Dysbiosis in the Gut Microbiome in Streptozotocin-Induced Diabetes Rats and Follow-Up During Retinal Changes

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Received: April 28, 2021
Accepted: July 23, 2021
Published: August 25, 2021

Citation: Padakandla SR, Das T, Sai Prashanthi G, et al. Dysbiosis in the gut microbiome in streptozotocin-induced diabetes rats and follow-up during retinal changes. *Invest Ophthalmol Vis Sci.* 2021;62(10):31. https://doi.org/10.1167/iovs.62.10.31

Purpose. To analyze the gut bacterial microbiome of streptozotocin-induced diabetic rats and rats with retinal changes.

Methods. Induction of diabetes was confirmed by an increase in blood sugar (>150 mg/dL), and the progression of diabetes with retinal changes was assessed by histology and immunohistochemistry of retinal sections. Microbiomes were generated using fecal DNA, and the V3–V4 amplicons were sequenced and analyzed by QIIME and R.

Results. Dysbiosis in the gut microbiome of diabetic rats and diabetic rats with retinal changes was observed at the phylum and genus levels compared with the control rats. Heat-map analysis based on the differentially abundant genera indicated that the microbiomes of controls and diabetic rats separated into two distinct clusters. The majority of the microbiomes in diabetic rats with retinal changes also formed a distinct cluster from the control rats. β-diversity analysis separated the microbiome of control rats from the microbiome of diabetic rats and diabetic rats with retinal changes, but the microbiomes of diabetic rats and diabetic rats with retinal changes showed an overlap. Functional analysis indicated that the enhanced inflammation in diabetic rats showing retinal changes could be ascribed to a decrease in anti-inflammatory bacteria and an increase in pathogenic and proinflammatory bacteria.

Conclusions. This study showed that the gut bacterial microbiome in diabetic rats with retinal changes was different compared with control rats. The results could help develop novel therapeutics for diabetics and diabetic individuals with retinal changes.

Keywords: diabetes, diabetic retinopathy, microbiome, NGS, amplicon sequencing

Diabetes mellitus (DM) is a chronic metabolic disorder of major public health importance. Currently, 463 million adults are estimated to have DM, and it is expected that the number will increase to 700 million by 2045.[1,2] In India, the prevalence of diabetes in adults is 8.9%, and an estimated 77 million people in India have diabetes.[3] Diabetic retinopathy (DR) is the most common ophthalmic manifestation of people with DM. DR is similar in patients with type 1 DM (T1DM) and those with type 2 DM (T2DM).[4,5] About 30% of people with DM have DR, which is the leading cause of visual impairment in working-age people worldwide.[6,7] The prevalence of DR among diabetics in India varied from 9.6 to 21.7% over the last decade.[8]

The gut bacterial microbiome is a dynamic community of bacteria, and dysbiosis (changes in diversity and function) in the gut microbiome has been associated with several autoimmune and inflammatory diseases.[9–12] The association of gut microbiome dysbiosis with ocular diseases, such as Sjögren syndrome, uveitis, age-related macular degeneration, and fungal and bacterial keratitis,[13–15] has been described. However, very few studies have addressed changes in the gut microbiome during the progression of DM to DR. Recently, our group demonstrated dysbiosis in the gut microbiomes and mycobiomes (at both the phylum and genus level) in people with T2DM and DR compared with healthy controls.[16,17] We identified specific bacteria and fungi associated with T2DM and DR. Such changes in the gut microbiome could also be studied in rodent model systems.[18,19]

Streptozotocin (STZ)-induced diabetic mice and rats have been used as models to study the underlying mechanisms of DR.[18,9] Rodents mimic the development of DR and exhibit early cellular and vascular abnormalities, but subsequent retinal changes as a consequence of prolonged hyperglycemia do not manifest in rodents or other model animals (e.g., cats, dogs, zebrafish).[18,19] A recent study confirmed dysbiosis at the genus level in the microbiome of diabetic mice (db/db mice).[20] Simultaneously, these mice presented certain features of DR, such as acellular capillaries, activation of retinal microglia, and infiltration of peripheral immune

cells into the retina.[20] In the present study, we compared the gut bacterial microbiome (henceforth microbiome) of healthy rats with STZ-induced DM and diabetic rats with retinal changes (DRC). The study had two objectives: (1) to identify the microbiome associated with DM rats, and (2) to ascertain whether microbiome dynamics are responsible for the progression of DM to DRC. We also monitored longitudinal changes in the gut microbiome in DM rats to understand the temporal development of DM and DRC. The similarities and differences that highlight the novelty of the current study are highlighted in the discussion section.

Materials and Methods

Animals

Sprague Dawley rats were obtained from National Institute of Nutrition (Hyderabad, India) following approval by the Institutional Animal Ethics Committee (P9F/IAEC/NIN/5/2018/GBP/SD-98M). Individual animals were housed in polypropylene cages at 22°C ± 2°C at 50% humidity and under a 12-hour light/dark cycle. Food and water were provided ad libitum.

Induction of Diabetes and Retinal Changes

Forty-eight animals, 3 months old and with an average body weight of 230 ± 14 g, were recruited together. The control groups (CT1–CT4) were injected with 0.1-M sodium citrate buffer, pH 4.5, as a vehicle[21,22] and were sacrificed after 1, 2, 3, and 4 months, respectively. DM was induced in 24 rats by injecting STZ (35 mg/kg) intraperitoneally.[21,22] DM1 and DM2 rats were sacrificed 1 and 2 months, respectively, after STZ injection, and CT1 and CT2 served as the controls. DRC 1 and DRC2 rats were sacrificed 3 and 4 months, respectively, after STZ injection, and CT3 and CT4 served as the controls. Glucose levels were quantitated using a drop of blood from the tail vein using an ACCU-CHEK Active Blood Glucose Meter Kit (Roche Diagnostics, Basel, Switzerland).

Preparation of Retinal Sections

Animals in the eight groups (CT1–CT4, DM1 and DM2, and DRC1 and DRC2) were allowed to fast overnight and were sacrificed by CO_2 asphyxiation. Eyeballs were fixed in 10% buffered formalin, embedded in paraffin blocks, and sectioned and mounted on glass slides as described earlier.[21,22]

Hematoxylin and Eosin Staining

Retinal sections were deparaffinized and stained with hematoxylin for 10 minutes following our earlier protocol.[21,22]

Immunohistochemistry

Immunohistochemical detection of VEGF and rhodopsin in retinal sections using VEGF- and rhodopsin-specific antibodies (ab32152 and ab232934, respectively; Abcam, Cambridge, UK) was done according to our earlier procedure.[21,22]

Imaging

All sections were observed at 40× magnification using an Aperio AT2 slide scanner (Leica Biosystems, Wetzlar, Germany).

Immunoblotting of Hypoxia-Inducible Factor-1 Alpha

Retinal cellular protein was extracted according to Reddy et al.[23] and quantified by Lowry's method,[24] and equal protein concentrations were resolved by electrophoresis (12% sodium dodecyl sulfate–polyacrylamide gel). Proteins were then transferred onto a nitrocellulose membrane, and immunoblotting of hypoxia-inducible factor 1-alpha (HIF-1α) protein was performed with HIF-1α primary antibody as described earlier.[22] Tubulin served as a loading control.

Fecal Sample Collection and DNA Extraction

Fecal pellets (five or six) were collected from each animal in a cryotube and frozen at –80°C for future use. Genomic DNA was extracted from ~150 mg of homogenized fecal pellets, and the quality of DNA was checked as described earlier.[13–17]

Illumina Library Preparation and Sequencing of the Bacterial Microbiome

Amplification of the V3–V4 region of bacterial 16S rRNA genes was performed as described earlier.[13] Bacterial microbiomes were generated following the standard Illumina (San Diego, CA, USA) protocol[13,25] and sequenced using 2 × 250-bp chemistry on an Illumina MiSeq system with a paired-end protocol at Xcelris Genomics Pvt. Ltd. (Ahmedabad, India).

Taxonomic Classification of Sequenced Reads

Paired-end reads were demultiplexed and merged using FLASH (https://ccb.jhu.edu/software/FLASH/). Reads with a mean Phred score < 25 and chimeric sequences were removed using Prinseq-lite (http://prinseq.sourceforge.net/) and Usearch61 (http://www.drive5.com/usearch/download.html), respectively. Only high-quality (HQ) reads were used to select operational taxonomic units (OTUs) using QIIME (http://qiime.org/). SILVA 138 SSU (https://www.arb-silva.de/documentation/release-138/) was used as the reference OTU database to identify bacteria at 97% identity, and the assignment of taxonomy was made as described in our earlier studies.[17,25] Sparse OTUs (<0.001% of the total HQ reads) were not considered.

Diversity Analyses of Bacterial Microbiome Samples and Statistical Analysis

Rarefaction curves and alpha diversity indices (Shannon diversity index, Simpson index, observed number of OTUs, and Chao1) for the microbiomes were generated using the vegan 2.4-2 package (http://vegan.r-forge.r-project.org/) in R (R Foundation for Statistical Computing, Vienna, Austria). The microbiome data were analyzed using R 3.3.2.

Identification of Differentially Abundant Taxonomic Groups

Kruskal–Wallis and Wilcoxon signed-rank tests (with $P < 0.05$ being significant) were implemented to identify significantly different genera in the CT, DM, and DRC microbiomes.

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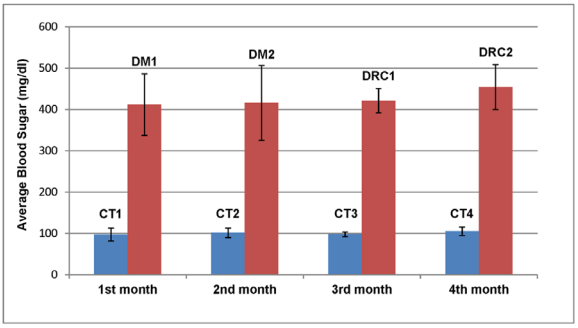

Figure 1. Average blood sugar levels of control rats (CT1–CT4), diabetic rats (DM1 and DM2), and diabetic rats showing retinal changes (DRC1 and DRC2).

A two-dimensional heat map, with rank-normalized abundances of the differentially abundant bacterial genera, was plotted in R. Differences in the gut bacterial microbiomes were also visualized using a non-metric multidimensional scaling (NMDS) plot based on Bray–Curtis distance dissimilarity.

Interaction Networks Among Bacterial Genera in the Gut Microbiomes

Interaction networks were generated separately for the CT, DM, and DRC microbiomes using CoNet (https://raeslab.org/software/conet.html) in Cytoscape (https://cytoscape.org/).

Results

Induction of Diabetes and Fasting Blood Sugar

Diabetes was induced in Sprague Dawley rats through intraperitoneal injection of STZ. The average fasting blood glucose in the DM and DRC rats was >150 mg/dL after 72 hours and persisted at >150 mg/dL after 1, 2, 3, and 4 months of STZ injection (Fig. 1). CT animals did not exhibit any significant changes in the blood glucose levels.

Histology and Immunohistochemistry

In this study, the third- and fourth-month DRC1 and DRC2 rats had increased levels of blood glucose (>400 mg/dL) (Fig. 1) and also showed a decrease in the number of cells in the inner nuclear layer and the outer nuclear layer compared with the CT3 and CT4 rats, respectively (Fig. 2). Further, HIF-1α increased significantly in the retina of DRC1 rats compared with CT3 rats, whereas VEGF increased in DM2, DRC1, and DRC2 rats compared with CT2 to CT4 animals. Intense staining of VEGF was also visible in the inner plexiform layer, outer plexiform layer and the layer between the photoreceptor layer and outer nuclear layer (Figs. 3A, 3B). A decrease in rhodopsin in the photoreceptor layer was observed in the DM and DRC groups compared with the CT rats (Fig. 3C).

Analysis of OTUs of the Gut Bacterial Microbiomes of Control, Diabetic, and Diabetic Rats Showing Retinal Changes

Average HQ reads per microbiome from the CT, DM, and DRC rats were 393,670, 464,123, and 441,727, respectively. From these microbiomes, 3036 OTUs were identified (1307 reference and 1729 de novo OTUs) (Supplementary Table S1). The saturation of rarefaction curves indicated that the sequencing depth and coverage were adequate to capture the total diversity in the 47 microbiomes (Fig. 4A). Alpha diversity analysis indicated that the Shannon diversity index and Simpson index differed significantly ($P < 0.05$) between the DM and DRC cohorts compared with the control but the number of observed OTUs and the Chao1 index were not significantly different (Fig. 4B).

Bacterial Community Composition

In the gut bacterial microbiomes of rats, 16 different phyla, excluding unclassified phyla, were detected (see Tables 2 and 3). The abundance of Bacteroidetes, Firmicutes, Euryarchaeota, Spirochaetes, and Proteobacteria was >0.50% in the eight cohorts, and Bacteroidetes and Firmicutes were the most abundant (Fig. 5; see also Tables 2 and 3). We observed longitudinal changes in abundance at the phylum level for CT1 to CT4, DM1 and DM2, and DRC1 and DRC2 (Figs. 5A–5C). DM1 differed from CT1 in the median abundance of Actinobacteria, Planctomycetes, Proteobacteria, Spirochaetes, and WPS2 (Table 1). However, after 2 months, DM2 differed from CT2 only in the median abundance of Lentisphaerae (Table 1). Further, in DRC1, four phyla (namely, Cyanobacteria, Euryarchaeota, Firmicutes, and Spirochaetes) showed a significant difference, whereas in DRC2 Euryarchaeota and Spirochaetes continued to show a significant difference in abundance, along with

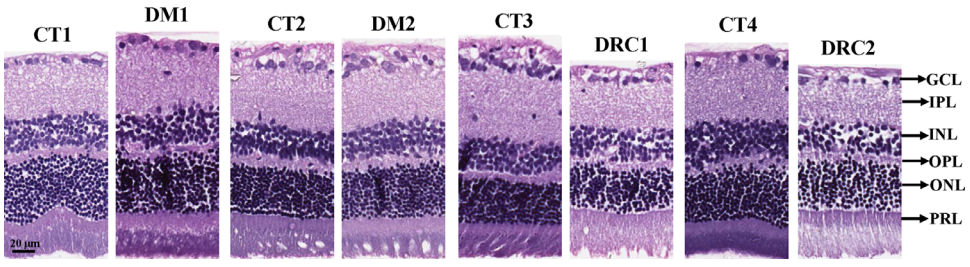

Figure 2. Hematoxylin and eosin–stained retinal sections of control (CT1–CT4), diabetic rats (DM1 and DM2), and diabetic rats showing retinal changes (DRC1 and DRC2). *Scale bar*: 20 µm. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PRL, photoreceptor layer.

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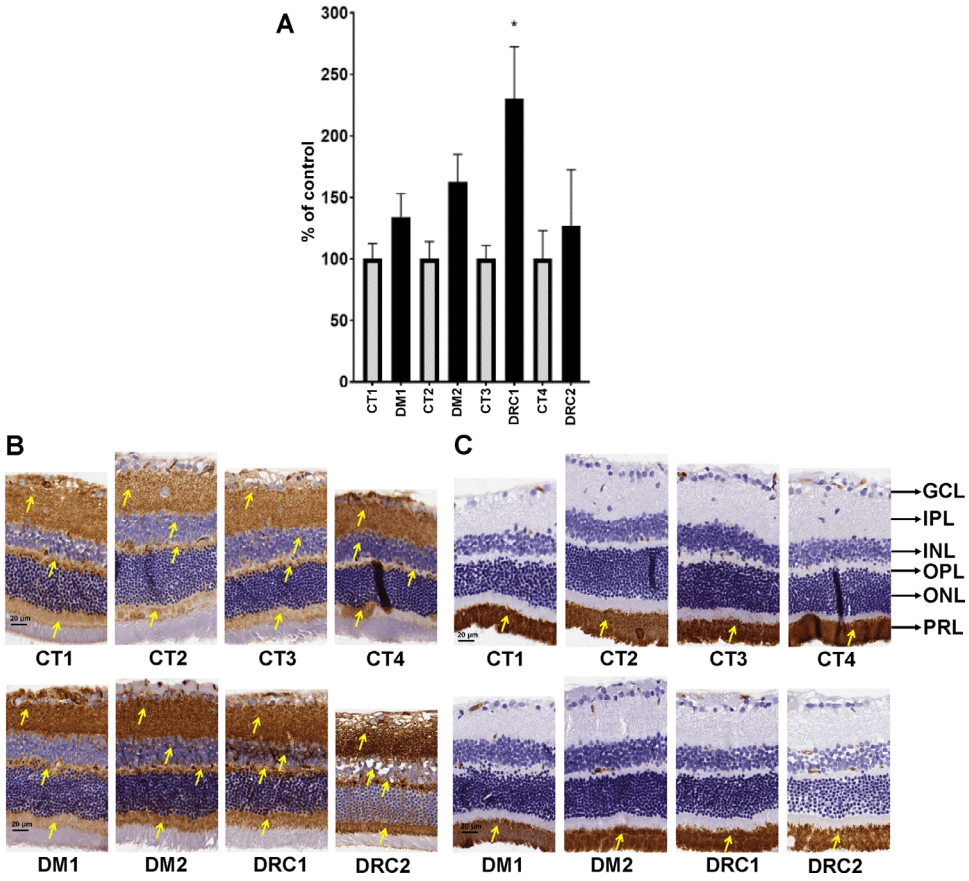

Figure 3. (**A–C**) Quantitative expression of HIF-1α by immunoblotting (**A**) and VEGF (**B**) and rhodopsin (**C**) by immunohistochemistry using specific antibodies in the retina of control rats (CT1–CT4), diabetic rats (DM1 and DM2), and diabetic rats showing retinal changes (DRC1 and DRC2). In **A**, levels of HIF-1α were calculated using tubulin as a control, and the data (mean $\pm$ SEM of three independent experiments) are represented as percentage of expression compared with control rats. In **B** and **C**, VEGF- and rhodopsin-specific antibodies were used for immunohistochemical staining. *Arrows* indicate either the increased production of VEGF or decreased production of rhodopsin. *Scale bar*: 20 µm. $P < 0.05$ was considered significant.

Actinobacteria and Proteobacteria, when compared with CT3 and CT4, respectively (Table 2).

At the genus level, 136, 137, and 136 genera were identified in CT1 to CT4, DM1 and DM2, and DRC1 and DRC2 rats (Supplementary Table S1). The most abundant genera, with average abundance of >1% in any one of the three groups, are listed in Supplementary Table S1. Comparison of the median abundance of the genera showed that no two microbiomes were identical (Figs. 6A–6C). In DM1, 12 genera decreased and 12 genera increased in abundance compared with CT1 (Table 3). Further, in DM2, four and seven genera decreased and increased, respectively, in abundance compared with CT2 (Table 4). In contrast, the DRC1 and DRC2 microbiomes showed many similarities. For example, eight identical genera showed a decrease and three identical genera showed an increase in abundance in both DRC1 and DRC2 microbiomes compared with their respective controls (Tables 5, 6). Box plots also confirmed a significant difference in abundance in genera in DM and DRC compared with their respective controls (Supplementary Figs. S1, S2; Tables 3–6).

Heat-map analyses indicated that the microbiomes of CT1 and CT2 formed two subclades (a and b), whereas DM1 and DM2 together formed a distinct subclade c within which DM1 formed a subclade d, with the DM2 microbiomes dispersed on either side of subclade c (Fig. 7A). The CT3 and CT4 microbiomes also formed two subclades (e and f) (Fig. 7B). The majority of the DRC microbiomes, which included all of the DRC2 microbiomes and three DRC1 microbiomes, formed a subclade g distinct from CT3 and CT4 microbiomes; however, three DRC1 microbiomes formed a cluster with CT3 (subclade e) (Fig. 7B). Further, when all of the microbiomes were analyzed together by heat map, the majority of the CT microbiomes (17 of 24) formed subclade a, four formed subclade b, and the remaining three were in subclade c, which had five DM1 microbiomes. It is interesting that the majority of the DM and DRC microbiomes (20 of 23) did not group with subclade a, which had the majority of the control microbiomes. Five microbiomes of DRC2 and three of DRC1 formed a subclade d; the DM2 microbiomes appeared to be the most diverse and were distributed in subclades a, b, and c (Supplementary Fig. S3). The NMDS plot also indicated that the CT microbiomes formed one distinct cluster (green), separate from both the DM (red) and the DRC (purple) groups ($P < 0.05$). Further, the majority of DM and DRC microbiomes also showed separation at the OTU level (Figs. 8A, 8B).

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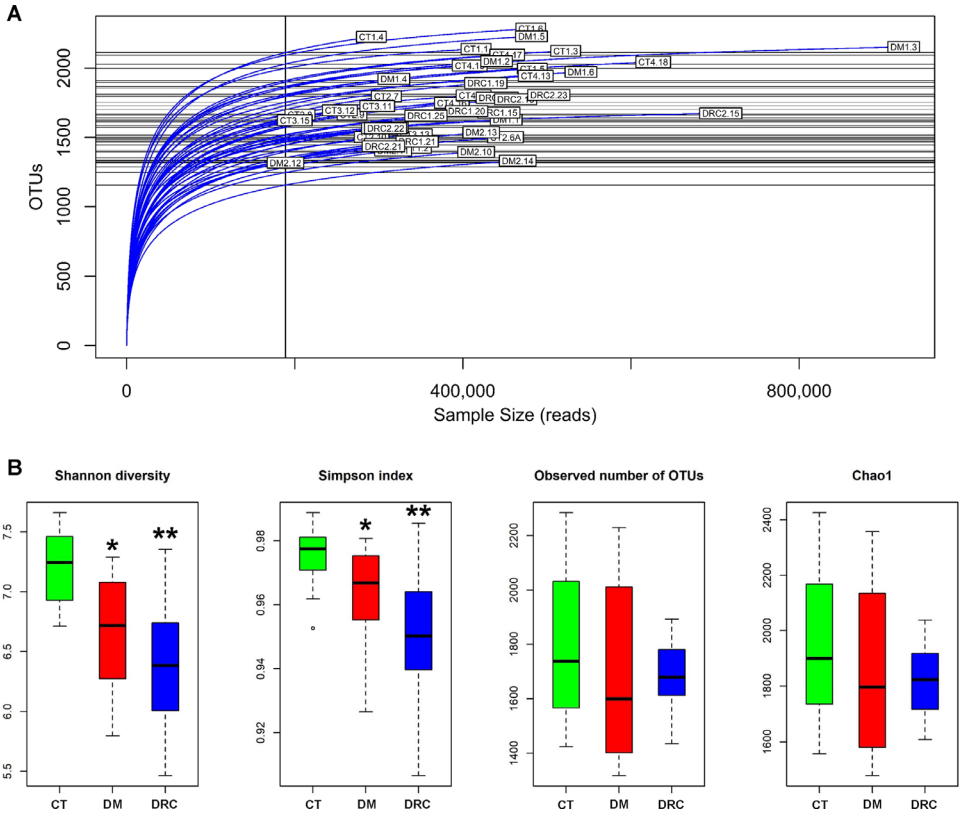

Figure 4. Rarefaction curves and (**B**) alpha diversity indices (Shannon diversity index, Simpson index, number of observed OTUs, and Chao1 index) of the gut bacterial microbiomes of control rats (CT, $n = 24$), diabetic rats (DM, $n = 12$), and diabetic rats showing retinal changes (DRC, $n = 11$). Asterisks indicate significant differences between CT and DM rats (*) and between CT and DRC rats (**), as determined by Student's *t*-test ($P < 0.05$).

Table 1. Median Abundance (%) of Phyla in Gut Bacterial Microbiomes of Control Rats After 1 Month (CT1, $n = 6$) and 2 Months (CT2, $n = 6$) and Diabetic Rats After 1 Month (DM1, $n = 6$) and 2 Months (DM2, $n = 6$)

	CT1		CT2		DM1*		DM2*		*P*	
Phylum	**Median**	**Present Out of 6 Samples**	**Median**	**Present Out of 6 Samples**	**Median**	**Present Out of 6 Samples**	**Median**	**Present Out of 6 Samples**	**CT1 vs. DM1**	**CT2 vs. DM2**
Actinobacteria	0.22	6	0.25	6	0.57	6	0.37	6	0.03	0.39
Bacteroidetes	41.12	6	45.08	6	35.51	6	39.38	6	0.18	0.09
Cyanobacteria	0.15	6	0.06	6	0.3	6	0.06	6	0.18	0.49
Deferribacteres	0.05	6	0.05	6	0.02	6	0.02	6	0.31	0.59
Elusimicrobia	0.09	6	0.25	6	0.21	6	0.16	6	0.94	0.07
Epsilonbacteraeota	0.06	6	0.04	6	0.11	6	0.04	6	0.07	1.00
Euryarchaeota	3.36	6	2.31	6	3.93	6	3.65	6	0.40	0.94
Firmicutes	27.78	6	43.43	6	29.14	6	38.17	6	1	0.70
Lentisphaerae	0.01	6	0.01	6	0.01	6	0	6	0.49	0.01
Patescibacteria	5.04	5	2.36	6	2.14	6	1.36	6	0.07	0.49
Planctomycetes	0.04	6	0.01	6	0.01	5	0.01	6	0.04	0.31
Proteobacteria	2.64	6	1.85	6	0.83	6	1.03	6	0.03	0.40
Spirochaetes	4.38	6	0.48	6	17.1	6	4.25	6	0.03	0.49
Tenericutes	0.16	5	0.23	6	0.19	6	0.32	6	0.49	0.59
Unclassified	0.7	6	0.01	6	1.65	6	0.02	6	0.13	0.39
Verrucomicrobia	0.01	6	0.03	6	0.02	6	0	5	0.09	0.07
WPS-2	0.04	6	0.12	6	0.13	6	0.06	6	0.03	0.39
Firmicutes:Bacteroidetes	0.68		0.96		0.82		0.97			
Firmicutes + Bacteroidetes	68.9		88.51		64.65		77.55			

The phyla Kiritimatiellaeota, Fusobacteria, and Nanoarchaeaeota, with median abundance of <0.01%, were not considered for comparison
* DM1 rats and DM2 rats were monitored 1 and 2 months, respectively, after the induction of DM using STZ.

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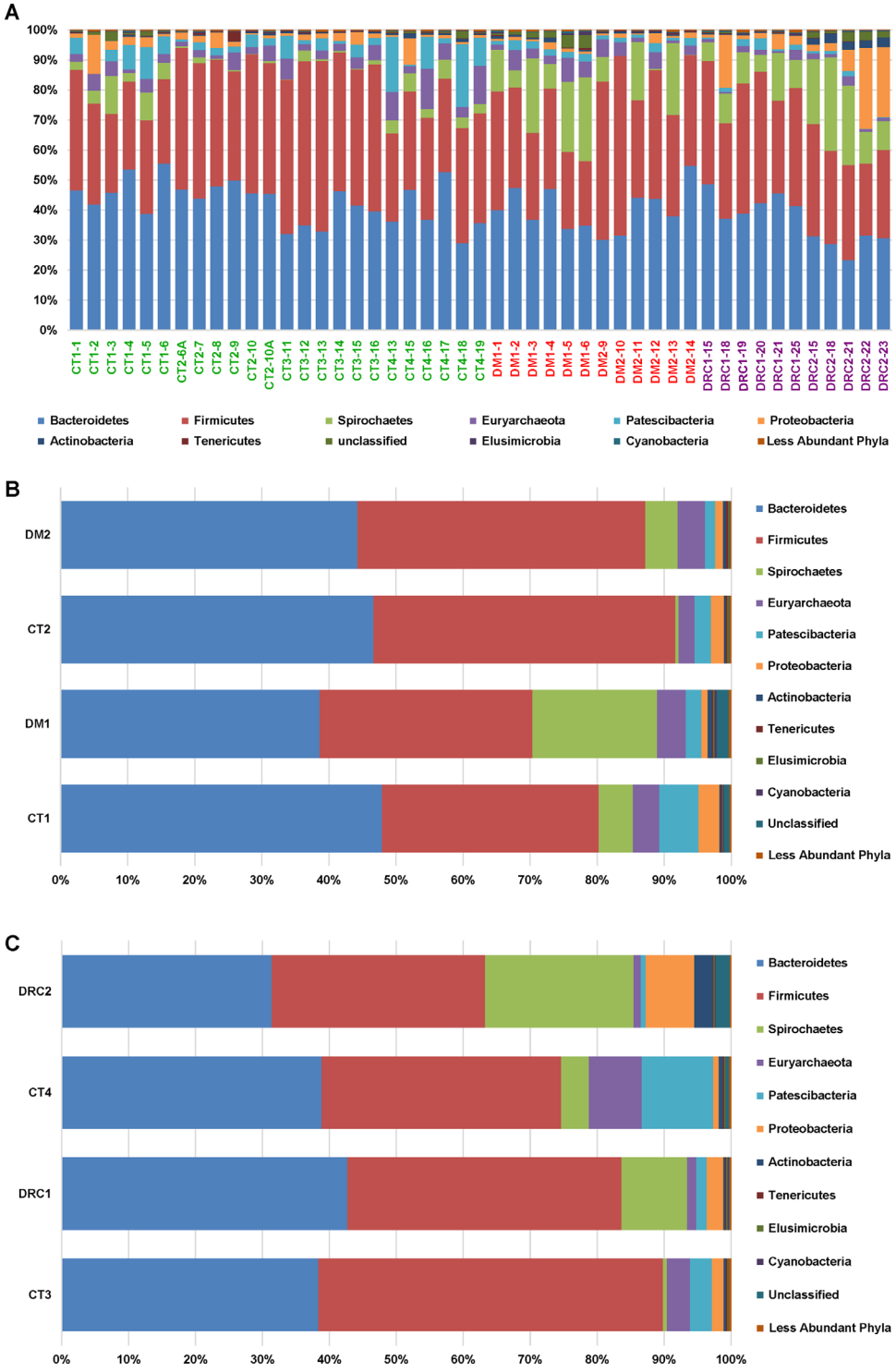

Figure 5. (**A**) Abundance (%) of phyla in the gut microbiomes of control rats (CT1, $n = 6$; CT2, $n = 6$; CT3, $n = 6$; CT4, $n = 6$), diabetic rats (DM1, $n = 6$; DM2, $n = 6$), and diabetic rats showing retinal changes (DRC1, $n = 6$; DRC2, $n = 5$). (**B**) Median abundance of bacterial phyla from gut microbiomes of control rats (CT1, $n = 6$; CT2, $n = 6$) and diabetic rats (DM1, $n = 6$; DM2, $n = 6$). (**C**) Median abundance of control rats (CT3, $n = 6$; CT4, $n = 6$) and diabetic rats showing retinal changes (DRC1, $n = 6$; DRC2, $n = 5$). CT1, CT2, CT3, and CT4 refer to control rats after 1, 2, 3, and 4 months, respectively; DM1 and DM2 refer to rats after 1 and 2 months, respectively, of STZ treatment; and DRC1 and DRC2 refer to rats after 1 and 2 months, respectively, of detection of retinal changes.

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Table 2. Median Abundance (%) of Phyla in Gut Bacterial Microbiomes of Control Rats After 3 Months (CT3, $n = 6$) and 4 Months (CT4, $n = 6$) and Diabetic Rats Showing Retinal Changes After 1 Month (DRC1, $n = 6$) and 2 Months (DRC2, $n = 6$)

	CT3		CT4		DRC1*		DRC2*		*P*	
Phylum	Median	Present Out of 6 Samples	Median	Present Out of 6 Samples	Median	Present Out of 6 Samples	Median	Present Out of 5 Samples	CT3 vs. DR1	CT4 vs. DR2
Actinobacteria	0.31	6	0.56	6	0.28	6	2.61	5	0.82	0.01
Bacteroidetes	36.41	6	33.77	6	40.53	6	29.47	5	0.24	0.05
Cyanobacteria	0.05	6	0.07	6	0.17	6	0.17	5	0.03	0.05
Deferribacteres	0.04	6	0.01	6	0.01	6	0.01	5	0.24	0.25
Elusimicrobia	0.18	6	0.17	6	0.17	6	0.17	5	0.82	0.93
Epsilonbacteraeota	0.05	6	0.03	6	0.11	6	0.02	5	0.59	0.54
Euryarchaeota	3.29	6	6.89	6	1.31	6	0.99	5	0.03	0.01
Firmicutes	48.9	6	31.08	6	38.86	6	29.93	5	0.03	0.66
Lentisphaerae	0.01	6	0.01	6	0.01	6	0.02	5	0.70	0.18
Patescibacteria	3.1	6	9.24	6	1.46	6	0.69	5	0.13	0.05
Planctomycetes	0.01	6	0.01	6	0.01	6	0	5	0.82	0.05
Proteobacteria	1.69	6	0.72	6	2.35	6	6.82	5	0.49	0.03
Spirochaetes	0.55	6	3.61	6	9.32	6	20.82	5	0.01	0.00
Tenericutes	0.18	6	0.16	6	0.17	6	0.08	5	0.82	0.33
Unclassified	0.02	6	0.42	6	0.08	6	1.96	5	0.09	0.03
Verrucomicrobia	0.01	6	0.01	6	0.01	6	0.02	5	1.00	0.13
WPS-2	0.08	6	0.07	6	0.09	6	0.05	5	0.49	0.54
Firmicutes:Bacteroidetes	1.34		0.92		0.96		1.02			
Firmicutes + Bacteroidetes	85.31		64.85		79.39		59.4			

The phyla Fusobacteria, Kiritimatiellaeota, and Nanoarchaeaeota, with median abundance of <0.01%, were not considered for comparison.
* DRC1 rats and DRC2 rats were monitored at 3 and 4 months, respectively, after the induction of DM using STZ.

Table 3. Median Abundance (>0.05% in Any One of the Groups) of Discriminatory Genera ($P \leq 0.05$) in Gut Bacterial Microbiomes From Control Rats (CT1, $n = 6$) and Diabetic Rats (DM1, $n = 6$)

		Median Abundance (%)		
No.	Genus	CT1	DM1	Function
Decreased in DM1 group				
1	*Ruminiclostridium*	2.14	0.77	Anti-inflammatory[38]
2	*Oscillibacter*	1.95	0.51	Anti-inflammatory[38]
3	*Rikenellaceae;g*_RC9	1.14	0.46	Anti-inflammatory/probiotic[38]
4	*Rikenellaceae;g*_dgA-11	0.74	0.03	Anti-inflammatory/probiotic[38]
5	*Bacteroides*	0.41	0.02	Anti-inflammatory/probiotic/antibacterial[38]
6	*Parasutterella*	0.15	0.01	Cholesterol metabolism
7	*Anaerovorax*	0.13	0.02	Inflammatory bowel syndrome[20,21]
8	Clostridiales XIII;*g*_AD3011	0.12	0.05	Not known
9	*Tyzzerella*	0.11	0	Pathogenic[41]
10	*Alistipes*	0.08	0.02	Anti-inflammatory[38]
11	*Rikenella*	0.05	0.01	Fat metabolism[39]
12	*Papillibacter*	0.05	0	Not known
Increased in DM1 group				
13	*Treponema* 2	4.22	16.95	Pathogen[17,33]
14	*Mitsuokella*	0.03	3	Anti-inflammatory[15,42,43]
15	*Faecalibacterium*	0.35	0.87	Anti-inflammatory/probiotic[38]
16	*Oribacterium*	0.14	0.58	Pathogen[46]
17	*Holdemanella*	0.06	0.31	Proinflammatory/pathogen[33,44,45]
18	*Oscillospira*	0.06	0.18	Associated with leanness and health[33,58]
19	*Coriobacteriaceae;g*_UCG-003	0.01	0.13	May be beneficial[49]
20	*Libanicoccus*	0	0.1	Not known[54]
21	*Escherichia* and *Shigella*	0	0.09	Pro-inflammatory/pathogen[33]
22	*Solobacterium*	0.01	0.08	Pathogen[33]
23	*Enterorhabdus*	0	0.07	Pathogen[33]
24	Firmicutes bacterium CAG:822	0	0.07	Not known

Sparse OTUs (with <0.001% of total high-quality reads) were not included.

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Figure 6. (**A**) Abundance (%) of genera in the gut microbiomes of control rats (CT1, $n = 6$; CT2, $n = 6$; CT3, $n = 6$; CT4, $n = 6$), diabetic rats (DM1, $n = 6$; DM2, $n = 6$), and diabetic rats showing retinal changes (DRC1, $n = 6$; DRC2 $n = 5$). (**B**) Median abundance of bacterial genera from gut microbiomes of control rats (CT1, $n = 6$; CT2, $n = 6$) and diabetic rats (DM1, $n = 6$; DM2, $n = 6$). (**C**) Median abundance of control rats (CT3, $n = 6$; CT4, $n = 6$) and diabetic rats showing retinal changes (DRC1, $n = 6$; DRC2 $n = 5$).

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Table 4. Median Abundance (>0.05% in Any One of the Groups) of Discriminatory Genera ($P \leq 0.05$) in Gut Bacterial Microbiomes From Control Rats (CT2, $n = 6$) and Diabetic Rats (DM2, $n = 6$)

No.	Genus	Median Abundance (%) CT2	DM2	Function
Decreased in DM2 group				
1	*Eubacterium*	2.88	0.81	Anti-inflammatory[38]
2	*Rikenellaceae;g*_dgA-11	0.81	0.16	Anti-inflammatory/probiotic[38]
3	*Parasutterella*	0.2	0.03	Cholesterol metabolism
4	*Anaerofilum*	0.17	0.04	Anti-inflammatory[38]
Increased in DM2 group				
5	*Phascolarctobacterium*	2.87	5.69	Probiotic/anti-inflammatory[46,47]
6	*Lachnospiraceae* group	0.7	1.92	Anti-inflammatory[46,47]
7	*Coriobacteriaceae;g*_UCG-003	0.02	0.11	May be beneficial[47]
8	*Megasphaera*	0	0.1	Probiotic/anti-inflammatory[46,47]
9	*Turicibacter*	0	0.07	Proinflammatory[38]
10	*Romboutsia*	0.01	0.06	Short-chain fatty acid producer/probiotic[42,43]
11	*Streptococcus*	0.01	0.05	Pathogen[33]

Sparse OTUs (with <0.001% of total high-quality reads) were not included.

Table 5. Median Abundance (>0.05% in Any One of the Groups) of Discriminatory Genera ($P \leq 0.05$) in Gut Bacterial Microbiomes From Control Rats (CT3, $n = 6$) and Diabetic Rats Showing Retinal Changes (DRC1, $n = 6$)

No.	Genus	Median Abundance (%) CT3	DRC1	Function
Decreased in DRC1 group				
1	*Ruminococcaceae* group	9.63	5.02	Anti-inflammatory[49]
2	*Methanobrevibacter*	3.13	1.24	Pathogen[60]
3	*Bacteroides*	0.68	0.03	Probiotic/antibacterial/anti-inflammatory[38]
4	*Rikenellaceae;g*_dgA-11	0.6	0.01	Anti-inflammatory/probiotic[38]
5	*Tyzzerella*	0.23	0	Pathogen[41]
6	*Anaerofilum*	0.21	0.03	Anti-inflammatory[38,50]
7	*Erysipelotrichaceae* group	0.19	0.1	Proinflammatory[51,54]
8	*Ruminococcaceae;g*_DTU089	0.14	0.01	Anti-inflammatory[38]
9	*Intestinimonas*	0.1	0.04	Anti-inflammatory[52]
10	*Pygmaiobacter*	0.09	0.03	Anti-inflammatory[53]
11	*Butyricimonas*	0.07	0.01	Proinflammatory[55]
12	*Papillibacter*	0.06	0	Not known
Increased in DRC1 group				
13	*Prevotella*	9.59	25.01	Pathogen[17]
14	*Treponema* 2	0.48	9.25	Pathogen[17,33]
15	*Sutterella*	0.08	0.18	Pathogen[17]
16	*Solobacterium*	0.05	0.14	Pathogen[33]

Sparse OTUs (with <0.001% of total high-quality reads) were not included.

Interactions Among the Gut Bacterial Genera of Control, DM, and DRC Rats

The gut bacterial microbiomes of CT, DM, and DRC rats (Supplementary Fig. S4) were connected through several "hub" genera or nodes (with a high degree of interaction >10) interacting either positively or negatively with other genera. The CT group with 11 hub genera shared one genera (*Ruminococcaceae;g*) with DM and seven (*Bradyrhizobium*, *Vibrio*, *Sphingomonas*, *Brevundimonas*, *Methylobacterium*, *Ralstonia*, and *Akkermansia*) with DRC, and the remaining three (*Oribacterium*, *Treponema 2*, and *Sutterella*) were unique to the CT group. No hub genera were shared between the DM and DRC microbiomes; the hub genera *Methanosphaera*, *Ruminiclostridium*, and *Subdoligranulum* were unique to the DM group, and the hub genera *Poterioochromonas* spp. DS, *Siccibacter*, *Coriobacteriaceae;g*_UCG003, *Slackia*, *Libanicoccus*, *Pseudomonas*, and *Enorma* were unique to the DRC group.

Discussion

Animal Model and Novelty of the Study

This study confirms that STZ-induced diabetic rats, 3 months after the induction of diabetes, exhibited changes in the retina, such as decreased retinal thickness, increased expression of HIF-1α and VEGF, and decreased rhodopsin expression (Figs. 2, 3), thus confirming earlier observations regarding the occurrence of DR 3 to 6 months after development of DM in STZ-induced DM rats.[21–23,26,27] A few studies also monitored gut microbiome changes in STZ-induced rats after varying periods of time from 1 week to 15 weeks[28–32] after the induction of DM. But, the novelty of this study

Table 6. Median Abundance (>0.05% in Any One of the Groups) of Discriminatory Genera ($P \leq 0.05$) in Gut Bacterial Microbiomes From Control Rats (CT4, $n = 6$) and Diabetic Rats Showing Retinal Changes (DRC2, $n = 5$)

No.	Genus	Median Abundance (%)		Function
		CT4	DRC2	
Decreased in DRC2 group				
1	*Methanobrevibacter*	6.72	0.99	Pathogen[60]
2	*Ruminococcaceae* group	5.26	2.31	Anti-inflammatory[49]
3	*Bacteroides*	0.36	0.01	Probiotic/antibacterial/anti-inflammatory[38]
4	*Rikenellacea;g*_dgA-11	0.14	0	Anti-inflammatory/probiotic[38]
5	*Anaerofilum*	0.09	0.04	Anti-inflammatory[38,50]
6	*Ruminococcaceae;g*_DTU089	0.06	0.03	Anti-inflammatory[38,49]
7	*Tyzzerella*	0.06	0	Pathogen[41]
8	*Intestinimonas*	0.05	0.02	Anti-inflammatory[52]
Increased in DRC2 group				
9	*Prevotella*	12.36	20.74	Pathogen[17]
10	*Treponema 2*	3.46	20.62	Pathogen[17,33]
11	*Clostridium sensu stricto 1*	0.01	0.2	Pathogen[17]
12	*Erysipelotrichaceae* group	0.07	0.12	Proinflammatory[51,52,54]
13	*Solobacterium*	0.03	0.12	Pathogen[33]
14	*Pseudomonas*	0.02	0.09	Pathogen[17]
15	*Turicibacter*	0	0.06	Proinflammatory[59]

Sparse OTUs (with <0.001% of total high-quality reads) were not included.

compared with the above studies includes the following: (1) Microbiome dynamics were monitored in STZ-induced diabetic rats for 4 months at regular monthly intervals, unlike earlier studies,[28–32] which monitored microbiome changes only at a single time point (e.g., 1 week, 30 days, 15 weeks). The first 2 months correspond with induction and progression of diabetes, whereas the third and fourth months coincide with the occurrence of retinal changes. (2) Zhou et al.[32] monitored Zucker diabetic fatty (ZDF) rats for 15 weeks, but these rats exhibit hyperglycemia at 8 weeks of age[32]; thus, changes in the microbiome were monitored at only 8 weeks[32] after the occurrence of diabetes compared with the 16 weeks monitored by us. (3) ZDF rats differ from STZ-induced diabetes rats in that the former has a mutated leptin receptor. (4) None of the studies simultaneously monitored retinal marker changes and gut microbiome changes, as was done in this study from 1 month to 4 months.

Gut Bacterial Microbiome in the Control Rats

In the current study, 10 abundant phyla were detected in the gut microbiomes of the control rats (Table 1), in accordance with earlier reports.[33,34] Further, at the genera level, 18 abundant genera were identified in the gut microbiome of the rats, and 11 of these genera had been reported earlier by Nagpal et al.[26] (Supplementary Table S1). The observed differences in the microbiome could be due to diet differences[35] or the influence of caging and bedding.[36] Longitudinal changes in abundance at the phyla level were also observed in the control, DM, and DRC microbiomes (Figs. 5A–5C).

Dysbiosis in Diabetic Rats

In agreement with earlier reports Firmicutes and Bacteroidetes[33,34] were the most abundant phyla and the ratio of Firmicutes to Bacteroidetes increased marginally only when DM1 was compared with CT1. This was not unexpected, as an increased ratio of Firmicutes to Bacteroidetes is known to be associated with inflammation, obesity, and insulin resistance.[28,37]

At the genera level in the DM1 and DM2 rats, a decrease in the abundance of *Ruminoclostridium*, *Oscillibacter*, Rikenellaceae;g_RC9, *Rikenellaceae;g*_dgA-11, *Bacteroides*, *Alistipes*, *Eubacterium*, and *Anaerofilum* was observed (Tables 3, 4). These bacteria are known to be anti-inflammatory,[36] reduce bacterial translocation across the intestine, maintain gut integrity,[36] and are associated with β-cell autoimmunity and insulin resistance.[38] Eventually, a decrease in their abundance is likely to be associated with DM, thus confirming earlier studies in diabetic rats and in T2DM subjects.[17,28,39] Other genera that decreased in abundance in DM rats could be implicated in several other physiological aspects, such as *Parasutterella* in bile acid maintenance and cholesterol metabolism, *Tyzzerella* in non-alcoholic fatty liver disease,[40] *Anaerovorax* in inflammatory bowel syndrome, and Rikenella and genus Rikenellaceae;g_RC9 in high-fat diets and lipid metabolism.

In addition to the above genera that decreased in abundance, 18 genera increased in DM1 and DM2 gut microbiomes (Tables 3, 4). These included eight pathogens, one proinflammatory bacteria, six anti-inflammatory/probiotic bacteria,[33,42,43] and three others. An increase in *Holdemanella*, for example, has been implicated in constipation and chronic kidney disease,[44,45] *Faecalibacterium* in inflammatory bowel syndrome, and *Oribacterium in* periodontal disease.[46] The increase in *Coriobacteriaceae;g*_UCG-003 in DM1 rats could be related to the beneficial effects of Roux-en-Y gastric bypass on type 2 diabetes.[47]

Gut microbiome dysbiosis has also been reported in people with diabetes[17] and in diabetic mice.[20] These diabetic mice (db/db), following intermittent fasting, showed a reduction in DR end points; thus, it was suggested that restructuring of the gut microbiome by intermittent fasting prevents retinopathy in db/db mice.[20] Other studies on mice have dealt with the effects of various antidiabetic drugs on the gut microbiome and were not considered for comparison in the current study.[33]

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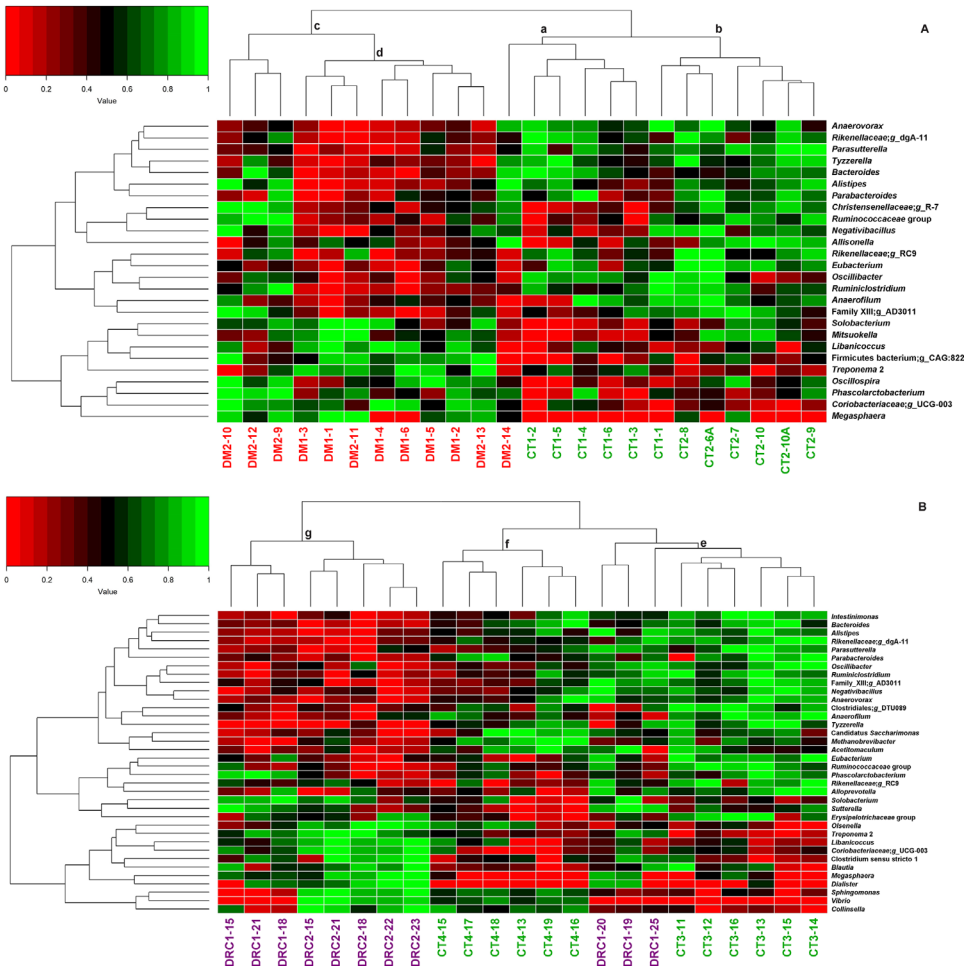

Figure 7. Two-dimensional heat map representing rank-normalized abundances (scaled between 0 and 1) of 20 differentially abundant bacterial genera (median abundance of >0.1% in at least one group) for (**A**) the gut bacterial microbiomes of control rats (CT1, $n = 6$; CT2, $n = 6$) and diabetic rats (DM1, $n = 6$; DM2, $n = 6$), and (**B**) the gut bacterial microbiomes of control rats (CT3, $n = 6$; CT4, $n = 6$) and diabetic rats showing retinal changes (DRC1, $n = 6$; DRC2 $n = 5$). The discriminating genera are arranged along two dimensions (axes) based on hierarchical clustering.

Gut Microbiome Longitudinal Changes in Diabetic Rats

Abundance changes in the DM2 rats compared with the DM1 rats (Tables 3, 4) at the genera level were not identical. Only two genera, *Rikenellaceae;g*_dgA-11, which has anti-inflammatory properties, and *Parasutterella* decreased in DM1 and DM2. A decrease in *Parasutterella* would be detrimental to DM subjects because *Parasutterella* has been correlated with reductions in low-density lipoprotein levels.[48] Further, *Coriabacteriaceae;g*_UCG-003 was the only genus that increased in both DM1 and DM2 microbiomes, and four other genera (*Phascolarctobacterium, Lachnospiraceae, Megasphaera*, and *Romboutsia*) that are either probiotic or anti-inflammatory in function increased only in DM2 microbiomes.[42,43]

Gut Microbiome Changes in Diabetic Rats With Retinal Changes

In DRC1 and DRC2 rats, the majority of the genera that decreased in abundance compared with CT3 and CT4 rats possess anti-inflammatory (producers of short-chain fatty acids) or probiotic properties (Tables 5, 6). These genera are associated with other diseases, including the *Ruminococcaceae* group and *Ruminococcaceae;g*_DTU089 in Crohn's disease[49]; *Anaerofilum* in increased intestinal permeability[50]; *Erysipelotrichaceae* in inflammation of gastrointestinal tract[51]; *Intestinimonas* in anti-inflammation[52]; Pygmaiobacter in hypertension[53]; and *Rikenellaceae;g*_dgA-11 in the maturation and development of gut microbiota[54] (Tables 5, 6). Several other bacteria that increased in abundance in DRC rats were either proinflammatory or pathogenic. The proinflammatory bacteria included the *Erysipelotrichaceae* group (earlier found to be involved in gastrointestinal tract disorders, colorectal cancer, and tumorogenic animals)[51] and *Turicibacter*. There were several pathogenic bacteria (Tables 5, 6), but *Treponema* was the only pathogen common to human and rat DRC gut microbiomes.[17] In DRC1 and DRC2, pathogens such as *Methanobrevibacter* and *Tyzrella* decreased. Earlier studies had indicated that *Tyzrella* was over-represented in Crohn's disease[49] and is associated with non-alcoholic fatty liver disease in rats.[41] *Butyricimonas*, a proinflammatory bacterium, also decreased in DRC1, and a decrease in *Butyricimonas* has been reported in T1DM patients.[55] Bacteroides, a probiotic, also decreased in

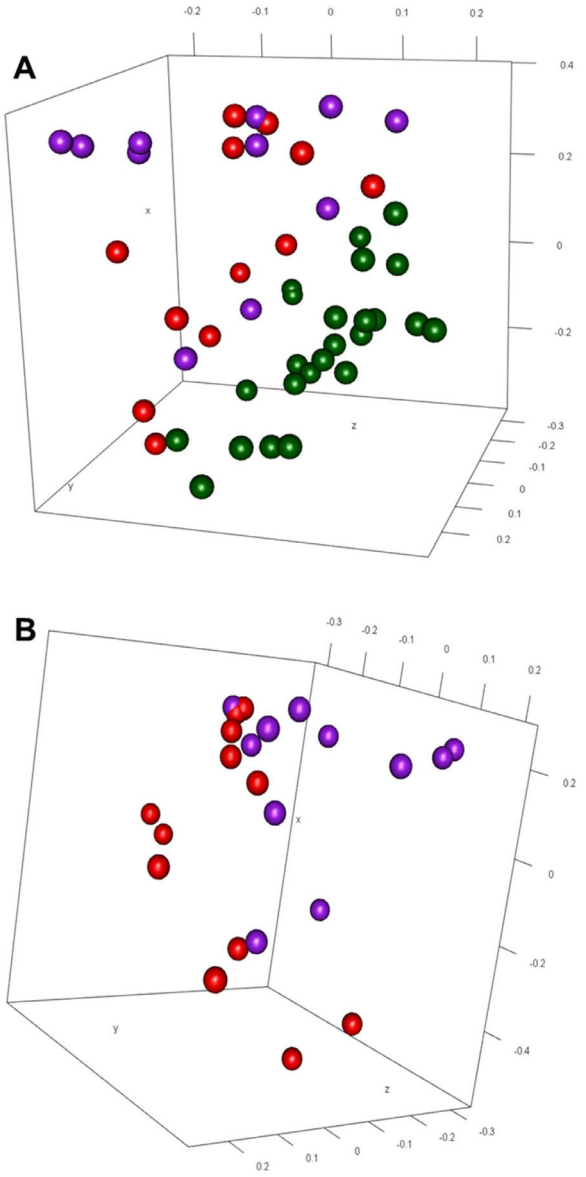

Figure 8. NMDS plots depicting β-diversity using Bray–Curtis dissimilarity in microbiomes at the OTU level for (**A**) control rats (CT1–CT4, $n = 24$, *green*), diabetic rats (DM1 and DM2, $n = 12$, *red*), and diabetic rats showing retinal changes (DRC1 and DRC2, $n = 11$, *purple*) and (**B**) diabetic rats (DM1 and DM2, $n = 12$) versus diabetic rats showing retinal changes (DRC1 and DRC2, $n = 11$).

DRC1. The relevance of the observed increase in Firmicutes bacterium CAG:822 and the gut bacterium *Libanicoccus*[54] in DM1 is not known.

Network and β-Diversity Analysis

Network analyses indicated that the gut bacterial microbiomes of the CT, DM, and DRC group rats were different. Further, β-diversity analysis (Fig. 8) also confirmed that the CT microbiomes were distinct from the DM and DRC groups, but the DM and DRC microbiomes showed an overlap (Fig. 8). Overall, the microbiomes of DM and DRC rats are distinct from those of the control microbiomes, and functionally the trend appears to be an increase in proinflammatory and pathogenic bacteria and a decrease in anti-inflammatory and probiotic bacteria in DM and DRC microbiomes. Dysfunction of the intestinal barrier, leading to enhanced permeability and translocation of microbial molecules into the systemic circulation, has been implicated in DM[56,57] obesity and hyperglycemia and increased risk of systemic infection.[57] Similar studies on the molecular and cellular orchestrators of diabetic retinopathy are lacking.

Study Limitations

Fecal pellets as the DNA source may not be representative of the entire gut microbiome. Also, metabolomics of blood samples could have revealed microbial metabolites that may have influenced the DRC rats.

Conclusions

We confirmed dysbiosis in the gut microbiomes of rats with DM at the phylum and genus levels compared with CT rats.

We also observed dysbiosis in the gut microbiomes of rats with DRC at the phylum and genus levels compared with CT rats. We observed a decrease in anti-inflammatory and probiotic bacteria and an increase in proinflammatory and pathogenic bacteria in the DRC rats.

Additional studies based on fecal microbial transplantation would help establish the connection between the gut microbiome and DRC unequivocally.

Future searches for novel therapeutics for people with DM and DR could benefit from the current information.

Acknowledgments

Supported by the Prof Brien Holden Eye Research Centre and Hyderabad Eye Research Foundation, L. V. Prasad Eye Institute, and by a grant from the Department of Science and Technology, Government of India (EMR/2017/000815). The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Disclosure: **S.R. Padakandla,** None; **T. Das,** None; **G. Sai Prashanthi,** None; **K.K. Angadi,** None; **S.S. Reddy,** None; **G.B. Reddy,** None; **S. Shivaji,** None

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