






Ocular Microbiota of Severe Meibomian Gland Dysfunction (Chronic Dry Eyes) after Intense Pulsed Light (IPL)

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Abstract

Ocular IPL therapy has recently been widely used for MGD, especially for patients not showing improvement with traditional therapies (warm compresses and lid scrubs) to clean debris and reduce bacterial overgrowth. Insights on the ocular microbiome and quantitative microbiome in MGD after a course of IPL could provide useful data on bacterial community monitoring and associated mechanisms linked with IPL. Ocular swabs were obtained from a severe MGD patient and age-sex matched healthy for metagenomics, followed by 16S rRNA gene sequencing and qPCR. Of 10 samples, including left and right eyes (el, er) of severe MGD females before (Db) and after 2-4 IPLs (Da2, Da3, and Da4) and the matched non-MGD females (H), both of ~40 years Using 16S rRNA gene sequencing as microbiota and combined 16S rRNA gene qPCR as quantitative microbiota revealed significant disperse in the microbiome structures of Db compared with Da and H (HOMOVA, $p < 0.001$). Bacterial *Propionibacterium acnes* and unclassified taxa in the family *Propionibacteriaceae* and order *Actinomycetales* represented the core Db microbiota and were reduced after 2-4 IPLs in Da, making the Da microbiome and clinical (mucocutaneous junction, corneal, and conjunctival fluorescein score) closer to H (NMDS with Pearson's correlation, $p < 0.05$). The recovery of the Da microbiome also allowed Da metabolic potentials to be closer to H. Our findings first demonstrated the ocular microbiome dysbiosis in severe MGD, dispersed from Da and H, in Thai subjects, correlated with bacterial quantity and clinical MGD, including the mucocutaneous junction process. The results additionally provided taxa representing Db vs. Da and H and preliminarily underlie the idea that IPL could improve dysbiosis in the MGD microbiome.

Keywords:

Ocular;
Microbiota;
Intense Pulsed Light (IPL) Laser;
Bacteria;
Meibomian Gland Dysfunction;
Dry Eye Therapy.

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1- Introduction

Meibomian gland dysfunction (MGD) is a common chronic eye disorder (~69.3% among Asians), characterized by blockage of the terminal duct that delivers lipid-rich secretion produced by meibomian glands (meibum) or other abnormalities of the meibomian glands affecting the quality or quantity of meibum [1]. Subsequently, this affects imbalanced tear film, eye irritation and dryness, blurred vision, and inflamed eyes as the blockage meibum accumulates

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and promotes microbial and mite infections [2]. MGD is classified into mild, moderate, and severe based on secreting meibum quantity and quality, symptom severity, mucocutaneous junction, corneal staining, and gland loss number [1, 3]. In severe MGD, gland loss is over half of the meibomian gland area, and severe MGD may show permanent gland atrophy, altered ocular microbial proliferation, and chronic eye inflammation. Risk factors include aging, fatty acid intake, contact lens use, Asean ethnicity, medications or antibiotics, hormones, and the ocular surface microbiome [1, 4–9]. In addition to the *Demodex* mite, microorganisms such as the bacteria genera *Propionibacterium*, *Corynebacterium*, and *Staphylococcus* were reported to be highly associated with MGD. The pathology of these bacteria in MGD remains uncertain [5-7, 9, 10].

Traditional therapy includes artificial tears, anti-inflammatory drugs, and warm compresses following lid scrubs to clean debris and reduce mite-bacteria overgrowth. These practices show temporary improvement (1-3 months), and recovery in severe MGD was variable [11, 12]. Intense pulsed light (IPL) has become an alternative therapy, with reported more permanent symptom improvement (>6 months) and time-efficient than traditional therapies, e.g., more improved meibum quality and quantity, decreased gland abnormality, increased tear film lipid layer thickness, and reduced mite-bacteria growth (i.e., inhibited *Corynebacterium macginleyi* growth) [13–15]. To date, no report was on the ocular quantitative microbiota and metabolic potentials in severe MGD without *Demodex* confounding factor (study subjects had no *Demodex*; Table 1) before and after 2-4 IPL versus healthy using metagenomics-derived 16S rRNA gene next-generation sequencing and qPCR. We thereby preliminarily characterized these, along with the clinical parameters, and firstly revealed the ocular quantitative microbiota of severe MGD in Thai ethnic groups.

Previously, Sagaser et al. [10] analyzed ocular rosacea with dry eye U.S. volunteers who were treated with cytokines TGF- β and IPL+TGF- β and reported that IPL+TGF- β treatment showed the improved dry eye symptoms than TGF- β treatment alone; however, the study investigated ocular rosacea causing dry eyes and did not investigate the quantitative bacterial counts and correlation with clinical parameters and microbial metabolic function potentials. Wang et al. [8] reviewed several causes (i.e., antibiotics and tear film problems) that could disturb the ocular surface bacterial community from homeostasis to dysbiosis, which could affect the host cellular and molecular pathways, such as ocular inflammation, cytokine activation, and harmful metabolic products. This supported an interplay between microbial metabolisms and host clinical pathogenesis. Jing et al. [16] reported the microbiota of Chinese with no, mild, moderate, and severe MGD, all showing different percent relative abundance taxon compositions; however, the study did not exclude a possible confounding factor for microbiota imbalance by *Demodex* and yeast infection, an effect of IPL recoverable treatment, and no quantitative bacterial counts and correlation with clinical parameters and microbial metabolic function potentials.

2- Methods

Two Thai females of ~40 years, one diagnosed as non-MGD and the other as severe MGD by physicians based on clinical signs and symptoms, had no *Demodex*. Ocular samples (left and right eye swabs of healthy and MGD patients before IPL) were collected using sterile cotton swabs to wipe the upper and lower conjunctival sac and eyelid at the diagnosis date. For the severe MGD, the patient was treated with IPL (Quantum™, Lumenis, USA), each 2-3 weeks apart, utilizing 10–12 J/cm² intensity (depending on local severity) and a 590-nm filter with a 6-mm SapphireCool cylindrical light for the upper and lower eyelids. The severe MGD thereby contained samples after the 2nd, 3rd, and 4th IPLs. Sample abbreviations were: for instance, her representing healthy eye right; Dbel, disease before IPL on eye left; and Da2el, disease after 2nd IPL on eye left (Table 1). These ocular swab sample collections and protocols were approved by the Institutional Review Board of Bumrungrad International Hospital; subjects were provided written informed consents, and data were anonymized before assessment. A summary flowchart of methodology is in Figure 1-a.

Table 1. Clinical characteristics, quality 16S rRNA gene sequencing reads, and total bacteria number results of healthy and severe MGD subjects

Sample IDs	Healthy subject Hel, Her ^a	Severe MGD subject			
		Before Dbel, Dber	After 2 nd IPL Da2el, Da2er	After 3 rd IPL Da3el, Da3er	After 4 th IPL Da4el, Da4er
MGD grading (0-4+) ^b	1+	3+	3+	3+	2+
Mucocutaneous junction (%)	0	70	20	20	20
Corneal and conjunctival fluorescein score (%)	<3	80	20	10	5
Conjunctival reaction (0-4+)	0.5+	0.5+	1+	1+	1+
Debris grading (0-4+)	0.5+	1+	1+	0	0
<i>Demodex</i> grading (0-4+)	0	0	0	0	0
Quality 16S rRNA gene sequencing reads	74727, 42780	69,641	78,580, 66,290	92,387, 91,912	89,940, 64,833
Good's coverage (%)	99.84, 99.86	99.95	99.97, 99.91	99.92, 99.96	99.96, 99.89
Total bacteria copies/eye(5.36×10 ⁶ , 1.49×10 ⁷	4.67×10 ⁷	1.49×10 ⁷ , 1.8×10 ⁷	1.71×10 ⁷ , 3.58×10 ⁷	7.29×10 ⁶ , 3.35×10 ⁷
α diversity					
Chao1	627.46, 335.21	163.62	221.07, 587.78	539.71, 368.38	309.64, 516.22
Shannon	4.12, 3.16	0.75	4.09, 4.46	3.60, 3.28	4.21, 3.73

^a Comma (“,”) represented where two samples and hence two sequencing reactions were performed for left and right eye swabs (el and er). ^b 0 = none, 1+ = minimal, 2+ = mild, 3+ = moderate, 4+ = severe; NA represents data not available.

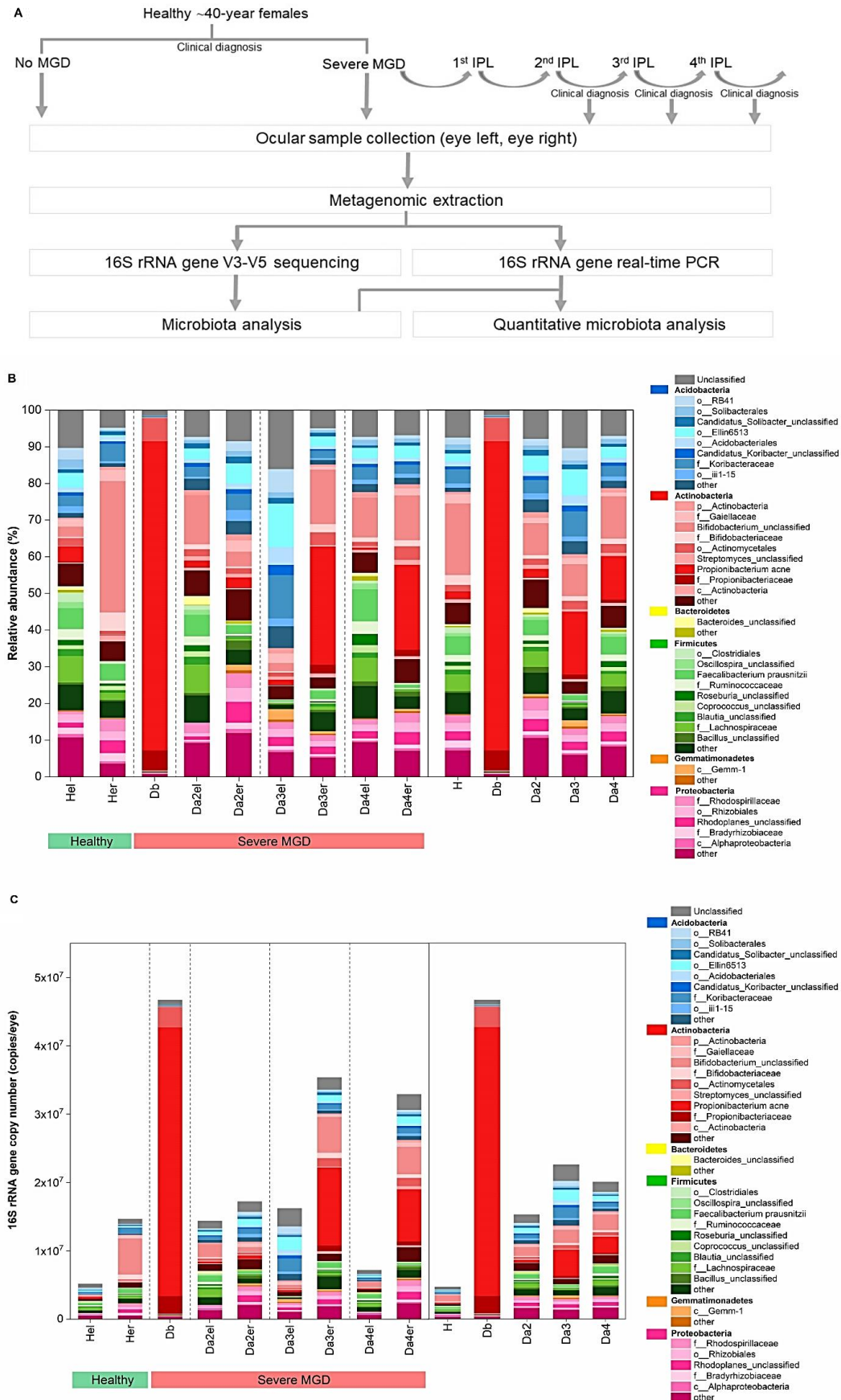


Figure 1. (a) Graphical abstract; and ocular surface microbiota of healthy and severe MGD before and after 2-4 IPLs, displayed as (b) percent relative abundance and (c) bacterial copy number. Bar charts are separated by gray line: left bar charts represent individual data (eye left, eye right), and right bar charts represent average data. Microbiota compositions are reported in species level, and OTUs where Mothur could not identify genus or species name were denoted by small letters (p_ abbreviates phylum; o_, order; c_, class; and f_, family) to the deepest taxonomic names that could be identified. Same color shade denotes same phylum, e.g., red color shade is for phylum Actinobacteria.

For quantitative microbiota analyses, metagenomics were extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany). Then, multiple displacement amplification (MDA) using the REPLI-g Mini Kit (Qiagen, Hilden, Germany) was performed, followed by 16S rRNA gene V3-V5 sequencing via universal prokaryotic primers 342F and 895R and the MiSeq600 platform (Illumina, California, USA) according to established protocols [17]. A non-swab sample was included as a negative control for the microbiota experiment, and no bacterial amplicon was found. A copy number of bacteria was quantified by 16S rRNA gene qPCR using the Rotor-Gene Q PCR system (Qiagen, Hilden, Germany) [17]. All sequences (NCBI SRA accession SRP269903) were analyzed following Mothur's standard operating procedures (SOP), which included processing for quality reads, operational taxonomic unit (OTU), good's coverage (percent coverage of sequences to estimate true diversity), alpha-diversity (Chao and Shannon), beta diversity (non-metric multidimensional scaling, NMDS) using Morisita-Horn dissimilarity indices, and Pearson's correlations [18]. Potential metabolic functions of bacteria were analyzed using PICRUSt [17, 18].

3- Results

The 16S rRNA gene V3-V5 library preparation and sequencing results were successful, as the number of quality sequencing reads was >42,000 reads/sample with an average 501 base length (Table 1, except samples Dbel and Dber sequencing numbers were low so were combined to make normalized sequencing depth). These covered 99.84–99.97% of Good's coverage of estimated bacterial species-level OTU diversity, which was sufficient for biodiversity analyses. Chao alpha-diversity showed relatively highest species richness in healthy subjects (Hel and Her), followed by the IPL-treated MGD (Da2-Da4), and much lower in the severe MGD before IPL (Db). Shannon alpha-diversity (species richness and evenness) emphasized the relatively greater diversity in H and Da than in Db. Nonetheless, the quantitative bacterial count of healthy and MGD (before and after) was found to be similar across samples: slightly higher in Db (Table 1: avg. 4.67×10^7 copies/eye), decreased in Da2-Da4, and lowest in H (1.17×10^7 copies/eye). Subsequently, the quantitative alpha-diversity suggested that the Db had low diversity but in high quantity, opposite to the Da2-Da4 that showed the increasing trend of alpha-diversity of species (closer to H alpha-diversity) with the decreasing trend of bacterial quantity from Da2 to Da4, provided that the H and Da4 demonstrated the relatively smallest bacterial quantity/eye.

For microbiome analyses as relative proportions (percentages), the bacterial phylum Actinobacteria represented major prevalence (Figure 1B: avg. 38.98%), followed by Acidobacteria (17.75%), Firmicutes (16.61%), and Proteobacteria (14.62%). *Propionibacterium acnes* (recently named *Cutibacterium acnes*) represented the dominant species (84.22%) in Db, while other species were rare. The percentages of *P. acnes* were lower in Da2-Da4 and H, suggesting that it might be involved in MGD clinical characteristics. Interestingly, the alpha-diversity distributions of taxa in Da2-Da4 (after the IPL treatments) showed close to H. For example, members of the phyla Acidobacteria, Actinobacteria, Firmicutes, and Proteobacteria increased in Da2-Da4, and each phylum became more equally proportional in the overall community composition. In Actinobacteria, the other genera than *P. acnes* became more proportionate, such as *Bifidobacterium* and an unclassified species in the order Streptomyces, resembling diversity to the H microbiome in which other members than *P. acnes* in the phylum Actinobacteria were dominant (Figure 1-b). For the phyla Acidobacteria, Firmicutes, and Proteobacteria, the diversity of members in percentages also became close between Da2-Da4 and H (Figure 1B: right bar charts). For analyses in bacterial quantity (quantitative microbiota), Db showed extensively high copies of *P. acnes* (Figure 1-c: 3.93×10^7 copies/eye), and this result explained its strictly small Shannon alpha-diversity in Table 1. For Da2-Da4 and H, smaller to minor *P. acnes* quantities were found, and the diversity was replaced by several other species and phyla.

The beta-diversity demonstrated that the Db microbiome was positioned far apart in NMDS from the Da3-Da4-Da2 and H, respectively (HOMOVA, $p = 0.001$), with clinical mucocutaneous junctions statistically correlated (Figure 2-a: Pearson's correlation, $p = 0.02$; Table 1: avg. 50% in Db vs. 20% Da vs. 0% H). The clinical corneal and conjunctival fluorescein scores demonstrated a Db trending correlation but no significant statistic. The result preliminary inferred that the mucocutaneous junction characteristic might reveal a clinical marker for Db, and this clinical marker aligned in the same direction as the representing species markers for Db (four bacterial species-level OTUs: unclassified in order Actinomycetales, unclassified in genus *Propionibacterium*, unclassified in family Propionibacteriaceae, and *Propionibacterium acnes*) (Figure 2-b: $p < 0.05$) (Figures 2-a and 2-b). In contrast, certain OTUs, including the unclassified families Veillonellaceae and Bifidobacteriaceae, the unclassified genus *Bifidobacterium*, the unclassified class Actinobacteria, and *Bifidobacterium thermacidophilum*, were statistically represented in H ($p < 0.05$), and some OTUs, including the unclassified genus *Roseburia* and the unclassified family Ruminococcaceae, were correlated in the same direction with both Da and H ($p < 0.05$). This finding supported close bacterial compositions among Da and H in Figures 1-b and 1-c and also suggested genus *Propionibacterium* is one determining factor for the MGD microbiome, while genus *Bifidobacterium* is for H. Next, the metabolic profiles corresponding to these prevalent OTUs were predicted from their individual quantitative microbiota. The metabolic profiles were generally close between Da and H groups; for instance, *Propionibacterium acnes* showed unique metabolic profiles in Db, and the genus *Bifidobacterium* showed similar metabolic profiles in Da and H. Yet, unclassified OTU in the genus *Propionibacterium* demonstrated a relatively close profile between Db and Da, and unclassified OTU in the class Actinobacteria demonstrated no difference among Db, Da, and H (Figure 2-c).

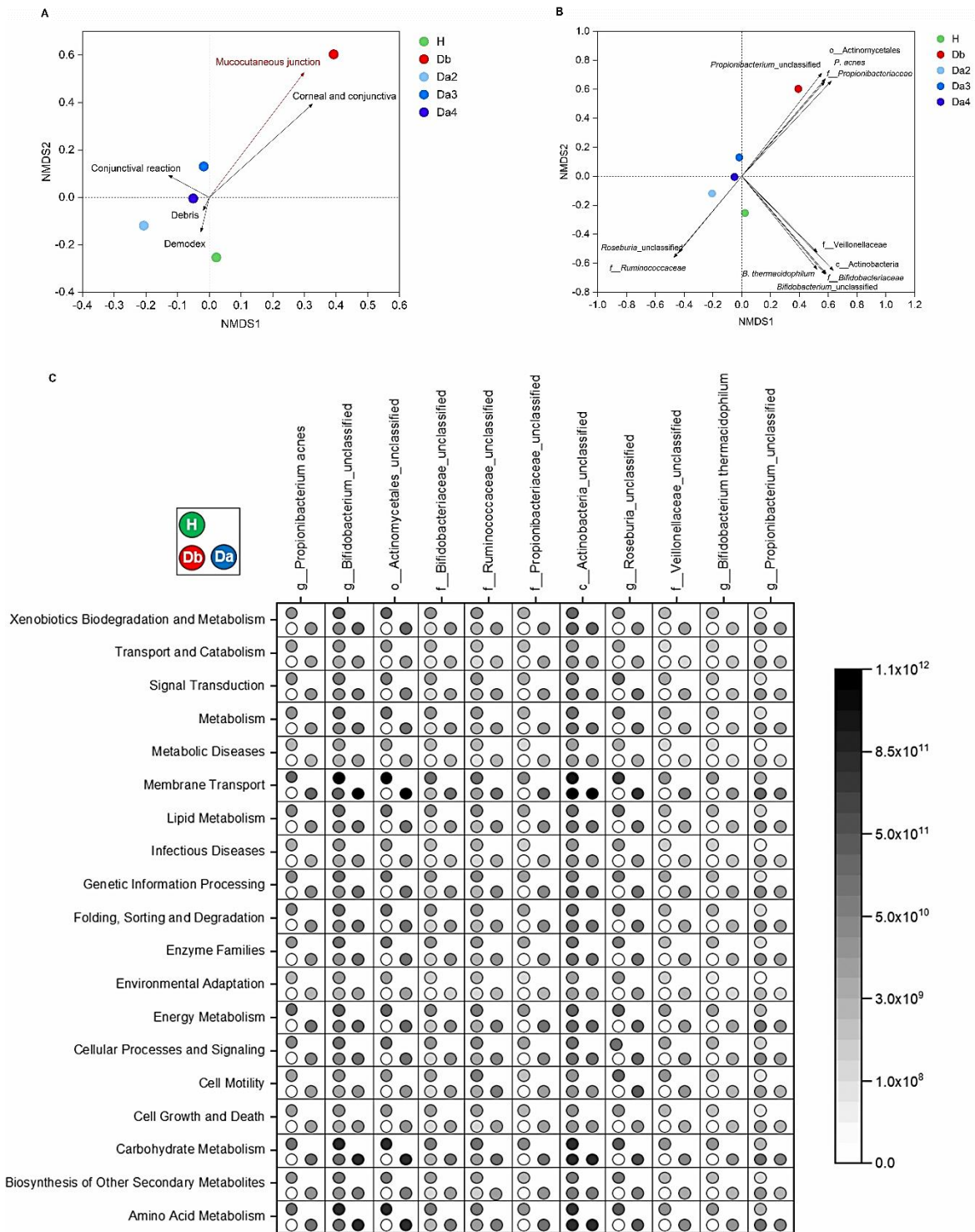


Figure 2. Analyses of microbiome beta-diversity and correlations with clinical eye characteristics, representing bacterial species and their metabolic potentials. (A) Non-metric multidimensional scaling (NMDS) plot and Pearson’s correlation with clinical eye characteristics (red arrow represented statistically significant correlation ($p < 0.05$)); (B) NMDS along representing bacterial species ($p < 0.05$), and (C) top abundant OTUs and their metabolic potential profiles for H, Db, and Da groups.

4- Discussion

Unstable meibum quality-quantity in MGD affected microbial community proliferation and diversity, and *vice versa* [9]. Specifically, *P. acnes* could degrade meibum lipids in meibomian glands and conjunctival sacs for growth, resulting in dysbiosis of the microbiome and lessened quality-quantity of meibum, a major cause of MGD clinical consequences (i.e., defective tear film and reduced tear film lipid layer thickness) [5]. *P. acnes* also releases lipase and esterase that induce inflammation of the cornea, conjunctiva, and epithelial cells, and the inflammation affects tear film. Overall, this gives rise to chronic dry eye and MGD [2, 19], and hence mucocutaneous junction and corneal-conjunctival fluorescein

gradings were noticed in the severe MGD compared to H (Table 1, Figures 1-b, 1-c, and 2-a) [3]. Supportively, Okonkwo et al. [20] systematically reviewed the ocular surface microbiome and reported. *Propionibacterium* as transient ocular surface bacteria capable of sight-threatening infections, and that the ocular microbiome was consistent between right and left eyes.

The microbiome alpha- and beta-diversities of Db differ from those in Da2-Da4 and H (Figures 1 and 2). The significantly decreased Chao and Shannon alpha-diversity in MGD was interpreted as decreased species richness and evenness, but the total count was interpreted as increased specific taxon growth. This data supported that bacterial dysbiosis is associated with severe MGD and dominates *P. acnes* (Db, 3.93×10^7 copies/eye) and suggested that *P. acnes* functions may correlate with MGD clinical symptoms [1, 5, 7, 19]. This is consistent with previous reports [5, 9, 21, 22] of the lower alpha-diversity in MGD and that *P. acnes* was significantly associated with increasing MGD severity ($p = 0.004$). Moreover, Pearson's correlation identified OTU in the family Propionibacteriaceae and genus *Propionibacterium* as correlated with MGD condition. However, after the IPL treatments, *P. acnes* decreased while commensal bacteria (similar to H) increased, highlighting that this dysbiosis of the bacterial community could be relieved by IPL. Moreover, the other core Db-representing unclassified taxon in Actinomecetales might include *Corynebacterium*, a common genus previously reported in ocular dry eye [15]. Benefits of IPL for MGD include (i) heating meibomian glands and meibum to less viscosity to support distributing throughout the cornea and unclogging; (ii) minimizing *Demodex* and bacteria quantity; (iii) stimulating collagen synthesis; (iv) improving cell turnover rate; (v) balancing pro- and anti-inflammatory cytokines; and (vi) generating reactive oxygen species that destroy bacteria [13, 14].

The microbial metabolic profiles differed between Db and H (also, Db and Da). *P. acnes* and family Propionibacteriaceae that had significant prevalence in MGD possessed relatively low or non-diverse metabolisms. Aragona et al. [19] reviewed microbiota changes that included *Propionibacterium* in experimented mice prior to the development of MGD symptoms and hypothesized that they also affected the regulation of mucin composition-production. Mucin is present in the ocular tear film, which functions to maintain hydration and lubrication on the ocular surface. On the contrary, the estimated functions of H and Da2-Da4 prevalent species possessed highly diverse profiles, e.g., microbial functions involved in the biosynthesis of other secondary metabolites and lipid metabolism. The bacterial functional potentials for high lipid metabolisms are consistent with our clinical records of improved dry eye symptoms, and the previously reported ocular IPL could increase tear film lipid layer thickness and inhibit *Corynebacterium* growth [15]. In parallel, we analyzed age-controlled Thai mild and moderate MGD before and after 2-4 IPL treatments and found the alpha-diversity in mild and moderate MGD was slightly higher than that in severe MGD, with the major bacterial taxa remaining similar (e.g., relative abundance of *Propionibacterium* before IPL and *Bifidobacterium* after IPL) [23]. In addition, we are analyzing eyelash microbiota in these healthy vs. MGD before and after IPL subjects to explore how eyelash bacteria are correlated with ocular surface bacteria involved in MGD and effect of IPL treatments (unpublished data). However, further investigation with sufficient statistical number of male and female subjects is required to determine the association between microbial metabolisms and dysbiosis microbiota.

5- Conclusion

This study represents the first comprehensive ocular surface quantitative microbiota comparison between Thai healthy and severe MGD to reveal the differing microbiota in relative percent abundances and quantitative bacterial counts, as well as the changing microbiome before and after 2-4 IPL treatments to reveal the recovering quantitative microbiota along the clinical diagnosis and microbial functional potential analyses. Our data highlighted the very low bacterial diversity in Db, with an extensive prevalence of family Propionibacteriaceae, especially *P. Acnes* (recently named *Cutibacterium acnes*), when compared to H and Da. After IPL, several bacterial taxa recovered, including decreasing Propionibacteriaceae and increasing the alpha-diversity for Da2-Da4. The Da2-Da4 microbiota compositions also showed many positively correlated taxa and correlated in the direction with clinical eye recovery (i.e., 0–20% mucocutaneous junction grading in H and Da, vs. 70% in Db; and <3-20% corneal and conjunctival fluorescein score in H and Da, vs. 80% in Db) and quantitative microbiota functional profiles with H. These data were consistent with our recent publication on Thai mild and moderate MGD after IPL treatments [23]. Therefore, our preliminary data provided insight into the severe MGD's disrupted quantitative microbiome and their correlated clinical characteristics, species, and metabolic potentials (vs. H and IPL-treated).

Study limitations include a relatively small sample size, and the findings encouraged an increasing sample size to assess the more accurate data and accuracy of the findings.

6- Declarations

6-1- Author Contributions

Conceptualization, N.S. and K.P.; methodology, N.S.; formal analysis, L.W., K.P., A.W., N.W., A.I., D.D., and N.S.; data curation, N.S.; writing—original draft preparation, L.W., K.P., A.W., N.W., A.I., D.D., and N.S.; writing—review and editing, L.W., K.P., A.W., N.W., A.I., D.D., and N.S.; supervision, K.P. and N.S.; project administration, L.W., K.P., A.W., N.W., A.I., D.D., and N.S.; funding acquisition, K.P. and N.S. All authors have read and agreed to the published version of the manuscript.

6-2-Data Availability Statement

The data presented in this study are available in the article.

6-3-Funding

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6-4-Institutional Review Board Statement

Ethic approval was not required for this study. The ethic in Thailand was exempted when fewer than four human subjects were involved.

6-5-Informed Consent Statement

Not applicable.

6-6-Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancies have been completely observed by the authors.

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