

# Dysbiosis of Ocular Surface Microbiota in Patients With Refractive Allergic Conjunctival Diseases

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**Purpose:** We investigated ocular surface microbiota dysbiosis in patients with refractory allergic conjunctival diseases (ACDs; stratified into mild and severe groups) treated with topical tacrolimus.

**Methods:** Patients (n = 21) with refractory ACDs (including vernal and atopic keratoconjunctivitis) actively treated with topical tacrolimus and 6 healthy controls were evaluated. Based on clinical scores and expression of specific cytokines on the ocular surface, patients with ACDs were divided into mild and severe groups using cluster analysis. The microbial composition of tear specimens collected from patients with mild and severe ACD and control subjects using the Schirmer test paper was determined through next-generation 16S rRNA sequencing analysis.

**Results:** Compared with healthy controls, patients with ACDs exhibited significantly decreased ocular surface microbiota  $\alpha$ -diversity. Ocular surface microbiota mainly comprised members of the phyla Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria in all groups. The relative abundance of ocular surface microbiota in patients with ACDs was increased for phylum Firmicutes and decreased for phylum Proteobacteria (compared with control subjects). The genera *Blautia* (vs. mild

ACD group) and *Morganella* (vs. control group) exhibited significantly increased abundance only in the severe ACD group.

**Conclusions:** The ocular surface microbiota in patients with severe ACD exhibited decreased diversity and exacerbation of dysbiosis compared with that in patients with mild ACD and control subjects. Patients with mild refractory ACD also exhibited decreased diversity of these microbiota. These alterations in microbiota indicated a change in the ocular surface of patients with refractory ACD (be it because of disease pathogenesis or topical immunomodulatory treatment).

**Key Words:** microbiota, ocular surface, next-generation sequencing, allergic conjunctival diseases, Schirmer test

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Vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC) are refractory forms of allergic conjunctival diseases (ACDs). VKC predominantly affects male children and young adults and repeatedly exacerbates in spring and early summer.<sup>1,2</sup> AKC is a chronic and severe ocular complication associated with atopic dermatitis (AD). The complication rate of AKC in Japanese patients with AD is 74.3%, and this rate is increasing.<sup>3</sup> Clinical characteristics observed in patients with refractory ACDs, including VKC and AKC, include conjunctival proliferative lesions with severe conjunctival eosinophilic inflammation, giant papillae, and limbal gelatinous infiltration and corneal involvement, including superficial punctate epitheliopathy with severe mucinous deposition and shield ulcer. In general, chronic ocular surface damage due to allergic inflammation causes various physiological alterations and, more specifically, dysbiosis in the conjunctival sac.<sup>4</sup>

Patients with refractory ACDs are treated medically with steroids, including betamethasone and dexamethasone, and immunosuppressive eye drops, such as tacrolimus ophthalmic suspension and cyclosporin ophthalmic solution. In particular, long-term use of the immunosuppressive drug tacrolimus to treat refractory ACDs has been reported to be beneficial.<sup>5</sup> However, long-term use of steroidal and immunosuppressive eye drops can disrupt the ocular surface homeostasis and defense mechanisms. Therefore, we hypothesized that understanding the alterations in the ocular surface microbiota that occur in response to treatment of ocular allergic inflammation associated with VKC and AKC is important for the long-term management and medical treatment of these diseases.

Indigenous bacterial flora associated with the ocular surface has been characterized using conjunctival smear

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samples and traditional microbiology techniques. However, difficult-to-culture bacteria have not been sufficiently examined owing to the limitations of standard microbial culture techniques. Previous studies using 16S rRNA gene amplicon sequencing, which avoids the limitations of culture techniques, identified several microbial genera in the human conjunctiva.<sup>6,7</sup> Therefore, we used 16S rRNA gene sequencing to conduct a comparative analysis of the microbiomes of patients with mild and severe ACD undergoing treatment, mainly topical application of tacrolimus, for AKC or VKC and healthy control subjects.

## MATERIALS AND METHODS

This was a prospective and cross-sectional study. The protocol was approved by the institutional review board of the Nihon University School of Medicine (approval number RK-171212-3) and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all the studied individuals or, in the case of minors, from parents after explanation of the nature and possible consequences of the study for sample collection and subsequent analyses.

### Patients

Twenty-one patients diagnosed with AKC or VKC who were undergoing treatment at the Department of Ophthalmology of Nihon University Itabashi Hospital (Tokyo, Japan) were included in this study. Patients enrolled in the ACD groups from June 2019 through March 2020 included those with VKC or AKC diagnosed according to the Japanese guidelines for ACDs.<sup>8</sup> These guidelines include having conjunctival proliferative change (giant papillae and/or limbal gelatinous infiltration) in patients with VKC and having facial AD in the case of patients with AKC. Patients with VKC or AKC also exhibited positive results for the allergen-specific IgE antibody in serum or total IgE in tears, and/or the conjunctival smears of these patients tested positive for eosinophils.

The exclusion criteria were as follows: 1) ocular surface disease other than ACDs, 2) history of treatment with systemic anti-inflammatory drugs such as corticosteroids and immunosuppressants, 3) use of contact lenses, 4) antimicrobial use during the 6 months before study initiation, and 5) history of ocular surface surgery or intraocular surgery during the 12 months before study initiation.

The control group consisted of 6 healthy volunteers with a mean age of  $41.0 \pm 13.0$  years (SD), including 4 men and 2 women who did not have ocular disorders and did not wear contact lenses.

### Clinical Score for VKC and AKC

Clinical findings for each patient with VKC and AKC were evaluated using a 5-5-5 exacerbation grading scale for ACDs.<sup>9</sup> The judgment guidelines for the scale were as follows: The 5 severe clinical findings (active giant papillae, gelatinous infiltrates of the limbus, exfoliative epithelial keratopathy, shield ulcer, and papillary

proliferation at the lower palpebral conjunctiva) were assigned 100 points each, the 5 moderate clinical findings (blepharitis, papillary proliferation with velvety appearance, Horner-Trantas spots, edema of the bulbar conjunctiva, and superficial punctate keratopathy) were assigned 10 points each, and the 5 mild clinical findings (papillae at the upper palpebral conjunctiva, follicular lesion at the lower palpebral conjunctiva, hyperemia of the palpebral conjunctiva, hyperemia of the bulbar conjunctiva, and lacrimal effusion) were assigned 1 point each. The sum of the points in each grade was considered as the clinical score on the 5-5-5 exacerbation grading scale.

### Conjunctival Microbiome Analysis Using the Schirmer Test Paper

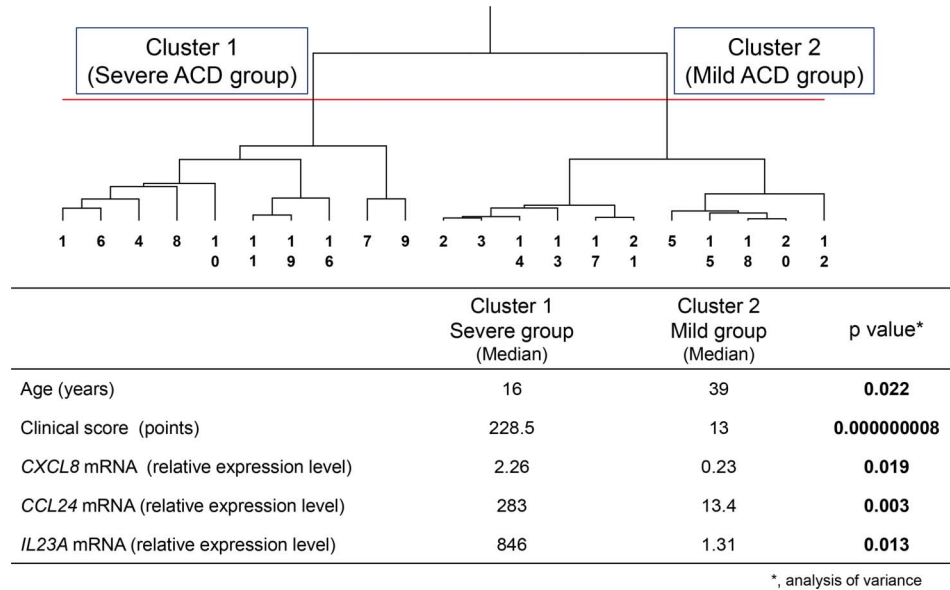
#### Tear Sampling and DNA Extraction Methods for Next-Generation Sequencing

Tear sampling was conducted according to the Schirmer test method<sup>10</sup> without topical anesthesia using Schirmer test papers (Schirmer Tear Production Measuring Strips; AYUMI Pharmaceutical Corporation, Tokyo, Japan). The test papers were placed in the lower conjunctival fornix in both eyes of a patient for 1 minute and then removed. DNA was extracted from the 5 mm tip of each Schirmer test paper for next-generation sequencing using the QIAamp MinElute Virus Spin Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The concentration and quality of purified DNA were analyzed with a QUBit fluorometer (Life Technologies Japan, Tokyo, Japan) and an Agilent TapeStation system (Agilent, CA).

#### Next-Generation Sequencing

16S rRNA libraries were constructed according to the "16S Metagenomic Sequencing Library Preparation protocol," recommended by Illumina, Inc, (San Diego, CA) and using unique barcode sequences. Polymerase chain reaction (PCR) was performed using a TaKaRa Cycler Dice Touch (Takara Bio, Shiga, Japan), 2× KAPA HiFi HotStart Ready Mix (Kapa Biosystems, Inc, Wilmington, MA), and the following conditions: initial denaturation at 95°C for 3 minutes; followed by 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds; and a final extension step at 72°C for 5 minutes. DNA concentration and size distribution of the libraries were analyzed with a QUBit fluorometer and Agilent TapeStation. PCR products were purified using AMPure XP magnetic beads (Beckman Coulter Inc, Indianapolis, IN), diluted to an equimolar concentration, and pooled according to their unique barcode sequence to enable multiplexing. Subsequently, Illumina dual-index barcodes were added to the pooled PCR products with the Nextera XT Index Kit (Illumina, Inc). The indexed PCR products were purified, pooled, and diluted to equimolar concentrations before paired-end sequencing with a MiSeq Reagent Kit v3 (600-cycle) (Illumina, Inc), following the manufacturer's directions.

**FIGURE 1.** Cluster analysis of patients with ACDs. Cluster analysis, based on clinical scores and chemokine mRNA expression levels, classified the 21 patients with ACDs included in this study into 2 groups: severe ACD ( $n = 10$ ) and mild ACD ( $n = 11$ ). The numbers indicate the patient's enrollment number. The red line indicates the point where the dendrogram divides into 2 clusters. The analysis of variance shows that these 2 clusters differ in the clinical score and in the mRNA expression levels of CXCL8, CCL24, and IL-23A. CXCL, C-X-C chemokine ligand; CCL, C-C chemokine ligand. (The full color version of this figure is available at [www.corneajrnl.com](http://www.corneajrnl.com).)



### Next-Generation Sequencing Analysis

For microbial 16S rRNA gene sequence analysis, the assembled reads were processed using the QIIME2 platform (v. 2019.10). Sequence reads were imported into QIIME2 and quality assessment; filtering, barcode trimming, and chimera detection were performed using the DADA2 pipeline. Taxonomic classification was assigned to amplicon sequence variants using the SILVA 132 release with taxonomic classification at >99% confidence. Sequencing data for phyla, family, and genus amplicon sequence variants were obtained by dividing the number of reads for each taxon by the number of reads in the sample. All amplicon sequence variants with an average relative abundance of >0.5% were included for data analysis. Alpha diversity index calculations, including Chao1, Shannon index, and observed operational taxonomic units, were performed, and data obtained were visualized using QIIME scripts. Cross-sectional difference in beta diversity between the groups was assessed by permutational analysis of variance of unweighted and weighted UniFrac distances and illustrated using principal coordinate analysis (PCoA) models.

### Real-Time Reverse Transcription PCR Quantification of CXCL8, IL-23A, and CCL24 Expression on the Ocular Surface

To assess allergic inflammation in the ocular surface, we measured CXCL8 (C-X-C chemokine ligand 8/interleukin [IL]-8), IL23A (IL 23 subunit alpha), and CCL24 (C-C chemokine ligand 24/eotaxin-2) expression levels in the ocular surface by real-time reverse transcription PCR (RT-PCR) using impression cytology specimens. The impression cytology of the ocular surface was conducted using the 5 mm tip of the Schirmer test paper (AYUMI Pharmaceutical Corp). The test paper was applied to the upper tarsal conjunctiva without local anesthesia, pressed gently using a glass rod, and then removed and preserved in RNAlater RNA stabilization reagent (QIAGEN, Hilden, Germany) until real-time RT-PCR analysis.

Total RNA from each impression cytology specimen was isolated using an RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. For real-time PCR, reversed transcription of RNA samples was performed using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies Japan) following the manufacturer's instructions.

Real-time RT-PCR was performed using the TaqMan Gene Expression Assay (Life Technologies Japan) and the following predesigned primers/probes: Hs99999034\_m1 (CXCL8), Hs00372324\_m1 (IL23A), and Hs00171082\_m1 (CCL24) (Life Technologies Japan) in a StepOnePlus system (Life Technologies Japan). Target comparative threshold values were calculated using the  $\Delta\Delta CT$  method and normalized to those of GAPDH (Hs99999905\_m1) from the same sample.

### Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics 24 (IBM Japan Ltd, Tokyo, Japan) and MAC Toukei-Kaiseki v.2 software (Esumi, Tokyo, Japan). Cluster analysis based on the Euclidean distance and Ward linkage was used to classify patients into mild and severe ACD groups using similar characteristics for clinical scores and expression levels of CXCL8, IL23A, and CCL24. In addition, the Mann-Whitney  $U$  test was used to compare clinical scores and chemokine mRNA expression levels between the mild and severe ACD groups. For next-generation sequencing analysis, the Steel-Dwass test was used for the 3-group comparison of results for the mild ACD, severe ACD, and control groups.

## RESULTS

### Cluster Classification of ACD Group Based on Clinical Scores and Cytokine/Chemokine mRNA Expression Levels

A total of 21 patients with AKC and VKC were classified by cluster analysis. Clustering identified 2 distinct groups, mild

ACD and severe ACD groups, based on clinical scores and cytokine/chemokine mRNA expression levels (Fig. 1). These refractory patients with ACD, including patients with AKC and VKC, were then further stratified into mild refractory ACD and severe refractory ACD groups. The clinical scores and chemokine mRNA expression levels of the mild and severe groups differed significantly (Fig. 1). The age and sex of the patients in the control group are given in Table 1. The control subjects were not administered any topical or systemic medication.

There were no significant differences in demographic data or allergological background between the mild and severe ACD groups, except for age ( $P = 0.018$ ; Table 2). Of note, topical tacrolimus was used by all patients; however, there was no significant difference in the number of patients with AKC and VKC, percentage of patients with allergic complications, and number of steroid users between the mild and severe ACD groups. Steroid eye drops were used in 2 patients in the severe group but not in any of the patients in the mild group. The details of the eye drops used are given in Table 2. At the time of their initial visit to our hospital, both patients were using steroid eye drops for 4 to 5 months in an add-on instillation protocol, using them for about a week during acute exacerbations of ACD. The steroid eye drops were discontinued after the initial visit. The tacrolimus ophthalmic dosage was divided into 3 categories: twice-daily, proactive instillation, and reactive instillation. Proactive instillations were defined as continuous usage at a dose of no more than once a day to prevent acute exacerbations, and reactive instillations were defined as administration twice a day on days when subjective symptoms worsened and no administration on days when the symptoms were improved.

## Alteration of the Conjunctival Sac Microbiome

### Diversity Analysis

Alpha diversity is evaluated on the basis of species richness and evenness in a microbiome search. Chao1 and Shannon indices were used to evaluate the  $\alpha$ -diversity of the ocular surface microbiota in mild ACD, severe ACD, and control groups. The Chao1 index indicates the expected value of the number of bacterial species and is used as a measure of richness. The Shannon index quantifies the degree of evenness, and it is a representative index for assessing alpha diversity based on evenness. Evenness refers to the similarity

**TABLE 1.** Demographic Data and Allergological Backgrounds of ACD and Control Groups

|                                | Control Group   | ACD Group       | <i>P</i> |
|--------------------------------|-----------------|-----------------|----------|
| Patients (eyes)                | 6               | 21              |          |
| Age (yr) (mean $\pm$ SD)       | 41.0 $\pm$ 13.0 | 27.3 $\pm$ 14.2 | 0.079    |
| Sex (male:female)              | 4:2             | 19:2            | 0.147    |
| Allergic complications (cases) |                 |                 |          |
| Allergic rhinitis              | 0               | 16              |          |
| Atopic dermatitis              | 0               | 17              |          |
| Bronchial asthma               | 0               | 5               |          |

**TABLE 2.** Demographic Data and Allergological Backgrounds of Mild and Severe ACD Groups

|   | Mild Group      | Severe Group    | <i>P</i>     |
|---|-----------------|-----------------|--------------|
| Patients (eyes)                         | 11              | 10              |              |
| Age (yr) (mean $\pm$ SD)                | 33.8 $\pm$ 12.4 | 20.1 $\pm$ 12.9 | <b>0.018</b> |
| Sex (male:female)                       | 10:1            | 9:1             |              |
| Allergic complications (cases)          |                 |                 |              |
| Allergic rhinitis                       | 8               | 8               | 0.926        |
| Atopic dermatitis                       | 8               | 9               |              |
| Bronchial asthma                        | 2               | 3               |              |
| Clinical form of ACD (eyes)             |                 |                 |              |
| AKC                                     | 3               | 5               | 0.284        |
| VKC                                     | 8               | 5               |              |
| Topical use of therapeutic drugs (eyes) |                 |                 |              |
| Antiallergic eye drops                  | 8               | 10              | 0.696        |
| DSCG                                    | 4               | 4               |              |
| Epinastin                               | 4               | 4               |              |
| Olopatadin                              | 0               | 1               |              |
| Steroid eye drops                       | 0               | 2               | 0.119        |
| Fluorometholone 0.1%                    | 0               | 1               |              |
| Betamethasone 0.1%                      | 0               | 1               |              |
| Topical tacrolimus                      | 11              | 10              | 0.961        |
| Eye drops* (twice/d)                    | 3               | 10              |              |
| Eye drops* (proactive)                  | 4               | 0               |              |
| Eye drops* (reactive)                   | 2               | 0               |              |
| Ointment† (lid skin)                    | 2               | 0               |              |

Bold indicates statistically significant difference.

\*Tacrolimus ophthalmic suspension 0.1%.

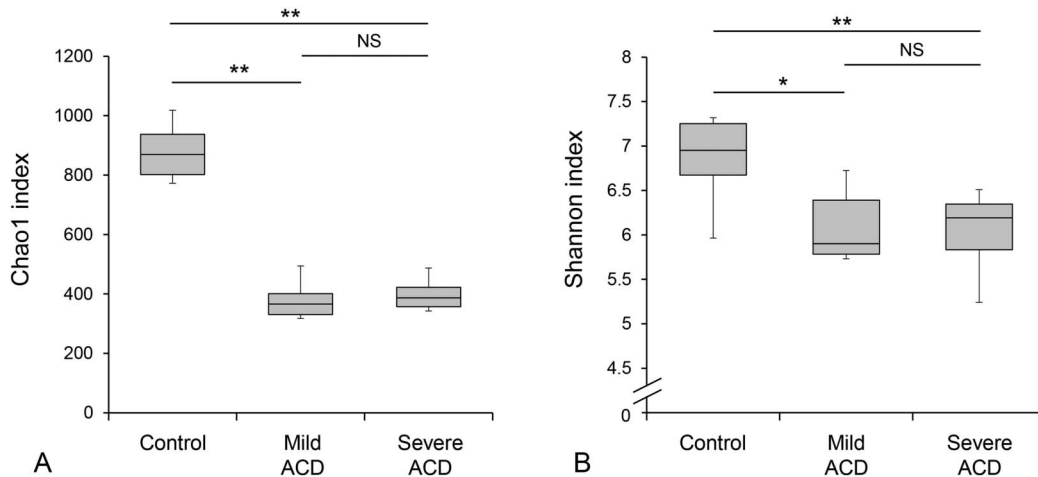
†Tacrolimus dermatological ointment 0.1%.

DSCG, disodium cromoglycate.

in the frequencies of the different species that make up a sample. The evaluation of evenness shows higher diversity if the composition of each species is equal.

In the Chao1 index evaluation, the median index levels in mild ACD, severe ACD, and control groups were found to be 336.1 (317.4–494.4) (range), 387.2 (342.8–487.0), and 869.3 (772.5–1018), respectively. The Chao1 index levels of mild and severe ACD groups were significantly lower than those of the control group (Steel–Dwass test,  $P < 0.01$ ; Fig. 2A). In the Shannon index evaluation, the median index levels in mild ACD, severe ACD, and control groups were 5.90 (5.73–6.72), 6.19 (5.24–6.51), and 6.95 (5.96–7.32), respectively. The Shannon index levels of the mild and severe ACD groups were significantly lower than those of the control group (Steel–Dwass test,  $P < 0.01$  and  $P < 0.05$ , respectively; Fig. 2B).

Beta diversity indicates the degree of difference in the diversity of 2 samples. PCoA, based on the unweighted UniFrac distance, was used to evaluate the  $\beta$ -diversities of the ocular surface microbiota among mild ACD, severe ACD, and control groups. The PCoA plot results for control, mild ACD, and severe ACD groups formed 3 different clusters (Ctrl, ACD1, and ACD2) (Fig. 3). The Ctrl cluster consisted of only control patients, whereas ACD1 included predominant patients with mild and some patients with severe ACD, and ACD2 was dominated by severe patients.



**FIGURE 2.** Measures of  $\alpha$ -diversity in mild ACD, severe ACD, and control groups. Chao1 (A) and Shannon (B) indices of diversity in mild and severe ACD groups are significantly lower than those in the control group. \* $P < 0.05$ ; \*\* $P < 0.01$  (Steel–Dwass test); NS, not significant.

### Relative Abundance of Bacterial Phyla and Genera in the Conjunctival Sac

By analyzing our 16S rRNA gene sequencing data, we were able to determine the composition of the bacterial microbiota on the ocular surface at the phylum and genus levels.

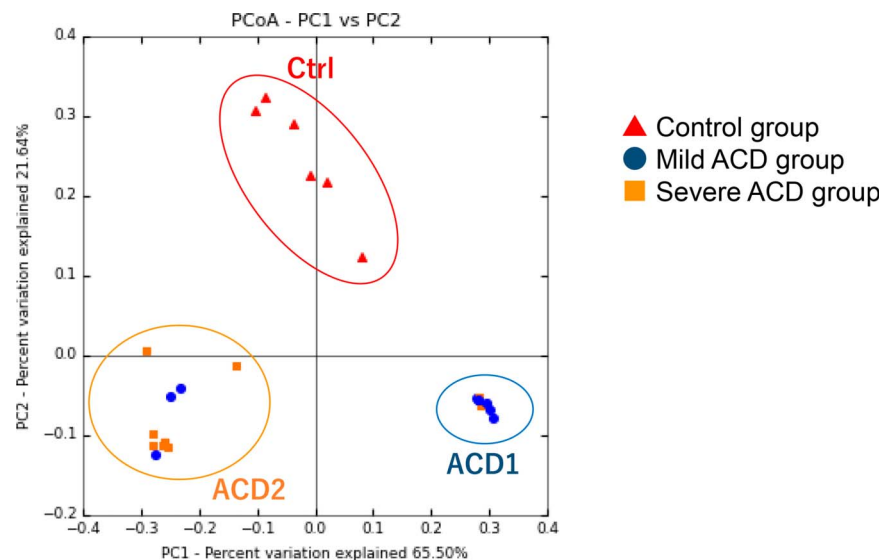
#### Phylum-Level Analysis

The core bacteria in the ocular surface microbiome included those from 4 phyla: Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria. The total relative abundance of conjunctival microbiota belonging to the phyla Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria was over 95% in the mild and severe ACD groups and over 85% in the control group (see Supplemental Figure, Supplemental Digital Content 1, <http://links.lww.com/ICO/B331>). Among the mild ACD, severe ACD, and control groups, there was no difference in the relative abundance of

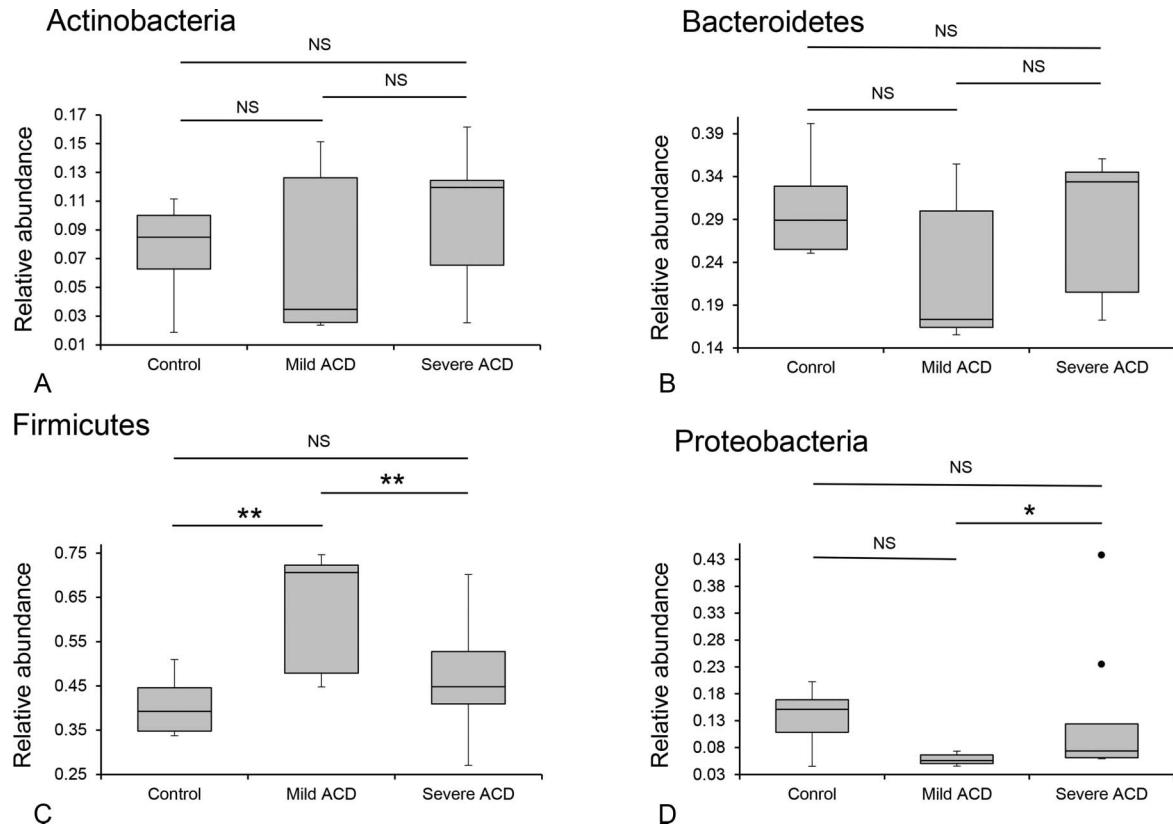
conjunctival microbiota belonging to the Actinobacteria and Bacteroidetes phyla (Figs. 4A, B). The relative abundance of phylum Firmicutes in the mild ACD group was significantly higher than that in the severe ACD ( $P = 0.007$ ) and control ( $P = 0.007$ ) groups (Steel–Dwass test, Fig. 4C). The relative abundance of phylum Proteobacteria in the mild ACD group was significantly lower than that in the severe ACD group ( $P = 0.013$ ; Steel–Dwass test; Fig. 4D).

#### Genus-Level Analysis

Genus-level comparisons were made among the mild ACD, severe ACD, and control groups for the phyla Firmicutes and Proteobacteria. The relative abundance of genera belonging to the Firmicutes and Proteobacteria phyla was different among the mild ACD, severe ACD, and control groups (Figs. 5 and 6). Genus-level statistical comparisons are



**FIGURE 3.** PCoA plots of the  $\beta$ -diversity of the ocular surface microbiota in mild ACD, severe ACD, and control groups. PCoA analysis shows that ACD and control groups differ in the  $\beta$ -diversity of bacterial community in the ocular surface. Three populations are seen: control, ACD1, and ACD2. (The full color version of this figure is available at [www.corneajrnl.com](http://www.corneajrnl.com).)



**FIGURE 4.** Statistical comparisons of relative abundance of ocular surface microbiota for 4 major phyla in mild ACD, severe ACD, and control groups. The relative abundance of the phylum Firmicutes in the mild ACD group was significantly higher than that in the severe ACD ( $P = 0.007$ ) and control ( $P = 0.007$ ) groups. The relative abundance of phylum Proteobacteria in the mild ACD group was significantly lower than that in the severe ACD group ( $P = 0.013$ ). \* $P < 0.05$ ; \*\* $P < 0.01$  (Steel–Dwass test); NS, not significant; ●, exaggerated value.

given in Table 3. The genus *Blautia* was the only genus that differed significantly in abundance between the severe ACD and mild ACD groups ( $P = 0.021$ ; Table 3). Bacterial genera that differed significantly in relative abundance between the 2 ACD groups and the control group included the Firmicutes *Staphylococcus*, *Streptococcus*, *Coprococcus*, *Ruminococcus*, and *Veillonella*, and the Proteobacteria *Delftia* and *Pseudomonas*. Among 2 other Proteobacteria genera, the relative abundance of *Morganella* was significantly increased only in the severe ACD group compared with that in the control group ( $P = 0.038$ ).

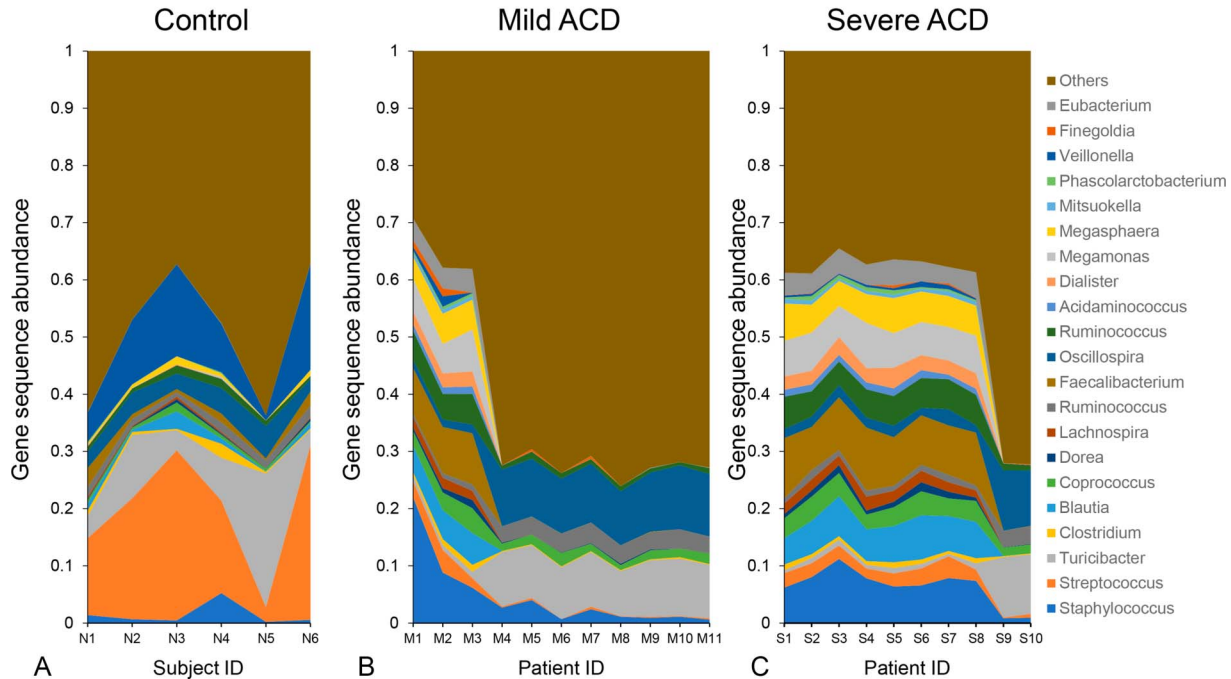
## DISCUSSION

We performed 16S rRNA gene sequencing of tear specimens to investigate the ocular surface microbiota in patients with refractory ACDs, including VKC and AKC, who were undergoing treatment with topical tacrolimus, and clarified the alteration in diversity and dysbiosis in the ocular surface. The 16S rRNA gene sequencing method is advantageous in detecting difficult-to-culture bacteria. Several studies have reported common core bacteria in the microbiomes of normal human conjunctivae based on 16S rRNA gene sequencing, including those from the following 6 genera:

*Pseudomonas*, *Cutibacterium*, *Corynebacterium*, *Acinetobacter*, *Staphylococcus*, and *Streptococcus*.<sup>6,7,11</sup> In addition, the following factors have been reported to cause alterations in the microbiota of the ocular surface: age and sex, contact lens wear, ocular surface infection, antibiotic use, and ocular surface diseases, including dry eye and ACDs.<sup>12–16</sup>

In previous reports, a decrease in the diversity and dysbiosis of gut microbiota has been showed in several immunological and allergic diseases, such as graft-versus-host disease, asthma, and AD.<sup>17–19</sup> In addition to dysbiosis in the gut microbiota, in allergic diseases, dysbiosis has been noted in the skin microbiota of patients with AD and in the airway microbiota of patients with asthma.<sup>20–22</sup> Furthermore, Liang et al<sup>4</sup> reported dysbiosis of conjunctival microbiota in patients with ACD. In this study, the Chao1 and Shannon indices evaluating  $\alpha$ -diversity levels in the mild and severe ACD groups were significantly decreased, compared with those of the control group. These alterations indicate a decrease in the number of bacterial species or an increase in the relative abundance of certain species of bacteria present in the ocular surface and may represent major characteristics of ocular surface microbiota in patients with VKC and AKC after tacrolimus ophthalmic treatment. However, it is unclear whether these alterations are primarily due to allergic

## Genus-level analysis in phylum Firmicutes



**FIGURE 5.** Bacterial genus composition of the ocular surface microbiota in the phylum Firmicutes in control (A) mild ACD (B), and severe ACD (C) groups. A–C, Show the differences in relative abundance of core conjunctival bacteria in the phylum Firmicutes among the control, mild ACD, and severe ACD groups, respectively. The relative abundances of the core bacteria that make up the microbiota of the ocular surface differed between the control and ACD groups. (The full color version of this figure is available at [www.corneajrnl.com](http://www.corneajrnl.com).)

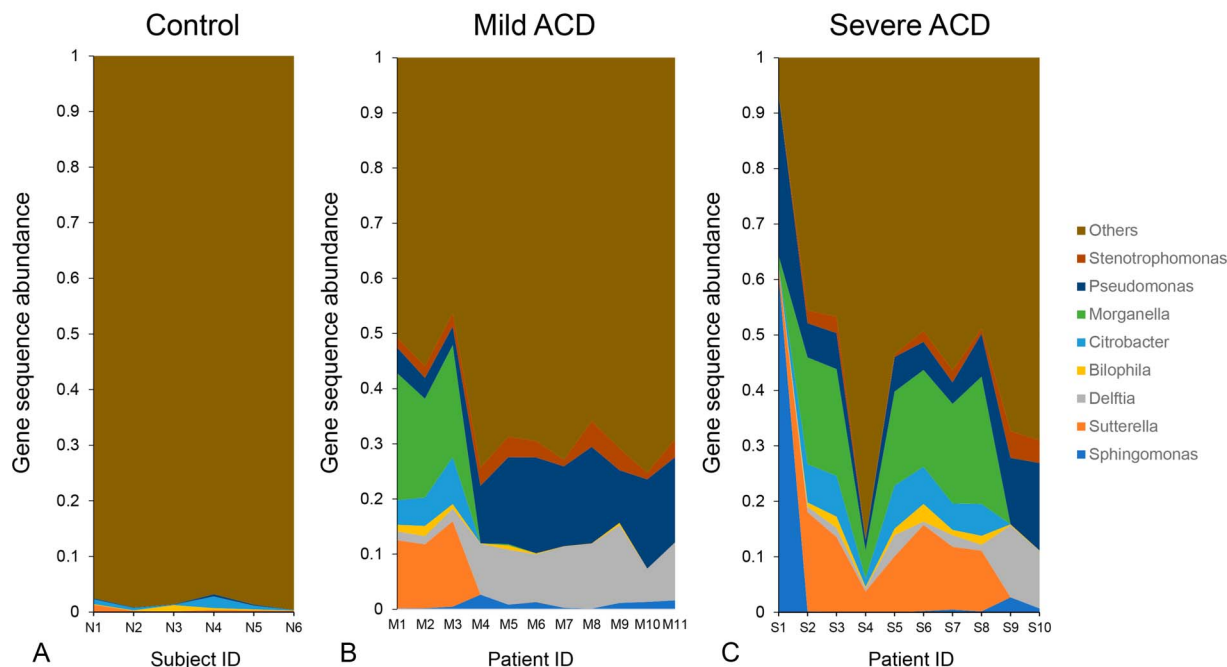
inflammation in the conjunctiva or administration of tacrolimus, which is an immunosuppressant drug with a chemical structure similar to that of macrolide antibiotics. Because tacrolimus is reported to have no antibacterial effect,<sup>23,24</sup> it is unlikely to affect the microbiota through antibacterial action. Therefore, alterations of the microbiota in the ocular surface of patients with ACDs are likely to be influenced by the atopic constitution of the host and allergic inflammation in the ocular surface. However, the immunosuppressive effects of tacrolimus and steroid ophthalmic solutions on the host ocular surface may disrupt the microbiota. Therefore, it is important to recognize that these alterations occur in the ocular surface microbiota in cases of ACD treated with tacrolimus or steroid eye drops. Further investigations are needed to determine the effects of immunosuppressant and steroid application on ocular surface microbiota.

The  $\beta$ -diversity of the ocular surface microbiota in patients with ACDs in this study was distinctly different from that of the ocular surface microbiota in the control. However, the ACD1 and ACD2 clusters were not clearly distinguished by disease severity alone. These results suggest that alterations in the relative abundance of taxa in the ocular surface microbiota are influenced by the atopic predisposition of the eye and partly by the severity of the ACDs.

The mild ACD group was characterized by an increase in the relative abundance of the phylum Firmicutes and a decrease in the relative abundance of the

phylum Proteobacteria in comparison with the control group. Among the Firmicutes, the increased relative abundance of the genera *Staphylococcus*, *Coprococcus*, and *Ruminococcus* and decreased relative abundance of the genera *Streptococcus* and *Veillonella* are representative of alterations in the ocular surface microbiome of patients with ACDs. Previously, an increased detection rate of *Staphylococcus aureus* in the skin microbiota of patients with AD and in the conjunctival microbiota of patients with ACDs was shown by microbiological culture techniques.<sup>25,26</sup> The increased relative abundance of the genus *Staphylococcus* in patients with ACDs found in this study may represent a similar alteration of the ocular surface microbiota as observed previously using the microbiological culture techniques. In addition, decreased relative abundance of both *Streptococcus* and *Veillonella* was shown in the gut microbiota of children with food sensitization in early life.<sup>27</sup> In the phylum Proteobacteria, the increased relative abundance of the genera *Morganella*, *Delftia*, and *Pseudomonas* represents alteration in the ocular microbiota of patients with ACDs. An increase or decrease in the relative abundance of a particular bacterial species is believed to lead to a decrease in diversity and to create a pathogenic microbiota.<sup>7,28</sup> Therefore, our investigation is consistent with the hypothesis that patients with ACDs have dysbiosis of the ocular surface microbiota.

### Genus-level analysis in phylum Proteobacteria



**FIGURE 6.** Bacterial genus composition of the ocular surface microbiota in the phylum Proteobacteria in control (A), mild ACD (B), and severe ACD (C) groups. A–C, Show the differences in relative abundance of core conjunctival bacteria in the phylum Proteobacteria among control, mild ACD, and severe ACD groups, respectively. Core bacteria in the microbiota of the severe ACD group showed low relative abundance compared with that of the control group. (The full color version of this figure is available at [www.corneajrnl.com](http://www.corneajrnl.com).)

We divided the patients with ACDs into 2 groups based on clinical scores and expression levels of *IL-8*, *CCL24*, and *IL-23A* in the ocular surface using cluster

analysis. Previously, we reported that *IL-8* and *CCL24* expression levels are useful markers of allergic inflammation in the ocular surface.<sup>29,30</sup> According to the cluster

**TABLE 3.** Alterations in Bacterial Taxa Isolated From the Ocular Surface in Mild ACD, Severe ACD, and Control Groups

| Genera                | Relative Abundance [Median (Range)] |                        |                        | Statistical Comparison (P)  |                              |                                |
|-----------------------|-------------------------------------|------------------------|------------------------|-----------------------------|------------------------------|--------------------------------|
|                       | Control Group                       | Mild Group             | Severe Group           | Mild Group vs. Severe Group | Mild Group vs. Control Group | Severe Group vs. Control Group |
| Phylum firmicutes     |                                     |                        |                        |                             |                              |                                |
| <i>Staphylococcus</i> | 0.002<br>(0.001–0.022)              | 0.017 (0.004–0.102)    | 0.029 (0.006–0.053)    | 0.414                       | <b>0.042</b>                 | <b>0.013</b>                   |
| <i>Streptococcus</i>  | 0.073<br>(0.013–0.105)              | 0.002 (0–0.019)        | 0.009 (0.001–0.016)    | 0.119                       | <b>0.004</b>                 | <b>0.007</b>                   |
| <i>Blautia</i>        | 0.004<br>(0.001–0.011)              | 0.008E-02 (0–0.025)    | 0.026 (0–0.036)        | <b>0.021</b>                | 0.279                        | 0.124                          |
| <i>Coprococcus</i>    | 0.003<br>(0.001–0.005)              | 0.012 (0.007–0.020)    | 0.014 (0.009–0.019)    | 0.678                       | <b>0.003</b>                 | <b>0.003</b>                   |
| <i>Ruminococcus</i>   | 0.004<br>(0.002–0.007)              | 0.007 (0.004–0.024)    | 0.020 (0.006–0.024)    | 0.138                       | <b>0.032</b>                 | <b>0.005</b>                   |
| <i>Veillonella</i>    | 0.039<br>(0.005–0.063)              | 0 (0–0.009)            | 0.0014 (0–0.005)       | 0.080                       | <b>0.003</b>                 | <b>0.005</b>                   |
| Phylum Proteobacteria |                                     |                        |                        |                             |                              |                                |
| <i>Morganella</i>     | 0 (0–0.0155)                        | 0 (0–0.0169)           | 0.0121 (0–0.0155)      | 0.159                       | 0.827                        | <b>0.038</b>                   |
| <i>Delftia</i>        | 0                                   | 0.0052 (0.0010–0.0077) | 0.0012 (0.0004–0.0077) | 0.183                       | <b>0.003</b>                 | <b>0.013</b>                   |
| <i>Pseudomonas</i>    | 0.0001<br>(0.0060E-02–0.0006)       | 0.0077 (0.0022–0.0106) | 0.0049 (0.0031–0.1275) | 0.840                       | <b>0.003</b>                 | <b>0.003</b>                   |

Bold indicates statistically significant difference.



analysis, the mild ACD group with low clinical scores and low cytokine expression levels included patients whose symptoms were relatively mild because of the effects of medical treatment, whereas the severe ACD group with high clinical scores and high cytokine expression levels included patients refractory to the treatment or those who exhibited acute exacerbations because of relapse. When the ocular surface microbiota was compared between the 2 groups with different severities of ACDs, an increased abundance of *Blautia* spp. was associated with ACD severity. Similar alterations in the abundance of *Blautia* spp. in the gut microbiota have been reported in Parkinson disease,<sup>31</sup> type 2 diabetes,<sup>32</sup> graft-versus-host disease,<sup>17</sup> early breast cancer,<sup>34</sup> and atopic disorders.<sup>33</sup> Therefore, the genus *Blautia*, a butyrate-producing taxon, may play a crucial role in blood glucose regulation, lipid metabolism, and regulation of T-cell differentiation in various diseases.<sup>32–35</sup> To analyze the pathogenesis of allergic inflammation in the ocular surface of patients with ACDs, it is necessary to take into account the abundance of *Blautia* spp. in the ocular surface microbiota. Furthermore, it is necessary to examine whether the abundance of *Blautia* spp. in the ocular surface is meaningful as a biomarker for ACDs.

Our study had several limitations. First, neither the microbiota of untreated patients with VKC and AKC nor the microbiota of patients before medical treatment was evaluated to reveal a more accurate ACD-induced dysbiosis. Second, because this was a cross-sectional study, we did not take into account age or seasonal variations in individual patients with VKC and AKC. Third, it is unclear whether bias due to individual differences in ocular surface microbiota was avoided in the statistical analysis. To help clarify some of these concerns, large-scale studies with specimens collected only during the season of symptom exacerbation should be conducted.

In conclusion, the ocular surface microbiota in patients with ACDs undergoing treatment mainly with topical application of tacrolimus showed decreased diversity and dysbiosis compared with that in control individuals. The relative abundance of some bacterial genera in the ocular surface microbiota differed according to the severity of ACD. Specific alterations of the microbiota may be characteristic biomarkers of changes in the ocular surface of patients with ACDs.

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