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Original Article

Potential correlation of oral flora with pemphigus vulgaris — A case control study

Bing-jie Li, Wen-xiu He, Hong Hua*, Pan Wei **

Department of Oral Medicine, Peking University School and Hospital of Stomatology & National Center for Stomatology & National Clinical Research Center for Oral Diseases & National Engineering Research Center of Oral Biomaterials and Digital Medical Devices, Beijing, PR China

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Abstract *Background/purpose:* Oral flora is related to various immune-related diseases. Herein we explored the characteristics of oral flora in patients with pemphigus vulgaris (PV) and analyzed the correlation between oral flora and PV.
Materials and methods: Twenty-two untreated patients with PV and 12 healthy controls (HC) were included in this case-control study. The characteristics of salivary microbiome were assessed by high-throughput sequencing using the 16S rRNA Illumina MiSeq approach, and differences between the PV and HC groups were determined. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was applied to screen key metabolic pathways and preliminarily explore potential mechanisms underlying PV occurrence and development.
Results: The abundance of oral flora in the PV group was significantly lower than that in the HC group, and there were characteristic changes. The relative abundance of *Prevotella* and *Agrobacterium* in the PV group was significantly higher than that in the HC group ($P < 0.05$) and that of *Neisseria*, *Lautropia*, and *Fusobacterium* was significantly lower ($P < 0.05$). There was a linear correlation between *Prevotella* and serum Dsg3 level in PV. KEGG pathway analyses indicated significant differences in nine metabolic pathways between the PV and HC groups ($P < 0.05$), namely carbohydrate metabolism, digestive system, neurodegenerative disease, glycan biosynthesis and metabolism, drug resistance: antimicrobial, infectious disease: viral, circulatory system, excretory system, and nervous system.
Conclusion: The oral flora of patients with PV presented characteristic changes, and several metabolic pathways were affected, including N-glycan biosynthesis and metabolism. *Prevotella* spp. appear to require the most attention in PV. We believe that oral flora dysbacteriosis contributes to PV occurrence and development.

* Corresponding author. Department of Oral Medicine, Peking University School and Hospital of Stomatology, No. 22, Zhongguancun South Avenue, Haidian District, Beijing, 100081, PR China.
** Corresponding author. Department of Oral Medicine, Peking University School and Hospital of Stomatology, No. 22, Zhongguancun South Avenue, Haidian District, Beijing, 100081, PR China.
E-mail addresses: honghua1968@aliyun.com (H. Hua), dent_wei@163.com (P. Wei).

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Introduction

Pemphigus is a rare autoimmune blistering disease, in which IgG autoantibodies inhibit the adhesion function of desmoglein (Dsg), which results in the loss of the cell adhesion ability of keratinocytes and induces the formation of blisters.[1,2] Pemphigus vulgaris (PV) and pemphigus foliaceus (PF) are the main subtypes of pemphigus,[3] responsible for approximately 65% and 90% of pemphigus diagnoses, respectively. PV is the most common type of pemphigus involving the oral mucosa.[4,5]

Bacterial flora is closely related to the occurrence and development of autoimmune diseases. Intestinal flora has been confirmed to be related to PV.[6,7] In 2019, Huang et al.[8] reported for the first time that patients with PV have gut microbial dysbiosis: the abundance of *Lachnospiracea_incertae_sedis* and *Coprococcus* was found to decrease, while that of *Granulicatella* and *Flavonifractor* was enriched in patients with PV. Furthermore, plasma levels of C5a, interleukin (IL)-2R, IL-6, IL-8, IL-7, IL-1β, IL-17A, IL-5, and IL-21 were significantly increased in patients with PV, with *Flavonifractor* exhibiting a positive correlation with C5a, IL-6, IL-8, IL-7, IL-1β, IL-17A, and IL-21. *Lachnospiracea_incertae_sedis* and *Coprococcus* showed a negative correlation with IL-17A.

The oral cavity, which has the second largest microbial community following gut, exhibits >700 bacterial species. Several studies have investigated the correlation between oral flora and oral diseases and immune disorders.[9,10] Moreover, oral flora is reportedly associated with various autoimmune diseases.[11–13] Deng et al. reported that the relative abundance of *Derxia*, *Haemophilus*, and *Pseudomonas* in the saliva of patients with oral lichen planus was lower than in that of healthy controls.[12] Zhang et al.[14] investigated salivary samples of patients with rheumatoid arthritis and found that the abundance of *Haemophilus* spp. was relatively low and that *Haemophilus* spp. negatively correlated with anticyclic citrullinated peptide antibodies; in contrast, the abundance of *Prevotella* spp. was relatively high and *Prevotella* spp. positively correlated with rheumatoid factor. Nevertheless, only a few studies have focused on oral flora in patients with PV.[10,15] For example, Scaglione et al.[16] analyzed the difference in oral microbiota between patients with PV and healthy controls at the phylum level, and Zorba et al.[17] analyzed the relationship between halitosis and bacterial flora in patients with PV.

To date, no studies have explored the correlation between oral flora and PV occurrence and development. Thus, in this study, we applied high-throughput 16S rRNA sequencing to characterize the oral flora of patients with PV and healthy controls. Our core objective was to explore the possible role of dominant bacteria in PV occurrence and development through pathway prediction.

Materials and methods

Ethical approval

This study was approved by the Biomedical Ethics Committee of Peking University School of Stomatology (Beijing, China) [PKUSSIRB-2021-02-62-21, approved on 2021.03.22]. Each subject agreed to participate in the study and provided written informed consent before the study commenced.

Participant recruitment

Thirty-four subjects [22 patients with PV and 12 healthy controls (HC; i.e., PV and HC groups, respectively)] who were referred to the Department of Oral Medicine, Peking University School and Hospital of Stomatology from June 2018 to June 2021 were enrolled in this case-control study.

Inclusion and exclusion criteria

Subjects who met the following criteria were included in this study: 1. aged between 18 and 65 years; 2. patients with PV diagnosed by clinical, histopathological, and immunological diagnoses according to the 2014 Japanese Dermatological Association guidelines for pemphigus management[18]; 3. able to collect saliva sample; 4. otherwise healthy patients; and 5. willing to provide signed informed consent (Fig. 1).

Subjects who met the following criteria were excluded: 1. pregnant patients; 2. patients with other known oral mucosal diseases; 3. patients with restricted activities, life-threatening systemic diseases, or other autoimmune diseases; 4. patients with a history of using immunomodulators or antibiotics within 1 month and those who had received treatment for pemphigus; 5. those who had used a mouthwash within 7 days; 6. tobacco consumers; and 7. patients with moderate to severe periodontitis, visible caries, or dentures.

Participant information

Basic information, such as gender and age, was collected for each patient from a patient filing system. The calculus index (CI), pemphigus disease area index (PDAI), and Dsg1 and Dsg3 indicators of patients were derived from previous medical records.

Laboratory methods

Sample collection, transportation, and storage

All participants were asked to avoid eating or drinking water within 2 h before oral sampling. Using standard

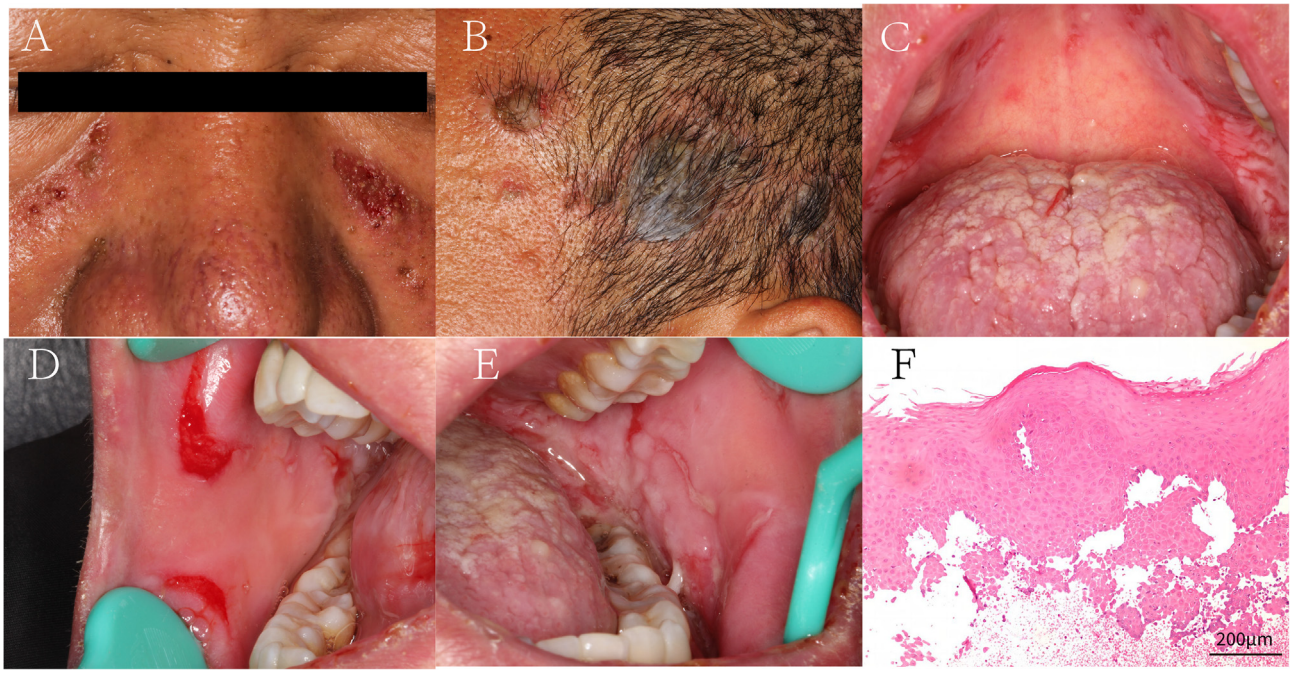

Figure 1 Patient with pemphigus vulgaris(PV). (A-E) clinical manifestations: bulls and erosions on the face and scalp, and erosions of the oral mucosa. (F) Histopathology showed acantholysis and intraepithelial blister formation.

techniques, 5 mL unstimulated complete saliva was collected from each subject in a sterile conical tube from 8 a.m. to 11 a.m. The tubes were then centrifuged at 10,000 rpm for 20 min, and the supernatant was collected and stored at −80 °C until needed. The samples were stored on dry ice during transportation.[19]

DNA extraction

Total genome DNA from samples was extracted using CTAB method. DNA concentration and purity was monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1 ng/μL using sterile water.

High-throughput 16S rRNA Illumina NovaSeq sequencing

To analyze microbial population, we amplified the V3—V4 region of the 16S rRNA gene. PCR was performed using the universal bacterial forward primer 338F (5′-ACTCCTACGG-GAGGCAGAG-3′) and reverse primer 806R (5′-GGAC-TACHVGGGTWTCTAAT-3′).

Amplicons were extracted from 2% agarose gels and purified using the Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany), according to manufacturer instructions. Sequencing libraries were generated using TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific, Waltham, MA, USA). Finally, the PCR products were sequenced and analyzed on an Novaseq 6000 platform (Illumina) according to the manufacturer's recommendations.

Bioinformatics and statistical methods

The analysis was conducted by following the "Atacama soil microbiome tutorial" of Qiime2docs along with customized program scripts (https://docs.qiime2.org/2019.1/). The original data FASTQ files were imported into a format that can be operated by the QIME2 system using qiime tools import program. Demultiplexed sequences from each sample were quality filtered and trimmed, de-noised, merged, and then the chimeric sequences were identified and removed using the QIIME2 dada2 plugin to obtain the feature table of amplicon sequence variant (ASV). The QIIME2 feature-classifier plugin was then used to align ASV sequences to a pre-trained GREENGENES 13_8 99% database (trimmed to the V3V4 region bound by the 338F/806R primer pair) to generate the taxonomy table. Any contaminating mitochondrial and chloroplast sequences were filtered using the QIIME2 feature-table plugin.

Further, to determine microbial richness and diversity, alpha diversity was assessed by measuring Chao 1 species richness and Shannon diversity index. Rarefaction curves were used to determine species richness based on the operational taxonomic units of each group and compared between the groups with MOTHUR. Besides, to determine microbial dissimilarity between the groups, beta diversity was assessed. We used principal coordinates analysis of UniFrac distances to evaluate beta diversity, and data were analyzed with Metastats. To detect bacterial component variation between the groups, we applied linear discriminant analysis effect size (LEfSe) was assessed. Finally, to determine functional changes, Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used.

Data were analyzed using SPSS v25.0 (IBM Corporation, Armonk, NY, USA). Graphs were created with OmicStudio (https://www.omicstudio.cn/tool/24), R (https://www.r-

Table 1 Baseline data. No statistical difference was found in gender, age, or CI between the PV and HC groups.

	PV group n = 22	HC group n = 12	*P*
Gender (M/F)	10/12	3/9	0.24
Age ± SD (year)	48.95 ± 11.86	43.00 ± 4.63	0.11
CI ± SD	1.41 ± 0.59	1.50 ± 0.52	0.66

Abbreviations: PV, pemphigus vulgaris. HC, healthy controls. M, male; F, female. CI, calculus index.

project.org/), and GraphPad Prism 9.0 (GraphPad Software Inc., La Jolla, CA, USA). Normality and homogeneity of variance were examined. Analysis of variance and *t*-test were used to compare age, gender, and CI; alpha diversity (Chao1 and Shannon indices) was analyzed using the Kruskal–Wallis test. LEfSe was assessed using the Kruskal–Wallis and Wilcoxon tests. Pearson's correlation coefficient was determined to analyze the correlation between certain bacteria and blood cells. $P < 0.05$ indicated statistical significance.

Results

Subject demographics

Th 34 subjects included in this study showed was no statistical difference in their age or gender (Table 1).

Overall characterization of microbiota structure

Using Illumina MiSeq sequencing, 3,540,166 merged sequences were obtained in total, with average of 104,123 sequences from each sample (minimum, 60,568; maximum, 117,627). These were clustered into 17,750 operational taxonomic units, representing 49 phyla, 130 classes, 212 orders, 282 families, 544 genera, and 324 species. Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria, and Actinobacteria represented 96.13% sequences.

Apparent variations in microbial communities

Chao 1 and Shannon indices indicated that oral flora abundance in the PV group was significantly lower than that in the HC group ($P < 0.05$) (Fig. 2A/B). Principal coordinates analysis revealed a clear separation between these groups, and microbial community difference between the groups was obvious (Fig. 3), indicating that patients with PV showed characteristic changes in their oral flora.

Changes in salivary microbiome

Metastats was applied to compare bacteria with relative abundance of >1% at the genus level between the groups. When LEfSe score was >3, the relative abundance of *Prevotella*, *Agrobacterium*, *Lactobacillus*, *Enhydrobacter*, *Moraxella*, *Serratia*, *Pseudomonas*, *Enterobacter*, *Coprococcus*, and *Candidatus* Koribacter in the PV group was significantly higher than that in the HC group ($P < 0.05$). On the other hand, the relative abundance of *Neisseria*, *Lautropia*, *Fusobacterium*, *Veillonella*, *Rotheia*, *Leptotrichia*, *Campylobacter*, *Actinomyces*, *Corynebacterium*, and *Cardiobacterium* showed a significant decrease ($P < 0.05$) (Fig. 4A/B).

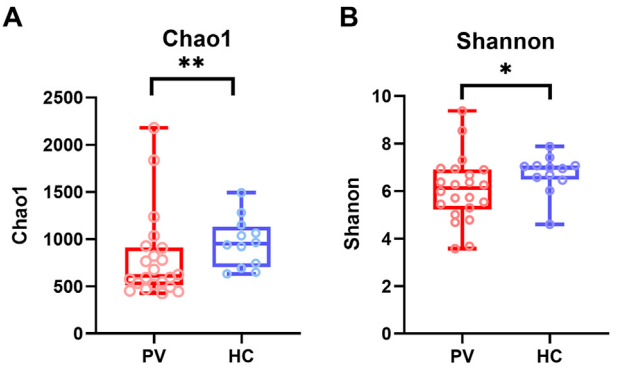

Figure 2 Differences of alpha diversity between the PV and HC groups. (A) The Chao1 of the alpha diversity index in the HC group was significantly higher than in the PV group (P< 0.01). (B) Shannon indice were significantly higher in the HC group than in the PV group (P< 0.05). PV: pemphigus vulgaris. HC: healthy controls. *: P < 0.05; **: P <0.01.

Redundancy analysis

Saliva of eight patients with pemphigus disease area index = 0 and treatment time >6 months was collected post-treatment and compared with that collected pretreatment. Redundancy analysis (RDA) was performed to analyze the relationship between Dsg1 and Dsg3 levels and bacterial abundance. In Fig. 4, arrow length represents the strength of the correlation between environmental variables and microbes: the longer the arrow length, the stronger the correlation. Further, in the plot, the perpendicular distance between the microbe and environmental variable axes reflects the correlations between them: the smaller the distance, the stronger the correlation. As evident (Fig. 4), *Prevotella* and Dsg3 showed the most significant correlation. *Leptotrichia*, *Rotheia*, and *Veillonella* showed higher negative correlation with Dsg3. *Neisseria* was the most significantly correlated with Dsg1; and *Haemophilus* was the most negatively correlated with Dsg1 (Fig. 5).

The Kyoto Encyclopedia of Genes and Genomes pathway analyses

KEGG pathway analysis was performed to identify putative functional pathways related to salivary microbiota. Overall, 401 metabolic pathways were identified, with nine showing significant differences between the PV and HC groups ($P < 0.05$): carbohydrate metabolism, digestive system, neurodegenerative disease, glycan biosynthesis and metabolism, drug resistance: antimicrobial, infectious disease: viral, circulatory system, excretory system, and nervous system (Fig. 6A). In particular, carbohydrate metabolism and glycan biosynthesis and metabolism were highly expressed in patients with PV. Further analyses revealed changes in few secondary metabolic pathways (Fig. 6B).

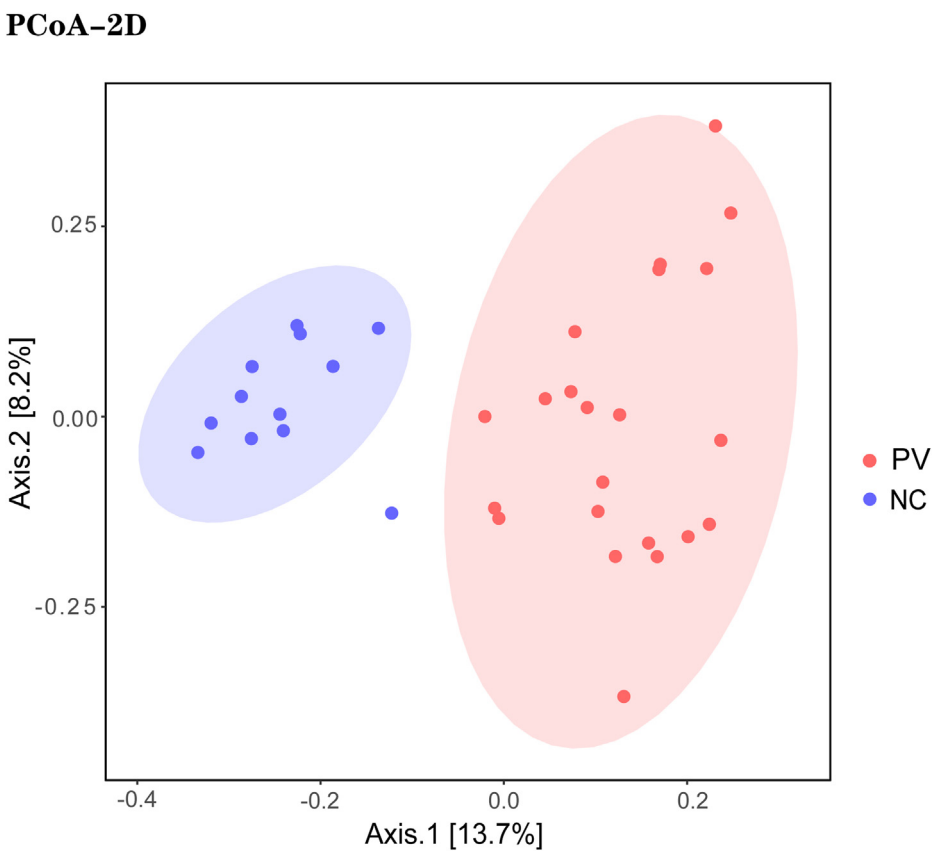

Figure 3 Principal coordinates analysis (PCoA) indicated the different community composition between the PV and HC groups. PV: pemphigus vulgaris. HC: healthy controls.

Discussion

A few previous studies have investigated the relationship between PV and intestinal microbiota. Huang et al.[8] reported for the first time that patients with PV experience gut microbial dysbiosis. They found that the abundance of *Lachnospiracea_incertae_sedis* and *Coprococcus* decreased, while that of *Granulicatella* and *Flavonifractor* was enriched in patients with PV. Morevoer, the plasma levels of C5a, IL-2R, IL-6, IL-8, IL-7, IL-1β, IL-17A, IL-5, and IL-21 showed a significant increase in patients with PV. *Flavonifractor* exhibited a positive correlation with C5a, IL-6, IL-8, IL-7, IL-1β, IL-17A, and IL-21, whereas *Lachnospiracea_incertae_sedis* and *Coprococcus* showed a negative correlation with IL-17A. Altogether, these data suggested that these organisms participate in the pathogenesis of PV by regulating T-cell differentiation and pertinent cytokines.

PV-based studies continue to focus on oral flora comparison.[10,15] Scaglione et al.,[16] for example, only analyzed differences in oral flora between patients with PV and HC at the phylum level. Further, Zorba et al.[17] investigated the role of oral microbiome in PV by applying deep sequencing of the bacterial 16S rRNA gene to oral smear samples and found *Fusobacterium nucleatum*, *Gemella haemolysans*, and *Parvimonas micra* to be statistically abundant in patients.

Herein alpha and beta diversity analyses indicated statistically significant differences in flora between the PV and HC groups, suggesting that the richness and diversity of oral flora in patients with PV were significantly different from that in HC. This finding demonstrated that the balance of oral flora in patients with PV had been destroyed. Considering the well-recognized roles of microbiome in systemic immunity, perturbation of healthy microbial community, i.e., dysbiosis, has been associated with the pathophysiology of numerous autoimmune diseases, including type 1 diabetes, multiple sclerosis, and rheumatoid arthritis.[20,21] As the second largest habitat in humans where microbiota can colonize, the oral cavity is also where lesions manifest in >90% patients with PV. We believe that changes in oral microbiota are related to factors that aggravate PV.

The relative abundance of *Prevotella*, *Agrobacterium*, *Lactobacillus*, *Enhydrobacter*, *Moraxella*, *Serratia*, *Pseudomonas*, *Enterobacter*, *Coprococcus*, and *Candidatus* Koribacter was higher in the PV group and that of *Neisseria*, *Lautropia*, *Fusobacterium*, *Veillonella*, *Rotheia*, *Leptotrichia*, *Campylobacter*, *Actinomyces*, *Corynebacterium*, *and Cardiobacterium* was lower. Periodontitis is associated with *Prevotella*, *Alloprevotella*, *Porphyromonas*, *Actinomyces*, *Fusobacterium*, *Haemophilus*, and *Aggregatibacter*, all of which comprise opportunistic pathogens.[22] Patients with PV showed increased abundance of *Prevotella* and decreased abundance of *Actinomyces*, *Rotheia*, and *Fusobacterium*, with the remaining flora showing no statistically significant differences. Besides, CI showed no significant differences between the groups. It can thus be considered that periodontitis did not contribute to the differences observed between the groups. *Lactobacillus*[23] provides a range of health benefits; for example, they promote the

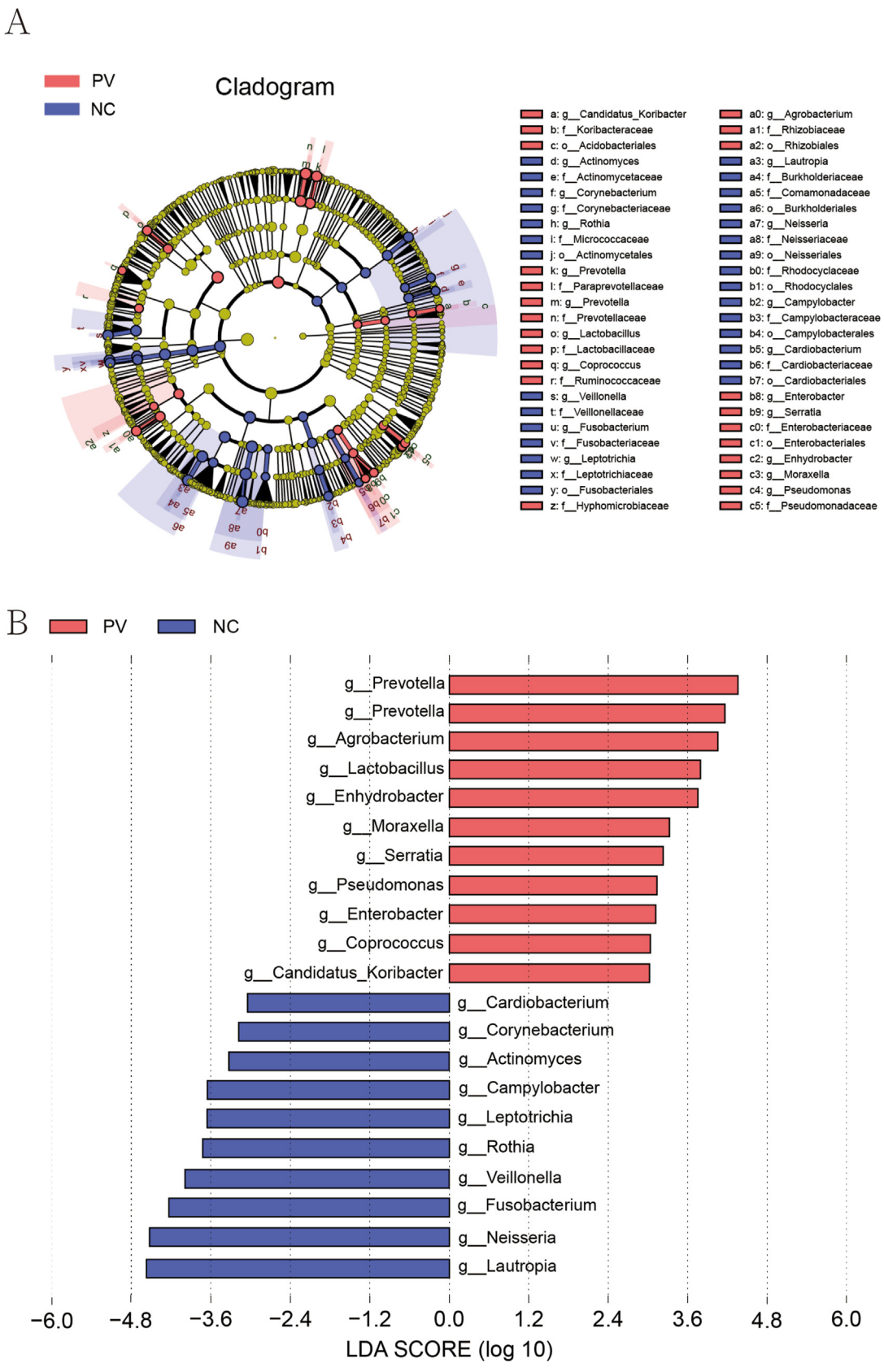

Figure 4 LDA Efect Size (LEfSe) analysis indicated differences between the PV and HC groups at the genus level. Cladogram (A) and the distinct bacterial species between the PV and HC groups, with a LAD score>2 (B). PV: pemphigus vulgaris. HC: healthy controls.

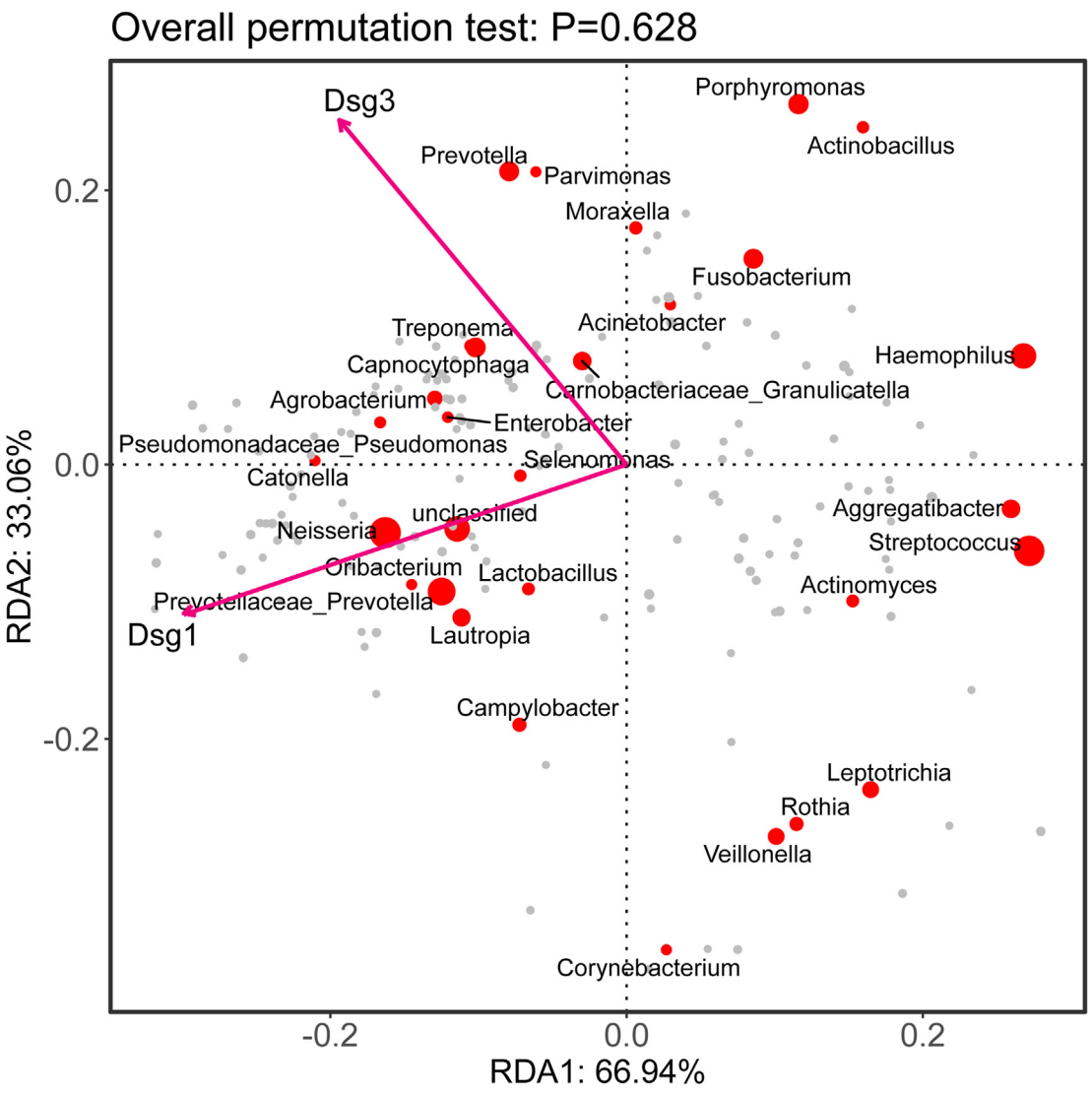

Figure 5 RDA. *Prevotella* showed the most significant correlation with Dsg3, while *Leptotrichia*, *Rotheia*, and *Veillonella* showed higher negative correlation with Dsg3. *Neisseria* showed the most significant correlation with Dsg1.

growth of oral and intestinal flora and are known to restore normal flora; moreover, they seem to be correlated to self-regulation of oral flora. *Moraxella*, *Serratia*, *Pseudomonas*, *Enterobacter*, and *Coprococcus* are associated with respiratory tract and digestive tract infections,[24–27] in addition to oral mucosal wounds and local infection. Similar to our findings, Zorba et al.[17] detected *Prevotella* and *Neisseria* in patients with oral PV lesions. *Fusobacterium* is highly prevalent in the oral cavity and plays a key role in adhesion. *Fusobacterium nucleatum* is regarded to be a "bridge organism" in the development of dental biofilms. The inconsistency of *Fusobacterium* between the results of this study and those reported by Zorba et al.[17] may be related to different sampling sites.

To our knowledge, herein we report for the first time that fructose and mannose metabolism and N-glycan biosynthesis and metabolism were markedly enriched in patients with PV, as predicted by KEGG pathway analyses. Several oligosaccharides (glycans) covalently attach to a protein to alter protein stability and activity; these constructs are known as glycoproteins.[28] According to proteomic studies, an increased abundance of high mannose-type oligosaccharides is characteristic of murine epidermis glycoproteins, including desmosomes (e.g. desmocollin 1, desmocollin 3, and DSG).[29] These findings indicate that oral flora can impact the function of desmosomes in patients with PV by affecting the N-glycosylation of epidermal and eudermal proteins.

Dsg3, the most important factor in PV, is related to disease severity. This is the first study to explore the correlation between oral flora and PV-related factors. According to studies based on RDA correlation, *Prevotella* shows the most significant correlation with Dsg3, while *Leptotrichia*, *Rotheia*, and *Veillonella* show higher negative correlation with Dsg3. Therefore, *Prevotella* spp. seem to require the most attention in patients with PV.

Prevotella is prevalent in periodontitis.[30] Gursoy et al. investigated human neutrophil defensins and their effect on epithelial cell monolayers and found that high concentrations of human neutrophil defensins increased *Prevotella*

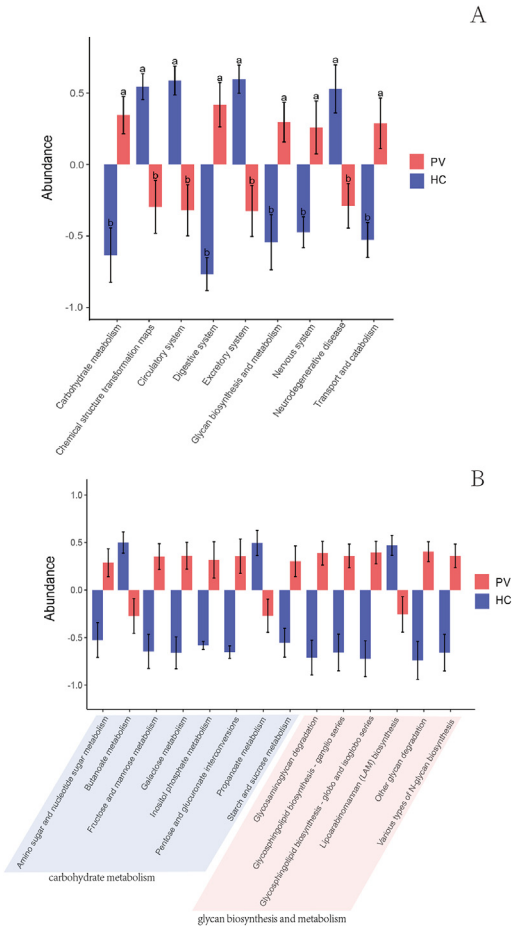

Figure 6 KEGG pathway analyses. A. Nine metabolic pathways showed significant differences between the groups: carbohydrate metabolism, chemical structure transformation maps, circulatory system, digestive system, excretory system, glycan biosynthesis and metabolism, nervous system, neurodegenerative disease, transport and catabolism ($P < 0.05$). B. Further analysis of carbohydrate metabolism and glycan biosynthesis and metabolism indicated changes in some secondary metabolic pathways. KEGG: Kyoto Encyclopedia of Genes and Genomes; PV: pemphigus vulgaris; HC: healthy controls.

colonization, and also affected the intercellular junctions of epithelial cells and induced the formation of periodontal pockets.[31] Herein *Prevotella* showed the highest correlation with Dsg3. *Prevotella* perhaps also affects desmosome junction in patients with pemphigus, but further studies are needed to elucidate the precise mechanism.

Some *Prevotella* strains are clinically important pathobionts that participate in various human diseases by promoting chronic inflammation.[32] IgA or IgG antibodies to *Prevotella* are present in the sera of patients with rheumatoid arthritis.[33] Further, oral and intestinal flora of patients with rheumatoid arthritis show increased abundance of *Prevotella*, which has the ability to promote local and systemic Th17 differentiation.[34] *Prevotella* is also elevated in bacterial vaginitis and is positively correlated with IL-23, IL-17, IL-12, and IFN-γ.[35] *In vitro* experiments have confirmed that *Prevotella* can induce epithelial cells to express higher levels of IL-1α, IL-1β, IL-6, IL-8, CCL20, CXCL2, CXCL3, and CCL5,[35–37] indicating that *Prevotella* has a high intrinsic ability to promote local IL-17-mediated immune responses via epithelial cells. Although the pathogenesis of PV is closely related to B cells, T-cell subsets also play an indispensable role.[38] Patients with PV evidently show significantly higher frequency of Th17 cells in peripheral blood mononuclear cells than HC, particularly during the acute onset and active chronic stages.[39] Yuan et al. found that T lymphocytes infiltrating PV lesions were mostly $CD4^+$ T cells expressing IL-21 and IL-17A, not typical T follicular helper cells expressing CXCR5. In addition, IL-21^+/IL-17^+ $CD4^+$ T cells might provide helper function for B-cell activation and differentiation, leading to pathogenic autoantibody production in PV lesions.[40] *Prevotella* may promote local inflammatory responses, leading to changes in local T-cell subsets, consequently affecting PV; however, further studies are warranted to validate this hypothesis.

To summarize, our preliminary results indicated that patients with PV have significantly different microbial diversity and composition than HC. These differences are related to disease condition, and the possible underlying mechanism involves a local inflammatory reaction or effects on desmosomes. This study has some limitations that the sample size is small, and more samples are needed to verify our results. Further studies are warranted to confirm whether changes in oral flora affect desmosome function.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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