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Distinct Ocular Surface Microbial Profiles in Corneal Transplant Candidates

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Purpose: To characterize the ocular surface microbiome in patients undergoing corneal transplantation and to evaluate microbial shifts associated with corneal endothelial decompensation and surgical history.

Methods: In this single-center case–control study, conjunctival swabs were collected from 54 adults scheduled for lamellar or penetrating keratoplasty and from 16 healthy controls. Sampling was performed under sterile conditions immediately before surgery. Bacterial DNA was analyzed by 16S rRNA gene sequencing targeting the V3–V4 regions. Alpha and beta diversity indices were calculated using the Shannon index and Bray–Curtis dissimilarity. Taxonomic composition was compared across groups stratified by clinical status and prior ocular surgery.

Results: Pretransplant patients showed significantly higher alpha diversity than healthy controls ($P = 0.04$), and beta diversity analysis confirmed distinct microbial community structures between groups ($P = 0.002$). The patient group exhibited enrichment of *Enterobacteriaceae*, *Pseudomonas*, and *Escherichia–Shigella*, whereas *Bacteroidota* and *Bacteroidia* predominated in healthy subjects. No significant differences in diversity or composition were observed between decompensated and nondecompensated cases. Patients with prior penetrating keratoplasty displayed higher microbial diversity than those with previous phacoemulsification ($P = 0.03$).

Conclusions: Corneal transplant candidates exhibit distinct ocular surface microbial profiles characterized by increased diversity and enrichment of opportunistic taxa. Although endothelial decompensation did not significantly alter microbial composition, prior surgical history appeared to influence diversity patterns. These exploratory findings provide preliminary evidence that the ocular surface microbiome may play a role in the preoperative assessment and postoperative outcomes of corneal transplantation.

Key Words: ocular surface microbiome, corneal transplantation, endothelial decompensation, microbial dysbiosis, alpha and beta diversity

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The human ocular surface contains a dynamic, complex microbiota (Ocular Surface Microbiome, OSM) consisting of an association of functionally important commensal microorganisms.[1,2] The use of culture-independent approaches and metagenomic techniques, have now demonstrated extensive taxonomic diversity with evidence that the OSM plays an active role in maintaining ocular surface homeostasis and in modulating local immunity.[3,4]

Studies have proven that microbial signatures, even in low abundance, play roles in immune surveillance, regulation of inflammatory cascades and prevention from opportunistic infections.[5] Disrupting this equilibrium—often termed as dysbiosis—is associated with various ocular surface diseases such as dry eye disease, allergic conjunctivitis, microbial keratitis, and more recently, keratoconus.[6] Aside from eye infections, there is growing recognition that systemic relevance of the human microbiota also extends to solid organ transplantation.[7] The microbiota of the gut has also been demonstrated to affect transplantation outcomes by modulating alloimmune priming, tolerance to the graft, immunosuppressive drug metabolism, and posttransplant complications.[8,9] Such considerations raise important questions regarding whether microbiota–host interactions at other

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mucosal niches, such as at the ocular surface, might have similar importance in corneal transplantation.[10]

To date, despite increasing interest in the human microbiome, the ocular surface microbial landscape in patients undergoing keratoplasty has not been systematically characterized, particularly in those with corneal endothelial decompensation—which is the leading indication for corneal transplantation.

Metagenomic sequencing verified that resident microbial communities of greater biodiversity indices than those reported previously inhabit the OSM and have an indispensable immunomodulatory function in defense against eye pathogens.[11] In systemic applications, novel evidence supports that the microbiota can modulate solid organ transplantation at various levels, such as donor/recipient immune regulation, priming and effect phase of alloimmune responses, availability or metabolism of immunosuppressive drugs, outcomes of transplantation, and postsurgical infections.[9,12] In the present study, we aimed to elucidate the composition and nature of the OSM in patients with corneal endothelial decompensation before keratoplasty to analyze possible microbial trends that correlate with this eye condition.

MATERIALS AND METHODS

This single-center case–control study was authorized by the local Ethical Committee (CE AVEC: 901/2022/Oss/AOUBo, approved on December 15, 2022, ClinicalTrials.gov ID NCT06830538) and performed in accordance with the Declaration of Helsinki. Informed consent was obtained from the subjects after explanation of the nature and possible consequences of the study.

Patient Population

The healthy control group included 16 adult volunteers with no history of ocular or systemic disease, recruited primarily from nonpatient-facing health care personnel and administrative staff of the Ophthalmology Unit. All participants met strict inclusion criteria: absence of current or previous ocular surface disorders, no history of ocular surgery, no contact lens wear, and no use of systemic or topical medications in the preceding 6 months. Each underwent a full ophthalmologic examination 1 week before microbiome sampling, which was performed under sterile conditions in a dedicated clinical setting.

The study group included 54 adult patients consecutively recruited from the Corneal Pathology Outpatient Clinic of the Ophthalmology Unit. All patients were candidates for either endothelial lamellar keratoplasty or penetrating keratoplasty because of one of the following clinical indications: Fuchs endothelial corneal dystrophy (FECD), pseudo-phakic bullous keratopathy (PBK), or graft failure after previous corneal transplantation. Demographic characteristics of this cohort are shown in Table 1, whereas clinical features, such as indication for transplantation and previous surgeries, are detailed in Table 2. Patients were further stratified based on lens status (phakic vs. pseudo-phakic), history of previous corneal transplantation (including type of prior graft), and the clinical presence of corneal decompensation.

TABLE 1. Demographic Characteristics of the Study Population Stratified by Group

Characteristic	Healthy Subjects (n = 16)	Corneal Transplant Candidates (n = 54)
Sex (M/F)	2/14	24/30
Mean age (±SD)	60.21 ± 14.32	69.28 ± 13.60
Median age (±IQR)	58.5 (±18.34)	66.30 (±12.75)
Age range (min–max)	27–80	24–93

For the purposes of microbiota comparison, the classification into "decompensated" and "non-decompensated" was based on clinical criteria. Patients with Fuchs endothelial corneal dystrophy were categorized as nondecompensated, as they typically presented with minimal stromal edema, reduced endothelial cell count, and mild corneal haze not preventing visualization of anterior chamber structures. In contrast, patients undergoing transplantation for pseudo-phakic bullous keratopathy or graft failure were categorized as decompensated, characterized by marked stromal edema, epithelial bullae, and/or corneal opacity severe enough to hinder anterior segment examination.

Microbiome sampling was performed in the operating room, under sterile conditions, immediately before the planned surgical procedure. All samples were collected in the morning, between 9:00 and 12:00 PM, to ensure consistency and minimize potential circadian variation in microbial composition.

As this was an exploratory study, no a priori sample size calculation was performed.

Ocular Examination and Sampling

All patients underwent a standardized, comprehensive ophthalmic assessment in the Corneal Service of the Ophthalmology Unit 4 to 6 weeks before the surgery. The underlying corneal disorder was diagnosed on the base of medical history, slit-lamp biomicroscope, and, when indicated, anterior-segment optical coherence tomography (AS-OCT). FECD was defined by the presence of corneal guttata and central or diffuse endothelial cell loss (confirmed by specular microscopy), together with mild stromal edema and increased pachymetry, but without visually significant corneal opacification. PBK was diagnosed in pseudo-phakic eyes showing diffuse corneal edema, epithelial bullae, and loss of endothelial function, with no alternative cause of decompensation.

Graft failure was identified as persistent or progressive corneal edema in eyes with a history of penetrating or lamellar keratoplasty, accompanied by diminished visual acuity and loss of graft clarity. Patients with FECD, PBK, or graft failure had received no topical or systemic antibiotic therapy, nor they worn contact lenses, in the 6 months preceding sample collection. All were routinely treated with hypertonic saline tear substitutes and a hypertonic ophthalmic

TABLE 2. Stratification of Corneal Transplant Candidates by Clinical Corneal Decompensation Status, Diagnosis, and Lens Status

Group	No. Patients (n)	Main Diagnosis	Lens Status/Anamnesis
Nondecompensated	26	Fuchs endothelial corneal dystrophy (26/26)	Phakic: 14/pseudophakic: 12
Decompensated	28	- Graft failure after previous keratoplasty (15/28) - Pseudophakic bullous keratopathy (13/28)	- PK: 8/lamellar: 7 (within graft failure group) - Pseudophakic: 28
Total	54		

PK, penetrating keratoplasty.

ointment, which were discontinued 1 week before sampling. The clinical rationale for these agents was to temporarily reduce corneal edema while the patients awaited definitive surgical intervention.

In all cases, sampling was performed on the day of surgery. Based on institutional practice, surgery was typically scheduled within 3 to 6 months from the onset of clinically significant corneal decompensation symptoms. Conjunctival swabs (E-swab, Copan, Murrieta, CA, USA) were collected from the inferior fornix of the eye to be operated before undergoing corneal transplantation, under one drop topical anesthesia with 0.4% oxybuprocain. Samples were placed on ice, promptly transported to the laboratory facilities, and stored in an −80°C freezer until DNA extraction.

DNA Extraction

All samples were processed for DNA extraction using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the "Pre-treatment for Gram-Positive Bacteria" protocol and then the "Purification of Total DNA from Animal Tissues (Spin-Column Protocol)." The extracted DNA was eluted twice in 30 μL in Buffer AE (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0), quantified by Qubit dsDNA High Sensitivity assay kit (Life Technologies, Milan, Italy), and stored at −20°C until library preparation.

16S rRNA Sequencing

The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified according to the 16S metagenomic sequencing library preparation protocol (Illumina, San Diego, CA) with minor improvements. Briefly, DNA samples were subjected to 27 cycles of PCR amplification, then purified with AMPure XP beads, and eluted in 30 μl of Tris-HCl pH 8.5. Last, 15 μL of amplified DNA were used in indexing PCR. Blank control libraries were produced from mock DNA extractions made on lysis buffer alone, to control environmental and technical contamination. Final indexed libraries were prepared by equimolar (2 nmol/L) pooling, denaturation and dilution to 6 pmol/L before loading onto the MiSeq flow cell (Illumina) with a 20% Phi-X control library spike in. Sequencing was performed at 300 bp in paired end.

Sequencing Data Processing

We performed sequence trimming to remove any residual adapters and low-quality sequences. After that, we merged the technical replicates of the samples. Then, we used DADA2 pipeline to perform taxonomy assignment. This pipeline is a widely used tool for processing amplicon sequencing data from 16S rRNA gene sequences, with the goal of accurately identifying amplicon sequence variants (ASVs). DADA2 then learns the sequencing error rates directly from the dataset, enabling it to distinguish between true biological variation and sequencing errors. After this, the pipeline performs dereplication, collapsing identical sequences into unique representatives to reduce redundancy. The core step of the pipeline involves denoising, where DADA2 applies its statistical model to infer the true sample sequences by correcting for observed errors. If paired-end sequencing was used, forward and reverse reads are then merged to reconstruct the original DNA fragments, provided there is sufficient overlap. Chimeric sequences, which are artifacts formed during PCR amplification, are identified and removed. Finally, the high-resolution ASVs obtained are assigned taxonomic classifications using reference databases such as SILVA or Greengenes. Once the taxonomic composition of the samples was estimated, we proceeded to remove potential contamination originating from the environment or the entire technical procedure. To achieve this, we used the R package *Decontam* along with 4 blank samples that were sequenced using the same protocol as the other samples in this study. We applied the prevalence-based method, testing different threshold values. We observed a plateau in the number of reads identified as contaminants for thresholds between 0.2 and 0.5. Based on this, we consider the samples decontaminated using these thresholds to be effectively free of the contamination represented by the blanks. We selected a final threshold of 0.5 to be as conservative as possible, while remaining within a reasonable parameter range.

Statistical Analysis

To assess within-sample diversity, we calculated alpha diversity metrics as the Shannon diversity index that recapitulates observed richness and evenness, using the Phyloseq utility *estimate richness.* Comparisons of Shannon index diversity index were done by *t* test after verification of normality (Shapiro–Wilk test) and homoscedasticity. Before beta diversity analysis, count data were normalized to relative abundances by transforming each sample's read counts to proportions. Between-sample diversity was then evaluated using Bray–Curtis dissimilarity to quantify compositional differences among microbial communities. We performed PERMANOVA from the Bray–Curtis dissimilarity matrix. The analysis was conducted first to compare pretransplant and

healthy subjects, then to compare the patients who had complications after transplant to those who did not, and last the patients treated with different surgical approaches. For visualization, Principal Coordinates Analysis was applied to the Bray–Curtis dissimilarity matrix. Comparisons of the relative abundance of different taxa were performed by *t* test corrected for multiple comparisons by Benjamini and Hochberg method. All statistical analyses were performed in R.

RESULTS

The conjunctival sampling procedure was well tolerated by all participants, with no reported discomfort or adverse effects. DNA extraction from ocular swabs yielded on average 0.56 ng/μL of DNA, with a range from 0.06 ng/μL to 1.9 ng/μL, supporting the overall low microbial biomass of the ocular surface. To account for the possible presence of environmental contaminants, paired blank control libraries were produced from mock DNA extractions made on lysis buffer alone, which were used to clean the results also from very low aspecific contaminations. 16S DNA sequencing yielded an average of 23339 ± 1777 reads per sample, with a mean of 1739 ± 823 bacterial ASVs identified per sample. Samples ranged from 630 (min) to 4332 (max) ASVs/sample.

Microbial Alpha and Beta Diversity

Bacterial sample alpha diversity was measured calculating Shannon index, which measures microbial diversity estimating both richness (the number of taxa within a community) and evenness (the relative abundance of taxa in each sample). The mean Shannon diversity index was 6.7 ± 0.5 in the whole series (range: 5.7–7.8), reflecting a moderately high microbiome diversity. The average Shannon index in the healthy controls was 6.5 ± 0.3 and 6.7 ± 0.5 in the pretransplant patients, with a statistically significant difference ($P = 0.04$, *t* test), thus showing that the ocular surface microbiome in corneal transplant candidates displays a slightly greater variety of microbial taxa relative to healthy individuals (Fig. 1A). This finding suggests that chronic corneal disease and/or prior ophthalmic interventions might be associated with alterations in microbial community structure, potentially contributing to the observed increase in α-diversity. Although higher diversity is often interpreted as a marker of microbial stability and health in other body sites, on the ocular surface, it may reflect dysbiosis, resulting from disrupted epithelial integrity, altered tear composition, or changes in ocular surface immunity.

Beta-diversity analysis was performed to evaluate overall differences in microbial community composition between healthy individuals and corneal transplant candidates. PERMANOVA analysis revealed a statistically significant difference in beta-diversity between the 2 groups ($P = 0.002$), representative of 2.5% of the total variation in microbial composition. The 3D Principal Coordinates Analysis plot (Fig. 1B), based on Bray–Curtis dissimilarity, illustrates a partial separation between the 2 groups, with red dots representing transplant candidates and blue dots representing healthy controls. Although there is notable overlap between the clusters, the pretransplant patient group shows a higher interindividual variability about microbial diversity, suggesting that different prior clinical courses could have a specific influence on ocular surface microbial composition. These results suggest that corneal disease and/or prior ocular interventions are associated with detectable shifts in ocular surface microbial profiles, albeit with considerable interindividual variability.

Alpha-diversity metrics were further analyzed by stratifying corneal transplant candidates into decompensated and nondecompensated subgroups. As shown in Figure 2A, no statistically significant difference was observed for the Shannon index, which showed comparable mean values in the decompensated and not-decompensated groups ($P = 0.73$, *t* test). This result suggests that clinical corneal decompensation status does not substantially influence microbial diversity within the pretransplant population.

To evaluate whether microbial community structure differed between decompensated and not-decompensated corneal transplant candidates, beta diversity was analyzed using PERMANOVA on Bray–Curtis dissimilarities. The analysis showed that no significant differences were observed between the 2 groups ($P = 0.72$), indicating that the variance in microbial composition was not correlated to decompensation status. These results altogether suggest that clinical decompensation does not significantly alter the overall structure of the ocular surface microbiome in corneal transplant candidates.

Within the decompensated group, alpha diversity (measured by the Shannon index) varied according to patients' surgical history. As shown in Figure 2B, patients with a history of penetrating keratoplasty (PK) exhibited the highest microbial diversity, with a mean Shannon index of 7.1 ± 0.5. Those who had undergone lamellar keratoplasty showed intermediate diversity values (mean = 6.7 ± 0.5), whereas the lowest alpha diversity was observed in patients with no history of corneal transplantation but who had received phaco-emulsification with intraocular lens implantation (phaco group), showing a mean Shannon index of 6.5 ± 0.4. Comparisons of Shannon index values among decompensated patients stratified by surgical history revealed a significant difference between the phaco group and those with prior penetrating keratoplasty (PK) ($P = 0.03$, *t* test). In contrast, no statistically significant differences were found between the phaco and lamellar keratoplasty groups ($P = 0.27$), nor between lamellar and PK groups ($P = 0.34$).

Microbial Community Composition

PERMANOVA taxonomic differential analysis revealed significant compositional differences between healthy subjects and pretransplant patients. Healthy subjects showed higher median relative abundances of *Bacteroidia* (5.7% vs. 1.7%, $P = 0.005$; Fig. 3A), whereas pretransplant patients had increased Enterobacteriaceae (1.4% vs. 4.4%, $P = 0.02$), *Pseudomonas* (1% vs. 3.4%, $P = 0.01$), and *Escherichia–Shigella* (0% vs. 1%, $P = 0.002$; Fig. 3B–D).

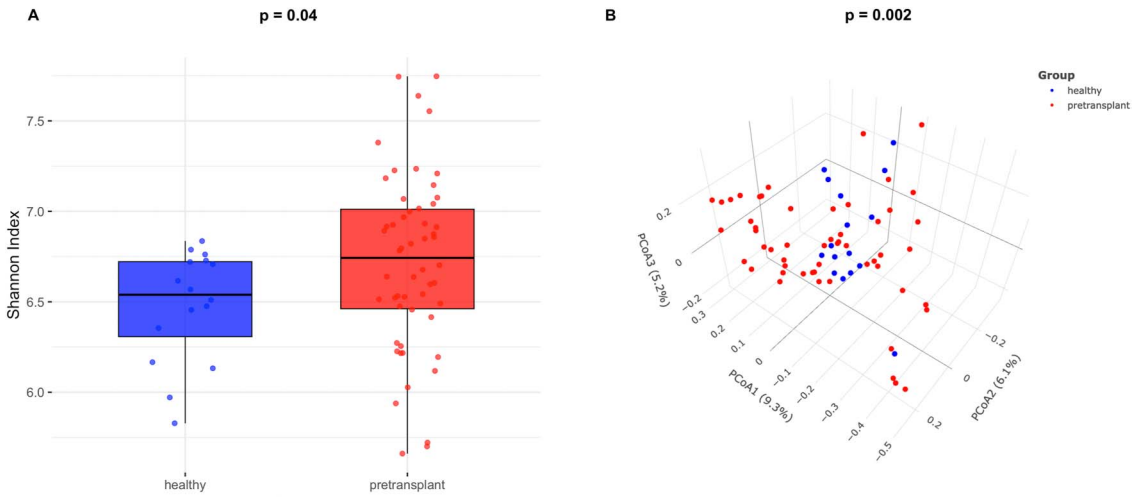

FIGURE 1. Microbial diversity in healthy and pretransplant subjects. A, Shannon diversity index in healthy subjects (blue) versus pretransplant patients (red). Boxplots represent the median and interquartile ranges. $P = 0.04$, t test. B, PCoA plot of beta diversity, healthy (blue) versus pretransplant (red) patients. Comparison of Bray–Curtis distances between the 2 groups is statistically significant ($P = 0.002$). PCoA, Principal Coordinates Analysis.

In the comparison between patients with and without corneal endothelial decompensation, the nondecompensated group showed higher *Firmicutes* (28.2% vs. 15.9%, $P = 0.001$) and *Bacilli* (23.4% vs. 13.7%, $P = 0.006$), whereas *Gammaproteobacteria* were enriched in decompensated cases (18.7% vs. 32.4%, $P = 0.004$; Figs. 4A–C). All analyses were corrected for multiple testing. No further differences were observed after stratification by clinical history or previous ocular surgery.

DISCUSSION

This study provides new evidence on the ocular surface microbiome in patients undergoing corneal transplantation, revealing distinct microbial diversity and compositional profiles compared with healthy controls. By applying 16S rRNA sequencing, we characterized microbial patterns associated with corneal endothelial dysfunction and prior ocular surgery, offering insight into the ocular surface microenvironment of patients requiring keratoplasty. Although the exact time from disease onset to sampling varied among patients, institutional scheduling typically occurs within 3 to 6 months from clinical decompensation, thereby minimizing variability in disease stage at the time of sampling.

These findings expand current understanding of how chronic corneal disease and surgical history may influence the ocular surface microbial ecosystem. The observed enrichment of opportunistic taxa and increased microbial diversity in

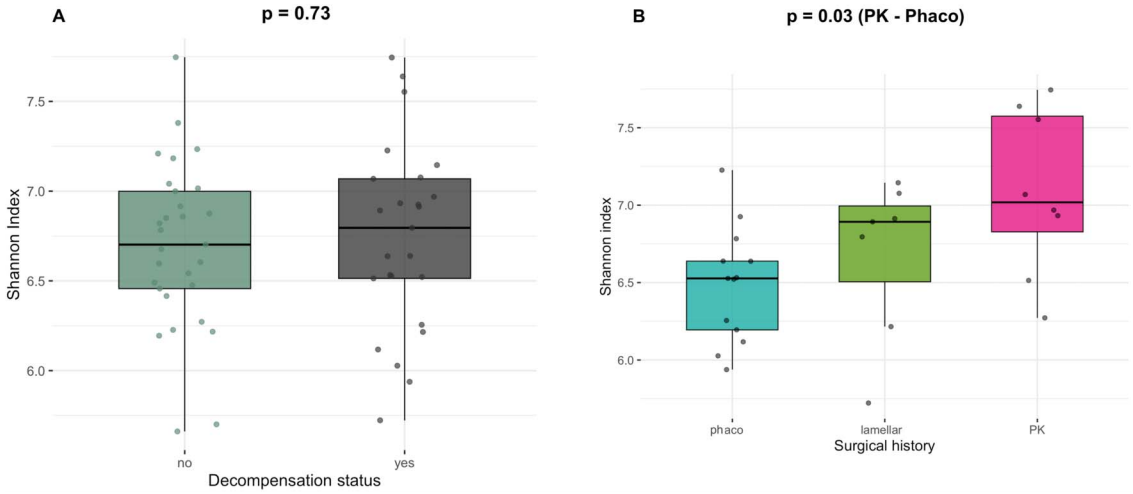

FIGURE 2. Microbial diversity within pretransplant patients. A, Shannon index in nondecompensated and decompensated patients ($P = 0.73$, t test). B, Shannon index comparison according to surgical history. PK, penetrating keratoplasty; Lamellar, lamellar keratoplasty; Phaco, phacoemulsification with intraocular lens implantation (PK vs. phaco, $P = 0.03$, t test).

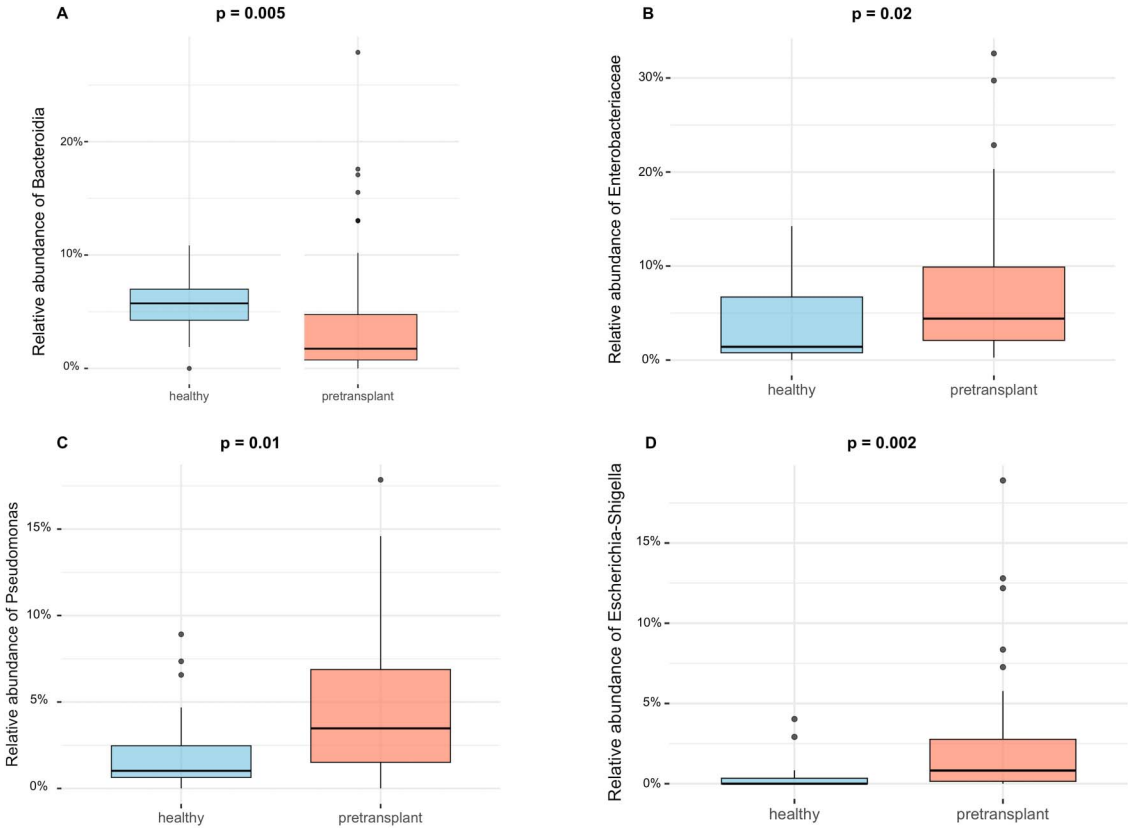

FIGURE 3. Taxonomic differential analysis between healthy subjects and pretransplant patients. Relative abundance is shown as percentage about the abundance of the entire taxonomic classification level. Higher relative abundance was observed in the healthy group for the phylum *Bacteroidota* and the class *Bacteroidia* (A). In contrast, the pretransplant patient group showed higher relative abundance for the family *Enterobacteriaceae* (B), and for the genera *Pseudomonas* (C) and *Escherichia–Shigella* (D).

pretransplant patients suggest that ocular surface dysbiosis could be part of the pathophysiological context leading to corneal failure and the need for keratoplasty.

Alpha-diversity analysis revealed a significantly higher microbial diversity in pretransplant patients compared with healthy subjects. This finding contrasts with the paradigm that increased microbial diversity is typically associated with a healthy microbiome, as observed in the gut or skin. On the ocular surface, however, elevated diversity may reflect dysbiosis, potentially driven by chronic disease–related alterations in epithelial integrity, tear film composition, or local immune responses. These results align with previous evidence suggesting that ocular surface disorders such as dry eye or keratoconus are linked to shifts in microbial community structure. Beta-diversity analysis further supported the presence of microbial community alterations in transplant candidates, with significant differences in overall community composition (PERMANOVA $P = 0.002$). Nevertheless, the relatively low effect size ($R^2 = 0.025$) suggests considerable interindividual biological variability and points to the complexity of OSM–host interactions, potentially influenced by clinical/therapeutic management.

Interestingly, no significant differences in either alpha or beta diversity were found between decompensated and nondecompensated patients. This suggests that although the presence of a corneal disease requiring transplantation may affect the ocular microbiome, the degree of clinical decompensation—characterized by stromal edema or epithelial bullae—does not further stratify microbial diversity in a relevant way. These findings may be explained by the fact that both patient subgroups experience similar chronic ocular surface stress or by potential ceiling effects in diversity disruption.

When stratifying the decompensated subgroup by surgical history, patients with a history of penetrating keratoplasty exhibited the highest microbial diversity, significantly greater than those who had undergone only phacoemulsification. This may reflect long-term changes in ocular surface physiology after full-thickness corneal transplantation, such as altered innervation, immune surveillance, or tear film dynamics. However, this difference did not extend to beta diversity, and no significant clustering by surgical history was observed.

Taxonomic analysis identified distinct microbial signatures in pretransplant patients compared with healthy controls. Transplant candidates showed enrichment of the family *Enterobacteriaceae* and the genera *Pseudomonas* and *Escherichia–Shigella*—all potentially opportunistic taxa frequently associated with ocular infections or disrupted epithelial barriers. Conversely, *Bacteroidota* and its class

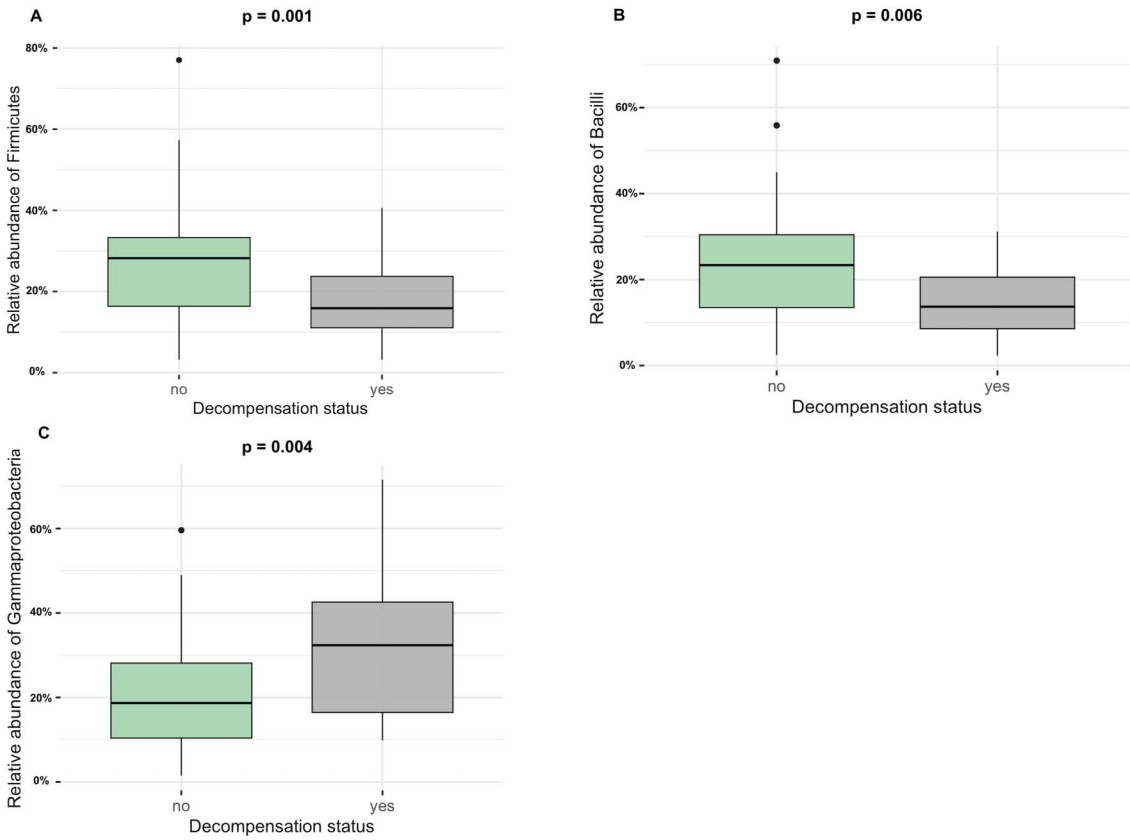

FIGURE 4. Taxonomic differential analysis between patients with and without corneal endothelial decompensation. Patients without decompensation exhibited higher relative abundance of the phylum *Firmicutes* (A) and the class *Bacilli* (B). Conversely, a higher relative abundance of the class *Gammaproteobacteria* was observed in the decompensated group (C).

Bacteroidia were more abundant in healthy subjects, suggesting a possible role for these taxa in maintaining ocular surface homeostasis.

In this context, it should be underlined that *Pseudomonas* genus includes the species *Pseudomonas aeruginosa*, one of the prevailing Gram-negative microorganisms consistently identified in most cases of ocular infections. *P. aeruginosa*–induced keratitis is one of the most severe and challenging forms of corneal infection, owing to its associated intense inflammatory reactions leading to corneal necrosis and dense corneal scar with loss of vision. Moreover, *P. aeruginosa* is classified within the ESKAPE pathogen group, frequently implicated in alarming upsurges of antimicrobial resistance.[13–15]

Similarly, *Escherichia*, a coliform bacterium that occurs as a commensal in the gut of humans and other warm-blooded mammals, is known to cause ocular infections such as conjunctivitis, keratitis, and endophthalmitis.[16] *Escherichia* strains can harbor different patterns of genes involved in antimicrobial resistance, toxin production, and biofilm formation, thus showing different levels of virulence for the ocular surface.[17]

When comparing decompensated to nondecompensated patients, we found increased relative abundance of *Gammaproteobacteria* in the decompensated group, whereas *Firmicutes* and *Bacilli* were enriched in nondecompensated patients. Although all differences reached statistical significance, the implications of these taxonomic shifts remain to be fully elucidated. *Gammaproteobacteria* include numerous opportunistic pathogens, and their enrichment may reflect an environment more prone to inflammation or infection.

On the other hand, *Bacilli* class comprises some "health-promoting" microorganism, such as *Bacillales* and *Lactobacillales*. Indeed, some species are employed as effective human probiotics, thanks to their ability to prevent the overgrowth of endogenous and exogenous pathogens, and to down-regulate the inflammatory factors. Thus, the higher abundance of *Bacilli* in nondecompensated patients could open the way to innovative microbe-based strategies for the prevention of corneal disorders.[18,19]

Importantly, no significant microbiome differences were observed in decompensated patients when stratified by previous surgical interventions or clinical history. This finding may indicate a degree of microbiome stability within this subgroup, or alternatively, it may highlight the dominant influence of the underlying disease state over procedural history.

This study has several limitations that should be acknowledged. The sample size, although sufficient for exploratory profiling, may have limited the ability to detect subtle subgroup differences, and the cross-sectional design prevents evaluation of temporal changes or causality. These aspects highlight the exploratory nature of the present

findings and emphasize the need for confirmation in larger, prospective cohorts. In addition, despite rigorous contamination control, the inherently low microbial biomass of ocular surface samples remains a technical challenge. Finally, taxonomic resolution was restricted to the genus level, as intrinsic to 16S rRNA V3–V4 sequencing, which cannot reliably discriminate closely related species, particularly within clinically relevant genera such as *Pseudomonas* and *Escherichia*.

Although the relatively small sample size may limit the generalizability of our findings, the statistically significant differences observed in microbial diversity and composition confirm the robustness of the main trends we identified. Therefore, our findings should be interpreted as exploratory, providing a basis for future multicenter studies with larger cohorts to validate and extend these observations.

Taken together, our results indicate that the ocular surface microbiome is altered in patients awaiting corneal transplantation, showing distinct taxonomic profiles associated with chronic corneal disease rather than with the degree of endothelial decompensation. These microbial patterns may reflect changes in the ocular surface environment that accompany corneal failure and surgical history. Further investigation is warranted to clarify how specific microbial taxa might influence graft integration, postoperative healing, and inflammation control. From a clinical standpoint, preoperative microbiome profiling could become a valuable component of patient assessment, helping to identify individuals at higher risk for postoperative complications and to guide tailored perioperative management aimed at improving graft success and visual outcomes.

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