# *Helicobacter Pylori* Infection Increases the Incidence of Erosive Oral Lichen Planus and Alters the Oral Microbiome Composition

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# Abstract

**Background** Oral lichen planus (OLP), a common clinical oral disease, is associated with an increased risk for malignant transformation. The mechanism underlying the pathogenesis of OLP is unknown. Oral dysbacteriosis is reported to be one of the etiological factors for OLP. Although *H. pylori* infection is associated with various oral diseases, the correlation between *H. pylori* infection and OLP is unclear. This study aimed to investigate the effect of *H. pylori* infection on OLP pathogenesis and oral microbiome composition in the Chinese population, which has a high incidence of *H. pylori* infection.

**Result** In this study, the saliva samples of 30 patients with OLP (OLP group) and 21 negative controls (NC group) were collected. *H. pylori* infection was detected using the carbon-14 urea breath test. The saliva samples were divided into the following four groups based on the *H. pylori* infection status: *H. pylori*-positive OLP (OLP+), *H. pylori*-positive NC (NC+), *H. pylori*-negative OLP (OLP−), and *H. pylori*-negative NC (NC−) groups. The oral microbiome composition was significantly different between the OLP and NC groups and between the OLP− and OLP+ groups. Compared with those in the OLP− group, the incidence of erosive OLP and the salivary levels of cytokines were higher in the OLP+ group. In contrast, the oral microbiome composition and cytokine levels were not significantly different between the NC− and NC+ groups.

**Conclusions** This is the first report demonstrated that *H. pylori* infection is significantly correlated with the pathogenesis of erosive OLP. The alleviation of *H. pylori* infection may decrease the incidence of erosive OLP.

# Background

The infection from *Helicobacter pylori*, which is classified as a primary carcinogen by the World Health Organization (WHO), can lead to the development of chronic gastritis, gastric ulcer, and gastric cancer[1, 2]. The human oral cavity is the temporary storage site for *H. pylori* and an extragastric *H. pylori* reservoir[3]. *H. pylori* infection was detected in the dental plaques and saliva using various molecular techniques[4, 5]. Additionally, *H. pylori* infection is associated with oral diseases, such as periodontitis and recurrent oral ulcer[5, 6].

The prevalence of oral lichen planus (OLP), a common chronic inflammatory oral mucosal disease, is 0.5–2.2% in general adult population[7]. The two main subtypes of OLP are reticular OLP and erosive OLP. The reticular type OLP is characterized by protruding white lacy lesions or papules[7, 8], while the erosive type OLP is characterized by erythema, erosion, or ulcerative lesions[7, 8]. OLP can cause different degrees of discomfort, including prolonged burning sensation, bleeding, tingling, and abnormal taste. Previous studies have demonstrated that OLP, which is defined as a precancerous condition by the WHO, is associated with an increased risk for malignant transformation[9].

Various factors, such as immunological, psychological, infectious, and genetic factors are reported to be involved in the pathogenesis of OLP[8, 10, 11]. Recent studies have demonstrated that oral microbiota is

involved in the development of oral diseases[12]. The oral microbiota of patients with OLP has increased microbial diversity, decreased *Streptococcus* abundance, and increased *Bacteroides* abundance[13–15]. Oral dysbacteriosis is reported to be one of the etiological factors of OLP[16].

The correlation between *H. pylori* infection and OLP has not been elucidated. *H. pylori* infection has been detected in the periodontal pocket of patients with OLP. Additionally, the presence of *H. pylori* in the oral cavity is associated with leukoplakia and OLP oral lesions[6]. One study reported the possible connection between *H. pylori* and erosive OLP[17]. However, recent studies have reported that *H. pylori* infection was not detected in the mucosal biopsies of patients with OLP, which indicated the lack of correlation between OLP and *H. pylori* infection[18].

Hence, the studies evaluating the correlation between OLP and *H. pylori* have reported contradictory findings. Additionally, most of these studies involved the Western population. In the Chinese population, the average *H. pylori* infection rate is high (more than 50%)[19]. However, the correlation between *H. pylori* and OLP has not been examined in the Chinese population.

In this study, the saliva samples collected from normal control volunteers and patients with OLP were grouped based on the *H. pylori* infection status. The clinical subtypes of OLP in *H. pylori*-positive and *H. pylori*-negative patients were determined. The findings of this study indicated that *H. pylori* infection increases the incidence of erosive OLP. However, the underlying mechanism was not elucidated. So, the oral microbiome composition of OLP patients with and without *H. pylori* infection was examined to evaluate the effect of *H. pylori* infection on the oral microbiome composition.

# Results

### Patients with OLP exhibit enhanced prevalence of *H. pylori* infection

The saliva samples of 30 patients with OLP (OLP group) and 21 normal control volunteers (NC group) were collected to analyze the correlation between *H. pylori* infection status and OLP clinical subtypes. Age and gender were not significantly different between the OLP and NC groups (Table 1).

*H. pylori* infection in the OLP and NC groups was detected using the urease breath test. The prevalence of *H. pylori* infection in the OLP group (70%) was markedly higher than that in the NC group (50%)(Table 1).

Erosive OLP was characterized by a red erosive mucosal surface (Figure S1 in Additional file). The lesion severity and the risk of malignant transformation in erosive OLP are higher than those in reticular OLP[9]. In the OLP group, the incidence of erosive OLP in *H. pylori*-positive patients (61.9%) was significantly (Chi-square test) higher than that in *H. pylori*-negative patients (11.1%) ($p$=0.0041). Additionally, *H. pylori* infection was correlated with the OLP subtypes (Table 2).

### Patients with OLP exhibit altered oral microbiome composition

Previous studies have demonstrated that the oral microbiome composition in patients with OLP was different from that in normal control individuals[20–22]. This study demonstrated that the α diversity of oral microbiota in the OLP group was significantly higher than that in the NC group (Fig.1A and B). Principal coordinate analysis (PCoA) revealed that the β diversity of oral microbiota in the OLP group was significantly different from that in the NC group (Fig. 1C).

The oral microbiome composition was significantly different between the OLP and NC groups at the phylum (Fig. 1D) and genus levels (Fig. 1E). The dominant phyla in the oral microbiota were *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* (Fig. 1D). Compared with the NC group, the OLP group exhibited a significantly decreased abundance of *Firmicutes* and a significantly increased abundance of *Bacteroidetes* (Fig. 1D). At the genus level, compared with the NC group, the OLP group exhibited a decreased abundance of *Streptococcus* and increased abundances of *Neisseria*, *Prevotella*, and *Prevotella7* (Fig. 1E).

The bacterial genera with an average relative abundance higher than 1% in the oral microbiome of the OLP and NC groups are listed in Fig. 2A. The volcano plot was used to represent the differences in the bacterial composition at the genus level between the OLP and NC groups (Fig. 2B). Bacteria with relative abundance greater than 1% and significant differences in relative abundance were screened out (Fig. 2B). Compared with the NC group, the OLP group exhibited decreased relative abundances of *Streptococcus* and *Rothia* and increased relative abundances of *Alloprevotella*, *Prevotella*, *Fusobacterium*, and *Porphyromonas* (Fig. 2B and C).

### *H. pylori* infection alters the salivary microbiome composition in patients with OLP.

To analyze the effect of *H. pylori* infection on the salivary microbiome composition of the OLP and NC groups, the saliva samples were divided into the following four groups based on the *H. pylori* infection status: OLP+ (n=21); OLP− (n=9); NC+ (n=10); NC− (n=11).

The α diversity of salivary microbiota in the OLP+ group was significantly higher than that in the OLP− group (Fig. 3A, B, C, D and E). In contrast, α diversity was not significantly different between the NC+ and NC− groups (Fig. 3A, B, C, D, and E). PCoA revealed that the β diversity of salivary microbiota was significantly different between the OLP+ and OLP− groups. However, the β diversity of salivary microbiota was not significantly different between the NC+ and NC− groups (Fig. 3F and G). Additionally, the bacterial composition at the genus and phylum levels was not significantly different between the NC+ and the NC− groups (Figure S2 in Additional file).

The salivary microbiome composition was significantly different between the OLP+ and OLP− groups at the phylum and genus levels (Fig. 4A and B). The predominant bacterial phyla were *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* (Fig. 4A), the relative abundance of *Bacteroidetes* was significantly high in the OLP+ group. At the genus level, the relative abundance of *Alloprevotella* in the OLP+ group was significantly higher than that in the OLP− group (Fig. 4B).

The bacteria in the salivary microbiota of the OLP+ and OLP− groups with an average relative abundance higher than 1% are listed in Fig. 4C. The volcano plot was constructed to determine the differences in the bacterial composition between the OLP+ and the OLP− groups at the genus level (Fig. 4D). The bacteria with relative abundance higher than 1% and significant differences in the relative abundance were screened out (Fig. 4D). Compared with the OLP− group, the OLP+ group exhibited significantly increased relative abundances of *Alloprevotella* and *Haemophilus* and a significantly decreased relative abundance of *Actinomyces* (Fig. 4D and E).

## Comparative analysis of salivary inflammatory factors

*H. pylori* infection can induce the gastric mucosa to secrete inflammatory factors, such as IL-6, IL-8, and IL-17[23]. Previous studies have reported the dysregulated expression of various inflammatory factors, such as IL-6, IL-8, IL-17, and TNF-α in patients with OLP[24]. In this study, the salivary levels of IL-6, IL-8, and IL-17 in the OLP and NC groups were analyzed using ELISA. The salivary levels of IL-6, IL-8, and IL-17 in the OLP group were significantly higher than those in the NC group (Fig. 5A).

Next, the effect of *H. pylori* infection on the salivary levels of inflammatory factors in the OLP and NC groups was evaluated. Additionally, the salivary levels of IL-6, IL-8, and IL-17 were comparatively analyzed between the following groups: OLP+ and OLP− groups; NC+ and NC− groups. Compared with those in the OLP− group, the salivary levels of IL-6, IL-8, and IL-17 were significantly upregulated in the OLP+ group (Fig. 5B). However, the salivary levels of IL-6, IL-8, and IL-17 were not significantly different between the NC+ and NC− groups (Fig. 5C).

Next, the correlation between key bacterial genera and inflammatory factors (IL-6, IL-8, and IL-17) was analyzed by constructing the heat map of Spearman's rank correlation coefficients (Fig. 6A and B). In the OLP and NC groups, the abundances of *Alloprevotella*, *Porphyromonas*, *Fusobacterium*, and *Prevotella* genera were positively correlated with IL-6 and IL-17, while the abundances of *Prevotella* and *Fusobacterium* genera were positively correlated with IL-8. Furthermore, the abundances of *Streptococcus* and *Rothia* genera were negatively correlated with IL-7, IL-6, and IL-8 (Fig. 6A). In the OLP+ and OLP− groups, the abundances of *Alloprevotella* and *Haemophilus* genera were significantly and positively correlated with IL-17, while those of *Actinomyces* genus were negatively correlated with IL-7, IL-6, and IL-8 (Fig. 6B).

## Correlation of salivary microbiome function with key bacterial genera

PICRUSt was used to predict the metagenome functional content based on 16S rRNA gene sequencing and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (Fig. 7). Compared with those in the NC group, the expression levels of genes involved in various metabolic pathways, such as histidine metabolism, phenylalanine metabolism, novobiocin biosynthesis, lipopolysaccharide (LPS) biosynthesis, LPS biosynthesis proteins, biotin metabolism, ubiquinone, and other terpenoid-quinone biosynthesis were upregulated, while those of genes involved in galactose metabolism, phosphotransferase system, and protein kinase were downregulated in the OLP group (Fig. 7A and B).

Furthermore, the levels of LPS biosynthesis proteins and LPS biosynthesis in the OLP+ group were upregulated when compared with those in the OLP– group (Fig. 7C and D).

LPS can induce inflammatory reaction[25]. PICRUSt analysis predicted that the microbial metabolic pathways involved in the pathogenesis of OLP are LPS biosynthesis proteins and LPS biosynthesis. The correlation between key bacterial genera and these two metabolic pathways was examined by constructing the heat map of Spearman's rank correlation coefficients (Fig. 8A and B). In the OLP and NC groups, the relative abundances of *Alloprevotella*, *Porphyromonas*, *Fusobacterium*, and *Prevotella* genera were positively correlated, while those of *Rothia* were negatively correlated with the levels of LPS biosynthesis proteins and LPS biosynthesis (Fig. 8A). In the OLP+ and OLP– groups, the abundances of *Alloprevotella* and *Haemophilus* genera were positively correlated, while those of *Actinomyces* were negatively correlated with the levels of LPS biosynthesis proteins and LPS biosynthesis (Fig. 8B).

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The oral microbiome composition was significantly different between the OLP and NC groups at the phylum (Fig. 1D) and genus levels (Fig. 1E). The dominant phyla in the oral microbiota were *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* (Fig. 1D). Compared with the NC group, the OLP group exhibited a significantly decreased abundance of *Firmicutes* and a significantly increased abundance of *Bacteroidetes* (Fig. 1D). At the genus level, compared with the NC group, the OLP group exhibited a decreased abundance of *Streptococcus* and increased abundances of *Neisseria*, *Prevotella*, and *Prevotella7* (Fig. 1E).

The bacterial genera with an average relative abundance higher than 1% in the oral microbiome of the OLP and NC groups are listed in Fig. 2A. The volcano plot was used to represent the differences in the bacterial composition at the genus level between the OLP and NC groups (Fig. 2B). Bacteria with relative abundance greater than 1% and significant differences in relative abundance were screened out (Fig. 2B). Compared with the NC group, the OLP group exhibited decreased relative abundances of *Streptococcus* and *Rothia* and increased relative abundances of *Alloprevotella*, *Prevotella*, *Fusobacterium*, and *Porphyromonas* (Fig. 2B and C).

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The salivary microbiome composition was significantly different between the OLP+ and OLP− groups at the phylum and genus levels (Fig. 4A and B). The predominant bacterial phyla were *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* (Fig. 4A), the relative abundance of *Bacteroidetes* was significantly high in the OLP+ group. At the genus level, the relative abundance of *Alloprevotella* in the OLP+ group was significantly higher than that in the OLP− group (Fig. 4B).

The bacteria in the salivary microbiota of the OLP+ and OLP− groups with an average relative abundance higher than 1% are listed in Fig. 4C. The volcano plot was constructed to determine the differences in the bacterial composition between the OLP+ and the OLP− groups at the genus level (Fig. 4D). The bacteria with relative abundance higher than 1% and significant differences in the relative abundance were screened out (Fig. 4D). Compared with the OLP− group, the OLP+ group exhibited significantly increased relative abundances of *Alloprevotella* and *Haemophilus* and a significantly decreased relative abundance of *Actinomyces* (Fig. 4D and E).

## Comparative analysis of salivary inflammatory factors

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and IL-17 in the OLP and NC groups were analyzed using ELISA. The salivary levels of IL-6, IL-8, and IL-17 in the OLP group were significantly higher than those in the NC group (Fig. 5A).

Next, the effect of *H. pylori* infection on the salivary levels of inflammatory factors in the OLP and NC groups was evaluated. Additionally, the salivary levels of IL-6, IL-8, and IL-17 were comparatively analyzed between the following groups: OLP+ and OLP− groups; NC+ and NC− groups. Compared with those in the OLP− group, the salivary levels of IL-6, IL-8, and IL-17 were significantly upregulated in the OLP+ group (Fig. 5B). However, the salivary levels of IL-6, IL-8, and IL-17 were not significantly different between the NC+ and NC− groups (Fig. 5C).

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# Discussion

The pathogenesis of OLP, a common clinical oral mucosal disease, is unknown[7, 8]. This study aimed to analyze the effect of *H. pylori* infection on the pathogenesis of OLP. The analysis of clinical data indicated that *H. pylori* infection increased the incidence of erosive OLP. Patients with OLP exhibited distinct salivary microbiome composition and enhanced salivary levels of inflammatory factors. *H. pylori* infection altered the composition and function of salivary microbiota and increased the salivary levels of inflammatory factors in patients with OLP.

Recent studies have demonstrated that the oral microbiome composition in patients with OLP is different from that in normal control individuals[20–22]. Consistent with the findings of previous studies, this study demonstrated that the salivary microbiome composition in patients with OLP was significantly different from that in normal control individuals. Compared with that in normal controls, the diversity of salivary microbiota was significantly higher in patients with OLP, which was consistent with the results of Kun *et al*[21].

The salivary microbiome composition at the genus level was comparatively evaluated between the OLP and NC groups. The relative abundance of several bacteria in the salivary microbiota was significantly different in patients with OLP. *Streptococcus* spp., which is one of the first microbes to colonize the oral cavity, determines the composition of oral microflora[26]. In the oral cavity, *Streptococcus* produces molecules that inhibit the growth of pathogens[27]. In this study, patients with OLP exhibited a significantly decreased relative abundance of *Streptococcus* in the salivary microbiota, which can result in the impairment of the growth-inhibitory effects of *Streptococcus* against oral pathogens. *Porphyromonas* spp. and *Fusobacterium* spp., which are the causative agents of periodontitis, modulate the T-cell-mediated immune responses[12, 28]. Recent studies have suggested that *Porphyromonas* spp. *and Fusobacterium* spp. infections can cause oral cancer[29, 30]. The relative abundances of *Porphyromonas* spp. *and Fusobacterium* spp. in the salivary microbiota of patients with OLP were significantly high, which suggested that these microbes are involved in the pathogenesis of OLP. *Prevotella* spp., a member of the oral microbiota, is abundant in patients with periodontal disease and periodontal abscesses[31]. Wang et al[21] and Du et al[32] reported that the relative abundance of *Prevotella* increased in the salivary microbiota of patients with OLP, which was consistent with the findings of this study. This indicated that *Prevotella* is an important pathogenic microorganism involved in the development of OLP. Additionally, the relative abundance of *Alloprevotella* increased in the salivary microbiota of patients with OLP. *Alloprevotella* increases the levels of $H_2S$ in oral microenvironment[33]. $H_2S$ is an inflammatory mediator that can promote the expression of pro-inflammatory factors, including IL-6, in the immune cells of humans and mice[34].

In OLP, various exogenous factors are reported to trigger the T lymphocyte-mediated inflammatory immune responses[8]. Previous studies have indicated that the secretion of inflammatory cytokines may play an important role in the OLP-associated immune dysregulation[24]. IL-6 promotes the proliferation of keratinocytes, which promotes epithelial hyperplasia in OLP[35, 36]. The enhanced production of IL-8 may

impair the tissue repair mechanism of the keratinocytes[37]. Recent studies have demonstrated that the expression of IL-17 was significantly upregulated in the mucosal tissue and peripheral blood of patients with OLP[38–40], which suggested that IL-17 mediates tissue damage in OLP lesions. IL-17 secreted by the keratinocytes at the lesion site of OLP may play a role in recruiting inflammatory cells to the lesion site[40].

In this study, the relative abundances of gram-negative bacteria, such as *Porphyromonas*, *Fusobacterium*, *Prevotella*, and *Alloprevotella*, were positively correlated with inflammatory cytokines, such as IL-6, IL-8, and IL-17. This indicated that these four bacterial genera mediate the pathogenesis of OLP by promoting inflammation.

The metabolic pathways of salivary microorganisms associated with LPS synthesis were significantly upregulated in patients with OLP and were positively correlated with the relative abundances of *Porphyromonas*, *Fusobacterium*, *Prevotella*, and *Alloprevotella*. LPS, which exerts immune-stimulatory and pro-inflammatory effects, can promote local inflammatory responses in tissues and upregulate the expression of inflammatory factors[25]. In the epithelial cells, LPS binds to TLR4 to promote the secretion of pro-inflammatory cytokines and chemokines, such as IL-6 and IL-8 through the JNK signaling pathway[41]. Additionally, LPS can stimulate the secretion of Th17-related cytokines, such as IL-17 in the immune cells[42]. Recent studies have demonstrated that LPS inhibits vitamin D receptors in the oral keratinocytes, which impairs the mucosal homeostasis and leads to epithelial barrier damage. Consequently, this may promote the occurrence and development of OLP[43].

This study, for the first time, demonstrated that *H. pylori* infection is significantly correlated with the pathogenesis of erosive OLP. Recent studies have reported that *H. pylori* infection is closely associated with oral diseases, such as periodontitis, aphthous stomatitis, and oral cancer[44–46]. Th17 cell-related pro-inflammatory cytokines, such as IL-6 and IL-17 are reported to play an important role in *H. pylori* infection-induced inflammation[47–50]. In the gastric mucosa, *H. pylori* infection can promote the secretion of IL-17[47]. IL-17 induces the secretion of IL-8, recruits neutrophils, and aggravates inflammation through the ERK 1/2 MAP kinase pathway[23]. Furthermore, the virulence factor of *H. pylori* can directly promote the secretion of IL-8 in the epithelial cells[51]. *H. pylori* infection-induced inflammatory cytokines in the stomach can enter the bloodstream and reach the oral cavity to modulate the immune microenvironment in the oral cavity and aggravate the inflammatory response[52].

The dysregulation of T lymphocyte balance may play an important role in the pathogenesis of OLP[8, 24]. The proportion of Th17 cells in the peripheral blood of patients with erosive OLP is significantly higher than that in patients with reticular OLP[38, 53]. The analysis of tissues derived from OLP lesions revealed that the erosive OLP lesions contained significantly high proportions of Th17 cells[39]. These results indicate that Th17-related cytokines, such as IL-6 and IL-17 are involved in the pathogenesis of erosive OLP[39, 53].

We speculated that *H. pylori*-induced IL-17 may spread to the oral cavity through the bloodstream and increase the proportion of Th17 cells, which may contribute to the development of erosive OLP.

In this study, the salivary levels of IL-6, IL-8, and IL-17 in the OLP + group were higher than those in the OLP – group. This indicated that *H. pylori* infection alters the oral immune microenvironment in patients with OLP, which can affect the microbiome composition and promote the development of diseases[54]. Additionally, *H. pylori* infection enhanced the synthesis of LPS in the salivary microbiota of patients with OLP, which may be one of the reasons for the increased incidence rate of erosive OLP.

This study demonstrated that the OLP + group exhibited a decreased relative abundance of *Actinomyces* in the salivary microbiota. *Actinomyces* spp., which are the core microorganisms of oral cavity[28], are essential for maintaining the balance of bacterial flora. The decreased abundance of dominant bacteria will lead to the dysregulation of microbiome composition and promote the colonization of other bacteria[55]. In this study, the relative abundances of *Alloprevotella* and *Haemophilus* were significantly high in the salivary microbiota of the OLP + group. *Haemophilus* spp., a gram-negative bacillus, is associated with various opportunistic infections[56–58]. Additionally, *Haemophilus* spp. can activate the macrophages and promote the secretion of pro-inflammatory cytokines, such as IL-6 and IL-8 [59]. Furthermore, *Haemophilus* spp. infection can induce Th17 cell differentiation and IL-17 secretion, and accelerate the recruitment of neutrophils[60].

In the NC group, *H. pylori* infection did not enhance the secretion of salivary inflammatory cytokines, as well as the microbial community structure. Previous studies have also reported that gastric *H. pylori* infection does not affect the oral microbiome composition[52]. There are conflicting reports on the colonization of *H. pylori* in the oral cavity. The number of *H. pylori* is low in the oral cavity[61]. A meta-analysis revealed that the number of *H. pylori* in the saliva was less than that in the dental plaque[62]. Chua *et al.* did not detect *H. pylori* sequences in the oral swab samples[52]. In this study, it was difficult to detect *H. pylori* in the saliva sample of subjects infected with *H. pylori* through 16S rRNA gene sequencing. This indicated that *H. pylori* infection was not enough to alter the oral immune microenvironment but might promote the development of OLP.

In patients with OLP, *H. pylori* infection may exacerbate the pathological condition of the oral immune microenvironment and disrupt the homeostasis of the salivary microbiota through the interaction with different members of the microbial community[63]. This may decrease the abundance of beneficial bacteria and increase the abundance of pathogenic bacteria in the oral cavity, which contribute to the development of severe clinical subtype of OLP.

The reason for increased incidence rates of erosive OLP in patients with *H. pylori* infection cannot be fully determined based on the findings of this study. This study elucidated the correlation of *H. pylori* infection with the oral microbiome composition and clinical phenotype of OLP. Hence, we hypothesize that the eradication of *H. pylori* may relieve the clinical symptoms of OLP and reduce the incidence of erosive OLP.

## Conclusions

This study elucidated the correlation of *H. pylori* infection with the oral microbiome composition and clinical phenotype of OLP. We firstly demonstrated that *H. pylori* infection is significantly correlated with the pathogenesis of erosive OLP Hence, we hypothesize that the eradication of *H. pylori* may relieve the clinical symptoms of OLP and reduce the incidence of erosive OLP. However, further clinical studies are needed to verify this hypothesis.

# Methods

## Sample collection

Patients with OLP(OLP group, n=30) who underwent comprehensive clinical and histopathological examinations at the Stomatology Hospital of Shandong University were randomly recruited in this study. According to the clinical diagnosis and clinical classification of OLP by WHO (2005)[64], OLP is divided into the following two subtypes: reticular OLP, characterized by a white papular lesion, white reticular lesion, or white plaque; erosive OLP, characterized by erythema and erosive lesions. According to the follow-up clinical examination, age-matched and gender-matched normal control volunteers were recruited (NC group, n=21). All study subjects signed the informed consent form. The study subjects did not have a history of periodontitis, dental caries, systemic diseases, smoking, and alcohol consumption. Additionally, the subjects had not undergone antibiotic therapy or received OLP treatment before the sample was collected. This study was approved by the Medical Ethics Committee of School of Stomatology of Shandong University (Protocol Number: 20161001).

The saliva samples were collected following the guidelines of the Manual of Procedures for Human Micro-biome Project (http://hmpdacc.org/resources/tools_protocols.php). Briefly, the unstimulated saliva samples were collected from each patient between 8:00 am and 11:00 am in a sterile DNase/RNase-free conical tube. The study subjected did not consume alcohol or food for at least 2 h before sampling. The samples were transported to the laboratory and stored at −80 °C until further use.

*H. pylori* infection was detected in the OLP and NC groups using the urea breath test (14C-UBT) kit (Headway, Shenzhen, China). Based on the *H. pylori* infection status, the samples were divided into the following four groups: *H. pylori*-positive NC (NC+ n=10), *H. pylori*-negative NC (NC−, n=11), *H. pylori*-positive OLP (OLP+, n=21), and *H. pylori*-negative OLP (OLP−, n=9) groups.

## Enzyme linked immunosorbent assay (ELISA)

The saliva samples (5 mL) were centrifuged at 4 °C and 3500 $g$ for 20 min. The supernatant was stored in a DNase/RNase-free EP tube at −80 ° C until further use. The concentrations of IL-6, IL-8, and IL-17 were examined using the ELISA kit (Neobioscience Technology Co., Ltd. Shenzhen, China), following the manufacturer's instructions.

## DNA extraction and 16S rRNA gene amplification

The genomic DNA was isolated from the saliva samples using the QIAamp DNA micro kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. The V3 and V4 regions (336F-806R) of the 16S rRNA gene were amplified using polymerase chain reaction.

## Sequencing and data analysis

High-throughput sequencing was performed at CloudSeq Biotech, Inc. (Shanghai, China). Briefly, the raw sequence data were obtained from samples sequenced using the Illumina MiSeq sequencer were subjected to base calling and quality filtering. The samples were separated based on the barcode. The adaptors were trimmed and the low-quality reads were removed using Trimmomatic. The paired ends were merged using FLASH. The optimized reads were used for operational taxonomic unit (OTU) clustering and the OTU matrices were generated. The most abundant sequence in each OTU was selected as the representative sequence. The sequences of representative OTU were compared with those listed in the Greengenes database and those of samples. The taxonomic abundance matrices were generated. The determination of alpha and beta diversities, as well as statistical analysis and mapping, were performed using Mother and R environment. The microbial gene function was predicted using PICRUSt.

## Statistical analysis

All data are represented as mean ± standard deviation. The differences in the cytokine levels between two groups were examined using the $t$-test. The Kruskal-Wallis test was used to analyze the differences in microbiome composition between two groups. The correlation between variables was examined using Spearman's correlation test. All statistical analyses were performed using GraphPad Prism version 7.01 (GraphPad Software, Inc. CA, USA).

# Declarations

## Acknowledgements

## Author's Contributions

YS & SG: conceptualized and supervised this study; YS & SL: study design and original draft preparation; WL, YS, JJ & SG: acquired funding; SL, ZY, JL, HL & YL: analyzed the data; YS & SL: involved in writing and editing.

## Funding

## Data availability

The sequencing data generated in this study are submitted to the Sequence Read Archive of the National Center for Biotechnology Information database (accession number: SRP133987). All data supporting the findings of this study are available in the manuscript. Supplementary material can be obtained from the corresponding author upon reasonable request.

## Ethics approval and consent to participate

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Medical Ethics Committee of School of Stomatology of Shandong University (Protocol Number: 20161001). Written informed consent was obtained from individual or guardian participants.

## Consent for publication

Not applicable.

## Conflict of Interest

The authors declare no conflict of interest.

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# Tables

Table 1 Demographic and clinical parameters of the saliva study samples found no statistically significant differences in age and gender of each group.

| Characteristics | NC- (n=10) | NC+ (n=11) | OLP- (n=9) | OLP+ (n=21) | p value |
|---|---|---|---|---|---|
| Age (mean ± SD) | 46.44±3.772 | 48.30±3.211 | 48.30±3.211 | 46.71±2.618 | 0.45[1] |
| Male/female | 7/3 | 11/0 | 8/1 | 16/5 | 0.5[2] |
| Smoking habit; | 0 | 0 | 0 | 0 | |
| Alcohol drinking; | 0 | 0 | 0 | 0 | |
| Antibiotics usage; | 0 | 0 | 0 | 0 | |
| Systemic disease; | 0 | 0 | 0 | 0 | |

[1]ANOVA; [2]Fisher's test.

Table 2  Chi-sauared test showed statistically significant association of infection of *H. Pylori* and the alteration of OLP subtype (*p*< 0.005).

| OLP subtype (%) | OLP- (n=9) | OLP+ (n=21) | p value |
|---|---|---|---|
| Reticular OLP (n=16) | 8 (88.9) | 8 (38.1) | 0.0041[1] |
| Erosive OLP (n=14) | 1 (11.1) | 13 (61.9) | |

[1]Chi-square test.

# Figures
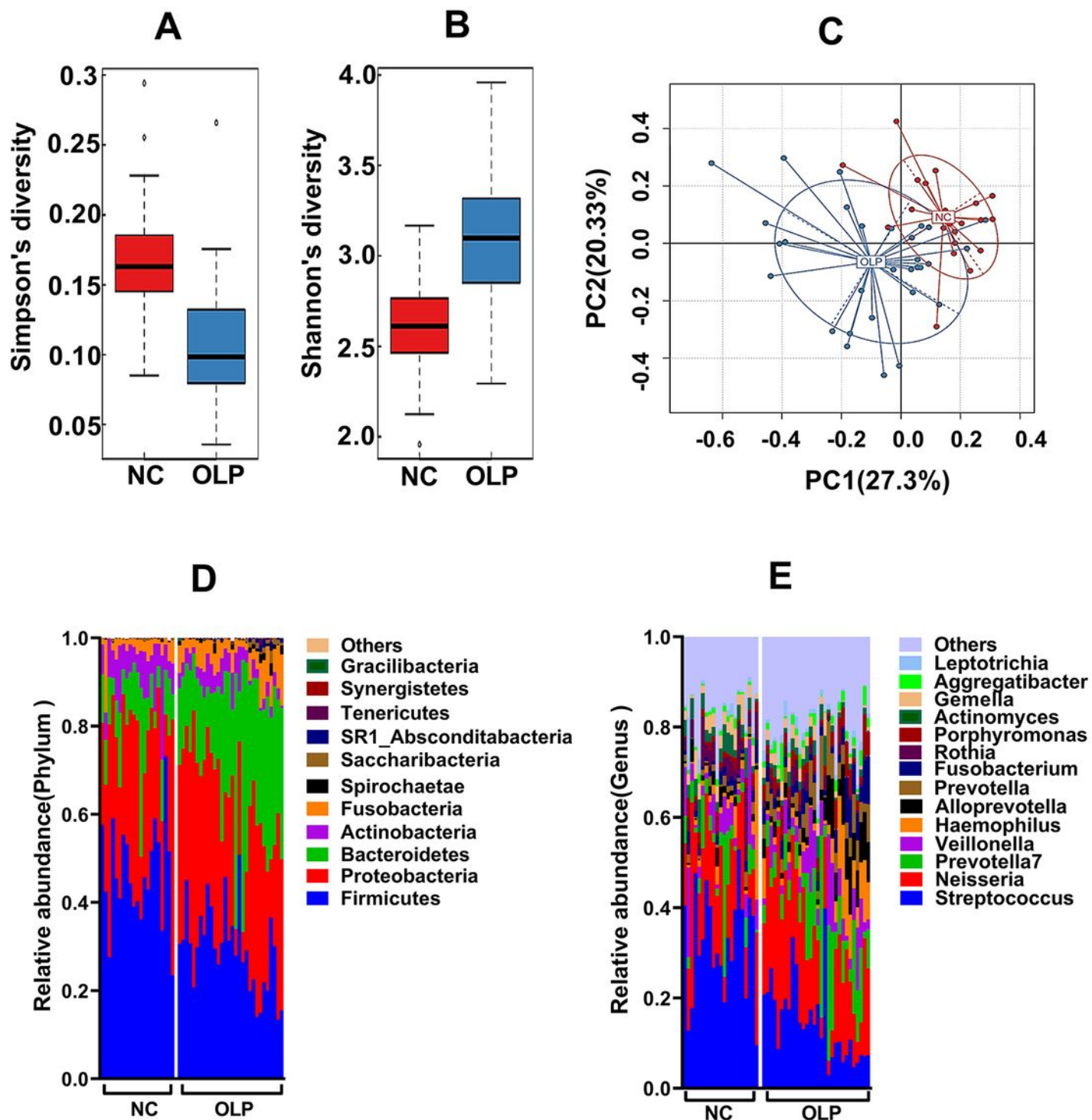
**Figure 1**

Comparison of the salivary microbiome profiles between the NC (n=21) and OLP groups (n=30). (A) Simpson's diversity index of salivary microbiome in the NC and OLP groups (p=0.00003). (B) Shannon's diversity index of salivary microbiome in the NC and OLP groups (p=0.0002). (C) Principal coordinate analysis (PCoA) plots of unweighted UniFrac distances of oral microbiota in the NC and OLP groups.

Comparative analysis of the oral microbiome composition at the phylum (D) and genus (E) levels between the NC and OLP groups.
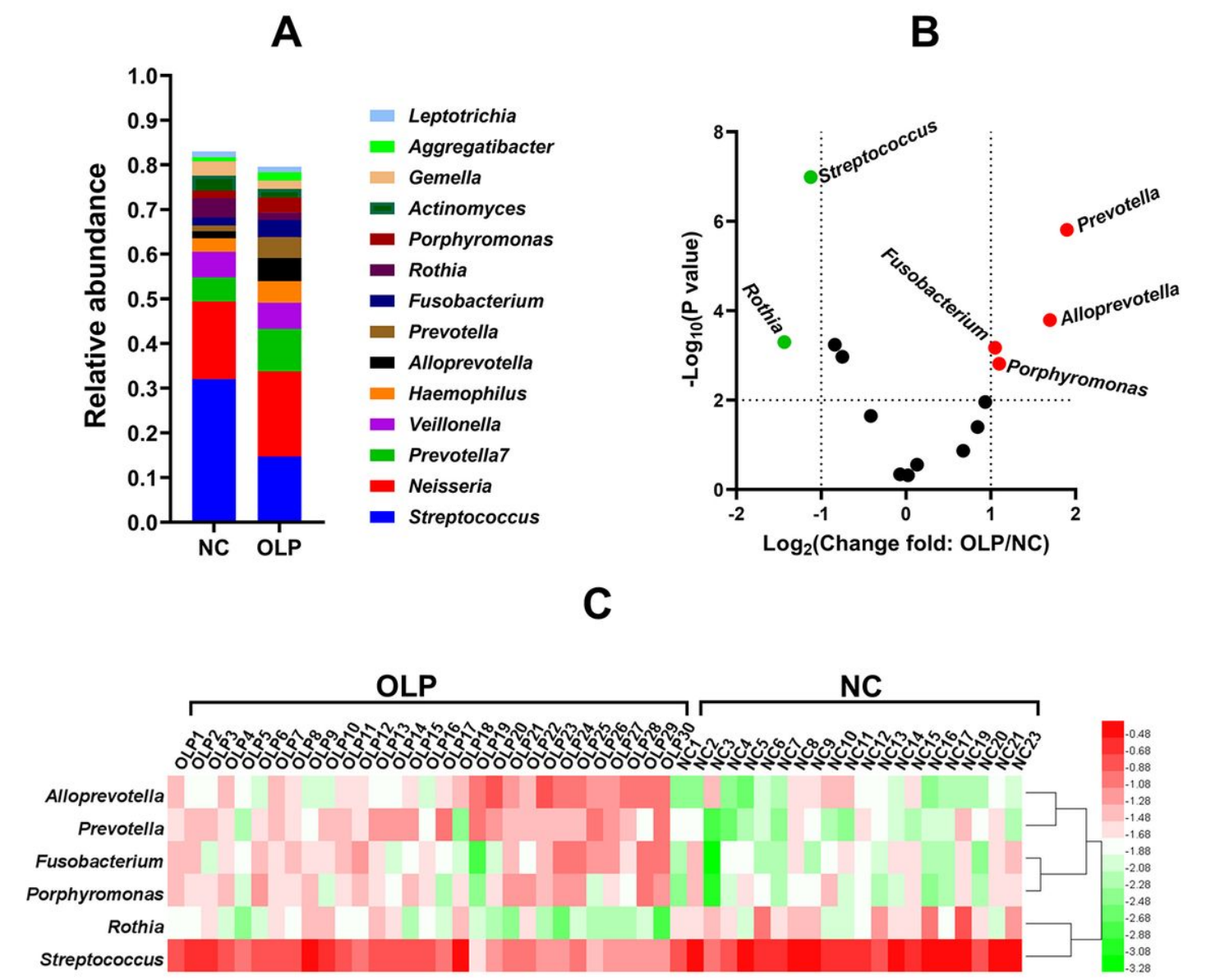


**Figure 2**

Comparison of the salivary microbiome composition at the genus level between the NC and OLP groups. (A) Comparative analysis of the salivary microbiome composition between the NC (n=21) and OLP (n=30) groups (average relative abundance >1% in the two groups). (B) Volcano plot analysis of salivary microbiome composition between the NC (n=21) and OLP (n=30) groups. The p-value was calculated using the t-test. According to the results shown in panel (A), bacteria with a relative abundance greater than 1% were selected and marked as key bacteria. (C) Heat map showing the relative abundances of the key bacteria for sampled individuals of the community subgroups.
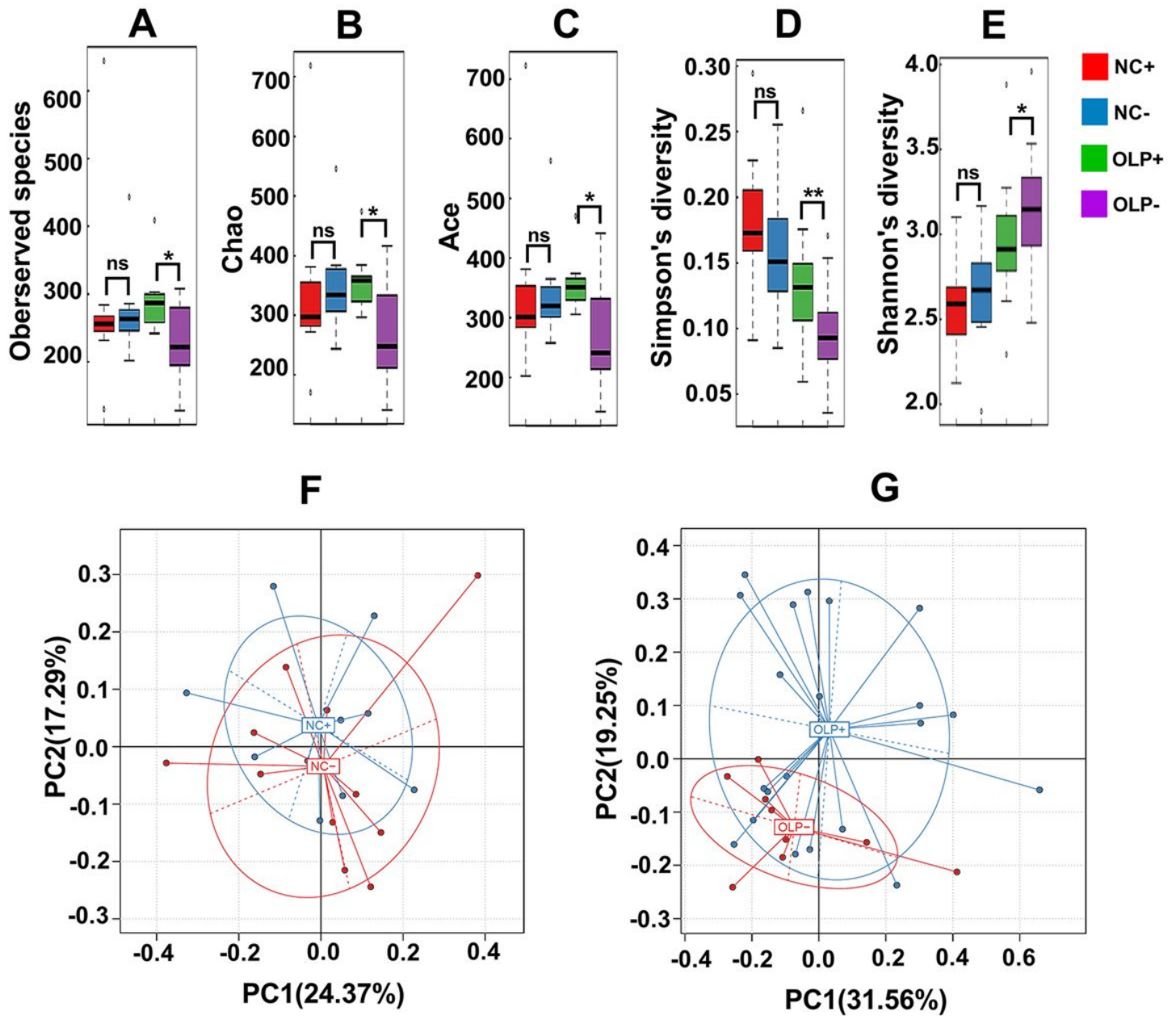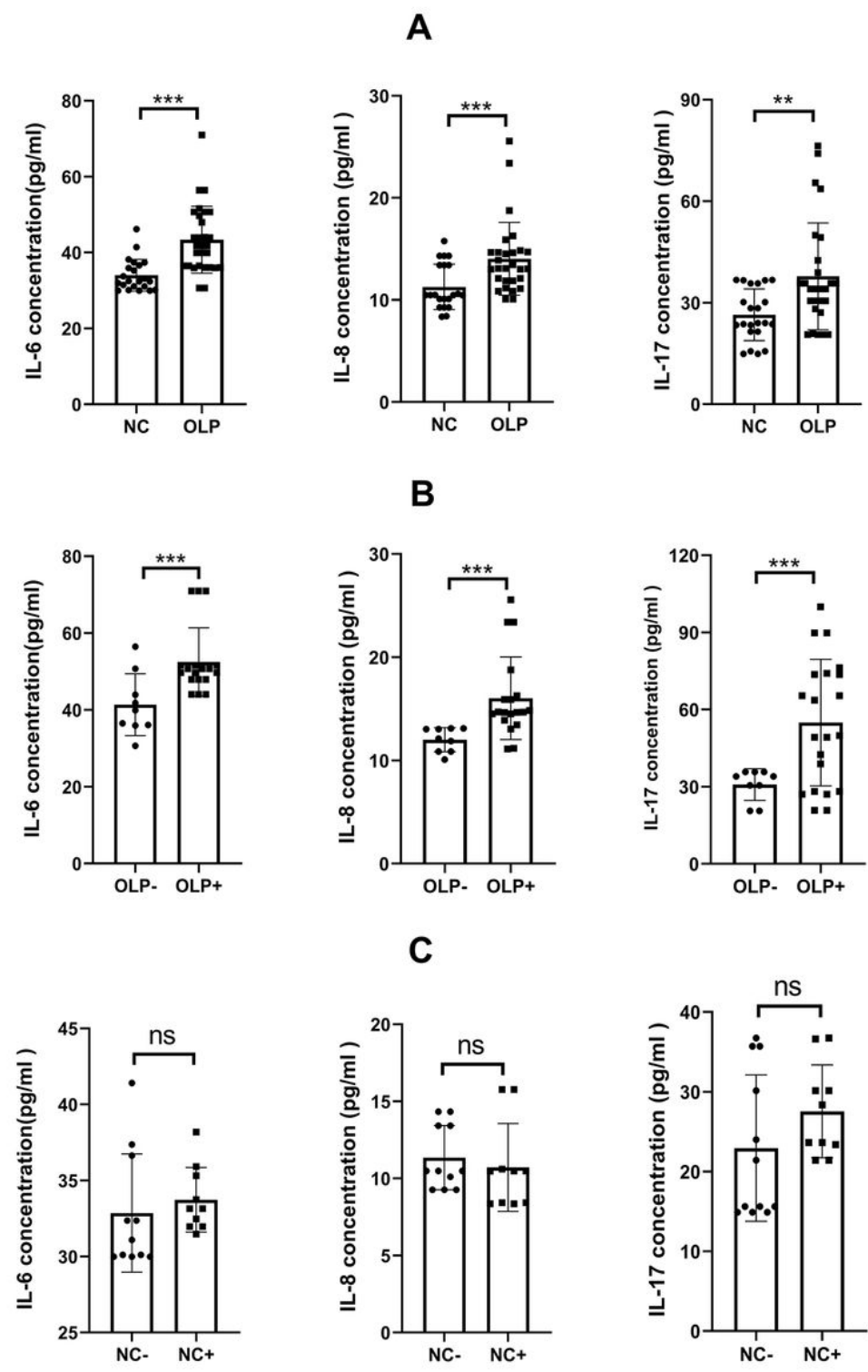
## Figure 3

Comparison of the salivary microbiome composition between the NC−, NC+, OLP−, and OLP + groups. (A) Observed species, (B) Chao diversity index, (C) Ace diversity index, (D) Simpson's diversity index, and (E) Shannon's diversity index between the NC− (n=11) and NC+ (n=10) groups, as well as between the OLP− (n=9) and OLP+ (n=21) groups. Principal coordinate analysis (PCoA) plots of unweighted UniFrac distances of saliva microbiota between the NC− (n=11) and NC+ (n=10) groups (F) and between the OLP− (n=9) and OLP+ (n=21) groups (G). *p < 0.05; **p < 0.01; ns, no significance.
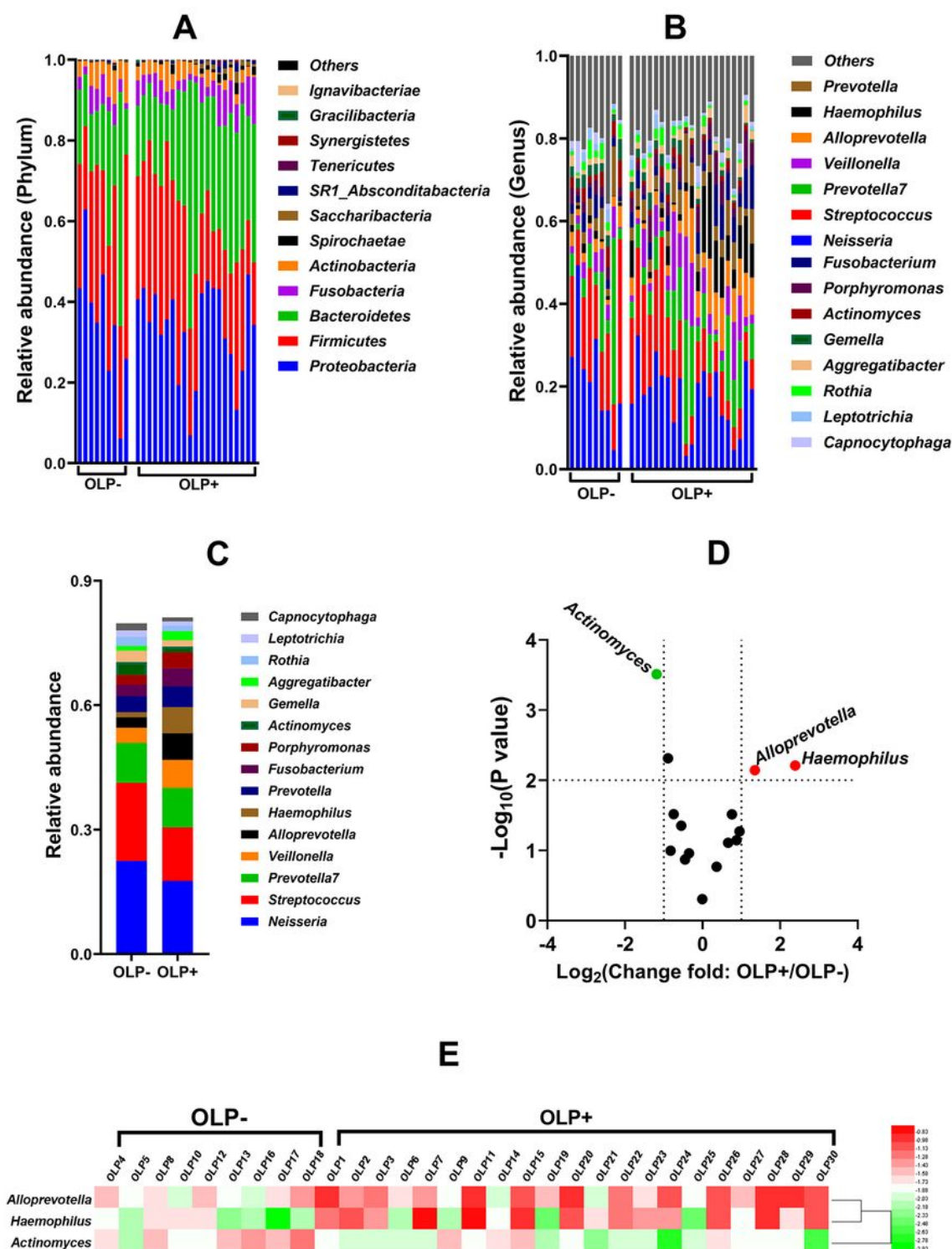
**Figure 4**

Comparative analysis of the salivary microbiome composition between the OLP− (n=9) and OLP+ (n=21) groups. (A) Comparative analysis of the salivary microbiome composition at the phylum and (B) the genus levels between the OLP− and OLP + groups. (C) Comparative analysis of the salivary microbiome composition between the OLP− and OLP+ groups (average relative abundance >1% in the two groups). (D) Volcano plot analysis of salivary microbiome composition between the OLP− and OLP+ groups. The

p-value was calculated using the t-test. According to the results shown in panel (C), bacteria with a relative abundance greater than 1% were selected and marked as key bacteria. (E) Heat map showing the relative abundance of the key bacteria for sampled individuals of the community subgroups.



**Figure 5**

The salivary levels of cytokines (IL-6, IL-8, and IL-17) were measured using ELISA. Comparative analysis of the salivary cytokine levels between the following groups: NC (n=21) and OLP (n=30) groups (A); OLP−

(n=9) and OLP+ (n=21) groups (B); NC− (n=11) and NC+ (n=10) groups (C). *p < 0.05; **p < 0.01; ***p < 0.001; ns, no significance.
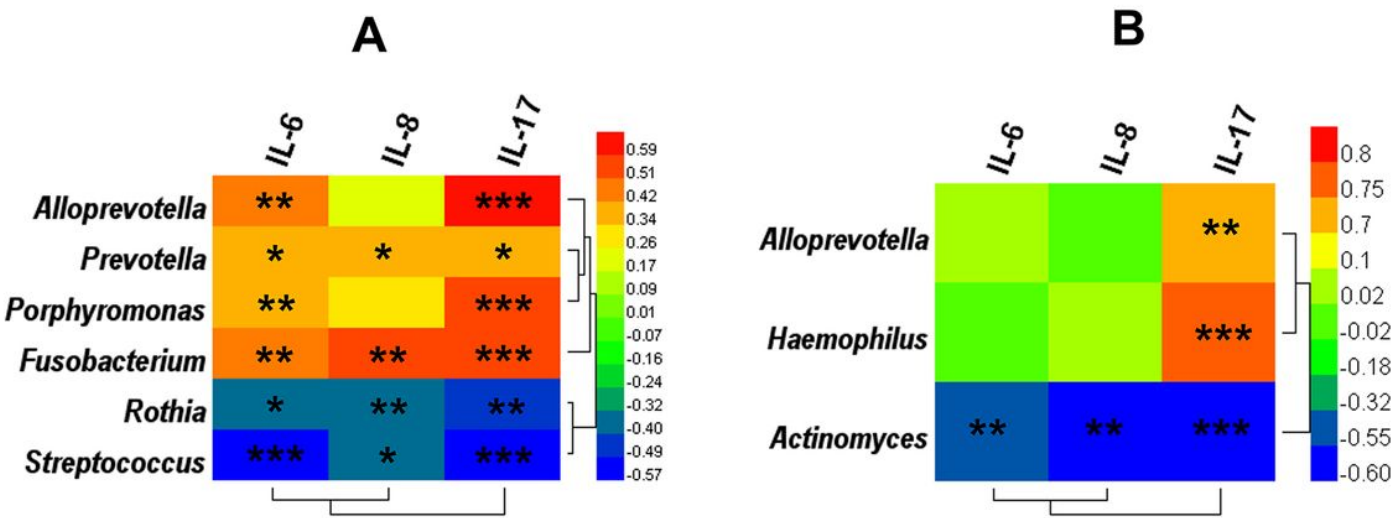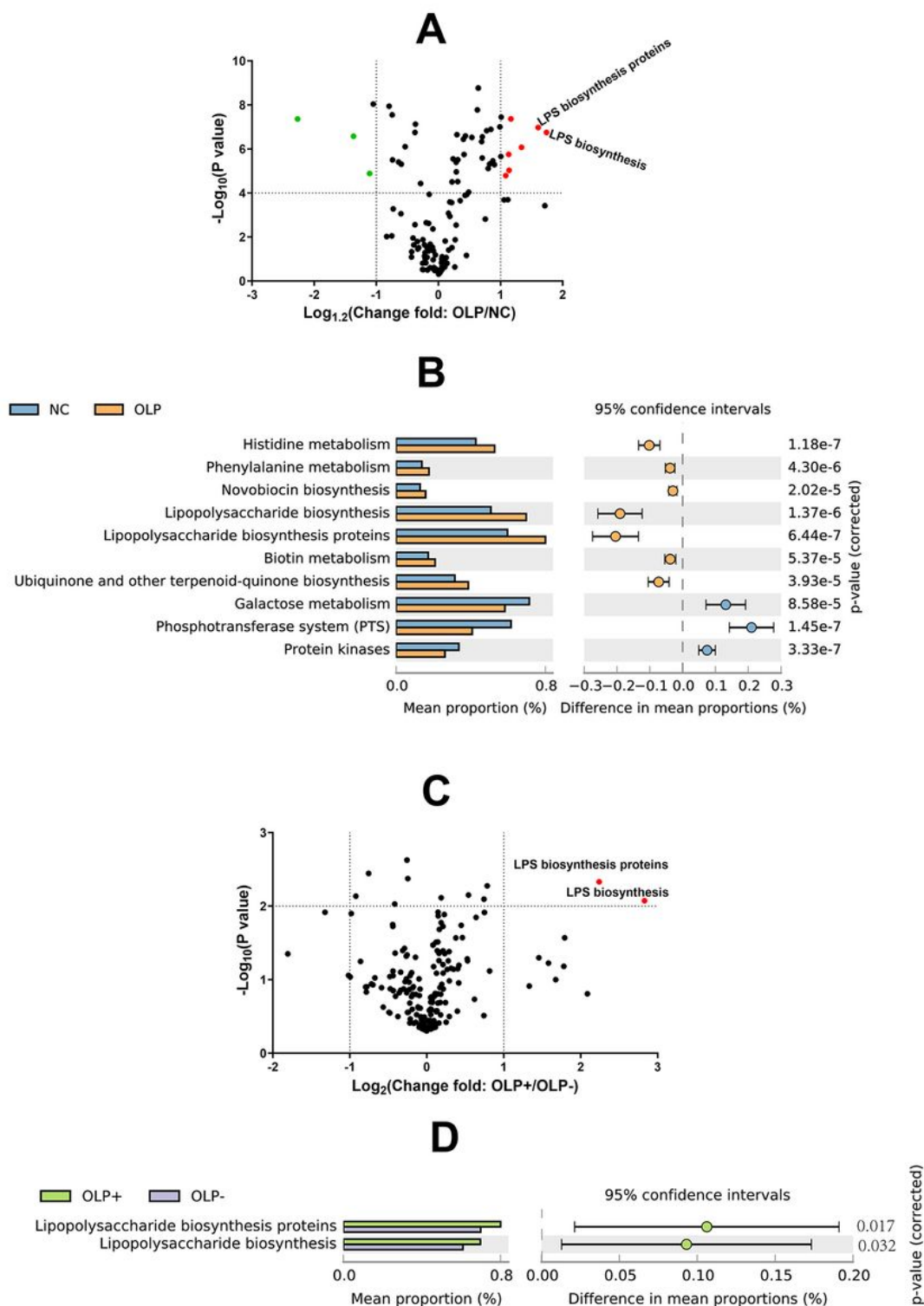


# Figure 6

Correlation of cytokine production with the relative abundance of salivary key bacteria at the genus level . (A) NC (n=21) and OLP (n=30) groups; (B) OLP− (n=9) and OLP+ (n=21) groups. The color of cells represents the correlation coefficient (R) values. Cells marked with an asterisk show significance after multiple comparisons of Spearman's correlation, *p < 0.05; **p < 0.01; ***p < 0.001

**Figure 7**

Comparative analysis of the relative abundance of PICRUSt-generated functional profiles of salivary microbiota. Volcano plot analysis of altered Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways between the following groups: NC (n=21) and OLP (n=30) groups (A); OLP− (n=9) and OLP+ (n=21) groups (C). KEGG pathways with an average relative abundance greater than 2% in the two groups were included. The p-value was calculated using the two-tailed paired t-test (B) and (D) based on volcano

plot analysis (Figure A and Figure C). The distinct gene categories were selected according to significant differences in gene categories at level 3 (t-test, $p < 0.05$). The bar plots on the left show the mean proportion of each KEGG pathway. The dot plots on the right show the differences in mean proportions between the two indicated groups using p-values.
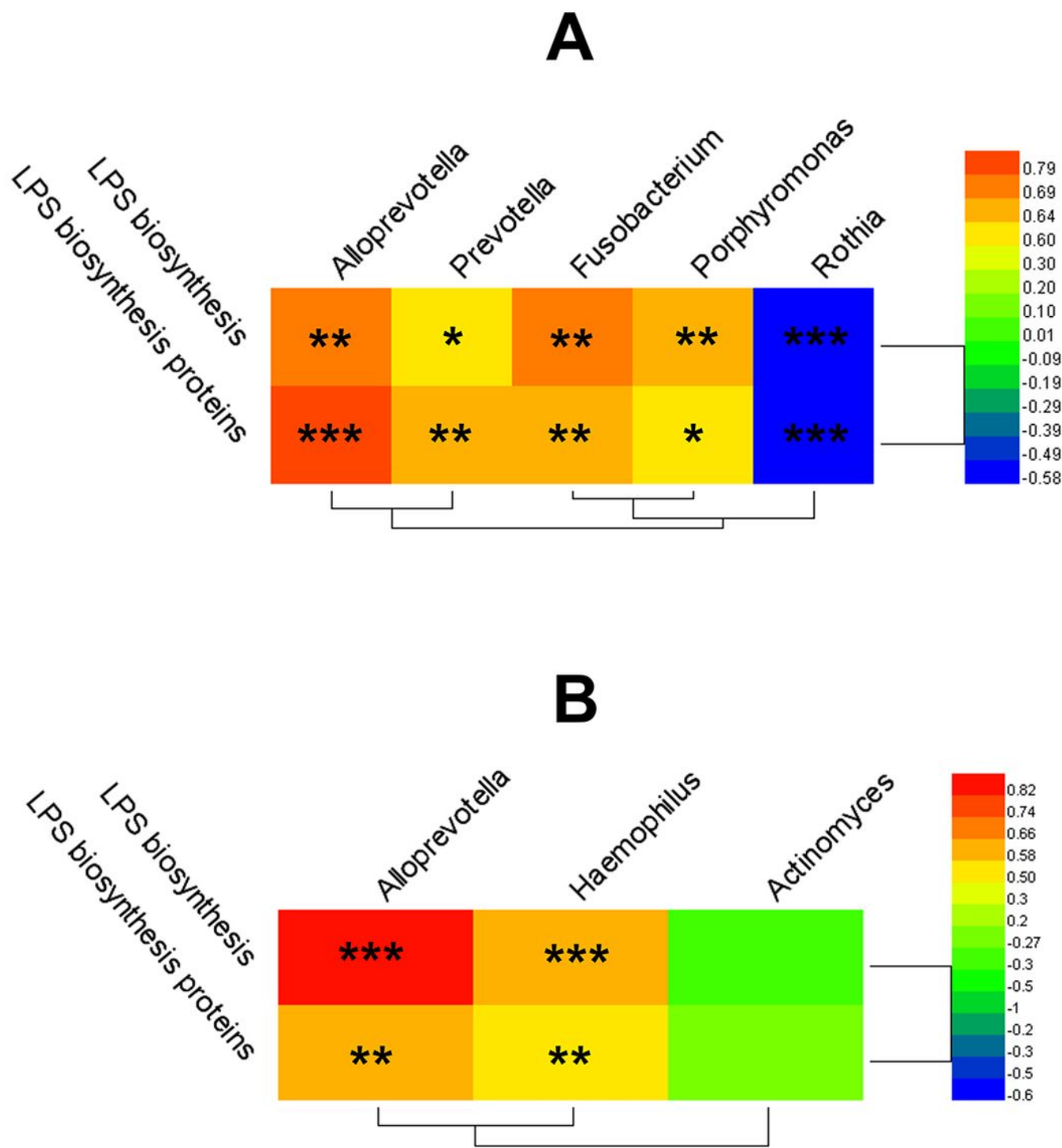


Figure 8

Correlation of PICRUSt-generated key pathway with the relative abundance of salivary key bacteria at the genus level. (A) NC (n=21) and OLP (n=30) groups; (B) OLP− (n=9) and OLP+ (n=21) groups. The color of cells represents the correlation coefficient (R) values. Cells marked with an asterisk show significance after multiple comparisons using Spearman's correlation, *p < 0.05; **p < 0.01; ***p < 0.001.

## Supplementary Files

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