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AIMS AND SCOPE

The primary aim of *World Journal of Diabetes* (*WJD*, *World J Diabetes*) is to provide scholars and readers from various fields of diabetes with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

WJD mainly publishes articles reporting research results and findings obtained in the field of diabetes and covering a wide range of topics including risk factors for diabetes, diabetes complications, experimental diabetes mellitus, type 1 diabetes mellitus, type 2 diabetes mellitus, gestational diabetes, diabetic angiopathies, diabetic cardiomyopathies, diabetic coma, diabetic ketoacidosis, diabetic nephropathies, diabetic neuropathies, Donohue syndrome, fetal macrosomia, and prediabetic state.

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Basic Study

Alteration of intestinal microbiota is associated with diabetic retinopathy and its severity: Samples collected from southeast coast Chinese

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Abstract

BACKGROUND

Current approaches for the therapy of diabetic retinopathy (DR), which was one of leading causes of visual impairment, have their limitations. Animal experiments revealed that restructuring of intestinal microbiota can prevent retinopathy.

AIM

To explore the relationship between intestinal microbiota and DR among patients in the southeast coast of China, and provide clues for novel ways to prevention and treatment methods of DR.

METHODS

The fecal samples of non-diabetics (Group C, $n = 15$) and diabetics (Group DM, $n = 30$), including 15 samples with DR (Group DR) and 15 samples without DR (Group D), were analyzed by 16S rRNA sequencing. Intestinal microbiota

compositions were compared between Group C and Group DM, Group DR and Group D, as well as patients with proliferative diabetic retinopathy (PDR) (Group PDR, $n = 8$) and patients without PDR (Group NPDR, $n = 7$). Spearman correlation analyses were performed to explore the associations between intestinal microbiota and clinical indicators.

RESULTS

The alpha and beta diversity did not differ significantly between Group DR and Group D as well as Group PDR and Group NPDR. At the family level, *Fusobacteriaceae*, *Desulfovibrionaceae* and *Pseudomonadaceae* were significantly increased in Group DR than in Group D ($P < 0.05$, respectively). At the genera level, *Fusobacterium*, *Pseudomonas*, and *Adlercreutzia* were increased in Group DR than Group D while *Senegalimassilia* was decreased ($P < 0.05$, respectively). *Pseudomonas* was negatively correlated with NK cell count ($r = -0.39$, $P = 0.03$). Further, the abundance of genera *Eubacterium* ($P < 0.01$), *Peptococcus*, *Desulfovibrio*, *Acetanaerobacterium* and *Negativibacillus* ($P < 0.05$, respectively) were higher in Group PDR compared to Group NPDR, while *Pseudomonas*, *Alloprevotella* and *Tyzzelerella* ($P < 0.05$, respectively) were lower. *Acetanaerobacterium* and *Desulfovibrio* were positively correlated with fasting insulin ($r = 0.53$ and 0.61 , respectively, $P < 0.05$), when *Negativibacillus* was negatively correlated with B cell count ($r = -0.67$, $P < 0.01$).

CONCLUSION

Our findings indicated that the alteration of gut microbiota was associated with DR and its severity among patients in the southeast coast of China, probably by multiple mechanisms such as producing short-chain fatty acids, influencing permeability of blood vessels, affecting levels of vascular cell adhesion molecule-1, hypoxia-inducible factor-1, B cell and insulin. Modulating gut microbiota composition might be a novel strategy for prevention of DR, particularly PDR in population above.

Key Words: Intestinal microbiota; Diabetic retinopathy; Occurrence; Progression; Southeast coast of China

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Core Tip: Current approaches for the therapy of diabetic retinopathy (DR) have their limitations. Our study revealed that alteration of gut microbiota was associated with DR and its progression, and further, this association was mediated by multiple mechanisms including producing short-chain fatty acids, influencing permeability of blood vessels, affecting levels of vascular cell adhesion molecule-1, hypoxia-inducible factor-1, B cell and insulin. Hence, reconstruction of gut microbiota might be a promising strategy for prevention of DR.

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INTRODUCTION

For the moment, diabetes is one of the fastest developing and worldwide metabolic diseases, with multiple complications such as diabetic retinopathy (DR). Global pool analysis of DR in 2010 revealed the proportion of DR, and vision-threatening DR in diabetics was 34.6%, 10.2% respectively[1]. DR will cause visual impairment and even blindness in adults aged 20 to 74 years old, and is considered as one of the primary causes[2]. DR is subdivided into non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). PDR is less common in patients but more threatening to vision compared with NPDR[3]. The treatment of PDR and visually threatening diabetic macular edema (DME) is a main research topic on DR. Laser therapy, anti-angiogenic therapy, anti-inflammatory therapy and surgery are major treatments for PDR. Laser therapy is a classic tool for severe NPDR and PDR, aiming to preserve visual acuity[4]. However, laser would impair the central vision and night vision[5]. As a main anti-angiogenic therapy, anti-vascular endothelial growth factor (VEGF) is a recommended therapy of DR refractory to laser treatment and DME. Contrast to laser monotherapy, anti-VEGF therapy can improve visual acuity in inpatients with DME or PDR[6,7]. However, considering limited half-life time of anti-VEGF agents, the repetitive injections of anti-VEGF are

required at one or two months intervals, causing increased financial burden, increased occurrence of endophthalmitis and elevated intraocular pressure[8]. Besides, the long-term therapy of anti-VEGF would reduce patient compliance, increase the incidence of treatment interruption, and result in deterioration finally[9]. Almost nearly 50% patients are insensitive or even non-responsive to anti-VEGF therapy, but new approved anti-angiogenic therapies as well as effective and evidence-based replacement treatments are absent[10,11]. Although glucocorticoids can be used in patients who failed to respond to anti-VEGF, the role in treating PDR still need further confirmation and the side effect of elevating blood glucose constrains its application in diabetics[9]. Other treatments including anti-protein kinase C, angiotensin receptor blockers, fenofibrate have their own flaws[12-16].

Chronic low-grade inflammations are already recognized as pivotal players in the development of diabetes and its complications including DR. Besides the anti-inflammatory effect *via* generating short chain fatty acid such as butyrate[17], intestinal microbiota also plays a pro-inflammatory role by increasing intestinal permeability, releasing lipopolysaccharide (LPS) which was relevant with distant inflammatory response and impacted cytokines such as TNF- α and IL-6[18,19]. The roles of microbiota on inflammation may explain its possible contribution on occurrence and development of DR. An animal experiments showed that intermittent fasting can prevent the occurrence of DR[10]. Only four studies aiming at the relationship between human intestinal microbiota and DR have been found[20-23]. Jayasudha *et al*[21] performed Illumina sequencing of the internal transcribed spacer 2 region which mainly detects fungus. Three other studies performed 16S rRNA sequencing to distinguish the microbiota between diabetics with DR and without DR[20,22,23]. Moubayed *et al*[20] only analyzed fecal genus *Bacteroides* among healthy volunteers, diabetic patients with DR and without DR, lack of analysis of the other microbial community types. As for the other two studiers, one of their limits was that diabetics enrolled are always treated with metformin[22,23]. Metformin can reduce the severity of DR and incidence of NPDR independently and the mechanisms might be anti-angiogenesis and anti-inflammation[24-27]. Notwithstanding no studies have demonstrated that intestinal microbiota involves in the effect of metformin on DR, metformin should also be considered as a confounding factor which may affect the accuracy of the conclusion about relationship between intestinal microbiota and DR stated by Huang *et al*[22]. Moreover, the effect of metformin on different microbiota were inconsistent in type 2 diabetes mellitus (T2DM) patients, which possibly be impacted by duration of diabetes, gender and race[28]. Thus, metformin taken by whole T2DM subjects still probably complicate the analysis of gut microbiota[24]. The abundance of intestinal microbiota was obviously affected by diet and geographic proximity[29,30]. Different intestinal microbiota may be relevant to same diseases among different persons from different areas and with different dietary habits. Our study is focus on exploring the differential bacteria between diabetic patients with DR and without DR, as well as diabetic patients with PDR and NPDR in south Zhejiang and north Fujian in China, aiming to unravel the link between intestinal microflora and DR, and find a new therapeutic target for DR, especially PDR.

MATERIALS AND METHODS

Study population and sample collection

For this study, 45 samples were obtained from patients who are hospitalized in the department of endocrinology in the 1st Affiliated Hospital of Wenzhou medical university from August, 2018 to September, 2020. Patients were divided into non-diabetics (Group C, $n = 15$) and T2DM patients (Group DM, $n = 30$), which was further divided into patients with DR (Group DR, $n = 15$) and patients without DR (Group D, $n = 15$). Further, Group DR was divided into patients with PDR (Group PDR, $n = 8$) and patients without PDR (Group NPDR, $n = 7$). The enrolled patients are aged between 30-80 years old without conditions as pregnant, lactation, current smoker, current drinker, BMI ≥ 27 , prescribed for metformin, alpha glycosidase inhibitor, antibiotics, probiotics, glucocorticoids, cathartics or PPI within 3 mo, rheumatoid arthritis, inflammatory bowel disease, or gastrointestinal tract operation. After admitted into our department, demographic, medical history, physical examination data were collected and several biochemical tests were performed. Participants self-collected a fecal sample, which were collected by patients, and stored at -80 °C later in less than 24 h. This study was approved by the Ethics Committee of the 1st Affiliated Hospital of Wenzhou medical university. All participants gave their informed consent. The trial register number is 2018-129.

DNA extraction and amplification

Fecal samples were snap frozen and stored at -80 °C after collection. Bacterial DNA was isolated from the fecal samples using MagPure Soil DNA LQ Kit (Magen, United States) following the manufacturer's instructions. DNA concentration and integrity were measured by Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and agarose gel electrophoresis, respectively. Polymerase chain reaction (PCR) amplification of the V3-V4 hypervariable regions of the bacterial 16S rRNA gene was carried out in a 25 μ L reaction using universal primer pairs (343F: 5'-TACGGRAG-GCAGCAG-3'; 798R: 5'-AGGGTATCTAATCCT-3'). The reverse primer contained a sample barcode and both primers were connected with an Illumina sequencing adapter.

Library construction and sequencing

The amplicon quality was visualized using gel electrophoresis. The PCR products were purified with Agencourt AMPure XP beads (Beckman Coulter Co., United States) and quantified using Qubit dsDNA assay kit. The concentrations were then adjusted for sequencing. 16S rDNA sequencing were performed using Illumina MiSeq platform at Shanghai OE Biotech Co., Ltd.

Bioinformatic analysis

Paired-end reads were preprocessed using Trimmomatic software to detect and cut off ambiguous bases(N)[31]. It also cut off low quality sequences with average quality score below 20 using sliding window trimming approach. After trimming, paired-end reads were assembled using FLASH software [32]. Parameters of assembly were: 10 bp of minimal overlapping, 200 bp of maximum overlapping and 20% of maximum mismatch rate. Sequences were performed further denoising as follows: Reads with ambiguous, homologous sequences or below 200 bp were abandoned. Reads with 75% of bases above Q20 were retained using QIIME software (version 1.8.0)[33]. Then, reads with chimera were detected and removed using VSEARCH[34]. Clean reads were subjected to primer sequences removal and clustering to generate operational taxonomic units (OTUs) using VSEARCH software with 97% similarity cutoff[34]. The representative read of each OTU was selected using QIIME package. All representative reads were annotated and blasted against Silva database (Version 123) using Ribosomal Database Project classifier (confidence threshold was 70%)[35]. The microbial diversity in fecal samples was estimated using the alpha diversity that include Chao1 index, Shannon index and Simpson index. The Unifrac distance matrix performed by QIIME software was used for weighted Unifrac principal coordinates analysis (PCoA) construction. The 16S rRNA gene amplicon sequencing and analysis were conducted by OE Biotech Co., Ltd. (Shanghai, China).

Statistical analysis

Data with normal distribution and homogeneity of variance were compared using independent samples *t* test, otherwise, were compared using Wilcoxon test. Comparisons between groups were performed with the clinical characteristics. For associations between clinical characteristics and gut microbial, Spearman correlation analysis were performed using R version 3.6.1. Correction for multiple testing was performed using false discovery rate with the Benjamini-Hochberg. False discovery rate values < 0.05 were considered statistically significant.

RESULTS

Clinical and biochemical characteristics

The clinical and biochemical characteristics were compared between Group DM and C as well as Group D and DR, Group PDR and NPDR (Tables 1 and 2). The age, sex proportion and BMI did not differ between the three pairs mentioned above. Further, the other indexes were comparable between Group DM and C except for fasting blood glucose, glycated hemoglobin A1c (Tables 1 and 2). Compared to Group D, neutrophil to lymphocyte ratio, CD4⁺ T cell count were significantly increased in Group DR ($P < 0.05$, $P < 0.05$), whereas, B cell count, CD8⁺ T cell count, NK cell count, percentage of NK cell were decreased ($P < 0.01$, $P < 0.05$, $P < 0.01$, $P < 0.05$, Table 2). Patients in Group PDR had a significantly lower level of estimated glomerular filtration rate (eGFR) and B cell count ($P < 0.05$, $P < 0.01$), and a higher level of fasting insulin compared with NPDR ($P < 0.05$, Table 2).

Sequencing summary

Total 890469 sequences read with an average of 19788.2 reads per sample were obtained among the 45 samples. 46551 OTUs were observed totally, with a mean of 1034.467 OTUs. The phyla *Bacteroidete* was the dominant intestinal microbiota with approximately 40% (Figure 1). The other three phyla dominated in microbiota were *Firmicutes*, *Proteobacteria* and *Actinobacteria*, with average relative abundances of 29.1%, 19.6% and 5.2% respectively (Figure 1).

Fecal microbiota diversity

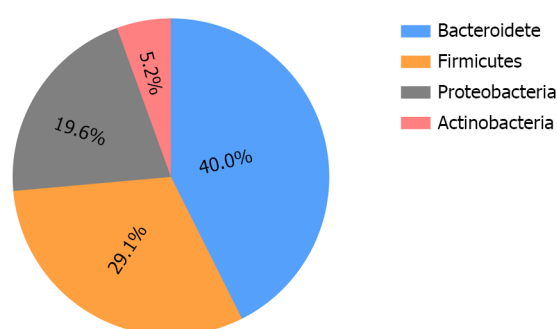
The Chao1, Shannon, simpson indexes were significantly higher in Group C compared with Group DM ($P < 0.001$, $P < 0.001$, $P < 0.001$, Figure 2A-C). However, they did not differ significantly between Group D and Group DR as well as between Group PDR and Group NPDR ($P > 0.05$, Figure 2D-I).

Weighted and unweighted PCoA showed a distinct distance between Group C and Group DM (Adonis, $P < 0.01$, $P < 0.01$, Figure 3A and B), whereas Group D and Group DR had no distinction ($P > 0.05$, $P > 0.05$, $P > 0.05$, $P > 0.05$, Figure 3C and D). However, the microbiota community in Group PDR was not differed from Group NPDR ($P > 0.05$, $P > 0.05$, Figure 3E and F).

Table 1 Basic characteristics compared between Group DM and Group C

Characteristic	Group DM vs Group C		
	Group DM (n = 30)	Group C (n = 15)	P value
Age	55.93 ± 9.58	59.73 ± 16.46	NS
Gender, male/female	19/11	9/6	NS
Height	165.70 ± 8.07	162.20 ± 7.78	NS
Weight	64.75 ± 7.67	62.53 ± 8.29	NS
Body mass index	23.47 ± 2.21	23.73 ± 2.27	NS
Waist circumference	84.75 ± 7.78	84.67 ± 8.36	NS
Hip circumference	91.42 ± 7.32	92.07 ± 7.29	NS
Waist hip ratio	0.93 ± 0.05	0.92 ± 0.08	NS
Waist height ratio	0.51 ± 0.05	0.52 ± 0.05	NS
Systolic blood pressure	128.43 ± 11.98	127.80 ± 14.03	NS
Fasting glucose	8.29 ± 2.79	5.34 ± 0.95	< 0.01
HbA1c	9.94 ± 1.96	5.63 ± 0.31	< 0.01
Triglycerides	1.81 ± 1.82	1.72 ± 0.77	NS
LDL-C	3.00 ± 0.97	2.94 ± 0.88	NS
eGFR	92.29 ± 27.65	87.21 ± 30.00	NS

Data are presented as mean ± SD. HbA1c: Glycosylated hemoglobin A1c; LDL-C: Low-density lipoprotein cholesterol; eGFR: Estimated glomerular filtration rate; Group C: Samples with non-diabetics; Group DM: Samples with diabetics; NS: Not significant.



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Figure 1 The pie shows average relative abundances of the most four dominated phyla. Relative abundances are presented as percentage.

The composition of fecal microbiota

At the phylum level, *Proteobacteria* was the most abundant in Group C followed by *Bacteroidetes* (Figure 4A). The abundance of *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* varied between Group DM and Group C (Figure 4A). The *Firmicutes* to *Bacteroidetes* ratio was slightly higher in Group DM compared with Group C, however, there was no significant difference ($r = 0.86$ vs $r = 0.81$, $P = 0.53$).

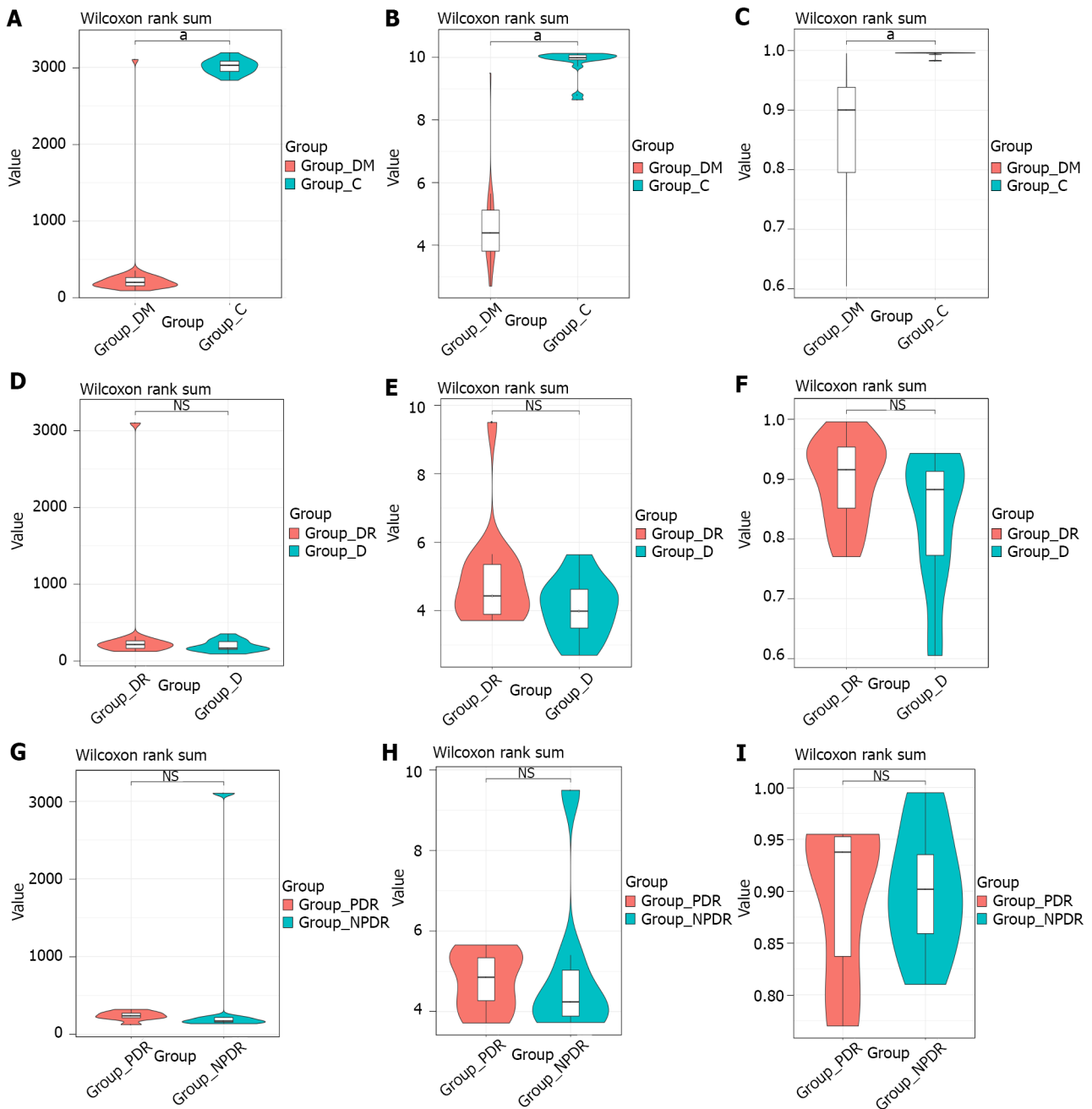
At the phylum level, the majority composition of microbiome in Group D and DR were *Bacteroidetes* and *Firmicutes* (Figure 4B). The relative mean abundance of phylum *Bacteroidetes* and *Firmicutes* as well as *Firmicutes* to *Bacteroidetes* ratio between Group D and Group DR were similar ($P = 0.33$, $P = 0.37$, $P = 0.52$, Figure 4B). The relative mean abundance of phylum *Bacteroidetes*, *Firmicutes* and *Firmicutes* to *Bacteroidetes* ratio showed a similarity between Group PDR and NPDR ($P = 0.71$, $P = 0.33$, $P = 0.54$, Figure 4C).

The linear discriminant analysis effect size revealed that *Fusobacteriaceae*, *Fusobacteriales*, *Fusobacteriia*, *Fusobacteriaceae*, *Desulfovibrionales*, *Delta Proteobacteria*, *Burkholderiaceae* and *Beta Proteobacteriales* were dominant in Group DR (Figure 5A). Meanwhile, *Eubacteriaceae* and *Pseudomonadaceae* were dominant in PDR and Group NPDR respectively (Figure 5B).

Table 2 Basic characteristics compared between samples with diabetic retinopathy and samples without diabetic retinopathy, as well as patients with proliferative diabetic retinopathy vs patients without proliferative diabetic retinopathy

Characteristic	Group DR vs Group D			Group PDR vs Group NPDR		
	Group DR (n = 15)	Group D (n = 15)	P value	Group PDR (n = 8)	Group NPDR (n = 7)	P value
Age	55.87 ± 10.54	56.00 ± 8.90	NS	58.75 ± 11.85	52.57 ± 8.44	NS
Gender, male/female	9/6	10/5	NS	3/5	3/4	NS
Diabetes duration	14.27 ± 7.27	4.045 ± 4.67	NS	15.50 ± 4.99	12.87 ± 9.49	< 0.01
height	165.13 ± 9.26	166.27 ± 6.97	NS	165.75 ± 10.12	164.43 ± 8.90	NS
Weight	64.83 ± 9.11	64.67 ± 6.24	NS	65.19 ± 9.64	64.43 ± 9.22	NS
Body mass index	23.49 ± 1.99	23.45 ± 2.48	NS	23.07 ± 1.24	23.97 ± 2.62	NS
Waist circumference	83.47 ± 8.40	86.03 ± 7.17	NS	85.75 ± 9.77	80.86 ± 6.20	NS
Hip circumference	89.53 ± 8.83	93.30 ± 5.03	NS	92.88 ± 8.17	85.71 ± 8.50	NS
Waist hip ratio	0.93 ± 0.06	0.92 ± 0.05	NS	0.92 ± 0.07	0.95 ± 0.03	NS
Waist height ratio	0.51 ± 0.05	0.52 ± 0.05	NS	0.52 ± 0.07	0.49 ± 0.02	NS
Systolic blood pressure	131.93 ± 13.27	124.93 ± 9.74	NS	135.75 ± 13.79	127.57 ± 12.15	NS
Fasting glucose	9.24 ± 2.95	7.34 ± 2.35	NS	9.61 ± 1.83	8.81 ± 4.00	NS
HbA1c	10.01 ± 1.63	9.87 ± 2.31	NS	9.26 ± 1.62	10.87 ± 1.24	NS
Triglycerides	2.18 ± 2.50	1.44 ± 0.57	NS	2.42 ± 2.90	1.90 ± 2.14	NS
LDL-C	2.78 ± 0.90	3.22 ± 1.02	NS	2.51 ± 1.16	3.09 ± 0.34	NS
eGFR	88.08 ± 32.09	96.51 ± 22.72	NS	69.33 ± 33.84	109.51 ± 7.88	< 0.05
Urine albumin creatine ratio	527.22 ± 1055.30	30.90 ± 52.89	NS	508.40 ± 731.68	548.74 ± 1404.61	NS
Serum creatinine clearance value	97.59 ± 52.53	114.66 ± 41.75	NS	94.24 ± 60.68	101.41 ± 45.96	NS
Urea nitrogen	7.65 ± 5.66	5.87 ± 2.24	NS	9.60 ± 7.24	5.41 ± 1.58	NS
Fasting insulin	61.34 ± 24.06	70.91 ± 57.40	NS	73.31 ± 19.84	47.66 ± 21.96	< 0.05
HOMA-IR	3.82 ± 2.13	3.10 ± 2.00	NS	4.53 ± 1.62	23.00 ± 2.46	NS
HOMA-B	0.40 ± 0.25	0.80 ± 1.02	NS	0.36 ± 0.13	0.45 ± 0.36	NS
Platelet	217.60 ± 55.49	203.67 ± 63.63	NS	196.63 ± 43.45	241.57 ± 61.04	NS
Mean platelet volume	11.13 ± 0.85	10.99 ± 0.79	NS	11.40 ± 0.88	10.83 ± 0.76	NS
Platelet distribution width	13.79 ± 1.58	13.95 ± 1.55	NS	14.05 ± 1.55	13.49 ± 1.67	NS
Neutrophil to lymphocyte ratio	2.85 ± 0.99	2.10 ± 0.86	< 0.05	2.75 ± 0.90	2.97 ± 1.15	NS
T cell count	1165.87 ± 373.69	1518.00 ± 335.85	NS	1092.75 ± 504.02	1249.43 ± 119.08	NS
B cell count	249.67 ± 82.72	378.87 ± 124.49	< 0.01	198.00 ± 57.65	308.71 ± 66.81	< 0.01
CD4 ⁺ T cell count	733.87 ± 299.45	953.2 ± 226.40	< 0.05	684.13 ± 413.77	790.72 ± 49.25	NS
CD8 ⁺ T cell count	389.40 ± 116.23	490.47 ± 137.90	< 0.05	371.88 ± 145.83	409.43 ± 76.37	NS
CD4 ⁺ to CD8 ⁺ T cell ratio	1.91 ± 0.60	2.01 ± 0.42	NS	1.85 ± 0.79	1.98 ± 0.31	NS
NK cell count	220.20 ± 85.18	462.27 ± 250.54	< 0.01	240.00 ± 114.91	197.57 ± 20.12	NS
NK cell percent	12.66 ± 3.65	19.41 ± 9.48	< 0.05	14.05 ± 4.52	11.07 ± 1.33	NS

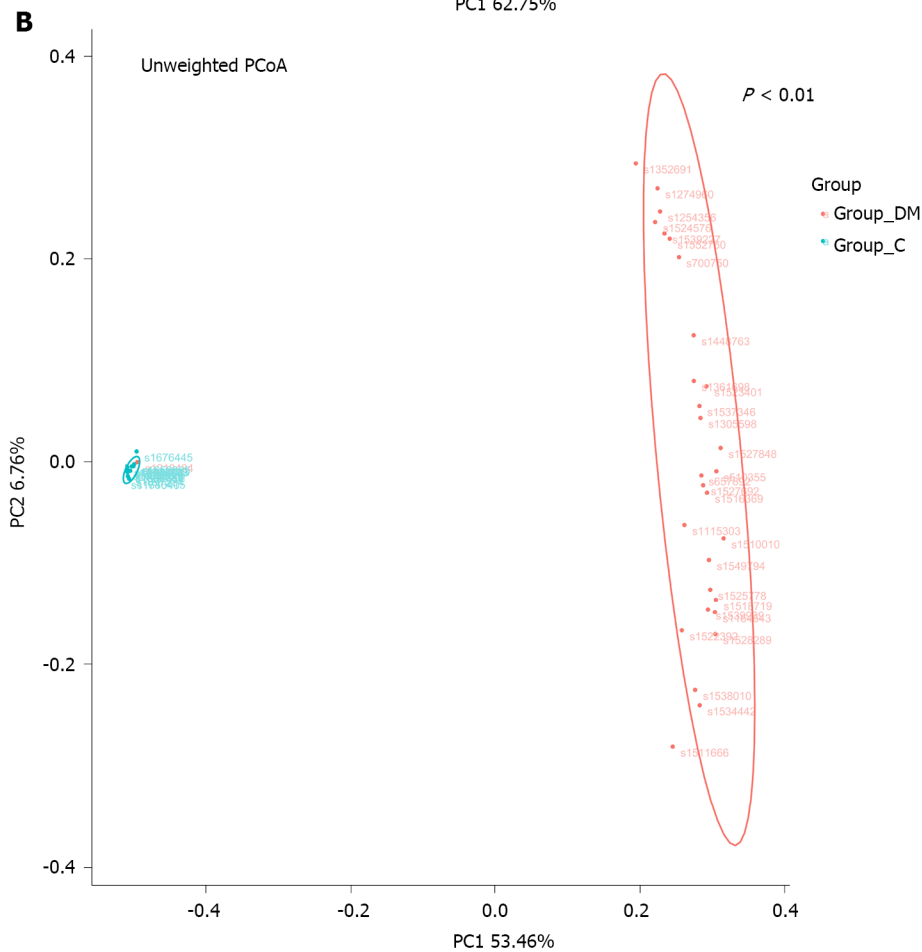
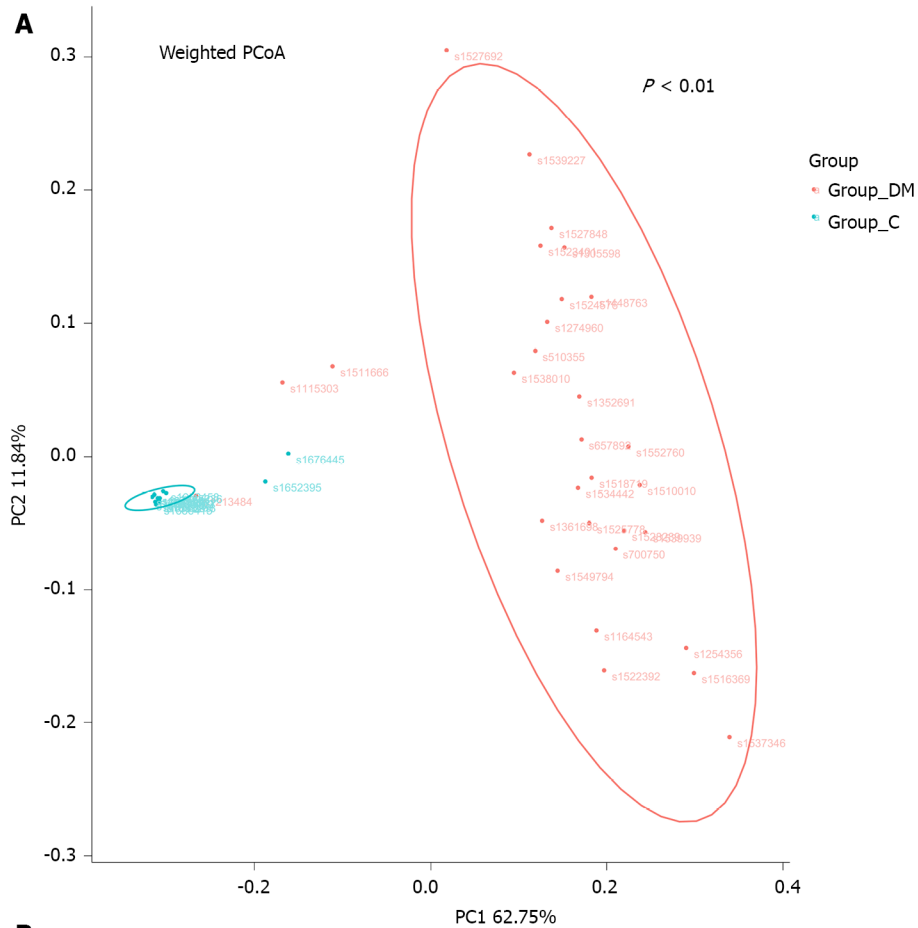
Data are presented as mean ± SD. HbA1c: Glycosylated hemoglobin A1c; LDL-C: Low-density lipoprotein cholesterol; eGFR: Estimated glomerular filtration rate; HOMA-IR: Homeostasis model assessment of insulin resistance; HOMA-B: Homeostasis model assessment of beta-cell function; NS: No significance; Group DR: Samples with diabetic retinopathy; Group D: Samples without diabetic retinopathy; Group PDR: Patients with proliferative diabetic retinopathy; Group NPDR: Patients without proliferative diabetic retinopathy.

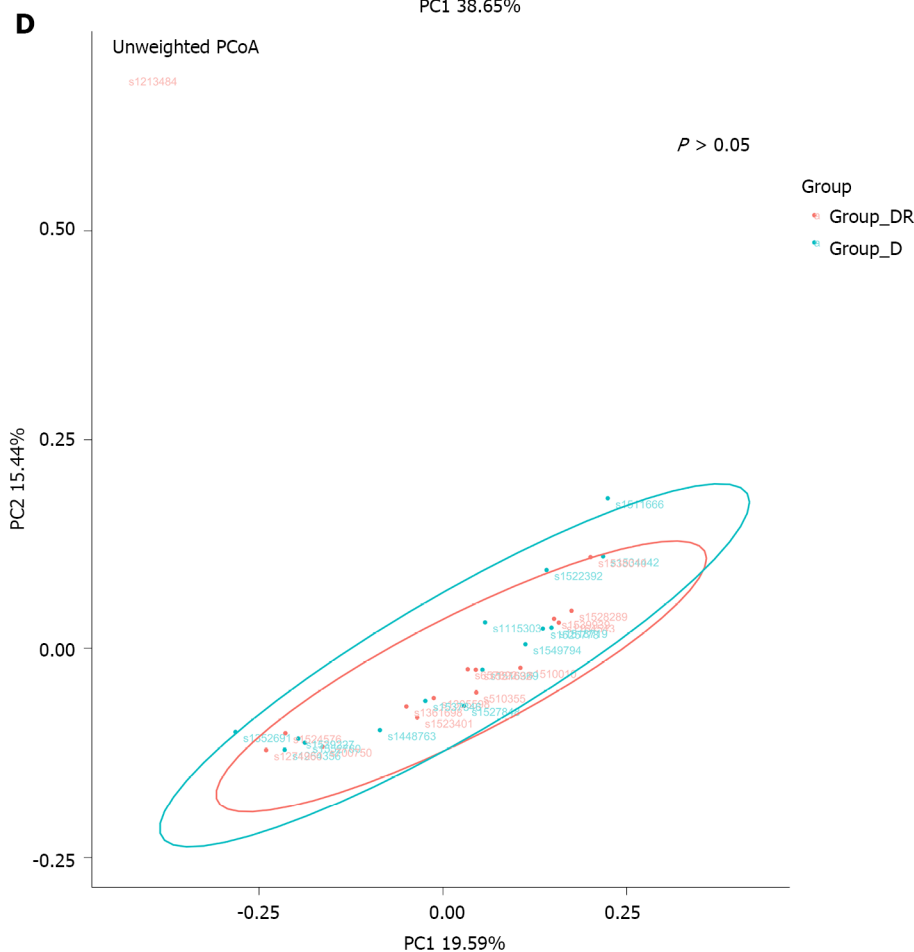
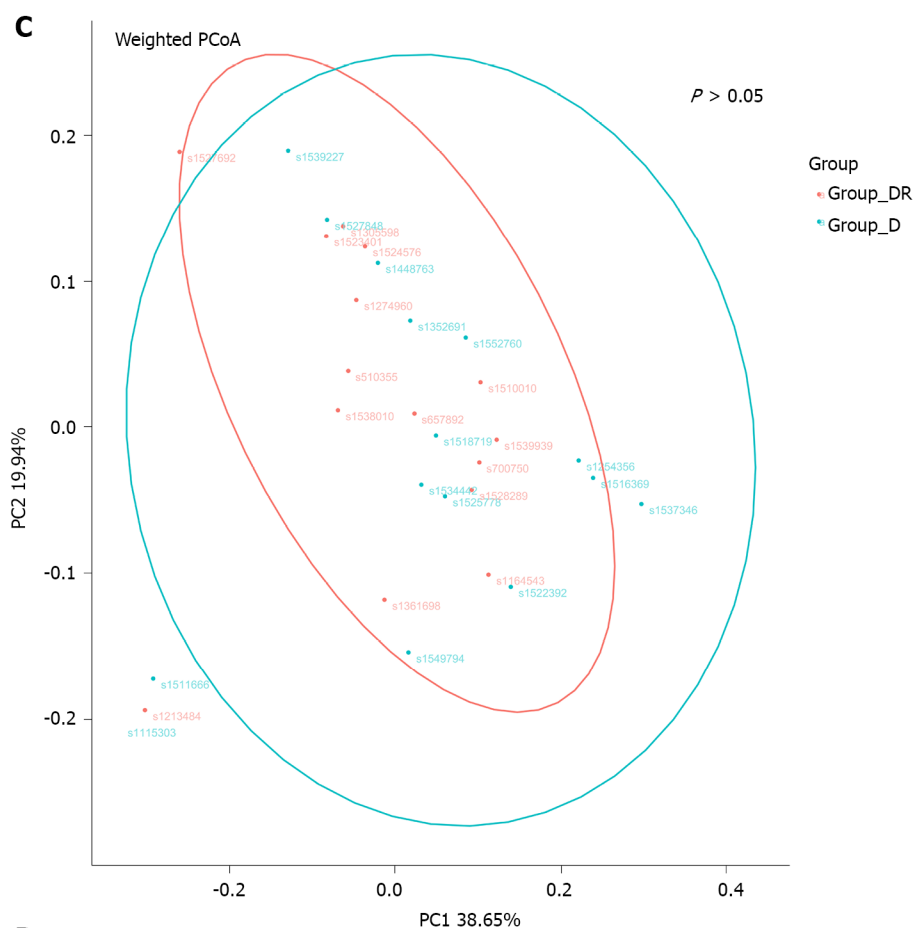


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Figure 2 Alpha diversity analysis of microbiota in the six groups. A: The Chao1 index between Group DM and Group C; B: The Shannon index between Group DM and Group C; C: The Simpson index between Group DM and Group C; D: The Chao1 index between Group DR and Group D; E: The Shannon index between Group DR and Group D; F: The Simpson index between Group DR and Group D; G: The Chao1 index between Group PDR and Group NPDR; H: The Shannon index between Group PDR and Group NPDR; I: The Simpson index between Group PDR and Group NPDR. * $P < 0.05$. NS: Not significant. Group C: Samples with non-diabetics; Group DM: Samples with diabetics; Group DR: Samples with diabetic retinopathy; Group D: Samples without diabetic retinopathy; Group PDR: Patients with proliferative diabetic retinopathy; Group NPDR: Patients without proliferative diabetic retinopathy.

At the phylum level, *Fusobacteria* was significantly higher in Group DR than in Group D ($P < 0.05$, Figure 6A). In addition, at the family level, *Fusobacteriaceae*, *Burkholderiaceae*, *Desulfovibrionaceae* and *Pseudomonadaceae* were significantly increased in Group DR than in Group D ($P < 0.05$, $P < 0.05$, $P < 0.05$, $P < 0.05$, Figure 6B). At the genus level, the abundance of *Senegalimassilia*, *S5-A14a* and *Lachnospiraceae_UCG-008* were significantly decreased in Group DR than in Group D ($P < 0.05$, $P < 0.05$, $P < 0.05$), whereas, *Fusobacterium*, *Pseudomonas*, *Lachnospiraceae_UCG-010* and *Adlercreutzia* were significantly increased ($P < 0.05$, $P < 0.05$, $P < 0.05$, $P < 0.05$, Figure 6C). Further, *Eubacterium*, *Peptococcus*, *Desulfovibrio*, *Acetanaerobacterium*, *Negativibacillus* and *Family_XIII_UCG-001* were significantly increased in Group PDR compared with Group NPDR ($P < 0.01$, $P < 0.05$, $P < 0.05$, $P < 0.05$, $P < 0.05$, $P < 0.05$), whereas, *Pseudomonas*, *Alloprevotella*, *Tyzzereella* and *Tyzzereella-3* had a reduction ($P < 0.05$, $P < 0.05$, $P < 0.05$, $P < 0.05$, Figure 6D).





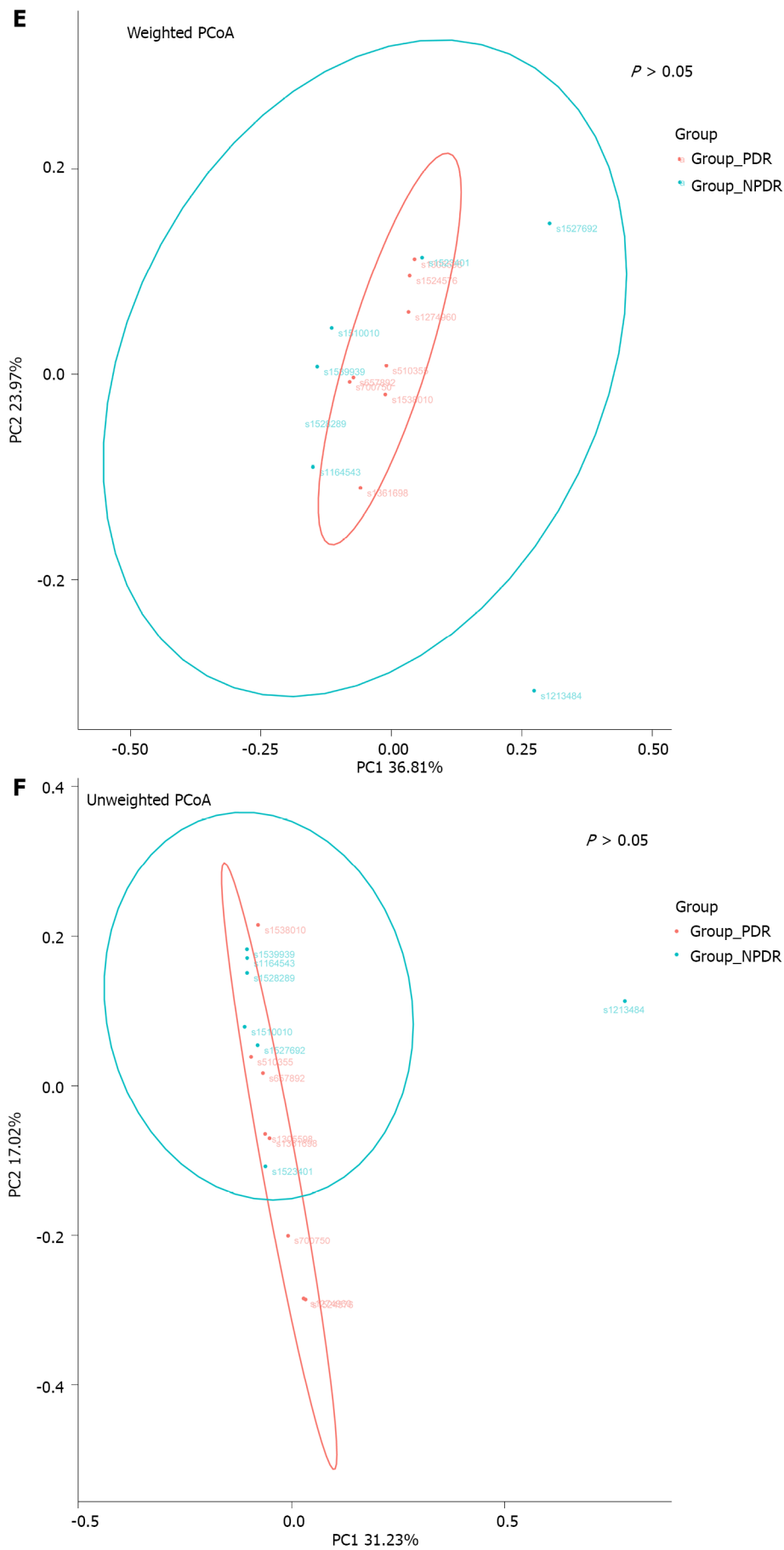


Figure 3 Beta diversity analysis of microbiota in the six groups. A and B: Weighted and unweighted PCoA between between Group DM and Group C; C

and D: Weighted and unweighted PCoA between between Group DR and Group D; E and F: Weighted and unweighted PCoA between between Group PDR and Group NPDR. Differences were assessed by Adonis. Group C: Samples with non-diabetics; Group DM: Samples with diabetics; Group DR: Samples with diabetic retinopathy; Group D: Samples without diabetic retinopathy; Group PDR: Patients with proliferative diabetic retinopathy; Group NPDR: Patients without proliferative diabetic retinopathy.

Spearman's correlations between the relative abundance of bacterial families, clinical indices and biochemical characteristics were performed between Group DR and Group D as well as Group PDR and NPDR. In Group D and Group DR, *Pseudomonas* had a negative correlation with NK cell count ($r = -0.39$, $P < 0.05$, Figure 7A). However, *Senegalimassilia* had a positive correlation with NK cell% ($r = 0.42$, $P < 0.05$, Figure 7A). Meanwhile, in Group PDR and Group NPDR, *Acetanaerobacterium* ($r = 0.53$, $P < 0.05$) and *Desulfovibrio* were positively correlated with fasting insulin ($r = 0.61$, $P < 0.05$, Figure 7B), when *Negativibacillus* was negatively correlated with B cell count ($r = -0.67$, $P < 0.01$) and eGFR ($r = -0.66$, $P < 0.01$, Figure 7C).

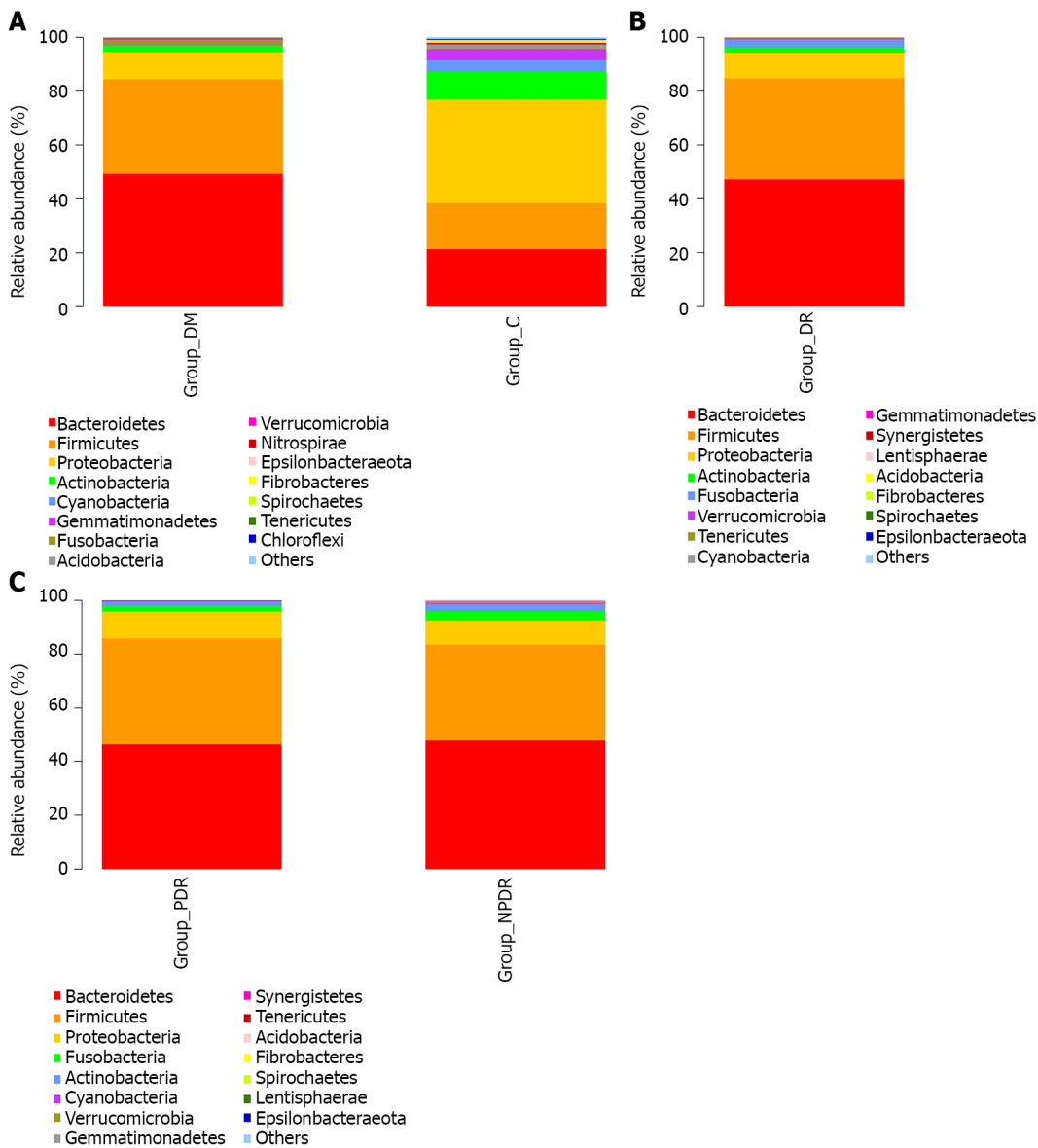
DISCUSSION

Disorder in intestinal microbiota composition has been implicated in occurrence and development of diabetes mellitus (DM)[36,37]. Intestinal microbiota dysbiosis induces oxidative stress, inflammation, insulin resistance and vascular permeability, which probably involves in progression of diabetic complication including DR[37,38]. However, the association between intestinal microbiota and DR remains unclear. Moubayed *et al*[20] found diabetic patients have higher relative abundance of *Bacteroides* than non-diabetic patients, however microbiota differences between patients with DR and without DR were not detected. A study presented microbiota biomarkers to help diagnosing DR, but not analyze the relation between them and clinical markers[22]. Considering existing mature examination to make a definite diagnosis of DR, microbiota biomarkers did not contribute much to the diagnosis. Besides, there was no evidence to prove that the relationship between biomarkers and DR was not accidental. The primary aim of the current study was to assess the gut flora differences of persons with DM and healthy controls, of diabetic patients with DR and without DR separately, applying 16S rRNA gene sequencing. In addition, the analysis of the correlation between the gut flora differences and clinical indexes was taken.

PCOA analysis revealed that the α diversity was decreased significantly in Group DM compared those in Group C, which was in line with previous study[22]. Lower bacterial richness was associated with several common metabolic markers including overall adiposity, insulin resistance and dyslipidaemia coexisted in T2DM[39]. Maintenance of gut nomobiosis played a protective role in glycolipid metabolism[40], on the contrary, gut dysbiosis characterized with reduced microbiota diversity induced expansion of pathogenic bacteria, gut inflammation and deterioration of diabetes[41,42]. Significant variations of gut microbiota between T2DM patients and nondiabetic controls revealed in PCoA was showed by a previous report[43], which was accordant with ours. In addition, in accordance with previous study, α diversity indexes did not differ significantly between Group DR and Group D[22,23]. The α and β diversity did not significantly change between Group DR and Group D as well as between Group NPDR and PDR in our study.

At phylum level, *Bacteroidota*, *Firmicutes*, *Proteobacteria* and *Actinobacteriota* occupied more than 80% of community abundance were regarded as the most dominant phyla in each group. A study found that the abundance of *Firmicutes* and *Bacteroidetes* increased in Group DM compared with Group C, with slightly higher *Firmicutes*/*Bacteroidetes* ratio in Group DM, consistent to our study[43]. Further, the investigation performed by Li *et al*[44] got the similar results in Han population. Of interests, we found that *Firmicutes* were more abundant in Group DR than Group D, while *Bacteroidetes* were less abundant in Group DR via Wilcoxon test. However, Huang *et al*[22] got an opposite result. Although the result in our study was lack of statistical difference, this distinction also got our attention. Metformin may influence *Firmicutes* abundance[45]. We guessed that metformin received by most diabetic patients without DR stated by Huang *et al*[22] might led to lower abundance of *Firmicutes* in Group DM without DR. Compared with Group D, there was a significant increase abundance of *Fusobacteria* in Group DR. However, the study performed by Sisinthy Shivaji was discordant with ours[23], which possibly due to the inconsistent effect of metformin on *Fusobacteria*[28].

Our results indicated that *Fusobacteriaceae*, *Desulfovibrionaceae*, *Burkholderiaceae* and *Pseudomonadaceae* at the family level increased in Group DR compared with Group D. Further, *Eubacteriaceae* and *Pseudomonadaceae* were predominant in Group PDR and Group NPDR respectively. *Fusobacteria* and *Fusobacteriaceae* produced short-chain fatty acids including acetate and propionate and their abundance increased in non-alcoholic steatohepatitis (NASH) subjects compared to nonalcoholic fatty liver and healthy controls[46]. Elevated and propionate in faeces of human NASH subjects were relevant to the increase of Th17 in peripheral blood[46]. An study in animal model showed that blocking the IL-23-Th17-IL-17A pathway would help alleviating DR in mice[47]. Therefore, our results suggested that



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Figure 4 The composition at phylum level in the six groups. A: The composition at phylum level in Group DM and Group C. The abundance of *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* varied between the two groups; B: The composition at phylum level in Group DR and Group D. The relative mean abundance of phylum *Bacteroidetes* and *Firmicutes* between Group D and Group DR were similar ($P = 0.33$, $P = 0.37$); C: The composition at phylum level in Group PDR and Group NPDR. The relative mean abundance of phylum *Bacteroidetes*, *Firmicutes* and showed a similarity between Group PDR and NPDR ($P = 0.71$, $P = 0.33$). Group C: Samples with non-diabetics; Group DM: Samples with diabetics; Group DR: Samples with diabetic retinopathy; Group D: Samples without diabetic retinopathy; Group PDR: Patients with proliferative diabetic retinopathy; Group NPDR: Patients without proliferative diabetic retinopathy.

increased *Fusobacteria* and *Fusobacteriaceae* may contribute to producing acetate and propionate, increasing Th17 and causing DR. *Fusobacteriaceae* is a gram-negative bacterium producing endotoxin, LPS[48]. A study found that *Fusobacteriaceae* increased and induced LPS in pigs with NASH[48]. And, in hyperglycaemic mice, elevation of systemic LPS contributed to the occurrence of DR[49]. Thus, higher abundance of *Fusobacteriaceae* possibly produce LPS and cause DR in our study. *Eubacteriaceae* is one of the bacteria that can metabolize aromatic amino acids to produce p-Cresyl sulfate (a prototype protein-bound uremic toxin)[50]. P-Cresyl sulfate induced renal cell carcinoma to overexpress hypoxia-inducible factor (HIF)-1 α [51]. In our study, eGFR was lower in Group PDR than NPDR. P-Cresyl sulfate which was one of metabolites of great uremic solutes produced by *Eubacteriaceae* may deteriorate DR by elevate the level of HIF-1 α in retina in our study.

Compared to Group D, the genera *Fusobacterium*, *Pseudomonas*, *Adlercreutzia* and *Lachnospiraceae*_UCG-010 were increased, but *Senegalimassilia*, *Lachnospiraceae*_UCG-008 and *S5-A14a* were decreased in Group DR. Huang *et al*[22] found that compared with patients with diabetic patients without DR, Group DR had decreased *Blautia* and *Lactobacillus* and less of them took metformin. Besides preventing the occurrence of DR, metformin also increase *Blautia* and *Lactobacillus*[27,52]. Accordingly, despite lack of statistical differences in the numbers of patients using metformin between the two groups stated by

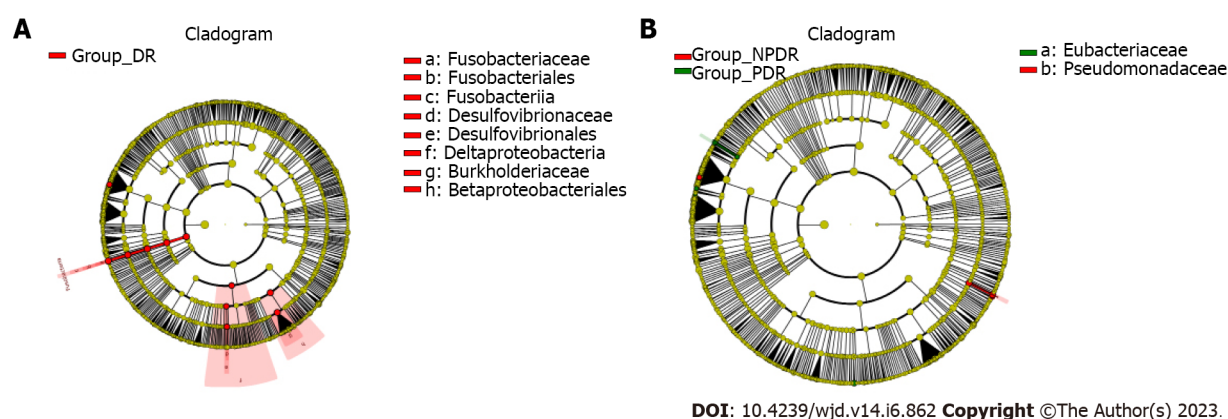
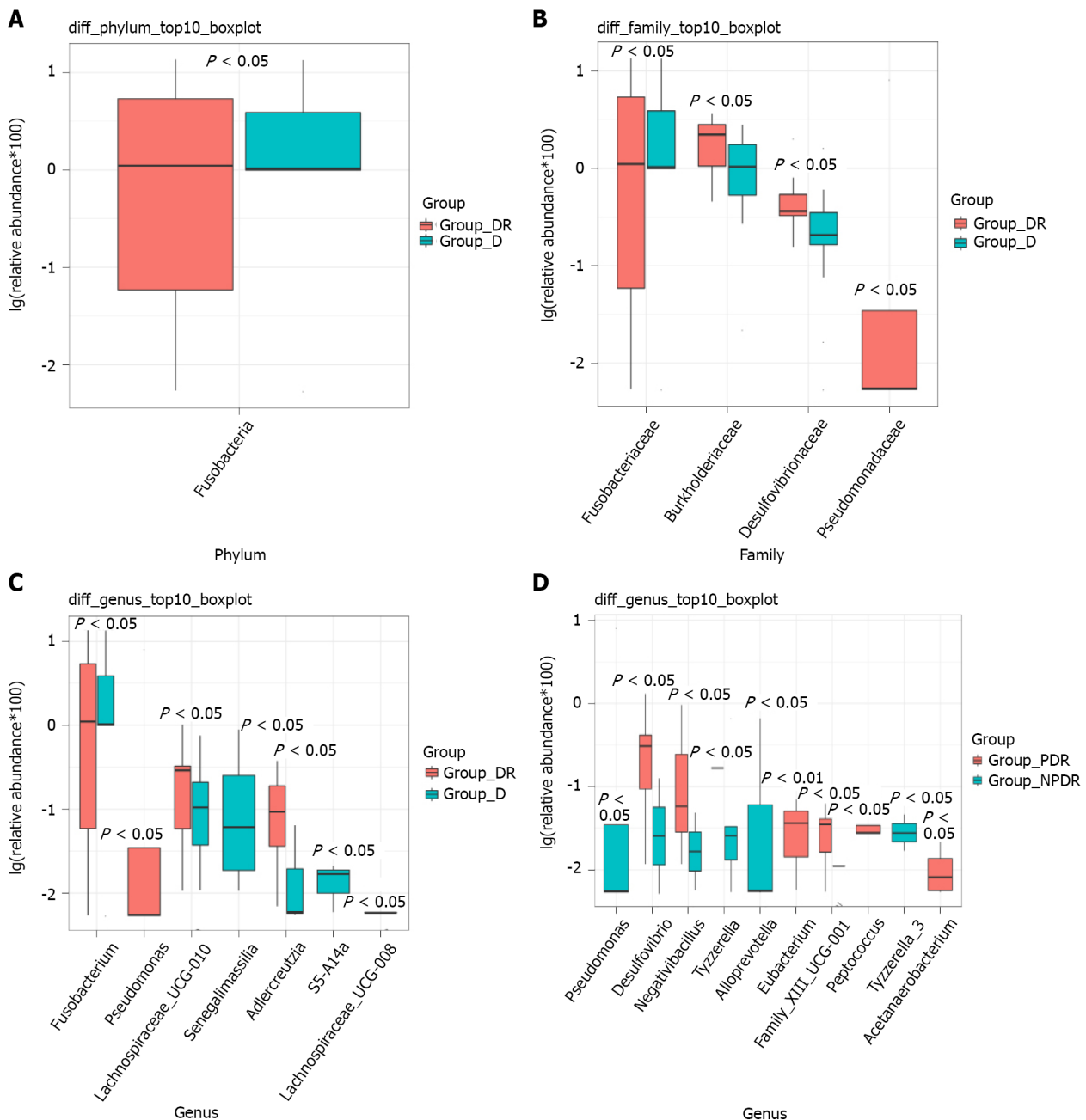


Figure 5 Results of linear discriminant analysis effect size in the six groups. Wilcoxon signed rank test was used for statistical analyzing. The threshold of linear discriminant analysis score was set to 2.0. A and B: Red nodes designated microorganism that only been detected in Group DR. The regions marked with yellow indicated no significant difference between Group DR and Group D as well as Group PDR and NPDR. Group DR: Samples with diabetic retinopathy; Group D: Samples without diabetic retinopathy; Group PDR: Patients with proliferative diabetic retinopathy; Group NPDR: Patients without proliferative diabetic retinopathy.

Huang *et al*[22], we speculated that increased *Blautia* and *Lactobacillus* and lower incidence of DR were relevant to metformin, and the causal relationship between *Blautia*, *Lactobacillus* and DR remained uncertain. Patients in Group DR had a larger proportion of *Fusobacterium*, suggesting a possible larger proportion of *Fusobacterium Nucleatum* (Fn) than in Group D. As a most frequent *Fusobacterium Specie*, FN secreted adhesins recognized vascular endothelial cell receptors and increased the vascular permeability contributing to the development of retinopathy possibly[53,54]. This may explain why diabetic patients with higher *Fusobacterium* are more likely to develop DR. *Pseudomonas aeruginosa* was one of *Pseudomonas species* which was a common pathogen in human body[55]. *Pseudomonas aeruginosa* could help secreting exotoxin to induce hyperpermeability and thrombosis of pulmonary vessels[56]. Besides, our study found that *Pseudomonas* was negatively correlated with absolute value of NK cells, consistent to other studies[57,58]. We presumed that *Pseudomonas* increased the permeability of retinal blood vessels and decreased NK cells, resulting in the occurrence of DR. Further experiments were needed to confirm the supposition. *Adlercreutzia* was positively correlated with leptin level which was positively correlated with the severity of DR[59,60]. Therefore, *Adlercreutzia* may promote the occurrence of DR by influencing leptin. *Senegalimassilia* had the genome that produced enterolactone, which was one of the two kinds of lignans in mammals and negatively correlated with white blood cells and C-reactive protein[50,61,62]. Increased *Senegalimassilia* would inhibit inflammatory response by producing enterolactone, and prevent DR consequently. The relationship between *Lachnospiraceae*_UCG-010, *Lachnospiraceae*_UCG-008, S5-A14a and diabetic complications had not been reported.

Eubacterium, *Peptococcus*, *Desulfovibrio*, *Acetanaerobacterium*, *Negativibacillus* and *Family_XIII_UCG-001* were higher in Group PDR compared to Group NPDR, while *Pseudomonas*, *Alloprevotella* and *Tyzzereella* were lower. *Eubacterium* is known as a butyrate producer[63]. Sodium butyrate in low concentration can promote angiogenesis whereas high concentration sodium butyrate has anti-angiogenic effect[64,65]. However, whether *Eubacterium* in our study promotes DR by generating low concentration sodium butyrate needs to be further clarified. Diabetes had a close relationship with cognitive impairment[66-68]. Diabetics with DR were more likely to suffer cognitive impairment (CI) and patients with higher severity of DR were more likely to have higher incidence of CI[69]. In addition, retinal vessel and cerebral small vessel had similar embryological origin, size and structure, suggesting that DR and CI may have similar pathophysiological basis[69]. A study showed patients with T2DM who had CI had higher level of *Peptococcus* and our study showed patients in Group PDR had a higher level of *Peptococcus* than Group NPDR[70]. *Peptococcus* may inspire both CI and DR progression in diabetic patients. *Desulfovibrio* desulfuricans was one of three species isolated from human faeces and could induce endothelial cell to produce vascular cell adhesion molecule-1 (VCAM-1) relating to the severity of DR[71,72]. Elevated level of *Desulfovibrio desulfuricans* may involve in the progression of PDR. The conclusion needs to be further explored due to the lack of analyzing species levels in genera *Desulfovibrio*. *Desulfovibrio* and *Acetanaerobacterium* were positively correlated with fasting insulin level and their abundance were higher in Group PDR than Group NPDR in our study. Insulin could induce HIF and neovascularization by PI3K and MAPK pathway[73]. Hyperinsulinemia may be the mechanism of *Desulfovibrio* and *Acetanaerobacterium* promoting PDR. Our study showed that *Negativibacillus* was negatively correlated with B lymphocyte. Considering immune cells including B lymphocyte inhibited the formation of pulmonary neovascularization by ischemi, *Negativibacillus* may promote retinal neovascularization by decreasing B lymphocyte[74]. As stated earlier, *Pseudomonas aeruginosa* increased the incidence of DR by promoting increased vascular permeability. However, *Pseudomonas aeruginosa*



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Figure 6 Relative abundance of microbiota displayed by Box and whiskers plots. A-C: Box and whiskers plots display relative abundance of microbiota in different level between Group DR and Group D; D: Box and whiskers plots display relative abundance of genera between Group PDR and NPDR. Group DR: Samples with diabetic retinopathy; Group D: Samples without diabetic retinopathy; Group PDR: Patients with proliferative diabetic retinopathy; Group NPDR: Patients without proliferative diabetic retinopathy.

decreased in Group PDR than Group NPDR. *Pseudomonas aeruginosa* inhibited HIF, a key molecule of developing PDR[75]. Reason for the phenomenon that *Pseudomonas aeruginosa* was related higher incidence of DR but lower incidence of PDR needed further exploration. Butyric acid exhibited anti-angiogenic effect by inhibit expression of *VEGF/KDR* gene, and the higher abundance of *Alloprevotella* in the Group NPDR may suppress angiogenesis *via* butyric acids, thus delay the onset of PDR[76]. *Tyzzerella* produced much propionate which was capable of reducing the expression of VCAM-1 and intercellular adhesion molecule-1 (ICAM-1) induced by cytokine[77,78]. The levels of VCAM-1 and ICAM-1 in serum and eyes of patients in Group PDR were elevated compared with Group NPDR[79]. Hence, *Tyzzerella* may have slowed the progression of DR by reducing VCAM-1 and ICAM-1. The effect of *Family XIII UCG-001* on DR was still unknown.

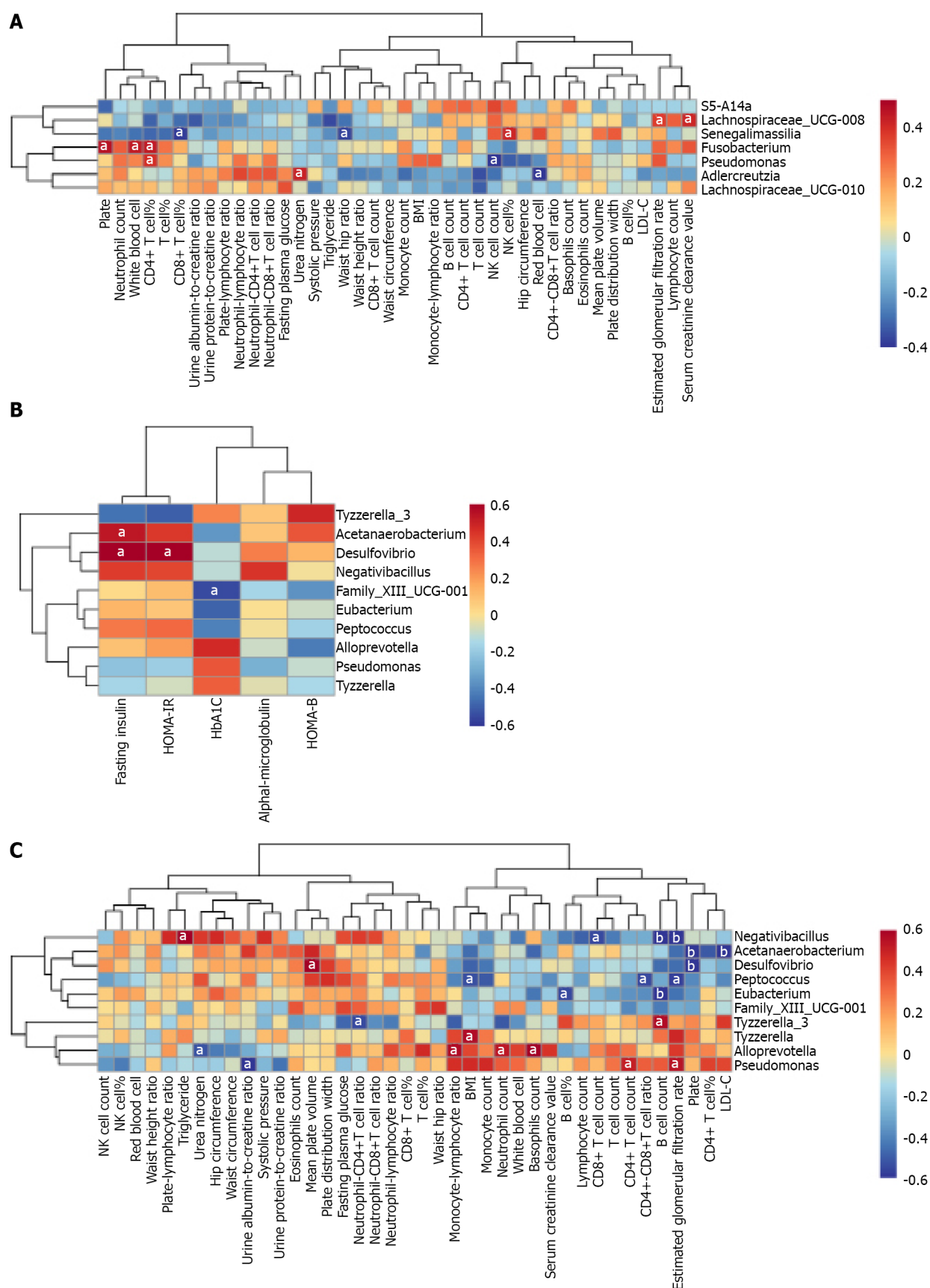


Figure 7 Correlation heatmap between gut microbiota and clinical indices. A: Correlation heatmap between gut microbiota and clinical indices in Group DM (Group DR vs Group D); B and C: Correlation heatmap between gut microbiota and clinical indices in Group DR (Group PDR vs Group NPDR). Different colors represent correlation level. Different colors represent correlation level (blue represents for negative correlation, red represents for positive correlation). ^a $P < 0.05$; ^b $P < 0.01$. Group C: Samples with non-diabetics; Group DM: Samples with diabetics; Group DR: Samples with diabetic retinopathy; Group D: Samples without

diabetic retinopathy; Group PDR: Patients with proliferative diabetic retinopathy; Group NPDR: Patients without proliferative diabetic retinopathy.

CONCLUSION

Our study explored the differences of intestinal microbiota between group DR and group D, as well as group PDR and group NPDR in the Chinese population of the southeast coastal region, rid of the interference of metformin. At the family level and genus level, much different microbiota was found between group DR and group D, and they may promote the occurrence of DR by affecting immune cells mediated by short-chain fatty acids, pro-inflammation response or anti-inflammation, inducing HIF and influencing permeability of blood vessels in the fundus. On the genus level, we found that besides *Pseudomonas*, the variation of microbiota composition between group PDR and group NPDR was completely different from that between group DR and group D. Some differential bacteria between group PDR and group NPDR may affect the level of butyrate or butyric acid, participate in the production of VCAM-1, decrease the level of HIF, affect the brain-eye barrier, promote insulin secretion and reduce B lymphocytes to promote or postpone the progress of DR. Accordingly, we speculated that the disorder of intestinal microbiota may be involved in the occurrence and development of DR, providing a possible novel therapeutic target for DR. However, our study lacked the detection at species level, as well as the measurement of microbial metabolites and related clinical indicators. The causal relationship between intestinal microbiota and the occurrence and development of DR remained unclear. Consider the limitation mentioned above, further investigation was required.

ARTICLE HIGHLIGHTS

Research background

For the therapy of diabetic retinopathy (DR), current approaches showed their own limitations. Modulation of gut flora was capable of preventing DR, which was revealed by animal experiment.

Research motivation

To provide clues for novel ways to prevention and treatment methods of DR.

Research objectives

This study aims to explore the relationship between intestinal microbiota and DR among patients in the southeast coast of China.

Research methods

By 16S rRNA sequencing, fecal samples of non-diabetics (Group C, $n = 15$) and diabetics (Group DM, $n = 30$) were analyzed. Spearman correlation analyses were performed to explore the associations between intestinal microbiota and clinical indicators.

Research results

The alpha and beta diversity did not differ significantly between Group DR and Group D as well as Group PDR and Group NPDR. At the genera level, *Pseudomonas*, *Fusobacterium* and *Adlercreutzia* were increased in Group DR than Group D while *Senegalimassilia* was decreased ($P < 0.05$, respectively). At the family level, *Pseudomonadaceae*, *Desulfovibrionaceae* and *Fusobacteriaceae* were significantly increased in Group DR than in Group D ($P < 0.05$, respectively). *Pseudomonas* was negatively correlated with NK cell count ($r = -0.39$, $P = 0.03$). In addition, the abundance of *Pseudomonas*, *Alloprevotella* and *Tyzzelerella* ($P < 0.05$, respectively) were lower in Group PDR compared to Group NPDR, while genera *Eubacterium* ($P < 0.01$), *Peptococcus*, *Desulfovibrio*, *Acetan-aerobacterium* and *Negativibacillus* ($P < 0.05$, respectively) were higher. *Desulfovibrio* and *Acetan-aerobacterium* were positively associated with fasting insulin ($r = 0.53$ and 0.61 , respectively, $P < 0.05$), when *Negativibacillus* was negatively associated with B cell count ($r = -0.67$, $P < 0.01$).

Research conclusions

Our research revealed that dysbiosis of gut flora was correlated with DR and its progression among diabetics in the southeast coast of China, probably *via* several mechanisms including producing influencing permeability of blood vessels, short-chain fatty acids, affecting levels of vascular cell adhesion molecule-1, hypoxia-inducible factor-1, B cell and insulin. Manipulating gut microbiota might be a novel way for prevention of DR, particularly PDR in population above.

Research perspectives

This research perspectives are as follow: (1) Current treatments for DR did not acquire satisfied effect; (2) Animal experiment revealed that reconstruction of gut microbiota could prevent DR; and (3) Does alteration of gut microbiota has connection with DR in human?

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FOOTNOTES

Author contributions: Gu XM and Lu CY have contributed equally to this work and share first authorship; Gu XM performed data analysis and co-wrote the first draft of the manuscript; Lu CY performed data analysis, specimen collection, and co-wrote the first draft of the manuscript; Pan J performed specimen collection and data collection; Ye JZ performed data analysis, experimental design, co-chief investigator of the study, and co-guarantor of this work; Zhu QH performed data analysis, experimental design, essay modification, co-chief investigator of the study, and co-guarantor of this work; all authors have provided substantial intellectual input and approved the final version for publication.

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Data sharing statement: The datasets presented in this study can be found in online repositories:

<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA786292>. The names of the repository/repositories and accession number(s) can be found below: NCBI SRA (accession: SRP349289).

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