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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Gut microbiota predict retinopathy in patients with diabetes: A longitudinal cohort study

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Abstract

The gut microbiota has emerged as an independent risk factor for diabetes and its complications. This research aimed to delve into the intricate relationship between the gut microbiome and diabetic retinopathy (DR) through a dual approach of cross-sectional and prospective cohort studies. In our cross-sectional study cross-sectional investigation involving ninety-nine individuals with diabetes, distinct microbial signatures associated with DR were identified. Specifically, gut microbiome profiling revealed decreased levels of *Butyricicoccus* and *Ruminococcus torques group,* alongside upregulated methanogenesis pathways among DR patients. These individuals concurrently exhibited lower concentrations of short-chain fatty acids in their plasma. Leveraging machine learning models, including random forest classifiers, we constructed a panel of microbial genera and genes that robustly differentiated DR cases. Importantly, these genera also demonstrated significant correlations with dietary patterns and the molecular profiles of peripheral blood mononuclear cells. Building upon these findings, our prospective cohort study followed 62 diabetes patients over a 2-year period to assess the predictive value of these microbial markers. The results underlined the panel's efficacy in predicting DR incidence. By stratifying patients based on the predictive genera and metabolites identified in the cross-sectional phase, we established significant associations between reduced levels of *Butyricicoccus*, plasma acetate, and increased susceptibility to DR. This investigation not only deepens our understanding of how gut microbiota influences DR but also underscores the potential of microbial markers as early indicators of disease risk. These insights hold promise for developing targeted interventions aimed at mitigating the impact of diabetic complications.

Key points

- *Microbial signatures are differed in diabetic patients with and without retinopathy*
- *DR-related taxa are linked to dietary habits and transcriptomic profiles*
- *Lower abundances of Butyricicoccus and acetate were prospectively associated with DR*

Keywords Diabetes · Diabetic retinopathy · Microbiome · Diet · Metabolite · Transcriptome

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Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by elevated blood glucose levels. In 2017, more than 451 million adults worldwide lived with DM, a number projected to rise to 693 million by 2045 (Cho et al. 2018). This condition poses significant health risks by affecting multiple organs and leading to various vascular and neurological complications. Diabetic retinopathy (DR), which affected 22.27% of diabetic individuals in 2020 (Teo et al. 2021), stands out as a leading cause of diabetes-related blindness (Cheung et al. 2010). Therefore, accurate identification of high-risk patients followed by prompt intervention is crucial. A thorough investigation into its unique pathophysiology and the discovery of new biomarkers for early detection are warranted.

Although hyperglycemia-related metabolic disruptions were considered central to DR development, long-term glycemic control has not effectively reduced DR incidence (Kim et al. 2022), highlighting the need to explore predisposing factors beyond high blood glucose levels. Modern sequencing techniques now allow characterization of microbial communities and their involvement in diseases like DM. Qin et al. initially reported a moderate microbial imbalance in diabetic patients, marked by decreased short-chain fatty acid (SCFA)–producing bacteria and increased opportunistic pathogens (Qin et al. 2012). Wang et al. introduced the concept of microbial age, which utilizes an unsupervised clustering approach to identify divergent microbial signatures associated with metabolic disturbances and aging, enlightening tailored management strategies (Wang et al. 2024). Ample evidence also linked altered gut microbiota to diabetic complications (Tanase et al. 2020), with specific species associations observed in DR cases, such as an increase in *Clostridium* and a decrease in *Roseburia* (van Heck et al. 2022). Additionally, shifts in *Bacteroidetes*, *Bifidobacterium*, and *Lactobacillus* have been noted in adults with DR (Li et al. 2022). Longitudinal studies are now emerging to establish causal relationships between microbial profiles and incident DM (Wang et al. 2022; Stewart et al. 2018). Moreover, Mendelian randomized studies and interventional studies utilizing fecal microbiota transplantation (FMT) strategies have further reinforced such causality (Sun et al. 2023; Zhang et al. 2024a; Wu et al. 2023; Zhang et al. 2022; Ng et al. 2022; Zhang et al. 2024b; de Groot et al 2021). Recent data have updated our understanding of the effectiveness of FMT in patients with diabetes, leading to improvements in blood glucose levels, lipid profiles, insulin resistance (as measured by the homeostasis model assessment), and blood pressure. Notably, FMT has shown promise in managing diabetes-related distal symmetric polyneuropathy and enhancing cardiometabolic outcomes independently of glycemic control (Yang et al 2023), while its impact on DR remains underexplored.

In this study, we leveraged 16S rRNA sequencing, dietary investigations, plasma metabolite analyses, and mRNA sequencing data from the *Shanghai Cohort Study of Diabetic Eye Disease* (SCODE, clinicaltrials.gov identifier: NCT03666052). Incorporating both cross-sectional and prospective cohort study designs, our primary objective was to scrutinize the associations between gut microbiota and DR, focusing on identifying potential biomarkers indicative of DR risk. Additionally, we explored associations between discriminatory genera and dietary components to elucidate upstream factors. Through the prospective cohort study, we demonstrated specific genera causally linked to DR incidence, providing insights for future mechanistic research into disease processes and potential therapeutic targets.

Materials and methods

Study population

This study adhered to the principles of the Declaration of Helsinki and received approval from the Medical Ethics Committee of Shanghai General Hospital (no. 2018KY209).

Participants were selected from our longitudinal cohort, *SCODE*, and provided written informed consent before enrollment. The *SCODE* cohort details have been previously published (Lin et al. 2022). Briefly, participants were tracked for health data annually at community health centers in Shanghai, including physical measurements, ophthalmic examinations, standardized questionnaires, and collection of blood and stool samples for laboratory tests.

Individuals included in this study had complete omics data and information for ascertainment of DR. Exclusion criteria were as follows: (1) eyes affected by conditions other than DR (e.g., hereditary retinopathy, high myopia, glaucoma, recent intraocular surgery within the past 6 months); (2) histories of systemic inflammatory diseases, infectious diseases, chronic gastrointestinal diseases, or severe organ injuries; and (3) antibiotic usage within 4 weeks prior to stool sample collection. Subjects attending the two-year follow-up were designated as the prospective cohort, while others comprised the cross-sectional analysis of this study. The conceptual framework of this study is illustrated in Fig. 1A.

Data collection

Demographic characteristics, medical history, current medications, and lifestyle information were gathered from all participants by trained technicians using standardized questionnaires at local community clinics. Anthropometric

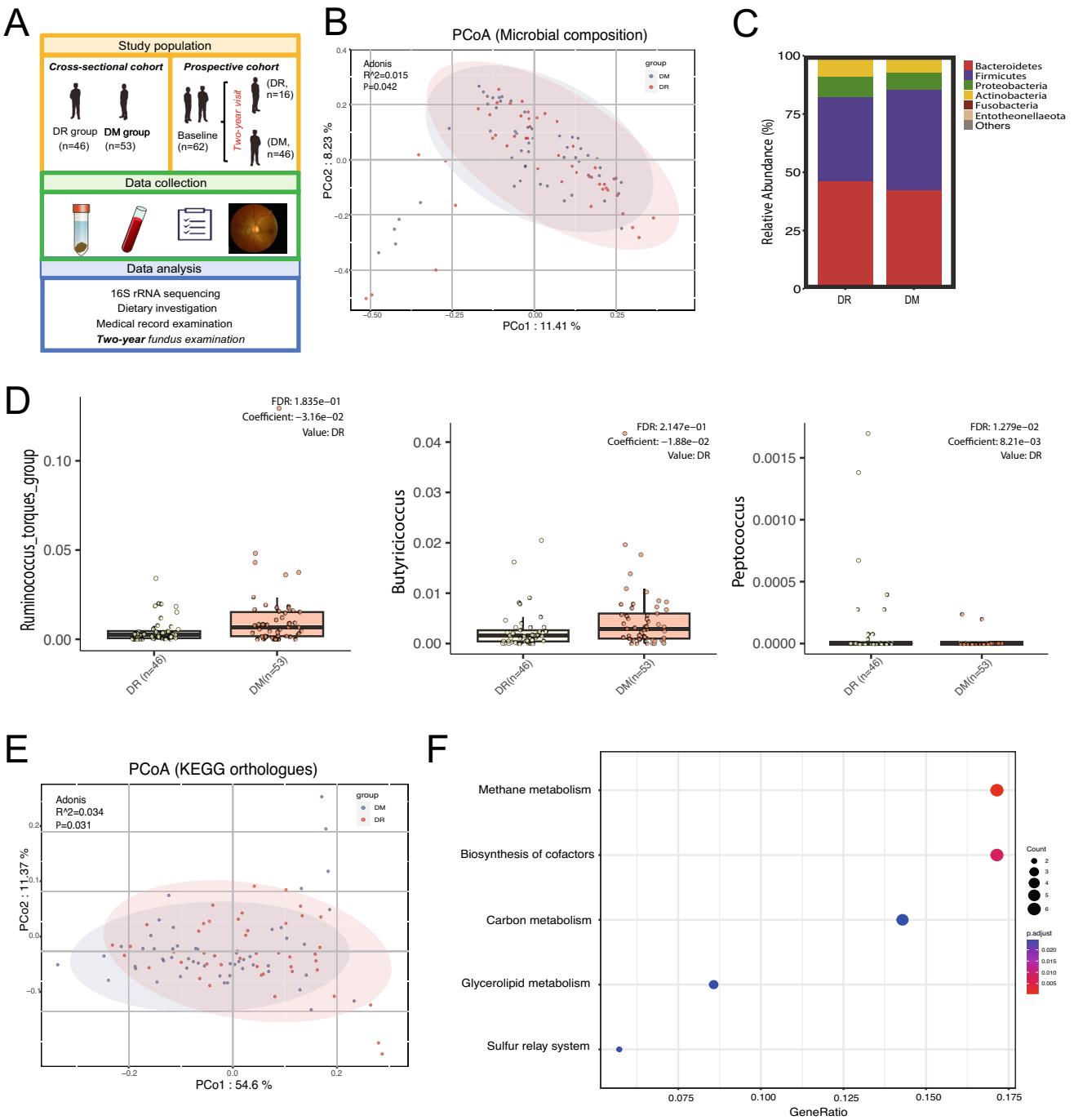

Fig. 1 Gut microbiome profiling in the cross-sectional study. **A** Illustration for the study designs. **B** Principal coordinates analysis (PCoA) to visualize Bray–Curtis distance–based beta diversity. **C** Bar plot of on the relative abundances of the phyla. **D** Bar plots to indicate the differential genera between the DR and DM groups. **E** PCoA to depict KEGG orthologue-based beta diversity. **F** Pathway enrichment analysis on the differentially expressed microbial genes

measurements were conducted following established protocols during participant visits.

Fecal samples were self-collected by participants at home as per detailed instructions and promptly transported on ice to the hospital clinical center within 24 h. Upon receipt, 10 g of feces was aliquoted into sterilized test tubes and stored at − 80 °C until processing. Each participant also provided two tubes of morning whole blood, which were centrifuged (3000 rpm, 20 min) to separate plasma and blood cells, both stored at − 80 °C until analysis.

Dietary intake was assessed using validated Food Frequency Questionnaires (FFQs), estimating consumption

frequency and portion sizes of 122 common food items over the preceding year. Average intakes were calculated based on the Chinese Food Composition Table (Yang 2005). Questionnaires with missing data or extreme reported calorie intake (< 600 or > 3500 kcal/day for women; < 800 or > 4200 kcal/day for men) were excluded ($n = 10$).

Disease ascertainment

Eye disease screenings utilized a remote system integrated with an artificial intelligence image-reading program at local community clinics (Peng et al. 2011). Two ophthalmologists independently verified DR events using the International Clinical Diabetic Retinopathy and Diabetic Macular Edema Disease Severity Scales (Wilkinson et al. 2003), resolving discrepancies through consensus with a third ophthalmologist. Baseline characteristics of participants remained undisclosed during assessments. Individuals identified with DR were advised to undergo detailed fundus evaluations at the hospital. DM diagnosis followed the American Diabetes Association criteria (ElSayed et al. 2023).

16s rrna sequencing and bioinformatics analysis

Fecal DNA extraction used the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA), with quality checked using a Nanodrop spectrophotometer and gel electrophoresis. The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified with primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) via polymerase chain reaction. After quantification with the Quantus™ Fluorometer (Promega, USA), DNA samples were subjected to library preparation and paired-end sequencing (2 × 250 bp) on an Illumina MiSeq PE300 platform.

Raw Illumina sequencing data were demultiplexed, quality-filtered, and spliced using Trimmomatic and FLASH. Quantitative Insights Into Microbial Ecology (QIIME) was employed for data analysis with default parameters. Operational taxonomic units (OTUs) were clustered at a 97% similarity cutoff using UPARSE, with chimeric sequences detected and removed by UCHIME. Intra- and inter-community diversity was calculated at a sequencing depth of 32,620 reads per sample. Alpha diversity metrics (ace, Chao1, Shannon, Simpson indices) assessed OTU richness, with statistical significance determined by the Wilcoxon rank-sum test. Beta diversity based on Bray–Curtis distance was visualized via principal coordinates analysis (PCoA), and differences were assessed by Adonis. Function prediction utilized PICRUSt, assigning Kyoto Encyclopedia of Genes and Genomes (KEGG)–annotated genes to algorithmically determined metabolic modules (www.kegg.jp/).

Taxonomic and functional differences were evaluated using Wilcoxon rank-sum tests, with multiple testing corrections applied using the Benjamini–Hochberg method. Microbiome multivariable associations were examined using linear models (MaAsLin) to scrutinize relationships between genus-level microbes, KEGG orthologs at the functional level, and DR development. Models adjusted for age, sex, BMI as fixed effects, and sample storage site as a random effect to mitigate potential confounding. Similar models substituted DR occurrence with dietary factors as fixed effects to explore their impact on gut microbiota composition. Significant KEGG orthologs identified by MaAsLin were further categorized into pathways.

SCFA quantification and data analysis

Metabolites were extracted by combining 0.03 mL of plasma sample with 0.05 mL of 50% sulfuric acid and 0.2 mL of extraction solution containing 2-methylvaleric acid. The mixture was vortexed in ice water for 30 s, followed by shaking for 10 min and sonication for another 10 min. After centrifugation (10,000 rpm, 15 min, 4 °C), the solution was stored at − 20 °C for 30 min, and the supernatant was used for gas chromatography–mass spectrometry analysis.

During data processing, single peaks were filtered, and peak area data with less than 50% nulls in all groups were retained. Missing values were imputed using numerical simulation, replacing them with half of the minimum observed value. Post-preprocessing, five peaks representing acetate, propionate, butyrate, valerate, and hexanoate were identified. Log2 transformation and UV formatting were applied to mitigate biological noise and potential batch effects. Differential metabolites were identified using least absolute shrinkage and selection operator (LASSO) regression, followed by logistic regression.

RNA sequencing

Total RNA was isolated from peripheral blood mononuclear cells (PBMCs) using the RNeasy mini kit (Qiagen, Germany). Paired-end sequencing was conducted using the TruSeq™ RNA Sample Preparation Kit (Illumina, USA), following the manufacturer's guidelines. Purified cDNA libraries were quantified and qualified using the Qubit® 2.0 Fluorometer (Life Technologies, USA) and Agilent 2100 bioanalyzer (Agilent Technologies, USA), respectively. Libraries were clustered by cBot and sequenced on the Illumina NovaSeq 6000 (Illumina, USA). Raw data underwent demultiplexing and alignment to the human reference genome GRCh37 using Hisat2 (version 2.0.5). SAM files were converted to BAM files and sorted with SAMtools (version 1.3.1). Stringtie software was used to count the number of fragments within each gene, and sequencing

depth normalization utilized the trimmed mean of M-values (TMM) algorithm. Differential gene expression analysis was performed using limma in R, with significance determined at adjusted $P < 0.05$. Pathway enrichment analysis of differentially expressed genes (DEGs) was conducted using Gene Set Enrichment Analysis (GSEA) and Gene Ontology (GO) analysis. Spearman correlation analysis explored potential interactions between gene expression patterns and gut microbiota. The mRNA expression analysis of the entire *SCODE* cohort has been detailed in a companion manuscript (Xiang et al. 2023).

Statistics

Continuous results were presented as mean ± standard deviation, and categorical variables were expressed as ratios. For general comparisons, two-sided Student's *t* tests (or Mann–Whitney tests) were used for continuous variables and Fisher's exact tests for categorical variables.

To assess the explained variance of 16S rRNA sequencing data at the genus and functional levels, we performed random-forest analysis with data split into train and test datasets at a 3:1 ratio. Only microbial genera and genes present in at least 30% of participants were analyzed. Initial variable selection used the minimum redundancy maximum relevance (mRMR) algorithm. Models were evaluated on test datasets using tenfold cross-validation, with accuracy measured by area under the curve (AUC) of receiver operating characteristic (ROC) curves. In the prospective cohort study, the random forest model incorporated discriminatory genera and genes identified from the cross-sectional study to predict subjects at risk of developing DR over a 2-year period.

Microbial co-abundance networks were constructed to validate the robustness of obtained associations between gut microbiota and DR. Participant clusters were identified based on the abundance of 30 genera within co-abundant groups using CCREPE (compositionality corrected by renormalization and permutation). Fisher's exact tests compared DR proportions among clusters.

Causal mediation analyses were used to comprehend relationships between dietary factors, microbial taxa, and DR development. The following models were used: (1) Mediator models: dietary component ~ genus + age + sex + BMI; (2) Outcome models: DR ~ dietary component + genus + age + sex + BMI. Significance was determined using nonparametric bootstrap procedures with multiple testing correction. Average causal mediation effects and average direct effects were reported.

Subgroup analysis was performed in the prospective cohort to assess the predictive ability of identified microbial taxa and metabolites. Subjects were stratified into high and low abundance groups based on ROC thresholds from the cross-sectional study. Fisher's exact tests compared DR incidences between these groups.

All statistical analyses and visualizations were performed using R version 4.3 (R Foundation, Vienna, Austria) and GraphPad Prism V.9.0 (GraphPad Software, San Diego, CA, USA). Results with a two-sided $P < 0.05$ or adjusted $P < 0.25$ were considered statistically significant unless otherwise specified.

Results

Descriptive characteristics of the study participants

In the cross-sectional study, we included a cohort of ninety-nine individuals aged between 36 and 92 years (median age: 70 years). All participants were diagnosed with type 2 diabetes, with a median duration of 13 years (range: 1 to 35 years). Among these, 46 patients showed DR-related lesions in their fundus images and were defined as the DR group, while the remaining fifty-three individuals were categorized into the DM group. Within the DR group, five patients had proliferative DR, with the others displaying mild to moderate non-proliferative DR. Compared to the DM group, participants in the DR group showed higher systolic blood pressure (SBP: 141.2 ± 12.76 mmHg) and a longer duration of diabetes (17.31 ± 7.93 years). Consistent with existing literature, glycemic profile worsened in the DR group, evidenced by significantly higher fasting plasma glucose and glycosylated hemoglobin (HbA1c). Other demographic and biochemical characteristics were similar between the two groups (Table 1).

Our prospective cohort study followed 62 participants over a 2-year period, all initially free of retinopathy. Over a span of 2 years, 16 participants had developed DR based on fundus imaging. Compared to those who remained free of DR, individuals who developed DR had lower levels of high-density lipoprotein. Detailed characteristics of all participants are outlined in Table 1.

Compositional and functional profiles of gut microbiota are altered in DR

In our cross-sectional study, we first profiled the gut microbiome to elucidate alterations associated with DR. Alpha-diversity analysis revealed no significant differences between the two groups (Supplementary Figure S1), indicating overall microbial community richness was relatively stable. However, principal coordinate analysis (PCoA) based on Bray–Curtis distance revealed a marginal difference in microbial community structure between the two groups (Adonis: $P = 0.042$; R-squared = 0.015; Fig. 1B).

Table 1 Participant characteristics

	DR-group ($n=46$)	DM-group ($n=53$)	*P*-value	Prospective cohort ($n=62$)
Demographics				
Sex (male:female)	19:27	31:22	0.108[a]	41:21
Age, year	70.63 ± 9.694	69.02 ± 6.323	0.077[b]	69.03 ± 5.665
BMI, kg/m^2	24.24 ± 2.512	23.74 ± 2.828	0.359[c]	24.89 ± 3.519
SBP, mmHg	141.2 ± 12.76	132.0 ± 12.72	**0.001[c]**	132.5 ± 11.57
DBP, mmHg	76.43 ± 7.432	76.62 ± 6.766	0.977[b]	76.52 ± 5.691
Waist circumference, cm	83.10 ± 7.358	83.98 ± 7.788	0.563[c]	87.26 ± 9.959
Diabetes duration, year	17.31 ± 7.928	13.36 ± 8.949	**0.023[b]**	11.72 ± 8.359
Hypertension, *N*(%)	33 (71.7%)	31 (58.5%)	0.208[a]	37 (59.7%)
Smoking status, *N* (%)	9 (19.6%)	7 (13.2%)	0.425[a]	15 (24.2%)
Alcohol drinking, *N* (%)	7 (15.2%)	13 (24.5%)	0.319[a]	17 (27.4%)
Laboratory data				
FPG, mmol/L	8.597 ± 2.598	7.474 ± 2.094	**0.015[b]**	7.418 ± 1.882
HbA1c, %	7.861 ± 1.522	7.096 ± 1.299	**0.001[b]**	7.103 ± 1.185
TG, mmol/L	1.626 ± 0.813	1.702 ± 1.188	0.656[b]	1.795 ± 1.244
HDL-c, mmol/L	1.348 ± 0.318	1.254 ± 0.344	0.161[c]	1.236 ± 0.3091
LDL-c, mmol/L	3.119 ± 0.938	2.907 ± 1.220	0.332[c]	2.855 ± 1.075
TC, mmol/L	5.099 ± 0.976	4.920 ± 1.353	0.447[c]	4.9 ± 1.237
ALT, U/L	18.35 ± 10.26	19.19 ± 9.729	0.666[b]	19.48 ± 10.21
AST, U/L	19.98 ± 6.126	19.68 ± 5.338	0.730[b]	20.48 ± 5.44
Bilirubin, mmol/L	12.58 ± 5.877	14.58 ± 8.537	0.301[b]	14.24 ± 8.189
Urea, μmol/L	6.588 ± 2.151	5.892 ± 1.702	0.095[b]	5.954 ± 1.595
Creatine, μmol/L	75.24 ± 17.94	84.06 ± 28.92	0.081[b]	84.68 ± 22.8
Uric acid, μmol/L	303.7 ± 77.16	331.0 ± 80.52	0.066[b]	334.4 ± 79.23
Neutrophil count	3.941 ± 1.535	3.772 ± 1.348	0.800[b]	3.816 ± 1.244
Lymphocyte count	1.970 ± 0.617	2.155 ± 0.957	0.330[b]	2.026 ± 0.9022
Monocyte count	0.386 ± 0.124	0.438 ± 0.188	0.221[b]	0.4395 ± 0.178
Neutrophil percentage, %	60.04 ± 7.577	57.29 ± 9.205	0.116[c]	58.88 ± 8.841
Lymphocyte percentage, %	31.32 ± 7.36	33.75 ± 9.108	0.155[c]	31.97 ± 8.788
Monocyte percentage, %	6.033 ± 1.32	6.72 ± 2.067	0.079[b]	6.824 ± 1.948
SCFA data				
Acetate, μg/ml	2.101 ± 1.126	3.253 ± 1.643	**< 0.001[b]**	3.117 ± 1.601
Propionate, μg/ml	0.335 ± 0.195	0.488 ± 0.286	**0.004[b]**	0.465 ± 0.324
Butyrate, μg/ml	0.212 ± 0.123	0.356 ± 0.159	**< 0.001[b]**	0.330 ± 0.033
Valerate, μg/ml	0.054 ± 0.044	0.065 ± 0.056	0.074[b]	0.060 ± 0.044
Hexanoate, μg/ml	0.125 ± 0.050	0.127 ± 0.050	0.086[b]	0.132 ± 0.055

[a]Fisher's exact test

[b]Mann–Whitney test

[c]Unpaired t test

Abbreviations: *BMI* body mass index, *DBP* diastolic blood pressure, *DR* diabetic retinopathy, *DM* diabetes mellitus, *FPG* fasting plasma glucose, *HbA1c* glycemic hemoglobin, *HDL-c* high-density lipoprotein-cholesterol, *LDL-c* low-density lipoprotein-cholesterol, *SBP* systolic blood pressure, *SCFA* short-chain fatty acid, *TC* total cholesterol, *TG* total triglyceride

Taxonomical profiling demonstrated a significantly lower abundance of *Firmicutes* at the phylum level and decreases in several SCFA-producing genera in the DR group compared to the DM group (Wilcoxon rank-sum test, $P < 0.01$, FDR < 0.25; Fig. 1C, D; Supplementary Table S1), recapitulating previous studies on DR. PCoA analysis of KEGG orthologues further highlighted significant functional differences between DR and DM (Adonis: $P = 0.031$; *R*-squared = 0.035; Fig. 1E), with pathway enrichment analysis indicating involvement in methane metabolism, cofactor biosynthesis, glycerol-lipid metabolism, carbon metabolism, and sulfur relay system (Fig. 1F; Supplementary Table S2).

We then explored microbial clusters associated with DR through co-abundance network analysis (Fig. 2A). Four distinct bacterial groups were identified: group 1 (predominantly *Bacteroidales* family members and *Lachnoclostridium*), group 2 (including canonical probiotic strains *Bifidobacterium* and *Lactobacillus*), group 3 (comprising cellulose-degrading genera like *Ruminococcus_2* and *Marvinbryantia*), and group 4 (represented by members of *Lachnospiraceae* such as *Blautia* and *Dorea*). Based on these taxonomic profiles, participants were classified into four clusters, labeled A to D. Cluster B exhibited a higher proportion of DR cases, characterized by an overabundance of group 1 bacteria, while clusters C and D showed higher levels of group 2, 3, and 4 bacteria, correlating with a lower incidence of DR. (Fisher's exact test: $P = 0.035$). Cluster A did not coalesce well, thus being excluded from the DR proportion comparison.

To assess the predictive capacity of omics data for DR, we compared the AUC values from ROC curves using random forest classifiers. Employing the mRMR algorithm, predictor variables were selected from 16S genus level, KEGG orthologue level, and their combined datasets (Fig. 2B; Supplementary Table S3). We identified 15 key genera that provided moderate discriminatory power, yielding an AUC value of 0.860 and explained variance of 18.12% (Fig. 2C). Adjusting for age, sex, and BMI through MaAsLin

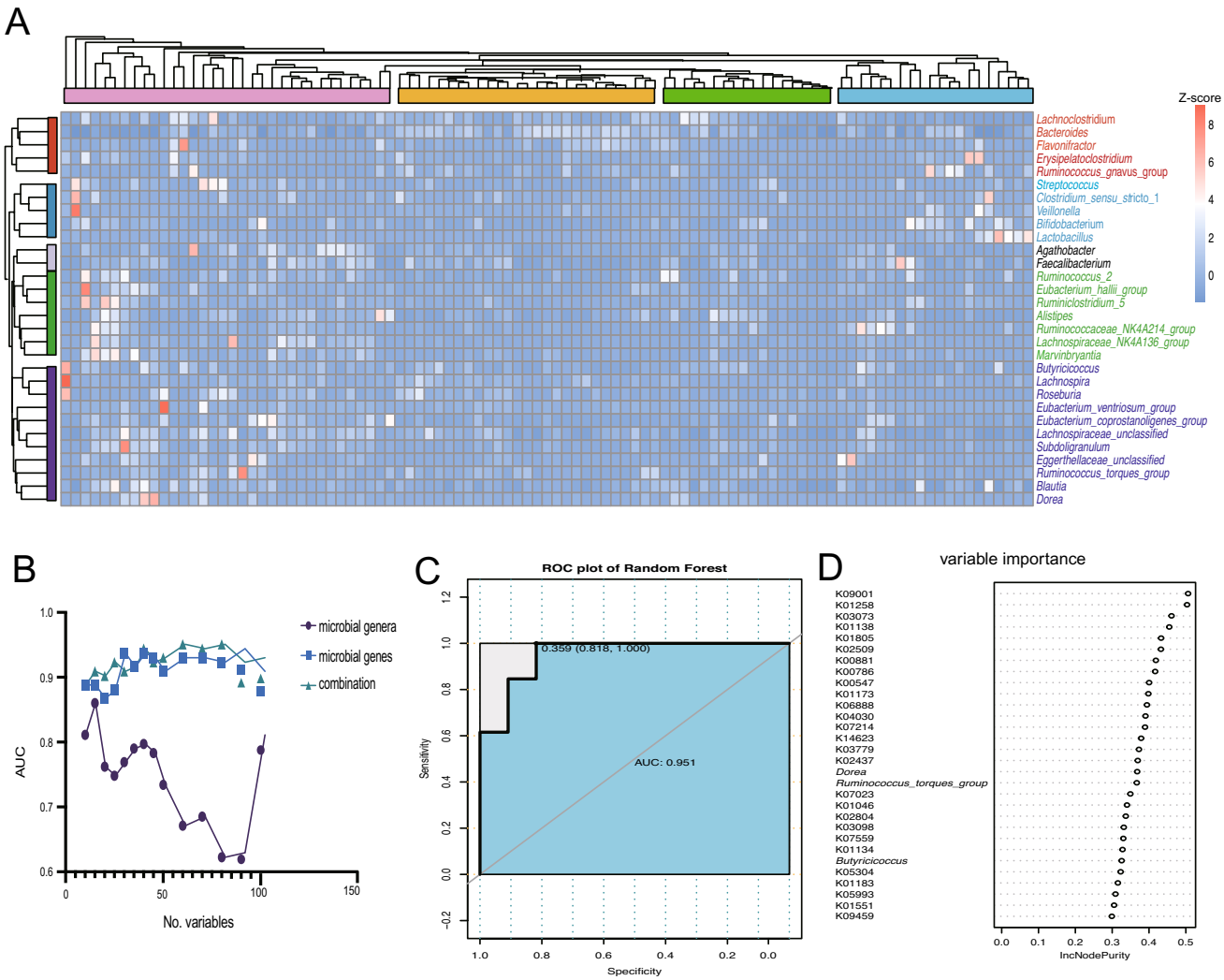

Fig. 2 Microbial signatures predict DR development. **A** Heatmap to show co-abundance groups of bacteria at the genus level and their abundance. Participants in the cross-sectional study were classified into four clusters, A to D, based on their taxonomic profiles. **B** AUC of random forest classifiers was used to discriminate DR based on 16S genus-level KEGG orthologue-level, and combined data. Each dot represents an AUC value of a random-forest classifier using a given number of selected features as predictor variables. The number of predictive variables selected from the datasets increases with the *x*-axis. **C** Receiver operating characteristic curve with the highest AUC value obtained by selected features from the combined datasheet. **D** Variable importance plot to indicate the predictive value of each selected features within the combined datasheet

analysis, we observed that lower levels of *Butyricicoccus* and *Ruminococcus torques group* were individually associated with increased DR risk (Supplementary Table S4). In terms of microbial genomic potential, a set of 30 key KEGG orthologues demonstrated stronger discriminatory power, achieving an AUC value of 0. 937 and explained variance of 19.54%. Combining 50 microbial genes and 10 genera yielded the highest discrimination, with an AUC value of 0.951 and explained variance of 20.56% (Fig. 2D). Specifically, impaired SCFA biosynthesis in the microbiota of DR patients correlated with reduced plasma levels of acetate and butyrate, as confirmed by LASSO-logistic regression analysis (Table 2). Collectively, these findings suggest that individuals with DR harbor a distinct microbiome characterized by metabolic shifts.

Dietary factors are associated with the altered gut microbiota in DR

Evidence substantiates the influence of dietary ingredients on gut microbiota composition, which is intricately tied to the onset of metabolic disorders (Gentile and Weir 2018). In this study, we examined dietary patterns to identify factors associated with microbiota changes in individuals with DR. Compared to those with DM but without retinopathy, adults with DR showed significantly higher cholesterol intake (Table 3). Logistics regression models corroborated significant associations of elevated cholesterol intake and reduced fat energy expenditure with increased DR risk. Moreover, MaAsLin analysis unveiled multiple correlations between dietary factors and discriminatory microbial genera (Supplementary Table S4). For instance, *Akkermansia* abundance was negatively associated with total calorie, fat intake, and cholesterol consumption, while the *Ruminococcus torques group* showed a negative correlation with carbohydrate energy expenditure. Subsequently, we delved whether the impact of dietary factors on DR was mediated by the gut microbiota. Causal mediation analysis indicated that *Akkermansia* modulated the effect of dietary cholesterol on DR development, while *Klebsiella* and *Dorea* mediated the effect of fat energy expenditure. (average causal mediation effect, estimate = −0.0002, 0.0651, and −0.2304, $P = 0.028$, 0.032, and 0.022; respectively; Supplementary Table S5).

Table 2 LASSO-logistics regression models of the cross-sectional study

	LASSO model	Sex, age-adjusted model		Fully adjusted model		
	Lambda.1se	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	AUC (95% CI)
Acetate	−0.0066	0.315 (0.162–0.565)	<0.001***	0.319 (0.158–0.593)	<0.001***	0.797 (0.709–0.885)
Propionate	/	0.518 (0.293–0.849)	0.016*	/	/	/
Butyrate	−5.5785	0.046 (0.006–0.174)	<0.001***	0.053 (0.006–0.203)	<0.001***	0.846 (0.764–0.928)
Valerate	/	0.806 (0.506–1.24)	0.336	/	/	/
Hexanoate	/	0.845 (0.409–1.74)	0.642	/	/	/

Fully adjusted models included LASSO-selected variable systolic blood pressure in addition to sex and age

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Table 3 Dietary investigation in the cross-sectional study

	DR-group ($n = 45$)	DM-group ($n = 51$)	*P*-value	Multivariate-adjusted logistic models		
				OR	95%CI	*P*-value
Total calorie intake, kcal/d	1625 ± 719	1603 ± 506.7	0.687	1	0.999, 1.00	0.683
Total protein intake, g/d	68.4 ± 45.21	64.56 ± 34.1	0.994	1.01	0.994, 1.02	0.366
Total fat intake, g/d	86.4 ± 43.34	94.21 ± 37.93	0.292	0.995	0.984, 1.01	0.331
Total carbohydrate intake, g/d	158.8 ± 80.11	147.8 ± 62.93	0.798	1	0.997, 1.01	0.31
Total fiber intake, g/d	9.695 ± 8.241	10.87 ± 6.127	0.171	1	0.947, 1.06	0.984
Total cholesterol intake, mg/d	343.3 ± 168.2	266.8 ± 108.8	0.011*	1.004	1.00, 1.01	0.008**
Protein energy contribution	0.164 ± 0.041	0.158 ± 0.052	0.518	276	0.030, 4E + 6	0.235
Fat energy contribution	0.421 ± 0.111	0.472 ± 0.121	0.034*	0.010	0.002, 0.407	0.019*
Carbohydrate energy contribution	0.398 ± 0.088	0.370 ± 0.094	0.135	49.6	0.475, 6753	0.107

Data were expressed as mean ± standard deviation. *T*-test and Mann–Whitney test were used for comparison between the two groups

*$P < 0.05$; **$P < 0.01$

Gut microbiota potentially regulates PBMC transcriptomics in DR

To investigate potential links between DR-related microbial changes and transcriptomic signatures of PBMCs, we conducted correlation analysis between the relative abundances of fifteen genera identified by random forest classifiers and DEGs in PBMCs. GSEA analysis revealed significant enrichment of pathways related to the spliceosome, ribosome, and viral protein interaction with cytokine and cytokine receptor in DR patients (Fig. 3A). Noteworthy downregulated DEGs included *C-X-C motif chemokine ligand 10*, *Otoferlin*, and *Matrix metallopeptidase 8* (log2 fold change = − 1.718, − 1.682, and − 1.562, respectively), while *Alpha-2-macroglobulin* was among the most upregulated DEGs in DR (log2 fold change = 1.258). Spearman correlation analyses revealed positive correlations of *Akkermansia* with *H3.5 histone* and *Marvinbryantia* to *ATP binding cassette subfamily A member 2* (Spearman rho = 0.438 and 0.341; adjusted $P < 0.001$; Fig. 3B; Supplementary Table S6). Conversely, *Granulicatella* showed negative correlations with *Selenoprotein N* and *Carbohydrate sulfotransferase 13*. GO pathway analysis of DEGs suggested enrichment in semaphorin receptor activity and proteoglycan binding (Fig. 3C), implying disturbances in extracellular matrix production and neural development in DR.

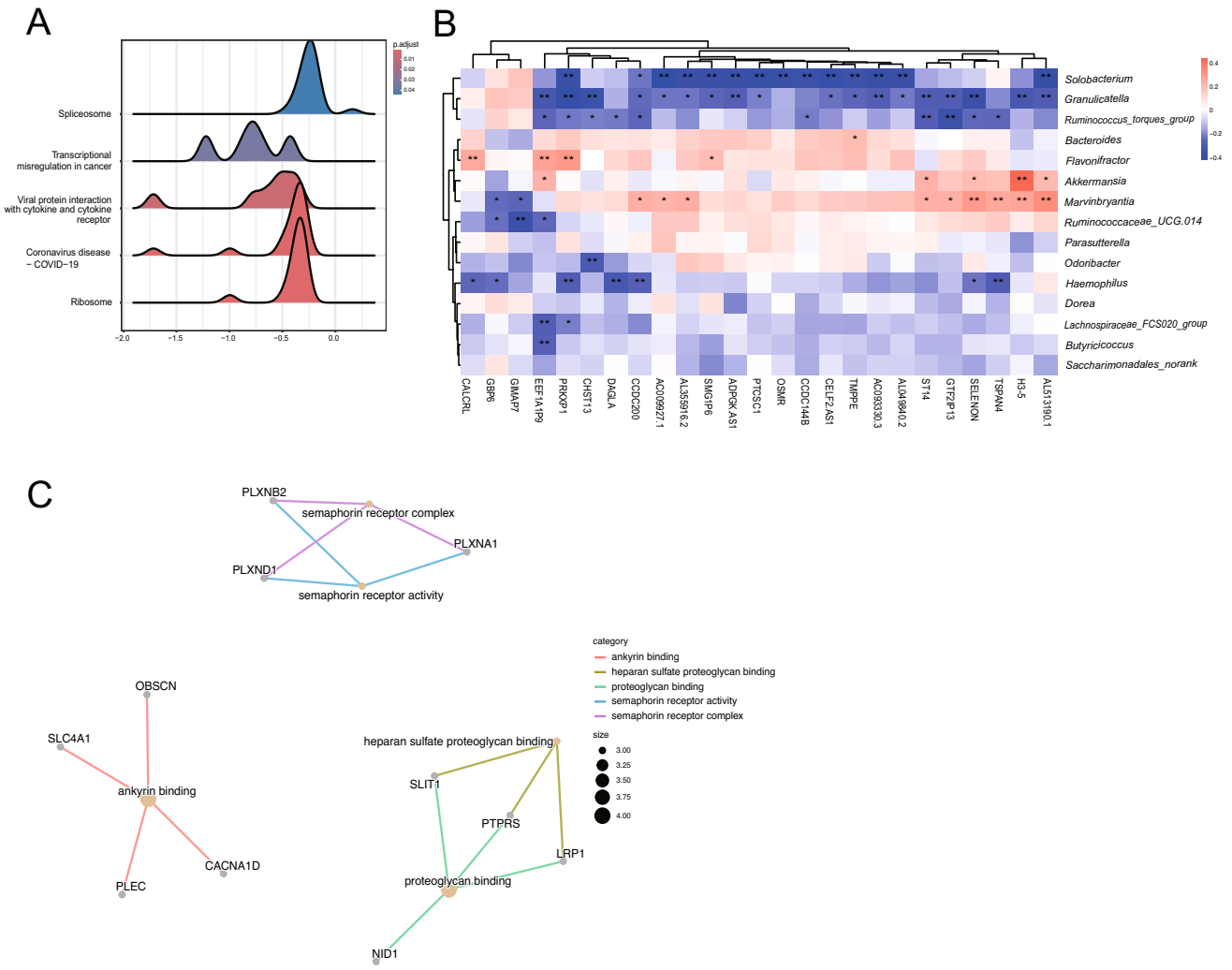

Fig. 3 DR-related microbiota associated with PBMC transcriptomics. **A** Gene set enrichment analysis on the differential expressed genes (DEGs) between the DR and DM groups to summarize the pathway enrichment. **B** Heatmap to show the correlation between the DEGs and the relative abundances of discriminatory genera for DR. **C** Gene Ontology pathway analysis on the genes correlated with discriminatory genera

Microbiome is prospectively associated with DR incidence

We then validated the predictive capacity of the fifteen discriminatory genera and microbial genes identified in our cross-sectional study by replicating the random forest analysis in our prospective cohort (Supplementary Figure S1B,C; Supplementary Table S7). Remarkably, this set of genera demonstrated significant discriminatory ability in identifying individuals at risk of developing DR, achieving an AUC value of 0.704. Furthermore, combining the 30 most predictive microbial genera and genes enhanced discriminatory power, yielding an AUC value of 0.864. Co-abundance analysis of these thirty most abundant genera revealed distinct patient clusters with varying DR risk trajectories: a small cluster with lower abundance of the *Bacteroides–Alistipes* group but relatively higher abundance of the *Lachnospiraceae* group remained free of DR during the 2-year follow-up, unlike other clusters where a higher proportion developed DR (Fig. S1D). We also confirmed negative correlations of *Granulicatella* with *CHST13* and *ST14*; *Haemophilus* with *DAGLA*, *GBP6*, and *CCDC200*; and *Ruminococcus torques group* with *TSPAN4*. *Flavonifractor* remained positively correlated with *CALCRL* (Supplementary Table S8). These findings mutually underscore the robust association of host microbial signatures with DR risk.

We further stratified this cohort based on the relative abundance of the two most discriminative genera, *Butyricicoccus* and *Ruminococcus torques group*. Using ROC analysis via the Youden index method, we divided the cohort into low-abundance and high-abundance groups and compared the incidence of DR between them. Individuals with a high abundance of *Butyricicoccus* at baseline exhibited a significantly lower incidence of DR compared to those with low abundance (12.1% versus 41.4%; $P = 0.018$; Fisher's exact test). Conversely, no significant difference in DR incidence was observed between the high and low abundance groups of *Ruminococcus torques group* in this cohort. Similar analyses using cutoff values for plasma acetate and butyrate levels revealed a significantly higher incidence of DR in individuals with low acetate levels at baseline (52.9% versus 13.9%; $P = 0.003$; Fisher's exact test). In conclusion, our findings suggest potential causal relationships between specific microbial signatures, plasma biomarkers, and DR incidence, highlighting new avenues for preventing DR.

Discussion

This combined cross-sectional and prospective cohort study delineated structural and functional changes in gut microbiota linked with DR. Our investigation identified a distinct set of microbial genera potentially serving as biomarkers to distinguish DR patients from those with diabetes. Furthermore, dietary components were broadly associated with these microbial genera, suggesting their possible role in DR development (Oliphant and Allen-Vercoe 2019; Gentile and Weir 2018). Integrating bacterial genera and genes improved model accuracy, shedding light on the interaction between host and microbiota. Leveraging the prospective cohort, we validated the predictive capacity of this biomarker panel for identifying individuals at high risk of DR and identified specific microbial taxa causally linked to DR incidence.

Building on prior research (Qin et al. 2012; Tanase et al. 2020; van Heck et al. 2022; Li et al. 2022), our study underscores significant decreases in the relative abundance of SCFA-producing genera, such as *Butyricicoccus* and *Ruminococcus torques group*, among DR patients. Conversely, an elevated presence of *Peptococcus*, recognized as a predictor for chronic inflammatory conditions (Ubachs et al. 2021; Zhang et al. 2020), was newly identified in individuals with DR. Moreover, our functional analysis is the first to highlight an enrichment of methanogenic pathways in DR patients. Methane production, derived from acetate conversion, aligns with the observed decrease in SCFA-producing genera, potentially influencing gut motility (Villanueva-Millan et al. 2022; Dimidi et al. 2017), thus exacerbating dysbiosis in DR patients.

Functional redundancy, which implies interchangeable bacterial species with similar functions, has been proposed to explain the taxonomically dispersed but functionally coherent microbiota observed in diabetic patients (Moya and Ferrer 2016). Using random forest analysis on omics datasets, we effectively identified microbial strains and genes that robustly discriminate DR cases (random forest classifiers, AUC = 0.951). Clustering based on co-abundance bacterial groups showed that individuals with higher *Bacteroidales* and lower *Lachnospiraceae* levels were more susceptible to DR. These findings highlight the role of a consortium of microbes and functional genes in DR pathophysiology, offering insights into potential microbiome-based strategies for DR prevention and intervention.

Whether bacteria alterations bona fide contribute to a hostile intestinal niche promoting DR or arise as consequences of diabetes-associated metabolic disturbances remain elusive. Addressing this gap, we included a longitudinal cohort with 2-year follow-up data to reinforced findings from the cross-sectional analysis. The microbial genes and genera selected by the cross-sectional cohort not only discriminated DR but also identified individuals prone to developing DR (random forest classifier, AUC = 0.864). Importantly, low abundance of *Butyricicoccus* and plasma acetate was prospectively associated with increased DR risk, aligning with prior studies reporting their metabolic benefits for diabetic patients. Beyond SCFA production, increased *Butyricicoccus* levels are

linked to decreased BMI, cholesterol conversion, improved glucose tolerance, and insulin sensitivity in response to prebiotic interventions (Chung et al. 2021; Tsai et al. 2022; Li et al. 2023; Rodriguez et al. 2020). Our study leverages diverse samples and robust findings validated across both cross-sectional and prospective cohorts, highlighting pronounced microbiome-retinopathy associations in diabetic individuals.

Assessing metabolic status in diabetes is complex due to multimorbidity, and gut microbial composition associated with host pathophysiology exhibits considerable divergence. Analyzing microbial metabolites offers insights into the gut microbiota's role in host physiology. For instance, Liu et al. demonstrated that the gut commensal *Christensenella minuta* influences host metabolism by producing secondary bile acids inhibiting intestinal farnesoid X receptor (Liu et al. 2024). Similarly, Zhai et al. identified pathogenic roles for *Ruminococcus gnavus*-derived tryptamine and phenethylamine in gut dysbiosis–induced insulin resistance in type 2 diabetes (Zhai et al. 2023). However, integrating findings from fecal and blood metabolite analyses presents challenges due to varying associations with taxonomic composition and microbial pathways. Deng et al. observed significant correlations between fecal butyrate levels and gut microbiota composition in prevalent type 2 diabetes, suggesting fecal metabolites better reflect microbial changes (Deng et al. 2023). In our study, integrating fecal microbiome and blood metabolite analyses robustly predicted DR among diabetic individuals. Yet, the low abundance of *Butyricicoccus* (<0.005) in our cohort suggests that a composite index combining bacterial abundances with plasma metabolites may offer a more suitable approach for risk assessment. Notably, fecal metabolites may directly reflect microbial changes, whereas blood metabolite analysis provides better insights into host responses and compensation for gut dysbiosis.

We rigorously explored various host characteristics potentially involved in gut microbiome signatures and the gut-retina axis. Investigation into dietary factors influencing gut microbiota composition revealed a cholesterol-enriched dietary pattern in DR patients and several correlations between dietary components and gut microbes. Despite recent emphasis on adjusting for dietary confounders in disease-microbiota crosstalk (Wang et al. 2022; Zhang et al. 2021), our study focused on participants recruited from community health care centers with presumed shared dietary habits. Although shared dietary practices and sample size limitations may obscure differences, causal mediation analysis suggests dietary variations may influence DR occurrence through alterations in gut microbiota composition. Furthermore, consistent microbiome–PBMC transcriptomics correlations across prospective and cross-sectional studies underscore their relevance to common alterations in host functions associated with DR.

There are several limitations in the present study. Our cohort study's small sample size may introduce potential for overfitting, necessitating replication in larger cohorts. Additionally, our microbiome profiling relied on 16S rRNA sequencing, providing lower resolution than metagenome shotgun sequencing (Ye et al. 2019). Furthermore, not quantifying the comprehensive metabolic output of altered gut microbiota limits the understanding of their functional correlation with DR pathogenesis. Untargeted metabolomic studies may be necessary to expand our knowledge of the gut microbiota's role in DR.

In conclusion, this cohort study elucidates compositional and functional profiles of the gut microbiota in DR patients. We identified microbial genera and genes highly discriminatory for DR individuals and specific genus and metabolite prospectively associated with the development of DR. Our results emphasize the benefits of omics strategies in exploring the biological plausibility of microbial metabolism in DR pathogenesis.

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Author contribution Y.X., L.L., and M.L. designed research. J.L. and S.C. conducted experiments. X.Q. and J.S. analyzed data. X.Q. wrote the manuscript. Y.M. and F.L. reviewed the manuscript. H.Z. reviewed the manuscript and obtained the funding. All authors read and approved the manuscript.

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Data availability All clinical data and data supporting Figs. 1–3 can be found in the Supplementary Tables. The name of the sequencing data repository and accession number are as below: https://www.ncbi.nlm.nih.gov/geo/; GSE221521.

Declarations

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of our institutional research committee and with the 1964 Helsinki declaration and its later amendments. Informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare no competing interests.

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