT mice were exposed to high glucose or an osmotic control in the presence and/or absence of SCFA for 12 hours. (A and B) High-glucose conditions induced upregulation of proinflammatory- and fibrosis-related genes two- to seven-fold in TECs and podocytes. Expression of IL6, fibronectin, and TGFβ were diminished after SCFA treatment in both TECs and podocytes. (A) Acetate reduced expression of chemokines (CCL2, CXCL10) in TECs. (B) Podocytes expressed higher levels of TNFα after glucose stimulation, which was diminished by butyrate and propionate. Data are shown as means±SD (n=6); *P<0.05; **P<0.01; ***P<0.001.

regulation of immune and inflammatory responses by commensal bacteria.[24,45] The importance of these receptors in mediating SCFA's effects in diabetic nephropathy was clearly evident, with the protective effects unable to be fully replicated in GPR43- and GPR109A-deficient mice. Although the benefit of acetate treatment was abrogated in $Gpr43^{-/-}$ mice, butyrate provided partial protection in $Gpr109A^{-/-}$ diabetic mice, likely because of its ability to also signal through GPR43. Studies of renal cell lines have supported the importance of GPR's, confirming their presence in kidney tissue, vessels, and podocytes.[46–48] Consistent with our findings *in vivo,* we demonstrated that under high-glucose conditions, treatment with SCFA was able to reduce inflammation in cultured TECs and podocytes *in vitro.* Similarly, Huang *et al.*[19] found incubation of glomerular mesangial cells induced under high glucose or LPS, with SCFA or a GPR43 agonist, inhibited proliferation and reduced production of reactive oxygen species and expression of MCP-1 and IL-1β, supporting this pathway as a target in diabetic nephropathy.

SCFAs also modulate the production of inflammatory mediators by macrophages independent of GPRs. Chang *et al.*[49] demonstrated suppression of LPS and cytokine-stimulated production of proinflammatory mediators (TNFα, IL6, and

NO) and enhanced release of the anti-inflammatory cytokine IL10 after treatment of macrophages with butyrate. Similarly, we found a significant reduction in macrophage accumulation within kidneys of diabetic mice treated with SCFA or high fiber, and lower expression of pathogenic cytokines and chemokines. Reduced expression of innate immune receptors TLR2 and TLR4 was also seen in our fiber-fed mice, supporting activation of innate immunity and TLRs as pathways through which the microbiota might modulate renal inflammation and injury.

Our studies were performed in a STZ model of type 1 diabetes. As STZ has antibiotic activity, confounding effects on gut microbiota were present. To exclude this, we studied the effects of STZ and found that gut microbiota was transiently altered after injection, as mice showed resilience to these perturbations,[50] with α diversity and microbial composition largely restored within 3 weeks. Our model has other potential limitations, including the lack of kidney failure after development of nephropathy. Thus, caution in extrapolating to human disease is advisable and replication in other murine models is warranted.

Our results provide evidence of the importance of the gut microbiota in diabetic kidney disease, and support the

hypothesis of a gut–kidney axis linking dietary fiber, the gut microbiota, SCFA, and kidney disease. Our study also provides mechanistic insights, demonstrating that dietary fiber and SCFAs protected against diabetic nephropathy through regulation of key pathways and genes involved in innate immunity, inflammation, and macrophage recruitment. Future studies are required to determine the ability of diet to not only delay progression, but also reverse established disease. Strategies targeting the modifiable capacity of the gut microbiota through dietary manipulation represent a novel and cost-effective therapeutic strategy that warrants further exploration.

SUPPLEMENTAL MATERIAL

This article contains the following supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2019101029/-/DCSupplemental.

Supplemental Figure 1. Rarefaction curve.

Supplemental Figure 2. HF diet attenuates the clinical and histological manifestations of diabetic nephropathy.

Supplemental Figure 3. Changes to gut microbiota before and after STZ injection.

Supplemental Table 1. Nutritional parameters of experimental diets.

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