Metagenomic analysis of the conjunctival bacterial and fungal microbiome in vernal keratoconjunctivitis

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Abstract

Purpose Vernal keratoconjunctivitis (VKC) is a severe form of ocular allergic disease possibly related to an altered ocular surface microbiota. The aim of the study was to determine the bacterial and fungal composition of conjunctival microbiota in VKC compared with healthy controls (HC). Methods Lower fornix conjunctival swabs were obtained from 22 VKC children and 20 age, sex and ethnicity-matched HC. Total DNA was extracted, and used for 16S rRNA and ITS2 gene amplification and sequencing. Results High-throughput sequencing of 16S rRNA and ITS2 amplicon libraries produced a total of 734,157 and 677,115 high-quality reads, respectively. Clustering of similar sequences (>97% of identity) resulted in 1,241 and 933 OTUs, respectively. Alfa and beta diversity metrics highlighted significant differences of conjunctival bacterial and fungal microbiota composition in VKC patients and HC. Proteobacteria, Firmicutes and Actinobacteria phyla were present in all subjects qualifying theme as a putative core microbiome of both HC and VKC groups. In addition, Bacteroidetes and Fusobacteria met the core microbiome's definition criteria in VKC patients. Of the 132 observed families, Moraxellaceae showed a higher abundance in VKC group than HC. Saccharomycetaceae, Malasseziaceae, and Dipodascaceae were present in all subjects, constituting the fungal core microbiome of both HC and VKC patients. OTUs referred to Malasseziaceae were significantly higher in VKC children compared to HC. Conclusion VKC patients and healthy controls have different conjunctival microbiomes. These results may provide new insights into the complex VKC pathogenesis.

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Abstract

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Methods

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Results

High-throughput sequencing of 16S rRNA and ITS2 amplicon libraries produced a total of 734,157 and 677,115 high-quality reads, respectively. Clustering of similar sequences (>97% of identity) resulted in 1,241 and 933 OTUs, respectively. Alfa and beta diversity metrics highlighted significant differences of conjunctival bacterial and fungal microbiota composition in VKC patients and HC.*Proteobacteria* , *Firmicutes* and *Actinobacteria* phyla were present in all subjects qualifying theme as a putative core microbiome of both HC and VKC groups. In addition, *Bacteroidetes*and *Fusobacteria* met the core microbiome's definition criteria in VKC patients. Of the 132 observed families, *Moraxellaceae* showed a higher abundance in VKC group than HC. *Saccharomycetaceae, Malasseziaceae* , and *Dipodascaceae* were present in all subjects, constituting the fungal core microbiome of both HC and VKC patients. OTUs referred to *Malasseziaceae* were significantly higher in VKC children compared to HC.

Conclusion

VKC patients and healthy controls have different conjunctival microbiomes. These results may provide new insights into the complex VKC pathogenesis.

Keywords: ocular surface microbiome, vernal keratoconjunctivitis, 16S rRNA gene amplicon sequencing, ITS2 rRNA gene amplicon sequencing, core microbiome

Introduction

Microorganisms colonize the ocular mucosa, where they promote pathogen immunity by facilitating inflammatory cell recruitment (1). Recent microbiome studies using DNA amplicon sequencing have revealed that the healthy human ocular surface (OS) has a more diverse and relatively consistent microbial population than previously determined by conventional culture-based studies (2-6). Similarly, the OS fungal microbiome of healthy individuals showed a greater degree of diversity than reported with conventional cultivable methods, observing that several fungal genera were associated with the healthy conjunctiva (7). Moreover, other studies have shown that microbiome on the conjunctival surface is different from the deeper tissue, suggesting that there is a spatially stratified conjunctival microbiota (8, 9).

There is an increasing interest in the role of the conjunctival microbiome in different ocular surface diseases, such as meibomian glans dysfunction (10), dry eye (11, 12), contact lens wear (13), Steven-Johnson Syndrome

(14), bacterial and fungal keratitis (15). An influence of the gut microbiota to the development of autoimmune diseases, including uveitis (16) and dry eye (17), has been also demonstrated. There is evidence that there is a link between microbes and the development of allergic diseases, suggesting that dysregulation of resident microbial communities (dysbiosis) might be associated with allergy risk (18-20). It is still uncertain if and how the ocular surface microbiome influences ocular allergy (OA).

Vernal keratoconjunctivitis (VKC) is a severe form of OA affecting mostly male children and young adults with a typical relapsing-remitting course, seasonal recurrences and potentially visual impairments. The aetiology of VKC may involve a variety of factors, such as genetic predispositions, environmental allergens, climate changes and both IgE- non IgE mediated hypersensitivities (21). Th2-cells, T2-type cytokines, pro-inflammatory cytokines, a variety of chemokines, growth factors, and enzymes are over-expressed in VKC patients (22, 23). We recently found and overexpression of multiple pattern recognition receptors (PRRs) in VKC patients suggesting that host-microbe interaction play a role in VKC pathogenesis (24). What is currently not known is whether VKC patients have a different microbial composition compared to healthy individuals and how these differences correlate with signs and symptoms. To investigate the VKC-associated ocular microbiome, we applied 16S and ITS2 amplicon sequencing. Understanding the patterns of the connection between the ocular microbiome and VKC development will help in understanding causes of this disease and will improve current therapies.

Materials and Methods

2.1 Tissue sampling

Twenty-two VKC patients (6 females, 16 males; mean age 10+3.5years) and 20 age, sex and ethnicity-matched healthy controls (HC) (7 females, 13 males; mean age 10+3.2 years) were recruited from the Ocular Allergy and Pediatric Ophthalmology services of the University of Padua between April 2019 and October 2019. Neither VKC patients nor healthy controls had a history of ocular diseases (other than VKC), infection, surgery or contact lens use. Diagnosis of VKC was based on the patient's history and presence of typical signs and symptoms (21). Type of VKC (tarsal, limbal or mixed), positive skin prick test and/or presence of serum allergen-specific IgE (IgE positive sensitization), and disease-specific ongoing treatment (topical 1% cyclosporine A, antihistamines and/or mast cell stabilizers) were recorded. All the healthy controls were free from any topical and systemic treatment. None of subjects enrolled received antibiotics, corticosteroids, or non-steroidal anti-inflammatory drugs within 6 months (Table 1).

Each patient was swabbed at the inferior fornix of the right eye with a single-use, dry, sterile nylon swab (eSwab, Copan diagnostics Inc., Murrieta, CA, USA), placed in 1 mL sterile liquid amies medium after use and stored at -20°C until processing. All samples were collected by the same expert operator (A.L.) by passing twice in the inferior fornix avoiding touching the lid margin and without using topical anesthetic to prevent any potential contamination. Each patient/parent was also provided with a simple 10-items non-validated questionnaire investigating the presence of some of the principal factors related to allergy development including allergy family history, vaginal or cesarean delivery, maternal breastfeeding and/or formula feeding, weaning age, residence in an urban or rural area, varied or restricted diet, atopic dermatitis within the first year of life, repeated and prolonged contacts with pets, and family's socioeconomic status. A validated, disease-specific questionnaire, the Quality of Life in Children with Vernal Keratoconjunctivitis (QUICK) questionnaire, was administered only to VKC (25). The study was approved by the Institutional Review Board and Local Ethical Committee, and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from a parent of all subjects enrolled.

2.2 16S rRNA and ITS2 amplification and sequencing

Total DNA was extracted from 200 µL of lysate using Cador Pathogen 96 QIAcube HT Kit (Qiagen, Hilden, North Rhine-Westphalia, Germany) according to the instructions provided by the manufacturer. The V3-V4 region of bacterial 16S rRNA was amplified using the primers Pro341F (5'-CCTACGGGNBGCASCAG -3') and Pro805R (Rev 5'-GACTACNVGGGTATCTAATCC -3')(26). ITS2 region of the fungal ribosomal small subunit RNA was amplified using the primers ITS3f (5'- GCATCGATGAAGAACGCAGC -3') and

ITS4r (5'- TCCTCCGCTTATTGATATGC -3'). PCR was performed with the following conditions: 94°C for 1 minute, 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 68°C for 45 seconds, followed by final extension at 68°C for 7 minutes. PCR products were analyzed by electrophoresis on 1.5% agarose gel. Negative controls (PCR reagents without template DNA and template from blank swabs) were included to verify the possible presence of contaminating DNA. The PCR-amplified amplicons were purified using Agencourt AMPure XP kit (Beckman Coulter, Inc., Brea, California, USA), quantified with a Qubit 2.0 instrument (Thermofisher, Waltham, Massachusetts, US) and sequenced with the Illumina MiSeq platform (MiSeq ver. 3, 600 cycles, Illumina, Inc., San Diego, California, USA) to generate paired-end reads with a nominal length of 300 bp in each direction.

2.3 Sequence data processing

Primer and adaptor sequences were trimmed using *cutadapt*implemented in *trimmomatic* (27), setting a minimal Phred score of 25. The two fragments of the paired-end reads were merged using*fastq-join* with default parameters and processed with the Quantitative Insights into Microbial Ecology (QIIME2 build 8_-2020) pipeline. In details, the Divisive Amplicon Denoising Algorithm 2 (DADA2) was used to denoise the reads (28), that were subsequently clustered into operational taxonomic units (OTUs) and taxonomically classified according to the bacterial Greengenes v.13.8 (29) and fungal UNITE version 7.0 reference databases (30). OTUs which were present in over 80% of samples with a minimum abundance of 0.01% were classified as "core OTUs". Core OTUs were grouped into three categories, based on their presence in HC, in VKC or in both groups.

QIIME2 pipeline was used for generating the rarefaction curves and for calculating the α diversity metrics (diversity of species within an ecosystem) including the Shannon diversity, Simpson index, observed number of OTUs and phylogenetic diversity.

Weighted and unweighted UniFrac distances, Bray-Curtis and Jaccard dissimilarity between samples were calculated and used for investigation of β diversity (diversity of species between ecosystems) through plotting principal coordinate analysis (PCoA). To determine the factors potentially influencing the conjunctival microbiome, bacterial and fungal communities were compared between groups while controlling for the other variables (age, sex, type of VKC, IgE sensitization, type of feeding, previous presence of atopic dermatitis, etc.).

2.4 Statistical analysis

Demographic data including age and sex were summarized using descriptive methods.

Microbiome data were analyzed using QIIME2 microbiome bioinformatics platform (31).

Sampling depth of the samples was evaluated to determine if the within-sample diversity was fully captured: saturation of the alpha refraction plots was inspected. Within-sample diversity was measured by Shannon's alpha diversity index. Influence of demographic characteristics (age and sex) and clinical variables (phenotype of VKC, positive skin prick test total and/or serum specific IgE, topical therapy, type of delivery and feeding, type of diet, contact with pets, allergy family history, living place, history of atopic dermatitis during the first year of life, history gastrointestinal disorders) were ascertained by Kruskal-Wallis statistical test. Pairwise comparisons were made when more than two modalities were present, and Benjamini-Hochberg adjustment procedure for type I error inflation due to multiple testing was used. Between-sample diversity was measured using Bray-Curtis index. Potential sample clusters were highlighted by 3D plots obtained from Principal Coordinates Analysis (PCoA) based on unweighted UniFrac as a distance metric.

The analysis of composition of microbiomes (ANCOM) (32) was used to identify differential abundant bacterial and fungal taxa between VKC and HC at various levels of hierarchy including phylum, family, genus, species, and OTUs. Volcano plot of the effect size difference vs. ANCOM test statistic W was used to visualize the result of differential abundance testing.

Results

3.1 Bacterial microbiome: community analysis

16S rRNA amplification was successfully carried out for 12/22 VKC samples (54.5%), and in 4/20 HC samples (20%). High-throughput amplicon sequencing produced a total of 734.157 high-quality reads (average of 45.885 reads per sample), which were clustered into 1.241 OTUs (97% sequence identity) and classified according to the Greengenes database. Rarefaction curves tended to saturation indicating that that the sequencing depth was adequate to capture the diversity in the microbiome in each conjunctival sample (Figure 1S).

Overall, α diversity was significantly higher in VKC patients compared to HC (p=0.05) (Figure 2S). Alpha diversity was significantly higher in IgE-negative patients than HC (p=0.03) as well as in tarsal VKC patients compared to the mixed type (p=0.02) and HC (p=0.03). Alpha diversity was also significantly higher in formula-fed than breastfed children (p=0.03) and in children with a history of atopic dermatitis (AD) during the first year of life (p=0.01) (Figure 1), but not significant considering the type of birth, socioeconomical condition, diet, or contact with pets.

Beta diversity metrics highlighted the differences of conjunctival microbiota composition in VKC patients and HC (Figure 3S). PCoA unveiled also the different clustering of bacterial microbiomes depending on different variables such as the presence of atopic dermatitis during the first year of life, the type of feeding and the severity of ocular surface inflammation according to the QUICK score (Figure 2).

3.2 Bacterial microbiome: Taxonomic classification

The number of OTUs taxonomically assigned at the different levels are shown in the Supplementary File (Table 1S). At the phylum level, conjunctival microbiomes were dominated by *Proteobacteria* (VKC 44.8%; HC 67.9%), *Firmicutes* (VKC 37.0%; HC 22;1%), A*ctinobacteria* (VKC 7.9%; HC 3.7%) and *Bacteriodetes*(VKC 4.2%; HC 2.7%) that accounted for $>$ 90% of all the reads (Figure 3).

Proteobacteria, Firmicutes and Actinobacteria were present in all subjects qualifying theme as a putative core OS microbiome of both HC and VKC patients. In addition, *Bacteroidetes* and*Fusobacteria* met the core microbiome's definition criteria in VKC patients (Table 2), being present in $>$ 80% of patients with a mean abundance $>$ 0.01%. At the family level,*Pseudomonadaceae* (VKC 25.8%; HC 54.9%), *Streptococcaceae*(VKC 15.6%; HC 4.1%), *Staphylococcaceae* (VKC 12.5%; HC 9.5%), and *Neisseriaceae* (VKC 6.5%; HC 9.1%) were dominant in both groups (Figure 4S). Of the 132 observed families, *Moraxellaceae*(W=15) showed a higher abundance in VKC group than HC (Figure 4). At the genus level, 10 genera including *Pseudomonas* (VKC 25.7%; HC 54.8%), *Staphylococcus* (VKC 15.6%; HC 4.1%),*Streptococcus* (VKC 12.5%; HC 9.4%), *Acinetobacter* (VKC 5.5%; HC 0.1%), *Neisseria* (VKC 4.2%; HC 0.4%),*Haemophilus* (VKC 3.1%; HC 0.5%), *Prevotella* (VKC 1.1%; HC 1.1%), *Corynebacterium* (VKC 2.4%; HC 1.4%),*Propionibacterium* (VKC 2.0%; HC 0.1%) and *Rothia* (VKC 2.0%; HC 0.1%) accounted for $>$ 70% of sequences in both groups (Figure 5).

3.3 Fungal microbiome: community analysis

Ten samples out of 22 (45,4%) from VKC patients produced a detectable ITS2 amplicon and were sequenced. A total of 677,115 high-quality reads (average of 48,365 reads per sample) were clustered into 933 OTUs and taxonomically classified against the UNITE ver. 7.0 database. As for the 16S rRNA samples, rarefaction curves tended to saturation indicating that that the sequencing depth was reasonable (Figure 5S).

Alpha diversity metrics of fungal community didn't substantially differ between HC and VKC children. However, Shannon index was significantly higher in IgE-negative than IgE-positive VKC patients (p=0.02), whereas the observed number of OTUs was significantly higher in males compared to females (p=0.01) (Figure 6S). Representing the microbiomes composition and structure through the PCoA plot, two distinct clusters emerged when comparing conjunctival microbiomes from VKC and HC samples as wells as from IgE-positive and IgE-negative subjects (Figure 7S).

3.4 Fungal microbiome: taxonomic analysis

At the phylum level, *Ascomycota* (mean abundance in VKC 79.2%; HC 71.9%) and *Basidiomycota* (VKC 19.4%; HC 27.4%) were the two dominant phyla accounting for > 98% of all sequences in both groups. At the family level, *Saccharomycetaceae* (VKC 55.8%; HC 62.7%), *Malasseziaceae* (VKC 15.6%; HC 3.3%),*Pleosporaceae* (VKC 12.4%; HC 2.6%) and *Cladosporiaceae*(VKC 4.0%; HC 3.0%) accounted for the vast majority of sequences (Figure 6). OTUs referred to *Malasseziaceae* were significantly increased in VKC children compared to HC (W=42).*Saccharomycetaceae, Malasseziaceae* , and *Dipodascaceae* were present in all subjects, thus constituting the fungal core microbiome of both HC and VKC patients. At the genus level, *Saccharomyces* (VKC 55.8%; HC 62.7%), *Malassezia* (VKC 15.6%; HC 3.3%),*Alternaria* (VKC 9.9%; HC 2.4%) and *Cladosporidium* (VKC 4.0%; HC 3.0%) were the prevalent genera.

Discussion

The application of high-throughput sequencing (HTS) techniques to metagenomics has drastically contributed to reveal a level of microbial diversity that was previously hidden by the selectivity of cultivable based methods (5). In our study, VKC patient showed a significantly higher bacterial α diversity than HC. Similar results have been already observed in patients with both ocular and extra-ocular inflammatory diseases (14, 33-35). Differently from bacteria, no significant difference in fungal α diversity metrics was observed between VKC and HC. Alpha diversity metrics were also evaluated according to other clinical variables showing that both bacterial and fungal richness and diversity were significantly higher in IgE-negative than IgE-positive VKC suggesting that atopic patients may have a different conjunctival microbiome. There is an accumulating evidence that dysbiosis precedes the development of allergic manifestations at least for food allergy and asthma (36). Interestingly, in our study children who had been formula-fed showed a significantly higher bacterial α diversity than those who had been breastfed as well as children with and without history of atopic dermatitis during the first year of life. Results of the 10-item questionnaire revealed that breastfeeding had been significantly more common among healthy children than VKC patients. Breastfeeding has been shown to be associated with a reduced risk of childhood asthma, atopic dermatitis and rhinitis (37). It has been suggested that breastfeeding shapes the early-life gut microbiota directly by transfer of the human milk microbiota (including lactobacilli and bifidobacteria) and indirectly by exposure to human milk oligosaccharides (HMOs), short-chain fatty acids (SCFAs), secretory IgA and components of the innate immune system which collectively influence and direct microbial growth and metabolism (36, 38, 39). If this can exert a remote and persistent influence on the composition of a local microbiota is unknown.

When exploring the effect of sex on conjunctival microbiome, we found that males had a significantly higher number of observed fungal OTUs than females. The preponderance of VKC in males suggested a potential role of sex hormones in its pathogenesis (40, 41), but a possible role of microbiome on the male prevalence in VKC hasn't been highlighted. Although the microbiome is known to be affected by age and sex, the influence of these factors on ocular surface microbiota remains controversial (9, 42, 43).

A normal conjunctival microbiome could be described by a core microbiome that contributes to maintain the functional stability and homeostasis of the ocular surface. It has been proposed the existence of a variable ocular surface microbiome, indicating that certain microbial types may always be present, while others are transient depending on age, sex, ethnicity, environment, lifestyle or food habits as well as tearing and blinking(6, 9). The temporal stability of ocular microbiota over a three months period has been assessed showing that, even though the microbial community was relatively stable, the ocular surface is not colonized consistently by any given OTU (6). We tried to define a core microbiome including in this definition all those taxa who were present with a minimum abundance >0.01% in at least 80% of samples.*Proteobacteria, Firmicutes and Actinobacteria* were identified as the 3 major bacterial phyla in all samples, while *Pseudomonas*sp., *Staphylococcus* sp., *Streptococcus* sp. and*Propionibacterium* sp. were ubiquitous among all the examined subjects at the genus level. All these core taxa are well-known potential ocular pathogens suggesting that the ocular surface is regularly exposed to opportunistic pathogens but the ocular surface can activate mechanisms suppressing microbial pathogenicity. Many other bacterial families (*Neisseriaceae, Fusobacteriaceae* ,*Moraxellaceae, Micrococcaceae, Gemellaceae, Paenibacillaceae* , and*Veilonnelaceae*) and genera (*Prevotella* sp.*, Bacillus* sp.*, Rothia* sp.*, Haemophilus* sp.) met the core microbiome's definition

criteria only in the VKC group. The VKC core microbiome included different species of gram-negative bacteria such as *Proteobacteria* (*Neisseria* sp., *Moraxella*sp., *Haemophilus* sp.) and *Bacteriodetes*(*Prevotella* sp.) able to induce an LPS-driven inflammatory response through the Toll-Like Receptor (TLR)4 /NF-ϰB pathway that may trigger the inflammation in these patients (24).

Differently from bacteria, the fungal core microbiome was similar in HC and VKC patients and included *Saccharomycetaceae, Malasseziaceae* , and *Dipodascaceae.* Interestingly, a significantly higher abundance of *Malasseziaceae* was found in VKC patients.*Malassezia* spp. have already been associated with many different disorders including bacterial keratitis (44, 45), dandruff, atopic dermatitis (46) and inflammatory bowel diseases (47). Atopic dermatitis patients are often sensitized to *Malassezia* with Th2- and IgE-specific responses or a mixed Th2/Th17 response, which is a possible mechanism also in VKC (24, 48). *Malassezia* spp.directly interacts with several PRRs, such as Dectin-1, Dectin-2, Mincle, TLR2 and TLR4 activating multiple signaling pathways (49-51). All these PRRs are over-expressed at gene and protein level in VKC patients (24). Therefore, we believe that glycan, phospholipid carbohydrate residues of allergens and microbes, can engage innate receptors on epithelial and dendritic cells priming a Th2/Th17 type response in VKC.

Fourteen of the 22 VKC children (64%) were under one or more topical treatments (cyclosporine A, antihistamines and/or mast cells stabilizers) at the time of swabbing, creating a potential bias in microbiome's analysis (Table 1). However, only 5 of the 10 VKC patients whose swabs were not sequenced because of the absence of the amplicons, were treated with topical medications. Furthermore, β diversity metrics didn't showed any significant difference of both bacterial and fungal communities according to the use and type of topical medications (data not shown) suggesting that factors other than topical eyedrops may alter the conjunctival microbiota. Similar results have been previously reported showing that topical cyclosporine didn't affect the richness and the diversity of the ocular bacterial flora (9, 12).

The main limitation of our study is the low number of patients and sequenced samples. VKC is a relatively rare disease in Western Europe with a prevalence of 1.1-10.5 cases per 10,000 inhabitants (52). On the other hand, in order to minimize the possible biases of age, gender and other variables, the two study groups were as homogeneous as possible. Only 4 out of 20 conjunctival swabs from healthy controls yielded 16S rRNA and ITS2 amplicons and allowed for the microbiome's analysis; on the contrary, a much higher proportion of swabs from VKC patients yielded sufficient genetic material to be analyzed. Even though this may represent the main concern, the minimum more common recommended sampling size to make a comparison between a pathological sample and normal samples is three to four, therefore sufficient in this case considering the very low microbial load in the healthy conjunctiva. A higher difficulty to obtain amplicon products from healthy subjects has been already described and attributed to the physiological antimicrobial activity and to the lower microbial load of healthy subjects (6, 53).

In conclusion, we showed that VKC patients have a different and more diverse ocular microbiota compared to normal subjects. The major challenge in microbiome studies is to translate metagenomic data into biological knowledge in order to understand how the microbiome may affect the host or vice versa. The comprehension of the relations between dysbiosis and human diseases will pave the way to more focused studies and new therapies.

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Figure legends

Figure 1. Boxplots illustrating Shannon diversity in bacterial microbiomes of healthy controls (HC) and vernal keratoconjunctivitis (VKC) patients in relation to the presence of serum IgE (A), type of VKC (B), type of feeding (C) and history of atopic dermatitis in the first year of life (D). Median values (horizontal line) and interquartile ranges have been indicated in the plots.

Figure 2. Three-dimensional Principal Coordinates Analysis (PCoA) based on weighted UniFrac distances. A: subjects with (yellow circles) and without history of atopic dermatitis in the first year of life (red circles); B: subjects with a history of breastfeeding (blue circles) and formula feeding (red circle); C: VKC patients with the lowest QUICK scores (0-45; yellow circles), intermediate scores (45-60; orange circles) and highest score (>90; red circles).

Figure 3 . Pie charts showing the mean relative abundance of the predominant bacterial phyla in VKC (A) and HC samples (B).

Figure 4. ANCOM volcano plot. The W value represents the number of times of the null-hypothesis (the average abundance of a given species in a group is equal to that in the other group) was rejected for a given species. The x-axis value represents the centered log ratio (clr) transformed mean difference in abundance of a given species between VKC and HC groups. A positive x-axis means a species is abundant in HC group and a negative x-axis value means a species is abundant in VKC group.

Figure 5 . Pie charts showing the mean relative abundance of the predominant bacterial genera in VKC (A) and healthy controls (B) samples. "Other" includes genera with <1% mean abundance.

Figure 6 . Pie charts showing the mean relative abundance of the predominant fungal families in VKC (A) and healthy control (B) samples. "Other" includes genera with <1% mean abundance.

Tables

Table 1. Demographic data.

	VKC patients (n = 22)	**Healthy controls (n = 20)**
Age (years)	**Age (years)**	**Age (years)**
Mean (range)	10 (5 – 17)	10 (5 – 15)
Median	9	9
Male / Female	16 / 6	13 / 7
VKC type (%)	**VKC type (%)**	**VKC type (%)**
Tarsal	6 (27%)	
Limbal	5 (23%)	
Mixed	11 (50%)	
IgE sensitization (%)	**IgE sensitization (%)**	**IgE sensitization (%)**
Present	10 (45%)	
Absent	12 (55%)	20 (100%)
Topical treatment (%)	**Topical treatment (%)**	**Topical treatment (%)**
CsA	2 (9%)	
Antihistamine ± Mast cell stabilizer	11 (50%)	
None	9 (41%)	20 (100%)

VKC = Vernal Keratoconjunctivitis. CsA = Cyclosporine A.

Table 2. Complete list of assigned OTUs which were present in over 80% of samples with a minimum abundance of 0.01% grouped into three categories as core OTUs in both HC and VKC groups, in VKC group and in HC group.

	Bacterial core microbiomes	**Bacterial core microbiomes**	**Bacterial core microbiomes**
	Phylum	**Family**	**Genus**
HC and VKC groups	Proteobacteria	Enterobacteriaceae	Pseudomonas
	Firmicutes	Neisseriaceae	Streptococcus
	Actinobacteria	Streptococcaceae	Staphylococcus
		Staphylococcaceae	Propionibacterium
		Propionibacteriaceae	
VKC group	Proteobacteria	Neisseriaceae	Haemophilus Rothia
	Actinobacteria	Moraxellaceae	Corynebacterium
	Bacteroidetes	Pasteurellaceae	Prevotella Bacillus
	Fusobacteria Firmicutes	Micrococcaceae	
		Corynebacteriaceae	
		Paraprevotellaceae	
		Fusobacteriaceae	
		Gemellaceae	
		Paenibacillaceae	
		Veillonellaceae	
		Bacillaceae	

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