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Correlation of gut microbial diversity to sight-threatening diabetic retinopathy

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Abstract

Purpose To determine the association of gut microbiome diversity and sight-threatening diabetic retinopathy (STDR) amongst patients with pre-existing diabetes.

Methods A cross-sectional study was performed, wherein 54 participants selected in total were placed into cases cohort if diagnosed with STDR and those without STDR but had a diagnosis of diabetes mellitus of at least 10-year duration were taken as controls. Statistical analysis comparing the gut microbial alpha diversity between cases and control groups as well as patients differentiated based on previously hypothesized Bacteroidetes/Firmicutes(B/F) ratio with an optimal cut-off 1.05 to identify patients with STDR were performed.

Results Comparing gut microbial alpha diversity did not show any difference between cases and control groups. However, statistically significant difference was noted amongst patients with B/F ratio ≥1.05 when compared to B/F ratio < 1.05; ACE index [Cut-off < 1.05:773.83 ± 362.73; Cut-off > 1.05:728.03 ± 227.37; p-0.016]; Chao1index [Cut-off < 1.05:773.63 ± 361.88; Cut-off > 1.05:728.13 ± 227.58; p-0.016]; Simpson index [Cut-off < 1.05:0.998 ± 0.001; Cut-off > 1.05:0.997 ± 0.001; p-0.006]; Shannon index [Cut-off < 1.05:6.37 ± 0.49; Cut-off > 1.05:6.10 ± 0.43; p-0.003]. Sub-group analysis showed that cases with B/F ratio ≥ 1.05, divided into proliferative diabetic retinopathy (PDR) and clinically significant macular edema (CSME), showed decreased diversity compared to controls (B/F ratio < 1.05). For PDR, all four diversity indices significantly decreased ($p < 0.05$). However, for CSME, only Shannon and Simpson indices showed significant decrease in diversity ($p < 0.05$).

Conclusions Based on clinical diagnosis, decreasing gut microbial diversity was observed among patients with STDR, although not statistically significant. When utilizing B/F ratio, the decreasing gut microbial diversity in STDR patients seems to be associated due to species richness and evenness in PDR when compared to decreasing species richness in CSME.

Keywords Diabetes mellitus, Sight-threatening diabetic retinopathy, Bacteroidetes, Firmicutes, Gut microbial diversity, Gut dysbiosis

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Background

The human body is host to tens of billions of microbes - bacteria, viruses, fungi, and protozoa - and their largest populations reside in the gut (small and large intestine), known as gut microbiota, and the microbiome refers to all the genes inside these microbial cells [1]. These microbes, in particular bacteria, live within humans from birth and create an ecosystem that is integral to health, such as digestion and immunity, both innate and adaptive [2]. Recently, there has been an interest to study the influence of aberrant composition or function of these microbes (dysbiosis) on several acute or chronic diseases such as diabetes mellitus (DM), inflammatory bowel disease, Alzheimer's disease, Crohn's disease, multiple sclerosis, muscular dystrophy, fibromyalgia and ocular diseases like diabetic retinopathy (DR), age related macular degeneration, uveitis, and Sjogren's disease [3–10].

DR progresses from mild non-proliferative diabetic retinopathy (NPDR), to moderate and severe NPDR and ultimately proliferative diabetic retinopathy (PDR). Sight-threatening diabetic retinopathy (STDR), which primarily comprises of clinically significant macular edema (CSME) and proliferative diabetic retinopathy (PDR) with and without macular edema [11]. It is well known that the control of DM is important for reducing the risk of STDR, as suggested by several studies including the UK Prospective Diabetes Study and Diabetic Retinopathy Clinical Research [12]. Patients with STDR need an immediate referral for ophthalmological treatment to prevent loss of vision. All STDR patients need routine comprehensive diagnosis, monitoring, and eventually treatment by an ophthalmologist. Identifying this cohort is essential and timely screening, referral and treatment can prevent or slow down loss of visual acuity [13].

A growing body of evidence shows that type 2 DM is associated with alteration in gut microbiota, dysbiosis; the underlying mechanism is increased inflammation, increased oxidative stress, increased vascular permeability, increased obesity, and insulin resistance, and altered glycemic control, however the role of gut microbiome with respect to ocular diseases is limited [10, 14, 15]. In humans, there have been only a couple of studies reported by Nadine et al. and Das et al. [16, 17], which confirm a role of gut dysbiosis in terms of varying abundance of bacterial genera within patients with DR compared to Type 2 diabetic patients and healthy patients without DR. Similar findings in terms of abundance were identified in our pilot study [3], wherein we also identified Bacteroidetes to Firmicutes relative abundance ratio (B/F Ratio) with an optimal cut-off point of 1.05 above which it presents as a potential biomarker for STDR. However, these studies have not reported the role of gut microbial diversity in terms of species richness and evenness using all available diversity measures in the development of STDR.

The present study therefore investigates the association of dysbiosis in the gut microbiome, with respect to microbial diversity, in subjects with type 2 DM who do not have a diagnosis of DR (controls, with duration of DM of 10 years or more) versus those diagnosed with STDR (cases, any duration of DM). In addition, the association of gut microbial diversity in diabetic patients with a B/F ratio <1.05 compared to B/F ratio ≥ 1.05 was also studied.

Materials and methods

Subject recruitment

Between April 2019 and October 2019, 58 eligible subjects were recruited in our initial pilot study from patients presenting to the tertiary eye care centre, Sankara Nethralaya, Chennai, India. Detailed methodology of that study has been discussed in detail in our previous paper [3]. After adjusting for missing data by deletion and utilizing a strict inclusion and exclusion criteria, in total 54 (21 controls and 33 cases) eligible participants were chosen. Subjects with type 2 DM underwent a comprehensive eye examination and were divided into two groups, controls if there was no evidence of DR but at least a 10 year or greater history of DM or cases if the presence of STDR (Clinically significant macular edema (CSME) and/or PDR) was diagnosed. Subjects with ocular pathologies that included but not limited to non-sight threatening DR, vascular retinopathy, ocular inflammatory or degenerative disorders were excluded. In addition, patients with recent antibiotic use within the last six months, as well as presence of any pre-existing systemic or neoplastic disease were also excluded. Of the study participants selected, initial demographic study variables were collected that included age, gender, height and weight, duration of DM, HbA1c (Glycosylated Hemoglobin), dietary preference (vegetarian or non-vegetarian), and associated systemic diseases, such as hypertension, coronary artery disease or dyslipidemia, based on history or medications. Vegetarians were those who were taking dairy and plant-based diets, and eggs, but no intake of fish, meat, or poultry.

The study was approved by the Institutional Review Board, Vision Research Foundation, Chennai. A written informed consent was obtained from study subjects, and the study complied with the tenets of the Declaration of Helsinki.

Fecal sample collection and sample processing for purified DNA (deoxyribonucleic acid)

After complete ophthalmic examination was conducted, a fecal swab was collected from each patient sample and the gut microbial DNA was isolated using Norgen

Microbiome DNA isolation Kit (Catalogue number 64100) using spin column chromatography. Homogenized fecal sample was incubated for 5 min at 65^0C using Lysis additive. The supernatant of the homogenized sample was centrifuged, and the supernatant was collected. Binding buffer I was added to the supernatant. The lysate was incubated in ice for 10 min and centrifuged for 2 min. The supernatant was collected, and equal volume of 70% ethanol was added and loaded to the spin column. Binding Buffer B and Wash solution A was run through the column and the DNA was eluted out using 50 µl Elution Buffer B.

Genomic sequencing

V4 region of 16S rRNA (Ribonucleic acid) were targeted for amplification using the primer pair were 515F (5'-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5' GGACTACHVGGGTWTCTAAT-3′). Illumina MiSeq with 250*2 paired end chemistry was used for sequencing and was performed by Npedia technologies. A FASTQ file generated from the sequencing was used for downstream analysis (Supplementary Fig. 1).

DADA2(1.14.1) [18], pipeline was used to process the FASTQ files in R version 3.6.3. SILVA v132 was used as a reference database and the downstream analysis was done using R package phyloseq [19]. Generated microbial presence and diversity was taxonomically classified at the phylum level.

Definitions used to study gut-microbial diversity

Alpha Diversity is the term used to measures the variance or diversity of microbes present within a particular sample. Alpha diversity values represent, 'species richness' and 'species evenness.'

Species richness – a count of the number of different species presents in a sample.

Species evenness – a measure of relative abundance of different species that make up the richness. So, relative abundance measures the prevalence of different phyla in the gut.

There are several indices which are currently used in literature to represent these measures as there is no consensus on which is the most accurate and significant index to use [20]:

(a) ACE index: estimates species richness, using sample coverage (sum of the probabilities of the observed species).
(b) Chao1 index: estimates species richness; it gives more weight to the low abundance species.
(c) Simpson's index: for species richness and species evenness; it gives more weight to the species evenness.
(d) Shannon index: for species richness and species evenness; it gives more weight to the species richness.

B/F ratio in cases and controls [3]

A two-sample t-test was done to compare the B/F abundance ratio between cases and controls. Multivariate linear regression analysis, adjusted for predetermined cofactors, was conducted to assess the significance of the B/F ratio in distinguishing between cases and controls. Employing Youden's J statistics method, we determined an optimal B/F ratio cutoff point to differentiate the two study groups. Comparison of the most common gut phyla, Bacteroidetes (B) and Firmicutes (F), revealed a significantly higher B/F ratio in cases than controls (cases, ≥1.05; controls, <1.05; $P=0.049$).

Statistical analysis

A microbiome R package [21], and phyloseq R package were used to analyse alpha diversity; all the R visualization was done using ggplot2 (v 3.3.2). Further statistical analyses were performed using a standard software package (Stata, version 16.1, StataCorp). Descriptive statistics on patient characteristics were summarized and compared using univariate analysis. After testing for non-normality of data, Wilcoxon signed-rank sum test was performed to compare the median alpha diversity values between the cases and control groups as well as patients with a B/F ratio < 1.05 versus B/F ratio ≥1.05. Subgroup analysis of clinically diagnosed STDR grouped into PDR with/without Diabetic Macular Edema (DME) and CSME group were compared with the control group that comprised of diabetic patients with no presence of DR. Similarly, sub-group analysis to compare the median alpha diversity values between those with B/F ratio < 1.05 versus B/F ratio ≥1.05 was performed by separating the B/F ratio ≥1.05 group into patients diagnosed with PDR with or without the presence of DME and CSME as well as utilizing the B/F ratio < 1.05 as its own subgroup. These comparisons within each subgroup were once again performed using a wilcoxon signed-rank sum test. Differences between two independent means were calculated (two-sided test; $p<0.05$) and a post hoc test was done to calculate power analysis.

Results

Baseline characteristics

Based on strict inclusion and exclusion criteria as well as accounting for complete data availability, a total of 54 sample participants with previously diagnosed type-2 DM were identified. Out of these 33 patients were diagnosed with STDR (CSME and/or PDR) and 21 patients were diagnosed with the absence of DR and with at least

a 10-year prior history of clinically diagnosed DM. In addition, the total sample population was divided based on B/F ratio < 1.05 and B/F ratio ≥1.05 to compare baseline characteristics. No statistically significant difference was noted between the baseline characteristics of cases and controls and the only statistically significant difference between the groups divided based on B/F ratio cut-off of 1.05 was noted in terms of dietary intake. Of those cases with B/F ratio < 1.05, 20.83% were vegetarian while of those cases with B/F ratio ≥1.05, 53.33% were vegetarian ($p=0.016$), (Table 1).

Comparison of alpha diversity values

When comparing median alpha diversity values, there seems to be a decreasing trend of diversity in the cases compared to controls in the ACE and Chao1 indices corresponding to species richness. In all four indices, the variations in median diversity values when comparing cases and controls were not found to be statistically significant, (Fig. 1).

However, when comparing median alpha diversity values between patients with respect to B/F ratio, those with B/F ratio ≥1.05 seemed to have a decreasing trend of diversity in all four indices when compared to patients with B/F ratio < 1.05. This association of decreasing alpha diversity values among STDR patients with respect to B/F ratio was found to be statistically significant as well, (Fig. 2).

Sub-group analysis of clinically diagnosed cases (STDR) grouped into PDR and CSME also showed a decreasing diversity among ACE and Chao 1 indices, with no discernible variation in diversity within the Simpson and Shannon indices, when compared to the control group. (Table 2). The results were not found to be statistically significant.

Sub-group analysis based on B/F ratio showed that the PDR and CSME groups with a B/F ratio ≥ 1.05 exhibited decreased diversity across all four indices compared to patients with a B/F ratio < 1.05. In the PDR group, the decreased alpha diversity values in all four indices were statistically significant ($p<0.05$). However, in the CSME group, a statistically significant decrease in diversity was noted only in the Shannon and Simpson indices ($p<0.05$) (Table 3).

Discussion

Recent studies have focused on studying the human gut microbiota and its relevance to health and disease especially in relation to obesity and a hyperglycemic state by causing chronic inflammation [16, 22]. How could gut microbiome influence the host immune system or cause chronic inflammation? Normally, the intestinal lining prevents the migration of microbes and their metabolites from the gut lumen to the bloodstream. However, a change in the intestinal milieu, intestinal dysbiosis, may deregulate the barrier effect of gut lining, and cause – leaky gut syndrome. Predominant gram-negative bacterial phyla such as Bacteroidetes releases bacterial endotoxin, lipopolysaccharides (LPS), and triggers an innate or natural immunity, and thereby contribute to pro-inflammatory pathways resulting in vascular dysfunction. In a study done on a mice with DM (db/db mice) showed its fecal bacterial composition (predominantly in Bacteroidetes and Firmicutes), presenting with

Table 1 Baseline characteristics of study population with respect to cases and controls as well as B/F ratio cut-off

N **= 54**	**Controls (*n* = 21) [DM without DR]**	**Cases (*n* = 33) [STDR]**	***p***	**B/F Ratio < 1.05 (*n* = 24)**	**B/F Ratio ≥1.05 (*n* = 30)**	***p***
Age, mean ± SD	57.50 ± 7.60	57.45 ± 8.19	0.982	60.04 ± 6.68	56.27 ± 8.51	0.082
Men N (%)	13 (61.90)	22 (66.66)	0.724	15 (62.50)	20 (66.67)	0.752
Duration of DM, mean ± SD	13.96 ± 5.99	14.17 ± 9.52	0.929	14.38 ± 5.61	13.93 ± 9.51	0.845
FBS, mean ± SD	156.90 ± 65.89	154.86 ± 70.61	0.916	149.29 ± 77.64	162.33 ± 58.29	0.484
PPBS, mean ± SD	207.64 ± 86.55	203.07 ± 88.48	0.853	200.50 ± 101.28	208.90 ± 72.36	0.724
HbA1c, mean ± SD	7.49 ± 1.48	7.48 ± 1.67	0.982	7.36 ± 1.70	7.64 ± 1.32	0.499
Height, mean ± SD	162.24 ± 12.79	162.74 ± 13.68	0.894	160.46 ± 11.04	164.93 ± 12.83	0.499
Weight, mean ± SD	69.34 ± 13.29	69.58 ± 13.91	0.950	67.48 ± 10.93	72.17 ± 13.76	0.180
BMI, mean ± SD	26.53 ± 5.52	26.44 ± 5.99	0.956	26.38 ± 4.68	26.76 ± 5.93	0.799
Vegetarian, N (%)	7 (33.33)	14 (42.42)	0.508	5 (20.83)	16 (53.33)	**0.016**
Associated systemic diseases (Based on history & medications)						
Hypertension, N (%)	9 (42.86)	22 (66.66)	0.088	13 (54.17)	18 (60.00)	0.670
Cardiovascular Disease, N (%)	6 (28.57)	8 (24.24)	0.726	4 (16.67)	10 (33.33)	0.169
Dyslipidemia, N (%)	3 (14.29)	3 (9.09)	0.557	4 (16.67)	2 (6.67)	0.250

SD: Standard Deviation; DM: Diabetes Mellitus; DR: Diabetic Retinopathy; B/F Ratio: Bacteroidetes/Firmicutes relative abundance ratio; BMI: Body Mass Index; HbA1c: Glycosylated Haemoglobin; FBS: Fasting Blood Sugar; PPBS: Post-Prandial Blood Sugar; Cases: Subjects with sight-threatening diabetic retinopathy (STDR); Controls: Subjects with diabetes mellitus, but no diabetic retinopathy

Patient characteristics were summarized and compared using descriptive statistics and univariate analysis

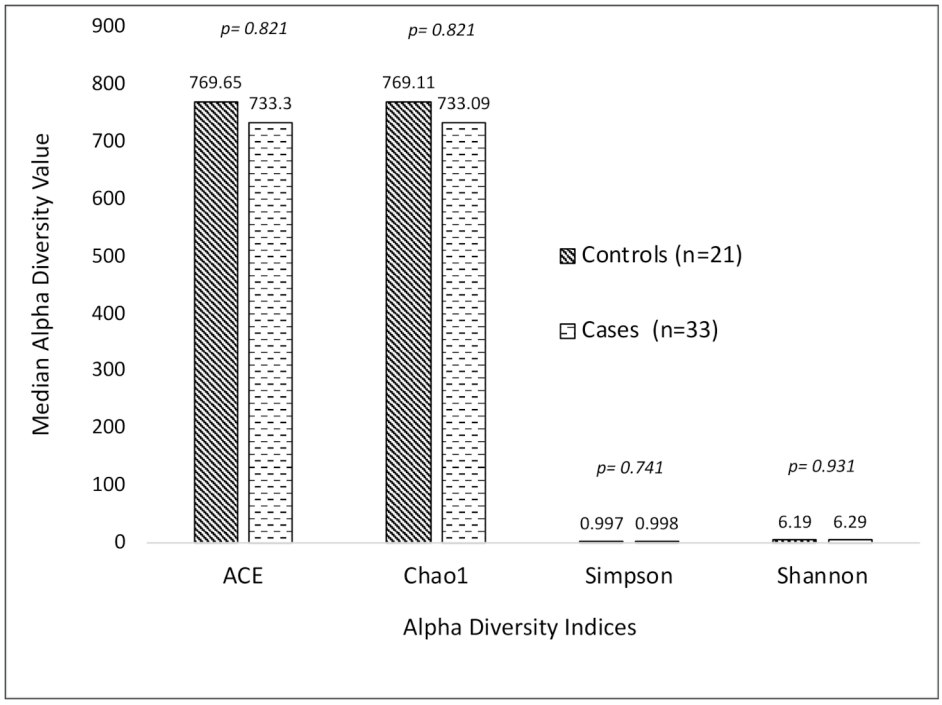

Fig. 1 Comparison of median alpha diversity values between cases and controls

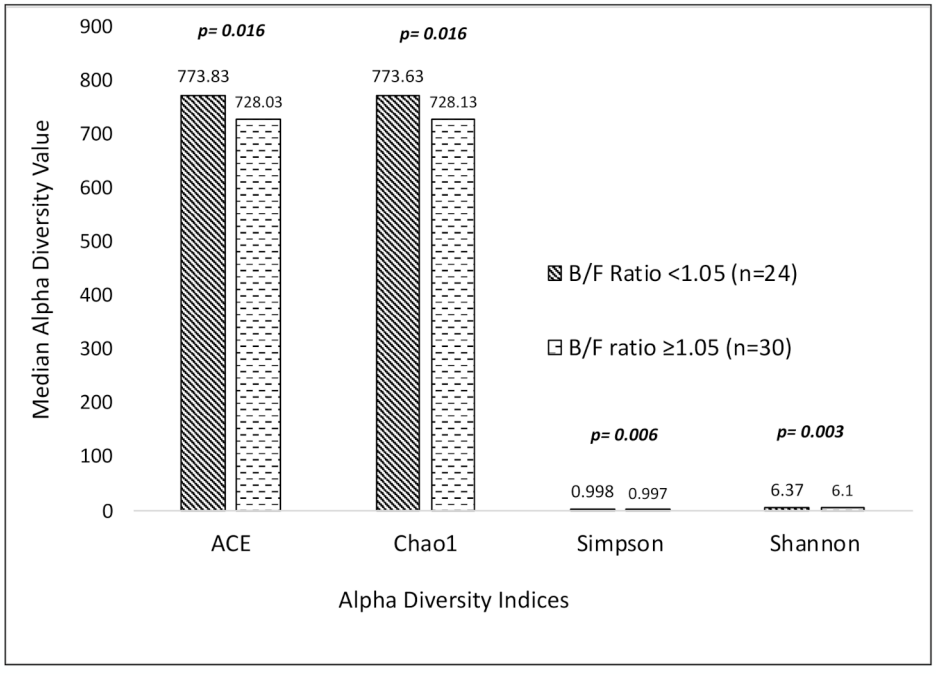

Fig. 2 Comparison of median alpha diversity values between Bacteroidetes/Firmicutes (B/F) ratio < 1.05 and B/F ratio ≥1.05

impaired intestinal barrier function and replicating some of the features of DR – acellular capillaries, activation of retina microglia, and infiltration of peripheral immune cells into the retina [23].

One of the strongest determinants of DR is the duration of hyperglycemia; however, in few of the subjects despite many years of DM, no DR is detected [24]. What protect these individuals remains an enigma? Is it due to the influence of gut microbiome? Can we establish a

Table 2 Comparison of alpha diversity values between clinically diagnosed STDR sub-grouped into PDR and CSME versus diabetic patients without DR

Alpha Diversity	Controls [DM without DR] (*n* = 21) median (95% CI)	CSME (*n* = 16) median (95% CI)	*p* (Control Vs CSME)	Controls [DM without DR] (*n* = 21) median (95% CI)	PDR (*n* = 17) median (95% CI)	*P* (Control Vs PDR)
ACE	769.65 (660.49–878.84)	753.98 (650.07–873.32)	0.774	769.65 (660.49–878.84)	728.07 (657.25–881.63)	0.488
Chao1	769.11 (647.60–866.10)	733.58 (649.98–873.01)	0.797	769.11 (647.60–866.10)	728.25 (657.21–881.87)	0.78
Simpson	0.997 (0.996–0.998)	0.998 (0.995–0.999)	0.916	0.997 (0.996–0.998)	0.998 (0.996–0.999)	0.601
Shannon	6.19 (6.06–6.35)	6.34 (6.03–6.39)	0.868	6.19 (6.06–6.35)	6.22 (5.97–6.37)	0.977

CI: Confidence Interval; DM: Diabetes Mellitus; DR: Diabetic Retinopathy; CSME: Clinically Significant Macular Edema; PDR: Proliferative Diabetic Retinopathy; STDR: Sight-Threatening Diabetic Retinopathy

Wilcoxon signed-rank sum test was performed to compare the median alpha diversity values between the cases and control groups

Table 3 Comparison of alpha diversity values between patients with B/F ratio ≥ 1.05 (sub-grouped into PDR and CSME) and those with B/F ratio < 1.05

Alpha Diversity	BF Ratio < 1.05 (*n* = 24) median (95% CI)	BF Ratio ≥1.05 (CSME) (*n* = 12) median (95% CI)	*p*	BF Ratio < 1.05 (*n* = 24) median (95% CI)	BF Ratio ≥1.05 (PDR) (*n* = 9) median (95% CI)	*p*
ACE	773.83 (759.34–789.78)	738.91 (612.02–867.74)	0.497	773.83 (759.34– 789.78)	670.74 (484.23–698.20)	**0.032**
Chao1	773.63 (759.33–789.58)	738.58 (612.01–867.46)	0.497	773.63 (759.33–789.58)	670.60 (484.21–698.18)	**0.032**
Simpson	0.998 (0.996–0.998)	0.997 (0.995–0.999)	**0.018**	0.998 (0.996–0.998)	0.997 (0.994–0.998)	**0.003**
Shannon	6.37 (5.95–6.48)	6.06 (5.86–6.30)	**0.045**	6.37 (5.95–6.48)	5.94 (5.69–6.11)	**0.003**

CI: Confidence Interval; B/F Ratio: Bacteroidetes/Firmicutes relative abundance ratio; CSME: Clinically Significant Macular Edema; PDR: Proliferative Diabetic Retinopathy; STDR: Sight-Threatening Diabetic Retinopathy

Wilcoxon signed-rank sum test was performed to compare the median alpha diversity values between the patients with a B/F ratio < 1.05 vs. B/F ratio ≥1.05

relationship between gut microbiome and DR? Would it lead to a new target for therapeutic intervention? Hence, evaluating gut microbiotome in two groups, each group lying at the two ends of the spectra of DR (either no DR or STDR) might solve a part of this puzzle. This was the driving force that resulted in us conducting our initial pilot study to investigate dysbiosis in the gut microbiome with respect to relative abundance in subjects with type 2 DM and compared the result in those who did not have DR (controls, with duration of DM of 10 years or more) with those who had STDR (cases, any duration of DM). Based on the initial results of our study [3], we concluded that gut dysbiosis in terms of relative abundance of microbial species might play a role in the development and severity of STDR among diabetic patients of a South Indian cohort. Specifically, when reviewing the ratio of Bacteroidetes/Firmicutes (B/F ratio), diabetics with a B/F ratio ≥1 were indicative of the presence of STDR.

Of note, we also found that amongst a growing body of literature describing disease-associated gut microbiota, loss of microbial diversity appears as a common feature when representing gut dysbiosis [25–32]. Hence, in our present study, we wanted to perform a secondary analysis of our pilot study to determine if there is an association of gut microbial diversity and the presence of STDR in diabetic patients. We noted that, the relationship between gut microbial diversity and diseased state tends to have an inverse correlation, a conclusion similarly arrived by previous literature as well [17, 18].

Regarding diversity indices, the clinically diagnosed cases were further grouped into patients either with a diagnosis of CSME, or PDR with or without DME involvement noted that there was no statistically significant variation in gut microbial diversity compared to diabetics with no DR. However, subgroup analysis of the population based on B/F ratio ≥1.05 grouped into patients either with a diagnosis of CSME, or PDR with or without DME involvement showed that there was a decrease in microbial diversity in both subgroups compared to the group which had a B/F ratio < 1.05 in all four alpha diversity indices.

While this decrease in microbial diversity was statistically significant for all four indices with respect to PDR, statistical significance was only noted for Shannon and Simpson indices with respect to CSME. This shows that decreasing microbial diversity based on richness and evenness could be associated with PDR as well as CSME with more weightage to the decreasing species richness associated with PDR alone. We speculate that this difference could be due to the fact that chronic inflammation plays an important role in the pathophysiology for DME, whereas, in eyes with PDR, it is the retinal ischemia that might be a dominating factor compared to just inflammation alone [33–38]. Close monitoring is necessary for the DM without DR group with a B/F ratio ≥ 1.05, as they may be at an increased risk of developing retinopathy changes.

It is also important to ponder whether this observation in the gut-retina axis might be the consequence rather than cause of disease as it is well known that the gut microbiota and the brain have a bidirectional communication mediated via the hypothalamic-pituitary axis (HPA axis) [39, 40]. The dysregulation of HPA-axis has been noted to be significantly higher in patients with moderate-to-severe DR when compared with patients with minimal or no DR [41]. There is a possibility that future prospective studies with a larger sample size might better elucidate such a relationship of gut dysbiosis in terms of decreased diversity with respect to the different diseased states within STDR.

Though the number of STDR (n=33) subjects was less to correlate the gut microbial diversity, the post hoc power calculation showed a power of 93%, which suggested that the study has sufficient power. The strength of the study was our utilization of cutting edge next-generation sequencing technology in order to find the association between gut dysbiosis and diabetic retinopathy. However, the study has been limited by the fact that it is a cross-sectional study, and hence further prospective studies are required to strengthen this association. Additionally, the high costs involved limited our sample size, affecting the study's power. Beta diversity should have been included to measure the similarity or dissimilarity of two communities in our analysis. Furthermore, our study is limited to primarily a South Indian population. Involving a sample cohort with multiple races, ethnicity and nationality would help better determine the translation of our study findings to the population as a whole.

Conclusion

A eubiotic gut is essential to the maintenance of human health. Our study shows a novel relationship between changes in the gut microbiota with respect to decrease in diversity and STDR. This work highlights several findings with potential clinical significance. Attempts to increase the gut microbial richness and evenness might have a therapeutic benefit in STDR. The options of altering gut microbiome like intermittent fasting, fecal microbial transplant, pre- and probiotics and antibiotics may find a role in future therapeutics of STDR [22].

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-024-03496-x.

Supplementary Material 1

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Author contributions
R.R.N and S.S. contributed to conception and design of the study. R.K. helped in acquisition of the data. R.K. and A.S. wrote the main manuscript text and prepared all the tables. R.K. and A.S. assisted with statistical analyses. R.R assisted with genomic analysis and all authors reviewed the manuscript.

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Data availability
The sequence data generated during the current study are available in the Sequence Read Archive (SRA) at NCBI under Bioproject: PRJNA1150757.

Declarations

Ethics approval and consent to participate
The study was approved by the Institutional Review Board, Vision Research Foundation, Chennai. A written informed consent was obtained from study subjects, and the study complied with the tenets of the Declaration of Helsinki. All participants gave written informed consent to provide a stool sample and to the availability of the stored samples for additional bioassays after the study protocol was fully explained (Study reference number: 674 A-2018-P).

Consent for publication
Not Applicable.

Competing interests
The authors declare no competing interests.

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