# Enriched and Decreased Intestinal Microbes in Active VKH Patients

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**PURPOSE.** To determine the possible microbiome related to Vogt–Koyanagi–Harada (VKH) disease in comparison to patients with noninfectious anterior scleritis and healthy people.

**METHODS.** Fecal samples were extracted from 42 individuals, including 11 patients with active VKH, 11 healthy people, and 20 patients with noninfectious anterior scleritis. We amplified the V3 to V4 16S ribosomal DNA (rDNA) region to obtain the target sequence. Then, the target sequence was amplified by polymerase chain reaction. The obtained target sequences were sequenced by high-throughput 16S rDNA analysis.

**RESULTS.** At the genus level, there were three enriched (*Stomatobaculum*, *Pseudomonas*, *Lachnoanaerobaculum*) and two depleted (*Gordonibacter*, *Slackia*) microbes that were detected only in patients with VKH. There were 10 enriched and 12 depleted microbes that were observed in both patients with VKH disease and noninfectious anterior scleritis ($P < 0.05$). The interactions of these microbes were graphed. *Tyzzerella* and *Eggerthella* were the nodes of interaction between these microorganisms, which were regulated by both positive and negative aspects, but the expression level in patients with active VKH was upregulated.

**CONCLUSIONS.** Special or nonspecial enrichment and decreased intestinal microbes were observed in patients with active VKH. The action mechanism of these microbes needs further study.

Keywords: intestinal microbes, Vogt–Koyanagi–Harada disease, Stomatobaculum, Pseudomonas, Lachnoanaerobaculum

VKH disease is a systemic autoimmune disease characterized by bilateral granulomatous panuveitis, poliosis, vitiligo, alopecia, auditory signs, and central nervous system abnormalities.[1] VKH disease can lead to severe impairment of visual acuity and even blindness when associated with retinal edema, retinal detachment, or retinal pigment epithelial changes due to its recurrent and chronic course.[2] The pathogenesis of VKH is not completely clear. The autoimmune response is reported to play an important role in this process. Moreover, many other factors, including intestinal flora, viral infection, and genetic factors, are involved.[3] Among these factors, the intestinal flora has received increasing attention. It has been recognized that intestinal flora is related to some autoimmune diseases, such as Behcet disease, rheumatoid arthritis, and inflammatory bowel disease. A study characterizing the differences in intestinal flora between patients with VKH and healthy controls was carried out. In this study, depleted butyrate-producing bacteria, lactate-producing bacteria, and methanogens as well as enriched gram-negative bacteria were identified in patients with active VKH.[4] However, whether these microbes are specifically related to VKH remains unknown. To answer this question, we selected patients with noninfectious anterior scleritis as disease controls and compared the difference in intestinal flora between patients with these diseases. Our study found similar results to the above experiment

in patients with VKH. However, 3 enriched (*Stomatobaculum*, *Pseudomonas*, *Lachnoanaerobaculum*) and 2 depleted microbes (*Gordonibacter*, *Slackia*) were detected only in patients with VKH, while 10 enriched and 12 depleted microbes were observed in both patients with VKH disease and noninfectious anterior scleritis.

## METHODS AND MATERIALS

### Participants

Forty-two individuals were enrolled in the study, including 11 patients with initial-onset active VKH (V group, average age: $46.6 \pm 8.0$ years, male/female: 0.83:1), 11 healthy controls without autoimmune disease (N group, average age: $48.2 \pm 9.8$ years, male/female: 0.83:1), and 20 patients with active noninfectious anterior scleritis (G group, average age: $47.3 \pm 10.8$ years, male/female: 0.81:1). There was no significant difference in age or sex of the three groups in this study. The inclusion criteria for individuals enrolled in the study were as follows: (1) individuals without other immune system diseases, such as systemic lupus erythematosus, diabetes, or Crohn disease; (2) individuals without infectious disease; and (3) patients who should be in the active stage of disease and were not taking any medicine. The diagnosis of active VKH disease was based on the

published criteria.[5–7] All these patients had at least one extraocular manifestation, including meningismus (7/11), tinnitus (4/11), poliosis (3/11), hearing loss (2/11), alopecia (2/11), and vitiligo (1/11). The diagnosis of noninfectious anterior scleritis is based on characteristic clinical manifestations, including ocular tenderness to touch, painful inflammation radiating to the forehead, edema affecting episcleral and scleral tissues, and injections of both the superficial and deep episcleral vessels.[8] Informed consent were obtained from all participants. This study met the requirements of the Declaration of Helsinki and was approved by the Clinical Ethics Committee.

### Fecal Sample Collection and DNA Extraction

Fecal samples were collected from patients with initial-onset active VKH or noninfectious anterior scleritis and controls. All samples were frozen immediately and then stored at −80°C until testing. The method of extracting DNA was based on the E.Z.N.A. Stool DNA Kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's instructions. The extracted DNA quality was measured by 1% agarose gel electrophoresis and spectrophotometry (260 nm/280 nm optical density ratio). The target sequence that needed to be amplified was introduced in the sequencing of the 16S ribosomal RNA (rRNA) gene amplicon.

### Sequencing of 16S rRNA Gene Amplicon

The extracted DNA was sent to Beijing Aoweisen Gene Technology Co., Ltd. (Beijing, China). Then, the DNA was detected on an Illumina (Santiago, California, USA) MiSeq PE300 platform. The V3 to V4 16S ribosomal DNA (rDNA) region was amplified using the universal primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACNNGGG TATCTAAT-3′). Next, the target sequence was amplified by polymerase chain reaction.

### Sequence Analysis

The target sequences were subjected to paired-end sequencing on an Illumina MiSeq platform. The chimaeras were filtered, split, and removed using QIIME (Professor Gregory Caporaso, Flagstaff, USA) (version 1.8.0). Sequences whose scores were less than 20 or that had base ambiguity, primer mismatch, or sequencing length less than 150 bp were excluded. According to barcodes, we clustered the sequences and grouped them as operational taxonomic units (OTUs). In addition, OTU similarity was set to 97%. After that, we matched every OTU to the corresponding species classification information by comparison to the Silva database. Then, Mothur (Professor Patrick Schloss, Michigan, USA) (version 1.31.2; MI, USA) was used to calculate the microbial $\alpha$-diversity, including the Shannon, abundance-based coverage estimator (ACE), and Chao1 indices. Based on the weighted UniFrac distance, we calculated clustering using the pheatmap from TBtools (Chengjie, chen. Guangzhou, China) (version 1.098652). The data were drawn based on the row scale. The different species communities of each sample were compared, and $\beta$-diversity was calculated by UniFrac.

### Statistical Analysis

To explore the difference in each group, we used Mothur software (version 1.31.2) to perform Metastats analysis and chose a *P* value <0.05 as indicating significance. Spearman correlation analysis was used as the mapping parameter of the correlation diagram. The correlation $\mid R \mid > 0.6$, $P < 0.05$, and Cytoscape[9] and CoNet[10] were used to visualize and analyze the network.

## RESULTS

### Multi Sample Shannon–Wiener Curves and Microbiome Species Diversity

To check if the sequencing depth and coverage were sufficient to cover the total diversity of the microbiomes, we tested the number of reads sampled. After that, we graphed the results and obtained multi sample Shannon–Wiener curves (Fig. 1a). The rarefaction curves of all microbiomes reached a plateau, which indicated that the sequencing depth and coverage were sufficient to cover the total diversity of the microbiomes.

To compare the species diversity among these three groups, we analyzed the $\alpha$-diversity (Chao1, observed_species, PD_whole_tree, and Shannon) (Fig. 1b) and $\beta$-diversity (Analysis of Molecular Variance (AMOVA)) (Fig. 1c) of gut microbiomes in each group. We found no difference in the $\alpha$-diversity or $\beta$-diversity among these three groups. The species richness was similar in patients with VKH, patients with noninfectious anterior scleritis, and healthy people.

### Comparison of Microbes Among the Three Groups

There were differences in microbes among these three groups at different levels. The biggest difference was at the genus level. Three enriched and two depleted microbes were detected in the V group compared to the G and N groups (all *P* < 0.05, Fig. 2a). The enriched microbes included *Stomatobaculum*, *Pseudomonas*, and *Lachnoanaerobaculum*, and the depleted microbes included *Gordonibacter* and *Slackia*.

*Pseudomonas* existed only in patients with VKH and was not detected in patients with noninfectious anterior scleritis or healthy controls. *Stomatobaculum* existed in patients with VKH and noninfectious anterior scleritis but was not detected in healthy controls, and the abundance in patients with VKH was much higher than that in patients with noninfectious anterior scleritis. *Lachnoanaerobaculum* existed in all three groups of patients, but the content in patients with VKH was significantly higher than those in the other two groups.

*Slackia* was not detected in patients with VKH but only existed in healthy controls and patients with noninfectious anterior scleritis. The content of *Gordonibacter* was depleted in patients with VKH but was enriched in patients with noninfectious anterior scleritis.

At the family level, Flavobacteriaceae existed only in patients with noninfectious anterior scleritis and healthy controls but not in patients with VKH.

At the phylum level, Cyanobacteria existed in the V and G groups but not in the N group, and the content in patients with VKH was much higher than that in patients with noninfectious anterior scleritis.

### Enriched Microbes in VKH Disease

In addition to the above results, by comparing the intestinal flora between patients with active VKH and healthy controls, we obtained an elevated microbe in patients with VKH and inferred which microbes might cause VKH disease.
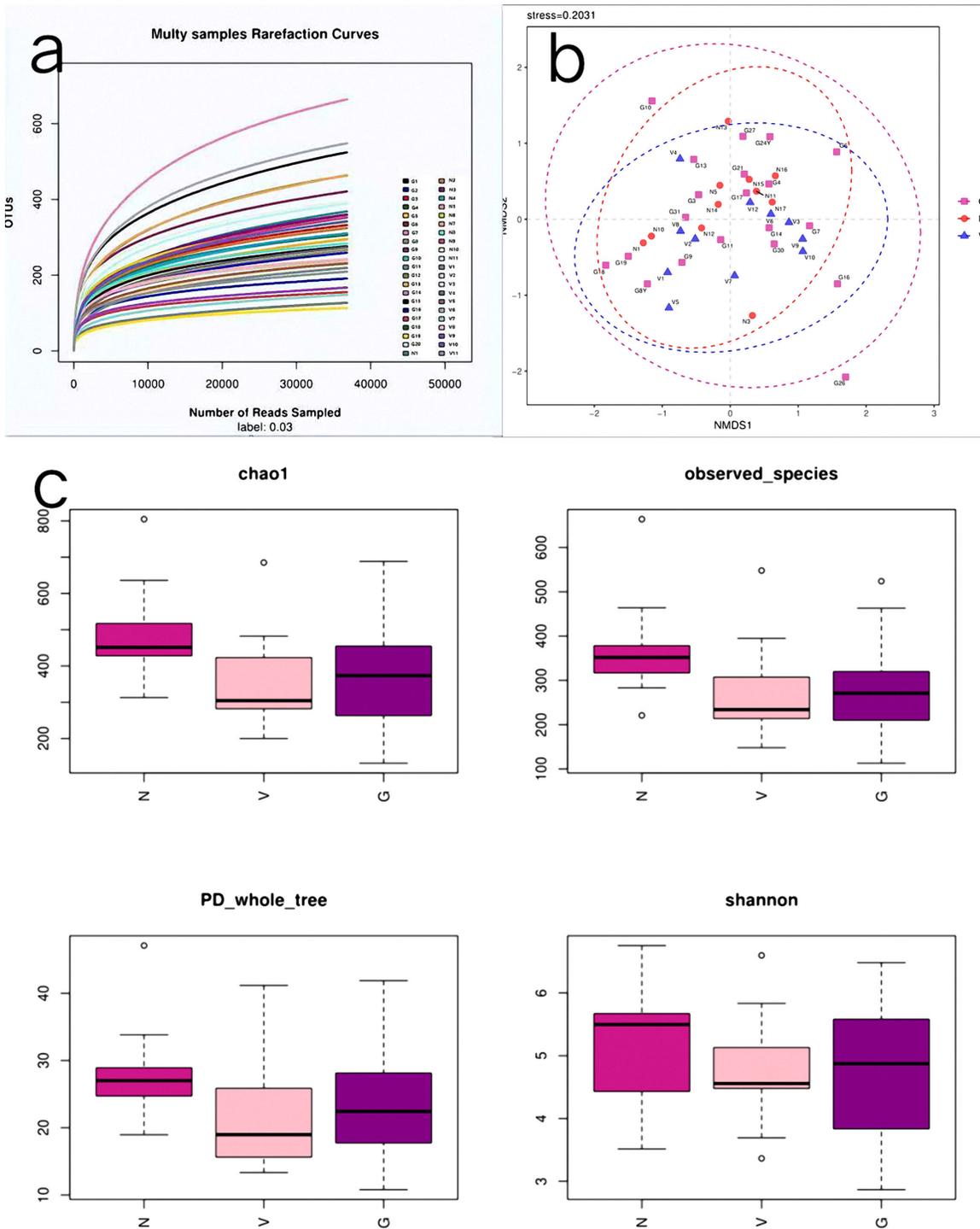
**FIGURE 1.** (a) Rarefaction curves of the gut microbes from 11 patients with VKH, 20 patients with noninfectious anterior scleritis, and 11 healthy controls. (b) α-Diversity with the measures Chao1, Observed OTUs, PD_whole_tree, and Shannon for patients with VKH, patients with noninfectious anterior scleritis, and healthy controls. (c) β-Diversity with the measures Analysis of Molecular Variance (ANOVA) for patients with VKH, patients with noninfectious anterior scleritis, and healthy controls.

At the genus level, there were 13 enriched microbes in patients with VKH compared to the healthy controls. There were three microbes in the family, one microbe in the order, one microbe in the class, and one microbe in the phylum. We divided these 19 enriched microbes into three types according to whether they were specifically increased in patients with VKH.

Type 1: As mentioned before, microbes specially related to VKH disease include *Pseudomonas*, *Stomatobaculum*, and *Lachnoanaerobaculum* at the genus level. These
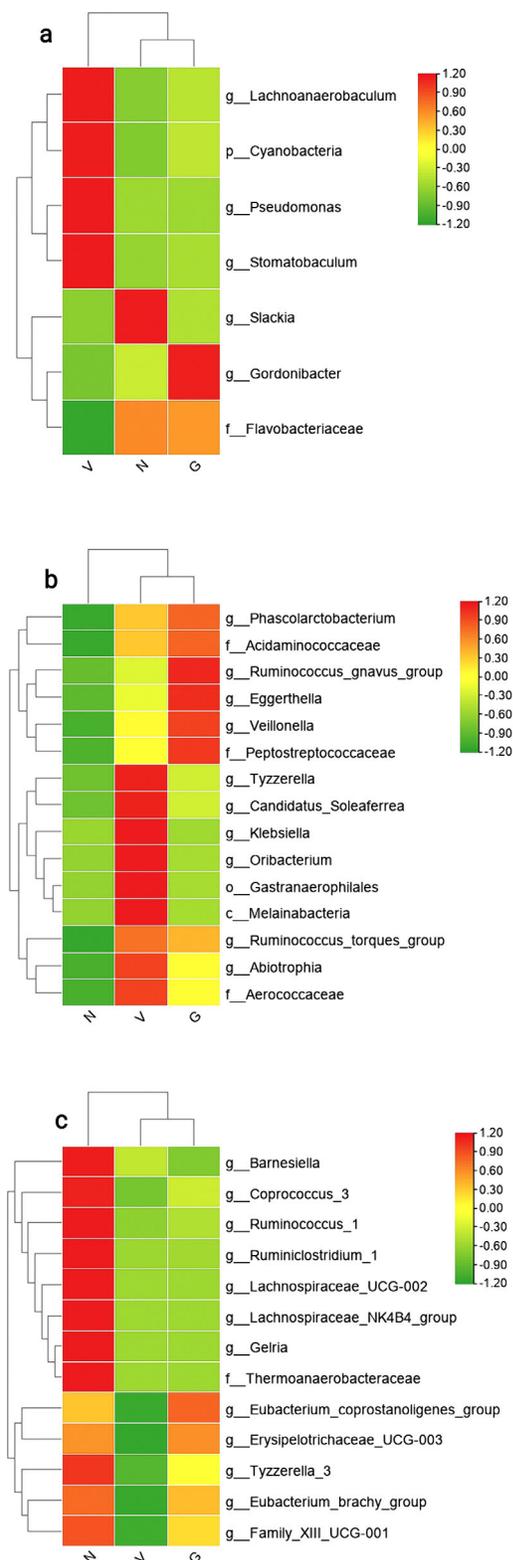
**Figure 2.** (a) The relative abundance of microbes related to VKH disease, specifically between 11 patients with VKH (V), 11 healthy controls (N), and 20 patients with noninfectious anterior scleritis (G). (b) The relative abundance of microbes that were enriched in VKH disease between 11 patients with VKH (V), 11 healthy controls (N), and 20 patients with noninfectious anterior scleritis (G). (c) The relative abundance of microbes that were depleted in VKH disease between 11 patients with VKH (V), 11 healthy controls (N), and 20 patients with noninfectious anterior scleritis (G).

microbes were significantly different between patients with VKH and healthy controls and between patients with VKH and noninfectious anterior scleritis. At the phylum level, Cyanobacteria was specifically related to VKH disease (all $P < 0.05$).

Type 2: Microbes related to immune-mediated disease. Some microbes were significantly different between patients and controls, but no significant difference was observed between patients with VKH and noninfectious anterior scleritis. We considered these microbes to be related to immune-mediated disease. *Tyzzerella*, *Ruminococcus gnavus* group, and *Eggerthella* at the genus level; Peptostreptococcaceae at the family level; Gastranaerophilales at the order level; and Melainabacteria at the class level were all related to immune-mediated diseases (all $P < 0.05$; Fig. 2b).

Type 3: Microbes might be related to immune-mediated disease. At the genus level, some microbes, such as *Oribacterium*, *Abiotrophia*, *Klebsiella*, *Ruminococcus torques* group, *Candidatus Soleaferrea*, *Veillonella*, and *Phascolarctobacterium* had significant differences between patients with VKH and healthy controls (all $P < 0.05$). Moreover, they were elevated in patients with noninfectious anterior scleritis, but the differences did not reach statistical significance between patients with noninfectious anterior scleritis and controls. The roles of these microbes need to be further studied using a larger sample size. At the family level, microbes might be related to immune-mediated diseases, including Aerococcaceae and Acidaminococcaceae (Fig. 2b).

## Depleted Microbes in VKH Disease

Using the same methods, we also obtained a specially depleted microbe in patients with VKH.

At the genus level, there were 14 depleted microbes in patients with VKH compared to healthy controls, along with 1 microbe at the family level and 1 microbe at the order level. We also divided them into three types according to whether they were specifically decreased in patients with VKH (Fig. 2c).

Type 1: As mentioned before, microbes specially related to VKH disease include *Gordonibacter* and *Slackia*. These microbes were significantly different between patients with VKH and healthy controls and between patients with VKH and noninfectious anterior scleritis. At the family level, Flavobacteriaceae was specifically related to VKH disease (all $P < 0.05$).

Type 2: Microbes related to immune-mediated disease, such as *Barnesiella*, *Lachnospiraceae* UCG-002, *Lachnospiraceae* NK4B4 group, and *Gelria*, were significantly different between patients with VKH and healthy controls as well as between patients with noninfectious anterior scleritis and healthy controls (all $P < 0.05$), but there were no significant differences between patients with noninfectious anterior scleritis and patients with VKH. At the family level, Thermoanaerobacteraceae was related to immune-mediated disease (Fig. 2c).

Type 3: Some microbes might be related to immune-mediated disease, such as *Ruminiclostridium* 1, *Eubacterium brachy* group, *Eubacterium coprostanoligenes* group, *Family XIII* UCG-001, *Ruminococcus* 1, *Coprococcus* 3, *Tyzzerella* 3, and *Erysipelotrichaceae* UCG-003. These microbes were significantly different between patients with VKH and healthy controls (all $P < 0.05$). They were also depleted in patients with noninfectious anterior scleritis, but the difference did not reach statistical significance
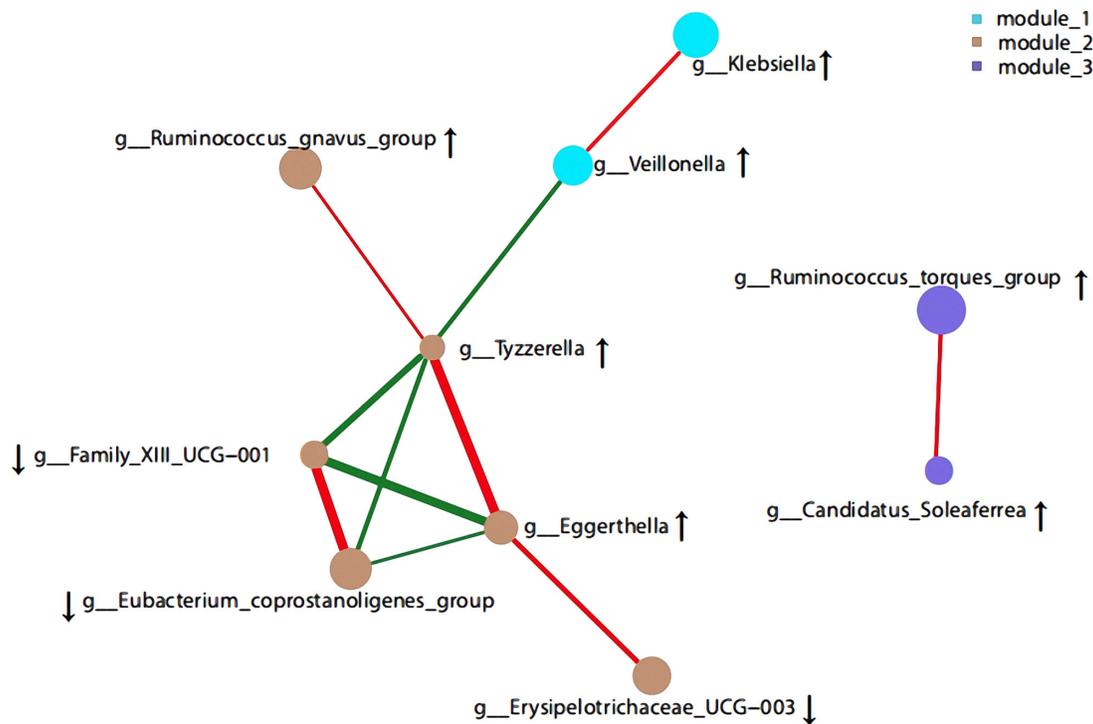
**FIGURE 3.** Bacterial interaction network in the gut microbes (the different microbes between patients with VKH and healthy controls) of patients with VKH (V) showing significant co-occurrence and coexclusion relationships at the genus level. Each node represents a genus, the node sizes in the network correspond to their content, and different colors of the nodes represent different modules. *Red* represents a positive correlation, and *green* represents a negative correlation. The darker the color is, the stronger the correlation.

between patients with noninfectious anterior scleritis and controls. The roles of these microbes in noninfectious anterior scleritis need to be studied using a larger sample size (Fig. 2c).

### Interaction of Intestinal Flora in Patients With VKH

We used Cytoscape and CoNet software to analyze the interactions of these changed microbes and found 3 modules, including 10 microbes that might have complex positive and negative interactions in patients with active VKH. Four major genera could be identified in the VKH network, including *Klebsiella* in module 1, *R. torques* group in module 2, and *E. coprostanoligenes* group and *Ruminococcus gnavus* group in module 3. For most microbes, the interactions between microbes might upregulate the pathogenic microbes and downregulate the protective microbes. However, *Tyzzerella* and *Eggerthella* were regulated by both positive and negative aspects. How they change depends on which side plays a stronger role. Five microbes interacted with *Tyzzerella*. Depleted *Family XIII* UCG-001 and *E. coprostanoligenes* group, as well as enriched *R. gnavus* group and *Eggerthella*, might enrich the content of *Tyzzerella*. However, *Veillonella* might cause the decrease of *Tyzzerella*. *Eggerthella* was affected by four microbes. Depleted *Family XIII* UCG-001 and *E. coprostanoligenes* group and enriched *Tyzzerella* might increase the content of *Eggerthella*. However, depletion of *Erysipelotrichaceae* UCG-003 might decrease the content of *Eggerthella*. In any case, our results showed that the expression levels of *Tyzzerella* and *Eggerthella* were

upregulated in patients with VKH. The hidden complex regulation mechanism needs further research (Fig. 3).

### DISCUSSION

In this study, we found five microbes related to VKH disease, including three enriched microbes (*Pseudomonas*, *Stomatobaculum*, *Lachnoanaerobaculum*) and two depleted microbes (*Slackia*, *Gordonibacter*). *Pseudomonas* was only enriched in the patients with VKH and did not change in patients with noninfectious anterior scleritis. *Stomatobaculum* and *Lachnoanaerobaculum* were enriched in patients with VKH and noninfectious anterior scleritis, and the contents of these two microbes were higher in patients with VKH. *Slackia* was not detected in the patients with VKH and was also decreased in patients with noninfectious anterior scleritis. The content of *Gordonibacter* decreased in patients with VKH but increased in patients with noninfectious anterior scleritis. In addition, there were 22 microbes related to VKH disease nonspecifically at the genus level.

The mechanisms of involvement of these microbes in the pathogenesis of VKH are not clear. We speculate that there are three possible mechanisms. In pathway 1, some microbes might disturb the balance between Th17/Tregs, which has been proven to be related to the pathogenesis of uveitis.[11] Fu et al.[12] found that dysbiosis of the gut microbe could break the balance of Th17/Tregs, contributing to VKH disease. Hironaka et al.[13] found that *Pseudomonas* might upregulate the expression of Th17 cells by increasing adenosine triphosphate.[12] Short-chain fatty acids (SCFAs) could promote Treg development and function by inducing expression of Foxp3, retinoic acids, or
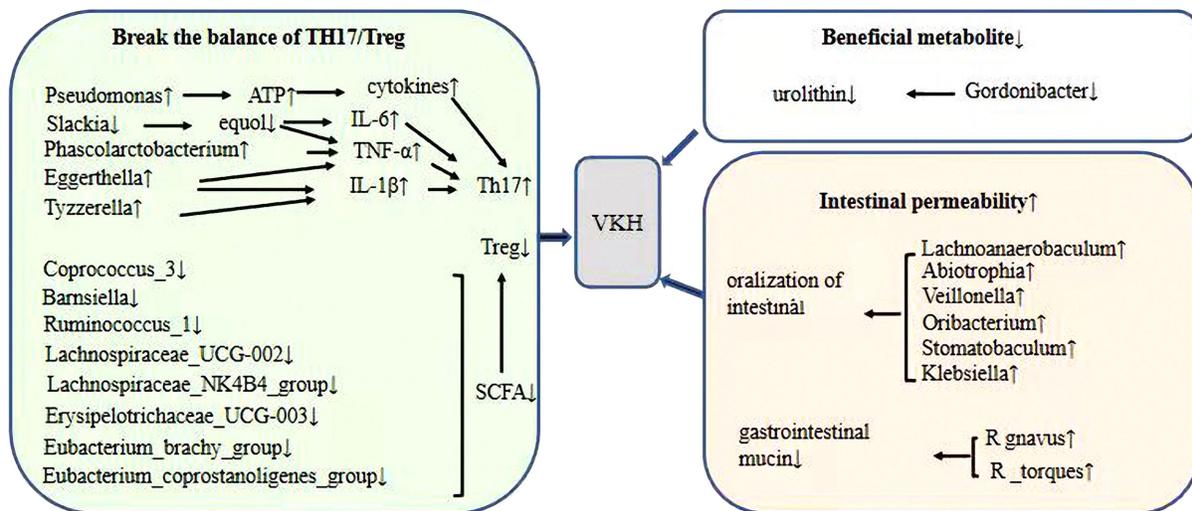
**Figure 4.** Diagram of the possible mechanism of intestinal microbes in VKH disease.

TGF-$\beta$1.[14] *Coprococcus* and *Barnesiella* were reported to be SCFA producers.[15] Decreases in *Coprococcus* 3 and *Barnesiella* might cause a decrease in SCFAs, which are related to the reduction in Tregs,[15] and butyrate is a kind of SCFA.[14] The decreased butyrate might result in the downregulation of Tregs. *Lachnospira, Ruminococcus, Erysipelotrichaceae, Eubacterium*, and *Ruminococcus* 1 were butyrate producers.[15,16] Our results showed that all these microbes were decreased. Therefore, we speculated that these decreased microbes cause VKH disease by downregulating Treg cells. The microbe also affects the balance of Th17 cells and Tregs by regulating the expression levels of cytokines. Depleted *Slackia* and enriched *Phascolarctobacterium, Eggerthella*, and *Tyzzerella* might increase TNF-$\alpha$, IL-1$\beta$, and IL-6 levels.[17–22]

In pathway 2, microbes might cause VKH by increasing intestinal permeability. In this case, microbiota and their products might spread out to the vascular system through the leaky gut and then translocate to the eye. If they are trapped or deposited in the uvea, they may become pathogens and trigger immune responses that cause or exacerbate uveitis. There was only indirect evidence for this. Horvath et al.[17] found that if the microbe (e.g., *Streptococcus*) commonly found in the oral cavity was detected in the intestine (oralization of the intestine), it might lead to increased intestinal permeability. *Stomatobaculum, Lachnoanaerobaculum, Oribacterium, Veillonella*, and *Abiotrophia* were microbes from the human oral cavity[18,19] and were enriched in patients with active VKH. Therefore, we speculate that these microorganisms might cause VKH disease by improving gut permeability. In addition, *R. gnavus* and *R. torques* could degrade human secretory mucin in monocultures.[20] It might be involved in the pathogenesis of VKH by degrading secretory mucin and increasing intestinal permeability.[21] Whether there is an antigen mimic between intestinal microbes and intraocular antigens is still unclear.

In pathway 3, microbes might also cause VKH disease by decreasing the production of beneficial metabolites ([Fig. 4](#)). Depleted *Gordonibacter* might cause VKH disease by downregulating beneficial metabolites. Opstelten et al.[22] detected the intestinal flora of current smokers and nonsmokers with Crohn disease. They found that *Gordonibacter* was a urolithin-producing bacterium that produced urolithins from the dietary polyphenols ellagic acid and ellagitannins. Urolithins are dibenzopyranone metabolites that exert anti-inflammatory activity in vivo. Depleted *Gordonibacter* might produce fewer urolithins, leading to a weakening of the anti-inflammatory response, which can ultimately cause VKH disease. However, the exact mechanism between *Gordonibacter* depletion and VKH disease still needs to be further studied. It was also reported that *Gordonibacter* was depleted in autoimmune diseases such as Crohn disease, which supported that the depletion of *Gordonibacter* might be related to autoimmune diseases.[23] It should be pointed out that intestinal bacteria do not exist independently. Their interaction and the environment determine the number and role of each microbe. Therefore, the onset of VKH is the result of the joint action of these microbes.

The limitation of our study is the small number of patients. In addition, all participants were Chinese people from Northeast China. The intestinal flora is affected by eating habits and the environment. Our results should be confirmed in other ethnic populations.

In conclusion, we found some microbes especially related to VKH disease by comparing patients with noninfectious anterior scleritis and healthy controls. We also found some microbes that were nonspecifically related to VKH. The mechanism of action of these gut microbes is still unclear. They may eventually lead to the onset of VKH through a complex regulatory network.

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