The Microbiome of the Meibum and Ocular Surface in Healthy Subjects

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PURPOSE. The purpose of this study was to investigate the microbiome in the meibum, conjunctival sac, and eyelid skin in young and elderly healthy subjects, and analyze the effect that age, sex, and region have on microbiome composition.

METHODS. This study involved 36 healthy subjects (young-age subjects: 9 men/9 women, age range: 20–35 years; elderly age subjects: 9 men/9 women, age range: 60–70 years). In all subjects, lower-eyelid meibum, lower conjunctival sac, and lower-eyelid skin specimens were collected from one eye, and then stored at –20°C. Taxonomic composition of the microbiome was obtained via 16S rRNA gene sequencing, and then analyzed.

RESULTS. The meibum microbiome showed a high α-diversity (within-community diversity), particularly in the young subjects. However, in approximately 30% of the elderly subjects, a low-diversity microbiome dominated by Corynebacterium sp. or Neisseriaceae was observed. In the young subjects, the microbiome of the meibum resembled that of the conjunctival-sac, yet in the elderly subjects, the microbiome of the conjunctival-sac became more similar to that of the eyelid skin. The eyelid-skin microbiome was relatively simple, and was typically dominated by Propionibacterium acnes in the young subjects, or by Corynebacterium sp. or Neisseriaceae in the elderly subjects. In both age groups, no significant difference was seen between the men and women in regard to the meibum, conjunctival-sac, and eyelid-skin microbiome.

CONCLUSIONS. Our findings confirmed that the meibum of healthy adult-age subjects harbors highly diverse microbiota, and revealed that the meibum microbiome, especially the decrease of its diversity, alters with aging and may affect the homeostasis of the ocular surface.

Keywords: microbiome, meibomian gland, meibum, conjunctiva, eyelid, Propionibacterium acnes, coagulase-negative Staphylococcus, Staphylococcus epidermidis, Corynebacterium sp., meibomian gland dysfunction, meibomitis-related keratoconjunctivitis (MRKC), ocular surface
(i.e. coagulase-negative Staphylococcus sp., Corynebacterium sp., and Propionibacterium acnes (P. acnes, recently renamed Cutibacterium acnes\(^{[19]}\)) as that in the lid margin were reportedly cultured from approximately 50% of the freshly expressed meibum of a normal subject. Thus, their findings indicated that commensal bacteria exist in the meibomian glands, yet did not indicate that the infection occurs in the meibomian glands. Recently, Zhang et al. confirmed that the predominant species isolated from the conjunctiva and meibomian gland secretion were Staphylococcus epidermidis (aerobes) and P. acnes (anaerobes), and they reportedly discovered more complex bacterial flora in the patients with MGD than in the controls.\(^{[20]}\) Furthermore, meibomitis, an inflammatory form of MGD, is thought to be caused by bacterial ingrowth, and studies have reported the importance of using a systemic antimicrobial treatment to effectively eliminate the ocular surface inflammation in meibomitis cases.\(^{[1,21–23]}\)

Since 2001, and especially over the past decade, a number of articles have been published regarding the analysis of the conjunctival microbiome via the use of 16S rRNA gene sequencing.\(^{[14,15,24–31]}\) However, and to the best of our knowledge, no previous studies have reported analyzing the detailed microbiome of meibum via 16S rRNA gene sequencing,\(^{[32,35]}\) so it has yet to be elucidated. Although one published study did investigate the meibomian gland microbiome via 16S rRNA gene sequencing for the strain once isolated from the culture of the meibum, some of the dominant anaerobic commensal bacteria, such as P. acnes, were not detected in that meibum.\(^{[32]}\)

Thus, the purpose of this present study was to perform a comprehensive, yet “first step,” analysis of the microbiome of human meibum, conjunctival sac, and eyelid skin via 16S comprehensive, yet “first step,” analysis of the microbiome of human meibum, conjunctival sac, and eyelid skin via 16S gene sequencing.\(^{[14,15,24–31]}\) However, and to the best of our knowledge, no previous studies have reported analyzing the detailed microbiome of meibum via 16S rRNA gene sequencing,\(^{[32,35]}\) so it has yet to be elucidated. Although one published study did investigate the meibomian gland microbiome via 16S rRNA gene sequencing for the strain once isolated from the culture of the meibum, some of the dominant anaerobic commensal bacteria, such as P. acnes, were not detected in that meibum.\(^{[32]}\)

**METHODS**

**Subjects**

The protocols used for the experiments in this study were approved by the Institutional Review Board of Kyoto Prefectural University of Medicine and the Kyoto City Hospital Organization, Kyoto, Japan, and in accordance with the tenets set forth in the Declaration of Helsinki, written informed consent was obtained from all subjects prior to their participation in the study.

This study involved 36 healthy volunteer subjects comprised of 9 young women (mean age: 25.9 ± 5.2 SD years), 9 young men (mean age: 31.8 ± 3.8 years), 9 elderly women (mean age: 64.0 ± 2.9 years), and 9 elderly men (mean age: 65.4 ± 2.6 years), and all subjects were native Japanese (i.e., of Asian ethnicity). The subjects in the young female group were healthy premenopausal women with a regular 28 to 30-day menstrual cycle (duration: 6–7 days), and they were seen in the follicular phase (i.e., before ovulation). The following subjects in both age groups were excluded from the study: tobacco smokers, contact lens wearers, and subjects with any eye and/or systemic disease, or who were taking medication at the time of the study.

**Sample Collection**

From one eye in each subject, microbiota at the following three sites were collected via single-use, clean, and sterile 3-mm diameter cotton swabs (JCB Industry Ltd., Tokyo, Japan): 1) a lower-eyelid skin sample from 3 mm below the eyelash line, 2) a lower conjunctival-sac sample, and 3) a lower-eyelid meibum sample. The specimens were collected in the following order. First, the skin microbiota was collected by rubbing with a swab moistened with sterile TET buffer (10 mM Tris-Cl [pH 8.0], 1 mM EDTA, 0.1% Tween 20). Next, the conjunctival-sac microbiota was collected by rubbing with a dry swab. The meibum sample was then collected via strict adherence to the following precautions in order to avoid any possible bacterial contamination from surrounding tissues. First, the lid margin was sterilized by use of 10% povidone-iodine, cleaned with sterile saline applied to a swab, and then wiped with a dry swab. The meibum was then squeezed out of the eyelid margin by use of a Yoshitomi Meibomian Gland Compressor (T.M.I. Co., Ltd., Saitama, Japan) under a surgical microscope, collected by use of a Daviel cataract spoon, and transferred to a dry swab. Each of the microbiota-sample swabs was then cut, with the head of the swab then being placed into a DNase-free Eppendorf Tube (Thermo Fisher Scientific, Waltham, MA, USA) and immediately stored at −20°C for later analysis.

**DNA Extraction and Sequencing**

DNA was isolated from each obtained specimen using a DNeasy PowerSoil Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer’s instructions. The V1-V2 region of the 16S rRNA gene was then amplified by polymerase chain reaction (PCR) using the following primers: 27modF: 5′-TCGTCGCGACGCTACAG-3′ and 338R: 5′-GTCTCCTGCGGCTGGAGATGTGTATAAGAGACAGTTGATYTGGTGTCAG-3′ and 338R: 5′-GTCTCCTGCGGCTGGAGATGTGTATAAGAGACAGTTGATYTGGTGTCAG-3′. The acquired paired-end reads were trimmed by Cutadapt version 1.8.1 (www.cutadapt.org).\(^{[35]}\) The adapted sequencers were trimmed by Cutadapt version 1.8.1 (www.cutadapt.readthedocs.io/en/v1.8.1/index.html).\(^{[36]}\) Next, high-quality reads that satisfy the following criteria were extracted using the USEARCH tool: (1) the expected number of errors in the read, which is calculated based on sequencing quality scores, does not exceed 0.5, (2) the length is 250 bp or longer, and (3) the sequence is not considered as a chimera of different species. Using the resultant high-quality reads, operational taxonomy units (OTUs) were generated by clustering the reads with 97% or higher similarity. The seed sequence of each OTU was then chosen and used for taxonomic classification by using UCLUST taxonomy assigner (www.drive5.com)\(^{[35]}\) and Greengenes reference sequence

**Sequence Processing and Taxonomic Classification**

Processing and analysis of the sequenced reads were conducted via the use of the QIIME analysis tool package version 1.9.0 (www.qiime.org).\(^{[35]}\) The acquired paired-end reads were merged into a single read by USEARCH version 8.0.1623 (www.drive5.com),\(^{[35]}\) and the adaptor sequences were trimmed by Cutadapt version 1.8.1 (www.cutadapt.readthedocs.io/en/v1.8.1/index.html).\(^{[36]}\) Next, high-quality reads that satisfy the following criteria were extracted using the USEARCH tool: (1) the expected number of errors in the read, which is calculated based on sequencing quality scores, does not exceed 0.5, (2) the length is 250 bp or longer, and (3) the sequence is not considered as a chimera of different species. Using the resultant high-quality reads, operational taxonomy units (OTUs) were generated by clustering the reads with 97% or higher similarity. The seed sequence of each OTU was then chosen and used for taxonomic classification by using UCLUST taxonomy assigner (www.drive5.com)\(^{[35]}\) and Greengenes reference sequence
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FIGURE 1. The composition of bacterial microbiota in the samples collected at the meibum, conjunctival sac, and eyelid skin sites. The 10 most abundant taxa are shown. Shannon index (SI), a measure of species diversity in a sample, is indicated by the gray-scale color. The subjects were sorted by SI of the meibum sample in ascending order, within each group of young male, young female, elderly male, and elderly female subjects. ID, subject identification number.

Data Analysis and Statistical Test

The statistical environment R was used for the principal coordinate analysis (PCoA) and statistical tests.

RESULTS

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The bacterial composition of each sample revealed by 16S rRNA analysis is shown in Figure 1, and the major 10 taxa, whose mean relative abundance is >1%, are depicted. It has been reported that, typically, the microbiome of human skin is relatively simple, and is dominated by Propionibacterium and Staphylococcus genera. Consistently, the eyelid-skin samples collected from the young subjects showed low alpha-diversity, or within-community diversity, index (Shannon index), and contained P. acnes and Staphylococcus epidermidis (S. epidermidis) as the dominant species, whose mean relative abundance was 43% and 18%, respectively. Interestingly, the meibum and conjunctival-sac samples obtained from the young subjects exhibited microbiomes distinct from that of skin. They were characterized by high alpha-diversity index, and consisted of a large number of bacteria species. Typically, the most abundant species is P. acnes or Pseudomonas sp. for meibum and P. acnes for the conjunctival sac, whose mean relative abundance are 22% or less. We also found that the microbiomes in the elderly subjects were different from those in the young subjects. On the eyelid skin, the community diversity was once again low, yet Corynebacterium sp. and the Neisseriaceae family (mostly a species that has no genus or species level affiliation) were the predominant taxa in many cases. The meibum and conjunctival sac microbiota were divided into high- and low-diversity types in the elderly subjects. In the low-diversity microbiota, either that of Corynebacterium sp. or the Neisseriaceae family was the most abundant taxon. Next, we assessed how interpersonal variation is affected by the age and sex of the subjects. Weighted UniFrac was used to measure the distance, or the degree of dissimilarity, between a pair of samples. At all of the sample collection sites, the weighted UniFrac pairwise distances between different age groups showed significantly higher values than those within the same age group (Fig. 2A). In contrast, the sex of the subject was found to have little impact on bacterial
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**FIGURE 2.** Analyses based on the weighted UniFrac pairwise distance between samples. (A) Distribution of the pairwise distances. All pairs of the samples from either of meibum, conjunctival sac (CS), or eyelid skin (skin) were divided into “within the same age groups” and “between the different age groups” (left) or “within the same sex groups” and “between the different sex groups” (right). The weighted UniFrac distances of each set of pairs are depicted in the box plot. Blue, pairs within the same age/sex groups; red, pairs between the different age/sex groups. The numbers above the box plots are P values of the Mann-Whitney U test. n.s., not significant (or P > 0.05). (B) A principal coordinate analysis plot based on the weighted UniFrac distance. Samples representing each of the collection sites, and subject age are shown by distinct symbols. Clusters of the samples with characteristic microbiomes are indicated. (C) Weighted UniFrac distances of the samples collected at the three different sites of the same subject. M–C, sample pairs between the meibum and the CS; M–S, sample pairs between the meibum and the skin; C–S, sample pairs between the CS and the skin. The numbers above the box plots are P values of the Wilcoxon signed-rank test. n.s., not significant (or P > 0.05).

communities, because no significant difference in weighted UniFrac pairwise distances was found between the within-group and between-group comparisons (Fig. 2A).

The similarity of the individual samples was visualized via PCoA of the weighted UniFrac distance matrix (Fig. 2B). The meibum and conjunctival-sac samples were basically distributed separately from the skin samples, indicating a different microbiome structure between meibum/conjunctival-sac and skin. However, several samples derived from the meibum, the conjunctival sac, and the skin of the elderly subjects formed clusters, and those clusters corresponded to a *Corynebacterium*- or Neisseriaceae-rich microbiome. This finding is consistent with the emergence of the low-diversity meibum/conjunctival-sac microbiota in the elderly subjects (Fig. 1).

We also evaluated the similarity of bacterial community among the three sites of the same individual, using the weighted UniFrac distance (Fig. 2C). In the young subjects, the meibum and conjunctival-sac microbiomes were most similar (i.e. showed the smallest weighted UniFrac value), whereas the meibum and skin microbiomes were most distant. However, in the elderly subjects, the distance between the conjunctival sac and the skin is closer than that between the meibum and the conjunctival sac.

Our findings indicated that the meibum, conjunctival-sac, and eyelid-skin microbiota in the young subjects differed from that in the elderly subjects, and we validated that indication via the statistical test results. At the meibum and conjunctival-sac sites, the mean of the Shannon α-diversity index was greatly reduced upon aging, and this change was found to be statistically significant (Fig. 3A). The mean of the relative abundance of *P. acnes* was also significantly reduced in the elderly subjects at all of the tested sites (i.e. meibum, conjunctival sac, and eyelid skin) (Fig. 3B). Among the other abundant taxa, *Corynebacterium* sp. showed considerable increase in the mean relative abundance at the conjunctival sac and skin (Fig. 3C), whereas *S. epidermidis* showed significant decrease at the skin in the elderly subjects (Fig. 3D).
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DISCUSSION

To the best of our knowledge, this present study is the first to investigate meibum microbiomes in detail and analyze their differences in relation to aging and sex in comparison with that of the adjacent conjunctival-sac and eyelid-skin microbiomes via the use of the 16S rRNA gene sequencing method. Our findings revealed that the pure meibum in healthy subjects harbors highly diverse microbiota (i.e., > 10 taxa) that usually cannot be recovered by a conventional culture technique. Furthermore, our findings show that there is a clear difference between young and elderly subjects in regard to the microbiome of the meibum, the conjunctival sac, and the eyelid skin.

In this present study, the Shannon α-diversity index of the meibum and conjunctival sac significantly decreased with aging. This finding is consistent with the previous reports in regard to the conjunctival microbiome, whereas another report showed no age difference or with a higher diversity in elderly subjects. One of the possible reasons for the difference in the Shannon index findings among the studies could be due to the different sequencing methods that were used (i.e., 16S genome sequence versus whole genome sequence) and/or the study design (i.e., the subjects’ age setting and the sample collection of each group, as Özkan et al. mentioned). Conversely, our eyelid-skin microbiome data was consistent with that of the skin microbiome, which is relatively simple and dominated by Propionibacterium and Staphylococcus genera. In fact, a low α-diversity with P. acnes and S. epidermidis was seen in the young subjects, and with Corynebacterium sp. or the Neisseriaceae family in the elderly subjects. Interestingly, no other changes in the microbiota associated with aging have been reported in other areas of the skin. Thus, whether or not this age-related change is a general feature of the eyelid needs further investigation. Corynebacterium sp. is reportedly noninflammatory under a steady-state condition; however, it could be an activator of skin immunity by expressing mycolic acid, which is required to mediate IL-23-dependent responses.

Interestingly, our microbiota findings indicated that the conjunctival-sac microbiome is close to that of meibum; yet distinct from that of the eyelid skin in the young, whereas it becomes more similar to that of the eyelid skin in the elderly. This might be explained by the fact that human microbiota, although personalized, varies systemically across specific body environments (habitats) and time (i.e. bacteria in the eyelid skin was intrapersonally transplanted to the conjunctiva upon aging by rubbing the eyelids with the fingers).

It should be noted that the diversity of the meibum/conjunctival-sac microbiome in elderly subjects is very low, and is occupied by either Corynebacterium sp. or Neisseriaceae. Corynebacterium sp., which reportedly has been found in the conjunctiva via the use of 16S RNA gene sequencing methods, is a causative bacterium of conjunctivitis, keratitis, and others, and greater attention is now being paid to its resistance to antimicrobial agents in the elderly. In fact, alteration of the microbiome due to the aging process might be the underlying background of these diseases.

In contrast to the age difference of the subjects, our study found that sex had no major impact on the microbiome at all three sites, which is consistent with previous findings. It is widely known that there is evidence indicating that the loss of gut microbiota diversity can occur and does affect the aging process, and that dysbiosis often drives infection and inflammation. Compared with S. aureus and S. epidermidis, P. acnes and Corynebacterium sp. reportedly produce relatively low lipase activity. Thus, a dysbiosis of meibum and/or conjunctiva could degrade meibomian lipids, results in meibomitis, unstable tear film, and ocular surface inflammation. Antimicrobial agents, such as minocycline and azithromycin, may contribute to a recovery from a dysbiosis of the ocular surface. Therefore, any change of the microbiome in meibum and conjunctiva could lead to a shift in ocular surface health, thus resulting in a diseased condition.

It should be noted that this present study did have some limitations. First, it should be noted that one limitation was that there was a possible risk of contamination. However, in all subjects, the meibum samples were obtained after careful cleaning of the eyelid border where the meibomian gland orifices are located, and far enough away from the conjunctival sac as well as from the eyelid skin. Second, although the sample size was not large, the microbiome, which was recurrently detected in each region and in each age group, proved the clear age-related difference. However, in order...
for the results to be deemed more conclusive, further study involving a larger sample size is needed. Third, the ethnicity of the subjects might have had an impact on the divergence of the microbiome.

In conclusion, the findings in this study confirmed diverse microbiota in the meibum of healthy human subjects, and revealed that it alters with aging, especially in regard to the decrease of its diversity. In the young subjects, the microbiome of the meibum closely resembled that of the conjunctival sac; yet in the elderly subjects, the microbiome of the conjunctival sac was found to have become more similar to that of the eyelid skin. Our findings and observations may possibly indicate one of the causes in the change of the meibum lipid composition in elderly subjects and patients with MGD.

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