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ORIGINAL RESEARCH

Causal Relationships Between Gut Microbiota, Metabolites, and Diabetic Nephropathy: Insights from a Two-Sample Mendelian Randomization Analysis

Xixi Song[1,2], Jingqiu Cui[1], Shiwei Li[1], Bo Huang[1]

[1]Department of Endocrinology and Metabolism, Tianjin Medical University General Hospital, Tianjin, People's Republic of China; [2]Department of Endocrinology and Metabolism, Baoding No.1 Central Hospital, Baoding, People's Republic of China

Correspondence: Jingqiu Cui, Department of Endocrinology and Metabolism, Tianjin Medical University General Hospital, No. 154 Anshan Dao, Heping District, Tianjin, 300052, People's Republic of China, Tel +8613920765604, Email cuijingqiu@tmu.edu.cn

Background: Previous studies have established a correlation between gut microbiota, metabolites, and diabetic nephropathy (DN). However, the inherent limitations of observational studies, including reverse causality and confounding factors, made this relationship uncertain.

Methods: In this study, we compiled summary statistics from a genome-wide association study (GWAS) conducted on gut microbiota, metabolites, and DN. We employed a two-sample Mendelian randomization (MR) approach, utilizing inverse variance weighted (IVW), MR-Egger, weighted median, and weighted mode methods.

Results: We detected the protective nature of genetically predicted representatives from the family Bacteroidaceae (OR: 0.716, 95% CI: 0.516–0.995, $p = 0.046$), family Victivallaceae (OR: 0.871, 95% CI: 0.772–0.982, $p = 0.026$), genus Bacteroides (OR: 0.716, 95% CI: 0.516–0.995, $p = 0.046$), genus Coprococcus 2 (OR: 0.745, 95% CI: 0.576–0.963, $p = 0.025$), and genus Lactococcus (OR: 0.851, 95% CI: 0.730–0.992, $p = 0.039$) against the development of DN. Conversely, we identified a positive correlation between the incidence of DN and entities, such as Phylum Bacteroidetes (OR: 1.427, 95% CI: 1.085–1.875, $p = 0.011$), class Bacteroidia (OR: 1.304, 95% CI: 1.036–1.641,$p = 0.024$), order Bacteroidales (OR: 1.304, 95% CI: 1.035–1.641, $p = 0.028$), genus Catenibacterium (OR: 1.312, 95% CI: 1.079–1.594, $p = 0.006$), genus Lachnoclostridium (OR: 1.434, 95% CI: 1.129–1.821, $p = 0.003$), and genus Parasutterella (OR: 1.270, 95% CI: 1.070–1.510, $p = 0.006$). In our analysis, none of the gut metabolites demonstrated a causal relationship with DN.

Conclusion: Our results substantiated the potential causal association between specific gut microbiota and DN. Therefore, our study offers novel insight into the mechanisms underlying DN. This finding provides a theoretical foundation for the future development of targeted strategies for the prevention and treatment of DN.

Keywords: Gut microbiota, diabetic nephropathy, gut metabolites, Mendelian randomization

Introduction

Diabetic nephropathy (DN) is the primary cause of chronic kidney disease (CKD), a prevalent microvascular complication caused by diabetes mellitus (DM), which is characterized by persistent proteinuria and decreased estimated glomerular filtration rate (eGFR).[1] Based on the International Diabetes Federation report, the global prevalence of diabetes in 2019 was estimated to be 9.3% (463 million people), with a projected increase to 10.2% (578 million) by 2030 and 10.9% (700 million) by 2045.[2] Approximately 20%-40% of people with DM develop DN.[3] DN significantly increases the risk of infection, end-stage kidney disease (ESRD), and cardiovascular diseases, such as coronary heart disease, heart failure, and hypertension. Based on 2002 data from the United States, nearly 44% to 45% of ESRD cases are attributed to DN. In Heidelberg, located in Southwest Germany, nearly 60% of patients who underwent kidney

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replacement therapy in 1995 were diagnosed with diabetes.[3] DN increases all-cause mortality and imposes substantial economic burdens on patients with diabetes. Current management approaches for DN encompass meticulous control of blood glucose and blood pressure, modulation of lipid metabolism disorders, and mitigation of urinary protein excretion. RAAS blockers, SGLT-2 inhibitors, GLP-1 agonists, and mineralocorticoid receptor antagonists are the common medications used for treating DN.[4] In addition, aldose reductase inhibitors (ARIs) may help prevent the progression of DN.[5] Nonetheless, the persistent risk of DN underscores the existence of unidentified risk factors and mechanisms.

An increasing body of evidence highlights the involvement of gut dysbiosis in the pathogenesis of DN.[6] Numerous studies have demonstrated that DN alters gut microbial composition and affects microbial community richness and diversity.[7,8] Larsen et al found that the proportion of Firmicutes and Clostridium was significantly reduced in the intestinal flora of patients with diabetes, and class Betaproteobacteria was highly enriched and positively correlated with blood sugar.[9] A meta-analysis also found a decrease in the mean abundance of Firmicutes and an increase in the mean abundance of Actinomycetes in the gut microbiota of patients with DN. Furthermore, the meta-analysis reported a significant change in specific bacteria, such as Hungatella, Bilophila, and Escherichia, in patients with DM and DN compared to patients without DN.[10] The gut microbiota profoundly affects the homeostasis of the host's internal environment. It maintains the integrity of the intestinal barrier, regulates renal metabolism, induces anti-inflammatory responses, and fosters the homeostasis of the immune system.[11] Impaired renal function in patients with DN can lead to the accumulation of metabolic toxins, resulting in an imbalance in intestinal microbiota.[12] Abnormal intestinal flora also accelerates the progression of DN by triggering oxidative stress, promoting inflammation, aggravating insulin resistance, and activating the RAS system and other mechanisms.[13] Gut dysbiosis weakens the intestinal barrier, leading to the leakage of lipopolysaccharides (LPS) into the systemic circulation. LPS stimulates the release of various pro-inflammatory factors by binding to LBP, CD14, and TLRs, inducing systemic inflammation.[14] Chen et al investigated the effect of outer membrane vesicles (OMVs) released by intestinal gram-negative bacteria on renal tubules in DN rats and found that OMVS-derived LPS can induce inflammation and tubular damage by activating caspase-11.[15] Pedersen et al demonstrated that Prevotella copri can induce insulin resistance, worsen glucose intolerance, and affect host metabolism in mice.[16] Lu et al reported that excessive acetate produced due to gut dysbiosis can lead to kidney injury in rats with diabetic nephropathy by activating intrarenal RAS.[17] Specific microbial-derived-metabolites, such as lipopolysaccharide (LPS), short-chain fatty acids (SCFAs), bile acids (BAs), and trimethylamine-N-oxide (TMAO), are pivotal in shaping host-microbe interactions.[18] SCFAs exert protective effects on the kidneys of diabetic mice by activating GPR43 and GPR109A receptors. They downregulate inflammatory factors, chemokines, and pro-fibrotic proteins in the kidney.[19] On the other hand, TMAO exacerbates renal inflammation and fibrosis in rats with DN by activating pyrin domain-containing-3 (NLRP3) inflammatory vesicles.[20] Prebiotics, probiotics, fecal microbiota transplantation, and gut microbiota-modulating anti-diabetic medications enable the manipulation of intestinal microbial composition, thus ameliorating DN-associated kidney damage.[13] These findings highlight the importance of the gut-kidney axis as a therapeutic target to modulate the progression of DN.

However, the aforementioned studies predominantly relied on animal models or cross-sectional observational analyses. The results of animal experiments cannot be fully translated into human clinical practice. In addition, observational studies cannot completely exclude the effect of confounding factors, such as pre-existing diseases, diet, and medications on the results, limiting their capacity to establish causal links. Mendelian randomization (MR), using the aggregated data of genome-wide association studies (GWAS), can mitigate confounding effects and facilitate the assessment of causal relationships between exposures and outcomes.[21] Thus, this study aimed to investigate the causal effect of specific gut microbiota and metabolites on DN. Our findings can help identify novel biomarkers and therapeutic targets to facilitate the early diagnosis and treatment of DN.

Materials and Methods

Study Design

We conducted a two-sample MR study to explore whether there is a causal relationship between gut microbiota, metabolites, and DN. Additionally, we utilized a reverse MR approach to investigate whether there is a bidirectional

relationship between DN and gut microbiota and metabolites (Figure 1). This study adhered to the Strengthening the Reporting of Observational Studies in Epidemiology-Mendelian Randomization (STROBE-MR) guidelines (Supplementary Material).[22]

Data Source

The genetic information pertaining to gut microbiota was obtained from an extensive GWAS study conducted by the MiBioGen consortium. This study included 18,340 individuals, predominantly of European descent, across 24 cohorts.[23] Utilizing 16S rRNA gene sequencing profiles, we aimed to elucidate the genetic underpinnings of gut microbiota composition. We removed 12 genera and 3 families that were categorized as "unknown" from the initial pool of 211 taxa to ensure data integrity. Consequently, 196 were retained for the analysis. Additionally, summary data pertaining to gut microbial metabolites were obtained from a distinct human GWAS study focused on the human metabolome with 2076 European participants.[24] Based on previous studies,[25] we selected 16 metabolites that play a key role in the progression of DM and DN. Summary statistics pertaining to DN were obtained from the FinnGen consortium R9 release

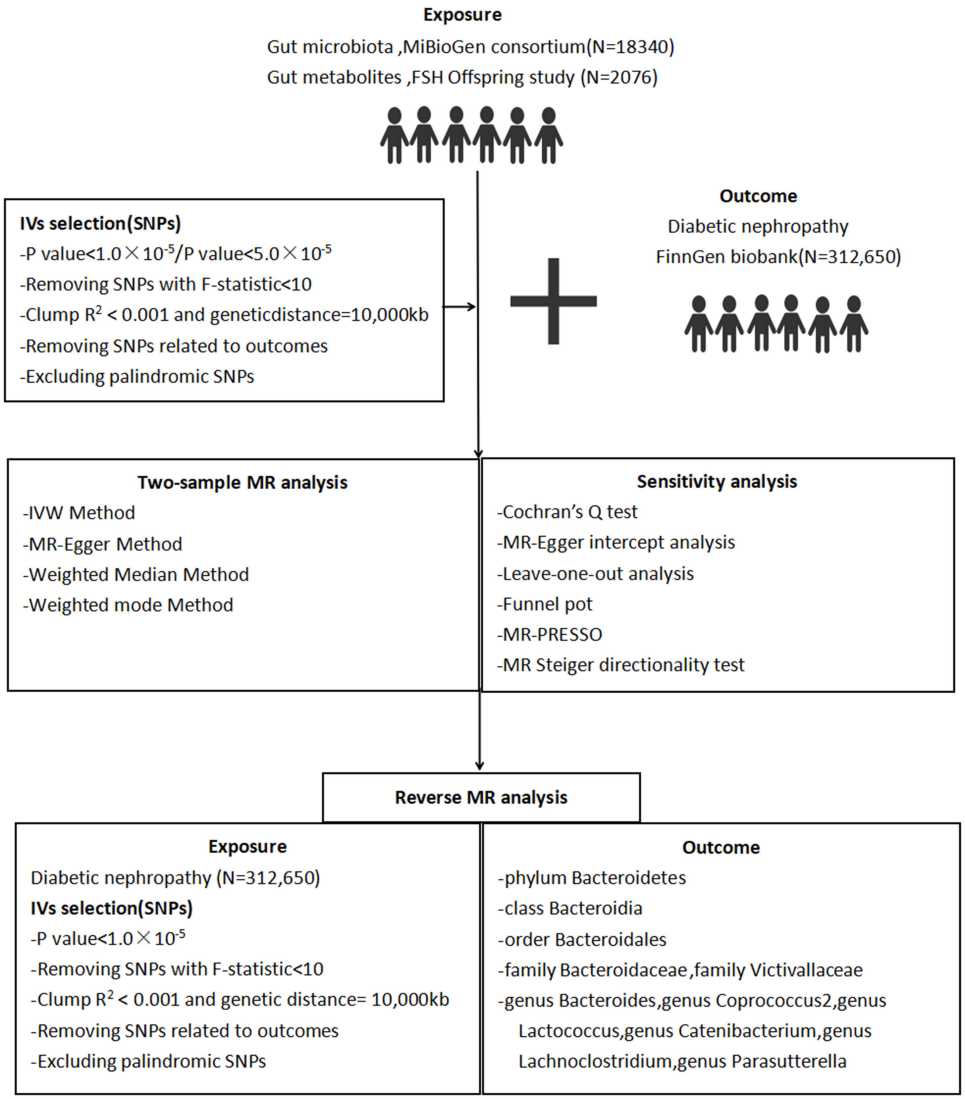

Figure 1 Flowchart of the study.
Abbreviations: SNP, single-nucleotide poly-morphism; MR, Mendelian randomization; IVW, inverse variance weighted median; MR-PRESSO, MR-Pleiotropy Residual Sum and Outlier.

data,[26] which consisted of a cohort of 312,650 European individuals. The study encompassed three endpoints: DM kidney failure, DM dialysis, and DM kidney transplantation.

Selection of Instrumental Variables

We organized the summary data from the gut microbiome GWAS into five different levels based on bacterial classes: phylum, class, order, family, and genus. We selected 16 key gut microbial metabolites for analysis. First, we ensured that the selected instrumental variables were strongly correlated with the exposure factors. Since the common gene loci associated with gut microbiota and metabolites exhibited limited genome-wide significance in GWAS, we opted to analyze SNPs at a significance level of $p < 1.0\times10^{-5}$ for gut microbiota. This criterion allowed us to capture an adequate number of instrumental variables. We extracted gut metabolite SNPs at significance levels of $P < 5.0\times10^{-5}$ and $P < 1.0 \times 10^{-5}$, respectively. The potency of the instrumental variables was assessed using the F-statistic. SNPs with F-statistic < 10 were deemed weak instrumental variables and were removed from our analysis. Second, the selected instrumental variables had to exhibit independence. To this end, we evaluated the linkage disequilibrium using an R2 threshold of 0.001 and ensured genetic separation of at least 10,000 kb between selected markers. Third, we excluded variables with p-values (pertaining to outcomes) less than 5.0×10^{-5} to ensure that the instrumental variables were not associated with the outcome. Additionally, palindromic SNPs were also excluded to mitigate potential sources of bias.

Mendelian Randomization Analysis

We conducted a two-sample MR analysis employing four main methodologies: inverse variance weighted (IVW), MR Egger, weighted median, and weighted mode. IVW is a canonical approach that combines Wald ratio estimates for each SNP through meta-analysis. MR Egger analysis can be utilized in the presence of horizontal pleiotropy. The weighted median approach computes the median of the inverse variance weighted ratio estimates, whereas the weighted mode approach determines the mode of the inverse variance weighted ratio estimates. These methods are resilient to potential instrumental variable violations and provide reliable results even when some instrumental variables fail to meet key assumptions. We also implemented the Bonferroni correction to reinforce the robustness of our findings. P-values more than the Bonferroni-corrected significance threshold, but less than 0.05, were regarded as nominally significant findings.

Sensitivity Analysis

We utilized numerous sensitivity analyses to detect potential gene pleiotropy and heterogeneity and assess the stability of our findings. These analyses included Cochran's Q statistic, MR-Egger intercept tests, leave-one-out (LOO) analyses, and funnel plots. These methods were collectively used to detect potential gene pleiotropy and heterogeneity within the MR analysis and evaluate the robustness of our results. Additionally, we conducted an MR-pleiotropy residual sum and outlier (MR-PRESSO) test. The MR-PRESSO Global test was employed to assess overall horizontal pleiotropy across instrumental variables, whereas the outlier test was used to evaluate the significance of pleiotropy for each SNP. Through an iterative process, we progressively removed outlier SNPs until the p-value of the global test was more than 0.05. The MR Steiger directionality test was employed to assess the robustness of the causal direction. All statistical analyses were conducted using R (version 4.2.3) and the associated MR software packages (TwoSampleMR, MendelianRandomization, Radial MR, MR-PRESSO).

Confounding Analysis

In addition to several methods of sensitivity analysis, we used the PhenoScanner database (http://www.phenoscanner.medchsl.cam.ac.uk/) to exclude SNPs associated with potential confounding factors, particularly hypertension, obesity, dyslipidemia, hyperuricemia, and smoking. Previous studies have identified significant associations between triglycerides and systolic blood pressure and the development of macroalbuminuria. Obesity has been linked to an increased risk of DN. Smoking was shown to be associated with an increased risk of albuminuria and declined eGFR in patients with T1D and T2D. Additionally, hyperuricemia, as a predictor of DN, may play a pathogenetic role in interstitial inflammation and

the progression of renal diseases.[27,28] Recognizing their potential effects on our analysis, we excluded SNPs associated with these factors. Next, we conducted a replication of the IVW method to corroborate the robustness of our results.

Reverse MR Analysis

For gut microbiota and metabolites causally associated with DN as screened by forward MR analysis, we conducted a reverse MR analysis to explore the possibility of reverse causality between DN and gut microbiota and metabolites. The method employed in this analysis mirrored the aforementioned protocol utilized for two-sample MR. The detailed steps are shown in the flowchart (Figure 1).

Results

Selection of Instrumental Variables

A refined set of instrumental variables was obtained after removing palindromic SNPs, excluding SNPs associated with the outcome, and conducting clustering and harmonization. Specifically, 2,036 SNPs originating from 196 different microorganisms ($p < 1\times10^{-5}$), 124 SNPs representing gut microbial metabolites ($p < 1\times10^{-5}$), and 524 SNPs representing gut microbial metabolites ($p < 5\times10^{-5}$) were selected for further analysis. The taxonomic delineation of the gut microbiome comprised five biological classification units, encompassing: phylum (103 SNPs), class (179 SNPs), order (217 SNPs), family (341 SNPs), and genus (1,196 SNPs). Simultaneously, 16 gut microbial metabolites were screened, comprising adipic acid (7/38 SNPs), alpha hydroxybutyric acid (5/25 SNPs), aminoadipic acid (6/28 SNPs), beta aminoisobutyric acid (15/46 SNPs), beta-hydroxybutyric (5/23 SNPs), gamma aminoisobutyric acid (10/36 SNPs), gentisic acid (6/33 SNPs), indole 3 propionate (13/33 SNPs), indoxyl sulfate (7/38 SNPs), kynurenine (12/43 SNPs), propionic acid (3/22 SNPs), trimethylamine N oxide (5/43 SNPs), tryptophan (3/25 SNPs), ureidopropionic acid (10/31 SNPs), phenylalanine (7/32 SNPs), and uric acid (10/28 SNPs). The F-statistics for all instrumental variables was more than 10, suggesting the absence of susceptibility to weak instrument bias (Supplementary Tables 1–3).

MR Analysis of Gut Microbiota and DN

The number of SNP considered for each gut microbial phenotype assessment ranged from 3 to 18. Utilizing the IVW method, we identified 15 bacterial taxa (including 1 phylum, 2 orders, 3 families, 2 orders, and 7 genera) exhibiting causal relationships with DN (Supplementary Tables 4). In alternative MR analyses, we noted a discrepancy between the MR Egger method and the IVW method regarding four bacterial taxa: class Verrucomicrobiae, order Verrucomicrobiales, family Verrucomicrobiaceae, and genus Akkermansia. Out of the 15 taxa, 11 demonstrated relevance to DN. Among them, five bacterial taxa displayed a negative correlation with the risk of DN, specifically family Bacteroidaceae (OR: 0.716, 95% CI: 0.516–0.995, $p = 0.046$), family Victivallaceae (OR: 0.871, 95% CI: 0.772–0.982, $p = 0.026$), genus Bacteroides (OR: 0.716, 95% CI: 0.516–0.995, $p = 0.046$), genus Coprococcus 2 (OR: 0.745, 95% CI: 0.576–0.963, $p = 0.025$), and genus Lactococcus (OR: 0.851, 95% CI: 0.730–0.992, $p = 0.039$). Conversely, six bacterial taxa were positively correlated with the risk of DN, including phylum Bacteroidetes (OR: 1.427, 95% CI: 1.085–1.875, $p = 0.011$), class Bacteroidia (OR: 1.304, 95% CI: 1.036–1.641,$p = 0.024$), order Bacteroidales (OR: 1.304, 95% CI: 1.035–1.641, $p = 0.028$), genus Catenibacterium (OR: 1.312, 95% CI: 1.079–1.594, $p = 0.006$), genus Lachnoclostridium (OR: 1.434, 95% CI: 1.129–1.821, $p = 0.003$), and genus Parasutterella (OR: 1.270, 95% CI: 1.070–1.510, $p = 0.006$) (Figures 2 and 3).

MR Analysis of Gut Microbiota Metabolites and DN

When the level of significance for the instrumental variables was set at $P < 1 \times 10^{-5}$, based on the results of the IVW analysis, each 1 unit increase in ureidopropionic acid corresponded to a 7.0% decrease in the relative risk of DN (OR: 0.926, 95% Cl: 0.859–0.998, $P = 0.046$). This association was verified across the three alternative methods of MR analysis. Importantly, our analyses did not reveal any causal relationship between the remaining gut microbial metabolites and DN (Figure 4, Supplementary Table 5).

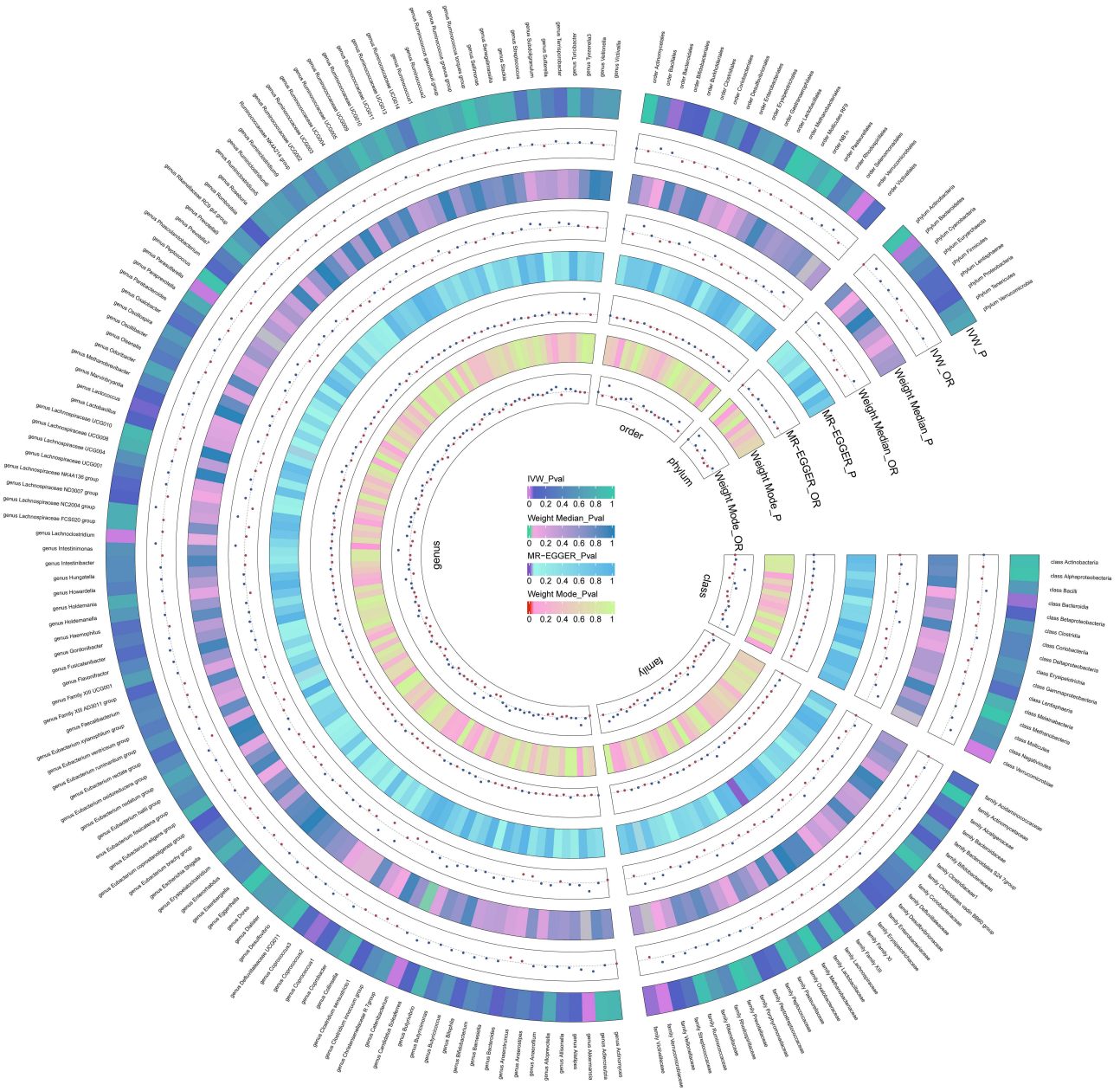

Figure 2 Preliminary Mendelian randomization results between all the gut microbiota and diabetic nephropathy.

Bonferroni Correction and Sensitivity Analysis

Our analysis indicated that none of the bacterial taxa reached the Bonferroni-corrected p-value threshold. Moreover, consistent with the MR-Egger and MR-PRESSO analyses (Table 1, Supplementary Tables 6 and 7), we could not identify any evidence of horizontal pleiotropy, evidenced by p-values > 0.05 across all tests. Notably, the Cochran's Q test revealed no discernible heterogeneity in study effects (Table 1 and Supplementary Table 8). All of the MR Steiger directionality tests consistently indicated a robust causal direction from the gut microbiota and metabolites to DN for all outcome measures. Subsequently, LOO analyses were conducted, demonstrating the robustness of our estimates against the effect of individual instrumental variables (Supplementary Figure 1A-K). The majority of funnel diagrams exhibited a symmetrical pattern, supporting the coherence of our findings (Supplementary Figure 2A-K).

Exposure	Outcome	No.of SNP	Method	OR(95% CI)	P
phylum Bacteroidetes	diabetic nephropathy	10	IVW	1.43 (1.09 to 1.88)	0.011
			MR Egger	1.48 (0.81 to 2.70)	0.243
			Weighted median	1.44 (1.00 to 2.07)	0.053
			Weighted mode	1.80 (1.02 to 3.17)	0.073
order Bacteroidales	diabetic nephropathy	13	IVW	1.30 (1.04 to 1.64)	0.024
			MR Egger	1.09 (0.69 to 1.73)	0.720
			Weighted median	1.37 (0.98 to 1.90)	0.063
			Weighted mode	1.75 (0.94 to 3.24)	0.103
order Verrucomicrobiales	diabetic nephropathy	11	IVW	1.40 (1.13 to 1.73)	0.002
			MR Egger	0.92 (0.45 to 1.89)	0.833
			Weighted median	1.39 (1.03 to 1.87)	0.032
			Weighted mode	1.57 (0.94 to 2.61)	0.115
class Bacteroidia	diabetic nephropathy	13	IVW	1.30 (1.04 to 1.64)	0.024
			MR Egger	1.09 (0.69 to 1.73)	0.720
			Weighted median	1.37 (0.99 to 1.89)	0.058
			Weighted mode	1.75 (0.90 to 3.39)	0.126
class Verrucomicrobiae	diabetic nephropathy	11	IVW	1.40 (1.13 to 1.73)	0.002
			MR Egger	0.92 (0.45 to 1.89)	0.833
			Weighted median	1.39 (1.03 to 1.87)	0.032
			Weighted mode	1.57 (0.90 to 2.73)	0.145
family Bacteroidaceae	diabetic nephropathy	8	IVW	0.72 (0.52 to 0.99)	0.046
			MR Egger	0.99 (0.16 to 6.35)	0.994
			Weighted median	0.77 (0.51 to 1.16)	0.213
			Weighted mode	0.88 (0.46 to 1.66)	0.701
family Victivallaceae	diabetic nephropathy	11	IVW	0.87 (0.77 to 0.98)	0.025
			MR Egger	0.87 (0.50 to 1.52)	0.643
			Weighted median	0.94 (0.80 to 1.10)	0.413
			Weighted mode	0.99 (0.77 to 1.28)	0.938
family Verrucomicrobiaceae	diabetic nephropathy	11	IVW	1.40 (1.13 to 1.73)	0.002
			MR Egger	0.92 (0.45 to 1.89)	0.829
			Weighted median	1.38 (1.03 to 1.86)	0.031
			Weighted mode	1.56 (0.90 to 2.69)	0.141
genus Bacteroides	diabetic nephropathy	8	IVW	0.72 (0.52 to 0.99)	0.046
			MR Egger	0.99 (0.16 to 6.35)	0.994
			Weighted median	0.77 (0.50 to 1.18)	0.229
			Weighted mode	0.88 (0.47 to 1.64)	0.695
genus Catenibacterium	diabetic nephropathy	4	IVW	1.31 (1.08 to 1.59)	0.006
			MR Egger	2.92 (0.25 to 3.34)	0.480
			Weighted median	1.35 (1.07 to 1.71)	0.012
			Weighted mode	1.37 (0.99 to 1.89)	0.156
genus Coprococcus2	diabetic nephropathy	8	IVW	0.74 (0.58 to 0.96)	0.025
			MR Egger	0.41 (0.05 to 3.15)	0.428
			Weighted median	0.71 (0.50 to 0.99)	0.042
			Weighted mode	0.69 (0.41 to 1.16)	0.208
genus Lachnoclostridium	diabetic nephropathy	13	IVW	1.40 (1.09 to 1.79)	0.008
			MR Egger	1.43 (0.62 to 3.30)	0.421
			Weighted median	1.38 (0.97 to 1.95)	0.072
			Weighted mode	1.27 (0.73 to 2.21)	0.408
genus Lactococcus	diabetic nephropathy	8	IVW	0.85 (0.73 to 0.99)	0.039
			MR Egger	0.61 (0.30 to 1.24)	0.224
			Weighted median	0.87 (0.72 to 1.06)	0.178
			Weighted mode	0.90 (0.66 to 1.24)	0.546
genus Parasutterella	diabetic nephropathy	14	IVW	1.34 (1.11 to 1.63)	0.003
			MR Egger	1.46 (0.59 to 3.61)	0.430
			Weighted median	1.32 (1.02 to 1.71)	0.036
			Weighted mode	1.32 (0.88 to 1.98)	0.199
genus Akkermansia	diabetic nephropathy	11	IVW	1.40 (1.13 to 1.73)	0.002
			MR Egger	0.92 (0.45 to 1.89)	0.832
			Weighted median	1.39 (1.03 to 1.86)	0.030
			Weighted mode	1.56 (0.90 to 2.68)	0.141

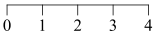

Figure 3 Forest plot summarizing the Mendelian randomization results of gut microbiota with a causal relationship to diabetic nephropathy.
Abbreviations: OR, odds ratio; CI, confidence interval; IVW, inverse variance weighted; MR, Mendelian randomization.

Exposure	Outcome	No.of SNP	Method	OR(95% CI)	P
adipic acid	diabetic nephropathy	7	IVW	1.01 (0.92 to 1.10)	0.855
alpha hydroxybutyric acid	diabetic nephropathy	5	IVW	0.93 (0.84 to 1.03)	0.189
aminoadipic acid	diabetic nephropathy	6	IVW	1.02 (0.93 to 1.11)	0.669
beta aminoisobutyric acid	diabetic nephropathy	15	IVW	1.03 (0.98 to 1.08)	0.194
beta hydroxybutyric acid	diabetic nephropathy	5	IVW	0.98 (0.87 to 1.11)	0.729
gamma_aminoisobutyric_acid	diabetic nephropathy	10	IVW	0.97 (0.91 to 1.04)	0.453
gentisic acid	diabetic nephropathy	6	IVW	0.98 (0.89 to 1.07)	0.611
indole 3 propionate	diabetic nephropathy	13	IVW	0.95 (0.89 to 1.02)	0.153
indoxyl sulfate	diabetic nephropathy	7	IVW	0.99 (0.91 to 1.07)	0.811
kynurenine	diabetic nephropathy	12	IVW	1.00 (0.94 to 1.06)	0.960
phenylalanine	diabetic nephropathy	7	IVW	0.99 (0.93 to 1.06)	0.761
propionic acid	diabetic nephropathy	3	IVW	0.95 (0.85 to 1.06)	0.349
trimethylamine N oxide	diabetic nephropathy	5	IVW	1.01 (0.99 to 1.04)	0.320
tryptophan	diabetic nephropathy	3	IVW	0.91 (0.78 to 1.06)	0.222
ureidopropionic acid	diabetic nephropathy	10	IVW	0.93 (0.86 to 1.00)	0.046
ureidopropionic acid(remove confounding factor)	diabetic nephropathy	9	IVW	0.94 (0.87 to 1.02)	0.139
uric acid	diabetic nephropathy	10	IVW	1.06 (0.98 to 1.15)	0.123

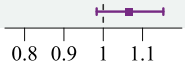

Figure 4 Forest plot summarizing the IVW method results of gut microbial metabolites to diabetic nephropathy (Instrumental variables with $p < 1.0\times10^{-5}$).

Analysis of Confounding Factors

Several factors, including hypertension, obesity, dyslipidemia, hyperuricemia, and smoking, could potentially affect the observed correlation between gut bacteria and the risk of DN. To determine the potential effects of such confounders, we searched the Phenoscanner database and identified instrumental variables that were associated with the aforementioned factors. Notably, a specific SNP (rs62273907) from the genus Parasutterella exhibited linkage with hypertension-related phenotypes. Remarkably, the causality remained robust even after the removal of this SNP (IVW OR= 1.360, 95% CI =1.090–1.700, p=0.007). We also found that an SNP (rs4738679) within the genus Lachnoclostridium and an SNP (rs6101934) associated with ureidopropionic-acid that were potentially associated with dyslipidemia-related phenotypes. After excluding these two SNPs, statistical significance persisted for genus Lachnoclostridium (IVW OR= 1.399, 95% CI =1.091–1.793, p =0.008), whereas ureidopropionic acid no longer showed statistical significance (IVW OR= 0.940, 95% CI =0.865–1.020, p =0.139). None of the other SNPs were correlated with any of the confounders (Supplementary Table 9).

Reverse Analysis

For results with nominal significance, we conducted a reverse MR analysis to identify potential reverse causality between DN and the flora species. This analysis identified no evidence of the presence of reverse causality between DN and the 11 gut flora species (Figure 5, Supplementary Table 10).

Discussion

This study represents the first MR study to investigate the causal implications of gut microbiota and metabolites in the context of DN. Using the MR framework, we identified the associations between gut bacterial taxa and the risk of DN. Specifically, we found that five bacterial taxa were associated with a reduced risk of DN, while six bacterial taxa were linked to an increased risk of DN. This finding is not exactly consistent with the conclusions of a previous MR study. This inconsistency may be related to our stricter criteria for selecting instrumental variables and the analysis of confounding factors.[29] These findings offer insights into potential avenues for DN prevention and treatment by targeting specific gut microbiota.

Our findings underscore the protective role of specific gut bacterial taxa. Notably, the family Bacteroidaceae and genus Bacteroides exhibited negative associations with the risk of DN. Conversely, the phylum Bacteroidetes, class Bacteroidia, and order Bacteroidales displayed positive correlations with the risk of DN. Previous MR analyses supported

Table 1 Sensitivity Analysis of the Causal Association Between Gut Microbiota and DN

Exposure	Outcome	Method	Heterogeneity			Horizontal pleiotropy	MR-PRESSO
			Q	Q_df	Q_*p*-value	Egger_Intercept _Pvalue	*P* value
Phylum Bacteroidetes	Diabetic nephropathy	Inverse variance weighted	8.8427	12	0.7163	0.9066	0.8576
		MR Egger	8.0902	11	0.7051		
Order Bacteroidales	Diabetic nephropathy	Inverse variance weighted	8.8427	12	0.7163	0.4042	0.67
		MR Egger	8.0902	11	0.7051		
Class Bacteroidia	Diabetic nephropathy	Inverse variance weighted	8.8427	12	0.7163	0.4042	0.6626
		MR Egger	8.0902	11	0.7051		
Family Bacteroidaceae	Diabetic nephropathy	Inverse variance weighted	8.5839	7	0.2839	0.7379	0.335
		MR Egger	8.4117	6	0.2094		
Family Victivallaceae	Diabetic nephropathy	Inverse variance weighted	7.1589	10	0.7103	0.9958	0.717
		MR Egger	7.1589	9	0.6205		
Genus Bacteroides	Diabetic nephropathy	Inverse variance weighted	8.5839	7	0.2839	0.7379	0.3193
		MR Egger	8.4117	6	0.2094		
Genus Catenibacterium	Diabetic nephropathy	Inverse variance weighted	0.5750	3	0.9021	0.5855	0.9153
		MR Egger	0.1602	2	0.9230		
Genus Coprococcus2	Diabetic nephropathy	Inverse variance weighted	4.6598	7	0.7013	0.5895	0.7366
		MR Egger	4.3352	6	0.6314		
Genus Lachnoclostridium	Diabetic nephropathy	Inverse variance weighted	8.4415	11	0.6732	0.9568	0.7023
		MR Egger	8.4384	10	0.5860		
Genus Lactococcus	Diabetic nephropathy	Inverse variance weighted	3.9804	7	0.7820	0.3890	0.8203
		MR Egger	3.1187	6	0.7938		
Genus Parasutterella	Diabetic nephropathy	Inverse variance weighted	4.2894	12	0.9776	0.8561	0.9813
		MR Egger	4.2550	11	4.2550		

our findings by linking class Bacteroidia to decreased renal function.[30] Another MR study found that phylum Bacteroidetes, class Bacteroidia, and order Bacteroidales significantly increase the risk of T1DM.[31] Chen et al analyzed clinical indicators and gut microbiota in 60 patients with DN, uncovering a positive correlation between genus Bacteroides and cholesterol and triglyceride levels. Conversely, they observed a negative correlation between genus Bacteroides and albumin and hemoglobin levels, suggesting potential adverse effects in the context of DN.[32] Similarly,

Exposure	Outcome	No.of SNP	Method	OR(95% CI)	P
diabetic nephropathy	phylum Bacteroidetes	28	IVW	1.00 (0.98 to 1.02)	0.793
diabetic nephropathy	order Bacteroidales	28	IVW	1.00 (0.97 to 1.02)	0.914
diabetic nephropathy	class Bacteroidia	28	IVW	1.00 (0.97 to 1.02)	0.914
diabetic nephropathy	family Bacteroidaceae	28	IVW	0.99 (0.95 to 1.03)	0.705
diabetic nephropathy	family Victivallaceae	24	IVW	1.00 (0.95 to 1.06)	0.915
diabetic nephropathy	genus Bacteroides	28	IVW	1.00 (0.97 to 1.03)	0.973
diabetic nephropathy	genus Catenibacterium	21	IVW	1.02 (0.96 to 1.09)	0.463
diabetic nephropathy	genus Coprococcus2	26	IVW	1.00 (0.97 to 1.04)	0.872
diabetic nephropathy	genus Lachnoclostridium	28	IVW	1.01 (0.98 to 1.04)	0.489
diabetic nephropathy	genus Lactococcus	24	IVW	1.05 (0.99 to 1.11)	0.110
diabetic nephropathy	genus Parasutterella	26	IVW	1.00 (0.96 to 1.05)	0.912

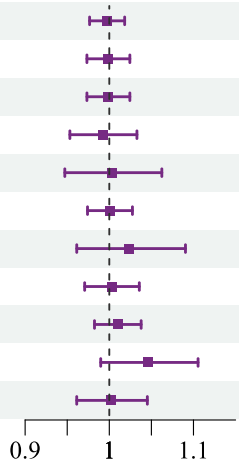

Figure 5 Forest plot summarizing the IVW method results of diabetic nephropathy to the specific gut microbiota.

studying patients with T2DM and DN revealed elevated levels of genus Bacteroides in the gut microbiome.[33] This observation aligns with the findings of Li et al,[19] who found a significant increase in genus Bacteroides among db/db mice with renal failure. The elevation of genus Bacteroides could be attributed to its association with the production of p-cresyl sulfate (PCS),[34] which is considered a uremic toxin that increases during DN.[35] Its accumulation activates the immune system, leading to increased production of inflammatory factors and kidney injury.[36] Notably, Bacteroides have been implicated in kidney inflammation via the LPS-Toll-like receptor 2/4 (TLR2/4) signaling pathway.[37,38] By analyzing alterations in the intestinal flora of patients with DN using macro-genome sequencing technology, He et al observed a reduction in the abundance of species, including Bacteroides coprocola, Bacteroides xylanisolvens, and Bacteroides sp D22 in the DN group.[7] Additionally, another study found that the phylum Bacteroidetes and the genus Bacteroides are negatively correlated with the urine albumin creatinine ratio (UACR) among patients with DN.[8] Animal experiments have also reported a lower abundance of genus Bacteroides in the DN db/db mouse model compared to control db/m mice.[39] Bacteroidetes can ferment dietary fiber into SCFAs, which can serve as potential therapeutic options for systemic inflammatory, immune, and metabolic disorders.[40] SCFAs exert their effects by activating G-protein coupled receptors (eg, GPR41, GPR43, and GPR109A) and inhibiting histone deacetylase (HDAC).[18,41] Activation of GPRs stimulates glucagon-like peptide-1 (GLP-1) secretion, enhancing blood glucose tolerance and insulin sensitivity.[41] The reduction in Bacteroidetes abundance can potentially inhibit SCFA generation and promote the progression of DN. The members of Bacteroidaceae also suppress inflammation by regulating cytokine expression and lymphocyte infiltration.[42] Moreover, Bacteroides fragilis was found to mitigate renal fibrosis by reducing lipopolysaccharide (LPS) and increasing 1.5-anhydroglucitol (1,5-AG) levels.[43] Bacteroides species are promising candidates for treating immune dysregulation and metabolic disorders.[42] It has also been suggested that certain species of Bacteroides may play both beneficial and pathogenic roles based on their location in the host. Upon translocation to extraintestinal organs, they may induce inflammation, diarrhea, etc.[44] The roles of phylum Bacteroidetes and its subclasses remain subjects of debate due to inconsistencies among previous studies. Our results indicated divergent causal associations between various levels of Bacteroides and DN. Specifically, the relationship at the family and genus levels is opposite to that observed at the phylum, class, and order levels. This discrepancy may be caused by other bacterial taxa under the classification of the order Bacteroidales. The cumulative effects of multiple taxa on DN can explain the observed effects. Further studies are needed to unravel the intricate mechanisms underlying the effect of various Bacteroides levels on the development of DN. We propose the following recommendations: First, future studies should investigate the individual effects of distinct subclasses of Bacteroides and also determine the combined effects of multiple bacterial taxa. Second, the effects of varying abundances of Bacteroides on the disease should be explored. Third, efforts should be made to assess the effects of drugs and diet on bacterial flora in clinical studies.

Genus Lachnoclostridium emerged as a risk factor for DN in our study. This finding aligns with previous studies[32] indicating a positive correlation between Lachnoclostridium, serum cholesterol, and triglycerides, implying its detrimental role in DN. Another study indicated that Lachnoclostridium is highly abundant in mice with DN. It positively correlated with the serum levels of Cr, IS, and IL-6, supporting its possible involvement in the pathogenesis of DN.[45] Additionally, Lachnoclostridium was abundant in the gut microbiota of individuals with DN.[46] In a case-control study, patients with stage 5 CKD exhibited a higher relative abundance of Lachnoclostridium than healthy controls.[47] Calorie restriction in the rat model of T2DM improved hyperglycemia, glucose tolerance, and insulin sensitivity, likely by reducing the abundance of pro-inflammatory bacteria, such as Bacteroides, Lachnoclostridium, and Bifidobacterium.[48] Lachnoclostridium has been identified as a trimethylamine (TMA)-producing genus. Specifically, L. saccharolyticum in the Lachnoclostridium genus effectively converted choline to TMA in vitro experiments. Moreover, in vivo studies have shown that the administration of L. saccharolyticum can markedly elevate TMAO levels in the serum of ApoE−/− mice.[49] TMAO, a bacterial uremic toxin, increases intestinal permeability and enters the bloodstream due to the impaired function of the intestinal barrier, potentially contributing to renal dysfunction.[50] Thus, we hypothesized that Lachnoclostridium may affect the development of DN by modulating TMAO levels. In addition, a recent study showed that Lachnoclostridium may increase the risk of type 2 diabetes by producing several metabolites, such as N-acetylglucosamine and hydroxyasparagine, which increase the risk of insulin resistance and obesity.[51]

Furthermore, this study identified the genus Coprococcus as a protective factor against DN, consistent with previous studies. Tao et al observed a significant reduction in the abundance of Coprococcus in the gut microbiota of patients with type 2 diabetes and DN.[8] Similarly, two studies reported decreased abundance of Coprococcus in the gut microbiome of patients with DN.[52,53] This might be attributed to Coprococcus being a butyrate-producing genus,[54] enhancing the integrity of the intestinal barrier by increasing the production of colonic mucins and tight junction proteins (ZO-1).[55] Additionally, it was demonstrated to mitigate kidney injury by activating GPRs or HDACs.[56,57] Notably, SCFAs, particularly butyrate, alleviate DN by suppressing high glucose-induced oxidative stress and NF-κB signaling through GPR43.[58] Furthermore, our study revealed that a higher abundance of the genera Catenibacterium and Parasutterella is associated with an increased risk of DN, whereas a greater abundance of genus Lactococcus and family Victivallaceae is associated with a lower risk of DN. Nosratola et al noted an increase in operational taxonomic units from Catenibacterium in patients with end-stage renal disease (ESRD).[12] Li et al found that altered levels of the microbiota genus Parasutterella are associated with estimated glomerular filtration rate and other clinical indicators of disease severity in CKD.[59] Likewise, another study found a significantly positive correlation between Parasutterella abundance and the risk of type 2 diabetes.[60] Our findings align to some extent with previous studies, the precise mechanisms remain uncertain and warrant further studies.

Notably, our analysis did not reveal any signs of reverse causation between these specific microbiota and DN, suggesting that DN is a consequence of gut dysbiosis, not an instigating factor behind gut dysbiosis.

Unfortunately, certain gut microbial metabolites, including SCFAs, tryptophan, TMAO, and BAs, play an important role in the development of DN.[18] However, our study did not identify a significant association, even when relaxing the filtering conditions for instrumental variables. This lack of significance may be attributed to various factors. First, the pathogenesis of DN is associated with numerous confounding variables that can affect the outcome. Hence, establishing statistical significance for individual metabolite-gene predictions becomes challenging without accounting for these confounding factors. Second, the MR analysis considers a linear association between gut microbial metabolites and DN, potentially overlooking true associations. There may be a nonlinear relationship between gut flora metabolites and DN. Therefore, future observational studies and MR studies should unveil the intricate relationships. Thirdly, DN may act as a cause rather than a consequence of gut dysbiosis. Regrettably, the reverse MR analysis conducted in this study did not yield any significant matches between the SNPs associated with DN and gut microbial metabolites, precluding further exploration.

The strength of this study lies in the use of the most recent and extensive GWAS dataset, employing a gene prediction approach to explore the association between gut microbiota and DN. This approach effectively minimized the effect of confounding factors and overcame the issues related to reverse causality. Nevertheless, several limitations should be considered. First, the GWAS dataset mostly comprised European participants, and due to differences in genetic background, disease susceptibility genes, environment, and lifestyles of various races, further validation is needed to determine the generalizability of these findings to non-European populations. Second, the classification of gut microbial data was constrained to the genus level and beyond, not allowing the establishment of a causal relationship between gut flora at lower taxonomic levels (eg, species or strains) and DN. Third, the composition of gut microbiota relies on various factors, including antibiotic use, diet, and geographical factors. This variability leads to a high degree of microbiome heterogeneity and inter-individual variability, potentially diminishing the statistical power of microbiome GWAS analyses. Fourth, the GWAS summary data on gut microbiota did not exclusively represent European populations, causing a disparity in ethnic proportions compared to the DN dataset. This disparity might lead to some levels of inconsistency in linkage disequilibrium correlations. Both risk factors of DN and the composition of gut microbiota are affected by age and gender. Unfortunately, we could not conduct stratified analyses, potentially affecting our results. Future studies can benefit from MR analyses within subgroups of the sample. Finally, despite efforts, a causal relationship could not be established between microbiota-related metabolites and DN, and reverse MR analyses could not be conducted due to data limitations. There is an urgent need for larger GWAS datasets of metabolites in the future, as they can facilitate more robust MR analyses of specific metabolites.

In conclusion, our study suggests a plausible cause-and-effect association between gut microbiota and DN. Notably, we identified six bacterial taxa with a positive causal relationship and five bacterial taxa with a negative causal

relationship with DN. These specific microbial strains hold promise as potential biomarkers for DN and offer novel therapeutic and preventive approaches. MR analyses are based on untestable hypotheses, which need further verification through experimental and clinical studies. The molecular mechanisms underlying the interactions between intestinal microbiota and metabolites and DN, and the reasonable application of specific microbiota in the treatment phase need further exploration.

Data Sharing Statement

All data relevant to this study were included in the article or were uploaded as supplementary information.

Ethics Approval

This study used publicly available deidentified data from studies that were approved by an ethics committee concerning human experimentation. This study was approved by the Medical Ethics Committee of Baoding No.1 Central Hospital (No. [2022]004).

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agreed to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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