Composition and diversity of meibum microbiota in meibomian gland dysfunction and its correlation with tear cytokines levels

Miss Ubonwan Rasaruck

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Clinical Sciences
FACULTY OF MEDICINE
Chulalongkorn University
Academic Year 2022

องค์ประกอบและความหลากหลายของไมโครไบโอมของไขมันที่เปลือกตา ในโรคต่อมไขมันที่เปลือกตาอุดตัน และความสัมพันธ์ระหว่างไมโครไบโอมกับระดับไซโตไคน์ในน้ำตา

น.ส.อุบลวรรณ รสารักษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเวชศาสตร์คลินิก ไม่สังกัดภาควิชา/เทียบเท่า
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2565

Thesis Title	Composition and diversity of meibum microbiota in meibomian gland dysfunction and its correlation with tear cytokines levels
By	Miss Ubonwan Rasaruck
Field of Study	Clinical Sciences
Thesis Advisor	TANITTHA CHATSUWAN, Ph.D.

Accepted by the FACULTY OF MEDICINE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the FACULTY OF MEDICINE
(Associate Professor CHANCHAI SITTIPUNT)

THESIS COMMITTEE

..... Chairman
(Professor WASEE TULVATANA)

..... Thesis Advisor
(TANITTHA CHATSUWAN, Ph.D.)

..... External Examiner
(Professor SUTHILUK PATUMRAJ, Ph.D.)

อุบลวรรณ รสารักษ์ : องค์ประกอบและความหลากหลายของไมโครไบโอมของไขมันที่เปลือกตา ในโรคต่อมไขมันที่เปลือกตาอุดตัน และความสัมพันธ์ระหว่างไมโครไบโอมกับระดับไซโตไคน์ในน้ำตา. (Composition and diversity of meibum microbiota in meibomian gland dysfunction and its correlation with tear cytokines levels) อ.ที่ปรึกษาหลัก : อ. ดร.ธนิษฐา ฉัตรสุวรรณ

โรคต่อมไขมันเปลือกตาอุดตันนั้นมีสาเหตุหลักมาจากการอุดตันของต่อมไขมันที่เปลือกตา ทำให้เกิดการเปลี่ยนแปลงทั้งปริมาณและคุณภาพของไขมันที่เปลือกตา องค์ประกอบของไขมันที่เปลี่ยนแปลงไปอาจจะสัมพันธ์กับการเปลี่ยนแปลงสมดุลของเชื้อแบคทีเรียบนเยื่อบุผิวตาและการเพิ่มขึ้นของระดับไซโตไคน์ในน้ำตา เกิดการหนาตัวขึ้นของเยื่อบุผิวและการอุดตันของต่อมไขมันที่เปลือกตาตามมาในที่สุด งานวิจัยนี้เป็นการศึกษาแบบตัดขวาง โดยทำการวิจัยในผู้ป่วยโรคต่อมไขมันเปลือกตาอุดตันระดับความรุนแรงปานกลางถึงมาก จำนวน 44 คน เปรียบเทียบกับอาสาสมัครตาปกติที่อายุเท่ากัน และเพศเดียวกัน จำนวน 44 คน โดยอาสาสมัครทั้งสองกลุ่มจะถูกเก็บไขมันที่เปลือกตาเพื่อวิเคราะห์ไมโครไบโอมโดย 16s rRNA sequencing และเก็บน้ำตาเพื่อวิเคราะห์ระดับไซโตไคน์ จากผลการศึกษาพบว่าในกลุ่มผู้ป่วยโรคต่อมไขมันเปลือกตาอุดตันระดับความรุนแรงมากนั้นมีความหลากหลายของเชื้อแบคทีเรียที่ลดลง นอกจากนี้แล้วยังพบว่าผู้ป่วยโรคต่อมไขมันเปลือกตาอุดตันมีเชื้อแบคทีเรีย *Bacteroides* และ *Novosphingobium* เพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเทียบกับอาสาสมัครตาปกติ และมีปริมาณของระดับไซโตไคน์ชนิด IL-17A ในน้ำตามากกว่าอาสาสมัครตาปกติ อย่างไรก็ตาม ถึงแม้ว่า *Bacteroides* จะเป็นตัวบ่งชี้ทางชีวภาพในกลุ่มผู้ป่วยโรคต่อมไขมันเปลือกตาอุดตัน ผู้วิจัยกลับไม่พบความสัมพันธ์ระหว่าง *Bacteroides* กับปริมาณของระดับไซโตไคน์ชนิด IL-17A ในน้ำตา และพบความสัมพันธ์แบบผกผันกันระหว่าง *Bacteroides* กับปริมาณของระดับไซโตไคน์ชนิด IL-1β ในน้ำตา จากงานวิจัยนี้ยังไม่สามารถสรุปความสัมพันธ์ระหว่างไมโครไบโอมของต่อมไขมันที่เปลือกตาและระดับไซโตไคน์ในน้ำตาได้แน่ชัด แต่ปริมาณแบคทีเรีย *Bacteroides* และ *Novosphingobium* ที่เพิ่มขึ้นน่าจะมีบทบาทสำคัญในกลไกการเกิดต่อมไขมันเปลือกตาอุดตัน การวินิจฉัยและรักษาโรคต่อมไขมันเปลือกตาอุดตันโดยเฉพาะก่อนการผ่าตัดตานั้นจะมีบทบาทสำคัญในการลดการติดเชื้อจาก

สาขาวิชา เวชศาสตร์คลินิก ลายมือชื่อนิสิต .....

ปีการศึกษา 2565 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

6378008930 : MAJOR CLINICAL SCIENCES

KEYWORD: Meibomian gland dysfunction (MGD), blepharitis, ocular surface microbiota, bacteria, 16s rRNA, meibum, Cytokines

Ubonwan Rasaruck : Composition and diversity of meibum microbiota in meibomian gland dysfunction and its correlation with tear cytokines levels. Advisor: TANITTHA CHATSUWAN, Ph.D.

Meibomian gland dysfunction (MGD) is commonly caused by obstruction of the terminal meibomian gland duct, which is associated with alterations in the quantity and quality of the meibum. This may affect the composition of meibum microbiota, causing aberrant cytokine production, epithelial hyperkeratinization, and meibomian gland blockage. This cross-sectional study included 44 patients with moderate to severe MGD and 44 healthy controls, to determine the meibum microbiota by next-generation sequencing (NGS) and its association with tear cytokines levels. We observed reduced bacterial diversity in the meibum microbiota of patients with severe MGD. Significantly higher abundance of *Bacteroides* and *Novosphingobium,* and substantially higher IL-17A levels were detected in the MGD group. Despite being a biomarker for MGD, *Bacteroides* showed no correlation with IL-17A but a moderate negative correlation with IL-1β. The relationship between core meibum microbiota and tear cytokines levels remains to be clarified. However, a higher abundance of *Bacteroides* and *Novosphingobium* is speculated to has a key role in the pathophysiology of MGD. To reduce the risk of this particular bacterial infection, timely diagnosis and treatment for MGD are recommended, especially before ocular surgery.

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

Academic Year: 2022 Advisor's Signature .....

ACKNOWLEDGEMENTS

This research was supported by Ratchadapisek Sompoch Endowment Fund, Faculty of Medicine, Chulalongkorn University (GA64/020), The 90th Anniversary of Chulalongkorn University Scholarship, Chulalongkorn University, and Research Grant of The Medical Council of Thailand. I was supported under H.M. the King Bhumibhol Adulyadej's 72nd Birthday Anniversary Scholarship in the Master's Degree program. This thesis would not be accomplished without many supports. First of all, I would like to express my deepest appreciation to my supervisors: Tanittha Chatsuwan, Ph.D., Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Assoc. Prof. Ngamjit Kasetsuwan, M.D., and Thanachaporn Kittipibul, M.D., Department of Ophthalmology, Faculty of Medicine, Chulalongkorn University for their invaluable contribution and considerable encouragement throughout this thesis. I would also like to extend my gratitude to the thesis committee: Prof. Wasee Tulvatana, M.D., Department of Ophthalmology, Faculty of Medicine, Chulalongkorn University, chairman of the committee, and Prof. Suthiluk Patumraj, Ph.D., Department of Physiology, Faculty of Medicine, Chulalongkorn University, the external committee for insightful comments and constructive advice. I am also grateful to Yutida Photabthai and Alita Yeunyon for assisting data collection, Wannika sratongjoung for helping me with tear cytokines extraction and Aschana Tirapattanun for contributing to DNA extraction. I would like to recognize the assistance that I received from Pisut Pongchaikul, M.D., Ph.D. for biostatistics and bioinformatic data analysis. I sincerely appreciate the staff and colleagues of the Cornea and Refractive Surgery Unit, Department of Ophthalmology, Faculty of Medicine, Chulalongkorn University for providing patients' information. Furthermore, I would like to thank my family for their unwavering support. Last but not least, I am forever indebted to my husband Janekij Yamkasikorn, M.D. for always providing me with encouragement and patience.

Ubonwan Rasaruck

TABLE OF CONTENTS

Page

ABSTRACT (THAI) iii

ABSTRACT (ENGLISH) iv

ACKNOWLEDGEMENTS v

TABLE OF CONTENTS vi

LIST OF TABLES 1

LIST OF FIGURES 2

Abbreviations 3

CHAPTER I Introduction 4

Research questions 6

Research objectives 6

Primary objective 6

Secondary objectives 6

Conceptual Framework 7

CHAPTER II Literature review 8

1. Human microbiota 8

2. The molecular biological method 8

3. The healthy ocular surface microbiota 11

4. The ocular surface microbiota in patients with MGD 13

5. MGD and tear cytokines levels 16

CHAPTER III Materials and methods 18

1. Research design 18

2. Population 18
2.1 Target Population 18
2.2 Control Population 18
3. Recruitment and sampling techniques 18
4. Inclusion criteria 19
5. Exclusion criteria 21
6. Sample size calculation 21
7. Research methodology 22
7.1 Data collection 23
7.1.1 Baseline characteristics 23
7.1.2 Ocular evaluation 23
7.1.3 Sample collection 24
7.1.3.1 Tear samples collection 24
7.1.3.2 Meibum samples collection 24
7.2 DNA extraction from meibum samples 24
7.3 Next-generation sequencing analysis (NGS) 26
7.4 Bioinformatic data analysis 26
7.5 Negative control 27
7.6 Tear cytokines extraction 27
7.7 Data Analysis and Statistics 28
7.8 Ethical approval and clinical trial registration 28
CHAPTER IV Results 29
1. Next-generation sequencing analysis (NGS) 32
1.1 The alpha-diversity 32

1.2 The beta-diversity 34

1.3 Taxonomic composition of meibum microbiota 35

1.4 The core meibum microbiota 41

1.5 Differential abundance analysis 42

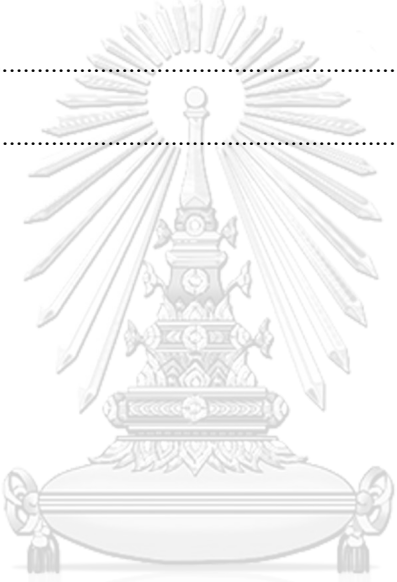
2. Correlation between core meibum microbiota and tear cytokines levels 43

CHAPTER V Discussion and Conclusion 46

Conclusion 51

REFERENCES 52

VITA 62



LIST OF TABLES

Page

Table 1. MGD severity grading scale 20

Table 2. Demographic information of patients with MGD and healthy controls 30

Table 3. Relative abundance of predominant meibum microbiota at phylum level 36

Table 4. Relative abundance of predominant meibum microbiota at genus level 36

Table 5. Relative abundance of significant bacteria at genus level 39

Table 6. Tear cytokines levels of MGD and HC 43

Table 7. Spearman correlations (r) between core meibum microbiota and tear cytokines levels 45

LIST OF FIGURES

	Page
Figure 1. Conceptual framework	7
Figure 2. The analysis of metagenomic sequencing	10
Figure 3. Sample size calculation	21
Figure 4. Ophthalmic parameters of patients with MGD and healthy controls	31
Figure 5. Rarefaction curve	32
Figure 6. α-diversity in meibum samples of MGD compared with HC	33
Figure 7. α-diversity in meibum samples of severe MGD, moderate MGD, and HC	33
Figure 8. Principal Coordinate Analysis (PCoA) of meibum samples of severe MGD, moderate MGD, and HC	34
Figure 9. Relative abundance at phylum level between MGD and HC	37
Figure 10. Relative abundance at phylum level between severe MGD, moderate MGD, and HC.	38
Figure 11. Relative abundance at genus level between MGD and HC	39
Figure 12. Relative abundance at genus level between severe MGD, moderate MGD, and HC.	40
Figure 13. Differential abundance analysis of meibum microbiota between MGD and HC	42
Figure 14. Heatmap of correlations between core meibum microbiota and tear cytokines levels	44
Figure 15. Composition and diversity of meibum microbiota in MGD and its correlation with tear cytokines levels	51

Abbreviations

ACE index	Abundance-based coverage estimator of species richness index
AMD	Age-related macular degeneration
ATD	Aqueous tear deficiency
DED	Dry eye disease
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
FGF2	Fibroblast growth factor 2
GVHD	Graft versus host disease
HC	Healthy controls
HIV	Human immunodeficiency virus
IFN-γ	Interferron gamma
IL	Interleukins
IPL	Intense pulsed light
MGD	Meibomian gland dysfunction
MIP-1α	Macrophage inflammatory protein 1 alpha
MMP-9	Matrix metalloprotease-9
NGS	Next-generation sequencing
NMOSD	Neuromyelitis optica spectrum disorder
OSDI	Ocular Surface Disease Index
OTU	Operational taxonomic unit
SJS	Stevens-Johnson syndrome
TBUT	Tear break-up time
16s rRNA	16s ribosomal ribonucleic acid

CHAPTER I Introduction

Modified sebaceous glands on the eyelid are called "meibomian glands." Meibomian glands are placed circumferentially along the eyelid margin, inside the tarsal plate. Each gland comprises a central excretory duct connected to multiple acini by short ductules. The opening of the central domain is at its distal end, located just anterior to the mucocutaneous junction of the eyelid margin. "Meibum" is the lipid component that is secreted by meibomian glands. It is an essential component of the outermost tear film layers. Healthy meibum slows tear evaporation, protects the eye from microbes, and preserves the clarity of the optical surface.[1] The chronic, diffuse abnormality condition of the meibomian gland is called "meibomian gland dysfunction (MGD)." MGD is a complex multifactorial disorder that consists of eyelid and conjunctival inflammation, corneal damage, tear film instability, and microbial changes. This condition is not only involved in alteration in meibum quality and quantity but also terminal duct obstruction.[2] The key pathophysiology of MGD is epithelial hyperkeratinization and more viscous meibum. Other relevant mechanisms are inflammatory process, bacterial overgrowth, progenitor cell differentiation and seborrhea. Multiple factors affect epithelial hyperkeratinization of the excretory duct and orifice of the meibomian gland, such as age, sex, hormone, and topical medication.

In patients with dry eye disease, increased evaporation of tear film from MGD occurs more often than dry eye from aqueous-deficient and decreased wettability.[3] Heiligenhaus, *et al*[4] stated that there were 77% of patients with dry eye disease that had evaporative dry eye and 11% of those that had aqueous tear deficient dry eye (ATD). Accordingly, Shimazaki, *et al*[5] also reported that 65% of patients with ocular discomfort symptoms had MGD. Moreover, 75% of those excluding aqueous tear deficiency dry eye were found to have evaporative dry eye. It may be concluded that patients with dry eye were mainly caused by MGD. The prevalence of MGD among Asian populations is higher than among Caucasians, which are 46% in the Bangkok study[6], 61% in the Shihpai study[7], 62% in the Japanese study[8], and

69% in the Beijing study[9]. On the contrary, the prevalence of MGD in Caucasians is 4% in the Salisbury study[10] and 20% in the Melbourne study[11].

When meibomian glands secrete abnormal meibum, the meibum often increases in lipid viscosity, leading to dysbiosis of microbiota on the ocular surface and pathogenic bacterial proliferation. Bacterial overgrowth produces lipid-degrading enzymes, toxic mediators, and inflammatory cytokines, which cause subclinical inflammation and epithelial hyperkeratinization.[1, 12] On the other hand, commensal bacteria on the ocular surface can also produce lipases and esterases, which are able to change the meibum composition[12], and subsequently lead to meibomian gland blockage.[13] Due to the fact that alteration of meibum composition can elevate the melting point of the lipid, meibum will be more viscous. Viscous meibum, together with desquamation of epithelial cells, finally leads to obstructive MGD.[1]

Due to some pathogenic bacterial proliferation, the diversity of meibum microbiota in patients with MGD may decrease compared with healthy controls. The alteration of the meibum composition may affect the proportion of meibum microbiota, which may activate the inflammatory response in the glandular environment. Glandular tissue inflammation affects glandular differentiation, lipid synthesis, and secretion.[14, 15] Understanding the diversity and composition of the meibum microbiota may help us clarify the pathophysiology of MGD. MGD severity is classified as mild, moderate, and severe.[16] Increased inflammatory products from specific types of bacteria may associate with the MGD severity. The previous study reported that the inflammatory process is strongly evident in moderate to severe MGD.[17] Meibomian gland obstruction elevates the intraglandular pressure and stimulates MAP kinase activity, producing inflammatory cytokines. Accordingly, tear inflammatory cytokines are potential biomarkers of MGD.[1] The specific tear inflammatory cytokines correlated with pain and clinical parameters of MGD were IL6, IL17A, and IL-1β.[17-20]

Until now, there has been a knowledge gap about "Does and how the pathogenic bacterial proliferation relates to the pathophysiology of MGD?". Thus, the research aims to find the meibum microbiota composition and diversity in patients

with meibomian gland dysfunction and its correlation with tear cytokines levels compared with healthy controls. Other factors that correlate with the alteration of ocular surface microbiota composition, which is also considered in this research, are divided into two groups. The first group is systemic host factors: age, geographic location, healthcare worker, diabetes mellitus, and oral antibiotics. The others are local host factors which are dry eye, contact lens use, ocular graft versus host disease (ocular GVHD), Stevens-Johnson syndrome (SJS), trachoma, and topical antibiotics. [21-24]

Research questions

Primary research question

What are the differences in meibum microbiota between patients with MGD and healthy controls?

Secondary research question

1. Does meibum microbiota in patients with MGD relate to tear cytokines release?
2. Does meibum microbiota affect the MGD severity?

Research objectives

Primary objective

To evaluate the composition and diversity of meibum microbiota in patients with MGD and healthy controls

Secondary objectives

1. To evaluate the correlation between core meibum microbiota / bacterial composition and tear cytokines levels
2. To evaluate the correlation between MGD severity and type of bacteria

Conceptual Framework

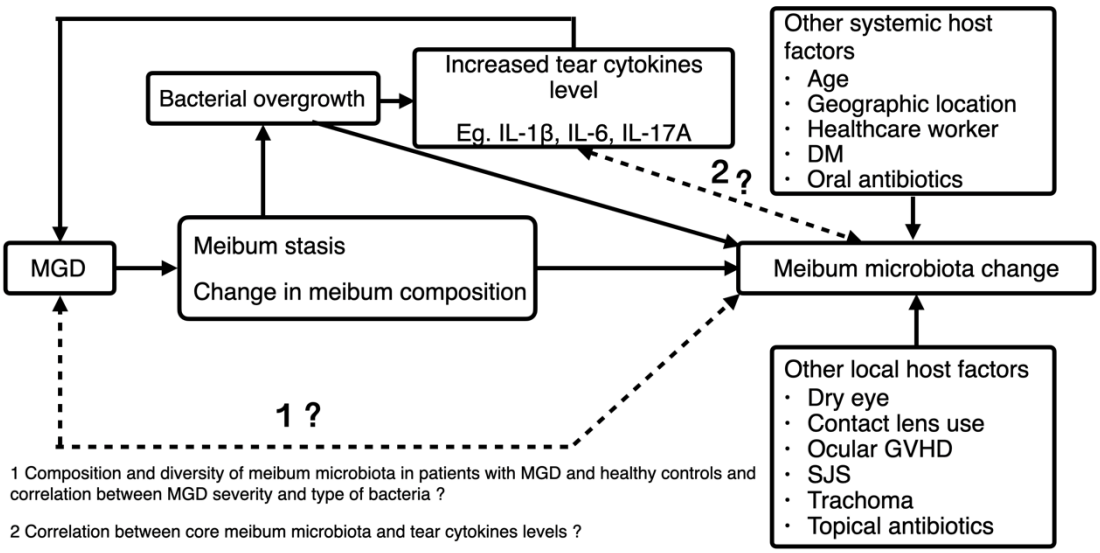


Figure 1. Conceptual framework

CHAPTER II Literature review

1. Human microbiota

A dramatic change in the study of microbial compositions occurred when 16S rRNA, a culture-independent molecular biological method, was applied.[25] To clarify, human microbiota is an array of microorganisms in the human body. A healthy microbiota contains richness and diversity, provides genetic, metabolic, and immunologic attributes, enhances metabolism, and diminishes infection and inflammation. The microbiome is the genetic component of the whole microorganism in that habitat.[26] Dysbiosis is qualitative and quantitative changes in microbial flora. Disruption of homeostasis will aggravate pathogenic bacterial proliferation.[27] Each human cell contains approximately 0.06 bacterium. However, 20–60% of human microbiota is unculturable due to the limited conditions, including atmospheric conditions, media types, and incubation period.[28]

The major ecosystems of the human body which contain the largest microbial communities are the skin, oral, nasal, gastrointestinal, and urogenital systems. The healthy microbiota should have high richness and diversity and composed of commensal bacteria that do not cause either infection or inflammation. Besides, it can inhibit pathogenic bacterial overgrowth.[26] Interestingly, the gut microbiota also affects the immunity of distant organs, including the ocular system. Many ocular conditions are related to the gut microbiota abnormalities, such as dry eye, infectious keratitis, diabetic retinopathy, scleritis, uveitis, glaucoma, NMOSD, and AMD.[29]

2. The molecular biological method

Apart from cultural technique, another method of bacterial identification is the molecular biological method, which shows a higher rate of bacterial detection and produces different results.[30] The molecular biological method is a culture-independent method which reported greater diversity of the ocular surface microbiota than previously known.[31] The whole genetic composition of the

microbiota is called metagenome. The metagenomic-based profiling of microbiota is the culture-independent sequencing-based approach composed of targeted approach and shotgun metagenomic sequencing. Targeted metagenomic sequencing will sequence specific amplified regions of the genome (16S and 18S rRNA). The 16S rRNA is the most frequently used target for bacteria. It consists of conserved and variable regions. Conserved regions are the PCR priming site associated with the bacteria's phylogenetic relationship. On the contrary, variable regions indicate the dissimilarity between species and benefits as amplifying regions.[32] The 16s rRNA sequencing method helps us clarify the bacterial community in the study population, but its drawback is low phylogenetic determination at the species level.[33] In contrast, shotgun metagenomic sequencing or whole genome sequencing analyzes total microbial strains, including bacteria, deep to species level, viruses, and fungi. Moreover, whole genome sequencing can explore functional and metabolic pathways as well. However, it is more expensive, less accessible, and unsuitable for the host DNA-riched biopsy specimen.[34]

The original method of 16s rRNA sequencing is capillary-based sequencing or Sanger sequencing. This method captures the total length of the 16s rRNA gene leading to very high accuracy. Its disadvantages are expensiveness, not cost-effectiveness, and time-consuming. Nowadays, Sanger sequencing has been replaced by next-generation sequencing (NGS) due to its high-throughput approach, reduced analytic time, and safe cost. NGS platform generates deeper sequencing of microbial communities. NGS processing is composed of library preparation, clonal amplification or cluster generation, and cyclic array sequencing. After sequencing, the RNA will be quality filtered and clustered in the operational taxonomic unit (OTU). Similarity more than 97% are classified as the same OTU. The accepted threshold level of dissimilarity is 3% for species and 5% for the genus. Many NGS platforms exist, such as Illumina, Ion-Torrent, and 454/Roche. The Illumina MiSeq platform can generate up to 250–300 bp reads, and samples total single region within a single read.[35]

The composition of the microbiota is defined as the taxonomic composition translated from the OTU. The ecological parameter consists of richness, diversity, and

evenness. Richness is defined as total bacterial types in the community. Diversity is the richness and relative abundance of the organism. Evenness is community equitability. The percent of relative abundance is used to compare the predominance of each type of bacteria. The α diversity index is used to compare diversity within one sample. Various parameters represent the α diversity index, such as the ACE index (Abundance-based coverage estimator of species richness), Chao1 index, observed-species index, Pielou index, Simpson's index, and Shannon diversity index. The more numbers of taxa and the more even abundance distribution result in, the more value of α diversity. β diversity index compares dissimilarity of the whole taxonomic composition between samples. The parameters for the β diversity index include Bray-Curtis dissimilarity indices, Jaccard's distance, weighted UniFrac distance, and unweighted UniFrac distance. [32] The results of β diversity are shown by principal coordinate analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA). The PCoA demonstrated that samples that cluster together have fewer different community structures. In comparison, PERMANOVA analysis shows how significant the difference in β diversity is. [36] (Figure 2.)

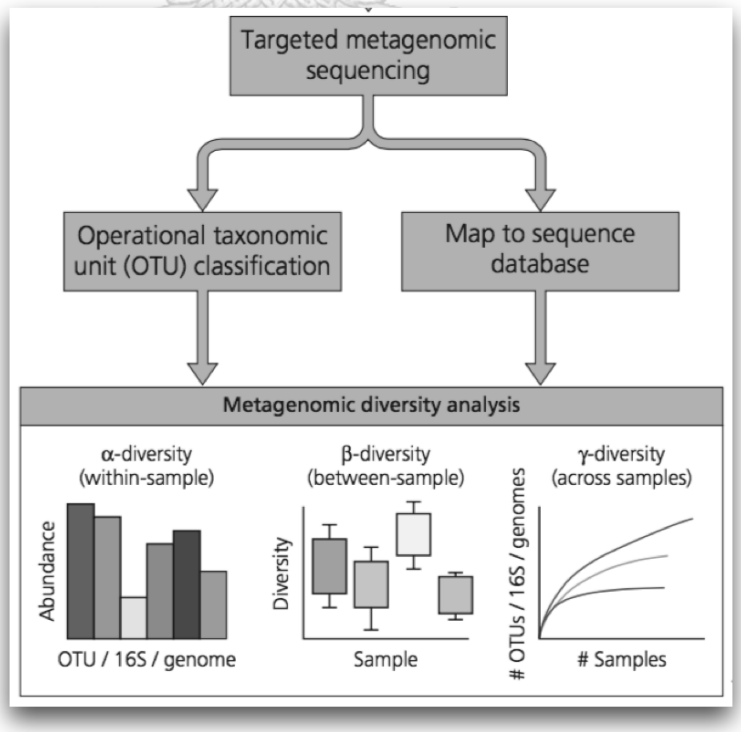


Figure 2. The analysis of metagenomic sequencing (reference: Mitreva 2017)

Core microbiota is defined as characteristics of microbial taxa in a particular environment, which may be different worldwide. There are three methods to quantify the core microbiota: occurrence only, relative abundance only, and abundance–occurrence. The occurrence only is commonly used in most of the studies, and any OTU detected in 30%-100% of samples, depending on various studies, is defined as core microbiota. This metric type includes rare taxa but does not provide much information. The following method is relative abundance only which taxa are determined to be core microbiota when it enriches in that environment. The controversy of this method is that some low abundance taxa may have an essential role in that community. Moreover, the relative abundance may change over time, so the metric type may miss some taxa that enrich in other time points. The abundance–occurrence combines the two previous methods, defining the minimal relative abundance threshold as 0.001% - 4.5% and the occurrence cut-off point as 50%-100%.[37]

3. The healthy ocular surface microbiota

The vast majority of microorganisms of ocular surface are bacteria. Additionally, studies showed that ocular surface has a paucibacterial microbiota which detected by conventional culture analysis.[38] Many factors correlate with the alteration of ocular surface microbiota composition, divided into systemic and local host factors. The systemic host factors included age, geographic location, healthcare worker, diabetes mellitus, and oral antibiotics. Local host factors are dry eye, contact lens use, ocular graft versus host disease (ocular GVHD), Stevens-Johnson syndrome (SJS), trachoma, and topical antibiotics.[21-24] Wen, *et al*[39] demonstrated that both age and sex affected the composition of healthy ocular surface microbiota, and age was the more substantial factor than sex. Deng, *et al*[40] reported that different geographic locations and travel habits also affected the composition of conjunctival microbiota in healthy subjects.[40]

By the culture-dependent method, the core ocular surface microbiota was composed of *coagulase-negative Staphylococci, Staphylococcus aureus Streptococcus, Propionibacterium* spp., and *Corynebacterium* spp. However, as

mentioned previously, unculturable bacteria can be missed. In 2007, 16S rRNA amplicon sequencing was introduced to ocular surface microbiota research for the first time. Graham, *et al*[41] found that *coagulase-negative Staphylococci* were the most common bacteria detected by the culture method. In comparison, 16s rRNA sequencing demonstrated various bacteria types, including *Rhodococcus erythropolis, Klebsiella oxytoca,* and *Erwinia* sp. Dong, *et al*[42] reported *Pseudomonas, Propionibacterium, Bradyrhizobium, Corynebacterium, Acinetobacter, Brevundimonas,* and *Staphylococci* as the most abundant genera of conjunctival microbiota in 4 healthy subjects. Zhou, *et al*[43] found that the most abundant genera in healthy conjunctival sacs were *Corynebacterium, Streptococcus, Propionibacterium, Bacillus,* and *Staphylococcus*. On the contrary, they reported less than 1% relative abundance of *Pseudomonas*, which were core microbiota in the conjunctival sac of the previous study.

Delbeke, *et al[44]*reported that the main microbial flora on the ocular surface of healthy population at the phylum level was composed of Actinobacteria (*Corynebacterium* and *Propionibacterium*), Proteobacteria (*Acinetobacter* and *Pseudomonas*) and Firmicutes (*Staphylococcus* and *Streptococcus*) with a relative abundance of 53%, 39%, and 8% respectively. At the genus level, the abundance microbiota were *Pseudomonas, Corynebacterium, Propionibacterium, Acinetobacter, Staphylococcus,* and *Streptococcus*.[2, 38, 45, 46] *Bacillus* was relatively abundant when present, although not incorporated in the core ocular surface microbiota.[30, 47-49] Sites of ocular surface microbiota which were eyelid skin, conjunctiva, and meibum, also had diversity in microbiota composition.[30, 50, 51] Factors influencing ocular surface microbiota included age and geographic location. However, sex had no significant impact on the ocular surface microbiota. Ocular surface microbiota changes from birth to adulthood.[2, 52] On the contrary, a previous study (Wen et al. 2017) demonstrated the different β diversity between male VS female and younger VS old adults. The aging group also contained greater antibiotic resistance genes. They concluded that the aging process is associated with changes in ocular surface microbiota.[39] The other study found that sex-steroid imbalance in menopause is

related to the alteration of the ocular surface microbiota and predisposed to allergic, autoimmune, and inflammatory-related ocular conditions.[53]

Suzuki, *et al*[54] found that aging causes in reduction of ocular surface microbiota diversity which may result in dysbiosis. The meibum had a high microbiota diversity, especially in young subjects (20-35 years old). But 30% of subjects in aging groups (60-70 years old) had low diversity of the meibum microbiota, predominated by *Corynebacterium* sp. or *Neisseriaceae*. The microbiota in the meibum was similar to that of the conjunctival sac in young subjects. However, the microbiota in the conjunctival sac resembled eyelid skin in aging.[54]

A healthy ocular surface is protected from microbial invasion via immune tolerance. This mechanism is the crosstalk between the microbiota and the epithelial cell's immune receptor that controls innate and adaptive immune responses. Consequently, microbiota diversity and composition alteration can aggravate the inflammatory immune response. The primary innate immune response is located at the epithelial receptor, pathogen-associated molecular pattern (PAMP) receptors. This receptor regulates the gene's transcription factor associated with cytokine production, such as IL-1, IL-17, and TNF- α. Regulatory T cells (Treg), the adaptive immune response, control immune tolerance to specific microbial antigens. The tolerance mechanism results in no inflammatory response on a healthy ocular surface.[26]

4. The ocular surface microbiota in patients with MGD

The ocular surface is vulnerable to contamination with microbes because it is persistently exposed to the external environment. There is no inflammatory response from the cornea and conjunctival epithelium in healthy subjects, supporting the evidence of colonization by commensal microbiota.[55] However, the alteration of microbiota composition leads to proinflammatory states and is associated with many ophthalmic conditions such as dry eyes, contact lens wearers, trachoma, diabetes mellitus, and Stevens-Johnson syndrome.[56-58] In MGD, some bacterial overgrowth results in the production of lipases and esterases, increased

melting temperature, and viscosity of the meibum, causing meibomian gland blockage.[13]

Changes in the meibum composition may affect the composition of meibum microbiota, which may activate the inflammatory response in the glandular environment. Glandular tissue inflammation affects glandular differentiation, lipid synthesis, and secretion.[14, 15] Topical azithromycin and oral doxycycline were introduced in the treatment of MGD due to their anti-inflammatory and antibacterial effects. In respiratory diseases, macrolides were associated with reduced IL-8, IL-6, TNF- α release, neutrophil, macrophage function stimulation, and breakdown and prevention of microbial biofilm formation. Although multiple clinical studies supported topical azithromycin and oral doxycycline in MGD patients, their mechanisms in alleviating MGD symptoms are not yet clearly understood.[16]

Zhang, *et al*[59] studied microbiota in conjunctival sac and meibum in patients with MGD by conventional culture technique. They found that MGD patients had a higher positive bacterial isolation rate than healthy controls in both meibum and conjunctival sac samples. The predominant bacteria were *Staphylococcus epidermidis* and *Propionibacterium acne*. A more complex bacterial profile was found in the MGD group. Conversely, Watters, *et al*[60] found no difference in bacterial composition in patients with DED with and without MGD. Mostly, the ocular surface microbiota mentioned in global MGD populations were *coagulase-negative Staphylococci, Staphylococcus aureus, Propionibacterium acnes,* and *Corynebacterium sp.* Still, the proportions varied across studies. [60]

Jiang, *et al*[61] studied microbiota in conjunctival sac and meibum in patients with MGD. Genomic DNAs of isolated strains from the bacterial culture were extracted and processed by 16s rRNA sequencing. They found that three components were associated with increased MGD severity: a higher isolation rate, a larger number of bacterial species, and greater bacterial severity. Accordingly, MGD might be related to changes in bacterial composition. They demonstrated a higher rate of bacterial isolation in the meibomian gland secretions compared to that in the conjunctival sac. They also found that some bacteria, *Staphylococcus,* Microbacteriaceae*, Micrococcus luteus,* and *Bacillus* had a significantly higher positive

bacterial identification rate in the meibum than in the conjunctival sac. *Corynebacterium macginleyi* was only found in the severe MGD group, associated with corneal ulcer and conjunctivitis. The bacteria that were isolated only from meibum consisted of *Bacillus, Paenibacillus,* and *Lysinibacillus*, indicating that the meibum had a more complex bacterial composition than the conjunctival sac. In addition, they also performed segmental meibomian gland secretion analysis and found that gram-negative bacterium Xanthomonadaceae was only found in the deep layers. This finding confirmed that the meibum environment differed from the conjunctival sac environment. The culture-based method can identify the bacteria deep to species level and measure the bacterial density. However, unculturable organisms are missed.

There are small amounts of published papers that study ocular surface microbiota in patients with MGD by the molecular biological method. Lee, *et al*[62] studied the microbiota in the eyelashes and tears samples of patients with blepharitis by 16s rRNA sequencing. They found a higher abundance of *Streptophyta*, *Corynebacterium*, and *Enhydrobacter* and a lower abundance of *Propionibacterium* in the blepharitis group, especially in tears samples. However, the study involved anterior, posterior, and mixed blepharitis.

Dong, *et al*[2] investigated the microbiota in conjunctival sacs of patients with MGD via 16S rDNA sequencing. They observed no significant difference in the **α** diversity index, but the PCoA showed distinct clusters between severe MGD and the other groups. A higher abundance of *Staphylococcus* and *Sphingomonas* and a lower abundance of *Corynebacterium* were detected in patients with MGD. Moreover, MGD severity was correlated with the abundance of *Staphylococcus* and the meiboscore.

Zhao, *et al*[14] used shotgun metagenomic analysis to evaluate the meibum microbiota. They found that the predominant genera in the MGD meibum were *Rubrobacter, Novibacillus, Campylobacter, Geobacillus, Sphingomonas, Corynebacterium, Sphingobium, Pedobacter, Fictibacillus,* and *Enterococcus*. Moreover, meibum was composed of a large amount of *Campylobacter coli, Campylobacter jejuni,* and *Enterococcus faecium,* which had immune invasion properties and were rarely found in healthy controls. They also found that the

meibum in patients with MGD had less pathogen diversity than the healthy, which was different from the previous study.[61] In addition, most of the microbiota in patients with MGD could metabolize benzoate, which can survive in an unstable oxygen environment. The composition of ocular surface microbiota was quite different across the studies, which may be affected by diverse populations, climates, and geographic locations.

5. MGD and tear cytokines levels

Potential markers of inflammation in DED are tear cytokines levels. Other factors that affect the increment of tear cytokines levels include aging, HIV infection, and diabetic retinopathy, but there is no sex predilection.[63] The majority of patients with dry eye are in moderate form.[64] From a previous study, elevated tear cytokines levels was often found in the severe form of DED and consistently failed to be detected in the moderate form of DED.[65, 66] Amalia, *et al*[17] studied tear cytokines and chemokines levels in patients with moderate MGD. Four of the 15 molecules were elevated: EGF, IL-1Ra, IL-6, and IL-8. The amounts of tear cytokines elevation corresponded with pain and clinical parameters that evaluate tear film stability, tear production, and corneal and conjunctival integrity. They concluded that the pathophysiology of both moderate and severe forms of MGD involves the inflammatory process. Landsend, *et al*[18] also found a high prevalence of DED and MGD in patients with aniridia. The MGD parameters, which consisted of glandular atrophy and short tear break-up time (TBUT), were associated with multiple tear cytokines levels, which were FGF2, IL-1β, IL-9, IL-17A, and MIP-1α. Zhang, *et al*[19] found that treatment of MGD with 1% azithromycin eye drop also reduced various tear cytokines levels, including IL-1β, IL-8, and MMP-9. These reductions were reversed to pretreatment levels after cessation of azithromycin treatment. Accordingly, Liu, *et al*[20] studied the effect of IPL (intense pulsed light) on MGD treatment and its correlation with tear cytokines levels. They found that IL-6 and IL-17A levels were correlated with ocular surface parameters of the lower eyelid before IPL treatment. The treatment also caused a reduction in the amounts of IL-6 and IL-

17A. Tong, *et.al[63]* found that IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17α and TNF-α were correlated with eyelid crusting. Nevertheless, they did not use other parameters to make a definite diagnosis of MGD. As previously reviewed, common tear cytokines associated with MGD are IL-6, IL-17A, and IL-1β.

Several methods for tear sample collection were proposed, including water eye wash [67], Weck Cell Sponge[66], capillary tubes[68], and cellulose acetate absorbent filters.[69] The tear samples collection by Schirmer strips was recently introduced because we routinely use these strips for tear volume measurement. Luminex multiplex analysis is the technology that is based on flow cytometry which allows for measuring multiple cytokines in a single well. This method is not only comparable to cytokines measurement by ELISA assay but also capable of measuring small-volume samples. A previous study reported numerous cytokines and MMPs detection in tears samples collected via Schirmer strips.[70]

CHAPTER III Materials and methods

1. Research design

A cross-sectional, observational analytic study

2. Population

Participants with moderate to severe MGD and healthy controls

2.1 Target Population

Patients with moderate to severe MGD from outpatient clinic, Department of Ophthalmology, King Chulalongkorn Memorial Hospital

2.2 Control Population

Healthy controls which were non-dry eye participants from an outpatient clinic, Department of Ophthalmology, King Chulalongkorn Memorial Hospital

3. Recruitment and sampling techniques

The participants were recruited from patients at outpatient clinic, Department of Ophthalmology, King Chulalongkorn Memorial Hospital. The investigators broadcasted the research project to the ophthalmologist in the clinic to help collect patients. The poster was also utilized to give information about the research and contact details. The participants were recruited via convenient sampling through consecutively participant collection that matched the study criteria.

4. Inclusion criteria

Patients with moderate to severe MGD [16, 71, 72]

1. Patients aged 40-80 years with OSDI ≥ 13
2. Tear break up time (TBUT) < 10 seconds OR ocular surface staining by fluorescence was consistent with the diagnosis of dry eye (> 5 corneal spots or > 9 conjunctival spots or lid margin staining ≥ 2mm length and ≥ 25% width)
3. Schirmer test without anesthesia ≥ 5mm/5min
4. A diagnosis of moderate to severe MGD (MGD stage 3-4), according to the International Workshop on Meibomian Gland Dysfunction: Report of the Subcommittee on Management and Treatment of Meibomian Gland Dysfunction 2006 (See Table.1)
5. If both eyes were involved, the investigator (U.R.) randomly selected only one eye by coin tossing.

Healthy controls: Non-dry eye participants

1. Age- and sex-matched volunteers aged 40-80 years with OSDI scores < 13
2. Had never been diagnosed with dry eye
3. TBUT ≥ 10 seconds
4. Ocular surface staining by fluorescence was not consistent with the diagnosis of dry eye (≤ 5 corneal spots or ≤ 9 conjunctival spots or lid margin staining < 2mm length and < 25% width).
5. Lid and meibomian gland evaluation were not consistent with the diagnosis of MGD. (See Table.1)
6. If both eyes were involved, the investigator (U.R.) randomly selected only one eye by coin tossing.

Table 1. MGD severity grading scale (Modified from[16])

Severity	Stage	Symptoms	Clinical signs	Meibum quality*	Meibum expressibility**	Oxford grading system
Mild	1	No discomfort, itching, or photophobia	Based on gland expression	2 to 3	1	No staining
Mild	2	Mild symptoms of ocular discomfort, itching, or photophobia	Scattered lid margin features	4 to 7	1	0 to 3
Moderate	3	Moderate symptoms of ocular discomfort, itching, or photophobia with limitations of activities	Lid margin vascularity Plugged meibomian orifice	8 to 12	2	4 to 10
Severe	4	Marked symptoms of ocular discomfort, itching, or photophobia with definite limitation of activities	Displacement of the mucocutaneous junction	$\geq$ 13	3	11 to 15

* Meibum quality is assessed at 8 glands in central third of lower eyelid : grade 0 = clear, grade 1 = cloudy, grade 2 = cloudy with granular debris, grade 3 = thick, like toothpaste

**Meibum expressibility is assessed at 5 glands in central third of lower eyelid : grade 0 = all, grade 1 = 3-4, grade 2 = 1-2, grade 3 = 0

5. Exclusion criteria

Both patients with MGD and healthy controls[73]

1. History of topical or systemic antibiotics treatment within three months

2. History of topical ophthalmic drops within three months (except for preservative-free artificial tears)

3. History of contact lens wear within three months

4. Ongoing ocular allergy, infection, or inflammation irrelevant to dry eye or MGD

5. History of ocular surgery within six months

6. History of systemic conditions that had an effect on the ocular surface

7. Healthcare workers, pregnancy, mentally ill person

6. Sample size calculation

Calculate the sample size of participants in each group

Compare 2 Means: 2-Sample, 2-Sided Equality [74]

$$n_A = \kappa n_B \text{ and } n_B = \left(1 + \frac{1}{\kappa}\right)\left(\sigma \frac{z_{1-\alpha/2} + z_{1-\beta}}{\mu_A - \mu_B}\right)^2$$

$$1 - \beta = \Phi\left(z - z_{1-\alpha/2}\right) + \Phi\left(-z - z_{1-\alpha/2}\right) \quad , \quad z = \frac{\mu_A - \mu_B}{\sigma\sqrt{\frac{1}{n_A} + \frac{1}{n_B}}}$$

where

$\kappa = n_A/n_B$ is the matching ratio
σ is standard deviation
Φ is the standard Normal distribution function
Φ^{-1} is the standard Normal quantile function
α is Type I error
β is Type II error, meaning $1 - \beta$ is power

Figure 3. Sample size calculation

Relative abundance of most predominant bacteria, which is *Staphylococcus aureus*[2]

Healthy controls : 8% (0.1-45.9%)

Clinical significance = 8%

Pooled SD = 11.89

α = 0.05, β = 0.20

N = 35 with clinical significance = 8%

If we calculate dropout rate at 20%, the sample size will become 44 per group

N, MGD patients = 44

N, Healthy controls = 44

7. Research methodology

The investigators conducted this study according to the tenets of the Declaration of Helsinki. The study was approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University. The study was conducted at the Department of Ophthalmology, King Chulalongkorn Memorial Hospital. The investigator (U.R.) gave the information sheet and explained the process of specimen collection, benefits, and risks of enrollment to the participants who met the inclusion and exclusion criteria. The investigator answered all questions until they clearly understood. Voluntary informed consent was obtained from patients with MGD and healthy controls before the enrollment. The volunteers could take time to make an independent decision before signing the consent. The investigator used the OSDI questionnaire to evaluate the ocular symptoms of the volunteers. All volunteers were examined by a slit lamp biomicroscope. Tear break-up time was measured by fluorescence instillation. Fluorescence staining was graded by Oxford grading. Lid features, meibum expressibility, and quality were recorded sequentially. All volunteers were appointed for the collection of tears for cytokines detection and meibum for 16s rRNA sequencing by NGS method.

7.1 Data collection

7.1.1 Baseline characteristics

- Age
- Sex
- Laterality
- Underlying ocular and systemic disease
- Current medications
- Geographic location (Province)
- Career

7.1.2 Ocular evaluation

- Ocular symptoms by OSDI score (Allergan Inc, Irvine, Calfornia, holds the copy-right) (0 to 100) [75]

Normal	0 to 12
Mild	13 to 22
Moderate	23 to 32
Severe	33 to 100

Clinical significance was defined as equal to or more than 7 unit change in score

- TBUT: by commercial fluorescence, blue exciter, yellow filter seconds (sensitivity 82%, specificity 86% to differentiate normal VS dry eye)[72]
- Schirmer test without anesthesia
- Conjunctiva and corneal staining by Oxford grading system (0 to 15)
- Lid assessments

- Lid margin irregularities
- Lid margin vascularities
- Plugged meibomian orifices
- Displacement of mucocutaneous junction (MCJ)

- MG assessments: meibum quality, meibum expressibility

7.1.3 Sample collection

7.1.3.1 Tear samples collection

Tear samples were collected by Schirmer strip in sterile technique. The investigator, with sterile gloves, placed a Schirmer strip over the lower eyelid margin without anesthesia. The proper location was at the lateral one-third of the lower eyelid. The volunteers were requested to close their eyes for 5 minutes. After that, the Schirmer strip was removed. The investigator recorded tear volume in a millimeter unit. The strip was stored in a sterile 2-ml centrifuge tube. Then the examiner kept the tube in the ice-filled container for 20-60 minutes. The container was stored at -80 degrees Celsius until the process of cytokines extraction.

7.1.3.2 Meibum samples collection

Meibum sample was collected after tear sample collection at the same visit with a sterile glove. The eyelid margin was sterilized with 10% povidone-iodine, cleaned with sterile saline, and wiped with a dry swab. Meibum was squeezed using a meibomian gland compressor and collected with a dry sterile swab. The swab was rolled from the innermost to the outermost corners of the eyelid and vice versa, starting with the lower eyelid margin and upper eyelid margin consequently. After that, the swab was placed into a DNase-free tube with DNA/RNA shield solution (Zymo, CA, USA). The sterile procedure was carefully done to ensure that the specimen would not be contaminated. The samples were stored at –20°C for further analysis.

7.2 DNA extraction from meibum samples

The DNA extraction process was performed at the laboratory unit of the Department of Microbiology, Faculty of Medicine, Chulalongkorn University. The QIAmp® DNA Microbiome Kit (QIAGEN, Hilden, Germany) was utilized in the DNA extraction process followed this protocol

1. 1 ml of sample was placed in a 2 ml tube with the addition of 500 µL Buffer AHL. The sample was incubated with end-over-end rotation at room temperature for 30 minutes.

2. The tube was centrifuged at 10,000 x g for 10 minutes. After that, the supernatant was removed.

3. 190 µL of Buffer RDD and 2.5 µL of Benzonase were added and mixed well. The tube was incubated at 37 °C for 30 minutes at 600 rpm in a heating block.

4. 20 µL of Proteinase K was added. The incubation process was performed at 56 °C for 30 minutes at 600 rpm in a heating block or water bath and was briefly spun at slow speed.

5. 200 µL of Buffer ATL (containing Reagent DX) was added, mixed well, and transferred into Pathogen Lysis Tube L. The Pathogen Lysis Tube L was placed into a Fastprep® 24 instruments, applying a velocity of 6.5 m/s twice for 45 seconds with a 5 minutes intermission, while samples were stored on ice.

6. The Pathogen Lysis Tube L was centrifuged at 10,000 x g for 1 minute. The supernatant was transported to a fresh microcentrifuge tube. After adding 40 µL of Proteinase K into the microcentrifuge tube, the tube was vortexed to mix and incubated at 56 °C for 30 minutes at 600 rpm in a heating block.

7. 200 µL of Buffer APL2 was added and mixed by pulse-vortexing for 30 seconds. The tube was incubated at 70 °C for 10 minutes and briefly spun.

8. 200 µL of ethanol was added to the lysate and mixed by pulse-vortexing for 15-30 seconds. Up to 700 µL of this mixture was applied to the QIAamp UCP Mini spin column without wetting the rim. The cap was closed, and the centrifuge was performed at 6,000 x g for 1 minute. The flow-through was removed, and the column was put back into the same collection tube. The procedure was repeated with any remaining ethanol-lysate mixture.

9. The QIAamp UCP Mini spin column was transferred to a fresh collection tube. The cap was opened, and 500 µL of Buffer AW1 was added without wetting the rim. The cap was closed, and the centrifuge was performed at 6,000 x g for 1 minute. The QIAamp UCP Mini spin column was placed into a fresh 2ml collection tube, and the filtrate was discarded.

10. The QIAamp UCP Mini spin column was opened, and 500 µL of Buffer AW2 was added without wetting the rim and centrifuged at 20,000 x g for 3 minutes.

11. The QIAamp UCP Mini spin column was placed into a fresh 2ml collection tube, and the filtrate was discarded. The centrifuge was performed at 20,000 x g for 1 minute.

12. The QIAamp UCP Mini spin column was placed into a fresh 1.5ml collection tube, and 50 µL of Buffer AVE was added directly onto the center of the membrane. The lid was closed, and the incubation process was performed at room temperature for 5 minutes. The tube was centrifuged at 6,000 x g for 1 minute to elute the DNA.

Consequently, the extracted specimens were transferred to the Omics Sciences and Bioinformatics Center, Chulalongkorn University as soon as possible with no longer than 1 week period. The next step was 16s rRNA sequencing via the Next-generation sequencing method (NGS).

7.3 Next-generation sequencing analysis (NGS)

The NGS method was performed by an Illumina MiSeq (Illumina, CA, USA). After 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). The amplification process involved an initial denaturation step for 2 minutes at 98 °C, 30 cycles of 98 °C for 20 seconds, 55 °C for 30 seconds, and 72 °C for 1 minute, accompanied by a final extension step at 72 C for 1 min. Afterward, the 16S amplicon was purified by sparQ Puremag Beads (QuantaBio, USA), indexed by 2.5 µl of each Nextera XT index primer in a 25 µl PCR reaction and ten cycles of PCR condition above. The final PCR products were cleaned, pooled, and diluted to the final loading concentration at 4 pM. Lastly, Cluster generation and 250-bp paired-end read sequencing were conducted on an Illumina MiSeq (Illumina, CA, USA) at Omics Sciences and Bioinformatics Center, Chulalongkorn University, Bangkok, Thailand.[54]

7.4 Bioinformatic data analysis

Paired-end reads were qualified and quantified using the FastQC software (Version 0.11.8, Babraham Institute, Cambridge, UK). Chimeric sequences were removed using VSearch software (version 2.21, http://drive5.com/usearch/). OTU were assigned and used to perform alpha diversity, beta diversity, and differential

abundance analyses. Alpha diversity, including rarefaction curve, ACE, Chao1, Pilou, Shannon, and Simpson indices, and the beta diversity were calculated using the MicrobiotaProcess package in R (version 1.8.2; R Foundation for Statistical Computing, Vienna, Austria). Differential abundance was analyzed using a negative binomial distribution-based model in the DESeq2 package, implemented in R. Briefly, the tables containing OTUs, taxa, and meta-table were combined and converted into phyloseq format, which is the standard format for microbiome analysis. Analysis was conducted using the DESeq2 function with an adjusted p-value of 0.01.

7.5 Negative control

The negative control was collected from the sterile cotton swab, placing in the DNase-free tube with DNA/RNA shield solution (blank swab). It was collected during the period of the sample collection. The negative control showed no bacterial detection.

7.6 Tear cytokines extraction

The process of tear cytokines extraction was performed at the central laboratory unit, Faculty of Medicine, Chulalongkorn University. The assay buffer used in this process was 200 µL of 1% BSA in phosphate-buffered saline (PBS) with the addition of sodium azide for preservation. The PBS with sodium azide was put into each centrifuge tube. Subsequently, the tube was incubated on a rocker at 25 °C for 3 hours and kept in an ice-filled container. Each Schirmer strip was moved to a new centrifuge tube. The strip was placed at the 25 mm mark at the sealed tube cap. The new centrifuge tube with Schirmer strip was centrifuged at 100g for 10 seconds. Afterward, the new tube was mixed with the old one containing residual tears and buffer. The concentration of tear cytokines (pg/ml) was analyzed by Luminex using a cytokine kit.[70]

7.7 Data Analysis and Statistics

1. Descriptive statistics for baseline characteristics (age, sex, laterality, underlying ocular, and systemic disease)

2. Student's t-test for comparison of OSDI score, TBUT, Schirmer test, Oxford score, and meibum quality between patients with MGD and healthy controls

3. Mann-Whitney U test for comparison of α diversity index and relative abundance of dominant phyla and genera between patients with MGD and healthy controls

4. One-way ANOVA was used to compare OSDI score, TBUT, Schirmer test, Oxford score, meibum quality, α diversity index, relative abundance of dominant phyla and genera, and tear cytokines levels between severe MGD, moderate MGD, and healthy controls.

5. Fisher's exact test for comparison of lid margin irregularities and plugged MG orifices between severe MGD, moderate MGD, and healthy controls.

6. Chi-squared test for comparison of lid margin vascularities and displacement of MCJ between severe MGD, moderate MGD, and healthy controls.

7. Spearman correlation for correlation between core meibum microbiota and tear cytokines levels

8. P-values less than 0.05 are considered statistically significant

All statistical analyses were performed in R.

7.8 Ethical approval and clinical trial registration

The authors conducted this study according to the tenets of the Declaration of Helsinki. This study was approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University with IRB number 289/64 and COA number 920/2021 and was registered in Thai Clinical Trials Registry with TCTR number TCTR20210221002.

CHAPTER IV Results

Eighty-eight meibum and tear samples were collected from 44 eyes of 44 patients with moderate to severe MGD (MGD) and 44 eyes of 44 age-sex-matched healthy control participants (HC). We enrolled 29 males (33%) and 59 females (67%), with a mean age of 61.2 in the MGD and 59.8 in HC. There were no statistically significant differences in underlying ocular and systemic diseases between groups. The demographic data are shown in Table 2. and Figure 4.

The mean OSDI score was 32.6 in severe MGD, 18.3 in moderate MGD, and 6.8 in HC, which was statistically significantly higher in severe MGD compared with moderate MGD, severe MGD compared with HC, and moderate MGD compared with HC. The mean TBUT was 4.6 sec in severe MGD, 4.9 sec in moderate MGD, and more than 10 sec in HC, which was statistically significantly higher in severe MGD compared with HC, and moderate MGD compared with HC. The mean Schirmer score between the three groups was not statistically significant difference which was 10.1 in severe MGD, 9.6 in moderate MGD, and 15.3 in HC. The mean Oxford staining score was 3.9 in severe MGD, 3.4 in moderate MGD, and 0,3 in HC, statistically significantly higher in severe MGD compared with HC and moderate MGD compared with HC.

Focusing on lid assessment, we detected lid margin irregularities in 46% of severe MGD, 17% of moderate MGD, and none of HC. Lid margin vascularity was detected in all patients with MGD and 11% of HC. Plugged MG orifices were detected in 27% of severe MGD, 22% of moderate MGD, and none of HC. These three parameters were detected as statistically significantly higher in severe MGD compared with HC and moderate MGD with HC. Displacement of MCJ was only found in severe MGD in 65% of patients. The mean meibum quality score was 18.3 in severe MGD, 10.3 in moderate MGD, and 0 in HC, which was statistically significantly higher in severe MGD compared with moderate MGD, severe MGD compared with HC, and moderate MGD compared with HC.

	Severe MGD	Moderate MGD	Healthy controls	p-value (HC vs. MGD)	p-value* (3 groups)
No.	26	18	44		
Age (mean ± SD)	61.9 ± 9.8	60.1. ± 9.9	59.8 ± 10.3		
Sex (female:male)	12 : 14	17 : 1	30 : 14		
Laterality LE RE	 11 15	 11 7	 20 24		
OSDI (Mean ± SD)	32.6 ± 16.7	18.3 ± 3.5	6.8 ± 4.6	< 0.001	<0.001
TBUT (Mean ± SD)	4.6 ± 1.3	4.9 ± 1.1	> 10	< 0.001	<0.001
Schirmer test (Mean ± SD)	10.1 ± 8.6	9.6 ± 4.5	15.3 ± 10.6	0.006	0.022
Oxford (Mean ± SD)	3.9 ± 1.5	3.4 ± 1.9	0.3 ± 0.7	< 0.001	<0.001
Lid assessment - Lid margin irregularities - Lid margin vascularity - Plugged MG orifices - Displacement of MCJ	 12 26 7 17	 3 18 4 0	 0 5 0 0		 < 0.001 < 0.001 0.002 < 0.001
Meibum quality (Mean ± SD)	18.3 ± 5.2	10.3 ± 1.7	0	< 0.001	< 0.001

TBUT, tear film break-up time

* One-way ANOVA

Table 2. Demographic information of patients with MGD and healthy controls

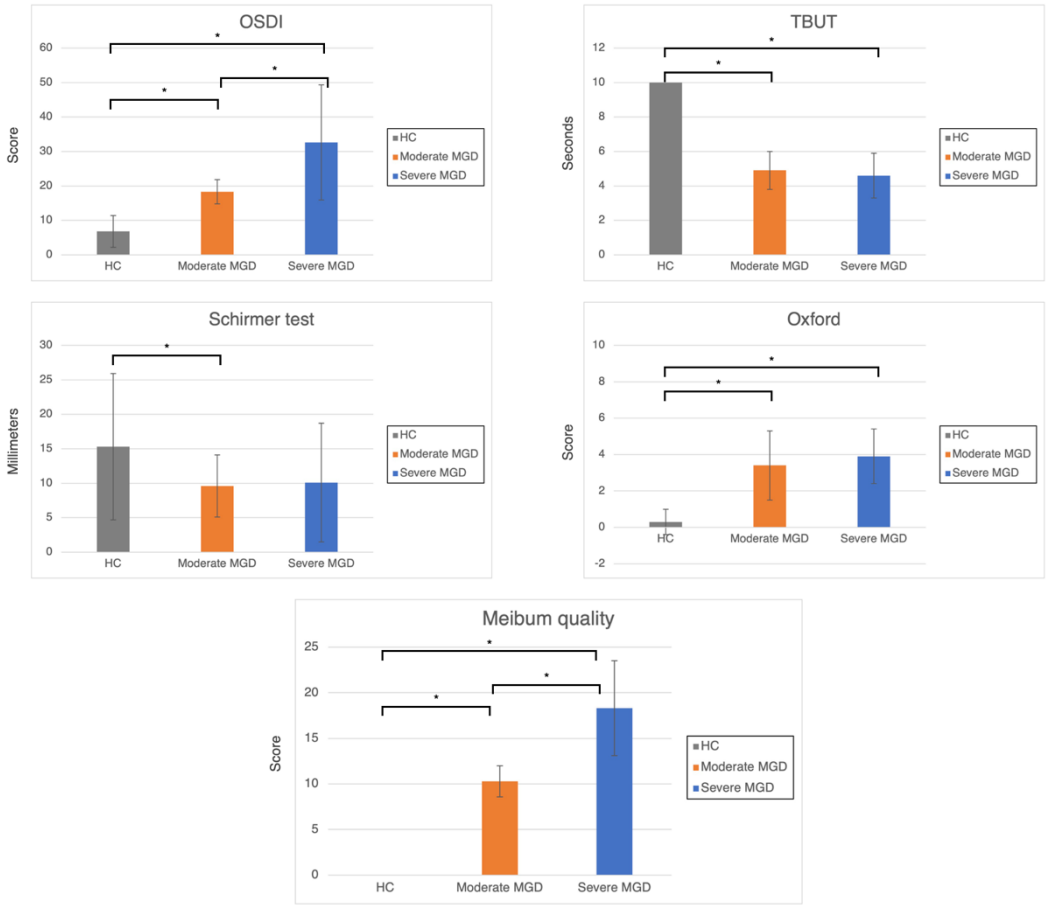


Figure 4. Ophthalmic parameters of patients with MGD and healthy controls (*p < 0.05, One-way ANOVA with post-hoc Tukey test)

1. Next-generation sequencing analysis (NGS)

1.1 The alpha-diversity

The rarefaction curves of the MGD and HC end up at the saturation platform, demonstrating the appropriate sequencing data size (Figure 5.). The **α**-diversity was compared between the MGD and HC group by ACE index, Chao1 index, observed-species index, Pielou index, Simpson's index, and Shannon diversity index; however, no significant differences were observed ($p > 0.05$) (Figure 6.). The Simpson's index showed significantly decreased **α**-diversity in patients with severe MGD compared to those with moderate MGD ($p = 0.045$) (Figure 7.).

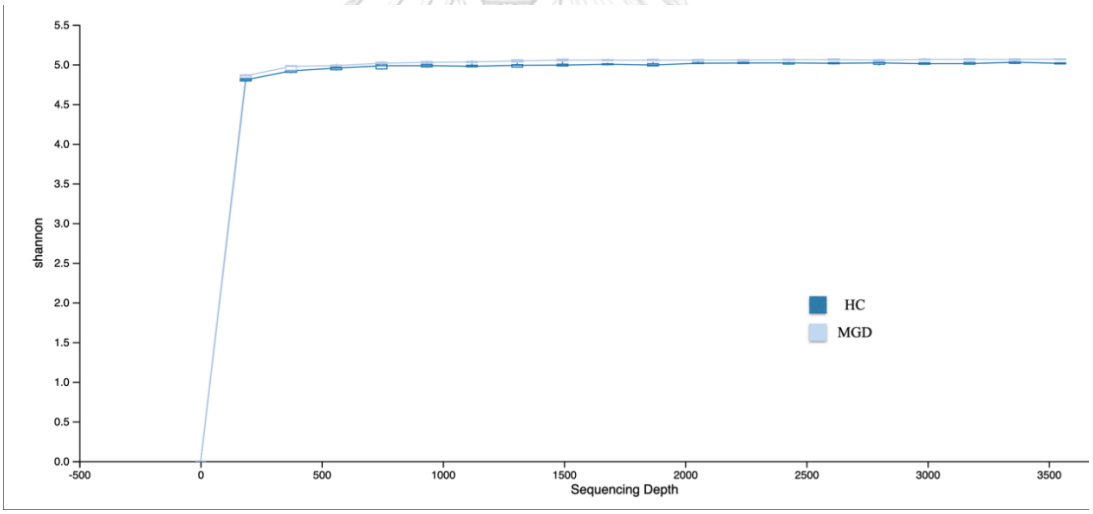


Figure 5. Rarefaction curve. This figure shows the rarefaction curves of the MGD and HC end up at the saturation platform, demonstrating the appropriate sequencing data size.

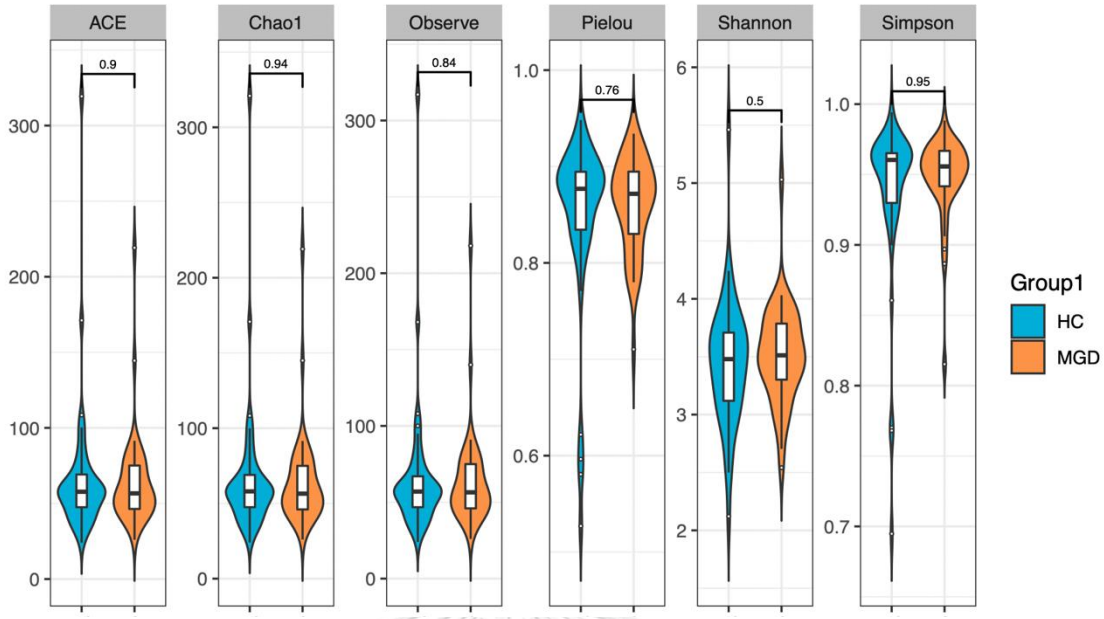


Figure 6. **α**-diversity. This figure demonstrates no significant difference in **α**-diversity in meibum samples of MGD compared with HC. ($p > 0.05$)

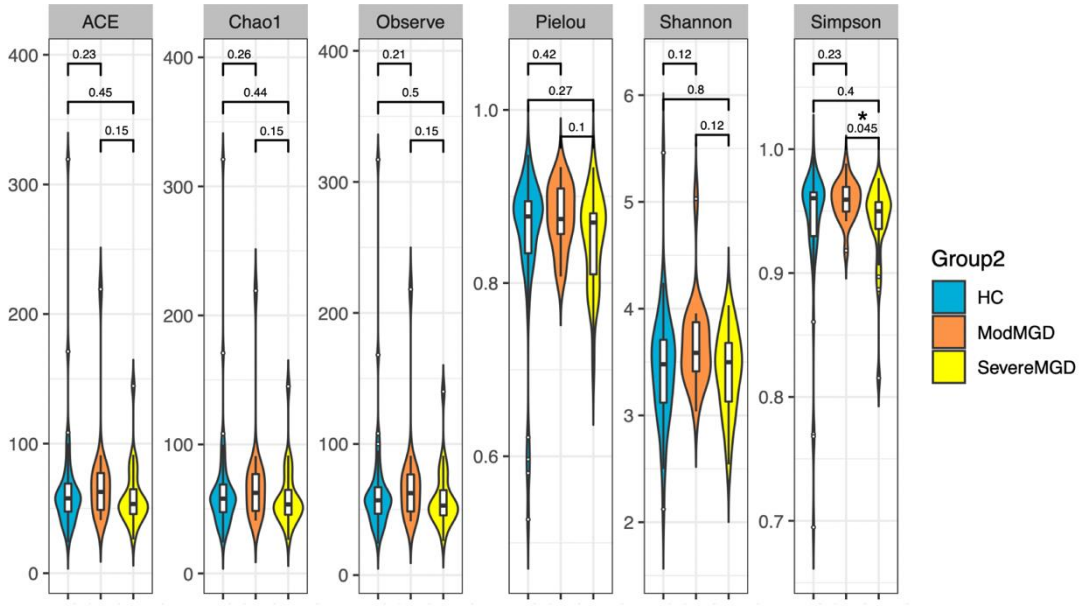


Figure 7. **α**-diversity. This figure demonstrates a significantly decreased **α**-diversity in meibum samples of severe MGD compared with moderate MGD by Simpson's index. ($p = 0.045$)

1.2 The beta-diversity

The Bray–Curtis dissimilarity indices, Jaccard's distance, weighted UniFrac distance, and unweighted UniFrac distance were used to compare the differences in the entire taxonomic composition. Principal Coordinate Analysis (PCoA) showed no clear distinction between the meibum samples from the MGD and HC groups ($p > 0.05$; permutational multivariate analysis of variance (PERMANOVA) analysis)(Figure 8.).

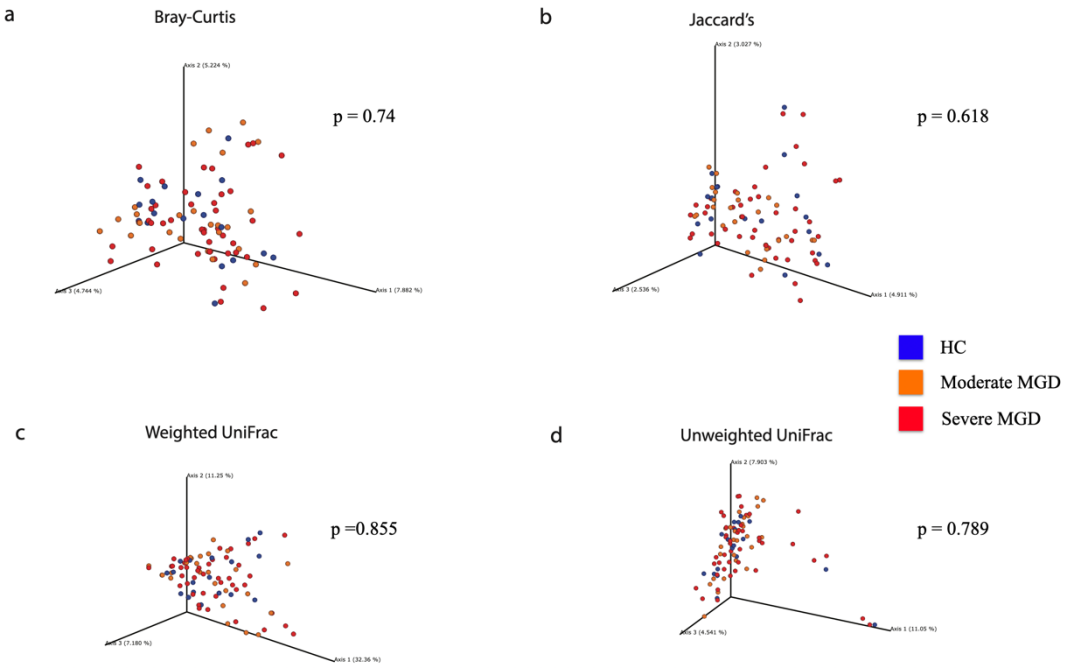


Figure 8. Principal Coordinate Analysis (PCoA). This figure shows no significant difference in β-diversity in meibum samples from severe MGD, moderate MGD, and HC by a. Bray-Curtis dissimilarity indices, b. Jaccard's distance, c. weighted UniFrac distance, d. unweighted UniFrac distance ($p > 0.05$, PERMANOVA analysis)

1.3 Taxonomic composition of meibum microbiota

At the phylum level, 31 phyla were observed from 88 eyes. The most predominated bacteria in both MGD group and healthy controls were Firmicutes (*Blautia, Dorea, Faecalibacterium, Streptococcus*) (46.73% vs 45.99%), Actinobacteria (*Collinsella, Streptomyces, Bifidobacterium*) (20.15% vs 21.40%), Proteobacteria (*Escherichia-Shigella, Acetobacter*) (17.26% vs 18.10%), and Bacteroidota (*Bacteroides*) (12.46% vs 10.47%), sequentially (Table 3.). There was no statistically significant difference in relative abundance at the phylum level between the MGD group and healthy controls (Figure 9.). There was also no statistically significant difference in relative abundance at phylum level between the severe MGD, moderate MGD, and HC (Figure 10.).

At the genus level, the most predominated bacteria in MGD group were *Blautia* (15.95 ± 7.62%), followed by *Bacteroides* (8.54 ± 5.17%), *Escherichia-Shigella* (7.35 ± 4.24%), *Streptomyces* (5.30 ± 5.54%), *Corynebacterium* (5.14 ± 7.17%), *Bifidobacterium* (4.89 ± 3.47%), *Lactobacillus* (3.98 ± 4.65%), *Streptococcus* (3.55 ± 4.00%), *Faecalibacterium* (3.35 ± 2.96%) and *Acetobacter* (3.20 ± 5.17%) (Table 4.).

Compared with HC, the MGD had a statistically significant higher relative abundance of *Bacteroides* (8.54% VS 6.00%, p = 0.015) and *Novosphingobium* (0.14% VS 0.004%, p = 0.012) (Figure 11.). The severe MGD had a statistically significant higher relative abundance of *Bacteroides* than HC (9.00% VS 5.99%, p = 0.045). Nevertheless, the Post-hoc test showed no statistically significant difference in the relative abundance of *Novosphingobium* between the three groups (p > 0.05). (Figure 12. ;Table 5.)

	MGD	Severe MGD	Moderate MGD	HC	p-value (HC vs. MGD)	p-value (3 groups)
P__ Firmicutes	46.75 ± 12.63%	46.59 ± 13.02%	46.98 ± 12.41%	45.99 ± 15.16%	0.799	0.964
P__ Actinobacteria	20.15 ± 11.48%	21.17 ± 13.35%	18.67 ± 8.22%	21.40 ± 13.36%	0.638	0.582
P__ Proteobacteria	17.26 ± 10.29%	17.13 ± 11.38%	17.44 ± 8.80%	18.09 ± 12.59%	0.732	0.943
P__ Bacteroidota	12.46 ± 6.13%	12.78 ± 6.14%	11.99 ± 6.27%	10.47 ± 6.00%	0.127	0.297
P__ Synergistota	0.82 ± 2.45%	0.59 ± 1.81%	1.14 ± 3.19%	1.08 ± 1.98%	0.570	0.547
P__ Patescibacteria	0.55 ± 1.24%	0.55 ± 1.24%	0.54 ± 1.27%	0.86 ± 1.67%	0.327	0.625
P__ Fusobacteriota	0.42 ± 1.15%	0.42 ± 1.26%	0.42 ± 1.01%	0.33 ± 0.86%	0.674	0.914
P__ Acidobacteriota	0.18 ± 0.77%	0	0.44 ± 1.17%	0.27 ± 1.00%	0.643	0.072
P__ Chloroflexi	0.10 ± 0.43%	0.04 ± 0.20%	0.19 ± 0.62%	0.27 ± 0.85%	0.258	0.180
P__ Planctomycetota	0.29 ± 1.14%	0.08 ± 0.33%	0.59 ± 1.73%	0.26 ± 1.06%	0.911	0.308

Table 3. Relative abundance of predominant meibum microbiota at phylum level.

	MGD	Severe MGD	Moderate MGD	HC	p-value (HC vs. MGD)	p-value (3 groups)
g__Blautia	15.95 ± 7.62%	15.49 ± 7.64%	16.62 ± 7.76%	13.65 ± 8.36%	0.181	0.377
g__Bacteroides	8.54 ± 5.17%	9.00 ± 5.02%	7.86 ± 5.44%	6.00 ± 4.42%	**0.015**	0.045*
g__Escherichia-Shigella	7.35 ± 4.24%	7.76 ± 4.68%	6.74 ± 3.54%	6.38 ± 4.42%	0.299	0.483
g__Streptomyces	5.30 ± 5.54%	4.30 ± 4.49%	6.74 ± 6.67%	3.34 ± 4.04%	0.614	0.134
g__Corynebacterium	5.14 ± 7.17%	6.47 ± 8.56%	3.23 ± 3.97%	7.59 ± 13.33%	0.286	0.072
g__Bifidobacterium	4.89 ± 3.47%	4.95 ± 3.71%	4.79 ± 3.19%	5.82 ± 4.96%	0.286	0.588
g__Lactobacillus	3.98 ± 4.65%	2.71 ± 3.52%	5.83 ± 5.51%	3.79 ± 4.73%	0.841	0.113
g__Streptococcus	3.55 ± 3.99%	4.60 ± 4.61%	2.04 ± 2.04%	3.47 ± 3.79%	0.930	0.029
g__Faecalibacterium	3.35 ± 2.96%	3.76 ± 3.19%	2.75 ± 2.57%	4.65 ± 5.19%	0.154	0.162
g__Acetobacter	3.20 ± 5.17%	2.46 ± 4.40%	4.27 ± 6.09%	3.74 ± 5.20%	0.623	0.435

Table 4. Relative abundance of predominant meibum microbiota at genus level.

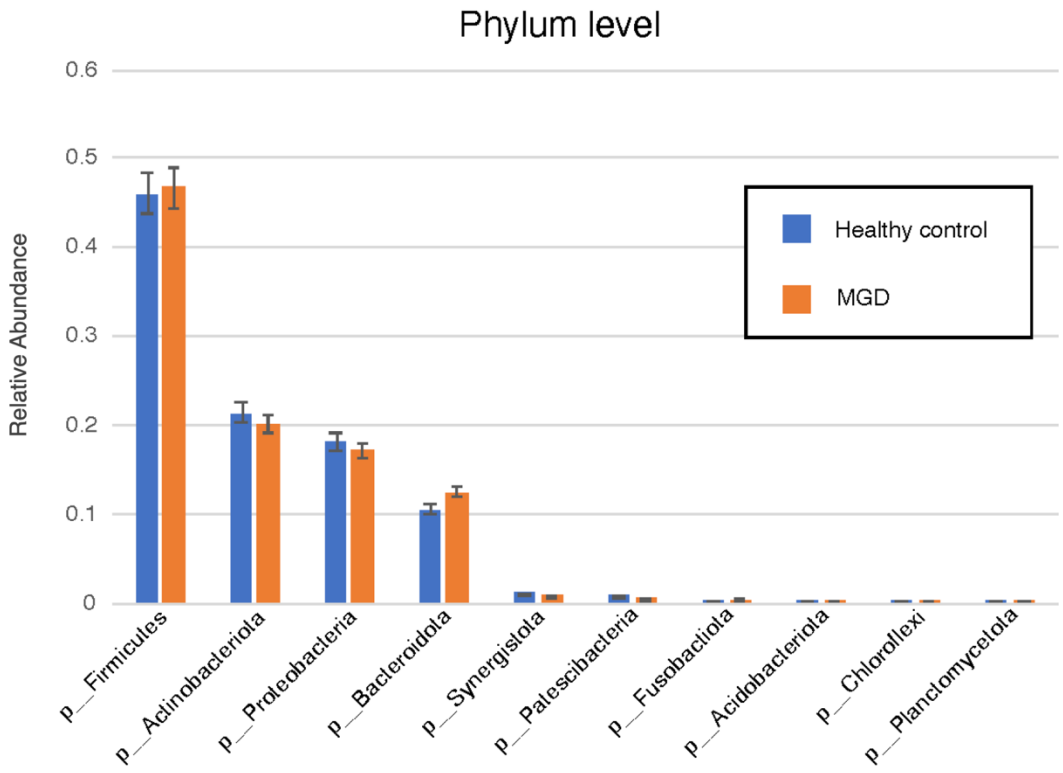


Figure 9. Relative abundance at phylum level between MGD and HC. This figure demonstrates no significant difference in relative abundance at phylum level in meibum samples of MGD compared with HC. ($p > 0.05$)

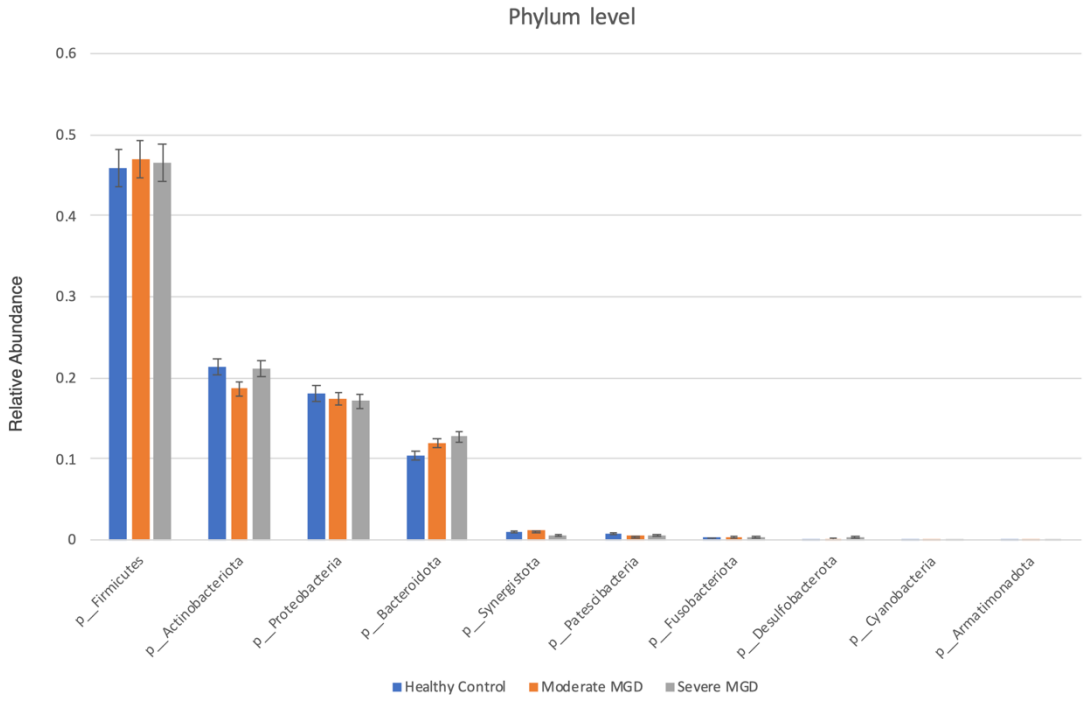


Figure 10. Relative abundance at phylum level between severe MGD, moderate MGD, and HC. This figure demonstrates no significant difference in relative abundance at phylum level in meibum samples between severe MGD, moderate MGD, and HC. ($p > 0.05$)

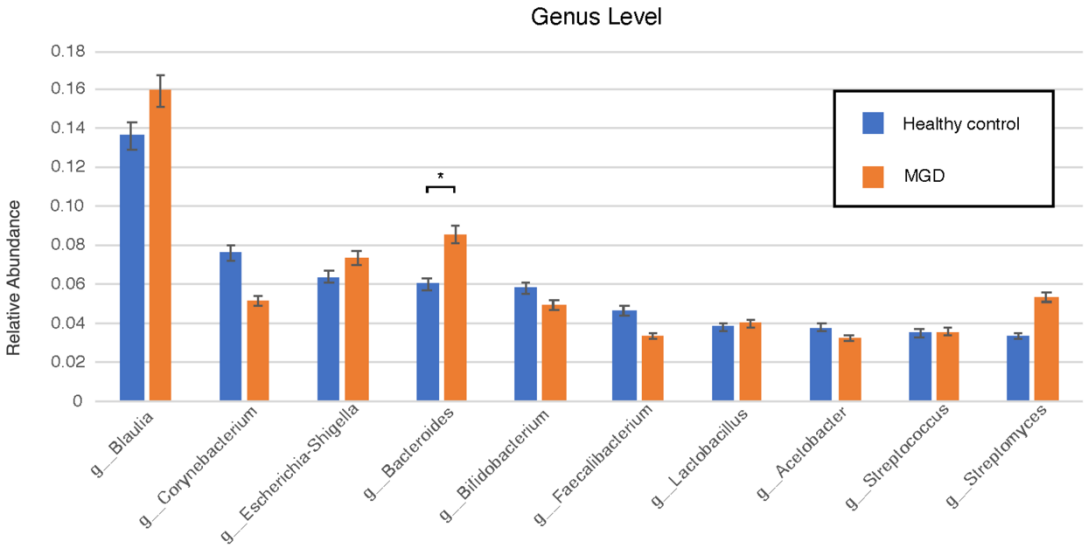


Figure 11. Relative abundance at genus level between MGD and HC. This figure demonstrates a statistically significant higher relative abundance of *Bacteroides* in MGD compared with HC (p = 0.015)

	MGD	Severe MGD	Moderate MGD	HC	p-value (HC vs. MGD)	p-value (3 groups)
g__Bacteroides	8.54 ± 5.17%	9.00 ± 5.02%	7.86 ± 5.44%	5.99 ± 4.42%	**0.015**	0.045*
g__Novosphingobium	0.14 ± 0.34%	0.14 ± 0.30%	0.13 ± 0.40%	0.004 ± 0.03%	**0.012**	0.041

* Post-hoc test using Tukey's test shows a significant difference between the severe MGD group and healthy controls (adjusted P-value = 0.035)

Table 5. Relative abundance of significant bacteria at the genus level

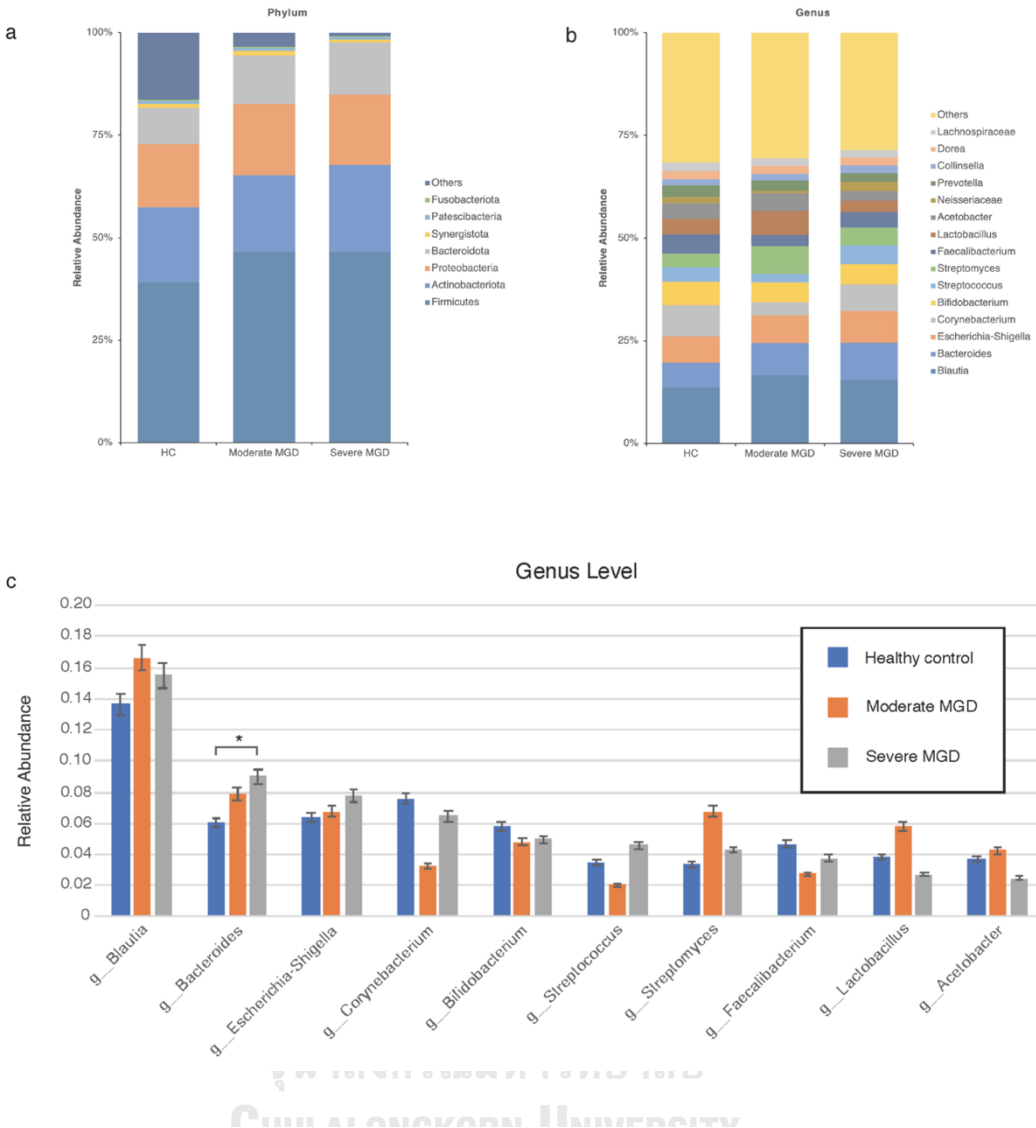


Figure 12. Relative abundance at genus level between severe MGD, moderate MGD, and HC. This figure demonstrates the relative abundance of meibum microbiota at a. Phylum level b. Genus level c. Statistically significant higher relative abundance of *Bacteroides* in severe MGD than HC (9.00% VS 5.99%, $p < 0.05$)

1.4 The core meibum microbiota

The core meibum microbiota was determined by the abundance–occurrence method, defining the minimal relative abundance threshold as 0.001% and the occurrence cut-off point at 50%.[37] The core meibum microbiota at genus level in both MGD and HC groups were *Blautia*, *Bacteroides*, *Escherichia-Shigella*, *Streptomyces*, *Corynebacterium*, *Bifidobacterium*, *Lactobacillus, Streptococcus, Faecalibacterium, Acetobacter, Prevotella, Lachnospiraceae, Dorea, Collinsella, Staphylococcus, Pseudomonas,* and *Eubacterium hallii* group.

1.5 Differential abundance analysis

To investigate the biomarkers of MGD, the results of differential abundance analysis were determined (Figure 13). Compared with HCs, the *Bacteroides* were significantly higher ($p < 0.001$), whereas *Nocardia* and *Obscuribacteraceae* were significantly lower in the MGD group compared to the HC group ($p < 0.001$).

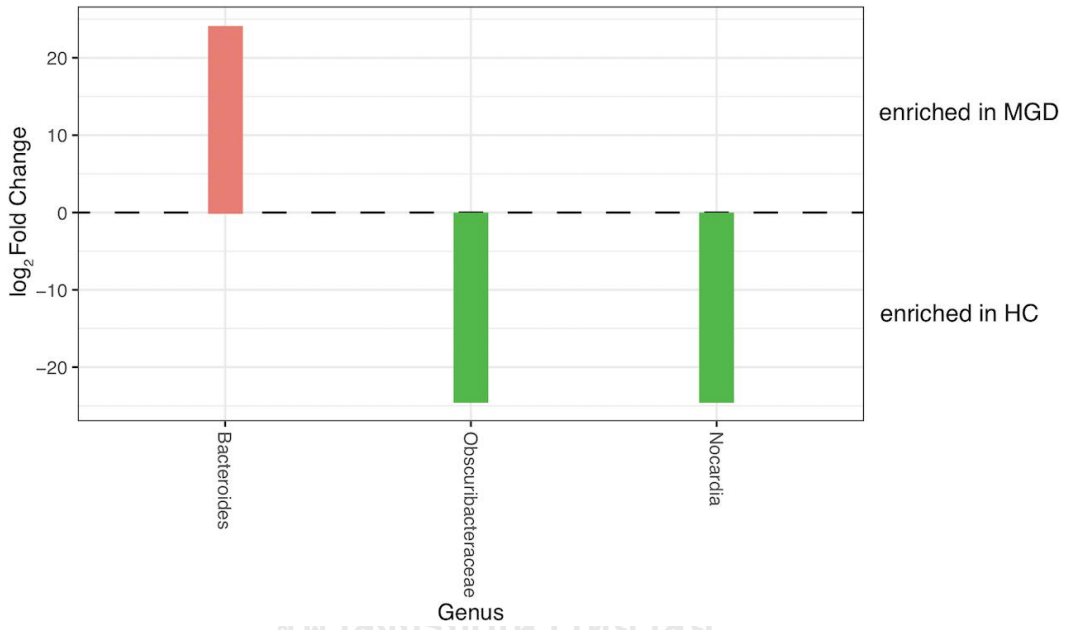


Figure 13. Differential abundance analysis. The figure demonstrates that *Bacteroides* was enriched in MGD, while *Nocardia cyriacigeorgica* and *Obscuribacteraceae* were enriched in HC

2. Correlation between core meibum microbiota and tear cytokines levels

This study focused on tear cytokines which were IL-1β, IL-6, and IL-17A. In the severe MGD group, the IL-1β, IL-6, and IL-17A levels were 12.24, 345.80, and 55.68 pg/ml sequentially. In the moderate MGD group, the IL-1β, IL-6, and IL-17A levels were 9.57, 210.87, and 46.21 pg/ml. The IL-1β, IL-6, and IL-17A levels in healthy controls were 10.03, 178.03, and 33.68 pg/ml. Significant higher IL-17A was detected in the MGD than in HC, especially in the severe MGD group ($p = 0.008$). (Table 6.)

Focusing on the correlation between core meibum microbiota and IL-17A levels, we find a moderate positive correlation between *Acetobacter* and IL-17A (Spearman's rho = 0.37, p-value < 0.05) and a moderate negative correlation between *Escherichia-Shigella, Faecalibacterium* and IL-17A (Spearman's rho = -0.38, -0.33 $p < 0.05$). Although a biomarker for MGD, Bacteroides showed no positive correlation to IL-17A but a moderate negative correlation to IL-1β. (Spearman's rho = -0.31, $p < 0.05$) (Figure 14. ;Table 7.)

Although IL-1β and IL-6 showed no significant correlation to MGD, they demonstrated a correlation to some core meibum microbiota. There were moderate negative correlation between *Escherichia-Shigella* and IL-1β and IL-6 (Spearman's rho = -0.39, -0.37 $p < 0.05$), *Collinsella* and IL-1β (Spearman's rho = -0.31 $p < 0.05$). Apart from the positive correlation to IL-17A, *Acetobacter* also showed a moderate positive correlation with IL-1β (Spearman's rho = 0.34, p-value < 0.05). (Figure 14. ;Table 7.)

Parameter	MGD		HC	p-value (ANOVA)	p-value (HC vs. MGD)
	Severe	Moderate			
IL-1β (pg/ml) (Mean ± SD)	12.24 ± 10.78	9.57 ± 7.53	10.03 ± 10.69	0.61	0.61
IL-6 (pg/ml) (Mean ± SD)	345.80 ± 389.42	210.87 ± 200.99	178.03 ± 324.33	0.13	0.12
IL-17A (pg/ml) (Mean ± SD)	55.68 ± 36.16	46.21 ± 31.68	33.68 ± 27.62	0.02*	0.008*

* Post-hoc test using Tukey's test shows a significant difference between the severe MGD group and healthy controls (adjusted P-value = 0.0144)

Table 6. Tear cytokine levels of MGD and HC.

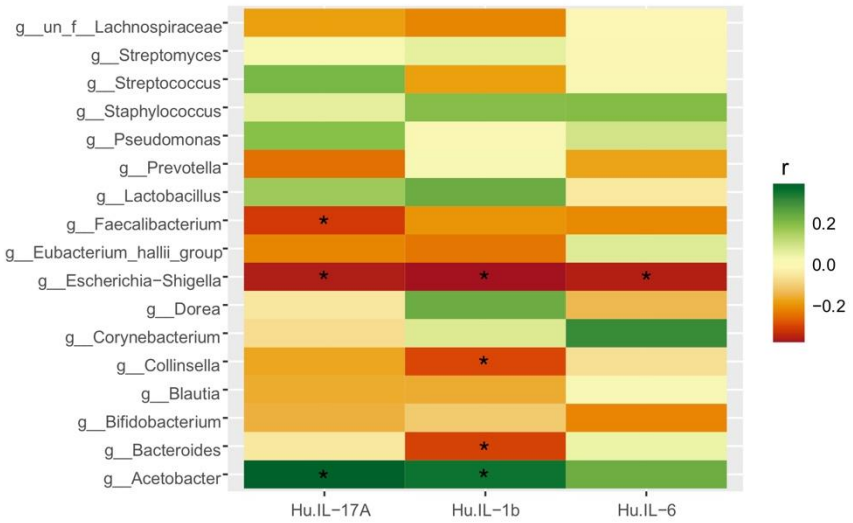


Figure 14. Heatmap of correlations between core meibum microbiota and tear cytokines levels. *p < 0.05 is considered statistically significant.

	IL-1β	p-value	IL-6	p-value	IL-17A	p-value
g__Streptococcus	-0.19	0.21	-0.01	0.94	0.20	0.20
g__Streptomyces	0.04	0.81	-0.03	0.85	0.01	0.99
g__Escherichia-Shigella	**-0.39**	**0.01**	**-0.37**	**0.01**	**-0.38**	**0.01**
g__Blautia	-0.18	0.25	-0.02	0.90	-0.18	0.25
g__Bifidobacterium	-0.12	0.43	-0.23	0.13	-0.17	0.28
g__Collinsella	**-0.31**	**0.04**	-0.08	0.60	-0.19	0.23
g__Acetobacter	**0.34**	**0.02**	0.21	0.16	**0.37**	**0.01**
g__Dorea	0.22	0.16	-0.15	0.32	-0.07	0.67
g__Faecalibacterium	-0.21	0.17	-0.22	0.14	**-0.33**	**0.03**
g__Bacteroides	**-0.32**	**0.04**	0.03	0.86	-0.07	0.67
g__Eubacterium_hallii_group	-0.26	0.09	0.05	0.74	-0.23	0.13
g__Corynebacterium	0.06	0.71	0.29	0.06	-0.09	0.58
g__Lactobacillus	0.22	0.15	-0.06	0.69	0.15	0.35
g__Prevotella	-0.001	0.99	-0.19	0.22	-0.27	0.08
g__Pseudomonas	-0.01	0.94	0.08	0.63	0.17	0.26
g__Staphylococcus	0.18	0.25	0.18	0.24	0.04	0.79
g__un_f__Lachnospiraceae	-0.23	0.13	-0.02	0.89	-0.19	0.21

Table 7. Spearman correlations (r) between core meibum microbiota and tear cytokines levels.

CHAPTER V Discussion and Conclusion

To the best of our knowledge, this is the first study to analyze meibum microbiota in patients with MGD using 16s rRNA gene sequencing. Our study found no differences in the diversity of microbial communities between the MGD and HC groups. Nevertheless, the Simpson's index detected a significantly decreased **α**-diversity in the patients with severe MGD compared to those with the moderate form. Our findings suggest the relationship between reduced bacterial diversity and disease severity. However, the PCoA plot demonstrated that the samples from the MGD group were relatively well clustered compared to those from the HC group. In contrast to previous research based on shotgun metagenomic analysis (Zhao, *et al*)[14], the community diversity was similar, but distinct clusters were detected between the MGD and HC groups. The different sequencing methods may affect the different results. Moreover, Zhao, *et al* did not describe MGD severity details, which may differ from our study.

In this study, the predominant phyla in both MGD and HC groups were Firmicutes, Actinobacteria, Proteobacteria, and Bacteroidetes. In contrast, previous metagenomic sequencing studies have reported that the most predominant phyla were Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes. In addition, there was a significantly decreased abundance of Proteobacteria in MGD compared with the HC.[14] A previous study in Northwestern China reported that the predominant phyla in the eyelid margin and conjunctival sac of patients with blepharitis were Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria.[36] Apart from the sites of collection and sequencing method, the geographic location also affected the composition of the microbiota.[44] For instance, the healthy Australian population had a lower abundance of Actinobacteria and a significantly higher abundance of Firmicutes.[30]

We reported *Blautia, Bacteroides, Escherichia-Shigella, Streptomyces, Corynebacterium, Bifidobacterium, Lactobacillus, Streptococcus, Faecalibacterium, Acetobacter, Prevotella, Lachnospiraceae, Dorea, Collinsella, Staphylococcus,*

Pseudomonas, and *Eubacterium hallii* groups as the core meibum microbiota at genus levels in both the MGD and HC groups. The previous study of meibum microbiota using whole genome sequencing reported that the predominant genera in the MGD meibum were *Campylobacter, Corynebacterium, Enterococcus*, *Fictibacillus, Geobacillus, Novibacillus, Pedobacter, Rubrobacter, Sphingobium,* and *Sphingomonas*[14] *Furthermore, Blautia, Bifidobacterium, and Faecalibacterium* were reported in the meibum of patients with internal hordeolum treated with hypochlorous acid eyelid wipes.[76] These probiotic organisms are capable of producing butyrate, inhibiting NF-kB signal transduction, and exerting anti-inflammatory effects. Thus, *Blautia, Bifidobacterium,* and *Faecalibacterium* may play a significant role in maintaining equilibrium in the meibomian gland microenvironment.[77]

Studies on the microbiota in the eyelid skin and conjunctival sac showed that *Pseudomonas, Corynebacterium, Propionibacterium, Acinetobacter, Staphylococcus,* and *Streptococcus* are the abundant microbiota in the healthy population.[2, 38, 45, 46] Only one published study has reported a healthy meibum microbiota using 16s rRNA gene sequencing. (Suzuki, *et al*)[54] The most abundant meibum microbiota was *Propionibacterium acnes* or *Pseudomonas sp.* However, the meibum was highly diverse in microbiota, in young individuals and the diversity decreased with increasing age. The aging population had a mean age of 60–70 years, similar to that of our study group. The predominated meibum microbiota during aging is *Corynebacterium sp.* and *Neisseriaceae*. In elderly individuals, the microbiota of the conjunctiva resembles the eyelid skin more than the meibum. Therefore, the conjunctival microbiota does not reflect the meibum microbiota in the aging population.

Corynebacterium is considered to be a commensal ocular surface microbiota that prevents pathogenic bacterial infection by stimulating IL-17 secretion from mucosal $\gamma\delta$T cells.[78] According to the previous study[2], *Corynebacterium* was the most abundant genus in a healthy conjunctival sac microbiota. *Corynebacterium* was more abundant in the healthy controls in our study, but the difference was not statistically significant. *Streptococcus* was the core meibum microbiota in our study

group. Our study reported no difference in the abundance of *Streptococcus, Neisseriaceae, Staphylococcus, Pseudomonas*, and *Bacillus* between the MGD and HC groups (3.55% VS 3.48%, 1.53% VS 1.55%, 1.3% VS 2.1%, 0.76 VS 1.03%, 0.15% VS 0.29%, $p > 0.05$). The *Propionibacterium* spp. was not detected in any of the specimens.

Surprisingly, we found that patients with MGD had a higher relative abundance of *Bacteroides* than HC, especially in the severe MGD group. Li, *et al*[79] showed that the conjunctival microbiota in the dry eye group was enriched by *Bacteroides* and that *Pseudomonas* was dominant in the non-dry eye group. Nevertheless, the *Bacteroides* were the dominant microbiota in the non-MGD, whereas, the *Bacilli* were the dominant microbiota in the MGD. However, the criteria used to differentiate the type of dry eye differed from that used in our study. Our study compared patients with MGD without aqueous tear deficiency (ATD)-induced dry eye and healthy controls without a dry eye to exclude the effect of ATD. In contrast, the former study did not exclude those with ATD from the study population. *Bacteroides* are the normal flora in the human digestive tract. As obligate anaerobes, they may be scarcely found on the ocular surface and are rarely detected using conventional culture methods.[80] The obstructed meibomian gland may alter the glandular environment, leading to an increased proportion of this microbe. Its virulence is due to its encapsulation as well as endotoxin-producing and highly antibiotic-resistant properties.[81] As an opportunistic pathogen, *Bacteroides* can penetrate the submucosal tissue and cause infection through the damaged mucosa.[36] There were few reports on *Bacteroides*-associated ocular infections, including blebitis, keratitis, and endophthalmitis after uneventful trabeculectomy[80] and endophthalmitis after uncomplicated extracapsular cataract extraction.[82] In patients with post trabeculectomy bleb failure, *Bacteroides fragilis* were also detected in conjunctival swabs by conventional culture methods compared to the nonfailure group.[83]

Novosphingobium, although not the core meibum microbiota, was more abundant in the patients with MGD. Recently, a study on conjunctival microbiota in patients with ocular Demodex infection (Liang, *et al*)[84] found a higher relative

abundance of *Novosphingobium* in patients with ocular Demodex infections. (1.1% vs 0.4%, p = 0.012). Ocular Demodex infection is associated with MGD. Demodex itself can destroy the meibomian and lacrimal glands, depleting lipids and the aqueous layers of the tear film resulting in DED. MGD may be a key factor in the alteration of ocular surface microbiota caused by Demodex infection. Sluch, *et al*[85] identified the pathogenic microbiota in corneal epithelial samples using 16s rRNA gene and metagenomic sequencing. They reported *that Acinetobacter, Cloacibacterium,* and *Novosphingobium* are strongly associated with keratitis caused by *Pseudomonas aeruginosa.* We analyzed the operational taxonomic unit (OTU) number using differential abundance analysis, which confirmed that *Bacteroides* are enriched in the MGD group, and *Nocardia* and *Obscuribacteraceae* are enriched in the HC group. In contrast to a previous study, they also reported a higher abundance of *Campylobacter coli, Campylobacter jejuni,* and *Enterococcus faecium* in the meibum of patients with MGD.[14]

Tear cytokines levels are potential markers of inflammation in DED. Other factors that increase tear cytokines levels include aging, HIV infection, and diabetic retinopathy, with no predilection to sex.[63] The common proinflammatory tear cytokines associated with MGD are IL-6, IL-17A, and IL-1β.[17-20] As expected, a significantly higher IL-17A was detected in the MGD group, especially in the severe MGD group, compared to the HC group. Moreover, IL-6 levels were increased in the MGD group but the difference was not statistically significant. IL-17 and IL-6 promote Th17 function, which activates corneal epithelial barrier disruption and is thus associated with the pathogenesis of DED.[86]

As far as we know, our study is the first study about the correlation between ocular surface microbiota and tear cytokines levels. Despite being a biomarker for MGD, *Bacteroides* showed no correlation with IL-17A, but a weak negative correlation with IL-1β. We demonstrated a moderate positive correlation between *Acetobacter* and IL-17A and a moderate negative correlation of IL-17A with *Escherichia-Shigella* and *Faecalibacterium*.

Although our study showed no significant correlation between IL-1β and IL-6 to MGD, they demonstrated correlation to some core meibum microbiota. IL-1β showed moderate negative correlation to *Escherichia-Shigella* and *Collinsella* and moderate positive correlation to *Acetobacter.* In comparison, IL-6 showed moderate negative correlation to *Escherichia-Shigella.* According to the alteration of tear cytokines levels, *Faecalibacterium*[76]*, Escherichia-Shigella* and *Collinsella* may be the probiotics, while *Acetobacter* may be the pathogenic bacteria in the meibomian gland. *Faecalibacterium prausnitzii* induced TNF-α, IL-4, IL-8, and IL-10 expression and reduced IL-1, IL-2, IL-6, IL-12, IL-17a, IFN-γ expression in the cell culture of colon carcinoma, supporting evidence that this bacterium is the commensal gut microbiota.[87] The study of gut microbiota in patients with rheumatoid arthritis demonstrated that *Collinsella* is strongly associated with IL-17A production.[88] In vitro study, *Escherichia-Shigella* induced TNF, IL-1 and IL-10 production by macrophages.[89] *Acetobacter* is a gram-negative bacteria that can produce cellulose β-(1,4)glucan, which stimulated the production of TNF-α and IL-12 by macrophages in vitro study.[90] However, there is still no evidence from the previous ophthalmic study that supports our hypothesis.

Further studies are required to evaluate the relationship between the core meibum microbiota and tear cytokines levels as a primary disease outcome. Moreover, apart from our included cytokines, other tear cytokines such as IL-1Ra, IL-8, IL-9, EGF, FGF2, MIP-1a, MMP-9, and PGE2 may also be considered.[17-20] The knowledge about the correlation between tear cytokines and core meibum microbiota in MGD may lead to therapeutic benefits in the future. We may utilize the targeted therapy to the specific cytokines in the MGD treatment.

Our study has some limitations. First, we did not focus on Demodex infestation in patients with MGD; therefore, its correlation with bacterial changes could not be analyzed. Secondly, the 16s rRNA gene sequencing method helps determine the bacterial community in the study population, although it has some drawbacks due to its weak phylogenetic ability at the species level.[33] However, this method is more cost-effective compared to whole genome sequencing. Lastly,

factors that may affect the different results were study population, degree of disease severity, geographic location, seasonal change, location and depth of the swab, method of specimen collection, and sequencing method.

Conclusion

The NGS analyses showed decreased bacterial diversity in the meibum microbiota of patients with severe MGD and a significantly higher abundance of Bacteroides and Novosphingobium in the MGD group. The precise association between the meibum microbiota and tear cytokine levels is yet to be elucidated. However, MGD diagnosis and treatment are recommended before ocular surgery to reduce the risk of potential bacterial infections. (Figure 15.)

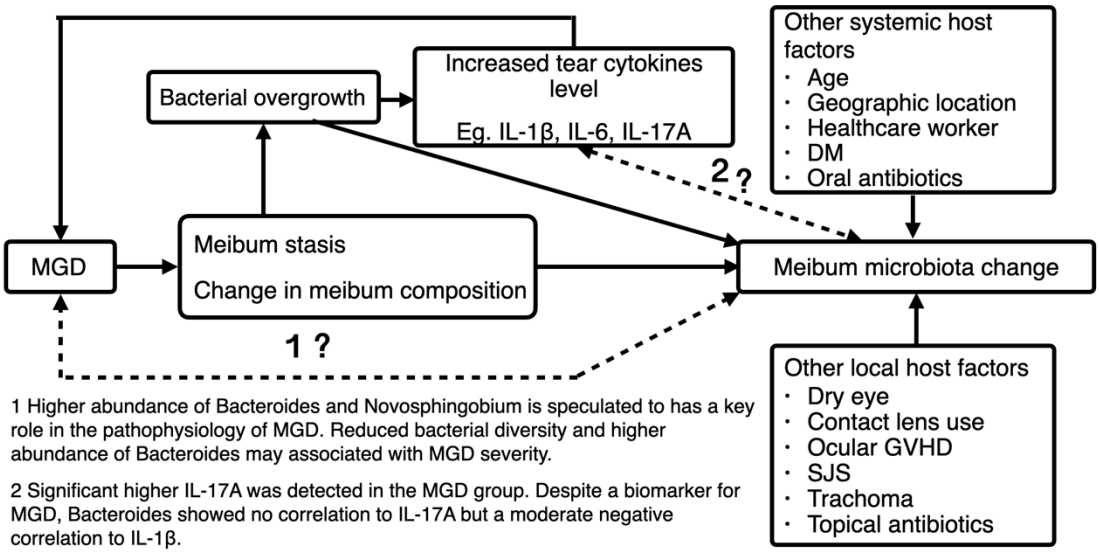


Figure 15. Composition and diversity of meibum microbiota in MGD and its correlation with tear cytokines levels

REFERENCES

1. Knop, E., Knop, N., Millar, T., Obata, H. & Sullivan, D. A. The international workshop on meibomian gland dysfunction: report of the subcommittee on anatomy, physiology, and pathophysiology of the meibomian gland. *Invest Ophthalmol Vis Sci* **52**, 1938-1978, doi:10.1167/iovs.10-6997c (2011).
2. Dong, X., Wang, Y., Wang, W., Lin, P. & Huang, Y. Composition and diversity of bacterial community on the ocular surface of patients with meibomian gland dysfunction. *Invest Ophthalmol Vis Sci* **60**, 4774-4783, doi:10.1167/iovs.19-27719 (2019).
3. Tsubota, K. *et al.* A new perspective on dry eye classification: proposal by the Asia Dry Eye Society. *Eye Contact Lens* **46** Suppl 1, S2-S13, doi:10.1097/ICL.0000000000000643 (2020).
4. Heiligenhaus A KJ, K. D., Kruse FE & Waubke TN. Therapy in tear film deficiencies. *Klin Monatsbl Augenheilkd* **204**, 162-168 (1994).
5. Shimazaki, J., Sakata, M. & Tsubota, K. Ocular surface changes and discomfort in patients with meibomian gland dysfunction. *Arch Ophthalmol* **113**, 1266-1270, doi:10.1001/archopht.1995.01100100054027 (1995).
6. Lekhanont, K., Rojanaporn, D., Chuck, R. S. & Vongthongsri, A. Prevalence of dry eye in Bangkok, Thailand. *Cornea* **25**, 1162-1167, doi:10.1097/01.ico.0000244875.92879.1a (2006).
7. Lin, P. Y. *et al.* Prevalence of dry eye among an elderly Chinese population in Taiwan: the Shihpai Eye Study. *Ophthalmology* **110**, 1096-1101, doi:10.1016/S0161-6420(03)00262-8 (2003).
8. Uchino, M. *et al.* The features of dry eye disease in a Japanese elderly population. *Optom Vis Sci* **83**, 797-802, doi:10.1097/01.opx.0000232814.39651.fa (2006).
9. Jie, Y., Xu, L., Wu, Y. Y. & Jonas, J. B. Prevalence of dry eye among adult Chinese in the Beijing Eye Study. *Eye (Lond)* **23**, 688-693, doi:10.1038/sj.eye.6703101 (2009).

10. Schein OD MB, T. J., Bandeen-Roche K & West S. Prevalence of dry eye among the elderly. *Am J Ophthalmol* **124**, 723-728 (1997).
11. McCarty, C. A., Bansal, A. K., Livingston, P. M., Stanislavsky, Y. L. & Taylor, H. R. The epidemiology of dry eye in Melbourne, Australia. *Ophthalmology* **105**, 1114-1119, doi:10.1016/S0161-6420(98)96016-X (1998).
12. Dougherty, J. M. & McCulley, J. P. Bacterial lipases and chronic blepharitis. *Invest Ophthalmol Vis Sci* **27**, 486-491 (1986).
13. Baudouin, C. *et al.* Revisiting the vicious circle of dry eye disease: a focus on the pathophysiology of meibomian gland dysfunction. *Br J Ophthalmol* **100**, 300-306, doi:10.1136/bjophthalmol-2015-307415 (2016).
14. Zhao, F. *et al.* Metagenomic profiling of ocular surface microbiome changes in meibomian gland dysfunction. *Invest Ophthalmol Vis Sci* **61**, 22, doi:10.1167/iovs.61.8.22 (2020).
15. Nichols, K. K. *et al.* The international workshop on meibomian gland dysfunction: executive summary. *Invest Ophthalmol Vis Sci* **52**, 1922-1929, doi:10.1167/iovs.10-6997a (2011).
16. Geerling, G. *et al.* The international workshop on meibomian gland dysfunction: report of the subcommittee on management and treatment of meibomian gland dysfunction. *Invest Ophthalmol Vis Sci* **52**, 2050-2064, doi:10.1167/iovs.10-6997g (2011).
17. Enriquez-de-Salamanca, A. *et al.* Tear cytokine and chemokine analysis and clinical correlations in evaporative-type dry eye disease. *Mol Vis* **16**, 862-873 (2010).
18. Landsend, E. C. S. *et al.* The level of inflammatory tear cytokines is elevated in congenital aniridia and associated with meibomian gland dysfunction. *Invest Ophthalmol Vis Sci* **59**, 2197-2204, doi:10.1167/iovs.18-24027 (2018).
19. Zhang, L. *et al.* Effects of azithromycin on gene expression profiles of proinflammatory and anti-inflammatory mediators in the eyelid margin and conjunctiva of patients with meibomian gland disease. *JAMA Ophthalmol* **133**, 1117-1123, doi:10.1001/jamaophthalmol.2015.2326 (2015).
20. Liu, R. *et al.* Analysis of cytokine levels in tears and clinical correlations after

intense pulsed light treating meibomian gland dysfunction. *Am J Ophthalmol* **183**, 81-90, doi:10.1016/j.ajo.2017.08.021 (2017).

21. Zilliox, M. J. *et al.* Assessing the ocular surface microbiome in severe ocular surface diseases. *Ocul Surf* **18**, 706-712, doi:10.1016/j.jtos.2020.07.007 (2020).
22. Grzybowski, A., Brona, P. & Kim, S. J. Microbial flora and resistance in ophthalmology: a review. *Graefes Arch Clin Exp Ophthalmol* **255**, 851-862, doi:10.1007/s00417-017-3608-y (2017).
23. Zhu X WL, R. X. *et al.* Conjunctival microbiota in patients with type 2 diabetes mellitus and influences of perioperative use of topical levofloxacin in ocular surgery. *Front Med (Lausanne)* (2021).
24. Doan, T. *et al.* Post-antibiotic ocular surface microbiome in children: a cluster-randomized trial. *Ophthalmology* **127**, 1127-1130, doi:10.1016/j.ophtha.2020.02.014 (2020).
25. Woese CR, F. G. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proc Natl Acad Sci USA*, 5088-5090 (1977).
26. Pasquale Aragona, M. *et al.* The ocular microbiome and microbiota and their effects on ocular surface pathophysiology and disorders. *Survey of Ophthalmology* **66**, 907-925 (2021).
27. Zhang, Y. J. *et al.* Impacts of gut bacteria on human health and diseases. *Int J Mol Sci* **16**, 7493-7519, doi:10.3390/ijms16047493 (2015).
28. Hugenholtz, P., Goebel, B. M. & Pace, N. R. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J Bacteriol* **180**, 4765-4774, doi:10.1128/JB.180.18.4765-4774.1998 (1998).
29. Kara M. Cavuoto, S. B. & Anat Galor. Relationship between the microbiome and ocular health. *The Ocular Surface* **17**, 384-392 (2019).
30. Ozkan, J. *et al.* Temporal stability and composition of the ocular surface microbiome. *Sci Rep* **7**, 9880, doi:10.1038/s41598-017-10494-9 (2017).
31. Gomes, J. A. P., Frizon, L. & Demeda, V. F. Ocular surface microbiome in health and disease. *Asia Pac J Ophthalmol (Phila)* **9**, 505-511, doi:10.1097/APO.0000000000000330 (2020).
32. Makedonka, M. *The Microbiome in Infectious Disease* 68-74 (Elsevier, 2017).

33. Janda, J. M. & Abbott, S. L. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J Clin Microbiol* **45**, 2761-2764, doi:10.1128/JCM.01228-07 (2007).
34. Allaband, C. *et al.* Microbiome 101: studying, analyzing, and interpreting gut microbiome data for clinicians. *Clin Gastroenterol Hepatol* **17**, 218-230, doi:10.1016/j.cgh.2018.09.017 (2019).
35. Punetha J, H. E. Short read (next-generation) sequencing: a tutorial with cardiomyopathy diagnostics as an exemplar. *Circ Cardiovasc Genet* **6(4)** (2013).
36. Wang, C. *et al.* Composition and diversity of the ocular surface microbiota in patients with blepharitis in Northwestern China. *Front Med (Lausanne)* **8**, 768849, doi:10.3389/fmed.2021.768849 (2021).
37. Neu, A. T., Allen, E. E. & Roy, K. Defining and quantifying the core microbiome: challenges and prospects. *Proc Natl Acad Sci USA* **118**, doi:10.1073/pnas.2104429118 (2021).
38. Doan, T. *et al.* Paucibacterial microbiome and resident DNA virome of the healthy conjunctiva. *Invest Ophthalmol Vis Sci* **57**, 5116-5126, doi:10.1167/iovs.16-19803 (2016).
39. Wen, X. *et al.* The influence of age and sex on ocular surface microbiota in healthy adults. *Invest Ophthalmol Vis Sci* **58**, 6030-6037, doi:10.1167/iovs.17-22957 (2017).
40. Deng, Y. *et al.* Geographic difference shaped human ocular surface metagenome of young Han Chinese from Beijing, Wenzhou, and Guangzhou cities. *Invest Ophthalmol Vis Sci* **61**, 47, doi:10.1167/iovs.61.2.47 (2020).
41. Graham, J. E. *et al.* Ocular pathogen or commensal: a PCR-based study of surface bacterial flora in normal and dry eyes. *Invest Ophthalmol Vis Sci* 48, 5616-5623, doi:10.1167/iovs.07-0588 (2007).
42. Dong Q, B. J. et al. Diversity of bacteria at healthy human conjunctiva. *Invest Ophthalmol Vis Sci* 5408-5413 (2011).
43. Zhou, Y. *et al.* The conjunctival microbiome in health and trachomatous disease: a case control study. *Genome Med* **6**, 99, doi:10.1186/s13073-014-0099-x (2014).

44. Delbeke, H., Younas, S., Casteels, I. & Joossens, M. Current knowledge on the human eye microbiome: a systematic review of available amplicon and metagenomic sequencing data. *Acta Ophthalmol*, doi:10.1111/aos.14508 (2020).
45. Huang, Y., Yang, B. & Li, W. Defining the normal core microbiome of conjunctival microbial communities. *Clin Microbiol Infect* **22**, 643 e647-643 e612, doi:10.1016/j.cmi.2016.04.008 (2016).
46. Ham, B. *et al.* Distribution and diversity of ocular microbial communities in diabetic patients compared with healthy subjects. *Curr Eye Res* **43**, 314-324, doi:10.1080/02713683.2017.1406528 (2018).
47. Li S YG, P. H. *et al.* How ocular surface microbiota debuts in type 2 diabetes mellitus. *Front Cell Infect Microbiol* **9**, 202 (2019).
48. Li Z GY, C. S. *et al.* Comparative portrayal of ocular surface microbe with and without dry eye. *J Microbiol* 2019 **57**, 1025-1032 (2019).
49. Zhang, H. *et al.* Conjunctival microbiome changes associated with soft contact lens and orthokeratology lens wearing. *Invest Ophthalmol Vis Sci* **58**, 128-136, doi:10.1167/iovs.16-20231 (2017).
50. Ozkan, J., Coroneo, M., Willcox, M., Wemheuer, B. & Thomas, T. Identification and visualization of a distinct microbiome in ocular surface conjunctival tissue. *Invest Ophthalmol Vis Sci* **59**, 4268-4276, doi:10.1167/iovs.18-24651 (2018).
51. Ozkan, J. & Willcox, M. D. The ocular microbiome: molecular characterisation of a unique and low microbial environment. *Curr Eye Res* **44**, 685-694, doi:10.1080/02713683.2019.1570526 (2019).
52. Delbeke, H., Younas, S., Casteels, I. & Joossens, M. Current knowledge on the human eye microbiome: a systematic review of available amplicon and metagenomic sequencing data. *Acta Ophthalmol* **99**, 16-25, doi:10.1111/aos.14508 (2021).
53. Versura, P., Giannaccare, G. & Campos, E. C. Sex-steroid imbalance in females and dry eye. *Curr Eye Res* **40**, 162-175, doi:10.3109/02713683.2014.966847 (2015).
54. Suzuki, T., Sutani, T., Nakai, H., Shirahige, K. & Kinoshita, S. The microbiome of the meibum and ocular surface in healthy subjects. *Invest Ophthalmol Vis Sci*

61, 18, doi:10.1167/iovs.61.2.18 (2020).

55. Ueta, M. Innate immunity of the ocular surface and ocular surface inflammatory disorders. *Cornea* **27** Suppl 1, S31-40, doi:10.1097/ICO.0b013e31817f2a7f (2008).
56. Petrillo, F. *et al.* Current evidence on the ocular surface microbiota and related diseases. *Microorganisms* **8**, doi:10.3390/microorganisms8071033 (2020).
57. Okonkwo, A. *et al.* Next-generation sequencing of the ocular surface microbiome: in health, contact lens wear, diabetes, trachoma, and dry eye. *Eye Contact Lens* **46**, 254-261, doi:10.1097/ICL.0000000000000697 (2020).
58. Kittipibul T, P. V. & Chatsuwan T. Comparison of the ocular microbiome between chronic Stevens-Johnson syndrome patients and healthy subjects. *Sci Rep* **10**, 4353 (2020).
59. Zhang, S. D. *et al.* Bacteriological profile of ocular surface flora in meibomian gland dysfunction. *Ocul Surf* **15**, 242-247, doi:10.1016/j.jtos.2016.12.003 (2017).
60. Watters, G. A., Turnbull, P. R., Swift, S., Petty, A. & Craig, J. P. Ocular surface microbiome in meibomian gland dysfunction. *Clin Exp Ophthalmol* **45**, 105-111, doi:10.1111/ceo.12810 (2017).
61. Jiang, X. *et al.* Pathogens in the meibomian gland and conjunctival sac: microbiome of normal subjects and patients with meibomian gland dysfunction. *Infect Drug Resist* **11**, 1729-1740, doi:10.2147/IDR.S162135 (2018).
62. Lee, S. H., Oh, D. H., Jung, J. Y., Kim, J. C. & Jeon, C. O. Comparative ocular microbial communities in humans with and without blepharitis. *Invest Ophthalmol Vis Sci* **53**, 5585-5593, doi:10.1167/iovs.12-9922 (2012).
63. Tong, L., Wong, T. Y. & Cheng, Y. Level of tear cytokines in population-level participants and correlation with clinical features. *Cytokine* **110**, 452-458, doi:10.1016/j.cyto.2018.05.013 (2018).
64. Lemp, M. A. & Nichols, K. K. Blepharitis in the United States 2009: a survey-based perspective on prevalence and treatment. *Ocul Surf* **7**, S1-S14, doi:10.1016/s1542-0124(12)70620-1 (2009).
65. Narayanan, S., Miller, W. L. & McDermott, A. M. Conjunctival cytokine expression in symptomatic moderate dry eye subjects. *Invest Ophthalmol Vis Sci* **47**, 2445-2450, doi:10.1167/iovs.05-1364 (2006).

66. Acera, A., Rocha, G., Vecino, E., Lema, I. & Duran, J. A. Inflammatory markers in the tears of patients with ocular surface disease. *Ophthalmic Res* **40**, 315-321, doi:10.1159/000150445 (2008).
67. Argueso, P. *et al.* Decreased levels of the goblet cell mucin MUC5AC in tears of patients with Sjogren syndrome. *Invest Ophthalmol Vis Sci* **43**, 1004-1011 (2002).
68. Massingale, M. L. *et al.* Analysis of inflammatory cytokines in the tears of dry eye patients. *Cornea* **28**, 1023-1027, doi:10.1097/ICO.0b013e3181a16578 (2009).
69. Esmaeelpour M, C. J., Watts P, Boulton M, Murphy PJ. Tear sample collection using cellulose acetate absorbent filters. *Ophthalmic Physiol Opt* **28**, 577-583 (2008).
70. Karl R. *et al.* A method to ectract cytokines and matrix metalloproteinases from Schirmer strips and analyze using Luminex. *Molecular vision* **17**, 1056-1063 (2011).
71. Wolffsohn, J. S. *et al.* TFOS DEWS II diagnostic methodology report. *Ocul Surf* **15**, 539-574, doi:10.1016/j.jtos.2017.05.001 (2017).
72. Tomlinson, A. *et al.* The international workshop on meibomian gland dysfunction: report of the diagnosis subcommittee. *Invest Ophthalmol Vis Sci* **52**, 2006-2049, doi:10.1167/iovs.10-6997f (2011).
73. Loretta B. Szczotka-Flynn, Eric Pearlman & Mahmoud Ghannoum. Microbial contamination of contact lenses, lens care solutions, and their accessories: a literature review. *Eye Contact Lens* **36**, 116- 129 (2010).
74. Chow S SJ, Wang H. & Shao J. *Sample Size Calculations in Clinical Research* (Chapman & Hall/CRC Biostatistics Series 2008).
75. Miller, K. L. *et al.* Minimal clinically important difference for the ocular surface disease index. *Arch Ophthalmol* **128**, 94-101, doi:10.1001/archophthalmol.2009.356 (2010).
76. Yang, S. *et al.* The microbiome of meibomian gland secretions from patients with internal hordeolum treated with hypochlorous acid eyelid wipes. *Dis Markers* 2022, 7550090, doi:10.1155/2022/7550090 (2022).
77. Wei Z, C. S. *et al.* Could gut microbiota serve as prognostic biomarker associated with colorectal cancer patients' survival? a pilot study on relevant mechanism.

Oncotarget **7**, 46158- 46172 (2016).

78. St Leger, A. J. *et al.* An ocular commensal protects against corneal infection by driving an interleukin-17 response from mucosal gammadelta T cells. *Immunity* **47**, 148-158 e145, doi:10.1016/j.immuni.2017.06.014 (2017).
79. Li, Z. *et al.* Comparative portrayal of ocular surface microbe with and without dry eye. *J Microbiol* **57**, 1025-1032 (2019).
80. Parker, J. S. *et al. Bacteroides* Spp. blebitis, keratitis, and endophthalmitis following uncomplicated trabeculectomy. *J Glaucoma* **28**, e118-e120; 10.1097/IJG.0000000000001235 (2019).
81. Nguyen, M & Vedantam, G. Mobile genetic elements in the genus *Bacteroides*, and their mechanism(s) of dissemination, *Mobile Genetic Elements*, **1:3**, 187-196; 10.4161/mge.1.3.18448 (2011).
82. Kelly, L. D. & Gaynon, M. *Bacteroides fragilis* endophthalmitis: a case report. *Can J Ophthalmol* **25**, 208-209 (1990)
83. Boiko, E.V. *et al.* Latent infections as a risk factor for posttrabeculectomy bleb failure. *J Glaucoma* **25**, 306-311 (2016).
84. Liang, X. *et al.* Demodex infection changes ocular surface microbial communities, in which meibomian gland dysfunction may play a role. *Ophthalmol Ther* **10**, 601-617; 10.1007/s40123-021-00356-z (2021).
85. Sluch, I. *et al.* NGS analysis of corneal pathogens: can we use it in a clinical setting? *Investigative Ophthalmology & Visual Science* **59**, 3684-3684 (2018).
86. Jones, L. *et al.* TFOS DEWS II management and therapy report. *Ocul Surf* **15**, 575-628; 10.1016/j.jtos.2017.05.006 (2017).
87. Rabiei, N. *et al.* Induction effects of *Faecalibacterium prausnitzii* and its extracellular vesicles on toll-like receptor signaling pathway gene expression and cytokine level in human intestinal epithelial cells. *Cytokine* **121**, 154718 (2019).
88. Chen, J. *et al.* An expansion of rare lineage intestinal microbes characterizes rheumatoid arthritis. *Genome Med* **8(1)**, 43 (2011).
89. Silvia Y Bando. *et al.* Expression of bacterial virulence factors and cytokines during in vitro macrophage infection by enteroinvasive *Escherichia coli* and

Shigella flexneri: a comparative study. *Mem Inst Oswaldo Cruz* **105(6)**, 786-791 (2010).

90. Kimika Saito, T.Y. *et al.* Soluble branched β-(*1,4)*glucans from *Acetobacter* species show strong activities to induce interleukin-12 in vitro and inhibit T-helper2 cellular response with immunoglobulin E production in vivo. *The Journal of Biological Chemistry*, **278**, 38571-38578 (2003).

VITA

NAME	Ubonwan Rasaruck
DATE OF BIRTH	20 Oct 1988
PLACE OF BIRTH	Bangkok
INSTITUTIONS ATTENDED	2012 Faculty of Medicine, Chulalongkorn University 2018 Department of Ophthalmology, Faculty of Medicine, Chulalongkorn University 2021 Cornea and Refractive Surgery Unit, Department of Ophthalmology, Faculty of Medicine, Chulalongkorn University
HOME ADDRESS	130/5, Mu.5, Bangkruay-sainoi Rd., Banglane, Bangyai, Nonthaburi, 11140
PUBLICATION	-
AWARD RECEIVED	-