Ocular Surface Microbiome in Diabetes Mellitus

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การศึกษาไมโครไบโอมของเยื่อบุตาในผู้ป่วยเบาหวาน

นางอรทัย สุวจนกรณ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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อรทัย สุวจนกรณ์ : การศึกษาไมโครไบโอมของเยื่อบุตาในผู้ป่วยเบาหวาน. (Ocular Surface Microbiome in Diabetes Mellitus) อ.ที่ปรึกษาหลัก : ดร.ธนิษฐา ฉัตรสุวรรณ

การเปลี่ยนแปลงของเยื่อบุตาและระบบภูมิคุ้มกันในโรคเบาหวาน อาจนำมาซึ่งการเปลี่ยนแปลงของไมโครไบโอมที่เยื่อบุตา โดยเฉพาะในภาวะเบาหวานขึ้นจอตาและภาวะที่มีการควบคุมระดับน้ำตาลเลือดได้ไม่ดี การศึกษานี้เป็นการศึกษาไมโครไบโอมของเยื่อบุตาในผู้ป่วยเบาหวาน โดยศึกษาตามระดับความรุนแรงของภาวะเบาหวานขึ้นจอตา และตามระดับการควบคุมน้ำตาลสะสมในเลือด (HbA1c) โดยวิธีการเพาะเชื้อและ NGS (Next-generation sequencing analysis) ทำการศึกษาแบบตัดขวาง (Cross-sectional study) ในผู้ป่วยเบาหวาน 60 ตาและผู้ที่ไม่เป็นเบาหวาน 20 ตา ทำการเก็บสิ่งส่งตรวจโดยป้ายที่บริเวณเยื่อบุตา ส่งตรวจเพาะเชื้อและวิธี NGS ซึ่งผลการศึกษาจากการเพาะเชื้อ พบเชื้อในกลุ่มผู้ป่วยเบาหวาน (15%) มากกว่าผู้ที่ไม่เป็นเบาหวาน (5%) (p-value=0.437) โดยพบเชื้อดื้อยาเฉพาะในกลุ่มผู้ป่วยเบาหวานที่มีเบาหวานขึ้นจอตา ผลตรวจด้วยวิธี NGS พบว่าเชื้อที่เยื่อบุตาในผู้ป่วยเบาหวานต่างจากผู้ที่ไม่เป็นเบาหวาน โดยในผู้ป่วยเบาหวาน พบเชื้อในผู้ที่มีเบาหวานขึ้นจอตา ต่างจากผู้ป่วยที่ไม่มีเบาหวานขึ้นจอตาและผู้ที่ไม่เป็นเบาหวาน (p-value<0.05) และยังพบความแตกต่างของเชื้อระหว่างผู้ป่วยเบาหวานที่ควบคุมระดับน้ำตาลสะสมในเลือดได้ไม่ดี (HbA1c ≥7%) ต่างจากผู้ป่วยที่คุมได้ดี (HbA1c <7%) และผู้ที่ไม่เป็นเบาหวาน (p<0.001) อีกทั้งยังตรวจพบเชื้อก่อโรคมากขึ้นในผู้ป่วยเบาหวาน โดยเฉพาะในผู้ที่มีเบาหวานขึ้นจอตา การศึกษานี้เป็นการศึกษาแรกที่แสดงถึงไมโครไบโอมของเยื่อบุตาในผู้ป่วยเบาหวานตามความรุนแรงของเบาหวานขึ้นจอตา และตามระดับการควบคุมน้ำตาลสะสมในเลือด โดยวิธี NGS ซึ่งผลการศึกษาแสดงให้เห็นถึงผลของการมีภาวะเบาหวานขึ้นจอตาและการควบคุมระดับน้ำตาลในเลือดต่อไมโครไบโอมของเยื่อบุตา

สาขาวิชา เวชศาสตร์คลินิก ลายมือชื่อนิสิต .....

ปีการศึกษา 2564 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORD: Ocular surface microbiome, Conjunctival microbiome, Diabetes mellitus, Diabetic retinopathy

Orathai Suwajanakorn : Ocular Surface Microbiome in Diabetes Mellitus. Advisor: TANITTHA CHATSUWAN, Ph.D.

This cross-sectional, age and gender matched study included 20 eyes of non-diabetic subjects (non-DM group) and 60 eyes of type 2 diabetes mellitus (DM group). Subgroups of DM were classified by diabetic retinopathy (DR) staging into no DR (DM-no DR), non-proliferative DR (DM-NPDR), proliferative DR (DM-PDR), and by glycemic control (well-controlled DM; HbA1c<7% , poorly-controlled DM; HbA1c≥ 7%). Conjunctival swabs were performed for ocular surface microbiome analysis using conventional culture and next-generation sequencing analysis (NGS). A higher culture-positive rate was found in DM (15%) than in non-DM group (5%) (p-value= 0.437). Antibiotic-resistant organisms were only detected in the DR groups (DM-NPDR and DM-PDR). The NGS analysis showed that potentially pathogenic bacteria predominated in DM, especially in DR. There was dissimilarity in the ocular surface microbiome between DM and non-DM groups. The subgroup analysis showed that the DR group had significantly different microbial community from DM-no DR and non-DM groups (p-value<0.05). The microbial community in the poorly-controlled DM was also significantly different from well-controlled DM and non-DM groups (p<0.001). Using the NGS method, this study is the first to signify the importance of DR and glycemic control status, which affect the changes in the ocular surface microbiome.

Field of Study: Clinical Sciences Student's Signature .....

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Orathai Suwajanakorn

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Abbreviations

ASVs	Amplicon sequence variants
BCVA	Best corrected visual acuity
BMI	Body mass index
DM	Diabetes mellitus
DM-no DR	Diabetes mellitus with no diabetic retinopathy
DM-NPDR	Diabetes mellitus with non-proliferative diabetic retinopathy
DM- PDR	Diabetes mellitus with proliferative diabetic retinopathy
DNA	Deoxyribonucleic acid
DR	Diabetic retinopathy
HbA1c	Hemoglobin A1c
IOP	Intraocular pressure
NGS	Next-generation sequencing analysis
Non-DM	Non-diabetes mellitus
rRNA	ribosomal ribonucleic acid
TBUT	Tear film break up time

CHAPTER I

Introduction

The microbiome, which has long been recognized, was first defined by Whipps and colleagues in 1988.[1] The concept that a single microorganism could cause the disease in the 17th century has changed with advancing technology and knowledge.[2] Nowadays, microorganisms are found to be a part of complex community that interact and communicate with others and have a significant impact on human health.[2] Microbiome provide the metabolic, immunologic, protective functions to maintain healthy microenvironment and preserve normal functions of the organ. [3-6] Similar to other organs, the microbiome play a vital role in maintaining the homeostasis of the ocular surface environment, and alteration of microbiome composition (dysbiosis) in many ocular surface diseases can lead to infection and inflammation.[3,4,6] Thus, understanding the physiology and pathogenesis of microbiome in the ocular surface should widen our knowledge and bring forth new treatment modalities.[7]

The development of advancing technology using the molecular sequencing method such as next-generation sequencing (NGS) has expanded the knowledge of microbiome study. Generating a million reads per run, this high throughput method can reduce the cost and analytic time.[3,8,9] Since the majority of the human microbiome is bacteria, the study of the human microbiome has focused on 16S rRNA sequencing by the NGS method.[3]

Diabetes mellitus (DM), a metabolic disorder characterized by disturbance of glucose metabolism and chronic hyperglycemia, is a major threat to global health. The prevalence of DM is increased rapidly over the past few decades[10] and is estimated to rise from 463 million in 2019 to 700 million in 2045 worldwide.[11] In Thailand, the estimated number of DM may reach 4.3 million in 2035.[12] DM is a significant risk factor

for multiple organ dysfunction, including cardiovascular, kidney, and eye.[12] The most common eye complication in DM is diabetic retinopathy (DR). This retinal vascular complication is one of the leading causes of blindness worldwide[12] and the second most common diabetic complication in Thailand.[13] Other complications are cataract, glaucoma, and ocular surface changes.[14,15]

Previous studies showed that ocular surface changes were frequently associated with type 2 DM and proportional related to the severity of DR and HbA1c level.[14,16-18] Increased risk of infection such as conjunctivitis, corneal ulcer, and endophthalmitis[19-23] are found in diabetic patients due to alteration of protective immune response, including decreased cytokine production and impaired immune cellular functions.[24-26] Alteration of immune function together with ocular surface changes may have an influence on the ocular surface microenvironment and microbiome. Moreover, the treatment modalities of DR, especially in the advanced stages of the disease, usually involve intraocular management such as intravitreal injection or intraocular surgery, which may introduce the ocular surface microbes into the eye and result in intraocular infection.[22,27] The study of the microbiome in diabetic patients, especially DR, may provide special considerations for managing these patients in the future.

Although many studies reported the changes in the ocular surface microbiome in DM, especially in DR, most used the culture technique.[28-32] Only a few studies applied the NGS method, which were reported from Korea and China.[33-36] Additionally, other factors such as ethnicity, climate, and geographic location may affect the variation, density, and core microbiome.[37]

To the best of author's knowledge, there is no report on ocular surface microbiome in DM in South East Asia and ocular surface microbiome data in DM according to the stage of DR and glycemic control in the molecular biological method. Therefore, the purpose of this study is to identify the difference of ocular surface microbiome between diabetic (DM) and non-diabetic subjects (non-DM) using the NGS method in Thai population and to determine whether the staging of DR and glycemic control affect the ocular microbiome differently.

Research questions

Primary research question

Is there any difference in ocular microbiome between diabetes mellitus and non-diabetic subjects in Thai population?

Secondary research questions

1. Does the stage of diabetic retinopathy affect the ocular surface microbiome differently?
2. Does HbA1c level affect the ocular surface microbiome?
3. Does the stage of diabetic retinopathy increase microbial antibiotic resistance?

Research objectives

Primary objective

To evaluate the ocular surface microbiome in diabetes mellitus patients compared with healthy control using the NGS method

Secondary objectives

1. To evaluate the ocular surface microbiome in diabetes mellitus subjects according to the stage of diabetic retinopathy
2. To evaluate the ocular surface microbiome in diabetes mellitus subjects according to HbA1c level
3. To evaluate antibiotic sensitivity of the identified organisms

Conceptual framework

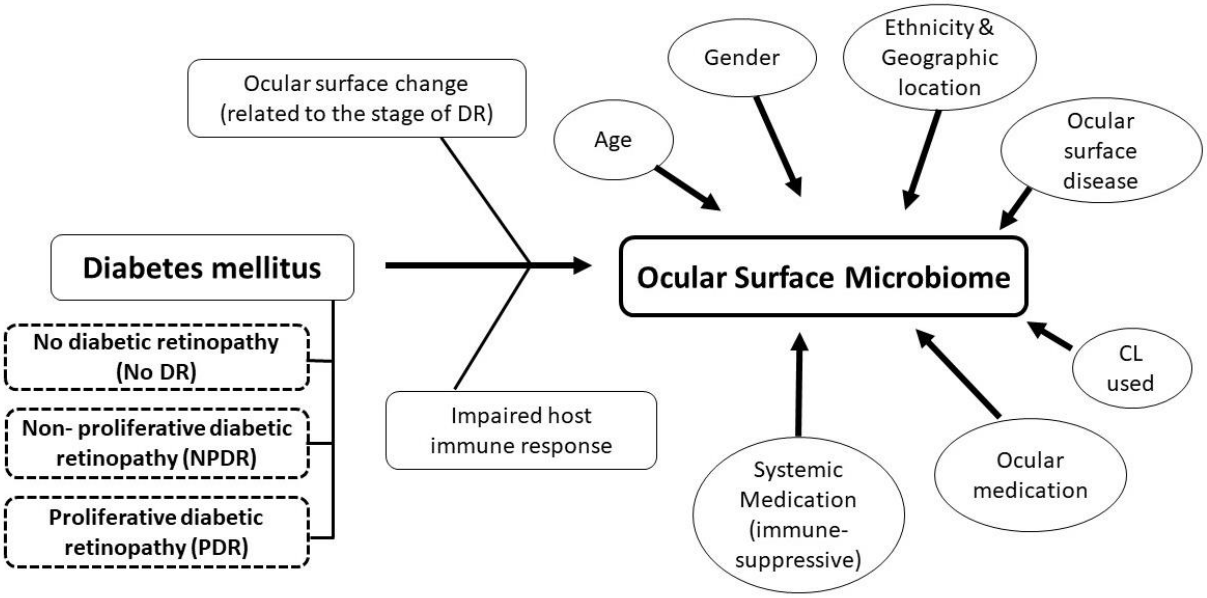

Figure 1 Conceptual framework

CHAPTER II

Literature review

1. Microbiome

The human microbiome project, launched in 2007, initially studied microbiome in the skin, oral, nasal, gastrointestinal, and urogenital systems.[38,39] Later studies expanded the scope of interest through other parts of the body, including the ocular surface.[37] These complex community of microorganisms affects the metabolic, immunologic, and protective functions in their resident organs to maintain a healthy microenvironment and preserve the normal function of those organs.[3-6] Imbalance of the microbiome composition (dysbiosis) may lead to the disease or prone to the disease state.[3,4,6]

Although the conventional method using culture technique is the gold standard for detecting microorganisms and antimicrobial susceptibility, it can determine only the live microorganisms. The major obstacles to this conventional technique are the detection of slow-growing, fastidious organisms or the limited amount of clinical specimens. Therefore, the culture method is not ideal for discovering the microbiome, which consists of a complex variety of microorganisms.[40]

The significant progression in microbiome study happens due to the development of molecular sequencing methods.[3] One of the molecular biological methods for identification and characterization of the microbiome is the targeted metagenomics sequencing, a DNA sequencing technique that focuses on a specific region, such as the 16S rRNA gene for bacteria and the 18S rRNA gene for fungus.[3] There are two common methods for targeted metagenomics sequencing, which are Sanger method and next-generation sequencing method (NGS). The Sanger sequencing method is capillary-based sequencing by using capillary electrophoresis. Though the

technique is very accurate, it can generate only one read per run, which is time-consuming and expensive.[3,9] Advanced technology using the NGS method has been developed to overcome these limitations. The technology is high throughout, which can generate a million reads per run, thus can reduce the cost and analytic times.[3,8,9] Since the majority of the human microbiome is bacteria, the application of the NGS technique focuses on 16S rRNA gene sequencing.[3]

2. Ocular surface microbiome

Recently, many studies have been focusing on ocular surface microbiome in healthy and in many diseases. Heleen D. et al. reviewed fourteen articles of ocular surface microbiome in healthy subjects and found that the core ocular surface microbiome were Actinobacteria (*Corynebaterium, Cutibacterium*), Proteobacteria (*Pseudomonas, Acinetoobacter*), and Firmicutes (*Staphylococcus, Streptococcus*)[37] Despite *Corynebacterium*, which was found in all studies [37], microbiome data of the ocular surface varied. Many studies demonstrated that age, gender, ethnicity, and geographic location might affect the ocular microbiome.[37,41-43] Children younger than 10 years old had more bacterial diversity and abundance of *Streptococcus*, and *Staphylococcus*, but less abundance of *Corynebacteruim*, *Cutibacterium*, and *Paracoccus* than the older population.[37,41] While the adult aged older than 60 years old significantly had more amount of bacteria than aged younger than 30 years old.[42] The effect of genders on the ocular surface microbiome is controversy.[37] Several studies found no significant impact,[41,42,44], while others found substantial differences in both bacterial components and diversity.[45,46] Doan T et al. reported the significant effects of age and specimen collection sites on the microbiome, in which lower conjunctiva had more bacterial composition than the upper but no significant impact on gender and eye laterality.[42] Regarding the regions, reports from Asian countries[33,46,47]

and USA[42] showed similar top 2 most common phyla of the ocular surface microbiome, which were Proteobacteria and Actinobacteria, whereas the report from Australia were Proteobacteria and Firmicutes.[48]

Several ocular surface diseases can also alter microenvironment and microbiome. Li Z. et al. found that the ocular surface microbiome in dry eye patients had lower diversity and more prominent of Bacteroidia and Bacteroidetes.[49] Microbiome composition was more similar to skin microbiome and had more prominent amount of Proteobacteria in contact lens wearers.[37,45,46] Apart from core microbiome changes in patients with allergic conjunctivitis, the microbial diversity was inversely proportional to its severity.[50] Recent study showed more diversity in chronic Stevens-Johnson syndrome compared to healthy subjects.[51]

3. Diabetes mellitus

Diabetes mellitus (DM) is a chronic metabolic disorder that impairs glucose metabolism and results in chronic hyperglycemia. The prolonged increase in blood glucose affects multiple organs throughout the body, commonly categorized as macrovasculopathy and microvasculopathy, eventually resulting in significant morbidity and mortality. Macrovasculopathy mainly involves cardiovascular and cerebrovascular systems and is the leading cause of death in DM patients. Additionally, microvasculopathy can result in chronic kidney disease, neuropathy, and diabetic retinopathy (DR), a significant cause of visual loss in DM patients.[10,12] Not only the most common ocular complication like DR, but DM can also cause cataracts, glaucoma, and ocular surface changes.[14,15]

The incidence of diabetic retinopathy is correlated with the duration of DM and glycemic control.[15] Chronic hyperglycemia is believed to be the initial cascade of the

alteration in the biochemical and physiologic function that leads to microvasculopathy and retinal pathology.[52] The hyperglycemic state affects several metabolic pathways, including the formation of advanced glycation end products (AGES), chronic inflammation, and increased oxidative stress resulting in vascular endothelial change and upregulating vascular endothelial growth factor (VEGF).[15,52] In diabetic retinopathy, the retinal vascular changes, including vascular endothelial dysfunction and increased vascular permeability, result in the clinical findings of retinal hemorrhage and exudate. In more advanced stages of diabetic retinopathy, the presence of macular edema (diabetic macular edema; DME), neovascularization (in proliferative diabetic retinopathy; PDR), vitreous hemorrhage, fibrous traction, and tractional retinal detachment can lead to significant visual impairment and usually require the management of intravitreal drug injection or intraocular surgeries.[53] These procedures may introduce the ocular surface microbes into the eye and result in intraocular infection.[22,27] The previous reports showed more bacterial colonization and presence of gram-negative bacteria on the ocular surface of the DM than in the non-diabetic (non-DM) groups.[28-32] Consistently, the most common organisms causing postoperative endophthalmitis reported in patients with DM were coagulase-negative *Staphylococcus* and gram-negative bacteria which correlates with conjunctival flora of the DM.[22,27,54]

4. Alteration of the ocular surface in diabetes mellitus

The formation of advanced glycation end products (AGEs) in the hyperglycemic state also affects the ocular surface by reducing proliferation and enhancing apoptosis of epithelial cells, inducing inflammation and oxidative stress in sub-basal nerve plexus.[55,56] Therefore, ocular surfaces in patients with DM may have increased conjunctival metaplasia, decreased conjunctival goblet cell density, decreased sub-

basal nerve density, decreased corneal sensitivity, compromised epithelium, and delayed wound healing.[55,56]

Sato E. et al. used fluorophotometry to detect corneal AGEs level in diabetic patients and reported that AGEs fluorescence level was significantly increased in patients with proliferative diabetic retinopathy (PDR) compared to the non-proliferative diabetic retinopathy (NPDR), no diabetic retinopathy (no DR) and non-diabetic patients (non-DM).[57] The increased corneal AGEs level may result from the rising of AGEs levels in aqueous and vitreous, especially in PDR, due to compromised blood-retinal barrier.[57,58]

Previous studies revealed that ocular surface changes were more commonly associated with type 2 DM and proportional to the severity of diabetic retinopathy and HbA1c level.[14,16-18] The meta-analysis found that tear function and corneal sensitivity were decreased in DM patients, especially in the PDR group.[59] A significant decrease in cell density and abnormal morphology of cornea epithelium were also found in DM patients, especially PDR.[14] Gekka M et al. reported the impaired cornea epithelial permeability function was correlated with the higher HbA1c level.[16]

Increased risk of infection are consistently reported in DM patients. Ansari AS et al. reported an increased incidence of conjunctivitis in type2 DM than in non-DM patients.[20] Chang YS et al. reported that diabetic patients had 1.3 times higher risk of corneal ulcer than non-DM.[19] Postoperative endophthalmitis was also commonly found in DM.[21-23,27,54] Moreover, endophthalmitis in diabetic patients was associated with a poorer visual outcome. Doft BH et al. reported that the final visual acuity (VA) after the endophthalmitis treatment was 39% in DM achieved VA 20/40, compared to 55% in the non-DM group.[60]

Increased risk of infection in DM patients due to alteration of protective immune response, including decreased cytokine production and impaired immune cell functions.[24-26] The alteration of immune functions together with ocular surface changes may influence the ocular surface microenvironment and microbiome and thus predispose diabetic patients to develop ocular infections.

5. Diabetes mellitus and microbiome

The association of microbiome and DM has been remarked by many reports.[61-63] Tanase DM et al. proposed the causal relationship of metabolic syndrome and gut microbiome dysbiosis,[61] which the alteration of bacterial components such as Bacteroidetes-to-Firmicutes ratio may be associated with blood sugar level.[62] While *Bifidobacterium sp.*, the bacteria with anti-inflammatory properties, was decreased, the pro-inflammatory bacteria, including *Escherichia* and *Enterobacter spp.*, were increased in diabetic patients compared with the healthy controls.[64,65] There was also a possible association between gut microbiome and DR. Das T. et al. demonstrated that DM patients had decreased gut probiotic bacteria and increased pro-inflammatory bacteria such as Shigella, and the magnitudes were more pronounced in DM patients with DR.[65]

Multiple ocular surface alterations in DM also play significant roles in microbiome changes. Higher positive culture rates of the ocular surface microbiome using different culture methods were reported in DM patients compared to the healthy controls.[28-32] Furthermore, a higher rate of isolated organisms was seen in DM patients with DR than in those without.[28,29] In general, the most common isolated organisms from ocular surface microbiome were *S. epidermidis* and *S. aureus*. However, gram-negative bacteria such as *Klebsiella* sp. was not uncommon in DM patients.[30,31]

Although many studies reported the changes in the ocular surface microbiome in DM, most used the conventional culture method.[28-32] Only a limited number of studies reported the results from the NGS.[33-36] Various factors such as ethnicity and geographic location may affect the variation, density, and core microbiome.[37] Currently, there were four reports using NGS to study the ocular surface microbiome in DM patients.[33-36] Previous studies showed higher alpha-diversity of the ocular surface microbiome in DM compared with non-DM, and beta-diversity showed significant differences in ocular surface microbiome composition between DM and non-DM.[33-36] In DM with dry eye, the alpha-diversity was higher than in DM only, dry eye only, and control.[35] Top four most common bacterial phyla found in both non-DM and DM were Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria.[34-36] The study from Korea reported the predominance of Proteobacteria, mainly *Acinetobacter*, *Burkholderia, Sphingomonas,* and *Ralstonia* in the DM group, compared to Firmicutes in the non-DM group.[33] The other study from China showed the increased abundance of Bacteroidetes and decreased abundance of Proteobacteria in the DM group.[34]

The knowledge of microbiome brings a paradigm shift in the new treatment concept such as probiotic, prebiotic, or bacterial transplantation. In the ophthalmologic field, there were reports of beneficial effects of probiotics in allergic conjunctivitis and dry eye disease.[66,67] To better understand the ocular surface microbiome and its possible relationship with DM, this study aims to find a difference of ocular surface microbiome in DM subjects classified by DR staging and glycemic control and non-DM subjects.

CHAPTER III

Materials and Methods

1. Research designs

A cross-sectional, observational analytic study

2. Population

Subjects with diabetes mellitus and with non-diabetes mellitus

Target Population

Diabetic and non-diabetic subjects who visited the out-patient clinic of the Department of Ophthalmology, King Chulalongkorn Memorial Hospital

3. Recruitment and sampling techniques

The subjects were recruited from the patients who visited the out-patient clinic of the ophthalmology department, King Chulalongkorn Memorial Hospital. The poster advertisements were used to recruit the patients for the study. This study used a convenience sampling technique by recruiting consecutive cases.

4. Inclusion criteria

1. Subjects older than 18 years old.
2. **Diabetes mellitus (DM group)**: Subjects who were diagnosed with type 2 diabetes mellitus according to the ADA (American Diabetes Association) diagnostic criteria[68] or those with diabetes mellitus with ongoing treatments by the physicians.

   The study focused on type 2 diabetes mellitus, which is the most common type and usually occurs during adulthood.[10]
3. **Non-diabetes mellitus (non-DM group):** Subjects who had no diabetes mellitus with documented normal blood sugar or HbA1c screening within 1 year.

5. Exclusion criteria

1. The presence of ocular surface diseases (For example, significant dry eyes, meibomian gland dysfunction, or Stevens-Johnson syndrome)
2. History of ocular and periocular infection/inflammation, allergic conjunctivitis within 3 months
3. History of previous contact lens use within 3 months
4. History of any ophthalmic medication use (except for non-preservative artificial tear) within 3 months (such as topical antibiotics, topical steroids, intravitreal drug injections, etc.)
5. History of ocular surgery within the past 3 months
6. History of systemic antibiotics, steroids, or immunosuppressive drugs used within the past 3 months
7. Health care workers

If both eyes met the criteria, only the right eye was included.

6. Diagnostic Criteria

Diabetes mellitus (DM) group

- Diagnosis of type 2 Diabetes mellitus according to the ADA (American Diabetes Association) diagnostic criteria[68]
    - Fasting blood sugar of greater than or equal to 126 mg/dl or
    - 2-hour blood sugar of greater than or equal to 200 mg/dl or
    - Random plasma glucose test greater than or equal to 200 mg/dl or
    - HbA1c greater than or equal to 6.5% or
    - Known history of diabetes mellitus for which the patients were under physician's care.
- DM group were divided into 3 subcategories by DR staging: according to the International Clinical Disease Severity Scale for Diabetic retinopathy[69]

1. No diabetic retinopathy (DM-no DR)
2. Non-proliferation diabetic retinopathy (DM-NPDR); including mild NPDR, moderate NPDR, and severe NPDR
3. Proliferative diabetic retinopathy (DM-PDR)

- DM group were divided into 2 subcategories by glycemic control (HbA1c level)
  1. HbA1c < 7%: well-controlled DM
  2. HbA1c ≥ 7% : poorly-controlled DM

7. Sample size calculation

Sample size calculation used two independent mean formula, with standard deviation 0.09 *(*adopted from the standard deviation of predominant bacterial phylum *(*Proteobacteria*)* of DM and non-DM group in the previous study.[34] The expert opinions suggested that mean clinical difference of 0.08 is considered significant.

$$n_1 = \frac{(z_{1-\frac{\alpha}{2}} + z_{1-\beta})^2 \left[\sigma_1^2 + \frac{\sigma_2^2}{r}\right]}{\Delta^2}$$

$$r = \frac{n_2}{n_1}, \Delta = \mu_1 - \mu_2$$

Figure 2 Sample size calculation

Ratio (r) = 3.00

Alpha (α) = 0.05, Z(0.975) = 1.959964

Beta (β) = 0.200, Z(0.800) = 0.841621

Sample size: Group1 (n_1) = 16, Group2 (n_2) = 48

The 1: 3 ratios of non-DM to DM group were applied as this study focused on the data from DM subjects. Adding 20% adjusted for data error, the sample size of

the non-DM group was 20 eyes, and DM group was 60 eyes. In the DM group, the subjects were divided into 3 subcategories which were no diabetic retinopathy (DM-no DR), non-proliferative diabetic retinopathy (DM-NPDR), and proliferative diabetic retinopathy (DM-PDR) with ratio 1:1:1 (20 eyes/group). If both eyes were eligible, only right eye was included. Overall, 80 eyes were enrolled in this study (20 eyes/group).

8. Methodology

This study was conducted in accordance with the tenets of the Declaration of Helsinki, and the protocol was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University. The study site was at the Department of Ophthalmology, King Chulalongkorn Memorial Hospital. The subjects who met the inclusions and exclusions criteria were provided with all the study information, including risks and benefits. The voluntary informed consent must be obtained before the enrolment. The subjects were divided into two main groups: the non-DM and DM group. The DM group was divided into DM-no DR, DM-NPDR, and DM-PDR groups. All groups were age- and gender-matched by frequency matching within 5 years to minimize confounders. Data of demographics and the Ocular Surface Disease Index (OSDI) questionnaire were collected together with complete ophthalmic examinations. The conjunctival swab was performed before any examinations contacting the ocular surface and the instillation of any drugs, except for topical anesthesia. All of the conjunctival swab, sample culture, DNA extraction, and 16S rRNA gene sequencing processes were done by masked investigators.

8.1 Data Collection

8.1.1 Demographic data

- Baseline characteristics: gender, age, geographic location, occupation, underlying medical condition, body mass index (BMI)
- Current medication use, history of allergy

Diabetic group:

- Duration of diabetes mellitus
- Fasting blood sugar (FBS) and Hemoglobin A1c (HbA1c) results within 3 months
- Diabetic complications

8.1.2 Ophthalmologic data

- Laterality of eye
- Previous ocular surgery
- History of ocular medications
- History of contact lenses used
- Ocular surface disease index (OSDI) questionnaire
- Complete eye examinations, including visual acuity, intraocular pressure (IOP), fluorescein tear break up time (TBUT), ocular staining (using Oxford staining score), lens status, DR staging, and thorough fundus examinations.

8.1.3 Slit-lamp examinations

1. The investigator, as the ophthalmologist, performed complete ophthalmic examinations by evaluating skin, eyelid disease, meibomian gland, ocular surface, anterior chamber, and lens.
2. The conjunctival swab was performed after the topical anesthesia was applied. (described below).
3. Ocular surface staining using fluorescein strip.

4. The sterile topical 1% Tropicamide (Mydriacyl®) eye drop 1 drop was administered to the inferior conjunctival fornix of the selected eye every 15 min until the pupil was fully dilated.
5. The fundoscopic examination was done by slit-lamp examination with a 90D lens to evaluate any retinal abnormalities and diabetic retinopathy staging.

8.1.4 Sample collection (Conjunctival swab)

The conjunctival swab was done in a clean examination room. Before the procedure, the subjects were required to wear a mask. Likewise, the investigator must wear a mask and sterile gloves. During the sample collection, the investigator and subjects were advised not to talk. All conjunctival swabs were done by the same investigator.

1. One drop of the sterile topical anesthesia drug (using 0.5% Tetracaine Hydrochloride eye drop solution, Alcon®, which is the only available topical anesthesia eye drop in Thailand) was administered to the inferior conjunctival fornix of the selected eye. Then, subjects were advised to close their eyes for 3 minutes.
    - The investigators respected the ethics of research involving human subjects. Topical anesthesia was instilled before the procedure to minimize patients' discomfort and blinking reflexes that may result in inadequate or contaminated sample collection.
    - Though topical anesthesia may interfere with the detection of microorganisms, the procedure was widely accepted in standard practice in several previous studies.[31,34,70]
2. The lower eyelid was pulled down to avoid touching the lid margin or eyelashes during specimen collection.

3. Sterile cotton swabs were swept from nasal to temporal part of inferior conjunctival surface 5 times without touching the eyelid or eyelashes (rotating the sterile cotton swab for each time for 360 degrees of swab collection).
4. The swab was placed in phosphate buffer saline (PBS) as a sterile transport medium (containing 1 ml).

For the sample preparation, the specimen and the sterile transport media were mixed using the vortex mixture (Vortex Genie 2) for 10 seconds. Then, the sterile pipette was used to transfer the sample onto the chocolate agar plate for 1 drop (approximately 0.1 ml). The sterile loop was used to streak the sample on the culture plate. Finally, the residual specimen was immediately transported in the ice pack in order to preserve the specimen for further DNA extraction. The processes of culture and DNA extraction were done at the Department of Microbiology, Faculty of Medicine, King Chulalongkorn Memorial Hospital.

8.2 Culture method:

Chocolate agar plates were incubated in a CO_2 incubator at 37°C, 5% CO_2, and checked daily for bacterial growth for a week. Bacterial identification was determined by automated identification system (VITEK®2XL) and mass spectrometry microbial identification system (VITEK®MS, USA).

The antibiotic susceptibility test was determined by using the Kirby-Bauer disk diffusion method and the VITEK®2XL system. The antibiotic included benzylpenicillin, ampicillin, oxacillin, piperacillin/tazobactam, cefazolin, cefuroxime, cefuroxime axetil, ceftriaxone, cefepime, ertapenem, imipenem, meropenem, amikacin, gentamycin, ofloxacin, ciprofloxacin, levofloxacin, moxifloxacin, erythromycin, clindamycin, chloramphenicol, linezolid, teicoplanin, vancomycin, tetracycline, tigecycline, fusidic

acid, rifampicin, and trimethoprim/sulfamethoxazole. The results were interpreted according to the Clinical Laboratory Standard Institute (CLSI) guideline.

Only chocolate agar was selected for the culture due to the limited amount of the specimen. However, most of the ocular surface organisms can grow in this medium, except for anaerobic bacteria, which only be detected by the NGS method.

8.3 DNA extraction

After the specimens were sent to the laboratory of the Department of Microbiology, Faculty of Medicine, Chulalongkorn University, the specimens were immediately processed for DNA extraction or were kept at -20°C for further DNA extraction within 1 week. The DNA extraction was performed using the QIAamp® DNA Microbiome kit (QIAGEN®, USA) using the following protocol.

1. A 500 uL Buffer AHL was added to 1 mL of sample in a 2-mL tube and incubated for 30 minutes at room temperature with an end-over-end rotator.
2. The tube was centrifuged at 10,000xg for 10 minutes, and the supernatant was discarded.
3. A 190 uL of Buffer and 2.5 uL of Benzonase were added and mixed well before being incubated at 37°C for 30 minutes at 600 rpm in a heating block or water bath.
4. A 20 uL Proteinase K was added and incubated at 56°C for 30 minutes at 600 rpm in a heating block or water bath. Then, the tube was briefly spun at a slow speed.
5. A 200 uL Buffer ATL (containing Reagent DX) was added and mixed well before being transferred into Pathogen Lysis Tube L. The Pathogen Lysis Tube L was then placed into a FastPrep®-24 instrument, with the application of a velocity of 6.5 m/s twice for 45 seconds with a 5-minute intermission while the sample was placed on ice.

6. The Pathogen Lysis Tube L was centrifuged at 10,000xg for 1 minute, and the supernatant was transferred into a new microcentrifuge tube. A 40 ul of Proteinase K was added and then vortexed to mix before being incubated at 56°C for 30 minutes at 6oo rpm in a heating block or water bath.
7. A 200 uL Buffer APL2 was added and mixed by pulse-vortexing for 30 seconds. Then, the sample was incubated at 70°C for 10 minutes, and the tube was briefly spun.
8. A 200 uL ethanol was added to the lysate and mixed by a pulse-vortexing for 15-30 seconds. Next, a 700 uL of this mixture was applied to the QIAamp UCP Mini spin column without wetting the rim. The cap was closed properly before being centrifuged at 6,000xg for 1 minute. Next, the flow-through (containing Buffer APL2 or Buffer AW1) was discarded, and the column was put back into the same collection tube. This step was repeated with any remaining ethanol-lysate mixture.
9. The QIAamp UCP Mini Spin column was transferred to a new collecting tube. A 500 uL Buffer AW1 was added without wetting the rim. The tube was centrifuged at 6,000xg for 1 minute. Then, the QIAamp UCP Mini spin column was placed into a fresh 2-mL collection tube, and the filtrate was discarded.
10. A 500 uL Buffer AW2 was added into the QIAamp UCP Mini spin column without wetting the rim. The column was centrifuged at 20,000xg for 3 minutes.
11. The QIAamp UCP Mini spin column was placed into a fresh 2-mL collection tube before being centrifuged at 20,000xg for 1 minute. The filtrate was discarded.
12. The QIAamp UCP Mini spin column was placed into a fresh 1.5-mL tube, and a 50 uL Buffer AVE was applied directly onto the center of the membrane. The tube was incubated at room temperature for 5 minutes before being centrifuged at 6,000xg for 1 minute to elute the DNA.

After DNA extraction, the specimens were preferred to be immediately sent to Omics Sciences and Bioinformatics Center, Chulalongkorn University, for 16S rRNA

sequencing by the next-generation sequencing method using Illumina MiSeq (Illumina, CA, USA). If the specimens could not be sent immediately, they were kept at -20°C with no longer than 1 week.

8.4 Next-generation sequencing method (NGS)

DNA was subjected to 16S metagenomic sequencing library preparation. The 16S rRNA gene was amplified using 341F and 805R primers, targeting V3-V4 variable regions, and 2X sparQ HiFi PCR Master Mix (QuantaBio®, USA) (Figure 1). The PCR amplification included an initial denaturation step of 2 minutes at 98°C, followed by 30 cycles of 98 °C for 20 seconds, 55 °C for 30 seconds, and 72 °C for 1 minute, followed by a final extension step at 72 °C for 1 minute for one time. Subsequently, 16S amplicons were purified using sparQ Puremag Beads (QuantaBio®, USA) and indexed using 2.5 µL of each Nextera XT index primer in a 25 µl PCR reaction, followed by 10 cycles of PCR amplification condition above. The final PCR products were cleaned, pooled, and diluted to the final loading concentration at 4 pM. Cluster generation and 250-bp paired-end read sequencing were performed on an Illumina MiSeq at The Omics Sciences and Bioinformatics Center (Chulalongkorn University, Bangkok, Thailand). The processes of 16S metagenomic sequencing are demonstrated in Figure 3.

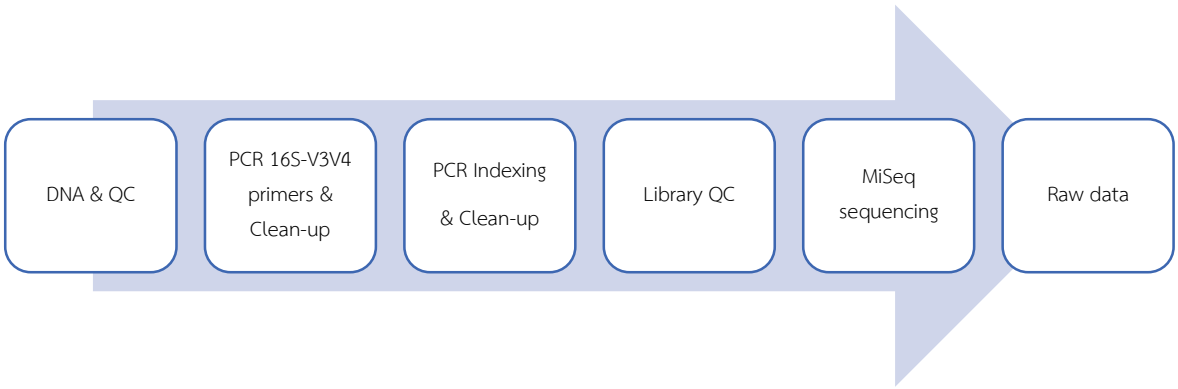

Figure 3 The processes of 16S metagenomic sequencing

16S V3-V4 Amplicon primers

Forward Primer =

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

Reverse Primer =

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

****Underline sequences are overhang adapter sequences that are trimmed automatically**

****Blue bold sequences are 16S V3V4 region**

8.5 Bioinformatics analyses

The raw sequences were categorized into groups based on the 5' barcode sequences. The sequences were processed following the DADA2 v1.16.0 pipeline (URL: https://benjjneb.github.io/dada2/). The microbial diversity and community structures using unique amplicon sequence variants (ASVs) were described by the DADA2 pipeline.[71] Microbial taxa were classified from Silva version 138 as a reference database.[72] Alpha diversity index (observed ASVs, Chao1 richness, Shannon, and PD

whole tree) was computed using DADA2 software. For Bata diversity, unweighted UniFrac distances were analyzed using box plot and principal coordinate analysis (PCoA) from Phyloseq data. Linear discriminant analysis effect size (LEfSe) was performed to identify the bacterial biomarkers.

Figure 4 The processes of bioinformatics analyses

Diversity and Taxonomic analysis [3,73]

1. **Alpha diversity**: Comparison of the microbial community within the sample using observed ASVs, Chao1 richness, Shannon, and PD whole tree
2. **Beta diversity**: Principal coordinate analysis (PCoA) and comparison of microbial community between sample using and box plot by unweighted UniFrac distances
3. **Taxonomic microbial composition**: Relative abundance of the species–composition of an organism relative to total organisms in the site (described in percentage), and core microbiome

8.6 Negative control

Negative control was derived from the sterile cotton swab placed in transport media without swabbing (blank swab), and the media were sent for culture and NGS analysis using the same protocol and at the same period of the sample collection.

After the culture and DNA extraction were performed, there were no detected bacteria in the negative control.

8.7 Data and Statistical analysis

Demographic data were analyzed using descriptive statistics. The Chi-square test was used for comparing categorical data variables between groups. Independent t-test or Mann-Whitney U test was performed for comparing continuous variables between two groups, while analysis of variance (ANOVA) or Kruskal-Wallis test was used for comparing more than two groups. Pairwise comparison of alpha diversity (observed ASVs, Chao1, Shannon, and phylogenetic diversity (PD) whole tree) was calculated using the Kruskal-Wallis test. Permutational multivariate analysis of variance (PERMANOVA) was performed to evaluate the differences in beta diversity among groups. Moreover, the Kruskal-Wallis sum-rank test was also used in LEfSe analysis to identify bacterial biomarkers that differed significantly in abundant taxon between sample groups. A p-value less than 0.05 was considered statistically significant. The data were processed by STATA version 15 and R version 4.0.4.

8.8 Ethical Consideration

The study was conducted in accordance with the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University (COA No.704/2021). In addition, the study was registered to the Thai Clinical Trial Registration (TCTR No. TCTR20210427010). This study strictly follows the ethical principles for research involving human subjects.

CHAPTER IV

Results

A total of 80 eyes (80 subjects) included 60 eyes of diabetes mellitus (DM group) and 20 eyes of non-diabetic control (non-DM group). The DM group was classified into 3 subgroups according to the diabetic retinopathy (DR) staging as no diabetic retinopathy (DM-no DR), non-proliferative diabetic retinopathy (DM-NPDR), and proliferative diabetic retinopathy (DM-PDR group) (20 eyes/group). The mean age of all subjects was 55.6 years old (range 37-79 years old), and 50% were male. Most of the subjects were office workers who resided in central areas of Thailand. The DM subjects were also classified by glycemic control into 2 subgroups, well-controlled DM (HbA1c <7%) (40%) and poorly-controlled DM (HbA1c ≥7%) (60%). The duration of DM ranges from 4 months to 36 years. Among subjects with DR (DM-NPDR and DM-PDR), 13 eyes had diabetic macular edema, and 12 eyes had vitreous hemorrhage. Demographics and clinical details are shown in Table 1.

1. Culture

This study revealed a higher rate of culture-positive in the DM group (15%) compared with the non-DM group (5%), without statistically significant difference (p-value = 0.437). There was no significant difference in culture-positive rate among non-DM and the three DM subgroups (p-value= 0.748). Seven organisms were identified. Most of the microorganisms identified were gram-positive cocci, which *Staphylococcus epidermidis* being the most common. *Kocuria palustris* and *Micrococcus luteus* were identified from the DM-PDR group. In addition, gram-negative bacilli, *Providencia rettgeri, was* isolated from the DM-NPDR group. There was no significant difference in the culture-positive rate between well-controlled and poorly-controlled DM subgroups (16.7% and 13.9 %, respectively, p-value = 0.571)

Table 1 Demographics data & baseline clinical characteristics of non-DM and DM groups

		Non-DM group N =20	DM group				p-value
			Total DM N = 60	DM-no DR N = 20	DM-NPDR N = 20	DM-PDR n= 20	
Age (yrs), mean (SD)		55.75 (9.54)	55.55 (8.93)	56.10 (8.81)	55.70 (9.15)	54.85 (9.24)	NA
Gender	Male	10 (50%)	30 (50%)	10 (50%)	10 (50%)	10 (50%)	NA
Laterality	RE	8 (40%)	32 (53.33%)	13 (65 %)	7 (35 %)	12 (60 %)	0.3†
Occupation	Office worker	9 (455)	18 (30%)	11 (55%)	6 (30%)	1 (5%)	0.67†
	General employee	5 (25%)	12 (20%)	4 (20%)	2 (10%)	6 (30%)	
	Business owner	3 (15%)	14 (23.33%)	2 (10%)	5 (25%)	7 (35%)	
	Farmer	1 (5%)	4 (6.67%)	0 (0%)	1 (5%)	3 (15%)	
	Others	2 (10%)	12 (20$)	3 (15%)	6 (30%)	3 (15%)	
Geographic location	Central	18 (90%)	43 (71.67%)	17 (85%)	15 (75%)	11 (55%)	0.52†
	East	1 (5%)	10 (16.67%)	1 (5%)	4 (20%)	5 (25%)	
	Others	1 (5%)	7 (11.67%)	2 (10%)	1 (5%)	4 (20%)	
BMI, mean (SD)		23.45 (3.15)	26.87 (4.25)	28.84 (5.20)	25.66 (3.26)	26.11 (3.49)	0.001†
BCVA logMAR, mean (SD)		0.08 (0.11)	0.45 (0.63)	0.10 (0.13)	0.31 (0.20)	0.93 (0.87)	0.011†
IOP, mean (SD)		15.00 (2.53)	15.17 (3.09)	16.55 (2.42)	14.95 (3.68)	14.00 (2.62)	0.83†
OSDI score, mean (SD)		1.70 (1.59)	1.45 (1.86)	0.89 (1.62)	1.89 (2.09)	1.57 (1.78)	0.6†
TBUT, mean (SD)		11.80 (1.24)	11.30 (1.52)	11.75 (1.68)	11.30 (1.45)	10.85 (1.35)	0.19†
Lens (n)	Phakia	20 (100%)	52 (86.67%)	20 (100%)	14 (70%)	18 (90%)	0.085†
	Pseudophakia	0 (0%)	8 (13.33%)	0 (0%)	6 (30%)	2 (10%)	
Duration of DM (yrs), median (IQR)		NA	7 (5, 13)	7 (6, 10)	10 (7, 16)	5 (3, 19)	0.13††
FBS, mean (SD)		91.7 (5.13)	145.52 (52.58)	124.5 (24.26)	149.3 (65.54)	162.75 (53.92)	<0.001† 0.063††
HbA1c (%), mean (SD)		5.06 (0.28)	7.69 (1.92)	6.99 (0.67)	8.02 (2.58)	8.07 (1.87)	<0.001† 0.13††
HbA1c Control (n)							
< 7 %; Well-controlled DM		NA	24 (40%)	9 (45%)	8 (40%)	7 (35%)	0.81††
≥ 7 %; Poorly-controlled DM		NA	36 (60%)	11 (55%)	12 (60%)	13 (65%)	
Diabetic macular edema		NA	13 (21.67%)	NA	6 (30%)	7 (35%)	NA
Vitreous hemorrhage		NA	12 (20%)	NA	NA	12 (60%)	NA

†: p-value comparison between non-DM and DM group (Chi-square test; laterality, occupation, geographic location, lens) (Independent t-test; BMI, BCVA, IOP, OSDI, TBUT, FBS, HbA1c), ††: p-value comparison between subgroup classified by DR staging (Kruskal-Wallis; duration of DM) (ANOVA; FBS, HbA1c) (Chi-square; HbA1c control)

(Abbreviations: DM=diabetes mellitus, Non-DM=non-diabetes mellitus, No DR= no diabetic retinopathy, NPDR=non-proliferative diabetic retinopathy, PDR=proliferative diabetic retinopathy, BMI=body mass index, BCVA=best corrected visual acuity, IOP=intraocular pressure, OSDI=ocular surface disease index, TBUT=tear film break up time, FBS=fasting blood sugar, HbA1c=hemoglobin A1c, NA=not available)

2. Antibiotic susceptibility

The DR subgroup (DM-NPDR, DM-PDR) had antibiotic-resistant organisms, which were not found in DM-no DR and non-DM groups. Resistance to benzylpenicillin, erythromycin, clindamycin, and fusidic acid in *Staphylococcus hominis and* resistance to ampicillin, cefazolin, and tetracyclin in *P.rettgeri* were found in the DM-NPDR subgroup. In addition, methicillin-resistant *S. epidermidis* (MRSE) was isolated from the DM-PDR subgroup. Antibiotic resistance organisms were found in both well-controlled DM (HbA1c <7) and poorly-controlled DM (HbA1c ≥ 7) groups. There was no data on the antibiotic susceptibility test for *K. palustris* and *M. luteus* because there was no standard value for interpreting the antibiotic sensitivity test of these two organisms from the Clinical and Laboratory Standards Institute (CLSI) standard guideline. The details of the culture and antibiotic susceptibility in non-DM and DM with subgroup classified by DR staging are shown in Table 2, and in non-DM and DM with subgroup classified by glycemic control are shown in Table 3.

Table 2 Results of culture and antibiotic susceptibility in non-DM and DM with subgroups classified by DR staging.

	Non-DM group	DM group		
		DM-no DR	DM-NPDR	DM-PDR
Growth	1/20 (5%)	3/20 (15%)	3/20 (15%)	3/20 (15%)
Gram-positive cocci				
Staphylococcus epidermidis	1	1		1[c]
Staphylococcus aureus		1		
Staphylococcus hominis		1	1[a]	
Streptococcus mitis			1	
Kocuria palustris				1
Micrococcus luteus				1
Gram-negative bacilli				
Providencia rettgeri			1[b]	

[a] Resistant to benzylpenicillin, erythromycin, clindamycin, fusidic acid

[b] Resistant to ampicillin, cefazolin, tetracyclin

[c] MRSE: methicillin-resistant staphylococcus epidermidis

(Abbreviations: DM=diabetes mellitus, Non-DM=non-diabetes mellitus, No DR= no diabetic retinopathy, NPDR=non-proliferative diabetic retinopathy, PDR=proliferative diabetic retinopathy)

Table 3 Results of culture and antibiotic susceptibility in non-DM and DM with subgroups classified by glycemic control.

	Non-DM group	DM group	
		HbA1c < 7	HbA1c ≥ 7
Growth	1/20 (5%)	4/24 (16.67%)	5/36 (13.89)
Gram-positive cocci			
Staphylococcus epidermidis	1	1[c]	1
Staphylococcus aureus			1
Staphylococcus hominis		1	1[a]
Streptococcus mitis		1	
Kocuria palustris			1
Micrococcus luteus		1	
Gram-negative bacilli			
Providencia rettgeri			1[b]

[a] Resistant to benzylpenicillin, erythromycin, clindamycin, fusidic acid

[b] Resistant to ampicillin, cefazolin, tetracyclin

[c] MRSE: methicillin-resistant staphylococcus epidermidis

(Abbreviations: DM=diabetes mellitus, Non-DM=non-diabetes mellitus, No DR= no diabetic retinopathy, NPDR=non-proliferative diabetic retinopathy, PDR=proliferative diabetic retinopathy, HbA1c=hemoglobin A1c)

3. Next-generation sequencing analysis (NGS)

In this study, Illumina sequencing of 16S rRNA genes generated a total of 1,786,948 high-quality reads, and the number of amplicon sequencing variants (ASVs) was 1359. The rarefaction curve demonstrated the number of ASVs representing the species richness within and between sequencing reads in each sample. Each curve represents each sample. The approximate saturation of microbial richness of all samples was 4,534 sequencing depths, as estimated by the rarefaction curves. The plateau curve in rarefaction was observed when approximately 2,000 sequencing depths were reached. This finding sufficiently estimated the true bacterial compositions of the ocular microbiome in human among the sample groups. The rarefaction are demonstrated in Figure 5.

3.1 Core ocular surface microbiome

The 100% core ASVs (amplicon sequence variants) sample matching was used to identify the core ocular surface microbiome. At the phylum level, Proteobacteria, Firmicutes**,** and Actinobacteria were the core microbiome of the non-DM group, and Proteobacteria and Firmicutes of the DM group. At the class level, Alphaproteobacteria, Gammaproteobacteria, Actinobacteria, and Bacilli were the core microbiome of the non-DM group, and Gammaproteobacteria and Bacilli of the DM group.

3.2 Taxonomic composition of ocular surface microbial community

The molecular biological method detected 23 bacteria phyla, 40 classes, 190 families, and 318 genera. The bacteria of phylum Proteobacteria were highly abundant in both non-DM and DM groups (40.56%, 42.18%), followed by Firmicutes (32.43%, 34.83%), Actinobacteria (13.43%, 10.71%), and Bacteroidetes (9.35%, 6.71%), respectively (Figure 6). The abundance of Actinobacteria was significantly decreased in DM compared to the non-DM group (p-value = 0.021) (Figure 7). Bacteria in the class of Alphaproteobacteria was significantly abundant in the non-DM group (p-value = 0.007), while Gammaproteobacteria was significantly abundant in the DM group (p-value = 0.036). At the family level, Enterobacteriaceae was significantly more abundant in DM than in the non-DM groups (p-value = 0.008). Neisseriaceae were significantly abundant in DM-NPDR compared to DM-no DR and the non-DM groups (p-value = 0.016, 0.029, respectively). At the genus level, *Escherichia-Shigella* dominated in DM groups compared to the non-DM group (p-value = 0.016). *Pseudomonas* was more abundant in the DM-PDR group (6.05%) when compared to the DM-NPDR (2.35 %), DM-no DR (2.42%), and non-DM groups (3.05%), with no statistical significance (p-value = 0.78). In subgroup of glycemic control, the relative abundance of *Escherichia-Shigella* was also significantly abundant in both well-controlled (10.38%) and poorly-controlled DM (10.02%) compared to the non-DM group (5.39%) (p-value = 0.028, 0.035, respectively) (Figure 8).

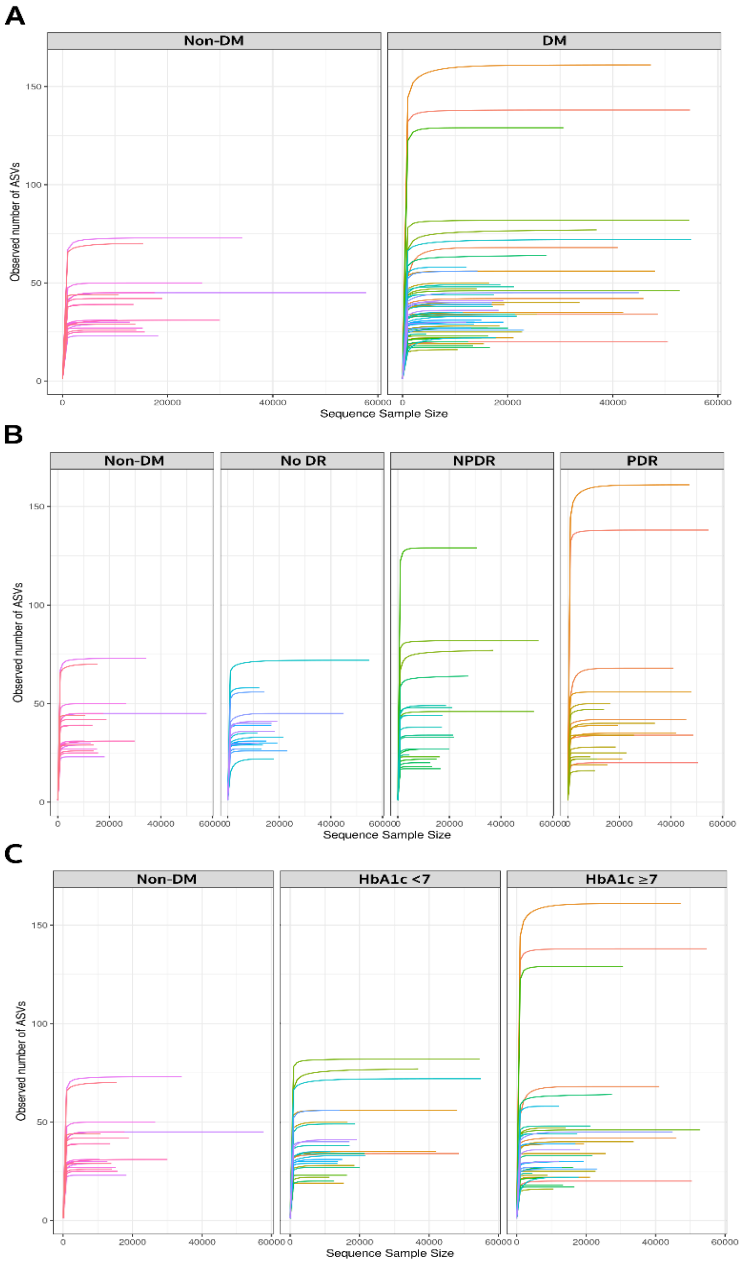

Figure 5 Rarefaction curve demonstrated the number of ASVs in each sample. Each curve represents each sample. (A) Comparison of rarefaction curve between non-DM and DM groups (B) between non-DM and subgroup of DR staging (C) between non-DM and subgroup of DM with glycemic control. (Abbreviations: ASVs=amplicon sequence variants, DM=diabetes mellitus, Non-DM=non-diabetes mellitus, No DR= no diabetic retinopathy, NPDR=non-proliferative diabetic retinopathy, PDR=proliferative diabetic retinopathy, HbA1c=hemoglobin A1c)

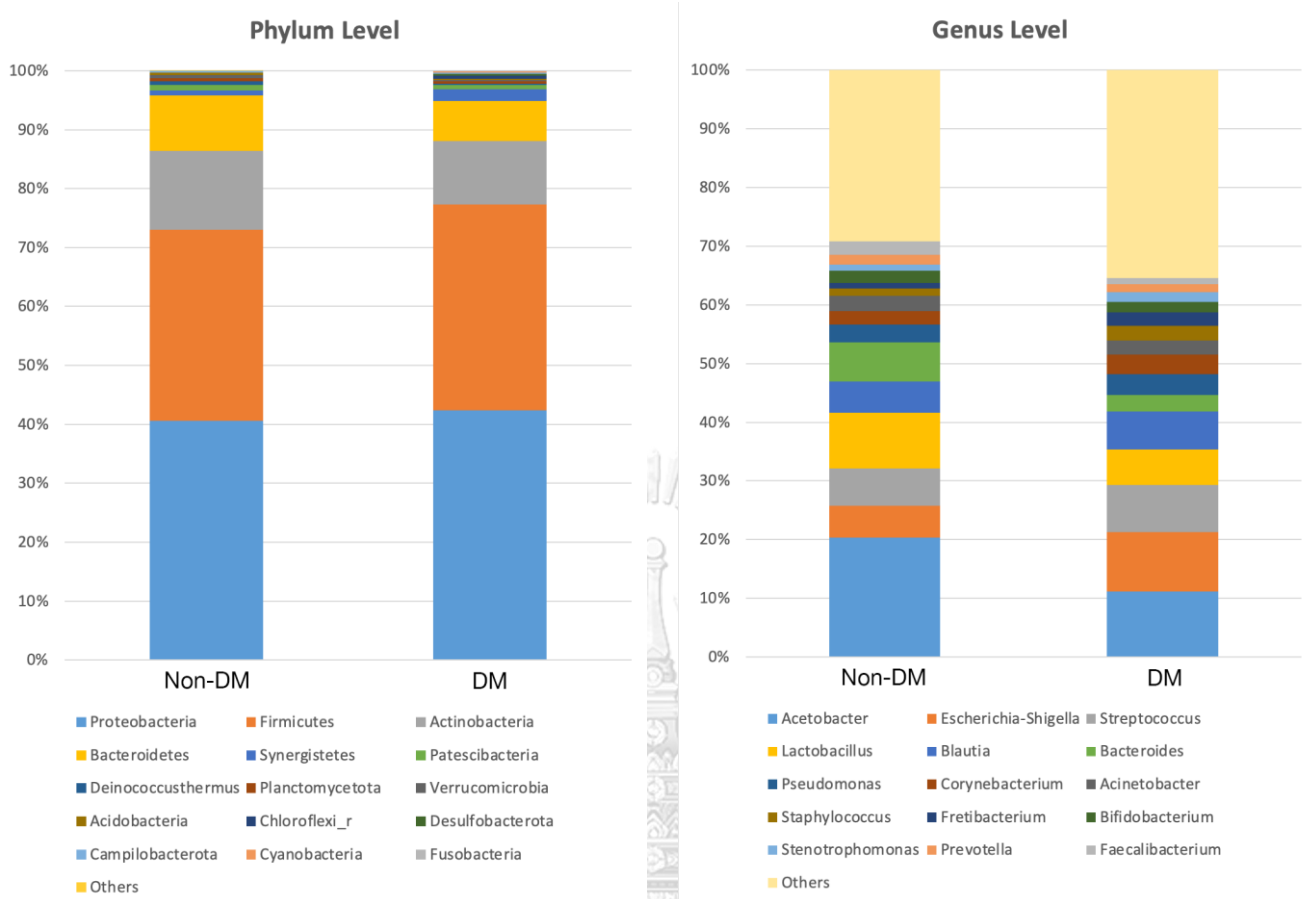

Figure 6 The relative abundance of top 15 phyla and genera level of ocular surface microbiome in non-DM and DM group

(Abbreviations: DM=diabetes mellitus, Non-DM=non-diabetes mellitus)

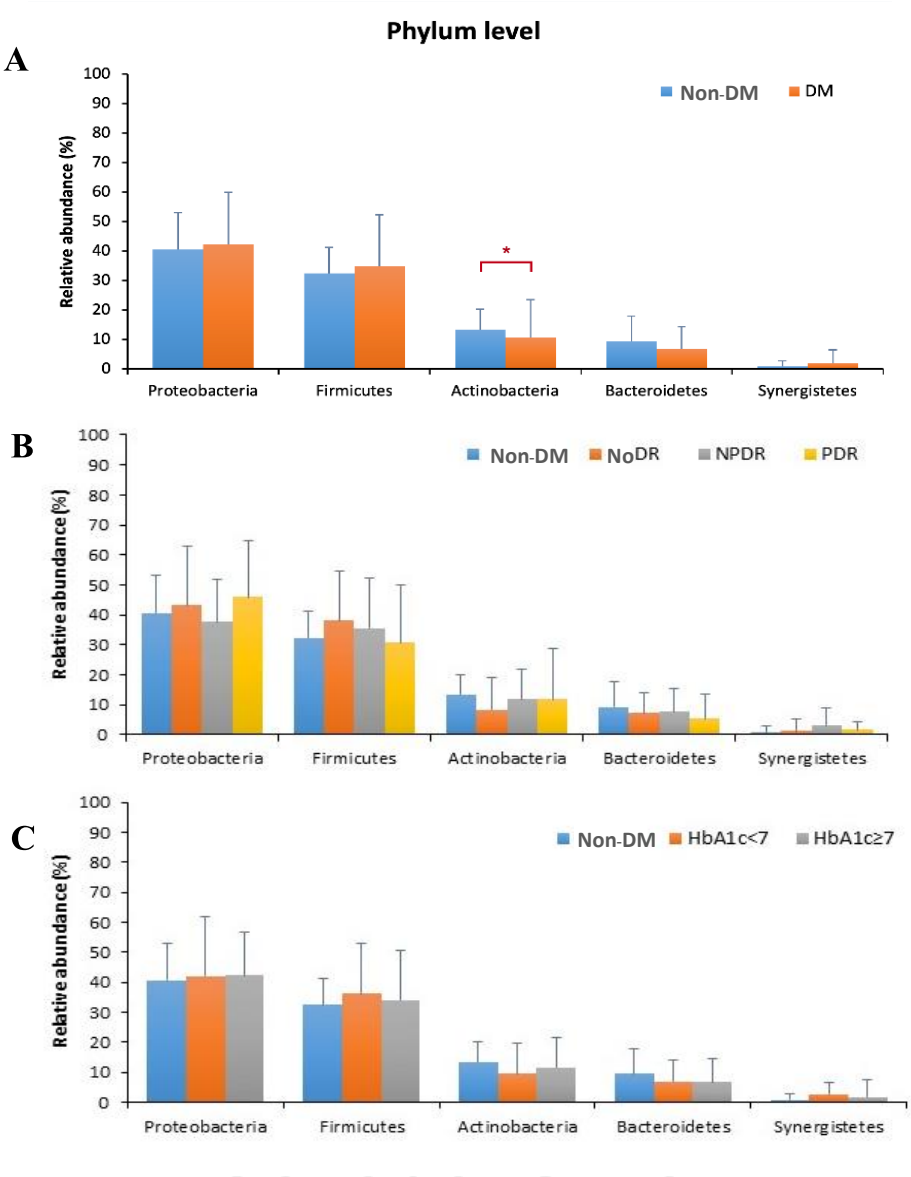

Figure 7 The relative abundance of top 5 phyla level of ocular surface microbiome (A) Comparison of top 5 phyla level between non-DM and DM groups, (B) between non-DM and DM subgroups classified by DR staging, and (C) between non-DM and DM subgroups classified by glycemic control. *p- value < 0.05 (Abbreviations: DM=diabetes mellitus, Non-DM=non-diabetes mellitus, No DR= no diabetic retinopathy, NPDR=non-proliferative diabetic retinopathy, PDR=proliferative diabetic retinopathy, HbA1c=hemoglobin A1c)

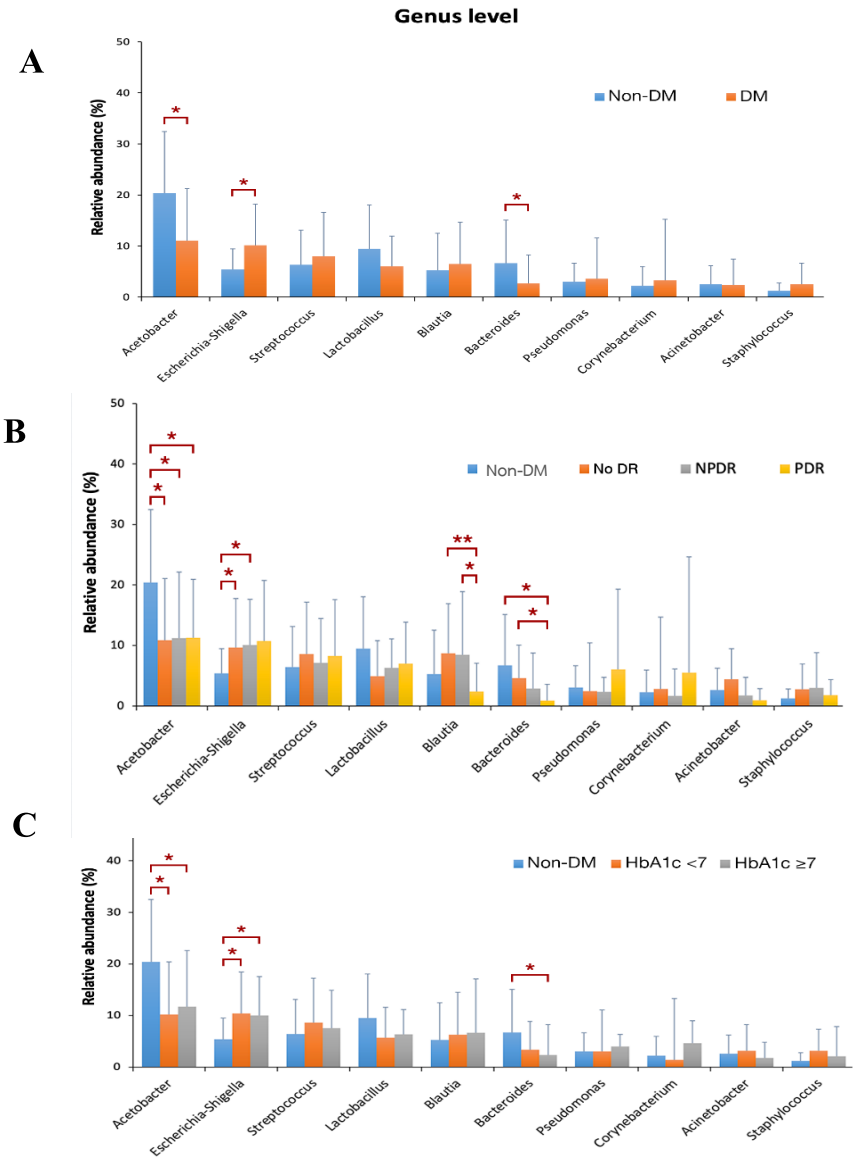

Figure 8 The relative abundance of top 10 genera level of ocular surface microbiome

(A) Comparison of top 10 genera level between non-DM and DM groups, (B) between non-DM and DM subgroups classified by DR staging, and (C) between non-DM and DM subgroups classified by glycemic control. *p- value < 0.05, **p- value < 0.001 (Abbreviations: DM=diabetes mellitus, Non-DM=non-diabetes mellitus, No DR= no diabetic retinopathy, NPDR=non-proliferative diabetic retinopathy, PDR=proliferative diabetic retinopathy, HbA1c=hemoglobin A1c)

To identify the possible biomarkers for differentiate the DM from non-DM groups, the Linear Discriminant Analysis Effect Size (LEfSe) analysis was performed to determine the significant difference in the bacterial distribution between groups. The bar plot represents the effect size LDA (LDA; Linear discriminant analysis) for a significant taxon in a particular group. Bacterial taxa with LDA scores greater than 2 was considered significant, and the results are shown in Figure 9.

Cladogram explains the differentially abundant taxonomic clades according to LEfSe analysis. The dot color and shading represent significantly higher abundance of taxon in a certain group. The significant phyla are presented as dots in the center, while the significant genera are shown in the outer circle. The results from both LEfSe and cladogram are similar. The result of LEfSe shows significant difference of single taxa level, while cladogram shows the different taxonomic clades (from phylum to genus) among groups. The cladograms are shown in Appendix B.

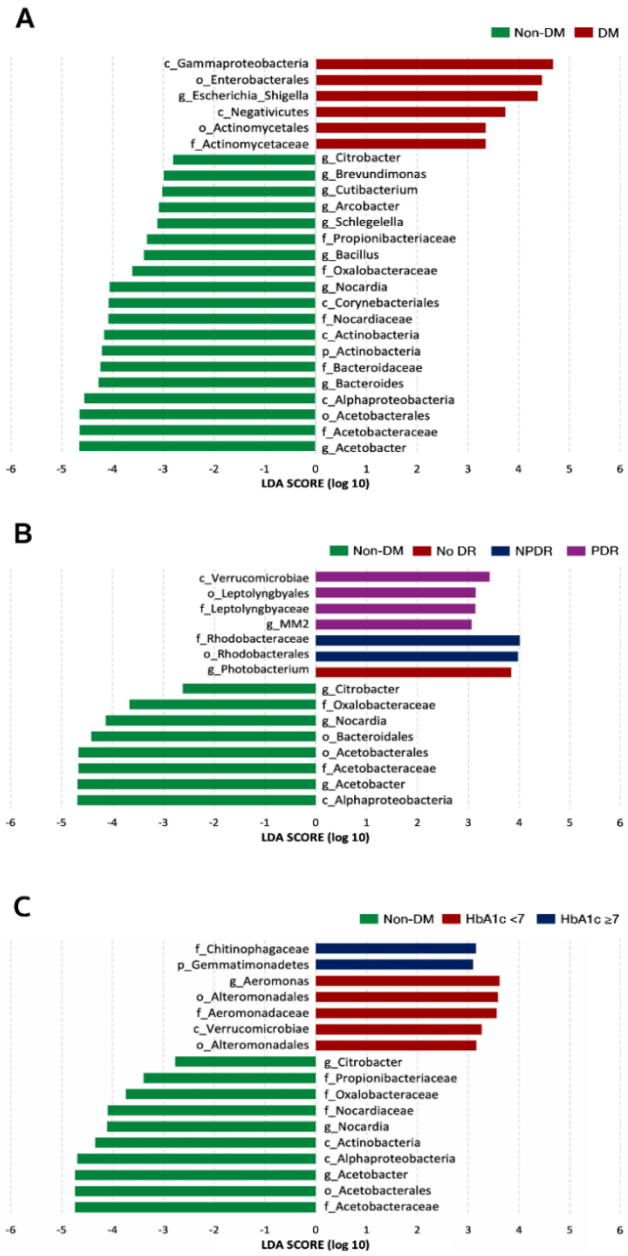

Figure 9 Linear discriminant analysis effect size (LEfSe)

The bar plot represents linear discriminant analysis scores (LDA) for a significant taxon in a certain group based on LEfSe results. (A) Comparison of taxa lists between non-DM and DM groups. (B) between non-DM and DM with subgroups classified by DR staging. (C) between non-DM and DM with subgroups classified by glycemic control. (Abbreviations: DM=diabetes mellitus, Non-DM=non-diabetes mellitus, No DR= no diabetic retinopathy, NPDR=non-proliferative diabetic retinopathy, PDR=proliferative diabetic retinopathy, HbA1c=hemoglobin A1c)

3.3 Alpha-diversity

Alpha-diversity was used to show the bacterial diversity within each community, which estimated the number of species in the microbial community and the abundance and diversity of species in environmental communities via statistical indices. Alpha-diversity was performed using observed ASVs, Chao1, Shannon, and phylogenetic diversity (PD) whole tree. All analyses showed no significant difference between the non-DM and DM groups (p-value > 0.05). In addition, for the subgroup of DR staging and glycemic control, there were no statistically significant differences in the alpha-diversity indexes between each subgroup. The box plots of alpha-diversity indices are demonstrated in Figure 10 (non-DM and DM groups), Figure 11 (non-DM and DM with subgroups classified by DR staging), and Figure 12 (non-DM and DM with subgroups classified by glycemic control).

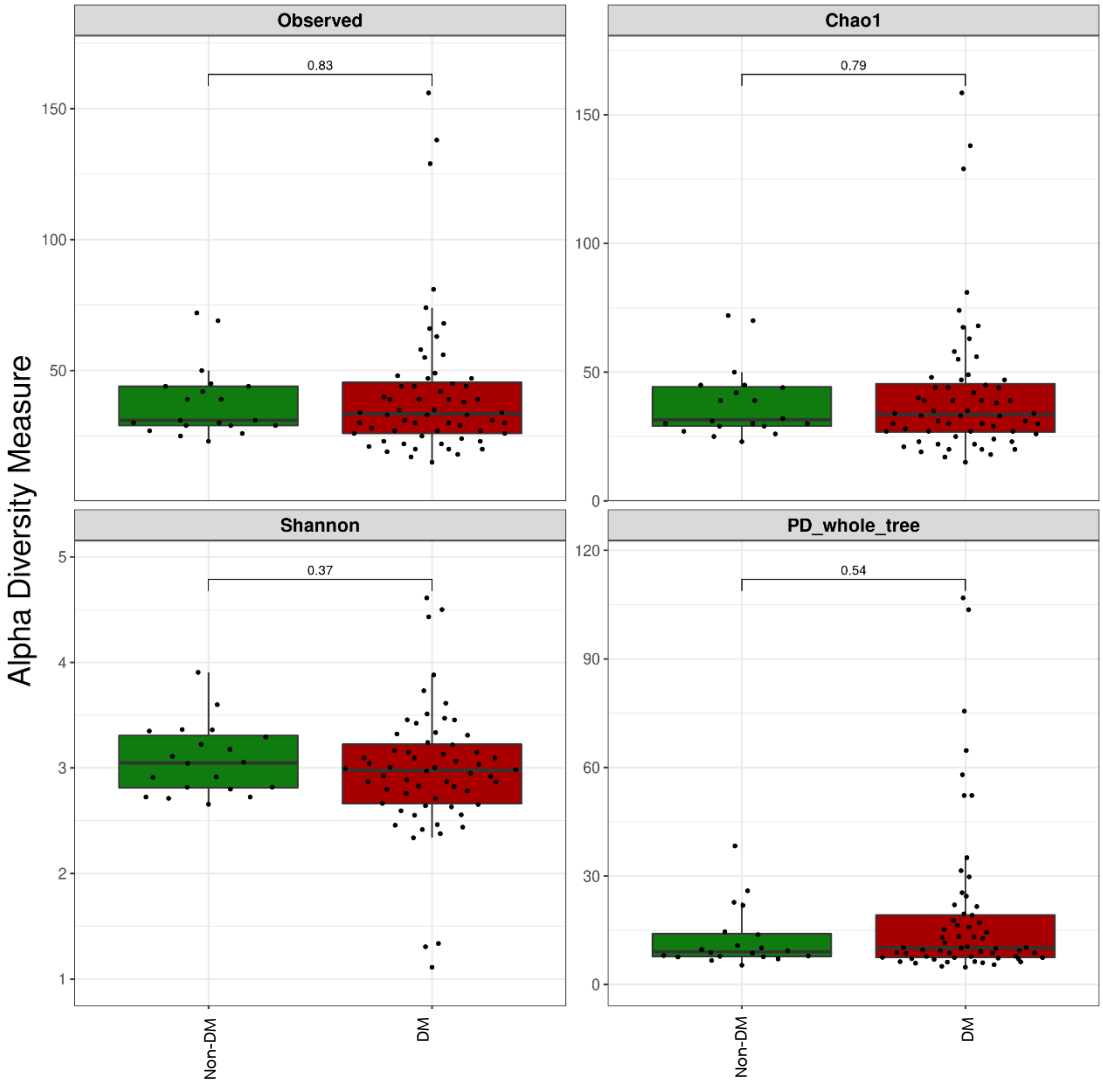

Figure 10 Alpha-diversity indices in non-DM and DM groups

(Abbreviations: DM=diabetes mellitus, Non-DM=non-diabetes mellitus)

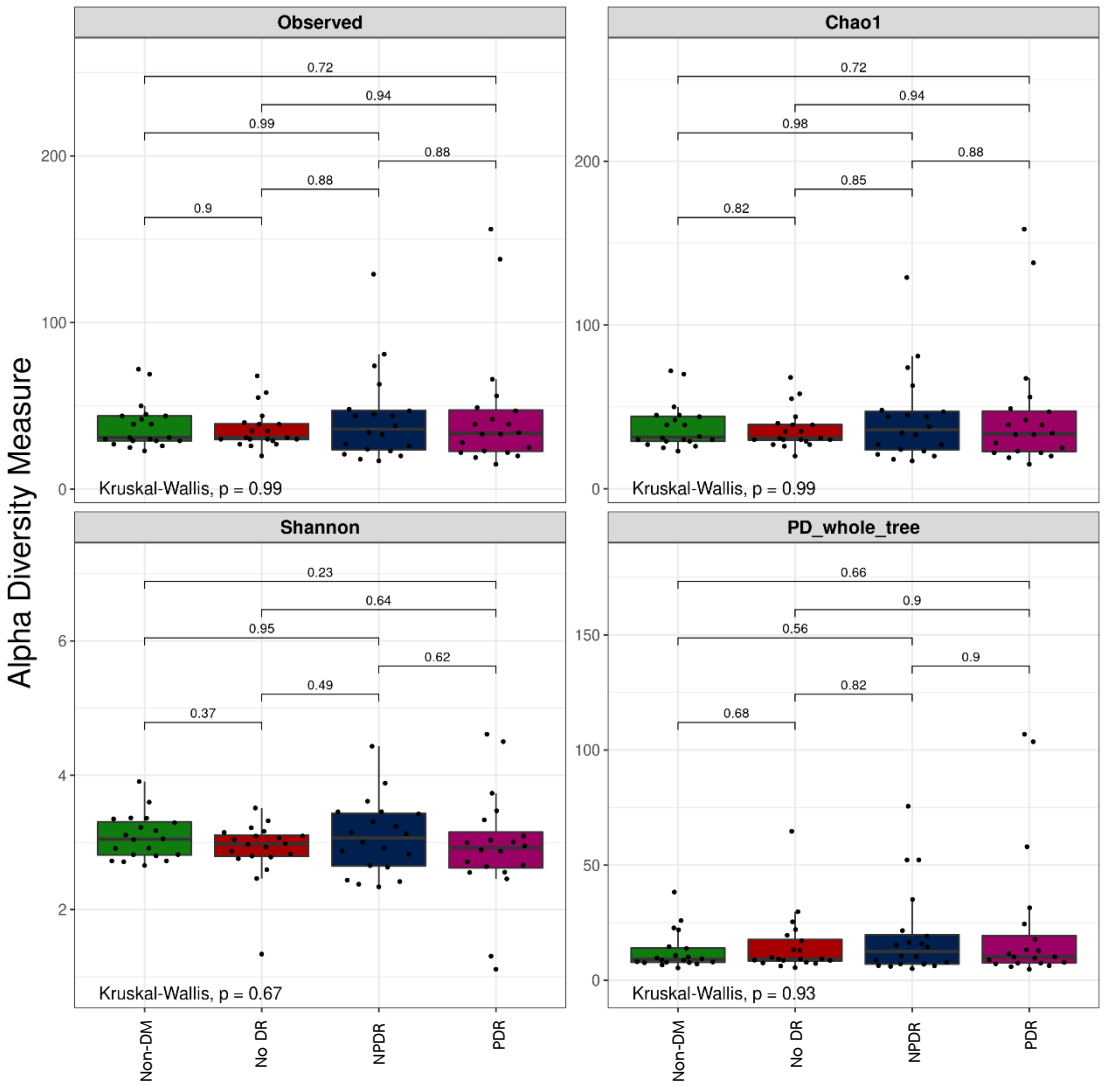

Figure 11 Alpha-diversity indices in non-DM and DM subgroups classified by DR staging.

(Abbreviations: DM=diabetes mellitus, Non-DM=non-diabetes mellitus, No DR= no diabetic retinopathy, NPDR=non-proliferative diabetic retinopathy, PDR=proliferative diabetic retinopathy)

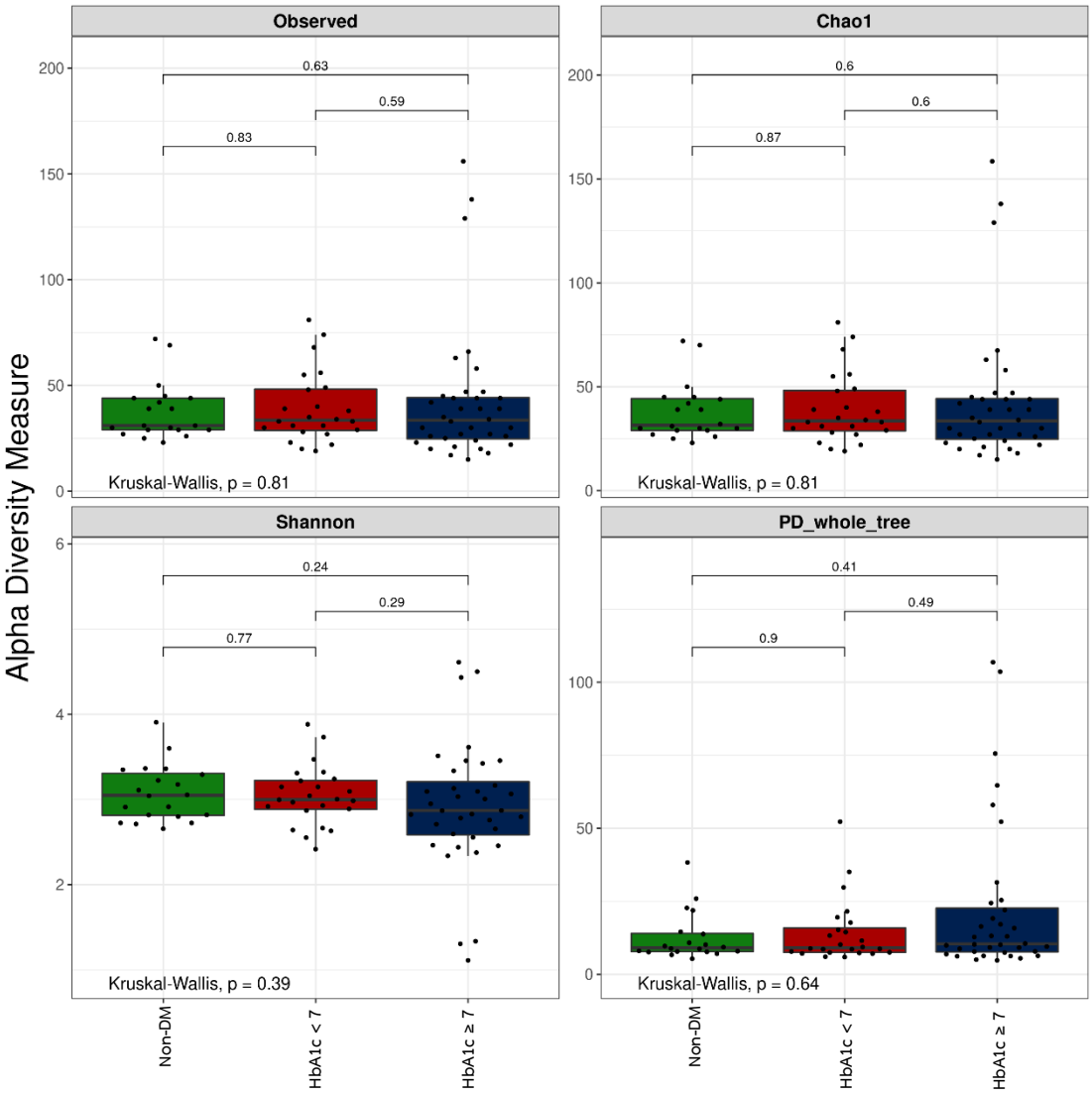

Figure 12 Alpha-diversity indices in non-DM and DM subgroups classified by glycemic control.

(Abbreviations: DM=diabetes mellitus, Non-DM=non-diabetes mellitus, HbA1c=hemoglobin A1c)

3.4 Beta-diversity

Beta-diversity was used to show the difference in the microbial community between the groups. This study used the principal coordinate analysis (PCoA), multidimensional scaling method to visualize the dissimilarity of the data, and box plot using unweighted UniFrac distances to calculate the distance between groups. The distance metric of unweighted UniFrac showed that the human ocular surface microbial community in the DM group was significantly different from the non-DM group (p-value = 0.0038) (Figure 13). In subgroup analysis, the results demonstrated that the microbial community in the DR subgroup (DM-NPDR and DM-PDR) significantly differed from DM-no DR and non-DM groups (p-value<0.05). However, the microbial community was not significant difference between DM-no DR and the non-DM group (p-value>0.05) as between the DM-NPDR and DM-PDR groups (p-value >0.05) (Figure 14). In subgroup of glycemic control, the beta-diversity showed that the ocular surface microbiome in the poorly-controlled DM group was significant difference from well-controlled DM and non-DM groups (p-value <0.001). There was no significant difference between the well-controlled DM and non-DM groups (p-value>0.05) (Figure 15).

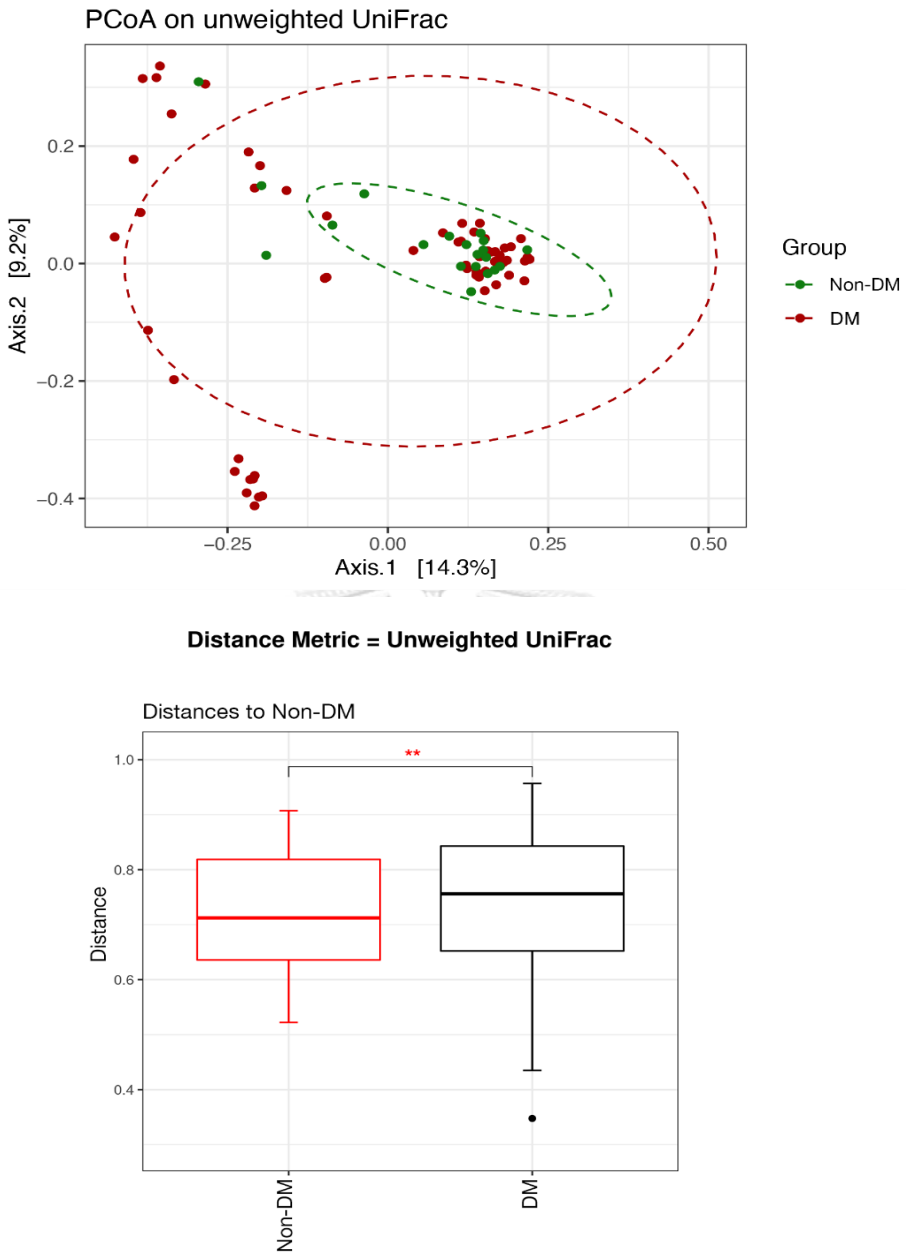

Figure 13 Principal coordinate analysis (PCoA) plot based on unweighted UniFrac distance and comparison of ocular surface microbiome (box plot) between the non-DM and DM groups.

The figure showed significant difference of microbial community between non-DM (green-dotted circle) and DM groups (red-dotted circle). (**p<0.001).

(Abbreviations: DM=diabetes mellitus, Non-DM=non-diabetes mellitus)

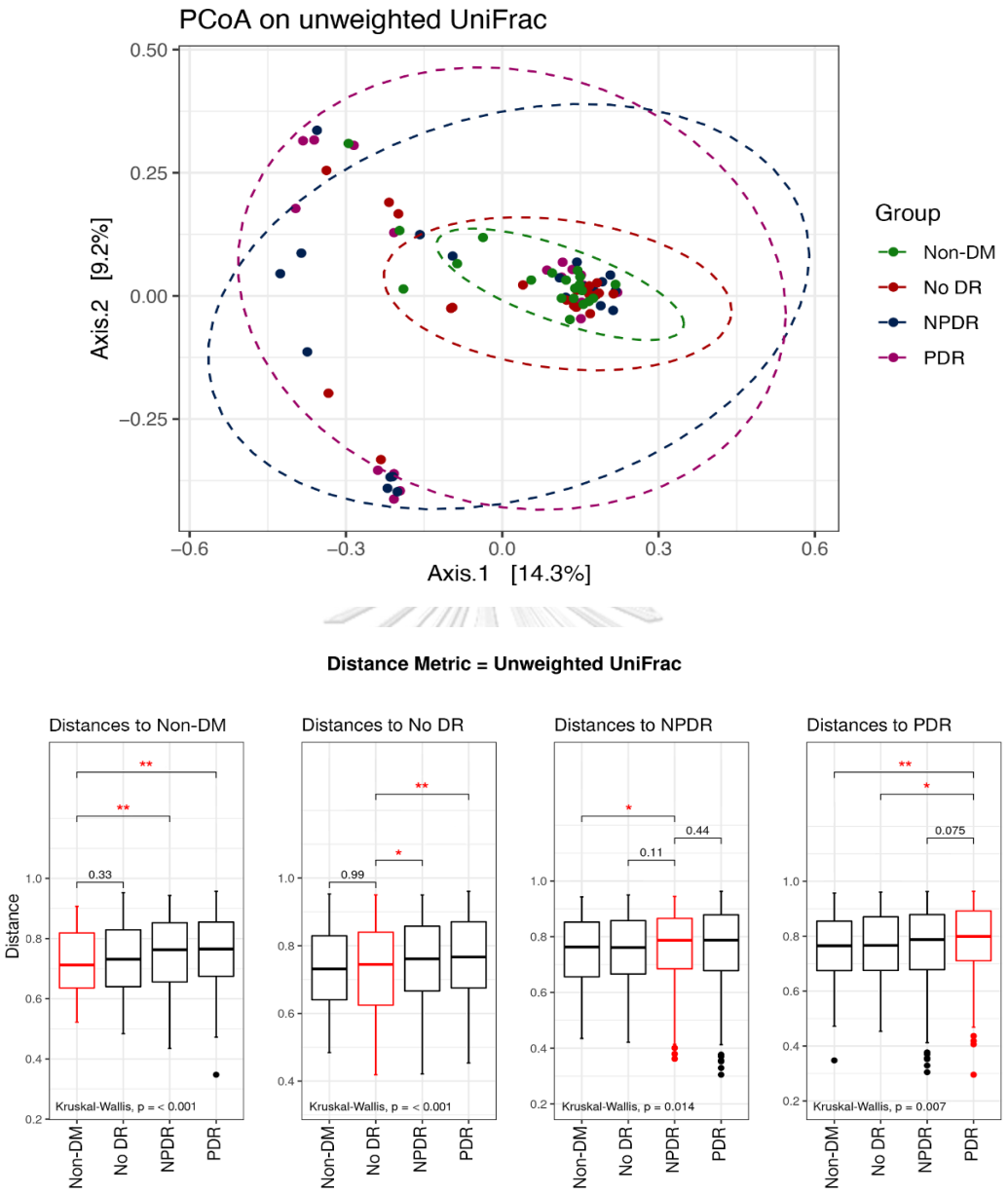

Figure 14 Principal coordinate analysis (PCoA) plot based on unweighted UniFrac distance and comparison of ocular surface microbiome (box plot) between the non-DM and the subgroups classified by DR staging.

(non-DM: green-dotted circle, no DR: red-dotted circle, NPDR: blue-dotted circle, and PDR: purple-dotted circle) *p- value < 0.05, **p- value < 0.001

(Abbreviations: DM=diabetes mellitus, Non-DM=non-diabetes mellitus, No DR= no diabetic retinopathy, NPDR=non-proliferative diabetic retinopathy, PDR=proliferative diabetic retinopathy)

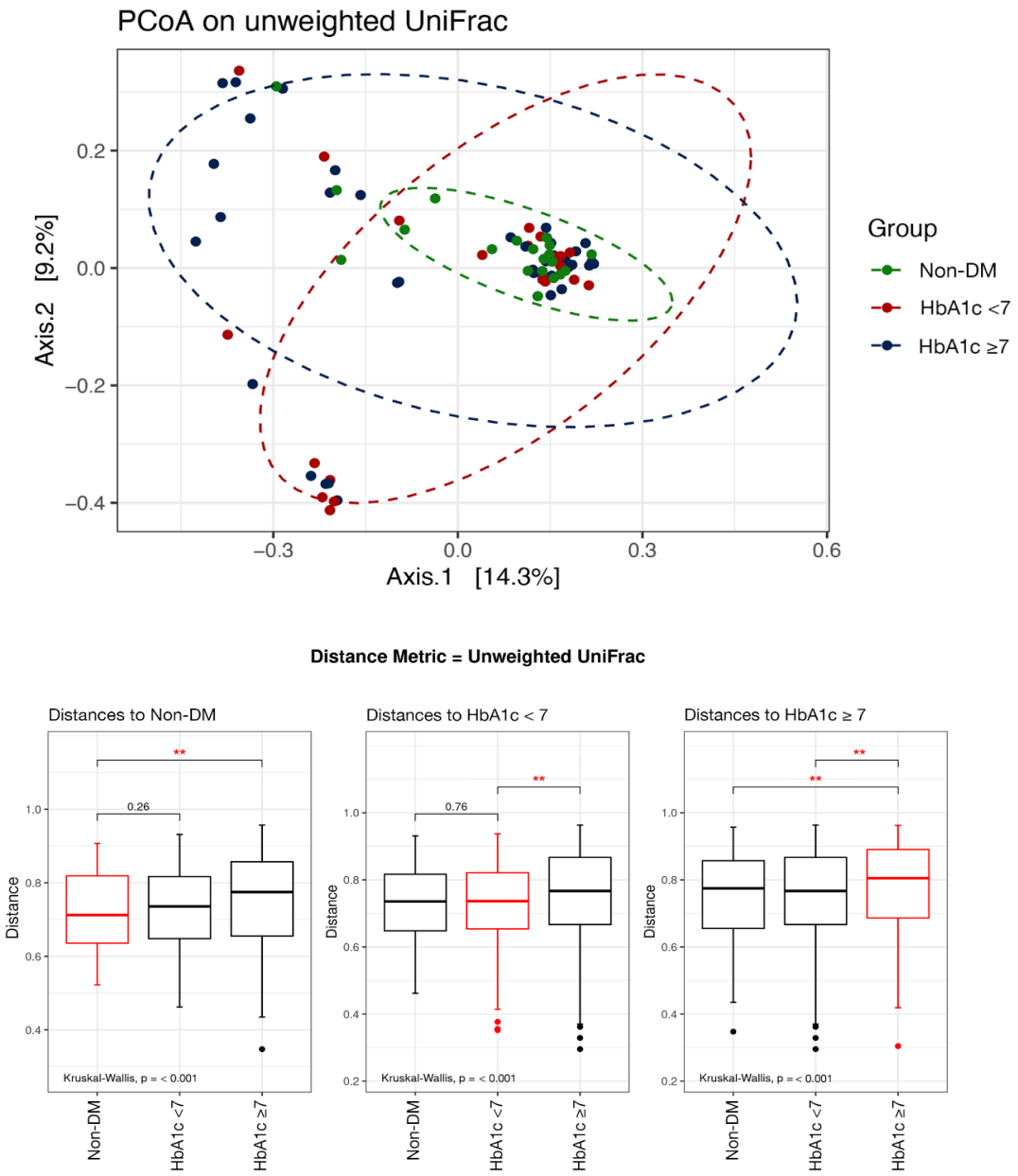

Figure 15 Principal coordinate analysis (PCoA) plot based on unweighted UniFrac distance and comparison of ocular surface microbiome (box plot) between the non-DM and the subgroups classified by glycemic control.

(non-DM: green-dotted circle, HbA1c <7%: red-dotted circle, HbA1c ≥7%: blue-dotted circle), *p- value < 0.05, **p- value < 0.001 (Abbreviations: DM=diabetes mellitus, Non-DM=non-diabetes mellitus, HbA1c=hemoglobin A1c)

CHAPTER V

Discussion and Conclusion

The microbiome contributes to the metabolic, immunologic, and protective properties to maintain a healthy microenvironment and preserve normal functions of the organs.[3-6] The ocular surface microbiome plays a vital role in maintaining the homeostasis of the ocular surface environment. Alterations of the ocular surface microbiome composition (dysbiosis) in many ocular surface diseases can lead to a higher rate of infection and inflammation.[3,4,6] Hyperglycemic condition in DM has been well-evidenced in altered immunologic response, leading to microenvironment changes.[24-26] However, there have been limited studies on the ocular surface microbiome of DM, particularly those with different DR staging and glycemic control status. Therefore, understanding the physiology of the ocular surface microbiome and their changes may enhance the preventive and treatment strategies, particularly in ocular inflammation and infections.[7]

Several studies described the ocular surface microbiome in DM, mainly using conventional culture technique. The culture-positive rate was shown to be higher in DM than in the non-DM group (21.7-94.13% and 4.3-73.3%, respectively).[28-32] The result was reinforced by our study, although not statistically significant (15% and 5%, respectively; p=0.437). The lower culture-positive rate in our study may be due to different specimen collection and culture techniques. In this study, the process of conjunctival swab was done by one masked investigator. Only one conjunctival swab was used for culture and NGS analysis to represent the same microbial community in the specimen. And due to the limited amount of specimen, this study used only chocolate agar plate, which can provide growth for most of the ocular surface organisms except for anaerobic bacteria, which can only be detected by the NGS

method. This process may affect the result of this study. However, previous studies used the same technique for sample collection and showed the culture-positive rate of healthy subjects was 10%.[51]

Most microorganisms identified in this study were gram-positive cocci with mainly coagulase-negative *Staphylococcus*. Gram-negative bacteria were more commonly found in the ocular surface microbiome of the DM group, consistent with previous reports.[28,31] Furthermore, the colonization of pathogenic bacteria was more detected in the DM than in the non-DM group.[28,29] These results showed a positive culture of *P. rettgeri*, gram-negative bacilli, in the DM-NPDR group. This organism can also be found in normal healthy gut but can lead to opportunistic infection. Koreishi et al. reported that *P. rettgeri* was a potential microorganism causing ocular infection such as conjunctivitis, keratitis, dacryocystitis, and endophthalmitis in immunocompromised patients, including DM.[74] *Kocuria* spp., gram-positive bacteria found in the DM-PDR group, was reported to cause canaliculitis, dacryocystitis, recurrent conjunctivitis, and severe marginal keratitis with corneal melt.[75,76] In addition, *M. luteus* was reported to cause infectious keratitis after microkeratome-assisted laser in situ keratomileusis (LASIK)[77], which was also found in the DM-PDR group.

Martin et al.[28] reported that DM with DR had more culture-positive rate than the DM-no DR subjects. However, this study did not find a significant difference in culture-positive rate between subgroup analysis by either the DR staging or the glycemic control. Remarkably, the antibiotic-resistant organisms were detected only in DM with DR group (including *P. rettgeri* and *S.hominis* in the NPDR group and MRSE in the PDR group). The previous study revealed more inflammation and intraocular tissue destruction in endophthalmitis caused by the resistant strain of *S. epidermidis* than the non-resistant strains.[78] Since the most common causative organism of endophthalmitis was ocular surface flora like coagulase-negative *Staphylococcus.*[22,54]

The finding is important to help in preoperative prophylaxis and management in these patients. One possible explanation for detecting antibiotic-resistant organisms in the DR group is that DM with DR subjects are more likely to receive the intraocular intervention.[79] Thereby, their ocular surface are more exposed to antibiotics. Hence, several studies reported the emergence of antibiotic-resistant organisms after multiple sessions of intravitreal drug injection using prophylactic antibiotics. [79-81]

The NGS showed more information on bacterial composition and diversity in the microbial community than the conventional culture method. The top four most common phyla found in the ocular surface microbiome in both non-DM and DM groups were Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes, which were similar to previous studies.[33,34] There was a lower relative abundance of phylum Actinobacteria in the DM group compared to the non-DM group. At the class level, Alphaproteobacteria predominated in the non-DM group, and Gammaproteobacteria predominated in the DM group, similar to Ham B. et al.[33] In contrast, Ham B. et al.[33] showed a higher abundance of *Acinetobacter* in the DM group, which was not found in Li S et al.[34] and this reports. The dissimilarity in the ocular surface microbiome between each study might be due to different age, gender, ethnicity, and geographic location of subjects.[37] Prior studies also demonstrated the abundance of potentially pathogenic bacteria in the DM group using NGS technique.[33,36] *Acinetobacter* and *Burkholderia* had higher proportion in the DM than in the non-DM group.[33] The other study found the abundance of *Pseudomonas, Haemophilus*, and *Empedobacter* on the ocular surface of DM than in the non-DM group.[36] This study likewise found higher proportion of family Enterobacteriaceae and Neisseriaceae, genus *Escherichia-Shigella* and *Pseudomonas* in the DM, especially in DM with the DR group.

The ocular surface microbiome was previously reported to be more diverse in the DM than in the non-DM group.[33-36] Nevertheless, this study using 4 indices for alpha-diversity analyses did not confirm such findings. The dissimilarity may be from the different sample collection techniques, analysis, and populations. However, from the beta-diversity analysis, this study can first demonstrate that the presence of DR and poorly-controlled DM status can significantly change the microbial community on the ocular surface.

The formation of advanced glycation end products (AGEs) in the hyperglycemic state affects the ocular surface by reduced proliferation and enhancing apoptosis of epithelial cells, inducing inflammation in the sub-basal nerve plexus.[55,56] These lead to compromised epithelium, delayed wound healing, and decreased corneal nerve fiber density in DM patients.[55,56] Sato E. et al. used fluorophotometry to detect corneal AGEs levels and reported that AGEs fluorescence level was significantly increased in patients with DM-PDR compared with DM-NPDR, DM-no DR, and non-DM groups.[57] The increased corneal AGEs level may result from the rising of AGEs levels in aqueous and vitreous, especially in PDR, due to compromised blood-retinal barrier.[57,82] Many groups have reported more severity of ocular surface changes in DR and poorly- controlled DM.[14,16-18] Together with alteration of protective immune response in DM,[24-26] these can lead to ocular surface microbiome dysbiosis and increase the abundance of potential pathogens in DM, especially in the DR subgroup.

This study is the first to report the ocular surface microbiome in DM according to DR staging and glycemic control using the NGS method. The results signify the effect of these factors on the ocular surface microbial community and might lead to further consideration regarding the prevention and management of ocular inflammation and infection in these particular groups of patients.

The limitations of this study were the small sample size and 16S rRNA gene sequencing incapability to detect viruses and fungi, which may be a part of the ocular surface microbiome. In addition, the data collection in a single time point may not represent the whole picture of ocular surface microbiome change with time.

Further studies are undoubtedly warranted to understand the cause-and-effect relationship. Additional assessments in the metagenomics profiling, protein expression, and metabolic activity may lead us to a better understanding of ocular surface microbiome change in this significant and common disease.

Conclusion

This study revealed a higher culture-positive rate in DM (15%) than in non-DM group (5%) (p-value=0.437). The results demonstrated definite differences in the ocular surface microbiome between the DM and non-DM groups, the DM with DR and no DR groups, and finally, poorly-controlled (HbA1c ≥7%) and well-controlled DM groups (HbA1c <7%). In addition, a higher prevalence of pathogenic bacteria and antibiotic-resistant strains was more commonly found in DR compared to the other groups. These results may provide further knowledge on ocular surface diseases and the pathogenesis of many infectious diseases related to the ocular surface microbiome in DM patients.

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APPENDIX A

Operational definitions

1. **Ocular surface:** The surface of conjunctiva, cornea, lacrimal gland, meibomian glands, tear, and lacrimal drainage system.[83] In this study, the investigators use the microbial information from inferior conjunctival swab to represent the ocular surface microbiome.
2. **Microbiome**: A complex community of microorganisms or microbial composition that interact with each other and the host to maintain homeostasis in their environment.[2]
3. **Core microbiome:** A set of microbial organisms that are characteristics of a host or environment of interest. Core microbiome are usually measured as microbial organisms shared among samples from a particular environment.[84] In this study, core microbiome is defined by using 100% core ASVs (amplicon sequence variants) samples matching in each groups.
4. **Dysbiosis:** The alteration in microbial diversity or microbial composition in the environment that can affect the functions of the organ, which can lead to infection or inflammation.[2]
5. **Amplicon sequence variants (ASVs)**[85]: A unit of analysis used to classify group of species based on DNA sequencing.
6. **Alpha-diversity**: To describe microbial community within a sample with consideration to the richness and evenness.[73]
    - Richness is defined as a number of different species in a sample.
    - Evenness is defined as the relative abundance of the species.
7. **Beta-diversity**: To describe the difference in the microbial community among samples.[73]
8. **Relative abundance:** Percentage of microorganisms relative to total microorganisms in the sample.

APPENDIX B

Cladogram

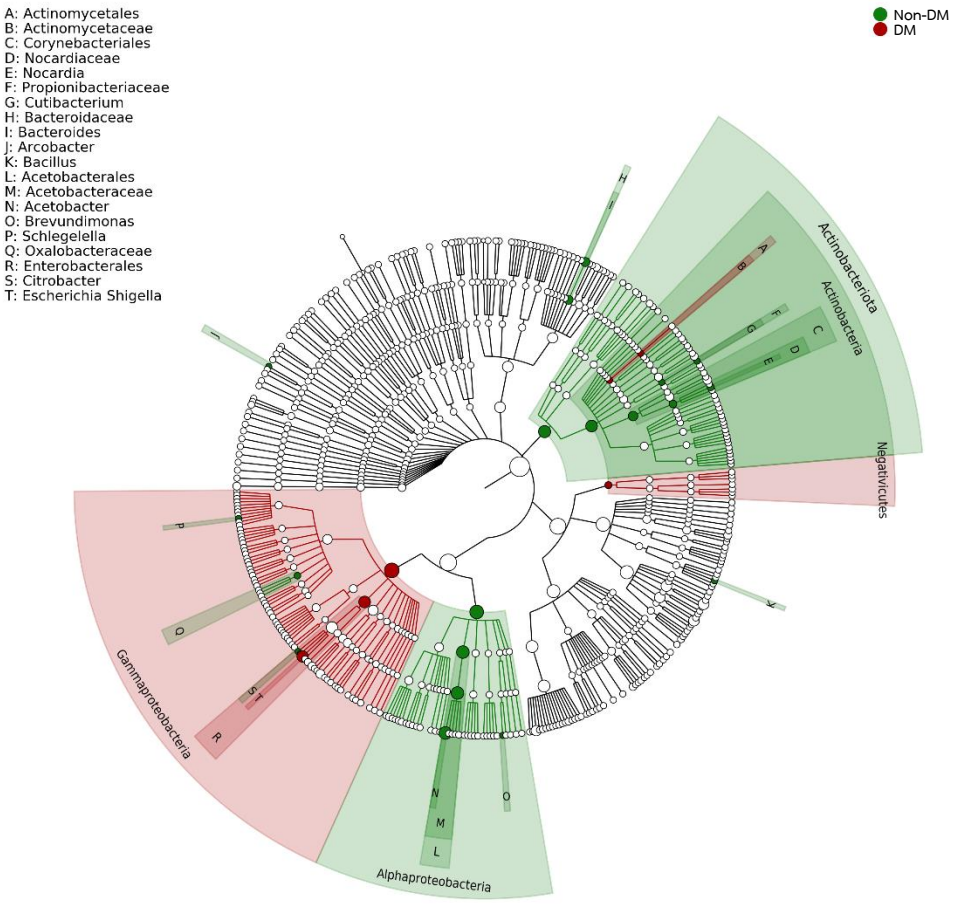

Figure 1 Cladogram shows the different taxonomic clades in non-DM and DM groups.

Figure 2 Cladogram shows the different taxonomic clades in non-DM and DM with subgroups classified by DR staging.

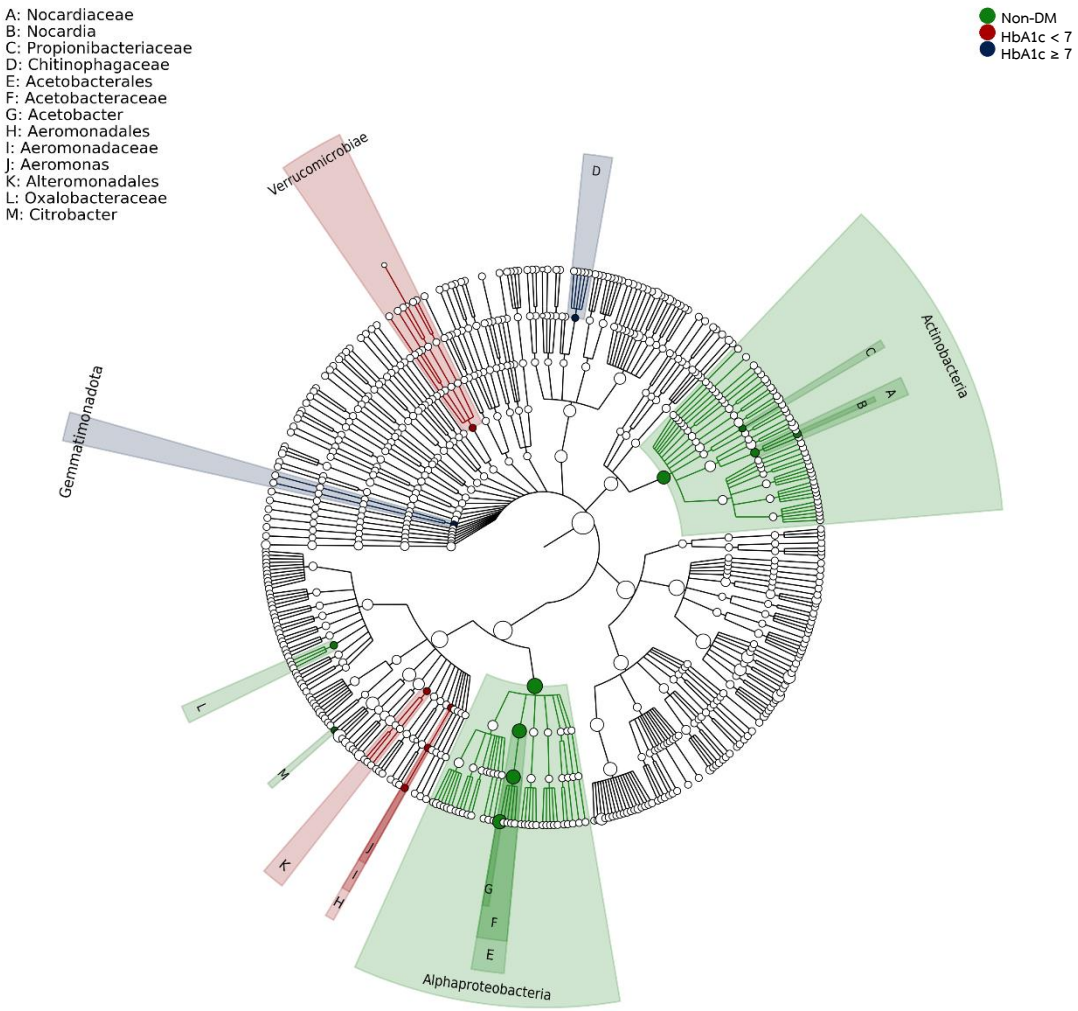

Figure 3 Cladogram shows the different taxonomic clades in non-DM and DM with subgroups classified by glycemic control.

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