

# Characterization of Vitreous Microbiota Dysbiosis Associated with Proliferative Diabetic Retinopathy

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**Purpose:** Emerging evidence suggests an association between ocular microbiota dysbiosis and ophthalmic diseases; however, the role of the posterior segment microbiome in diabetic retinopathy (DR) remains poorly characterized. In this study, we characterized the vitreous microbiome of patients with proliferative diabetic retinopathy (PDR) and systematically compared its microbial community structure with that of healthy controls.

**Methods:** A cohort of 19 PDR patients with type 2 diabetes mellitus and 19 non-DR controls were enrolled, with vitreous samples obtained through vitrectomy. Vitreous microbial composition was characterized using 2bRAD-M sequencing technology, enabling species-level taxonomic resolution. The comparison of dominant taxa, biomarker analysis and metabolic pathway differences between the two groups were further explored.

**Results:** The results of microbiome profiling revealed significant compositional differences in the vitreous core microbiome of PDR patients compared to controls, potentially associated with enhanced activity in membrane transport, nucleotide metabolism and carbohydrate metabolism pathways. LEfSe analysis identified 536 distinctive biomarkers of the two groups. At species level, the PDR group had significantly lower relative abundances of CAG-485\_sp009775375, Akkermansia\_muciniphila and Bacteroides\_acidifaciens, compared with control group.

**Conclusion:** This is the first study confirming the microbiota in human vitreous fluid samples by 2bRAD-M sequencing. These findings suggest a potential link between vitreous microbial dysbiosis and PDR, offering novel insights for future mechanistic investigations into DR.

**Keywords:** proliferative diabetic retinopathy, vitreous fluid, microbiota, 2bRAD-M

## Introduction

Diabetic retinopathy (DR) is the most common and well-known complication of diabetes in ophthalmology.<sup>1-3</sup> It is one of the leading cause of vision loss and even blindness, especially in developed countries.<sup>2,4,5</sup> Progressive damage to the retinal microvascular network can lead to tissue ischemia and proliferative diabetic retinopathy (PDR).<sup>6,7</sup> The vitreous fluid is a gel containing collagen and hyaluronic acid that is located between the lens and retina. When the retina undergoes pathological changes, the vitreous fluid components and biochemistry also change, which may in turn exacerbate PDR.<sup>8</sup> Current understanding of DR pathogenesis remains unclear, with clinical management limited primarily to laser photocoagulation and intravitreal administration of anti-angiogenic agents, both of which carry significant adverse effects.<sup>9</sup> It is necessary to elucidate the pathogenesis of DR to discover innovative therapeutic targets for DR intervention.<sup>8,10</sup>

The close relationship between gut microbes and a variety of human diseases has led to a reconsideration of the presence and function of microbiota in various parts of the human body.<sup>11-19</sup>

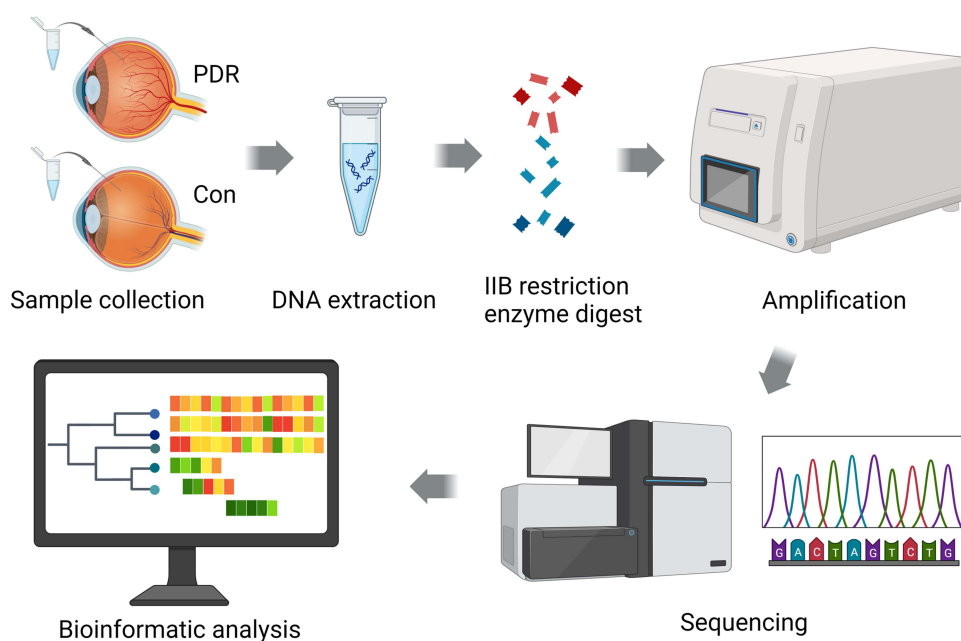
Patients with DR exhibited significant alterations in gut microbiota compositions compared to healthy controls.<sup>20</sup> Specifically, the proportions of beneficial bacterial genera such as *Lactobacillus*, *Roseburia*, and *Lachnospira* were

reduced, while the abundance of pro-inflammatory bacteria like *Bacteroidetes* and *Desulfobacterota* was markedly increased.<sup>20</sup> Furthermore, gut microbial metabolites such as GLP-1 and short-chain fatty acids (SCFAs) showed significant associations with DR progression, and GLP-1 treatment could alleviate retinal cell apoptosis and autophagy in type 2 diabetic mice.<sup>21</sup> In ophthalmology, the microbiome in the conjunctiva, cornea, and eyelid margins of individuals with or without eye diseases were previously detected and reported.<sup>22–25</sup> In particular, the validation of the human aqueous humor and vitreous fluid metagenome has subverted our perception of intraocular sterility.<sup>26</sup> However, the characteristics of posterior segmental microbiota in PDR patients have not yet been revealed.

The ocular surface microflora of individuals with different eye diseases is different from that with healthy status.<sup>27–31</sup> Li et al conducted 16S rRNA gene sequencing analysis on the ocular surface microbiota of patients with dry eye, which showed that *Bacteroidia* and *Bacteroidetes* were more abundant in the dry eye group, while *Pseudomonas* was significantly reduced.<sup>32,33</sup> The abundance of *Acinetobacter* and *Pseudomonas* in the ocular surface core flora of patients with T2DM (type 2 diabetes mellitus) increased significantly, while the abundance of *Corynebacterium* decreased, and the diversity of flora also increased significantly.<sup>34</sup> For diabetic children and adolescents with dry eye, the microbial diversity of the ocular surface was also higher with unique bacterial phyla and genera composition.<sup>30,31</sup> The above reports on the disturbance of the ocular surface microflora indicated a close relationship between ocular microbiota and eye diseases.

In recent years, it has been reported that the presence of resident bacteria on the ocular surface may be helpful to the immune barrier of the cornea.<sup>32</sup> *Corynebacterium* in the form of ocular surface symbionts can induce  $\gamma\delta$  T cells in ocular surface mucosa to secrete IL-17 to enhance ocular immune response.<sup>35</sup> Besides, germ-free SW (Swiss Webster) mice are more susceptible to *Pseudomonas aeruginosa* than wild SW mice. Transplanting coagulase-negative staphylococci isolated from conjunctival swabs from wild SW mice to the ocular surface of germ-free SW mice can help restore resistance to *Pseudomonas aeruginosa* infection.<sup>36</sup> This demonstrates the presence of ocular surface commensal bacteria (resident bacteria) and highlights the important role of ocular surface commensal microbiota in maintaining ocular immune defense homeostasis.<sup>36</sup> Therefore, the analysis of microbiota profile and function related to ocular disorders has profound significance for the clinical treatment of ophthalmic diseases.<sup>37</sup>

In the present study, vitreous fluid samples were obtained from patients with PDR and non-DR controls for comprehensive microbiome profiling using 2bRAD-M sequencing technology (Figure 1). This is the first investigation to reveal the characteristics and dysbiosis of the ocular posterior segmental microbiota in PDR patients.



**Figure 1** Schematic illustration of the study.

## Materials and Methods

### Sample Collection and Processing

The collection of vitreous fluid samples was approved by the ethical committee of the Eye Hospital of Shandong First Medical University, which was following the Association for Research in Vision and Ophthalmology (ARVO) Statement. Informed written consent was obtained from all the participants, ensuring their comprehensive understanding of the study's purpose, procedures, and potential risks.

Vitreous fluid samples were collected via vitrectomy from: (1) 19 T2DM patients with proliferative diabetic retinopathy (PDR) requiring surgical intervention (case group), and (2) 19 patients with idiopathic macular hole or epiretinal membrane (control group) who likewise required surgery. All the groups had no topical and systemic infection, glaucoma, age-related macular degeneration, retinal vein obstruction and uveitis, and the control group had no diabetes mellitus. The sample size was derived by the population proportion method. The parameters set for deriving the size included a 90% confidence interval and a 5% margin of error.

All procedures were performed under the standard manner of the planned management for surgery by the ophthalmologist. Before surgery, the operative eye was washed by the nurse and sterilized under standard disinfection manner of ocular surgery with 5% povidone-iodine eye drops by the retinal specialist, and it took 3 minutes to take effect. The patients were anesthetized using a bidirectional retrobulbar block. The operative eye was sterilized again, an eyelid speculum was inserted and 5% povidone-iodine eye drops were instilled into the cornea and conjunctival sac. After 3 minutes, the eye underwent three-port 25 gauge pars plana vitrectomy (PPV) using the Constellation Vision System and a new 25 gauge vitrectomy kit (Alcon, Fort Worth, TX, USA) by a single vitreoretinal surgeon. Approximately 500  $\mu$ L of vitreous fluid samples were firstly collected before perfusion fluid into the vitreous cavity. Then, the sample were sealed in a sterile bacterial culture tube and stored at  $-80^{\circ}\text{C}$  until use.

### Library Construction and Sequencing

The preparation of the 2bRAD-M library was conducted basically according to the original protocol.<sup>38</sup> In brief, genomic DNA was digested with BcgI enzyme (NEB) for 3 h at  $37^{\circ}\text{C}$ , and then ligated with the adaptors at  $4^{\circ}\text{C}$  for 12 h with T4 DNA ligase (NEB). The ligation products were then amplified, and the PCR products were treated with 8% polyacrylamide gel. Excise approximately 100 bp bands from the polyacrylamide gel and diffuse in nuclease-free aqueous solution at  $4^{\circ}\text{C}$  for 12 h. After introducing sample-specific barcodes, the products were purified and sequenced using the Illumina Novaseq PE150 sequencing platform. The adaptor and primer sequences involved are listed in [Supplementary Table S1](#).

### Bioinformatic Analysis

Approximately 173,165 microbial genomes, including bacteria, fungi and archaea, were downloaded from the NCBI RefSeq database. Following that, 16 types of type 2B restriction enzymes were used to sample restriction fragments from microbial genomes, which formed a huge database of 2bRAD microbial genomes. A comparison was made between all 2bRAD tags from each GCF occurring once within the genome. 2bRAD tags specific to species-level taxa were developed as 2bRAD markers, resulting in a 2bRAD marker database.

All sequenced 2bRAD tags after quality control were mapped to the constructed 2bRAD labeling database, the G score value of each species was calculated using the formula shown below, and species with G scores above threshold 5 were selected as candidate species to control false positives.

$$G\ score_{species\ i} = \sqrt{S_i \times t_i}$$

S: the number of reads assigned to all 2bRAD markers belonging to species i within a sample.

t: number of all 2bRAD markers of species i that have been sequenced within a sample.

The relative abundance of each species in the sample was calculated based on the following formula.

$$Relative\ abundance_{species\ i} = \frac{S_i/T_i}{\sum_{i=1}^n S_i/T_i}$$

S: the number of reads assigned to all 2bRAD markers of species *i* within a sample.

T: the number of all theoretical 2bRAD markers of species *i*.

## Statistical Analysis

All statistical analyses were performed using QIIME and R software (version 3.5.1). Microbiota alpha and beta diversity comparison was assessed by the Wilcoxon test and permutational multivariate analysis of variance (PERMANOVA). Kruskal Wallis analysis was performed for different comparison of microbial communities. The COG and KEGG pathways annotation was conducted using PICRUST2 software. The  $P < 0.05$  was considered statistically significant. The study schematic in [Figure 1](#) was drawn with the BioRender platform (BioRender, Toronto, Canada). Microbial sequencing raw data were deposited at the National Center for Biotechnology Information (NCBI) and publicly available as of the date of publication. The accession number is PRJNA1064640.

## Results

### Clinical Characteristics of Study Population

A total of 19 PDR patients with T2DM (case group) and 19 with idiopathic macular hole or epiretinal membrane (control group) were recruited for the study. Demographic and clinical characteristics of the enrolled patients were listed in [Table 1](#). The mean age of the case group was  $61.74 \pm 6.88$  years (range 47–81 years) and that of the control group was  $65.17 \pm 7.19$  years (range 52–85 years). The case group consisted of 10 males and 9 females, 12 in right eyes and 7 in left eyes. The control group consisted of 12 males and 7 females, 8 in right eyes and 11 in left eyes. The hemoglobin A1c (HbA1c) was 7.93%, and 13 patients in the case group and 8 in the control group had hypertension. The two groups were comparable for age, sex, eye, and hypertension ( $P = 0.74, 0.81, 0.78,$  and  $0.69$ , respectively). No patient developed vitritis, retinitis, or infectious endophthalmitis in the observation period after surgery.

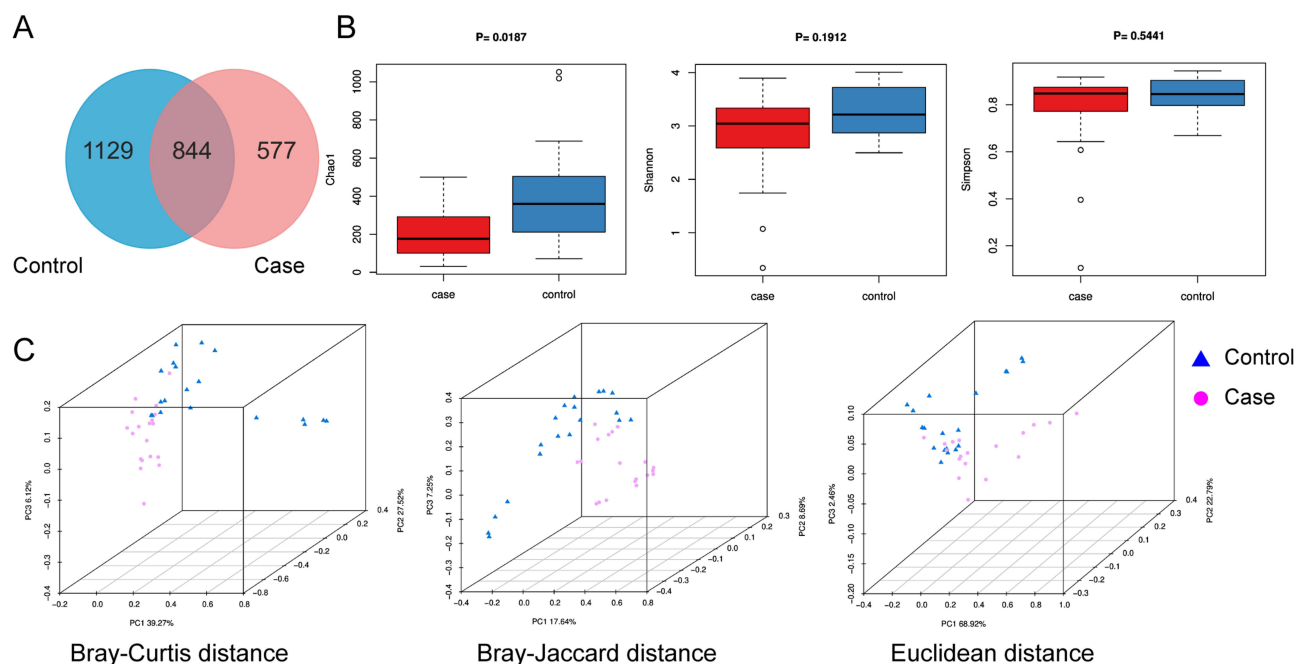
### Microbial Diversity of Vitreous Fluid in Control and PDR Group

To explore vitreous microbiome differences between PDR cases and controls, we performed 2bRAD-M sequencing analysis. Library construction and Illumina sequencing were carried out at OE Biotech Co., Ltd. (Shanghai, China). The details of quality control during sequencing are listed in [Supplementary Table S2](#). A total of 2550 species were identified, of which 844 were shared between the two groups, 1129 species were exclusively in the control group and 577 were unique to the case group ([Figure 2A](#)). For alpha diversity, the Chao 1 index, the measure of species richness, revealed that the case group had a significantly lower microbial richness than that of control. Although there was no significant difference in the Shannon index and Simpson index, the biodiversity trend of case group was the same as that of the Chao 1 index ([Figure 2B](#)). Moreover, the principal component analysis (PCA) indicated that the case group showed a much more significant structural shift than control, based on Bray–Curtis distance ( $P = 0.001$ ), Binary Jaccard distance ( $P = 0.001$ ), and Euclidean distance ( $P = 0.001$ ) ([Figure 2C](#)).

**Table 1** The Demographic Characteristics of the 38 Patients

Variable	Control Group	Case Group
Age (mean)	65.17 ± 7.19 years (52–85 years)	61.74 ± 6.88 years (47–81 years)
Sex		
Male	12 (63.16%)	10 (52.63%)
Female	7 (36.84%)	9 (47.37%)
Eye		
Right	8 eyes (42.11%)	12 eyes (63.16%)
Left	11 eyes (57.89%)	7 eyes (36.84%)
HbA1c	Not test	7.93%
Comorbidities		
Hypertension	8 (42.11%)	13 (68.42%)

**Abbreviation:** HbA1c, hemoglobin A1c.



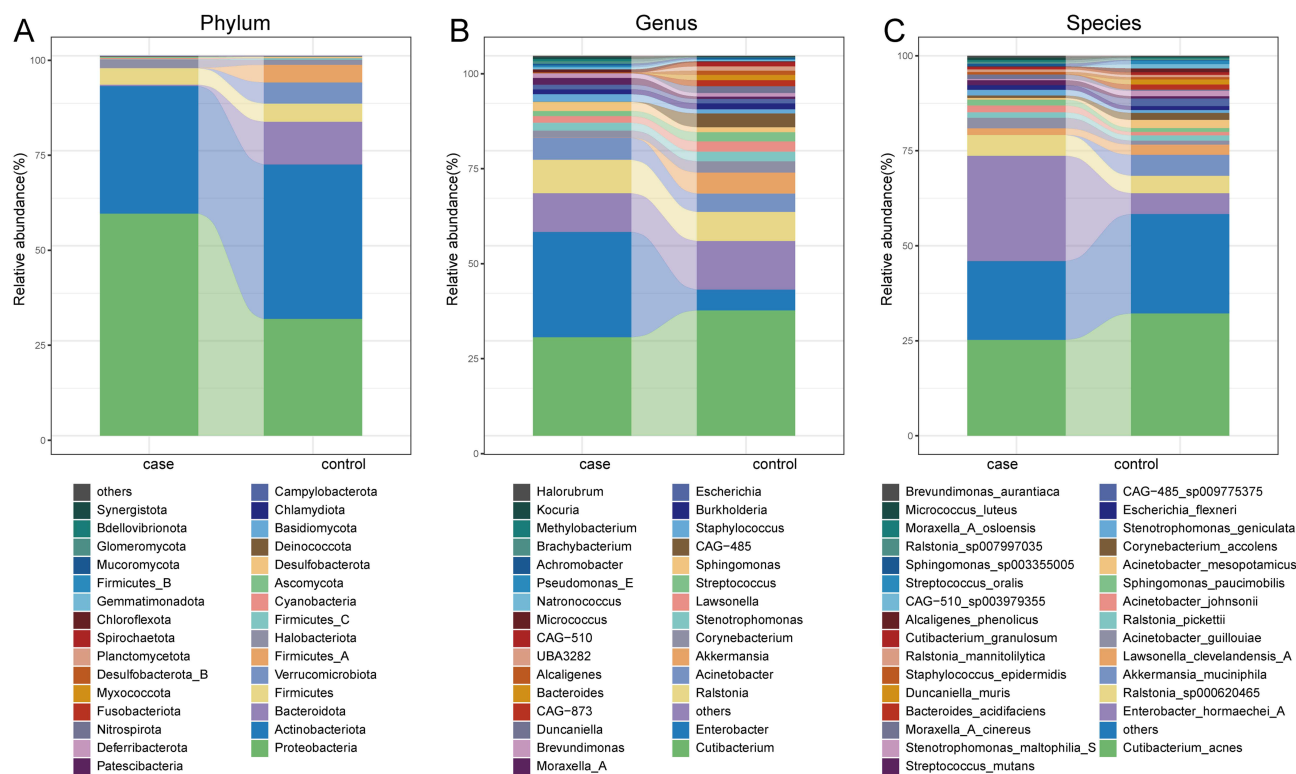
**Figure 2** Vitreous microbial diversity of PDR and control groups. **(A)** Venn diagram of overlapping species between the two groups. **(B)** Alpha diversity comparison between the two groups via ChaoI, Shannon index, and Simpson index analysis. **(C)** Beta diversity comparison between the two groups based on 3D-PCoA analysis. Bray-Curtis distance, Binary Jaccard distance and Euclidean distance metrics were used. Each point in the plot represents a sample.

## Microbiota Composition in the Vitreous Fluid

Microbiome composition analysis revealed significant differences in vitreous microbial profiles between PDR patients and non-DR controls. In total, 32 phyla were identified in the control and case groups, with Actinobacteriota (40.66% and 33.55%), Proteobacteria (30.73% and 58.47%), Firmicutes (4.79% and 4.35%) and Halobacteriota (1.30% and 2.18%) as the common dominant phyla (relative abundance higher than 1%) for the two groups (Figure 3A). Besides, Bacteroidota (11.26% and 0.36%), Verrucomicrobiota (5.56% and 0.02%) and Firmicutes\_A (4.64% and 0.08%) also account for a high proportion in control group, while rare in case group. In terms of genus level, 607 and 453 genera were identified in control and case group, respectively. *Cutibacterium* (32.99% and 25.93%), *Ralstonia* (7.67% and 8.81%), *Enterobacter* (5.48% and 27.72%) and *Acinetobacter* (4.80% and 5.80%) were the predominant genera common to both groups (Figure 3B). Besides, *Akkermansia* (5.56% and 0.02%), *CAG-485* (3.60% and 0.02%) were dominant genus in the control group while not in the case group. Based on the species level, *Cutibacterium\_acnes* (32.18% and 25.25%), *Enterobacter\_hormaechei\_A* (5.48% and 27.72%) and *Ralstonia\_sp000620465* (4.59% and 5.47%) were most in the two groups, and together with *Akkermansia\_muciniphila* (5.52% in control group), *Lawsonella\_clevelandensis\_A* (2.69% in control group), *Acinetobacter\_guillouiae* (2.75% in case group) and *Acinetobacter\_johnsonii* (1.81% in case group) constituted the top five species in the two group, respectively (Figure 3C).

## Differential Abundances of Vitreous Microbial Taxa Between Control and PDR Group

To explore the specific taxa that were differentially represented in control and PDR groups, Kruskal Wallis statistical analysis was conducted. Based on the data of Table 2, Bacteroidota, Verrucomicrobiota, Firmicutes\_A, Desulfobacterota, etc were significantly decreased in the case group compared with the control while Proteobacteria, Cyanobacteria, Halobacteriota and Deinococcota increased significantly. For genus level, *Duncaniella*, *CAG-485*, *UBA3282*, *CAG-873*, *Akkermansia*, *Bacteroides*, *Alcaligenes* and *Burkholderia* showed higher abundance in the control group but comparably lower in the case group, while *Enterobacter* and *Sphingomonas* exhibited increased significantly in the latter. At the species level, *CAG-485\_sp009775375*, *Akkermansia\_muciniphila*, *Bacteroides\_acidifaciens*, etc were more enriched in



**Figure 3** Vitreous microbial community composition. Relative abundance of the top 30 phyla (A) genus (B) and species (C) in the two groups.

the control group, whereas *Enterobacter\_hormaechei\_A*, *Acinetobacter\_guillouiae* and *Stenotrophomonas\_geniculata*, etc showed more enriched in the case group.

Furthermore, we performed LEfSe analysis to identify the distinctive biomarkers of the two groups (Figure 4). The results revealed 536 discriminative features with differential abundance between the two groups with linear discriminant analysis (LDA) score > 2 and  $P < 0.05$ . In terms of species, *Akkermansia\_muciniphila*, *CAG\_485\_sp009775375*, *Bacteroides\_acidifaciens*, *CAG\_510\_sp003979355*, and *Streptococcus\_oralis* were top 5 significantly enriched bacteria in the control group, while the *Enterobacter\_hormaechei\_A*, *Acinetobacter\_guillouiae*, *Acinetobacter\_johnsonii*, *Streptococcus\_mutans* and *Stenotrophomonas\_geniculata* were that tended to be enriched in case group.

## Difference of Vitreous Microbiota in Predicted Functions Between the Two Groups

To explore functional differences of vitreous microbiota between different samples and groups, we predicted microbial gene function abundance using PICRUSt2 software and analyzed by paired Wilcoxon test. A total of 392 metabolic pathways (at level 3) were predicted based on the KEGG database, of which 239 were significantly different between the two groups ( $P < 0.05$ ). The top 10 and top 30 metabolic pathways with the most significant differences are shown in Figure 5 and Supplementary Figure S1. Based on our data, the abundance of genes associated with metabolic pathways such as membrane transport, nucleotide metabolism, carbohydrate metabolism, signal transduction, cellular community –prokaryotes, among others, increased significantly in the case group.

We further evaluated the Spearman correlation between the top 30 relative abundance species and top 30 differential KEGG pathways of the two groups, the results suggested there was a significant overall correlation existed between them (Figure 6 and Supplementary Figure S2). Higher abundances of *CAG-485\_sp009775375*, *Akkermansia\_muciniphila* and *Bacteroides\_acidifaciens* in the control group were generally significantly negatively correlated with these pathways, while *Enterobacter\_hormaechei\_A*, *Acinetobacter\_guillouiae* and *Stenotrophomonas\_geniculata* with higher abundance in case group were generally significantly positively correlated with these pathways.

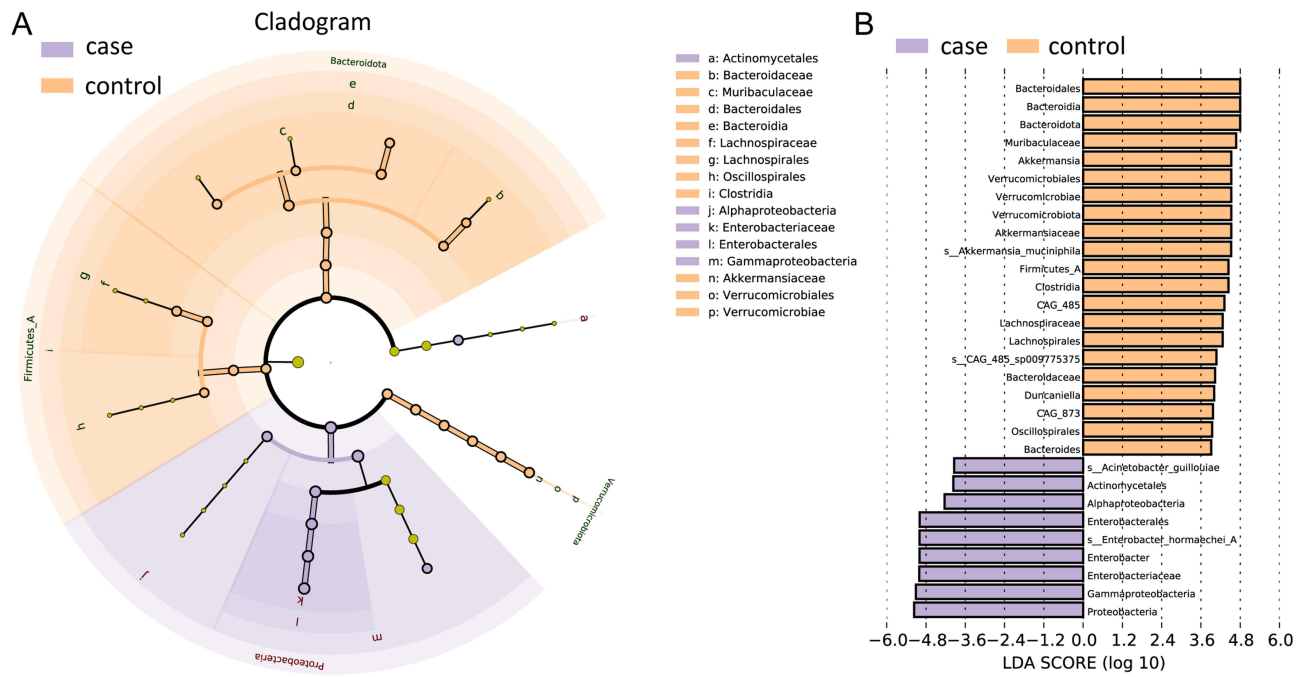
**Table 2** The Different Taxa Between the Two Groups with High Significance ( $P < 0.05$ )

Taxa		Average Relative Abundance			
		Control	Case	P-value	
Phylum	Bacteroidota	0.11257	0.00355	2.19E-07	
	Verrucomicrobiota	0.055552	0.00017	2.79E-06	
	Firmicutes_A	0.046404	0.000779	2.08E-05	
	Desulfobacterota	0.002852	0	5.43E-05	
	Proteobacteria	0.307327	0.584718	6.75E-05	
	Cyanobacteria	0.00092	0.002974	0.001842	
	Firmicutes_C	0.003914	0.000669	0.003088	
	Campylobacterota	0.000239	0	0.004091	
	Halobacteriota	0.013037	0.021795	0.042455	
	Deinococcota	0.000424	0.001282	0.046737	
Genus	<i>Duncaniella</i>	0.017728	3.46E-05	4.32E-08	
	<i>CAG-485</i>	0.036	0.000158	1.01E-07	
	<i>UBA3282</i>	0.011586	1.44E-05	6.45E-07	
	<i>CAG-873</i>	0.015738	0.000118	2.41E-06	
	<i>Akkermansia</i>	0.055552	0.000165	2.79E-06	
	<i>Bacteroides</i>	0.013955	2.55E-05	3.64E-05	
	<i>Enterobacter</i>	0.054757	0.27722	5.96E-05	
	<i>Alcaligenes</i>	0.010883	0.00206	0.000478	
	<i>Sphingomonas</i>	0.013231	0.024015	0.001022	
	<i>Burkholderia</i>	0.015493	0.012659	0.014765	
	Species	<i>CAG-485_sp009775375</i>	0.020004	8.56E-05	6.13E-07
		<i>Akkermansia_muciniphila</i>	0.055228	0.000165	2.79E-06
		<i>Bacteroides_acidifaciens</i>	0.013509	2.55E-05	3.64E-05
		<i>Enterobacter_hormaechei_A</i>	0.054753	0.277163	5.96E-05
<i>Acinetobacter_guillouiae</i>		0.01024	0.02749	0.000348	
<i>Stenotrophomonas_maltophilia_S</i>		0.014191	0.003	0.000387	
<i>Stenotrophomonas_geniculata</i>		0.007632	0.015232	0.001389	
<i>Acinetobacter_mesopotamicus</i>		0.021044	0.004235	0.002354	
<i>Acinetobacter_johnsonii</i>		0.008723	0.018068	0.010633	
<i>Streptococcus_mutans</i>		0.005958	0.012891	0.024542	

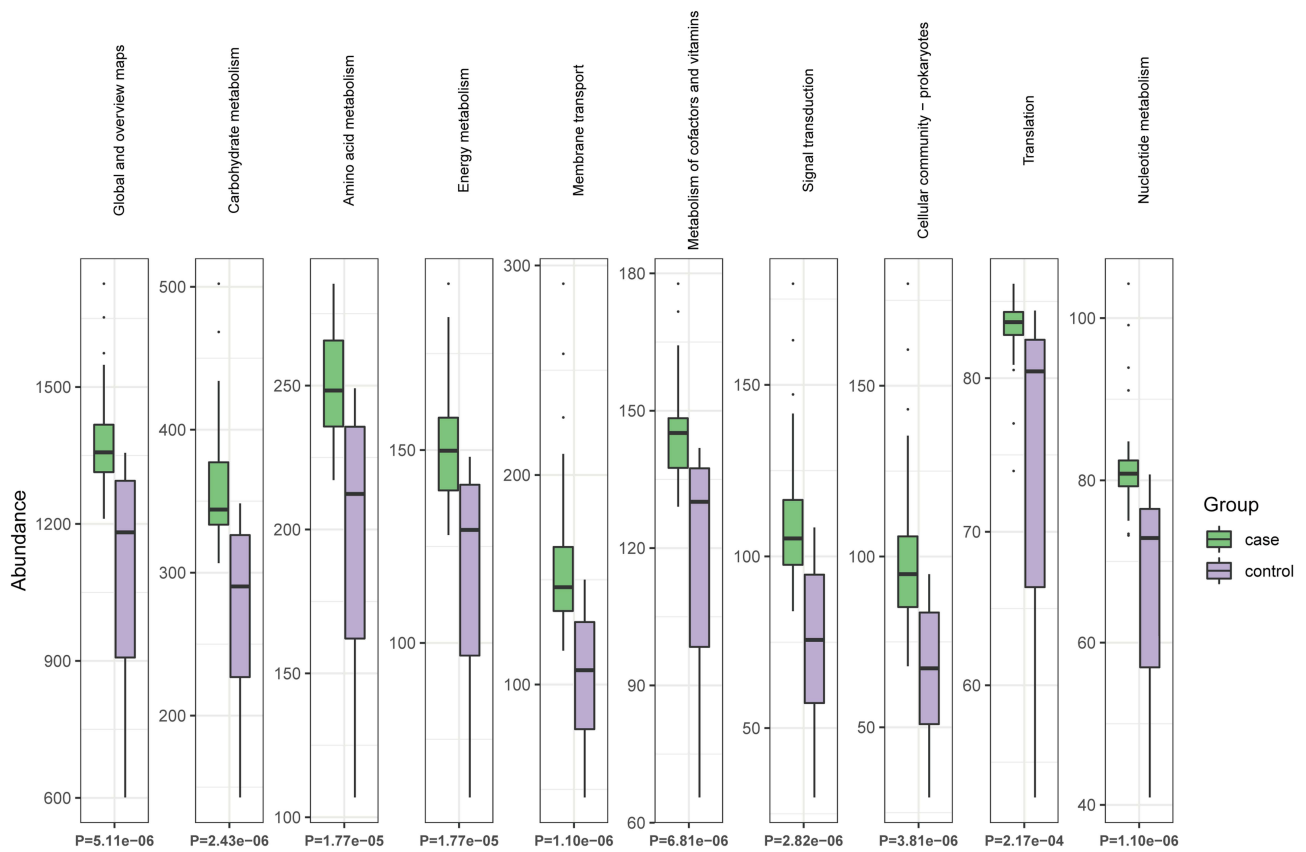
## Discussion

In this study, for the first time, we identified the composition of microbiota in human vitreous fluid samples and compared the differences between the vitreous microbiota between PDR and control individuals. There were significant differences in alpha diversity and beta diversity between the two groups. The vitreous microbiota in PDR patients was less diverse, and several species' relative abundances decreased significantly compared with the control. Further LEfSe analysis revealed 536 discriminatory character taxa between the two groups. Functional annotation analysis showed 239 related signaling pathways significantly different between the two groups. PDR group showed a significant enhancement in certain metabolic pathways especially in membrane transport, nucleotide metabolism and carbohydrate metabolism ( $P < 0.05$ ).

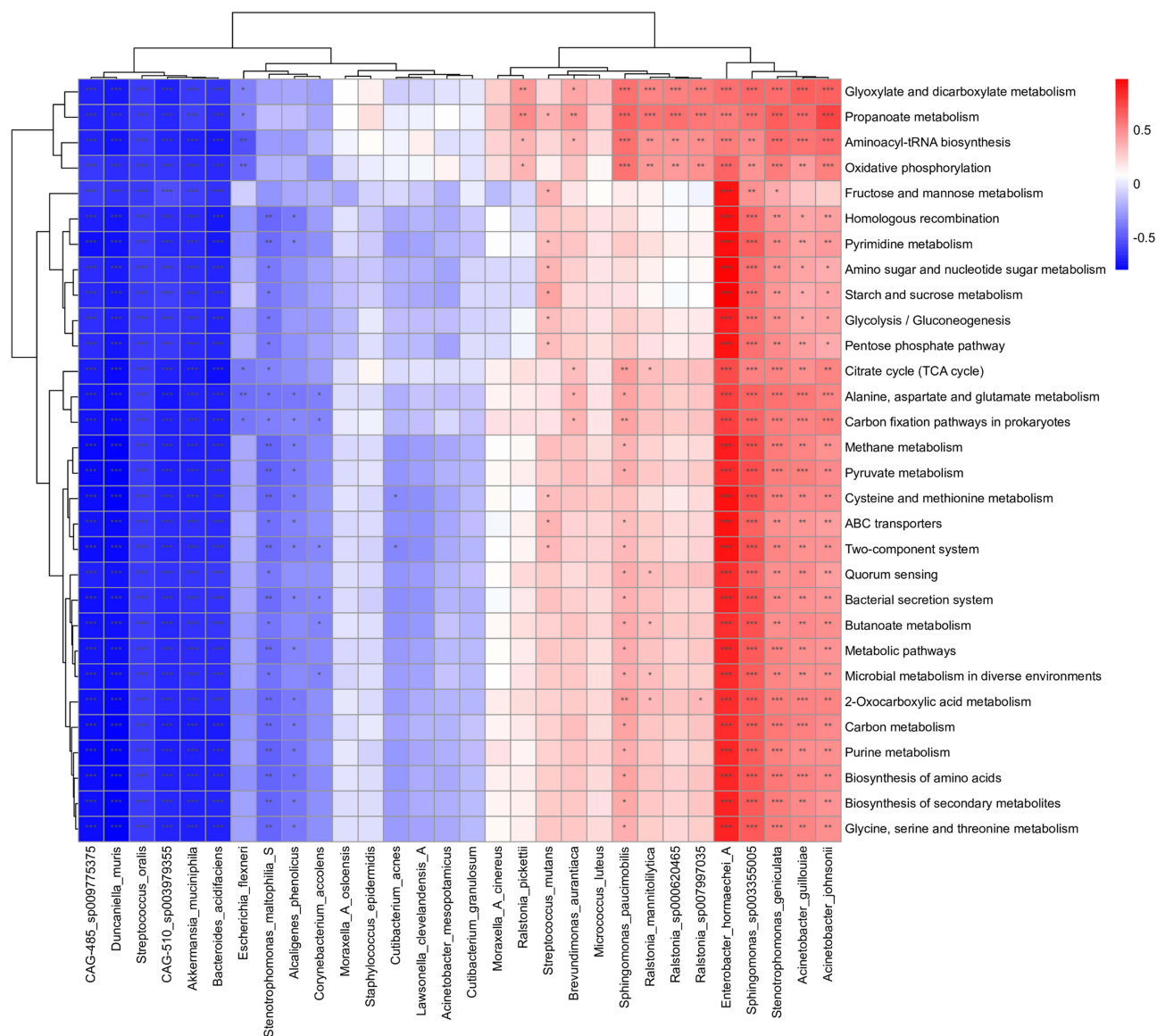
We have previously investigated several high-throughput sequencing technologies, including metagenomic sequencing and 2bRAD-M sequencing, and finally chose the latter, which is friendly to fewer biomass samples, to complete the vitreous microbiota project.<sup>39,40</sup> To eliminate the possibility of environmental contamination during sample processing, we collected 3 samples as negative controls, including reagents and magnetic beads involved in the process of DNA extraction and library construction. According to QC data, the negative control obtained readings several orders of magnitude lower than the experimental samples, indicating that the vitreous fluid samples in this study were highly unlikely to be contaminated during processing.



**Figure 4** LefSe analysis of differential abundances of microbial taxa between the two groups. **(A)** Cladogram of taxonomic hierarchical structure biomarkers of the two groups. **(B)** Differential species score plots present the species with relatively high abundance in each of the two groups.



**Figure 5** Results of KEGG function prediction for the top 10 most significant differences.



**Figure 6** Heatmap of Spearman correlation analysis of the top 30 relative abundance species and top 30 differential KEGG pathways ( $P$ -values: \* 0.01–0.05, \*\* 0.01–0.001, \*\*\* < 0.001).

Previously, Deng et al confirmed the existence of aqueous humor microbiome from four aspects: quantitative PCR, negative staining transmission electron microscopy, direct culture, and high-throughput sequencing technology, and proved that the composition of aqueous humor microbial communities of different species was different in four non-human mammals.<sup>26</sup> Arunasri et al reported differences in the vitreous microbiome between patients ( $n=9$ ) with post-fever retinitis and healthy controls through metagenomic sequencing approach.<sup>41</sup> In this study, we used the 2bRAD-M sequencing technology to characterize the vitreous microbiota for the first time and compared the differences in the microbiota between PDR patients and control patients at the species level.

Based on our data, a total of 32 phyla and 746 genera were identified in the two groups, which is much more than the 18 phyla and 301 genera in Arunasri's results.<sup>41</sup> The dominant phyla of vitreous microbiota in Arunasri's results for healthy control were Firmicutes and Proteobacteria, which is consistent with our results, but the relative abundances were not entirely consistent. In this study, Actinobacteriota had the highest relative abundance at 40.66%, while Firmicutes accounted for only 4.79% ([Supplementary Table S3](#)). At the genus level, the dominant genera *Clostridium*, *Klebsiella*, and *Lachnospirillum* in their healthy control had relative abundances well below 1% in this work, while the dominant genera *Cutibacterium*, *Akkermansia*, and *CAG-485* in our data had not been reflected in their paper ([Supplementary Table S3](#)). This discrepancy between the results of the two may be due to

the different races of people, or the sample size is not large enough to cover a wide range. Besides, Arunasri also characterized the mycobiome and virome associated with post-fever retinitis. Since this study was different from their sequencing methods, the sequencing results obtained here were vitreous fungi and archaea in the PDR group and control group. As shown in [Supplementary Figure S3](#), the proportions of fungi and archaea in the vitreous microbiome were relatively low, and the relative abundance percentages were less than 10%.

In the study by Deng et al, metagenomic analysis of aqueous humor samples from 41 patients undergoing cataract surgery identified 134 bacterial species, including 12 dominant species with relative abundance exceeding 1%.<sup>26</sup> Notably, two of these predominant species-*Staphylococcus epidermidis* and *Micrococcus luteus*-were also detected in both groups of our current study, albeit at lower abundance levels (>0.1%). These collective findings consistently demonstrated the existence of an intraocular microbial community.

The dominant phyla of conjunctival sac bacterial communities in diabetic patients identified by 16S rRNA gene sequencing were Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria, although their relative abundances were inconsistent across studies.<sup>34,42,43</sup> This was consistent with the dominant phyla of vitreous microbiota in the PDR group in our study. Whether there are certain similarities in the microbial communities of different eye tissues of diabetic patients needs more sample support and further analysis.

Current research on ocular microbiome dysbiosis in DR remains limited. Taraprasad et al conducted comparative analyses of aqueous humor microbiome profiles among DR patients, DM patients, and healthy controls ([Supplementary Table S4](#)).<sup>44</sup> Their findings demonstrated significantly reduced relative abundances of *Roseburia*, *Prevotellaceae* group, and *Haemophilus* in DR patients compared to healthy controls – microbial taxa functionally associated with either anti-inflammatory activity or pathogenic threaten. Orathai et al characterized conjunctival sac microbiota alterations across DR progression stages, demonstrating increased *Escherichia-Shigella* abundance in DM patients versus controls, with distinct microbial communities between DR and DM-no DR groups.<sup>42</sup> While our study shares consistent top three phyla with these studies, the observed discrepancies at the taxa levels may potentially attributed to sample types and limited cohort sizes.

In the present study, the microbiome composition of vitreous fluid in PDR group was significantly different from that in control group. In terms of species, the relative abundances of *CAG-485\_sp009775375*, *Akkermansia muciniphila* and *Bacteroides acidifaciens* were significantly decreased in the PDR group, while *Enterobacter hormaechei\_A* was significantly increased. *CAG-485\_sp009775375* belongs to the genus *CAG-485*, which remains uncultured and poorly characterized.<sup>45</sup> Its relative abundance in the intestinal flora was positively correlated with acute graft-versus-host disease in mice, and more research is needed to confirm the correlation between this bacterium and diseases.<sup>45</sup> *Akkermansia muciniphila* and *Bacteroides acidifaciens* were previously reported as important components of intestinal symbiotic bacteria in healthy mammalian individuals, and their reduced abundances were often associated with obesity, diabetes, liver injury, neurodegenerative disorders, among others.<sup>45–48</sup> In particular, *Akkermansia muciniphila* is considered one of the next generation of beneficial microorganisms and has entered the food regulatory framework.<sup>46</sup> The above evidence suggested *Akkermansia muciniphila* and *Bacteroides acidifaciens* might play a protective role in the vitreous body, and more work is needed on the specific details. *Enterobacter hormaechei* has been reported to be one of the most common nosocomial pathogens and can be isolated from samples of patients with diabetes bacteremia, pneumonia, urinary tract infections, biliary tract infections, colitis and cellulitis.<sup>47,48</sup> These clues point to the potential pathogenic role of *Enterobacter hormaechei* in the vitreous body, which needs to be further explored.

There were a few limitations that existed in the present study. The sample size was not large enough to explore more correlation between the dominant species of vitreous microbiota in the PDR group and the DR pathogenic mechanism. In addition, more work needs to be done to verify the predicted function of these dominant microorganisms.

In summary, this is the first report of characterization of the vitreous microbiota dysbiosis associated with PDR by 2bRAD-M. The composition of vitreous microbiota in PDR group was significantly different from that of control. *CAG-485\_sp009775375*, *Akkermansia muciniphila* and *Bacteroides acidifaciens* were more enriched in control group, whereas *Enterobacter hormaechei\_A*, *Acinetobacter guillouiae* and *Stenotrophomonas geniculata* showed high abundance in PDR group. Functional predictions showed significant differences in microbial KEGG pathways between the two groups. Hence, more research is needed to explore the potential relationship between vitreous microbiota disorder and the pathogenesis of DR.

## Ethics Approval and Consent to Participate

The study was approved by the ethical committee of the Eye Hospital of Shandong First Medical University (Medical Research Registration No. MR-37-24-037028), which was following the Association for Research in Vision and Ophthalmology (ARVO) Statement. Informed written consent was obtained from all the participants prior to study commencement. All the methods were carried out in accordance with the Declaration of Helsinki.

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## Disclosure

The authors report no conflicts of interest in this work.

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