Ocular Surface Microbiota in Diabetic Patients With Dry Eye Disease

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PURPOSE. To investigate the ocular surface (OS) commensal bacteria profiles of patients with diabetes mellitus (DM) and dry eye disease (DED).

METHODS. In the present study, subjects were assigned to four groups: 37 to the diabetic mellitus with dry eye disease (DM with DED) group, 22 to the diabetes mellitus (DM)-only group, 34 to the dry eye disease (DED)-only group, and 22 to the control group. Tear fluid was collected using Schirmer's tear secretion test paper. 16S ribosomal ribonucleic acid (rRNA) gene sequencing was used to analyze the bacterial microbiota.

RESULTS. The DM with DED group showed the highest operational taxonomic unit (OTU) numbers and alpha diversity and the most different beta diversity. The groups shared the four most abundant phyla, accounting for over 96% of the total abundance. At the genus level, there were 10 types of overlap in the core microbiota in the groups. They showed significant differences between the groups. Additionally, the DM with DED group and the control group showed four unique core genera, respectively. Unclassified *Clostridiales* and *Lactobacillus* were the core microbiota members of the DM with DED group, the DM-only group, and the DED-only group, but not the control group.

CONCLUSIONS. In the present study, our results showed that the patients in the DM with DED group had a more complex and comprehensive ocular surface microbial composition. To the best of our knowledge, this is the first study to reveal the microbial profile of dry eye disease in patients with diabetes mellitus.

Keywords: ocular surface, diabetes mellitus, dry eye disease, microbiota

Dry eye disease (DED), a multifactorial disease characterized by tear film instability and hyperosmolarity, inflammation, and neurosensory abnormalities, has a high prevalence worldwide. According to an epidemiology-related meta-analysis report from the Tear Film and Ocular Surface Society Dry Eye Workshop II (TFOS DEWS II), the global prevalence ranges from 5% to 50%.[1] The related symptoms such as burning, foreign body sensation, photophobia, and blurred vision would adversely affect daily lives.[2] Studies have reported that people with diabetes mellitus (DM) tend to have a higher risk of developing DED.[3] It is considered that DM is one of the risk factors.[4,5] Our previous community-based studies found that the prevalence of DED among children with DM was higher than that in controls and that people with poor metabolic control were more likely to present with DED.[6,7] To our knowledge, the increased prevalence of DED in DM patients is most likely due to corneal neuropathy caused by chronic hyperglycemia, which leads to tear film instability and decreased tear breakup time (TBUT).[8] Other possible causes include increased risk of lacrimal functional unit (LFU) dysfunction, abnormal enzyme metabolism, and decreased mucin secretion.[9–11]

With the advancement of bioinformatics techniques over the past 15 years, the relationship between the colonized microbiota, especially bacteria microbiota, and human diseases has attracted increasing attention.[12–14] Some examples are the link between gut microbiota and DM, Alzheimer's disease, and cancer.[15–17] Similarly, several studies have shown relationships between commensal microbiota and eye diseases, such as age-related macular degeneration (AMD), uveitis, and diabetic retinopathy (DR).[18–20] Normal OS has relatively stable, and low diversity colonized microbiota,[21–23] which help maintain

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homeostasis.[24] However, diseases may change the microbiota composition of the OS, negatively affecting the protective role of the microbiota and causing it to be harmful.[21,25–27]

Given the higher incidence of DED in patients with DM and the potential functions and changeability of commensal microbiota, the present study aimed to explore the microbiota profile of diabetic patients with DED, which has not yet been described. Considering the increasing number of patients with DM worldwide,[28] the number of DM patients with DED is unlikely to decrease unless the treatment landscape changes. In the present study, we collected tear fluid samples, considering the wide range of secretory pathways and an essential component of the tear film, and analyzed the microbiota composition using 16S rRNA gene sequencing. For comparison, three other groups were enrolled in the present study: the DM-only group, the DED-only group, and the control group.

METHODS

Subjects

The present study conformed to the ethical principles of the Declaration of Helsinki, and all participants received and signed written informed consent. The Medical Ethics Committee of the Shanghai General Hospital at Shanghai Jiaotong University approved the study protocol.

The participants in this study were patients who visited the outpatient clinics of Shanghai General Hospital and Shanghai Gonghui Hospital for dry eye-related symptoms from July 2017 to July 2018. Two ophthalmologists asked for relevant medical history, examined the eyes under slit-lamp microscopy, and completed relevant eye examinations. The participants were divided into four groups: DM with DED group, DM-only group, DED-only group, and control group. The four groups were closely matched in terms of age and sex.

The diagnosis of DM was based on the World Health Organization (WHO) diagnostic criteria.[29] The diagnosis of DED followed the revised Japanese DED diagnostic criteria described in our previous study.[7,30] The exclusion criteria were the same as those of our earlier studies on dry eyes,[31] which are as follows: ocular status that affected tear production or quality, including eyelid diseases and eyelid movement disorder(s) caused by facial paralysis, conjunctiva diseases, ocular surgeries within 6 months or refractive surgeries within 2 years; history of ocular chemical injuries or use of ocular medications or nutritional tear supplements; and systemic diseases such as Sjogren's syndrome, Parkinson's disease, rheumatoid arthritis, Grave's disease, and systemic lupus erythematosus.

Tear Fluid Collection and Microbial Diversity Analysis

All subjects underwent dry eye testing as described in our previous studies,[7,31] including TBUT, Schirmer's test, and corneal fluorescein staining (FL). Schirmer test strips (Jingming, Tianjin, China) were placed at the outer one-third of the temporal canthus of each eye for 5 minutes without anesthesia. The strips were then removed from the eyes, and the amount of wetting was recorded in millimeters. The strips were placed in centrifuge tubes and stored immediately at −80°C for further analysis.

Bacterial deoxyribonucleic acid (DNA) was extracted by DNA extraction kit (MP Biomedicals, Santa Ana, CA, USA). The Q5 High-Fidelity DNA Polymerase (NEW ENGLAND BioLabs, Ipswich, MA, USA) was used for PCR amplification. A Quant-It PicoGreen dsDNA Assay Kit (Invitrogen,Waltham, MA, USA) was selected for the fluorescent reagent and the quantitative instrument was a microplate reader (BioTek Q2 FLX800, BioTek, Winooski, VT, USA). The V3-V4 region of the 16S rRNA genes was amplified and sequenced. The sequencing libraries were prepared using the TruSeq Nano DNA LT Library Prep Kit (Illumina, San Diego, CA, USA). The libraries were quantified using the Quant-iT PicoGreen dsDNA Assay Kit on Promega QuantiFluor Fluorescence Quantification System (Promega, Madison, WI, USA). 2 × 300 base pair double-end sequencing was performed using a MiSeq sequencer, with the corresponding reagent, MiSeq Reagent Kit V3 (600 cycles).

Data Analysis

In the present study, the Illumina MiSeq platform (Illumina) was used for the double-end (paired-end) sequencing of community DNA fragments. QIIME software (Quantitative Insights Into Microbial Ecology, v1.8.0, http://qiime.org/) was used to identify questionable sequences and to check and reject chimeric sequences by calling USEARCH (v5.2.236). A clustering procedure (UCLUST) was used to group into operational taxonomic units (OTUs) the previously obtained sequences with 97% sequence similarity. The sequence with the highest abundance in each OTU was selected as the representative sequence for that OTU. Subsequently, a matrix file of OTU abundance in each sample was constructed based on the number of sequences in each OTU for each sample. OTUs with abundance values <0.001% (1 in 100,000) of the total sequenced samples were removed. The taxonomic information corresponding to each OTU was obtained by comparing the representative OTU sequence with the template sequence from the Greengenes database, using the default parameters in the QIIME software. Based on the OTU abundance matrix obtained, the number of OTUs common to each sample (group) was calculated using R software (R Foundation for Statistical Computing, Vienna, Austria). Data were analyzed using SPSS (V.22.0 SPSS Science, Chicago, IL, USA), and plots were created using GraphPad Prism v.8.0.1 (GraphPad Inc., La Jolla, CA, USA). One-way analysis of variance (ANOVA) was used to compare age profiles, the Kruskal-Wallis test was used to compare the Schirmer's test between groups, and chi-square tests were used to compare gender. For the comparison of continuous variables, the Mann-Whitney U test was used to test for differences between the two groups because there was always at least one group that did not conform to the normal distribution. Median and interquartile range (IQR) were used to represent the distribution of each group of continuous variables. Statistical significance was set at $P < 0.05$.

RESULTS

Population

A total of 115 individuals were invited to participate in the present study: 37 diabetic patients with DED (32.2%), 22 with DM-only (19.1%), 34 with DED-only (29.6%), and 22 controls (19.1%). Table showed the demographic and clinical

TABLE. Demographic and Clinical Characteristics: Distribution of Age, Sex, TBUT, Schirmer's Test, HbA1c, and the Duration of DM

Parameters		DM With DED	DM-Only	DED-Only	Control	P Value
		Mean ± SEM or Median [Lower Quartile, Upper Quartile]				
Sample size (n)		37	22	34	22	NA
Age (years)	Male	59.4 ± 3.5	61.3 ± 3.5	57.4 ± 4.5	56.8 ± 3.4	0.9930
	Female	62.5 ± 4.8	64.1 ± 5.8	55.0 ± 7.6	53.9 ± 4.5	0.1203
Sex, n (%)	Male	19 (50)	12 (52.2)	25 (62.5)	12 (54.5)	0.7134
TBUT test LE (s)		3.3 [2.9, 4.2]	10.5 [8.7, 11.5]	3.7 [3.5, 4.5]	11.8 [10.2, 13.0]	<0.0001[****a]
						0.0749[b]
						0.1167[c]
TBUT test RE (s)		3.3 [2.9, 4.2]	8.6 [6.5, 10.3]	4.1 [3.6, 4.5]	10.0 [7.5, 10.5]	<0.0001[****a]
						0.0604[b]
						0.2975[c]
Schirmer's test LE (mm)		4 [2, 4]	15 [12, 17]	4 [3, 5]	12 [10, 17]	<0.0001[****]
						0.3035[a]
						0.3390[b]
Schirmer's test RE (mm)		3.4 [3, 4]	15 [10,17]	4 [3,5]	14 [10,18]	<0.0001[****]
						0.3581[b]
						0.9851[c]
HbA1c (%)		6.9 [6.5, 8.1]	7.7 [6.7, 8.4]	NA	NA	0.3280
DM duration (years)		12 [6.5, 20]	14 [7.5, 20]	NA	NA	0.7242

[a] = comparison between all four groups; [b] = DM with DED group versus DED-only group; [c] = DM-only group versus control group; TBUT = tear breakup time; DM = diabetes mellitus; DED = dry eye disease; SEM = standard error of mean; LE = left eye; RE = right eye; NA = not available; $P^{****} < 0.0001$ (comparison of four groups).

characteristics of the participants. There were no significant differences in age or sex ($P > 0.05$) between the groups. The TBUT and Schirmer's test results were significantly lower in both groups with DED but were not statistically different between the two groups with or without DED. There was no significant difference in the duration of diabetes or glycosylated hemoglobin (HbA1c) levels between the two groups with DM.

OTU Numbers

Analyses showed that the DM with DED group had the largest OTU numbers at each classification level when compared to the other three, including phylum (1661; IQR, 1542–1714), class (1660; IQR, 1541–1714), order (1645; IQR, 1528–1702), family (1419; IQR, 1350–1483), and genus (1051; IQR, 984–1115); (all P values $< 0.0001^{****}$) (Fig. 1A). Additionally, the DM-only group had the second highest OTU numbers at each level (P values $< 0.01^{##}$–0.05^{*}), compared to the DED-only and control groups, except the genus level (Fig. 1A). There were no significant differences between the DED-only and the control groups at each level (Fig. 1A).

The Highest Alpha and Beta Diversities Are in the DM With DED Group

The alpha diversity results of the DM with DED group showed significant differences compared to the other three: the Shannon diversity index for the DM with DED group (7.53; IQR, 7.40–7.69) had P values of 0.0002^{***}, $<0.0001^{****}$, and 0.0013^{**}, versus the DM-only, DED-only, and control group, respectively, and the Simpson diversity index (0.975; IQR, 0.971–0.979) had P values of 0.0105^{*}, $<0.0001^{****}$, and 0.0227^{*}, versus the DM-only, DED-only, and control

group, respectively. These indices were highest in the DM with the DED group compared to all others. (Figs. 1B and C). The Chao1 index (1881; IQR, 1754–1943) had P values of $<0.0001^{****}$, $<0.0001^{****}$, and $<0.0001^{****}$, versus the DM-only, DED-only, and control group, respectively, and the ACE index (1954; IQR, 1822–2016) had P values of $<0.0001^{****}$, $<0.0001^{****}$, and $<0.0001^{****}$, versus the DM-only, DED-only, and control group, respectively, which showed that the DM with DED group had the highest richness (Figs. 1D and E). Additionally, the Shannon diversity (P values: DM-only versus DED-only, 0.3312; DM-only versus control, 0.5727; and DED-only versus control, 0.7805, respectively) and the Simpson diversity (P values: DM-only versus DED-only, 0.5657; DM-only versus control, 0.5263; and DED-only versus control, 0.1677) indices showed no significant differences between the other three groups (Figs. 1B and C). The DM-only group had significantly higher Chao1 richness and ACE richness than control (P values: $0.0239^{#}$ and $0.0111^{#}$), and all other comparisons showed no significant differences (Figs. 1D and E). The rarefaction curves for the individual sample and the groups were shown in Supplementary Data 1.

Weighted UniFrac Principal Coordinates Analysis (PCoA), parameters used to analyze beta diversity, showed an aberrant composition for the DM with DED group when compared with the DM-only, DED-only, and control group (P values: 0.001^{***}, 0.002^{**}, 0.001^{***}, respectively; adonis: permutational multivariate analysis of variance using distance matrices) (Figs. 1F and G). Compared to the control group, the microbiota compositions of the DM-only and DED-only groups all showed significant differences (P values: $0.022^{#}$ and $0.012^{#}$, respectively; adonis: permutational multivariate analysis of variance using distance matrices) (Figs. 1F and G). There was no difference in microbiota composition between the DM-only and DED-only groups (P value: 0.264) (Figs. 1F and G). Analysis of similarities (ANOSIM)

G.
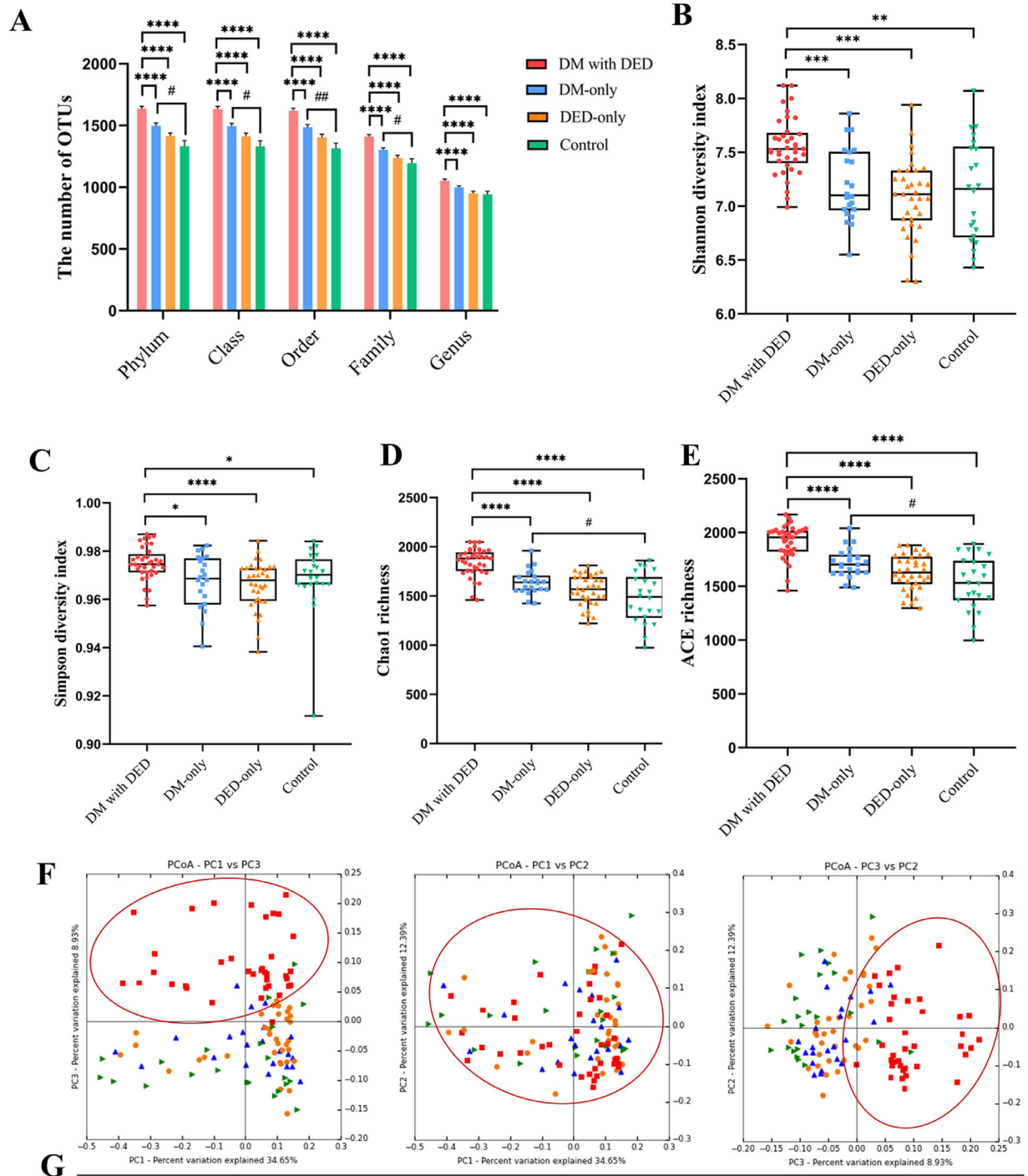
Comparisons		DM with DED vs. DM-only	DM with DED vs. DED-only	DM with DED vs. Control	DM-only vs. DED-only	DM-only vs. Control	DED-only vs. Control
Weighted Unifrac Adonis	R^2	0.09967	0.1061	0.1143	0.0225	0.0642	0.0572
	P-value	0.001***	0.002**	0.001***	0.264	0.022#	0.012#
Weighted Unifrac Anosim	R^2	0.1342	0.1725	0.2691	0.0238	0.0966	0.162
	P-value	0.015*	0.001***	0.001***	0.225	0.008##	0.006##

FIGURE 1. OTU numbers and the bacterial diversity of the four groups. **A.** The comparisons of OTU numbers at five classification levels. **B.** Shannon diversity index of the groups. **C.** Simpson diversity index of the groups. **D.** Chao1 richness index of the groups. **E.** ACE richness index of the groups. **F.** Weighted UniFrac PCoA of the groups. The area circled by the _red oval_ represents the DM with DED group. **G.**

ADONIS and ANOSIM analysis of the groups. * <0.05, ** <0.01, *** <0.001 (*represents the DM with DED group compared with the DM-only group, the DED-only group, and the control group); $^{\#}$ <0.05, $^{\#\#}$ <0.01, $^{\#\#\#}$ <0.001 ($^{\#}$ represents the DM-only and the DED-only group compared to the control group).
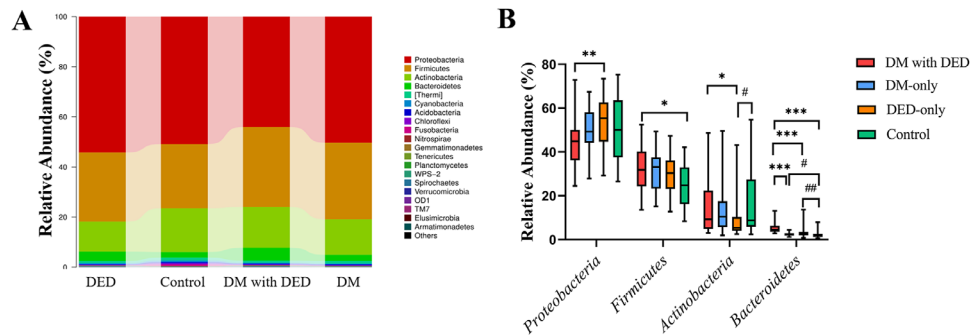
FIGURE 2. Bacterial compositions and comparisons at the phylum level. **A**. The phylum compositions in the four groups. **B**. The comparisons of the first four abundance phyla. * <0.05, ** <0.01, *** <0.001 (*represents the DM with DED group compared with the DM-only group, the DED-only group, and the control group); $^{\#}$ <0.05, $^{\#\#}$ <0.01, $^{\#\#\#}$ <0.001 ($^{\#}$ represents the DM-only and the DED-only group compared to the control group).

showed significant differences between groups (P values <0.05) except for the comparison of the DM-only and the DED-only group (P values = 0.225) and the correctness of grouping (all R^2 values > 0) (Fig. 1G).

Bacterial Taxonomy at the Phylum Level

The four groups showed the same bacterial phyla, Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes, accounting for >96% of the total abundance (Figs. 2A and B). The analysis for Proteobacteria showed significant differences between the DM with DED group (44.90%; IQR, 36.24%–50.02%) and the DED-only (55.37%; IQR, 44.68%–62.64%) (P values: 0.0041**). In the analysis for Firmicutes, a significantly higher level was detected in the DM with DED group (31.84%; IQR, 24.33%–40.26%) than in the control group (30.39%; IQR, 23.14%–36.07%) (P values: 0.0208*). In the analysis for Actinobacteria, the DED-only group showed the lower relative abundance (5.44%; IQR, 4.06%–10.31%), compared to the DM with DED (9.31%; IQR, 4.87%–22.34%) and the control group (8.77%; IQR, 5.91%–27.45%) (P values: 0.0383* and 0.0202$^{\#}$, respectively). In the analysis for Bacteroidetes, the DM with DED group (4.55%; IQR, 3.63%–6.27%) showed the highest relative abundance compared to the other three groups (all P values <0.0001****). Additionally, the relative abundance of Bacteroidetes in the DM-only (2.39%; IQR, 1.85%–2.97%) and DED-only group (2.60%; IQR, 1.97%–3.54%) was higher than the control group (1.73%; IQR, 1.22%–2.61%) (P values: 0.0391$^{\#}$ and 0.0060$^{\#\#}$, respectively). No statistical differences were found in other comparisons.

### Ocular Surface Core Microbiota at the Genus Level

More subtle statistical differences were found between the groups at the genus level. We used the "core microbiota" of the ocular surface as a parameter to compare the compositions of the groups. Here, we described the OS core microbiota as a bacterial microorganism, accumulates a mean relative abundance in one group of >76% in the present

study.[32,33] The results showed that the core compositions of the groups showed overlapping components and individual parts (Fig. 3).

Ten genera are the common to all groups: *Ochrobactrum, Bacillus, Corynebacterium, Cupriavidus, Lactococcus,* unclassified *Bacillaceae,* unclassified *Rhizobiales, Methylobacterium, Amycolatopsis,* and unclassified *Xanthomonadaceae*. The top five common core microbiota, the DM with DED group (*Ochrobactrum*: 20.59%; IQR, 16.59%–26.17%; *Bacillus*: 9.02%; IQR, 7.08%–12.52%; *Corynebacterium*: 5.50%; IQR, 1.79%–17.80%; *Cupriavidus*: 5.61%; IQR, 4.76%–7.14%; *Lactococcus*: 3.84%; IQR, 2.99%–5.36%) showed no statistical differences to the control group (all P values > 0.05). Additionally, the DM with DED group showed lower relative abundance of *Corynebacterium* and higher relative abundance of *Ochrobactrum, Bacillus, Cupriavidus, Lactococcus*, compared to the DM-only group and (or) DED-only group (P values range 0.05*–0.01**). The analysis of unclassified *Bacillaceae* showed no statistical differences between groups (all P values > 0.05). In the analysis for *Methylobacterium*, the DED-only group (1.90%; IQR, 1.39%–2.32%) showed a higher relative abundance than the control group (P value: 0.0254$^{\#}$), while no statistical differences were found in the other comparisons. In the analyses for unclassified *Rhizobiales* (0.67%; IQR, 0.49%–1.09%), *Amycolatopsis* (0.14%; IQR, 0.08%–0.30%), and unclassified *Xanthomonadaceae* (0.08%; IQR, 0.059%–0.135%), the control group showed the lowest relative abundance (all P values <0.0001) (Figs. 4A and B).

The Most Remarkable Difference in Core Composition Between the DM With DED Group and Control Group

Analysis for the core compositions of the groups showed the presence of unique core members in three: the DM with DED group, DED-only group, and control group. The DM with DED group and control group each had up to four types (unclassified *Ruminococcaceae, Bacteroides,* unclassified *Peptostreptococcaceae,* unclassified *Barnesiel-*
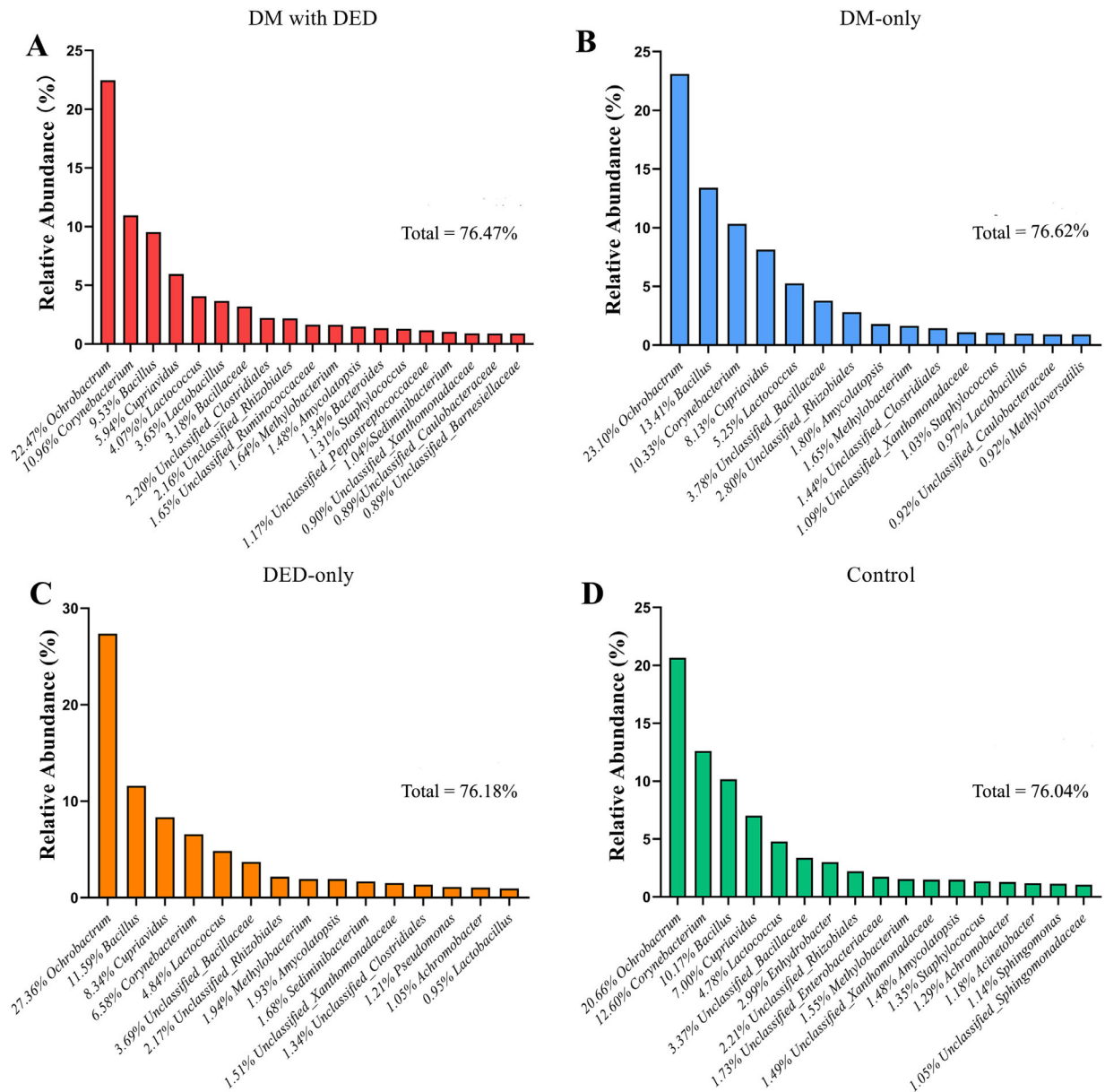
FIGURE 3. Mean relative abundance values of OS core microbiota in each group. **A.** The core microbiota of the DM with DED group. **B.** The core microbiota of the DM-only group. **C.** The core microbiota of the DED-only group. **D.** The core microbiota of the control group.

laceae for the DM with DED group, *Enhydrobacter*, unclassified *Enterobacteriaceae*, *Sphingomonas*, unclassified *Sphingomonadaceae* for the control group, respectively). The DED-only group had one (*Pseudomonas*), and the DM-only group did not have core microbiota that was only present in its group (Fig. 4C).

Unclassified *Clostridiale* and *Lactobacillus* Were the Core Members of the Disease Groups, With the Highest in the DM With DED Group

Interestingly, unclassified *Clostridiale* and *Lactobacillus* belong to the core microbiota members of the DM with DED group (unclassified *Clostridiale*: 1.84%; IQR, 1.29%–2.47%; *Lactobacillus*: 3.3%; IQR, 2.5%–4.6%), DM-only group

(unclassified *Clostridiale*: 1.42%; IQR, 1.17%–1.54%; *Lactobacillus*: 0.92%; IQR, 0.74%–1.1%), and DED-only group (unclassified *Clostridiale*: 1.22%; IQR, 0.80%–2.03%; *Lactobacillus*: 0.81%; IQR, 0.56%–1.32%), but not the control group (unclassified *Clostridiale*: 0.055%; IQR, 0.03%–0.1%; *Lactobacillus*: 0.05%; IQR, 0.037%–0.23%) (all *P* values <0.0001; each group compared with the control group). The DM with DED group showed the highest abundance of unclassified *Clostridiale* and *Lactobacillus* (all *P* values <0.05; each group compared with the DM with DED group) (Figs. 3 and 4D).

DISCUSSION

Several studies have described the commensal microbiota on the OS using the traditional microbial cultures and 16S rRNA
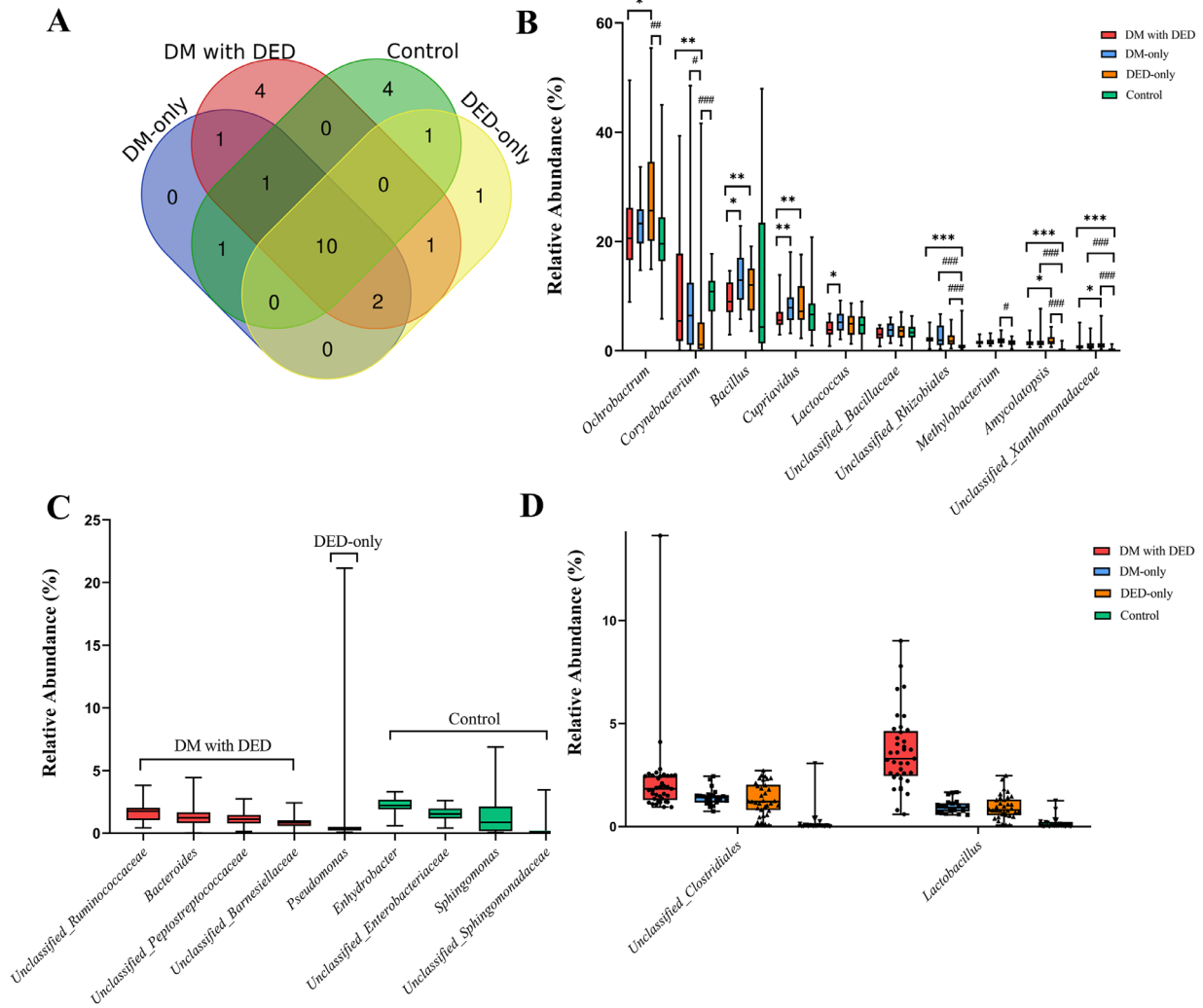
FIGURE 4. The characteristic of core microbiota for the groups. **A**. A Venn diagram shows the relationship of the core members of the groups. **B**. Ten core members in common of the groups. **C**. Unique core microbiota in the groups. **D**. Unclassified *Clostridiales* and *Lactobacillus* as the core microbiota of the DM with DED group, the DM-only group, and the DED-only group in the four groups. * <0.05, ** <0.01, *** <0.001 (* represents the DM with DED group compared with the DM-only group, the DED-only group, and the control group); # <0.05, ## <0.01, ### <0.001 (# represents the DM-only and the DED-only group compared to the control group).

gene sequencing.[23,34–37] Under normal physiological conditions, the microbiota is relatively stable, with low diversity and abundance, while still playing a crucial role in maintaining the homeostasis of the OS. Additionally, the composition of the microbiota appears to be different based on extrinsic factors. For example, the OS microbiota of individuals with DED and/or DM varies from that of healthy individuals, like those mentioned above. To explore the potential possible differences in microbiota for future research on the diabetic patients with DED, we collected tear fluid samples from three affected groups and one group of controls for analysis.

Compared to the other groups, the DM with DED group had the highest alpha and beta diversities, meaning that the samples contained a richer and more diverse microbial composition, which typically represents the hallmark of the disease, likely due to the decrease in lysosomal and antimicrobial compounds in tears.[38] We found the alpha diversity of the DED-only group did not differ from that of the control group. This is different from the results of Li et al.,[39] which

shows that the non-dry eye group had higher alpha diversity indices, Shannon and Simpson, than the dry eye group, and no significant differences in the other two indicators of alpha diversity, Chao1 and Observed species. Considering the different sampling sites between these two studies, it is difficult to compare the results directly. The DM-only group had higher alpha diversity than the control group, which is consistent with previous studies from Baknoon et al.[40] and Li et al.[41] The present study also showed conclusions consistent with their results on beta diversity. However, the sampling site, age, and geographic location of these studies were different. Consistent may do not mean that the connotation is the same, but it represents a trend of microbial alteration.

Further analyses on the microbial phylum and genus level are necessary to understand the OS microorganisms. We analyzed the four most abundant phyla, Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, as they accounted for over 96% of the sum of the microbial abundance in each group. In comparing the first four phyla in each group, our

results showed the highest Bacteroidetes in the DM with DED group and the lowest in the control group. While the comparisons of the first three did not exhibit specific characteristics. Interestingly, all these four phyla showed no significant differences between the DED- and DM-only groups.

The concept of core microbiota at the genus level was used to compare the microbiota population in the present study. The definition of the core microbiota in OS varies. Some studies have defined relative abundances of >1% in all samples[40] or as >1% of the 300 most abundant taxa,[42] or when microbiota accounted for >76% of the total abundance,[43] which is the definition we used in the present study. Our results showed that the core members of the four groups crossed and varied. The most remarkable difference existed between the DM with DED group and the control group, which each had up to four distinct and unique core members.

Compared to the control group, the relative abundance of unclassified *Clostridiale* and *Lactobacillus* at the genus level was higher in the DM with DED group, the DM-only group, and the DED-only group. The control patient samples contained the lowest abundance of them, while the DM with DED group had the highest, and the DED- and DM-only groups had abundances somewhere in between. A higher relative abundance of *Lactobacillus* in OS diseases is not particularly unusual. For example, Kittipibul et al.[26] showed a higher abundance of *Lactobacillus* in the OS of patients with Stevens-Johnson syndrome. However, the changes in and the influence of the microbiota on OS diseases remain to be explored.

Our results showed that the DM with DED group had the most considerable differences among the disease groups compared to the control group. We believe that these changes resulted from complex factors. The microbiota is one member of the OS microenvironment, which contains intractable and complex elements, such as cells, matrices, and hormones.[44] However, hyperglycemia is a crucial risk factor for the balance of the microenvironment. Hyperglycemia leads to corneal neuropathy, which causes patients not to notice when dry eye is happening. It can cause tissue damage that makes it favorable for microbiota to the be harbored. In our earlier study, our colleagues found that the tear samples from diabetic patients with DED had decreased lysozyme C and zinc-alpha-2-glycoprotein, both of which have molecular functions that include bacteriolysis and stimulation of lipid degradation.[31] We believe a potential correlation between microbiota changes and diabetic dry eye disease and identifying abnormalities in OS bacteria is essential for developing interventional treatments.

The present study had several limitations. First, because of the study design, the data of the ocular surface parameters are limited. For example, we did not divide meibomian gland dysfunction (MGD) and non-MGD patients into separate groups. Second, 16S rRNA gene sequencing has limitations. Although this method allows the entire microbial community to be characterized and defines the relative abundance of these communities spatially and temporally, it does not distinguish between viable and nonviable microorganisms. Third, microbiota on the ocular surface is not limited to bacteria, but also fungi, viruses, and chlamydia-like organisms, and so on. Hence, analysis of commensal bacterial cannot fully represent the composition of OS microbiota, although it is the most abundant and perhaps the most complex of them.

In summary, the present study results characterized the microbiota of tear fluid in diabetic patients with DED and showed the uniqueness of these microbial communities. It is the first time that the commensal microbiota has been explored in diabetic patients (who appear to have a higher incidence of DED) with DED. With the present study results, we hope to provide helpful, innovative, and evidence-based ideas to prevent and treat diabetic patients with DED.

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