

Pathogens in the Meibomian gland and conjunctival sac: microbiome of normal subjects and patients with Meibomian gland dysfunction

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Objective: To explore the composition of the ocular microbiome in normal subjects and patients with Meibomian gland dysfunction (MGD).

Subjects and methods: Seventy subjects (140 eyes) were enrolled in our study. Signs of dry eye were evaluated and bacterial species in the conjunctival sac (CS) and Meibomian gland (MG) secretions were then identified by 16S rRNA gene sequencing. Additionally, 17 subjects (34 eyes) were further evaluated to determine differences in the microbiomes in the surface and deep layers of MG using a segmental secretion analysis.

Results: The positive bacterial isolation rate was markedly higher in MG secretions than in the CS. The bacterial composition of the control and mild group was simple, whereas the composition of bacteria was more complex as the severity of MGD increased. The positive bacterial isolation rate and number of bacterial types were significantly higher in the severe MGD group than those in the control, mild and moderate MGD groups. *Corynebacterium macginleyi* was only detected in the severe MGD group, with an isolation rate of up to 26.3%. Furthermore, a new grading system for bacterial severity of MGD was proposed and the severity of MGD appeared to be positively correlated with a higher grade of bacterial severity. The segmental secretion analysis showed severe MGD had a significantly higher incidence of bacterial discordance rate.

Conclusion: The severity of MGD was positively correlated with a higher isolation rate, a greater number of bacterial species, and a higher grade of bacterial severity, which implied that MGD might be correlated with bacterial changes. This study provided some basis for the indications of antibiotic in clinical practice.

Keywords: Meibomian gland dysfunction, microbiome, pathogens, bacterial flora, 16S rRNA, scanning electron microscopy

Introduction

Dry eye is an ocular disease characterized by ocular discomfort due to an abnormal quantity or quality of tear film, which causes pathological changes in the ocular surface.¹ The prevalence of dry eye has increased in recent years, and the global prevalence ranges from 6.8% to 35.0% depending on ages and areas.^{2,3} There are two major kinds of dry eye, aqueous deficiency dry eye and evaporative dry eye. Evaporative dry eye includes Meibomian gland dysfunction (MGD) and other genetic diseases caused less mature Meibomian glands (MGs) such as *Eda*-deficient disease.⁴ MGD is the major form of evaporative dry eye, and, therefore, a large amount of research has been undertaken to better understand the pathogenesis of MGD and appropriate treatment methods.¹

A number of eye diseases have been reported to be related to ocular bacterial flora. Ocular microbes were reported to contribute to the occurrence of endophthalmitis after

cataract surgery in early studies.⁵ Recently, an increasing number of studies have focused on the relationship between bacterial flora and dry eye due to the frequent occurrence of various infectious diseases in cases of dry eye, such as anterior blepharitis,⁶ MG disease,⁷ and keratitis.⁸ In healthy people, the ocular surface usually only contains commensal bacteria, and one of the most common genus is coagulase-negative *Staphylococcus*.⁹ However, a broad range of bacteria, such as *Staphylococcus aureus* and *Klebsiella* spp., have been identified on the ocular surface of patients with the infectious diseases mentioned above.^{10,11}

Based on recent studies of ocular bacteria, antibiotics have been used as a treatment method for MGD; however, the precise pathologies of MGD remain largely unknown. Thus, the development of a more specific and feasible treatment method has been difficult. Currently, topical azithromycin alone or in combination with oral doxycycline has been reported to relieve the signs and symptoms of dry eye.^{12,13} However, the specific indications for the use of antibiotics to treat MGD in clinical practice remain poorly defined.

In previous studies, specimens of bacteria isolated from the ocular surface were primarily obtained from bacterial swabs of the conjunctival sac (CS) and eyelid margins,^{14–16} which may only represent a portion of the bacterial flora of the ocular surface. Few studies have explored the bacteria found in MG secretions.^{17,18} Therefore, the relationship between the bacterial flora found in MG secretions and the signs and symptoms of MGD remain unclear, and no study has yet established an appropriate method for defining the complete microbiome of the ocular surface. In this study, the microbiomes of the CS and MG from patients with MGD were investigated to establish a grading system for bacterial severity, which might provide a basis for clinical treatment of MGD.

Subjects and methods

This study was conducted according to the principles of the Declaration of Helsinki and was approved by the Human Research and Ethics Committee of Peking University Third Hospital. Written informed consent in Chinese was obtained from each participant (and a parent if the patient was under the age of 18) before enrollment.

Subjects

MGD patients were recruited from the outpatient department of the Department of Ophthalmology at Peking University Third Hospital consecutively between December 2015 and October 2017 and control volunteers were recruited

by advertisement between December 2015 and December 2017. The inclusion criteria for the MGD were as follows: 1) adult patients with a chief complaint of one of the following symptoms: dryness, foreign body sensation, burning, or tearing for more than 6 months; 2) a diagnosis of MGD with two or more of the following signs in both eyes: redness or thickening of the lid margin, telangiectasia, reduced or no secretions, poor quality secretions, and gland capping;¹⁹ and 3) a willingness to cooperate with the doctors during the examination procedure. Control volunteers consisted of 29 (58 eyes) subjects recruited by advertisement from the Physical Examination Center of Peking University Third Hospital. The inclusion criteria for the controls were as follows: 1) no chief complaint of any dry eye symptoms or tear break up time (TBUT) was equal to or longer than 5 seconds without obvious dry eye symptoms; 2) MG associated assessments were not able to meet the criteria for the diagnosis of MGD; 3) corneal staining was negative; 4) a willingness to cooperate with the doctors during the examination procedure. Exclusion criteria of MGD patients and healthy controls included the following: 1) patients who used of any type of antibiotic drops or systemic antibiotics within the past month; 2) patients who currently use treatments for dry eye or MGD (other than artificial tears); 3) patients with active allergies, infections, or inflammatory diseases of the ocular surface unrelated to dry eye or MGD; 4) a history of ocular trauma or surgery within one year; 5) patients with alterations in the lacrimal drainage system such as punctal occlusion; 6) patients who used systemic medications that might alter the tear film; 7) patients who used contact lenses within the past month; 8) patients with systemic diseases affecting the ocular surface; and 9) pregnant or nursing mothers. Forty-one patients (17 males, 24 females) with MGD (82 eyes) were enrolled in this study. The mean \pm SD age of MGD patients was 34.3 ± 10.8 years (ranged from 17 to 60). In the control group, 13 were males and 16 were females, with a mean \pm SD age of 31.8 ± 8.7 years (ranged from 21 to 55).

Clinical evaluation

The clinical assessments of the enrolled subjects were conducted in the following order: collection of demographic information (including age and sex) and physical signs including conjunctival injection, upper and lower tear meniscus height (TMH),^{20,21} TBUT,¹ corneal staining,^{22,23} lid margin,¹⁹ orifice, tear foam and MG assessments (detailed methodology is shown in Supplementary material).²⁴ An interval of 5 minutes was required between the different examinations. All examinations were conducted and the data

collected by two doctors (JXD and YJR), and the average values are presented as the final results.

Sample collection

Samples were collected in a sterile operation room by the same ophthalmologist (JXD) who was wearing sterile gloves and mask. Sterile transport swabs were used to collect bacterial samples. Sample collection was performed in the order described below. 1) The lower CS was swabbed at two different positions, and the swabs were swirled to obtain a specimen on each side of the swab. Care was taken to avoid contact with the eyelids and lashes when taking swabs from the CS. 2) After a drop of repivacaine hydrochloride (Benoxil; Santen Pharmaceutical Co., Ltd, Osaka, Japan) was applied to both eyes as topical anesthesia, each CS was successively irrigated with 0.9% medical saline, 0.025% iodophor and 0.9% medical saline for 1 minute. The eyelid margins (including the roots of the eyelashes) were sterilized using entoiodine. 3) A Meibomian massage was performed using a sterile eyelid plate in the upper eyelid to collect the MG secretions. A new sterile eyelid plate was used for the lower eyelid, after which the same procedure was repeated for the other eye. Segmental secretions were also collected from several patients using the same procedure described for the collection of normal MG secretions. The irrigation and sterilization procedures were performed again before the next MG secretion was collected. Additionally, sterile swabs were exposed in the air of the operation room for 10 seconds each time the sample was collected as blank control.

Strain isolation and 16S rRNA gene sequencing

After collecting the MG secretions, samples were stored at 4°C, and bacterial strains were isolated within 2 hours. The samples were plated onto agar plates containing 10 g of tryptone, 3 g of beef powder, 5 g of NaCl, 50 mL of defibrinated sheep blood, and 15 g of agar in 1 L of medium. After a 24–72-hour incubation at 37°C, colonies with different phenotypes were selected for further analysis. Bacterial morphologies were observed using an optical microscope (Olympus Corporation, Tokyo, Japan) and a high intensity focused ion beam/scanning electron microscopy (FIB/SEM, Zeiss Auriga Compact, Carl Zeiss Meditec AG, Jena, Germany). Genomic DNA was extracted from the isolated strains, and 16S rRNA genes were amplified using a previously described method.²⁵ The PCR products were purified and sequenced using an ABI3730 genetic analyzer (Beijing Genomics Institute, Beijing, China). After the products

were assessed for chimera formation using the Bellerophon server,²⁶ and the 16S rRNA gene sequences were compared with the GenBank database to search for related sequences using the BLAST program. Based on the 16S rRNA gene sequences, the phylogenetic tree of the isolated strains and their closest species were constructed with the maximum-likelihood method using MEGA5.²⁷ The bacterial culture of the blank controls showed no positive results.

Statistical analysis

ANOVA was used to compare the basic characteristics of patients with MGD and the number of isolated bacterial species of different severities. Fisher's least significant difference post hoc test was used to compare patients with moderate or severe MGD with mild MGD. Eyelid margin characteristics and the correlations between the severity of MGD and specific bacteria were analyzed using the chi-squared test. Comparisons of specific bacterial isolation rates between the CS and MG were conducted using McNemar's test. Segmental secretion analysis was performed using Student's *t*-test. $P < 0.05$ were considered statistically significant.

Results

Basic characteristics

Forty-one patients (17 males, 24 females) with MGD (82 eyes) were enrolled in this study; the mean \pm SD age of MGD patients was 34.3 \pm 10.8 years. Twenty-nine healthy volunteers (13 males, 16 females) were enrolled in the control group with a mean \pm SD age of 31.8 \pm 8.7 years (ranged from 21 to 55). The age and sex were matched between MGD and control ($P=0.305$, $P=0.779$, respectively). Of these 82 eyes with MGD, eight were subcategorized into the mild MGD group, 55 into the moderate MGD group and 19 into the severe MGD group. All patients completed the information collection process and examinations, and the results were shown in Table 1.

Analysis of the microbiomes of the CS and MG

The microbiomes isolated from CS and MG secretions are shown in Table 2. In the MG, *S. epidermidis* was the most frequently isolated bacterium and was observed in 46.4% of all eyes. By contrast, no bacteria were isolated from the CS of most patients (80.7%), and *S. epidermidis* was only detected in 10.7% of patients. In both the CS and MG, most of the isolated bacteria were Gram-positive, such as *Staphylococcus*, *Corynebacterium* and *Microbacteriaceae*. However, additional types of bacteria, including Gram-positive

Table 1 Basic characteristics of MGD patients and controls

Objectives	Control (n=58)	Mild (n=8)	Moderate (n=55)	Severe (n=19)	P-value
Upper TMH (mean ± SD)	0.183±0.065	0.125±0.046 [#]	0.131±0.054 ^{#,Δ}	0.126±0.045 [#]	<0.001*
Lower TMH (mean ± SD)	0.174±0.055	0.125±0.046 [#]	0.120±0.048 [#]	0.126±0.045 [#]	<0.001*
TBUT (mean ± SD)	6.50±1.79	2.88±1.55 [#]	2.63±1.57 [#]	2.47±1.39 [#]	<0.001*
Tear foam (mean ± SD)	0.29±0.46	0.75±0.71 [#]	0.80±0.49 [#]	1.11±0.32 ^{#,Π}	<0.001*
Conjunctival injection, n (%)	0 (0%)	2 (25.0%) [#]	4 (7.3%) [#]	2 (10.5%) [#]	0.001*
Corneal staining, n (%)	0 (0%)	0 (0%)	6 (10.9%) [#]	0 (0%)	0.117
Eyelid margin, n (%)					
Rounding of posterior margin	13 (22.4%)	1 (12.5%)	37 (67.3%) ^{#,Δ}	17 (89.5%) ^{#,Δ}	<0.001*
Irregularity	4 (6.9%)	3 (37.5%) [#]	43 (78.2%) ^{#,Δ}	18 (94.7%) ^{#,Δ}	<0.001*
Telangiectasia	1 (1.7%)	0 (0%)	15 (27.3%) [#]	7 (36.8%) [#]	<0.001*
Trichiasis	0 (0%)	0 (0%)	1 (1.8%)	1 (5.3%)	0.392
Hyperkeratinization	2 (3.4%)	3 (37.5%) [#]	26 (47.3%) [#]	12 (63.2%) [#]	<0.001*
Anterior blepharitis	7 (12.1%)	4 (50.0%) [#]	42 (76.4%) [#]	19 (100%) ^{#,Δ,Π}	<0.001*
Meibomian gland assessments (mean ± SD)					
Number of orifices	8.29±1.39	6.13±2.10 [#]	5.67±1.36 ^{#,Δ}	4.68±1.53 ^{#,Δ,Π}	<0.001*
Loss of orifices	0.41±0.50	0.75±1.04	1.25±0.65 ^{#,Δ}	1.89±0.74 ^{#,Δ,Π}	<0.001*
Degrees of obliteration	0.97±0.49	1.88±0.35 [#]	1.96±0.74 [#]	2.21±0.79 [#]	<0.001*

Notes: * $P<0.05$; [#] $P<0.05$ compared to control; ^Δ $P<0.05$ compared to mild; ^Π $P<0.05$ compared to moderate.

Abbreviations: MGD, Meibomian gland dysfunction; n (%): number of eyes (percentage in the group); TBUT, tear break up time; TMH, tear meniscus height.

bacteria such as *Bacillus*, *Paenibacillus* and *Lysinibacillus*, were only isolated from the MG, indicating that the bacterial constituents of the MG were more complex than the bacterial constituents of the CS. The Gram-negative bacterium *Moraxella osloensis* was detected in the CS in three eyes, and *Xanthomonadaceae* was detected in the MG secretions from one eye (Figure 1). Based on these results, a greater number of bacteria with a more complex composition were isolated from MG secretions.

Compositions of the microbiomes of the mild, moderate, severe MGD groups and healthy controls

The relationships between MGD severity and bacteria isolated from MG secretions and between MGD severity and all isolated bacteria (from MG secretions and the CS) were separately analyzed. According to the results obtained from both analyses, a significantly greater number of types of bacteria was detected in the severe and moderate MGD groups than in the control and mild MGD group ($P<0.05$), and all the bacteria were used as an illustration (Table 3). Only the Gram-positive bacteria *Staphylococcus* and *Lysinibacillus* were isolated from the control and mild MGD group.

As shown in the Figure 2, *S. epidermidis* was isolated from eyes of the healthy control and mild MGD. As observed by optical microscopy and scanning electron microscopy, they

showed similar morphology, such as round and agglomerated shape (Figure 2E, F, I and J), which is in accordance with the previous report.²⁸

However, many types of bacteria, including the Gram-positive bacteria *Staphylococcus* (Figure 2C, J, K), *Lysinibacillus*, *Microbacteriaceae* and *Bacillus*, and the Gram-negative bacteria *Moraxella osloensis* and *Xanthomonadaceae*, were isolated from the control and moderate MGD group. Figure 2G and K show the morphology of *S. aureus* and *S. hominis* from eyes of moderate MGD. They were round, agglomerated and Gram-positive under optical microscopy, similar to *S. epidermidis*. Interestingly, the surfaces of *S. hominis* have holes and were clearly different to those of *S. epidermidis* and *S. aureus*.

With the exception of the bacteria isolated from the control, mild and moderate MGD groups, other Gram-positive bacteria, including *Corynebacterium* and *Paenibacillus*, were identified in the severe MGD group. To our knowledge *C. macginleyi* has been isolated from the ocular site in the previous study.²⁹ *C. macginleyi* from eyes of severe MGD was rod-shaped and dispersed by optical microscopy and scanning electron microscopy (Figure 2H and L), which was significant different from microorganisms isolated from eyes of the healthy control, mild and moderate MGD. The incidence of *C. macginleyi* (Figure 2D, H and L) was significantly higher in the severe MGD group than in the

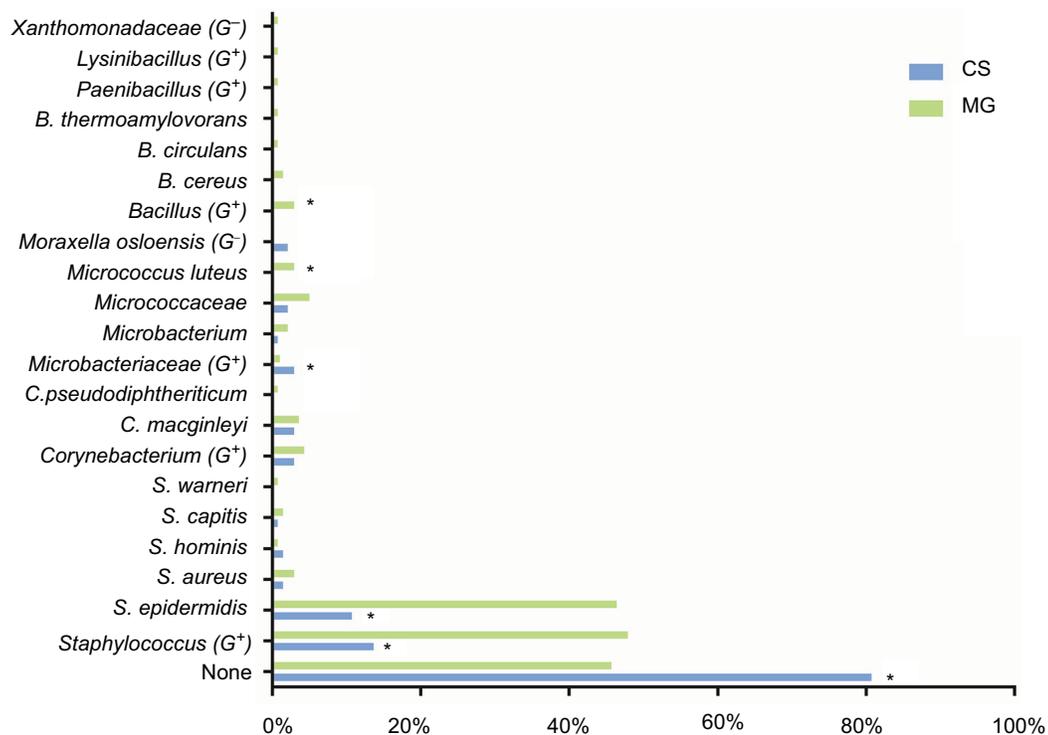


Figure 1 Bacteria isolated in conjunctiva sac and Meibomian gland among MGD and control.

Notes: In CS, most of eyes detected no bacteria (79.3%), while in MG, *Staphylococcus* made up the majority (56.1%). Between two groups, positive isolation rate of bacteria was remarkably higher in MG than CS, as well as *Staphylococcus epidermidis*. * $P < 0.05$. G+ and G- represent the Gram positive and negative bacteria, respectively.

Abbreviations: CS, conjunctival sac; MG, Meibomian gland; MGD, Meibomian gland dysfunction.

Table 2 Bacteria isolated in conjunctival sac and Meibomian gland among MGD patients and controls

Isolated bacteria	n (%)		P-value
	CS	MG	
None	113 (80.7%)	64 (45.7%)	<0.001*
<i>Staphylococcus</i> (G ⁺)	19 (13.6%)	67 (47.9%)	<0.001*
<i>S. epidermidis</i>	15 (10.7%)	65 (46.4%)	<0.001*
<i>S. aureus</i>	2 (1.4%)	4 (2.9%)	0.409
<i>S. hominis</i>	2 (1.4%)	1 (0.7%)	0.562
<i>S. capitis</i>	1 (0.7%)	2 (1.4%)	0.562
<i>S. warneri</i>	0 (0%)	1 (0.7%)	0.316
<i>Corynebacterium</i> (G ⁺)	4 (2.9%)	6 (4.3%)	0.520
<i>C. macginleyi</i>	4 (2.9%)	5 (3.6%)	0.735
<i>C. pseudodiphtheriticum</i>	0 (0%)	1 (0.7%)	0.316
Microbacteriaceae (G ⁺)	4 (2.9%)	14 (10.0%)	0.015*
<i>Microbacterium</i>	1 (0.7%)	3 (2.1%)	0.314
Micrococcaceae	3 (2.1%)	7 (5.0%)	0.198
<i>Micrococcus luteus</i>	0 (0%)	4 (2.9%)	0.044*
<i>Moraxella osloensis</i> (G ⁻)	3 (2.1%)	0 (0%)	0.082
<i>Bacillus</i> (G ⁺)	0 (0%)	4 (2.9%)	0.044*
<i>B. cereus</i>	0 (0%)	2 (1.4%)	0.156
<i>B. circulans</i>	0 (0%)	1 (0.7%)	0.316
<i>B. thermoamylovorans</i>	0 (0%)	1 (0.7%)	0.316
<i>Paenibacillus</i> (G ⁺)	0 (0%)	1 (0.7%)	0.316
<i>Lysinibacillus</i> (G ⁺)	0 (0%)	1 (0.7%)	0.316
Xanthomonadaceae (G ⁻)	0 (0%)	1 (0.7%)	0.316

Notes: * $P < 0.05$. G+ and G- represent the Gram positive and negative bacteria, respectively.

Abbreviations: CS, conjunctival sac; MG, Meibomian gland; MGD, Meibomian gland dysfunction; n (%): number of patients (percentage in CS or MG).

Table 3 Correlation between MGD severity and bacteria

Isolated bacteria	n (%)				P-value
	Control (n=58)	Mild (n=8)	Moderate (n=55)	Severe (n=19)	
None	31 (53.4%)	3 (37.5%)	23 (41.8%)	2 (10.5%) ^{#,¶}	0.012*
<i>Staphylococcus</i> (G ⁻)	23 (39.7%)	5 (62.5%)	29 (52.7%)	12 (63.2%)	0.218
<i>S. epidermidis</i>	23 (39.7%)	5 (62.5%)	28 (50.9%)	11 (57.9%)	0.358
<i>S. capitis</i>	2 (3.4%)	0 (0%)	0 (0%)	0 (0%)	0.412
<i>S. aureus</i>	0 (0%)	0 (0%)	3 (5.5%)	3 (15.8%) [#]	0.026
<i>S. hominis</i>	0 (0%)	0 (0%)	3 (5.5%)	0 (0%)	0.192
<i>S. warneri</i>	0 (0%)	0 (0%)	0 (0%)	1 (5.3%)	0.093
<i>Lysinibacillus</i> (G ⁺)	0 (0%)	1 (12.5%) ^{#,¶}	0 (0%)	0 (0%)	0.001*
<i>Microbacteriaceae</i> (G ⁺)	6 (10.3%)	0 (0%)	8 (14.5%)	3 (15.8%)	0.611
<i>Micrococcaceae</i>	3 (5.2%)	0 (0%)	5 (9.1%)	2 (10.5%)	0.660
<i>Micrococcus luteus</i>	1 (1.7%)	0 (0%)	2 (3.6%)	1 (5.3%)	0.796
<i>Microbacterium</i>	2 (3.4%)	0 (0%)	1 (1.8%)	1 (5.3%)	0.821
<i>Bacillus</i> (G ⁺)	1 (1.7%)	0 (0%)	2 (3.6%)	1 (5.3%)	0.796
<i>B. circulans</i>	0 (0%)	0 (0%)	1 (1.8%)	0 (0%)	0.669
<i>B. thermoamylovorans</i>	0 (0%)	0 (0%)	1 (1.8%)	0 (0%)	0.669
<i>B. cereus</i>	1 (1.7%)	0 (0%)	0 (0%)	1 (5.3%)	0.402
<i>Moraxella osloensis</i> (G ⁻)	0 (0%)	0 (0%)	3 (5.5%)	0 (0%)	0.192
<i>Xanthomonadaceae</i> (G ⁻)	0 (0%)	0 (0%)	1 (1.8%)	0 (0%)	0.669
<i>Corynebacterium</i> (G ⁺)	0 (0%)	0 (0%)	0 (0%)	6 (31.6%) ^{#,¶}	<0.001*
<i>C. macginleyi</i>	0 (0%)	0 (0%)	0 (0%)	5 (26.3%) ^{#,¶}	<0.001*
<i>C. pseudodiphtheriticum</i>	0 (0%)	0 (0%)	0 (0%)	1 (5.3%)	0.093
<i>Paenibacillus</i> (G ⁺)	0 (0%)	0 (0%)	0 (0%)	1 (5.3%)	0.093

Notes: n (%): number of patients (percentage in the group of MGD severity). G⁺ and G⁻ represent the Gram positive and negative bacteria, respectively. *P<0.05; [#]P<0.05 compared to control; [¶]P<0.05 compared to mild; [¶]P<0.05 compared to moderate.

Abbreviation: MGD, Meibomian gland dysfunction.

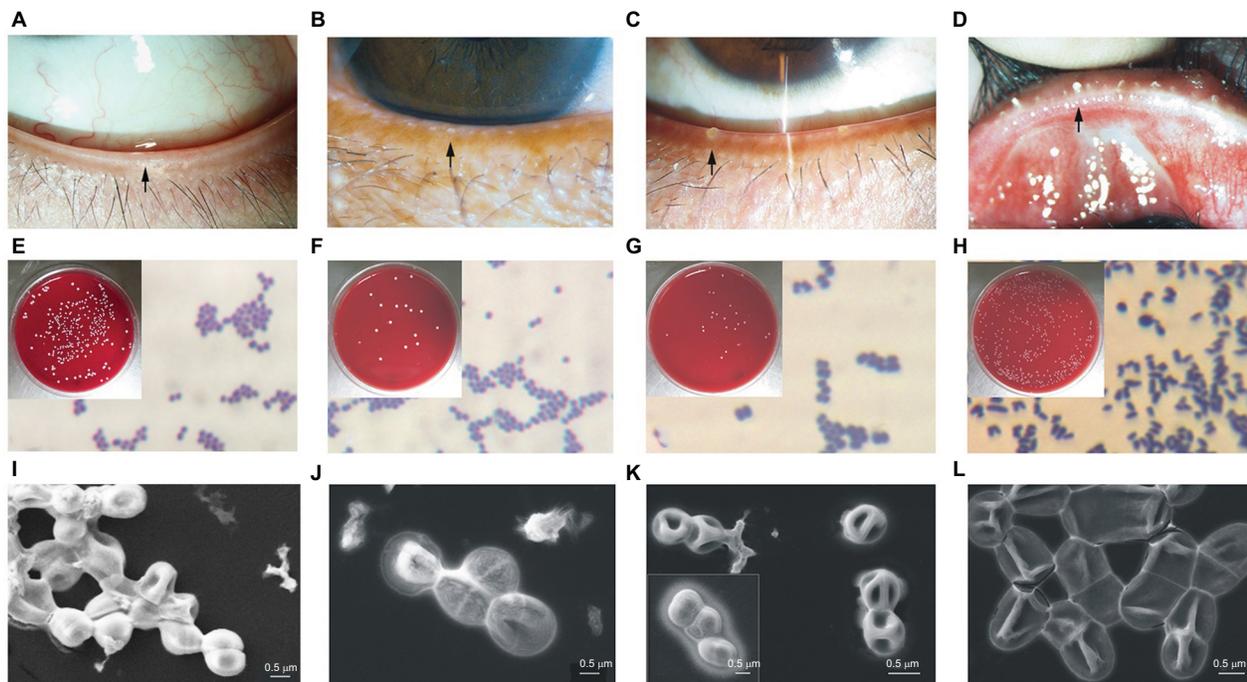


Figure 2 Culture and identification of bacteria in the control and different grades of MGD severity.

Notes: (A–D) Pictures of ocular surface taken before the samples of Meibomian gland secretion were collected: (A) healthy control; (B) diagnosed with mild MGD; (C) moderate MGD; (D) severe MGD. (E–H) Cultures of secretion samples from A, B, C and D respectively after incubation of 24–72 hours, and the Gram staining of bacteria under the microscope: (E) and (F) *S. epidermidis*; (G) *S. hominis* and *S. aureus*; and (H) *C. macginleyi*. (I–L) Images taken under optical microscopy and scanning electron microscopy to determine the morphologies of the isolated bacteria from E, F, G and H respectively: (I) and (J) *S. epidermidis*; (K) the bacterium in the framed space is *S. aureus*; others are *S. hominis*; (L) *C. macginleyi*.

Abbreviation: MGD, Meibomian gland dysfunction.

control and moderate MGD groups ($P<0.001$ and $P<0.001$, respectively). The incidence of *Lysinibacillus* (G⁺) showed a significant difference among the mild, moderate and severe MGD groups ($P=0.001$). Thus, the composition of the microbiome in the control and the mild MGD group was simple, whereas it was more abundant and complex as the severity of MGD increased (Figure 3).

Correlation between the microbiome composition and MGD severity

The characteristics of the isolated microbiome in groups with different severities of MGD were investigated to further explore the relationship between the microbiome composition and MGD severity (Table 4; Figure 2). Because *S. epidermidis* has been reported to be the most frequently isolated bacteria in the normal flora,⁹ the types of isolated bacteria and their isolation rates were analyzed with or without the inclusion of *S. epidermidis*. With the inclusion of *S. epidermidis*, the positive isolation rate of bacteria in the severe MGD group was 89.5%, which was significantly higher than the isolation rate observed in the control (46.6%) and moderate MGD group (58.2%) ($P=0.001$ and $P=0.006$, respectively). In addition, more types of bacteria were isolated from the severe MGD group than in the control ($P<0.001$) and moderate

MGD group ($P=0.021$). Although a higher positive bacterial isolation rate and more types of bacteria were identified in the severe MGD group than in the mild MGD group, the differences were not significant ($P=0.099$ and $P=0.065$, respectively). However, when the data were analyzed without *S. epidermidis*, the positive bacterial isolation rate was significantly higher for the severe group than that among the control ($P<0.001$), mild MGD groups ($P=0.033$), and moderate ($P=0.013$). Moreover, the types of isolated bacteria were also significantly more complex in the severe MGD group than those among the control ($P<0.001$) and the mild ($P=0.007$), and moderate MGD groups ($P=0.006$). Therefore, MGD severity was strongly correlated with an altered ocular microbiome that was specifically composed of non-*S. epidermidis* bacteria, such as *C. macginleyi*.

Relationship between commensalism and MGD severity

All of the eyes analyzed in this study were classified as grade 1 (no isolated bacteria), grade 2 (only *S. epidermidis*), grade 3 (*S. epidermidis* and other bacteria) and grade 4 (only other bacteria) to explore the relationship between bacterial commensalism and MGD severity. The overall P -value obtained from the chi-squared test was 0.004, and subgroup analyses

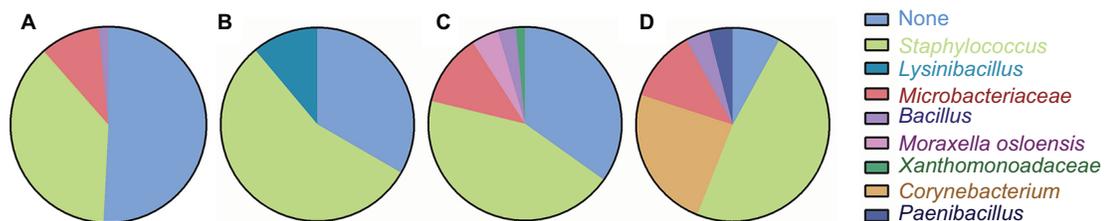


Figure 3 Composition of the microbiome in each group.

Notes: (A) Control; (B) mild MGD; (C) moderate MGD; (D) severe MGD. The size of area in pie chart represented the positive isolation rate of each bacterium.

Abbreviation: MGD, Meibomian gland dysfunction.

Table 4 Characteristics of isolated bacterial flora in control and different MGD severity

Objectives	Control (n=58)	Mild (n=8)	Moderate (n=55)	Severe (n=19)	P-value
Positive isolation, n (%)					
With S.e.	27 (46.6%)	5 (62.5%)	32 (58.2%)	17 (89.5%) ^{#¶}	0.012
Without S.e.	9 (15.5%)	1 (12.5%)	14 (25.5%)	12 (63.2%) ^{#,Δ,¶}	<0.001*
Types of isolated bacteria, mean ± SD					
With S.e.	0.62±0.697	0.75±0.707	0.89±0.975	1.42±0.961 ^{#¶}	0.005*
Without S.e.,	0.16±0.365	0.13±0.354	0.38±0.758	0.84±0.834 ^{#,Δ,¶}	<0.001*

Notes: * $P<0.05$; [#] $P<0.05$ compared to control; ^Δ $P<0.05$ compared to mild MGD; [¶] $P<0.05$ compared to moderate MGD.

Abbreviation: MGD, Meibomian gland dysfunction; n (%): number of eyes (percentage in the groups of control and MGD severity); S.e.: *Staphylococcus epidermidis*.

were performed to explore the detailed differences. First, differences between MGD groups were explored (Table 5A). A higher incidence of grades 3 and 4 was observed in the moderate (18.2%, 7.3%) and severe MGD groups (31.6%, 31.6%) than in the control (12.1%, 3.4%) and mild MGD group (12.5%, 0%), and the difference between the control and severe group was significant ($P<0.001$). A significantly higher incidence of grades 3 and 4 was also observed in the severe MGD group than in the moderate MGD group ($P=0.009$). Second, differences between different commensalism grades were analyzed (Table 5B). We observed a significantly higher incidence of severe MGD and a lower incidence of control in grade four patients than those in grade 1. And a significant difference between grade 4 and grade 1, grade 3 and grade 1 ($P<0.001$, $P=0.018$, respectively), between grade 4 and grade 2 ($P=0.018$) was observed. Based on these results, higher commensalism grades are related to the severity of the MGD.

Analysis of segmental MG secretions

Seventeen patients (34 eyes), including 12 eyes as control, 12 eyes with moderate MGD and 10 eyes with severe MGD, were randomly selected from all of the enrolled subjects to analyze segmental MG secretions. As shown in Table 6, *S. epidermidis* was the most frequently isolated bacterium, and

the Gram-negative bacterium *Xanthomonadaceae* was the only bacterium isolated from the surface layer of MG secretions in the moderate MGD group. Bacterial discordance was calculated: none (0%) indicated that bacterial results were consistent between the surface layer and the deep layer in both upper and lower Meibomian secretions; half (50%) indicated that bacterial results between the surface layer and the deep layer in one position (upper or lower Meibomian secretions) were consistent and in the other were different; and both (100%) meant bacteria between the surface layer and the deep layer in two positions (both upper and lower Meibomian secretions) were different. Severe MGD showed a significantly higher incidence of bacterial discordance ($P=0.035$).

Discussion

In this study, swabs of the CS and segmental MG secretions were confirmed. A correlation between the severity of MGD and the microbiome was observed, and we proposed a new grading system for bacterial severity which might be able to assess the severity of MGD.

Positive bacterial isolation rates for MG secretions (54.3%) were consistent with other published studies, in which the isolation rates for MG secretions ranged from 36.9% to 75.6%,¹⁸ which also implied that the sterilization procedure performed before sampling may not effectively

Table 5A Relationship between commensalism and MGD severity

Objectives n (%)	Grade 1 (n=58)	Grade 2 (n=46)	Grade 3 (n=24)	Grade 4 (n=12)	P1	P2	P3	P4
Control (n=58)	30 (51.7%)	19 (32.8%)	7 (12.1%)	2 (3.4%)				
Mild (n=8)	3 (37.5%)	4 (50.0%)	1 (12.5%)	0 (0%)		0.762		
Moderate (n=55)	23 (41.8%)	18 (32.7%)	10 (18.2%)	4 (7.3%)		0.558	0.720	
Severe (n=19)	2 (10.5%)	5 (26.3%)	6 (31.6%)	6 (31.6%)	0.004*	<0.001*	0.091	0.009*

Notes: * $P<0.05$.

Abbreviations: MGD, Meibomian gland dysfunction; n (%): number of eyes (percentage in the group of MGD severity); P1, P-value of overall chi-test; P2, P-value compared with control; P3, P-value compared with mild; P4, P-value compared with moderate.

Table 5B Relationship between commensalism and MGD severity

Objectives n (%)	Control (n=58)	Mild (n=8)	Moderate (n=55)	Severe (n=19)	P1	P2	P3	P4
Grade 1, (n=58)	30 (51.7%)	3 (5.2%)	23 (39.7%)	2 (3.4%)				
Grade 2, (n=46)	19 (41.3%)	4 (8.7%)	18 (39.1%)	5 (10.9%)		0.367		
Grade 3, (n=24)	7 (29.2%)	1 (4.2%)	10 (41.7%)	6 (25.0%)		0.018*	0.375	
Grade 4, (n=12)	2 (16.7%)	0 (0%)	4 (33.3%)	6 (50.0%)	0.004*	<0.001*	0.015*	0.450

Notes: * $P<0.05$.

Abbreviations: MGD, Meibomian gland dysfunction; n (%): number of eyes (percentage in the group of MGD severity); P1, P-value of overall chi-test; P2, P-value compared with grade 1; P3, P-value compared with grade 2; P4, P-value compared with grade 3.

sterilize the bacteria in the MG, particularly in patients with MGD. Thus, even when a standardized pre-operation sterilization procedure was implemented, the bacteria in the MG were not affected. During surgery, MG secretions containing bacteria might gradually contaminate the sterilized area and lead to severe consequences, such as endophthalmitis. Therefore, a stricter pre-operation evaluation of the MG may be required, as the bacterial isolation rate was highly correlated with MGD severity in our study, and severe MGD might increase the risk of infection. Stricter pre-operation preparation is also essential and should specifically include the use of a surgical adhesive membrane to cover the orifices of the MG. This preparation may reduce the risk post-operation infection, particularly in longer surgical procedures, such as vitreous and retina surgery.

According to our results, as the severity of MGD increased, the composition of the microbiome became more complex, and the bacterial abundance increased. However, mild MGD showed overall less abundance than the control as *Microbacteriaceae* and *Bacillus* were missing, and we thought the reason was the limitation of enrolled subjects in mild MGD. Meanwhile, the positive isolation rate of bacteria in mild MGD was still higher than the control, which indicated an altered microenvironment. *Corynebacterium*, particularly *C. macginleyi*, was only detected in the severe MGD group (31.6%), and this finding was significantly different between groups ($P < 0.001$). *Corynebacterium* is a Gram-positive bacterium that widely colonizes the mucous membranes and skin; however, *C. macginleyi* is a pathogen that causes corneal ulcers and conjunctivitis in the ophthalmic area.³⁰ Our study isolated *C. macginleyi* from MG secretions, and the positive rate was 26.3% in the severe MGD group. Thus, *C. macginleyi* isolation may be related to severe MGD and may be used as a biological marker for the evaluation of MGD severity; once *C. macginleyi* in MGD patients was isolated, it represented a more severe disease condition, which needs more attention from doctors.

Patients with MGD were previously shown to have a higher bacterial isolation rate in the MG than controls;¹⁸ however, our study is the first to analyze the number of types of isolated bacteria. Positive isolation rates and the number of different types of isolated bacteria were significantly higher in the severe MGD group than in the control and moderate MGD group. In the analysis that did not include *S. epidermidis*, positive bacterial isolation rates and the number of types of isolated bacteria were significantly higher in the severe MGD group than those among the control, mild and moder-

ate MGD groups. The presence of more types of bacteria might represent an altered commensal microenvironment that may be caused by the alteration of Meibomian functions and further contribute to disease progress. Moreover, non-*S. epidermidis* bacteria might better reflect the severity of MGD and provide the basis for the indication for antibiotic treatments in patients with severe MGD. These results also explain why previous studies of the use of antibiotics showed therapeutic effects.^{12,13,31,32}

A grading system for bacterial severity was first proposed and defined in our study, which might help to define the relative quantity of pathogens other than *S. epidermidis* in the MG commensal microenvironment. The grade of bacterial severity was a good indicator for the commensal microenvironment, according to our results. *S. epidermidis* was the bacterium that was most frequently isolated from the ocular surface in previous studies,^{14,15,18} and this bacterium is considered a component of the normal flora of the CS and MG. The presence of *S. epidermidis* with other bacteria might indicate that the commensal microenvironment has been partially altered, although the alterations were not extreme because *S. epidermidis* was still present. The presence of other bacteria in the absence of *S. epidermidis* indicates that the commensal microenvironment has been largely altered, and pathogens made up the majority of the bacterial community. When the concentrations of pathogens, such as *Staphylococcus aureus*, increase to a certain level, bacteria are able to initiate virulence and evade the host immune response,³³ which might explain why the severity of MGD increased when pathogens constituted most of the microbiome and commensalism and pathogen concentrations were increased. Based on our results, the grade of bacterial severity was significantly correlated with MGD severity, which might offer some meaningful insights for clinical practice.

The true etiology of MGD remains uncertain, although it is thought to be due to a number of factors, including bacterial infection.³⁴ Oral or topical antibiotics have been reported to be beneficial treatments for MGD.^{12,13} However, antibiotics are not effective for every patient, which is consistent with the results from our study, as not every patient in each group was present with a high grade of bacterial severity. Based on our results, a medical indication for antibiotics might be more suitable for patients with a high grade of bacterial severity (grade 3 or 4) or who have more types of isolated bacteria. Furthermore, a prospective study focusing on the effects of antibiotics on patients with MGD with high grades of bacterial severity or more types of isolated bacteria is required. We hope that precise medicines will

Table 6 Differences of bacterial flora isolated from segmental Meibomian secretions

Isolated bacteria	Control (n=12)		Moderate (n=12)		Severe (n=10)		P-value
	Surface	Deep	Surface	Deep	Surface	Deep	
<i>S. epidermidis</i>	5	4	8	5	4	6	
<i>S. warneri</i>						1	
<i>C. macginleyi</i>					2	3	
<i>C. pseudodiphtheriticum</i>					1		
Micrococcaceae	1	1	1				
Microbacterium				1			
<i>Micrococcus luteus</i>					1		
<i>Paenibacillus</i>					1		
Xanthomonadaceae			1				
<i>Bacillus thermoamylovorans</i>				1			
<i>Bacillus cereus</i>		1					
None	6	6	4	5	3	2	
Positive isolation, n (%)	6 (50.0%)	6 (50.0%)	8 (66.7%)	7 (58.3%)	7 (70.0%)	8 (80.0%)	
Bacterial discordance							
None (0%)	8 (50.0%)		4 (33.3%)		3 (30.0%)		
Half (50%)	4 (50.0%)		6 (50.0%)		2 (20.0%)		
Total (100%)	0 (0%)		2 (16.7%)		5 (50.0%)		0.035*

Notes: n (%): number of eyes (percentage in the groups of control and bacterial severity). *P<0.05.

become available for each patient in the future. Additionally, it is promising to explore the correlation between severity of other bacteria-correlated diseases and bacterial results using this grading system, which could give some implications for clinical practice.

Our study is the first to perform a segmental MG secretion analysis, and differences between the surface layer and deep layer of the MG were explored. Bacteria were isolated from both the surface layer and deep layer of the control and MGD groups. Some bacteria isolated from the deep layer were not detected in the surface layer, which strongly indicated that a bacterial microenvironment exists in the MG, rather than contaminated from the CS. Segmental secretions were less likely to be affected by the microbiome in the CS, which might be a reliable method for exploring the complexity of the microbiome of the MG in patients with MGD. Massage was performed to obtain secretions and proved to be effective in relieving MGD symptoms,^{32,35} which may help patients who are waiting for the bacterial results. Additionally, severe MGD showed a significantly higher incidence of bacterial discordance, which indicated that a more complex microbiome constitution and a deeper damage of MG occurred in severe MGD patients. More studies focusing on segmental bacterial analysis and the detailed bacterial species affecting the discordance in patients with different severities of MGD are required.

The severity of MGD is correlated with a higher bacterial isolation rate, a greater number of types of isolated bacteria, and a higher grade of bacterial severity. However, our study had some limitations. First, the numbers of enrolled eyes in mild MGD and patients who underwent the segmental secretion analysis were small. Second, due to the limitations associated with the detection device, we only explored aerobic bacteria isolated from the ocular surface, and further studies should also include anaerobic bacteria. Third, our study also lacked information regarding the patients' living environments, financial statuses and careers, which might also influence the results. Fourthly, although standard sterilization procedure was performed and matched among groups, bacteria flora in the eyelid margin might not be removed completely,³⁶ and the results could not fully represent the bacterial flora in MG. Last but not least, further studies are required to explore the use of antibiotic treatments for patients with high grades of bacterial severity and to investigate differences in the microbiomes in patients with different types of dry eye.

Conclusions

In this study, the microbiomes of the CS as well as the surface and deep layers of MG secretions were characterized by 16S rRNA gene sequencing. Bacterial isolation rates were significantly higher in MG secretions than in the CS and were higher in the severe MGD group than among

the control, mild and moderate MGD groups. Based on the grading system for bacterial severity proposed in this study, the types of bacteria isolated in each patient (with or without *S. epidermidis* included) were calculated. The number of bacterial types and the grades of bacterial severity are positively correlated with the severity of MGD, which may provide some insights into the appropriate treatment of MGD. Furthermore, *C. macginleyi* may have potential value as a biological marker for the evaluation of MGD severity.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Conjunctival injection and TMH

The degree of conjunctival injection was evaluated using a slit lamp microscope. The Institute for Eye Research Grading Scales¹ were used to assess bulbar conjunctival redness with a score of 0 representing grade 1 redness, and a score of 3 representing grade 4 redness, which indicates severe redness of the bulbar conjunctiva. The central upper and lower TMH were measured using a slit lamp microscope (with a graticule in 0.05 mm units).² Three consecutive readings were obtained, and the final results are presented as medians.

TBUT and corneal staining

A total of 5 µL of 2% sodium fluorescein was instilled onto the bulbar conjunctiva using a micropipette, without inducing reflex tearing. The patient was asked to blink naturally without squeezing 3–5 times and was then asked to stare straight ahead without blinking under the cobalt blue light until he or she received other instructions. A stopwatch was used to record the time between the last complete blink and the first appearance of a dry spot or disruption in the tear film.³ The procedure was repeated three times, and the final score is presented as an average value. For the corneal staining evaluation, the cornea was divided into five sectors.⁴ Each sector was graded from 0 to 3 using the following criteria: 0 representing no staining; 1 representing punctate or stippled staining; 2 representing ball and linear staining; and 3 representing coalesced staining.⁵

Eyelid margin, tear foam and MG assessments

According to the International Workshop on Meibomian Gland Dysfunction,⁶ six eyelid margin characteristics were assessed in this study: rounding of the posterior margin,

irregularity or notching of the margin, telangiectasia or vascularity of the lid margin, trichiasis, hyperkeratinization and anterior blepharitis. Each sign was assigned a score of 0 or 1. A score of 0 indicated the absence of the particular eyelid margin characteristic (i.e. normal), whereas a score of 1 indicated the presence of the characteristic (i.e. abnormal). Tear foam was scored from 0 to 2. A score of 0 represented normal tear foam, whereas scores of 1 and 2 represented mildly abnormal and severely abnormal tear foam, respectively. MG assessments included: 1) the average number of lower lid orifices; 2) the quality of the expressed secretion; and 3) the degree of orifice obliteration. The quality of the expressed secretion was scored as follows: 0=clear; 1=cloudy; 2=granular; and 3=toothpaste-like. The degree of orifice obliteration was expressed as follows: 1=light pressure; 2=moderate pressure; and 3=heavy pressure. The grade of MGD severity was also expressed according to criteria from the Meibomian Gland Dysfunction Workshop reported by A. Tomlinson et al.⁷

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