Infection and Drug Resistance

Dovepress
open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

Multiomics Analysis Reveals Significant Disparities in the Oral Microbiota and Metabolites Between Pregnant Women with and without Periodontitis

Min Zhao[1], Changyi Yang[1], Linhong Zhu[1], Xiaoqian Guo[1], Haiyan Ma[2], Yuanhao Luo[3], Qi Wang[4], Juanjuan Chen[4]

[1]Department of Periodontology, General Hospital of Ningxia Medical University, Yinchuan, Ningxia, 750004, People's Republic of China; [2]School of Stomatology, Ningxia Medical University, Yinchuan, Ningxia, 750004, People's Republic of China; [3]The Second Clinical Medical School, Lanzhou University, Lanzhou, Gansu, 730030, People's Republic of China; [4]Cuiying Biomedical Research Center, Lanzhou University Second Hospital, Lanzhou, Gansu, 730030, People's Republic of China

Correspondence: Juanjuan Chen; Qi Wang, Email ery_juanjuanchen@lzu.edu.cn; ery_wangqery@lzu.edu.cn

Introduction: Our study investigated the disparities and correlations between oral microbiota and metabolites in pregnant patients with and without periodontitis.

Methods: Subgingival plaque samples from all subjects were collected for shotgun metagenomic sequencing and broad-target metabolomics analysis.

Results: Forty pathogens, including *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Eubacterium saphenum*, *Gemella morbillorum*, *Tannerella forsythia*, *Streptococcus anginosus* group, Selenomonas sputigena etc, were significantly enriched in pregnant patients with periodontitis (PPP). Conversely, symbiotic species such as *Morococcus cerebrosus*, *Streptococcus vestibularis*, *S. salivarius*, *S. mitis*, and *S. pneumoniae* were significantly more abundant in healthy controls (HCs). A total of 87 predicted functional modules (PFMs) exhibited significant differences between the two groups; eight PFMs showed high enrichment in PPP with involvement of PPP-enriched species within these pathways. The remaining 79 PFMs encompassing ribonucleotide biosynthesis, carbohydrate, and amino acid metabolism were highly abundant in HCs. For oral microbial metabolome, a total of 105 metabolites related to 150 KEGG pathways displayed significant differences between the two groups. Pathways such as pyruvate metabolism, folate biosynthesis, vascular smooth muscle contraction, and AMPK/mTOR signaling pathway along with their associated metabolites were found to be enriched in PPP, while carbohydrate metabolism predominated among HCs. Spearman's rank correlation analysis revealed significant positive associations between species enriched in PPP and metabolites enriched in PPP, but significant negative associations between species enriched in PPP and metabolites enriched in HCs.

Discussion: Our findings provide potential biomarkers for distinguishing periodontitis during pregnancy while offering valuable insights into mechanisms exploration and clinical intervention.

Keywords: Pregnancy-induced periodontitis, oral microbiota composition, predicted functional modules, microbial metabolites profiling, biomarkers identification

Introduction

Pregnancy represents a distinct phase in a woman's life characterized by physiological changes including hormonal fluctuations. Oral microflora during pregnancy seems to be impacted by both local factors such as gestational diabetes mellitus, pre-eclampsia, as well as systemic conditions.[1] The human oral cavity harbors the second most intricate microbial community among all body sites,[2] encompassing 1089 high-quality genomes.[3] Total viable microbial counts are observed to be elevated in pregnant individuals compared to non-pregnant counterparts, particularly during the initial trimester of gestation.[2] Yang et al's investigation into full-term pregnant women's oral microbiota unveiled its involvement in regulating maternal immune responses and serving as a crucial mediator of local immune modulation at the placenta level.[4] Maintaining a balanced oral

Received: 13 June 2024
Accepted: 17 September 2024
Published: 25 October 2024

microbiome is imperative for ensuring a healthy pregnancy; disruptions in oral microbial composition may contribute to complications associated with gestation.

Periodontitis, caused by destructive host immune responses to pathogenic bacterial species resulting from the oral microbial dysbiosis in dental plaque,[5] is common and of high incidence in pregnancy. It is estimated that one woman in five could develop periodontitis during pregnancy.[6] Periodontitis is increased by the action of pregnancy hormones, vomiting, neglected oral hygiene, adequate diet, and nutritional changes. Periodontitis-associated oral pathogenic bacteria, such as *Fusobacterium nucleatum, Porphyromonas gingivalis, Filifactor alocis, Campylobacter rectus*, etc, have been implicated as potential contributors to adverse pregnancy outcomes such as preterm birth, preeclampsia, and low birth weight,[7–9] but are also risk factors of cardiovascular diseases, diabetes, rheumatoid arthritis, cancer, and chronic obstructive pulmonary disease.[10] Last two decades have witnessed unparalleled advances of the oral microecology in the transition from health to gingivitis and to destructive periodontal disease.[11] Abusleme et al has explored the ecology of oral subgingival communities in health and periodontitis and elucidated the relationship between inflammation and the subgingival microbiome by using 454-pyrosequencing of 16S rRNA gene libraries and quantitative PCR.[12] Griffen et al has revealed the distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing.[13] Zaura et al explored the ecobiological heterogeneity of the salivary ecosystem and relations between the salivary microbiome, salivary metabolome, and host-related biochemical salivary parameters in 268 healthy adults after overnight fasting, and revealed gender-specific differences in the microbiome and metabolome associated with salivary pH and dietary protein intake.[14] Li et al demonstrated the dysbiosis of oral microbiota and metabolite profiles associated with Type 2 Diabetes Mellitus (T2DM), and found that *Porphyromonas gingivalis* and *Prevotella melaninogenica* were significantly enriched, while the abundances of dental periodontal pathogens such as *Streptococcus mutans* and *Streptococcus sobrinus* were not significantly different.[15] Oral bacterial metabolites play a critical role in oral and systemically healthy maintenance since they are important mediators for oral bacteria growth and reproduction.[16] Lietzan et al[17] established oral β-glucuronidase (GUS) activity as a biomarker that captures both host and microbial contributions to periodontitis. Li et al reported that salivary levels of cadaverine and L-(+)-leucine, supragingival plaque levels of N-acetyldopamine and 3.4-dimethylbenzoic acid were significantly higher in T2DM patients than those of controls.[15] The association between periodontal pathogens and adverse pregnancy outcomes may involve the hematogenous spread of periodontal pathogens and multiple inflammatory mediators by the host and/or fetal immune response to pathogenic bacteria, as well as oral microbial pathogen transmission and colonization in the vagina.[7]

Although the oral microbiota and metabolites of periodontitis patients have been studied, somewhat, the oral microbial composition of pregnant patients with periodontitis and HCs remains expounding. The present study was executed to investigate the disturbances and associations of the oral microbiota and metabolites from subgingival plaque samples in pregnant women with and without periodontitis from China.

Materials and Methods

Subject Enrollment and Clinical Assessment

This study involves the use of human subjects and has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). The study was approved by the Medical Research Ethics Review Committee of General Hospital of Ningxia Medical University (No. 2020–682) on August 26th, 2020. Informed consent and questionnaire related to oral hygiene were obtained from each subject (Table S1).

The inclusion and exclusion criteria for pregnant women with periodontitis:

1. Patients aged between 20 and 39 years old, at pregnant metaphase (16 to 28 weeks), which is of low risk for miscarriage and preterm birth;
2. No dental discomfort before pregnancy and have never see a dentist;
3. Never received any periodontal treatment before;
4. See a dentist for symptoms such as bleeding or swelling of the gums after brushing the teeth after pregnancy;
5. Have not eaten fermented food such as yogurt for nearly a month;
6. No other systemic or infectious diseases;

7. No other serious oral diseases;
8. No orthodontic correction in progress;
9. No oral contraceptives in the last 6 months;
10. No oral antibiotics usage in nearly 2 months;
11. No smoking history or quit smoking for two years;
12. Clinical assessment: dental examination, probing depth (PD), attachment loss (AL), bleeding index (BL) were evaluated by the same professional dentist according to Armitage et al.[18]

For healthy controls (HCs), all were consistent with the patient group except for the fourth criterion.

Sample Collection

According to the above inclusion and exclusion criteria, a total of 36 patients with gestational periodontitis (Average duration of pregnancy: 21.26 ± 2.86 weeks) and 31 hCs from the department of obstetrics and stomatology from General Hospital of Ningxia Medical University were enrolled from September 2020 to August 2021. According to previously published literature, subgingival plaque samples are the most relevant specimens for investigating the pathogenesis of periodontal diseases due to their association with periodontitis microbiota.[19] Therefore, we have included subgingival samples in this study and collected them as follows:

1. Prepare an oral test kit, including a long-handled oral mirror, dental probe, surgical scissors, cotton tweezers, 2*2mm gauze pad, cotton ball, cotton gauze, and wood tongue depressor;
2. Prepare sample collection tubes, two 2 mL screw tubes (outer helix), one with 750 μL DNA sample preservation solution (for shotgun metagenomic sequencing, purchases from HCY Technology, Shenzhen, China) and another containing 750 μL PBS (for metabolomic detection);
3. Have the subjects lie in a standard dental chair with good lighting;
4. Rinse the teeth with an oral irrigator contains clean water to remove food debris from the mouth;
5. Clean the tartar, food debris, plaque and soft scale on the surface of the supragingival teeth with scraper and cotton balls. Subsequently use sterile cotton balls to isolate saliva around the sampling site and air dried to remove residual gingival plaque;
6. Subgingival plaque was collected on the mesiobuccal side from six of the Ramfjord index teeth (16, 11, 26, 31, 36, 46) using Gracey subgingival curette. If the index tooth has fallen out, teeth adjacent to the index tooth in the same area was collected. Plaques at least three sites from the six examining sites (buccal surface: mesial, intermediate, distal; lingual surface: mesial, intermediate, distal) of a tooth should be collected.
7. Submerge the curette tip into the solution in tube for 4 to 5s;
8. Scrape off the sample collection at the curette tip on the inside of the collection tube;
9. Cover the collection tube and shake for 4 to 5s to disperse the sample.
10. Mark the name of each sample clearly and store at −80°C. The samples were then transported to the laboratory by dry ice for shotgun metagenomic sequencing and metabolomic detection after all samples were collected.

It's worth noting that large amounts of blood samples (eg, haemorrhage) should be removed based on clinical diagnosis, and immediate resampling at the same site is not recommended. A second tooth should be chosen.

In the end, 134 samples, as well as 67 informed consents, and questionnaires from 67 subjects (36 patients and 31 healthy controls) were collected.

Shotgun Metagenomic Sequencing and Analysis

DNA Extraction

The non-commercial DNA extraction method using phenol-chloroform was reported to produce the highest total yield.[20] Cetyltrimethylammonium bromide (CTAB) is a modified method for rapid DNA isolation.[21] So, we used the CTAB method for total DNA extraction from the collected subgingival plaque samples. The details were as following:

1. Subgingival plaque sample was placed in 2 mL centrifuge tube, 2 sterilized steel balls were added and frozen in liquid nitrogen, and ground with frozen mixing ball mill (MM400, Retsch, Germany);
2. 800 μL CTAB lysate and 50 μL lysozyme (50 mg/mL, CAT#89833, Thermo Scientific, US) were added into the 2 mL tube, the liquid was water bathed at 65°C for 1 h, during which reversed the tube every 15 minutes;
3. Centrifuged at 12000 rpm for 10 min, added 800 μL phenol: chloroform: isopropyl alcohol (25:24:1), mixed completely, centrifuged at 12000 rpm for 10 min, transferred the upper water phase to a new 2 mL centrifuge tube;
4. Took the supernatant, added chloroform: isoamyl alcohol (24:1) and reversed to mix, centrifuged at 12000 rpm for 10 min;
5. Absorbed the supernatant into 1.5 mL centrifuge tube, added isopropyl alcohol and shake to mix, and precipitated at −20 degrees;
6. Centrifuged at 12000 rpm for 10 minutes and poured out the liquid, taking care not to pour out the precipitate. Wash with 1mL 75% ethanol after cleaning twice, the remaining small amount of liquid can be centrifugal again and collected, and then sucked out with the pipette tip;
7. Dried by blowing with an ultra-clean workbench or at room temperature.;
8. Added ddH_2O to dissolve the DNA sample;
9. Extracts were treated with 1 μL RNase A (CAT#EN0531, Thermo Scientific, US) to eliminate RNA contamination at 37 ° C for 15 min.

DNA quantity was determined by NanoDrop spectrophotometer (Thermo Scientific, US), Qubit Fluorometer (with the Quant-iTTMdsDNA BR Assay Kit), and gel electrophoresis.

A total of 53 qualified DNA samples containing 31 patients and 22 hCs were obtained for DNA library construction and shotgun metagenomic sequencing.

DNA Library Construction and Sequencing

DNA library was constructed as previously described.[22] Paired-end (PE) library with insert size of 350 bp for each sample was constructed, followed by a high-throughput sequencing with PE reads of length 2 × 150 bp based on DNBSEQ-T7 platform. Finally, 49 raw sequences from 29 patients and 20 hCs were used for omics and bioinformatics analysis. High-quality reads were obtained by filtering low-quality reads with ambiguous "N" bases, adapter, and human DNA contamination (mapped to the hg19 reference) from the DNBSEQ-T7 raw reads. Over 90% of the reads remained as high-quality reads.

Taxonomic and Functional Profiling Acquisition

Taxonomic profiles were generated from high-quality reads by using Metaphlan 3 (- input_type fastq - ignore_viruses - nproc 6) as described by Francesco Beghini et al.[23] MetaPhlAn is a computational tool for profiling the composition of microbial communities (Bacteria, Archaea, and Eukaryotes) from metagenomic shotgun sequencing data with species-level. Now MetaPhlAn 4 has been updated,[24] which relies on ~5.1M unique clade-specific marker genes identified from ~1M microbial genomes (~236,600 references and 771,500 metagenomic assembled genomes) spanning 26,970 species-level genome bins (SGBs, http://segatalab.cibio.unitn.it/data/Pasolli_et_al.html), 4,992 of them taxonomically unidentified at the species level (MetaPhlAn4 – The Huttenhower Lab (harvard.edu)).

HUMAnN 3.0 (-i input_clean_data -o output –threads 10 –memory-use maximum –remove-temp-output) was used to profile the abundance of microbial metabolic pathways and other molecular functions from the high-quality data (https://huttenhower.sph.harvard.edu/humann) according to previously published papers.[23]

Diversity Calculation

Alpha-diversity (within-sample diversity, R 4.0.3 vegan: diversity (data, index = "richness/shannon/Simpson/InSimpson")) was calculated using the richness, Shannon index, Simpson index, and Inverse Simpson index depending on the taxonomic profiles. Beta-diversity (between-sample diversity, R 4.0.3 vegan: diversity (data, index = "bray_curtis distance")) was calculated using the Bray–Curtis distance depending on the taxonomic profiles. P-values for PCoA are

calculated via Permutational Multivariate Analysis of Variance (PERMANOVA). P-values for alpha diversity were calculated by Wilcoxon rank sum test.

Metabolomic Test and Analysis

Sample Preparation and Extraction

Subgingival plaque samples were freeze-dried by vacuum freeze-dryer (Scientz-100F) and was crushed using a mixer mill (MM 400, Retsch) with a zirconia bead for 1.5 min at 30 hz. Dissolve 50 mg of lyophilized powder with 1.2 mL 70% methanol solution, vortex 30 seconds every 30 min for 6 times in total. Following centrifugation at 12000 rpm for 3 min, the extracts were filtrated (SCAA-104, 0.22 μm pore size; ANPEL, Shanghai, China, http://www.anpel.com.cn/) before UPLC-MS/MS analysis. Totally 66 samples from 35 patients and 31 healthy controls were successfully obtained for untargeted metabolomics.

UPLC Conditions

The sample extracts were analyzed by an UPLC-ESI-MS/MS system (UPLC, SHIMADZU Nexera X2, https://www.shimadzu.com.cn/; MS, Applied Biosystems 6500 Q TRAP, https://www.thermofisher.cn/zh/home/brands/applied-biosystems.html). The analytical conditions were as follows: UPLC: column, Agilent SB-C18 (1.8 μm, 2.1 mm * 100 mm); The mobile phase was consisted of solvent A, pure water with 0.1% formic acid, and solvent B, acetonitrile with 0.1% formic acid. Sample measurements were performed with a gradient program that employed the starting conditions of 95% A, 5% B. Within 9 min, a linear gradient to 5% A, 95% B was programmed, and a composition of 5% A, 95% B was kept for 1 min. Subsequently, a composition of 95% A, 5.0% B was adjusted within 1.1 min and kept for 2.9 min. The flow velocity was set as 0.35 mL per minute; The column oven was set to 40°C; The injection volume was 2 μL. The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (QTRAP)-MS.

Esi-Q Trap-Ms/Ms

The ESI source operation parameters were as follows: source temperature 500°C; ion spray voltage (IS) 5500 V (positive ion mode)/-4500 V (negative ion mode); ion source gas I (GSI), gas II (GSII), curtain gas (CUR) were set at 50, 60, and 25 psi, respectively; the collision-activated dissociation (CAD) was high. Instrument tuning and mass calibration were performed with 10 and 100 μmol/L polypropylene glycol solutions in QQQ and LIT modes, respectively. QQQ scans were acquired as MRM experiments with collision gas (nitrogen) set to medium. DP (decluttering potential) and CE (collision energy) for individual MRM transitions were done with further DP and CE optimization. A specific set of MRM transitions were monitored for each period according to the metabolites eluted within this period.

KEGG Annotation and Enrichment Analysis

Identified metabolites were annotated using KEGG Compound database (http://www.kegg.jp/kegg/compound/), annotated metabolites were then mapped to KEGG Pathway database (http://www.kegg.jp/kegg/pathway.html).

PERMANOVA Test of the Effects from Collected Phenotypes

To evaluate the effects of the collected clinical and lifestyle phenotypes, PERMANOVA test was used with the gene abundance files of the samples. The Bray–Curtis distance and 9999 permutations in R (3.2.5, vegan package) were used.

Correlation Analysis

Spearman's rank correlation was used to analyze the relationship between the significantly different species and metabolic pathways, as well as metabolites. To do this, the metagenomic and metabolomic data from the same patients and HCs including 20 hCs and 28 PPP were used.

Differential Analysis

No significant differences in the brushing habits, gravidity, and parity were observed between the two groups. The age at which the two groups were previously matched was adjusted using partial correlation analysis. For the significantly different phyla, genera, species and predicted microbial functional pathways between the two groups ($P < 0.05$), two-

tailed Wilcoxon rank sum test (effect size equal to (**eqn**), where n and m are the sample size of periodontal patients and HCs during pregnancy) was applied to the relative abundance data of taxonomic and functional profiles obtained from MetaPhlAn3 and HUMAnN3, respectively.

Results

Characterization of the Oral Microbial Structure in Pregnant Women with Periodontitis

MetaPhlAn 3.0 is used for profiling the composition of microbial communities from metagenomic shotgun sequencing data (Table S2A). PCoA analysis based on bray-distance of genera and species profiles showed that the plaque microbiome was significantly different between pregnant patients with periodontitis (PPP) and HCs (genera, $P = 0.020$, Figure 1A; species, $P = 0.025$, Figure 1C) although the oral microbiota differs between individuals. Alpha diversity analysis based on Shannon, Simpson, and inverse Simpson index showed that the richness and diversity of genera (Figure 1B) and species (Figure 1D) were significantly higher in PPP compared with HCs (Table S2B).

We have then analyzed the specific differences of oral microbiome between the two groups at phyla, genus, and species levels. Totally eight phyla were observed, among which Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, and Fusobacteria were the top five abundant phyla in both groups, Bacteroidetes and Fusobacteria were significantly enriched in PPP (Figure S1 and Table S2C). Sixty-eight genera were annotated, and 21 genera were significantly different between PPP and HCs (Figure 2A). The relative abundance (RA) of *Streptococcus* and *Morococcus* was significantly reduced in PPP while that of *Porphyromonas, Capnocytophaga, Fusobacterium, Leptotrichia, Tannerella, Filifactor, Peptostreptococcus, Parvimonas, Solobacterium, Ottowia, Desulfobulbus, Anaeroglobus, Eikenella, Bulleidia, Peptoanaerobacter, Dialister, Catonella*, and *Centipeda* were significantly enriched in PPP (Figure 2A and Table S2D). Two hundred and forty-five oral species were revealed, and 45 significantly different species were observed between two groups (Figure 2B). Thereinto, the RA of *Streptococcus vestibularis, Streptococcus mitis, Streptococcus pneumoniae, Streptococcus salivarius*, and *Morococcus cerebrosus* were significantly enriched in HCs, while the other 40 periodontal pathogens including *Anaeroglobus geminatus, Bulleidia extructa, Campylobacter showae, Capnocytophaga gingivalis, Catonella morbi, Centipeda periodontii, Desulfobulbus oralis, Dialister invisus, Eikenella corrodens, Eubacterium brachy, Eubacterium infirmum, Eubacterium nodatum, Eubacterium saphenum, Eubacterium yurii, Filifactor alocis, Fusobacterium nucleatum, Gemella bergeri, Gemella morbillorum, Leptotrichia hofstadii, Parvimonas micra, Peptostreptococcus stomatis, Porphyromonas gingivalis, Prevotella loescheii, Prevotella maculosa, Prevotella micans, Prevotella saccharolytica, Selenomonas sputigena, Solobacterium moorei, Streptococcus anginosus* group (SAG), *Tannerella forsythia, Treponema vincentii, Capnocytophaga gingivalis, Centipeda periodontii, Desulfobulbus oralis, Prevotella maculosa, Eubacterium infirmum, Selenomonas sputigena,* and etc were evidently abundant in the oral cavity of PPP (Figure 2B and Table S2E).

The abundance of species can provide insight into how a community functions, so we investigated the top abundant genera and species between PPP and HCs subsequently. During the top 15 abundant genera, five genera including *Porphyromonas, Capnocytophaga, Fusobacterium, Leptotrichia*, and Clostridiales Family_XIII_Incertae_Sedis_unclassified were significantly enriched in PPP, while *Streptococcus*, the most abundant genus in both groups, was significantly enriched in HCs (Figure S2). Among the top 20 abundant species, *Porphyromonas gingivalis* and *F. nucleatum* were significantly enriched in PPP, whereas *Streptococcus mitis* and *Streptococcus vestibularis* were definitely abundant in HCs (Figure S3).

Functional Characterization of the Oral Microbiota

HUMAnN 3.0 was used to efficiently and accurately profile the abundance of microbial metabolic pathways between PPP and HCs, which can also obtain the specific species contributing to the predicted functional modules (PFMs). Totally 9198 microbial metabolic pathway-microbiota interactions formed by 315 metabolic pathways and 195 oral microbiotas were observed (Table S2F). In which 840 interactions participated by 180 metabolic pathways and 57 oral microbiotas were significantly different. During the 315 PFMs, 87 pathways were significantly different between two groups ($P < 0.05$, Figure 3 and Table S2G). Eight pathways including beta-(1,4)-mannan degradation, superpathway of polyamine biosynthesis II,

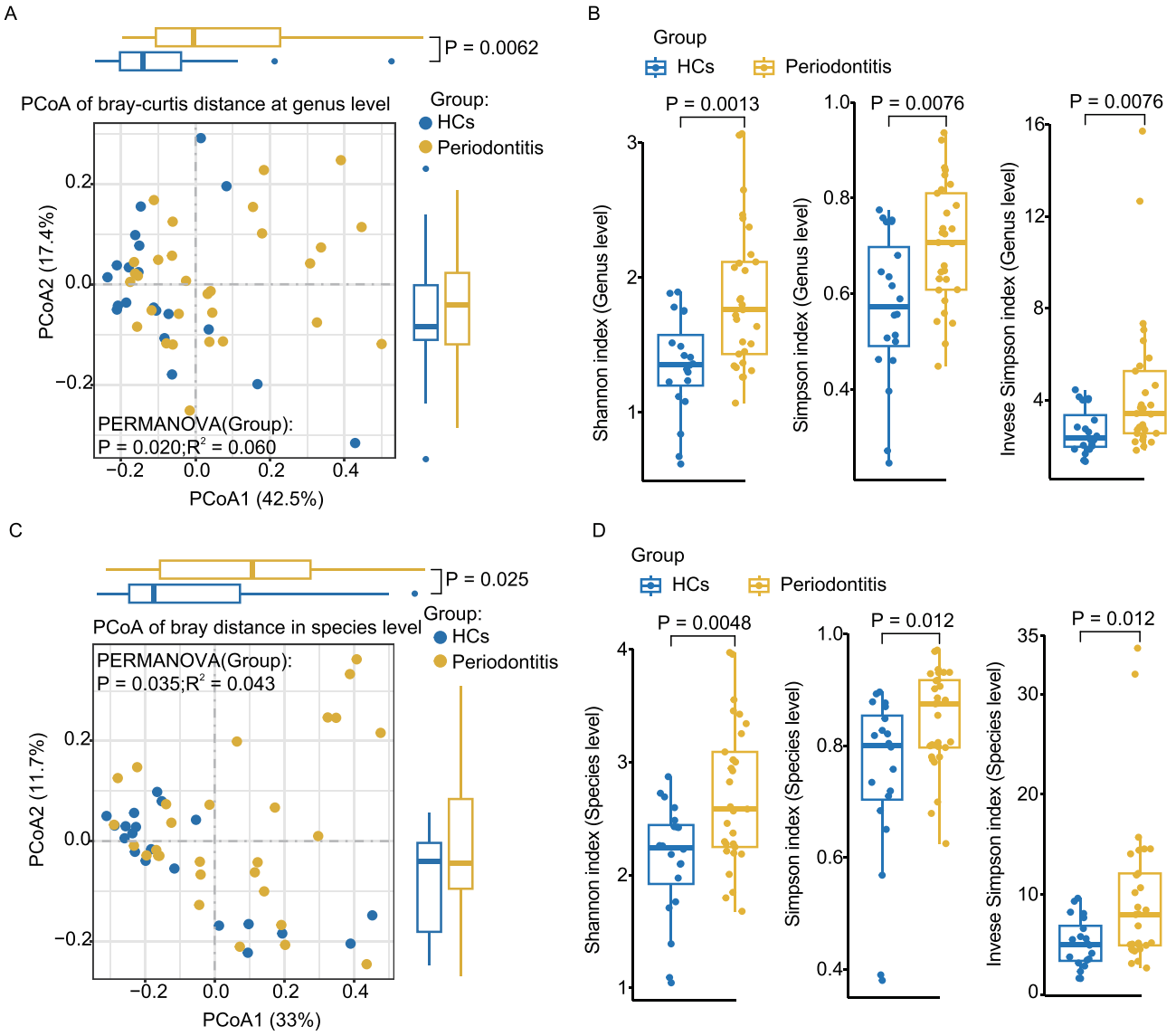

Figure 1 Diversity and richness of the oral microbiota in pregnant patients with periodontitis compared to HCs. (**A**) Genus-level beta diversity. (**B**) Genus-level alpha diversity. (**C**) Species-level beta diversity. (**D**) Species-level alpha diversity.

superpathway of menaquinol-8 biosynthesis II, superpathway of GDP-mannose-derived O-antigen building blocks biosynthesis, TCA cycle III (animals), Kdo transfer to lipid IVA III (Chlamydia), L-lysine fermentation to acetate and butanoate, 1.4-dihydroxy-6-naphthoate biosynthesis II were significantly enriched in PPP. Notably, no microbiota was involved in the first five pathways, while the last three pathways were participated by oral microbial species. The detailed interactions were shown in Table 1, from which we can easily observe that PPP-enriched species were significantly involved in these pathways. *P. gingivalis* was involved in Kdo transfer to lipid IVA III (Chlamydia); *F. nucleatum* was involved in L-lysine fermentation to acetate and butanoate; *Campylobacter showae, Centipeda periodontii, Selenomonas* sp. FOBRC6, *Selenomonas* sp. oral_taxon_126, *Selenomonas* sp. oral_taxon_138, *Selenomonas sputigena* were involved in 1.4-dihydroxy-6-naphthoate biosynthesis II. The other 79 microbial metabolic pathways including polysaccharides (sucrose, trehalose, and starch) degradation, glycogen degradation, glycolysis, TCA cycle, amino acids biosynthesis, ribonucleotides de novo biosynthesis, etc were significantly abundant in HCs. *S. australis, S. salivarius, S. pneumoniae, S. pneumoniae, S. mitis, S. vestibularis, H. sputorum, L. buccalis, M. cerebrums*, which were significantly enriched in HCs, were participated in the HCs-enriched pathways. These

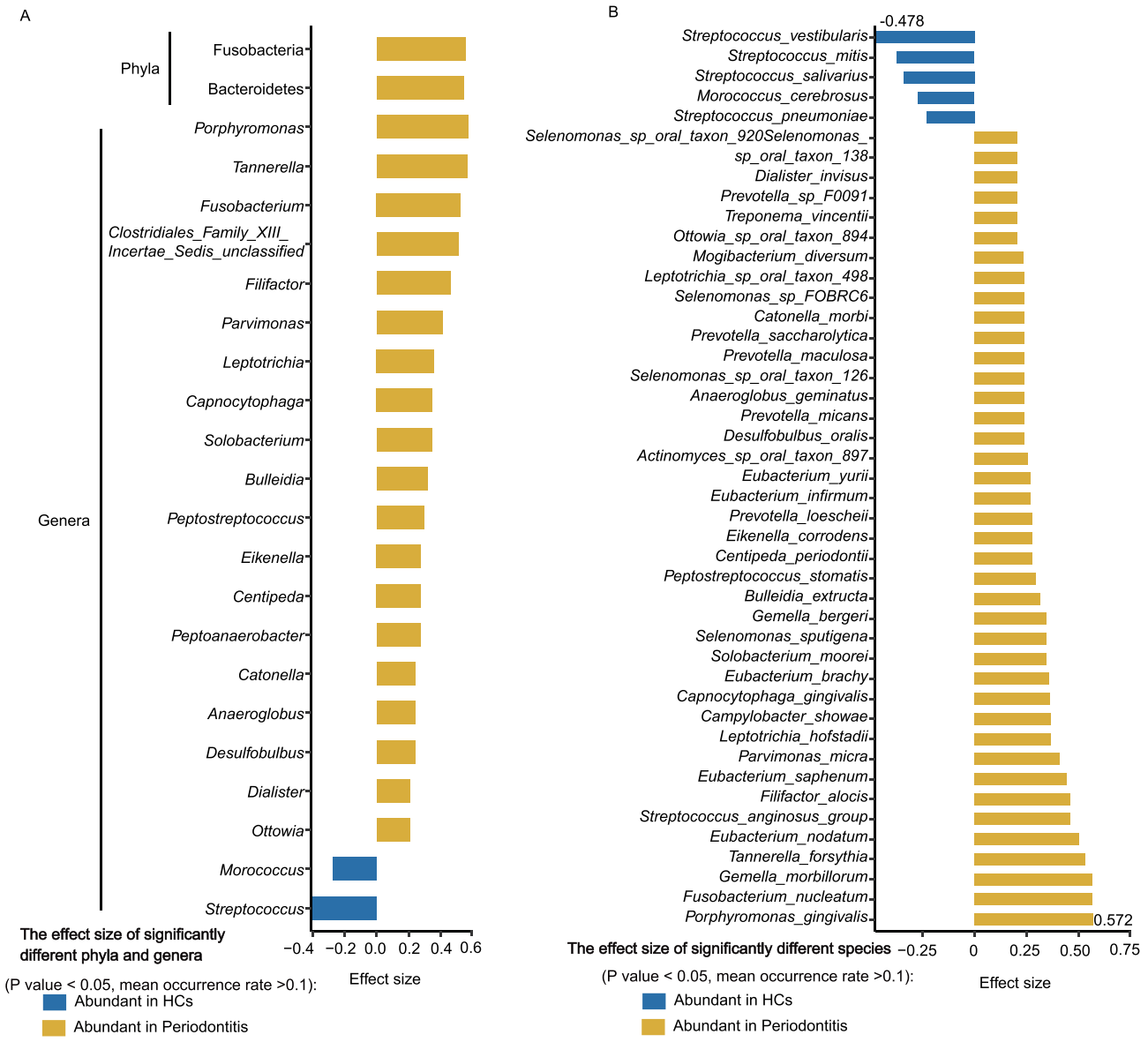

Figure 2 Pregnant patients with periodontitis exhibited significant differences in the composition of phyla, genera, and species compared to HCs. (**A**) Notable variations were observed in the phyla and genera between the two groups. (**B**) Distinct differences in species were noted between the two groups.

results suggested a dysfunction of the oral microbiota although their diversity and richness were significantly increased in PPP, suggesting that there might be more changes in metabolites in PPP.

We have also observed differences in the predicted functional pathways participated by the highly abundant species with significant differences between two groups, including *Fusobacterium nucleatum, Porphyromonas gingivalis, S. mitis, S. vestibularis*. Interestingly, the significantly different pathways participated by *F. nucleatum* and *P. gingivalis*, whose relative abundance was significantly higher in PPP, were all enriched in PPP (*S. vestibularis*, Figure S4; *P. gingivalis*, Figure S5). Conversely, the significantly different functional pathways participated by *S. mitis* and *S. vestibularis*, which were highly abundant in HCs, were all enriched in HCs (*S. mitis*, Figure S6; *S. vestibularis*, Figure S7). These results further substantiate the concordance across different species and functional pathways.

Oral Microbial Metabolites Examination and Analysis

To verify the changes in the oral microbial metabolites, untargeted metabolomics of the subgingival plaques was performed. PCoA analysis showed that the oral microbial metabolites were significantly different between the PPP

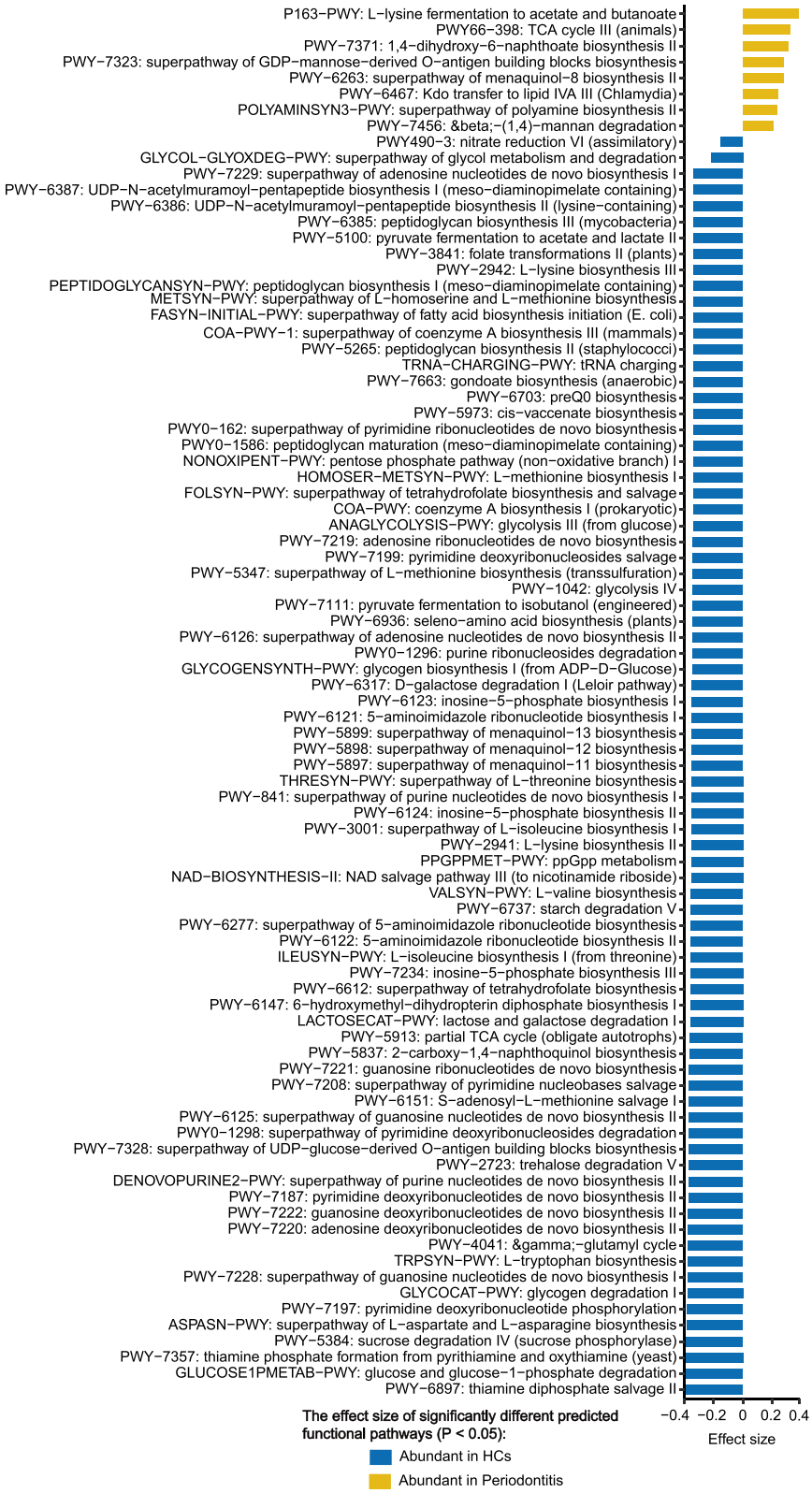

Figure 3 A total of 87 significantly altered predicted functional pathways were observed between the two groups, with only eight pathways being abundant in pregnant patients with periodontitis, while the remaining 79 pathways were enriched in HCs.

Table 1 The Microbial Metabolic Pathways Enriched in PPP and Their Interconnections with Associated Species

The HMP Unified Metabolic Analysis Network	P-value	Effect size	Mean occurrence in HCs	Mean occurrence in PPP	Mean Abundance of HCs	Mean Abundance of PPP	Enrichment
L-lysine fermentation to acetate and butanoate	0.0195	0.3793	0.4	0.6552	11.8303	74.1470	HCs < PPP
L-lysine fermentation to acetate and butanoate|*Fusobacterium hwasookii*	0.3528	0.0845	0.05	0.1379	1.1095	10.4848	HCs <= PPP
L-lysine fermentation to acetate and butanoate|*Fusobacterium nucleatum*	0.0109	0.3862	0.2	0.5517	3.7276	23.3747	HCs < PPP
L-lysine fermentation to acetate and butanoate|*Fusobacterium periodonticum*	0.0234	0.2724	0.05	0.3103	0.4844	5.4319	HCs < PPP
L-lysine fermentation to acetate and butanoate|*Fusobacterium* sp. oral_taxon_370	0.0906	0.1379	0	0.1379	0	3.5775	HCs <= PPP
TCA cycle III (animals)	0.0171	0.3241	0.1	0.4138	6.4702	63.0166	HCs < PPP
superpathway of GDP-mannose-derived O-antigen building blocks biosynthesis	0.0120	0.2759	0	0.2759	0	46.7112	HCs < PPP
superpathway of UDP-glucose-derived O-antigen building blocks biosynthesis	0.0310	−0.3672	0.95	0.8966	671.3250	486.4587	PPP < HCs
superpathway of UDP-glucose-derived O-antigen building blocks biosynthesis|*Actinomyces georgiae*	0.4302	0.0345	0	0.0345	0	0.0897	HCs <= PPP
superpathway of UDP-glucose-derived O-antigen building blocks biosynthesis|*Actinomyces* sp. oral_taxon_180	0.2473	0.0690	0	0.0690	0	3.9100	HCs <= PPP
superpathway of UDP-glucose-derived O-antigen building blocks biosynthesis|*Actinomyces* sp. oral_taxon_448	0.0909	−0.1	0.1	0	6.5036	0	PPP <= HCs
superpathway of menaquinol-8 biosynthesis II	0.0120	0.2759	0	0.2759	0	42.8798	HCs < PPP
andbeta;-(1,4)-mannan degradation	0.0336	0.2069	0	0.2069	0	32.3260	HCs < PPP
1,4-dihydroxy-6-naphthoate biosynthesis II	0.0070	0.3103	0	0.3103	0	30.2611	HCs < PPP
1,4-dihydroxy-6-naphthoate biosynthesis II|*Campylobacter concisus*	0.1487	0.1034	0	0.1034	0	2.0912	HCs <= PPP
1,4-dihydroxy-6-naphthoate biosynthesis II|*Campylobacter curvus*	NaN	0	0	0	0	0	HCs <≤ PPP
1,4-dihydroxy-6-naphthoate biosynthesis II|*Campylobacter gracilis*	0.1487	0.1034	0	0.1034	0	4.2221	HCs <= PPP
1,4-dihydroxy-6-naphthoate biosynthesis II|*Campylobacter rectus*	0.1487	0.1034	0	0.1034	0	1.4954	HCs <= PPP
1,4-dihydroxy-6-naphthoate biosynthesis II|*Campylobacter showae*	0.2473	0.0690	0	0.0690	0	0.5539	HCs <= PPP
1,4-dihydroxy-6-naphthoate biosynthesis II|*Centipeda periodontii*	0.4302	0.0345	0	0.0345	0	0.3463	HCs <= PPP
1,4-dihydroxy-6-naphthoate biosynthesis II|*Selenomonas artemidis*	0.4302	0.0345	0	0.0345	0	1.2879	HCs <= PPP
1,4-dihydroxy-6-naphthoate biosynthesis II|*Selenomonas infelix*	0.0906	0.1379	0	0.1379	0	1.6291	HCs <= PPP
1,4-dihydroxy-6-naphthoate biosynthesis II|*Selenomonas noxia*	0.1487	0.1034	0	0.1034	0	3.6595	HCs <= PPP
1,4-dihydroxy-6-naphthoate biosynthesis II|*Selenomonas* sp. FOBRC6	0.4302	0.0345	0	0.0345	0	0.1232	HCs <= PPP
1,4-dihydroxy-6-naphthoate biosynthesis II|*Selenomonas* sp. oral_taxon_126	0.1487	0.1034	0	0.1034	0	1.7529	HCs <= PPP
1,4-dihydroxy-6-naphthoate biosynthesis II|*Selenomonas* sp. oral_taxon_138	0.2473	0.0690	0	0.0690	0	0.4970	HCs <= PPP
1,4-dihydroxy-6-naphthoate biosynthesis II|*Selenomonas sputigena*	0.0553	0.1724	0	0.1724	0	4.1975	HCs <= PPP
superpathway of polyamine biosynthesis II	0.0405	0.2362	0.05	0.2759	0.7768	17.4912	HCs < PPP
Kdo transfer to lipid IVA III (Chlamydia)	0.0202	0.2414	0	0.2414	0	3.9337	HCs < PPP
Kdo transfer to lipid IVA III (Chlamydia)|*Porphyromonas gingivalis*	0.0202	0.2414	0	0.2414	0	3.7331	HCs < PPP

Figure 4 Differential microbial metabolome profiles were observed between the two groups. (**A**) PCoA analysis revealed significant differences in oral microbial metabolites. (**B**) Significantly distinct KEGG pathways were annotated for significantly altered metabolites. (**C**) There were notable differences in oral microbial metabolites between the two groups.

and HCs ($P = 1.3E^{-05}$, PCoA 1 = 22.2%, Figure 4A). A total of 1059 metabolites were measured for subjects from two groups, in which 482 significantly different metabolites were involved (Table S2H). Three hundred and eighty-four metabolites including carbocyclic thromboxane A2, glycine deoxycholic acid, 5-oxoETE, glycochenodeoxycholic acid, butyrate, etc were significantly abundant in PPP, while 98 metabolites including Inositol 1,3,4-trisphosphate, Indole-3-phosphate, 2.4-dihydroxy benzoic acid, D-galacturonic acid, 3-methoxytyramine, etc were evidently enriched in HCs (Figure S8).

To further explore the metabolites, we have annotated the metabolites to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Totally 452 metabolites were annotated with KEGG compound ID (C_num), and 151 metabolites were significantly different between two groups. Thereinto, 105 metabolites were highly abundant in PPP, while 46 metabolites were highly abundant in HCs (Table S2I and Figure 4C). During the 151 significantly different annotated metabolites, 105 metabolites were mapped with 82 KEGG pathways (ko_num, Figure 4B). Totally 638 metabolites and pathways counterparts were revealed (Table S2J). The top 10 pathways were explored, and we found that nine pathways including Metabolic pathways, Biosynthesis of secondary metabolites, Microbial metabolism in diverse environments, Biosynthesis of amino acids, Biosynthesis of cofactors, Purine metabolism, Nucleotide metabolism, 2-Oxocarboxylic acid metabolism, Protein digestion and absorption were highly enriched in PPP, while Microbial metabolism in diverse environments was similar in two groups (Figure 4B).

Subsequently, we have created schematic metabolic pathways that incorporate metabolites and their levels, including corresponding p-values, to intuitively demonstrate the relationship between metabolites and the pathways they participate in. During the 82 significantly different mapped KEGG pathways, we have removed pathways that do not belong to prokaryotes including Organismal Systems, the first-class KEGG pathways including metabolic pathways, and the overview pathways including metabolism and overview; carbon metabolism. Finally, 100 significantly different metabolites were annotated to 77 KEGG pathways (Table S2I). We can easily find that most of the metabolites were involved in substrates metabolism and signal transduction. ABC transporters, Purine metabolism, Arachidonic acid metabolism, Tryptophan metabolism, Tyrosine metabolism, etc were the pathways that more metabolites participate in. Adenosine 5-Monophosphate D-Glucose, L-Glutamine, L-Cysteine, Pyruvic Acid were involved in multiple metabolic pathways.

Correlation Analysis Between Oral Microbiota and Metabolic Contents

To further reveal the relationship between the oral microbiota and its metabolic function, both the composition of the oral microbiome and its functional modules were widely associated with the oral metabolites. For correlations between oral microbial composition and metabolites, significantly different species with an occurrence rate greater than 0.1 (n = 27) were correlated with significantly different metabolites annotated to the KEGG pathway (n = 105) (Table S2K and Figure 5). Interestingly, 22 periodontitis-enriched species including *E. nodatum, P. gingivalis, T. forsythia, E. saphenum, F. alocis, C. showae, F. nucleatum, P. micra, E. brachy, E. yurii* and etc were significantly positively correlated with PPP-abundant metabolites, such as amino acid and metabolites, glycerophospholipids, nucleotide and metabolites, carbohydrates and metabolites, organic acids, and fatty acyl. Accordingly, HCs-abundant species including *S. pneumoniae, M. cerebrosus, S. mitis, S. vestibularis*, and *S. salivarius* were positively correlated with HCs-enriched metabolites, but were negatively associated with the PPP-enriched metabolites. These results reminded us a bi-direction between the oral microbiota and microbial metabolites of periodontitis patients during pregnancy.

For correlations between oral microbial function and metabolites, significantly different functional modules (n = 87) were correlated with significantly different metabolites annotated to the KEGG pathway (n = 105) (Table S2L). We have focused on the relationship between the PPP-enriched eight pathways and their related metabolites (Table S2M). Interestingly, most of these pathways including PWY-6467: Kdo transfer to lipid IVA III (Chlamydia), PWY-7323: superpathway of GDP-mannose-derived O-antigen building blocks biosynthesis, PWY-7371: 1.4-dihydroxy-6-naphthoate biosynthesis II were significantly positively correlated with PPP-enriched metabolites such as 9-oxoODE, 13-oxoODE, Thiamine, 8.9-EET, butyrate, etc, but were significantly negatively correlated with the HCs-enriched metabolites. These results reminded us of the correlation between the oral microbial function and the metabolites.

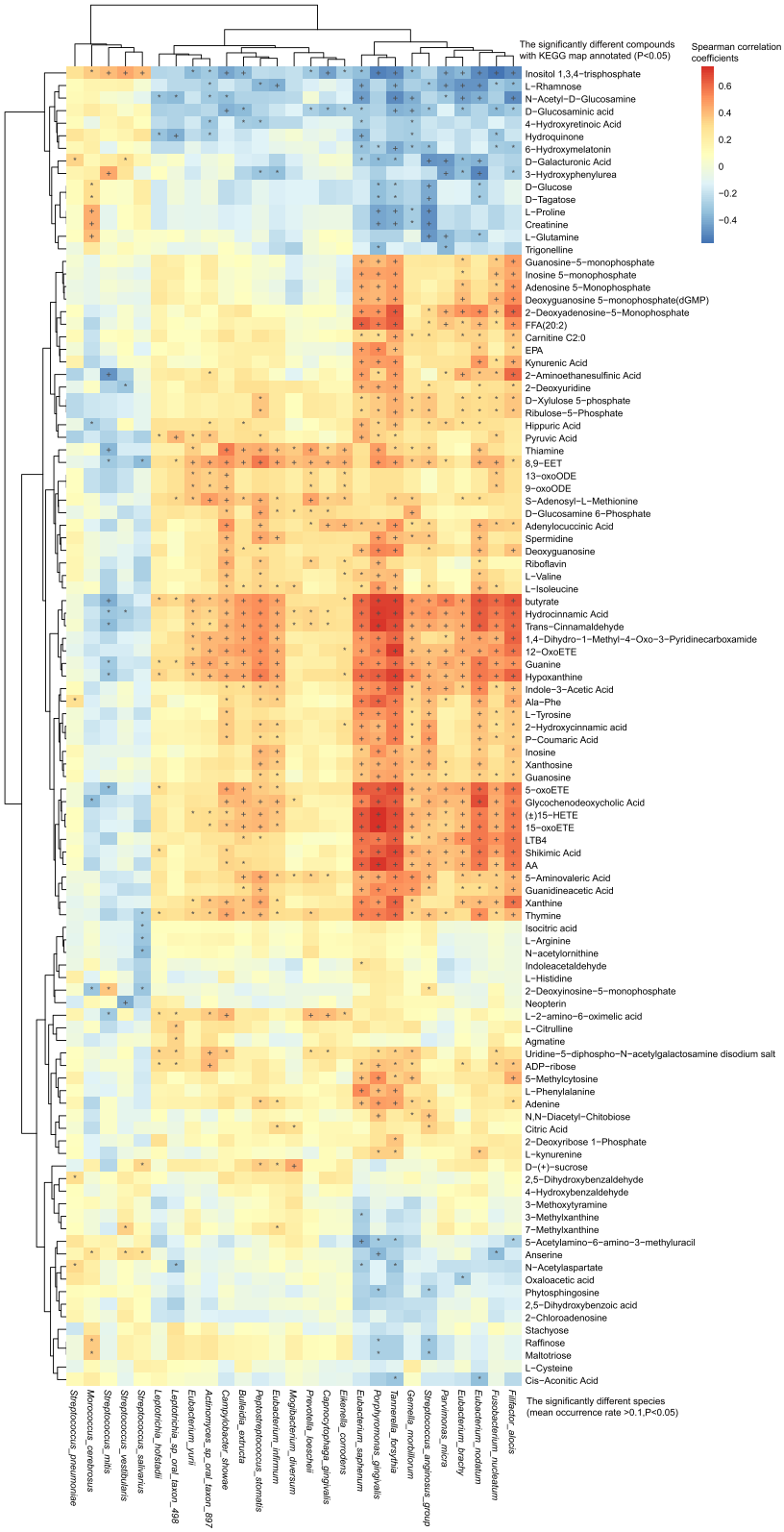

Figure 5 Correlation analysis was conducted between 105 significantly different metabolites and 27 significantly different species with a mean occurrence rate exceeding 0.1 (in at least 1 sample out of 10). The periodontitis-enriched species (depicted in black) exhibited positive associations with periodontitis-abundant metabolites, as did the healthy controls (depicted in blue). + and * denote the P-value. +P < 0.01; *P < 0.05.

Discussion

Oral microbial dysbiosis has been linked to the presence of oral diseases such as chronic periodontitis and adverse pregnancy outcomes. However, at present, the oral microbiota and metabolites in pregnant women with periodontitis remain unexplored at present. This study aimed to elucidate the oral microbiota and metabolome in pregnant women with and without periodontitis by integrating shotgun metagenomic sequencing and untargeted metabolomic analysis of 49 subgingival plaque samples from 29 pregnant patients with periodontitis (PPP) and 20 hCs. The findings revealed a significant increase in the oral microbial diversity and richness, as well as altered microbial metabolites, alongside a decrease in predicted functional modules (PFMs) in PPP, thus providing potential biomarkers for distinguishing PPP from HCs.

This study enrolled pregnant patients with periodontitis in the second trimester of pregnancy, a period that is typically associated with greater physical comfort for most women and a relatively low risk to the fetus. An appreciable increase in the diversity and richness of oral microbial communities was observed in PPP compared to HCs, potentially attributed to the influence of pregnancy and periodontitis. The observed phenomenon aligns with the findings of a previous study conducted by Wu et al, wherein they reported a significant reduction in alpha diversity of the plaque microbial community at the 1-week follow-up ($p < 0.05$) after Prenatal Total Oral Rehabilitation (PTOR) compared to baseline.[25] In the subsequent analysis, a total of two phyla, twenty-one genera, and forty-five distinct species were found to be significantly different between the two groups. Genera including *Streptococcus* and *Morococcus* were highly abundant in HCs, while *Porphyromonas* was enriched in PPP. *Porphyromonas* was also found enriched in pregnant women with gingivitis.[26] However, we have also identified distinct genera in periodontitis that differ from those found in gingivitis during pregnancy. This suggests that while both are oral diseases occurring during pregnancy, there are similarities and differences in the bacterial composition between these conditions. These variations may be attributed to differences in sample collection methods; for instance, the investigation of salivary and supragingival plaque microbiomes for gingivitis versus the exploration of subgingival microbiota for periodontitis. This underscores the importance of careful consideration when selecting and collecting samples.

For species, five normal oral commensals of the human oral microbiota, including *Streptococcus pneumoniae*,[27] *S. salivarius*,[28] *S. mitis*,[29] *S. vestibularis*,[30] and *Morococcus cerebrosus*,[31] exhibited a significant reduction in PPP. This marked decrease in these commensals suggests an oral microbial dysbiosis. Conversely, there was a significant enrichment of 40 periodontal pathogens, such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, in PPP compared to HCs. *P. Gingivalis* has been linked to preterm birth.[1] Gram-negative anaerobic bacteria, including *Tannerella forsythia* and *Fusobacterium nucleatum*, have been identified as keystone periodontal pathogens.[32,33] Interestingly, a lower relative abundance of *Tannerella forsythia* and *Treponema denticola* was found at 2 weeks after Prenatal Total Oral Rehabilitation (PTOR) compared to the baseline, which was consistent with our results.[25] Significantly, we have also demonstrated the presence of *Treponema* spp. including *Treponema vincentii* in the human oral cavity, where it is frequently associated with periodontal disease.[34] The facultative anaerobe *Gemella morbillorum* is a rarely isolated bacterium in the oral cavity but is increasingly implicated in infectious endocarditis.[35] *Eubacterium nodatum, E. brachy, E. infirmum*, and *E. yurii* were primarily isolated from subgingival samples taken from patients with moderate and severe periodontitis.[36] The *Streptococcus anginosus* group (SAG) was initially recovered from dental abscesses causing oral infections and is frequently isolated from dental caries and periodontal diseases.[37] *Eubacterium saphenum* is an unculturable bacterium related to periodontitis.[38] *Parvimonas micra*,[39] subgingival plaque species *Leptotrichia hofstadii, Campylobacter showae*,[40] *Capnocytophaga gingivalis*,[41] *Bulleidia extructa*,[42] *Peptostreptococcus stomatis*,[43] *Centipeda periodontii*[44] have all been reported as human periodontal pathogens. *Selenomonas sputigena*, helps to form biofilms and produce damaging acid to cause tooth decay,[45] was reported to be associated with the production of volatile sulphur compounds (VSCs) and can be found in subgingival plaques of deep periodontal pockets. Dental plaque species *Eikenella corrodens* is a facultative gram-negative bacillus which is a common inhabitant of the oral cavity both in periodontally healthy individuals and in periodontitis patients.[46] *Prevotella* strains, including *P. loescheii, P. maculosa, P. micans, P. saccharolytica*, may be clinically important pathobionts that can participate in human disease by promoting chronic inflammation.[47] *Anaeroglobus geminatus* is a relatively newly discovered putative pathogen, with a potential role in the microbial shift associated with periodontitis.[48] *Haemophilus parainfluenzae* could be biomarkers of periodontitis.[49,50] *Desulfobulbus oralis*, significantly associated with

periodontitis.[51] *Selenomonas* spp. including *S. sputigena* and *S. noxia* were observed increased in the subgingival plaque of young periodontal patients. Oral anaerobe *Mogibacterium diversum* is associated with initial periodontitis in adults.[52] *Dialister invisus* is a periodontal pathogen after enrichment from the biofilms of healthy adults.[53] In conclusion, our findings have unveiled substantial alterations in the oral microbial composition in PPP. The majority of our results align with prior studies, underscoring that the oral microbiota of periodontal patients during pregnancy closely resembles that of non-pregnant periodontal patients, despite the potential impact of pregnancy on periodontitis.

We have then analyzed the oral microbial functional potential. Eighty-seven significantly different functional pathways were observed between PPP and HCs, in which eight pathways including L-lysine fermentation to acetate and butanoate, TCA cycle III (animals), superpathway of GDP-mannose-derived O-antigen building blocks biosynthesis, superpathway of menaquinol-8 biosynthesis II, β-(1,4)-mannan degradation, 1.4-dihydroxy-6-naphthoate biosynthesis II, superpathway of polyamine biosynthesis II, Kdo transfer to lipid IVA III (Chlamydia) were associated with PPP. Thereinto, periodontal pathogen *P. gingivalis* was involved in Kdo transfer to lipid IVA III. *F. nucleatum* was involved in L-lysine fermentation to acetate and butanoate. *Campylobacter showae, Centipeda periodontii, Selenomonas sputigena* etc were involved in 1.4-dihydroxy-6-naphthoate biosynthesis II. The other 79 pathways including superpathway of L-methionine biosynthesis, glycogen biosynthesis and degradation, carbohydrate metabolism, polysaccharide degradation, amino acids metabolism, and adenosine/guanosine ribonucleotides/deoxyribonucleotides de novo biosynthesis were significantly abundant in HCs. Remarkably, our functional findings were in line with previous research. In a study by Wu et al, significant changes in the *Actinomyces defective*-associated carbohydrate degradation pathway and *Streptococcus Gordonii*-associated fatty acid biosynthesis pathway were observed in pregnant patients with oral disease who received Prenatal Total Oral Rehabilitation compared to baseline.[25] In another relevant study, Manzoor et al found associations of L-methionine, glycogen, adenosine-5'-phosphate biosynthesis with generalized gingivitis/initial periodontitis (GG/IP).[26] In addition to energy metabolism, nitrate reduction VI, pyruvate fermentation to acetate and lactate II, peptidoglycan biosynthesis was also observed to be increased in HCs. These results suggested a reduction of the subgingival microbiome function in PPP although the oral microbial richness and diversity were significantly increased in PPP.

To further explore the functional changes of the oral microbiota in PPP, the oral microbial metabolites were detected using ultra lipid chromatography-mass spectrometry (UPLC-MS). Notably, metabolomics analysis combined with enrichment analysis showed that 482 significantly different metabolites were identified, including 98 metabolites mainly involved in metabolic pathways, biosynthesis of secondary metabolites, nucleotide metabolism; purine metabolism, biosynthesis of amino acids, biosynthesis of cofactors, ABC transporters microbial metabolism in diverse environments, 2-oxocarboxylic acid metabolism, were significantly abundant in HCs. These findings were in line with the literature, indicating that periodontitis had a significant impact on the levels of metabolites involved in amino acid biosynthesis (eg, alanine, leucine, isoleucine, and valine), galactose metabolism (eg, myo-inositol, galactose, glucose, and hexitol), and pyrimidine metabolism (eg, uracil, uridine, beta alanine, and thymine).[54] The remaining 384 metabolites such as organic acid, butyrate, isobutyric acid, 8.9-EET, Trp-Pro, 16-HDoHE, Trp-Ile, 11/15-HEDE, Pro-Gln, Sphingosyl-phosphocholine, LPC, PC, 15-oxoETE, Gly-Phe, etc, mainly participated in metabolic pathways, microbial metabolism in diverse environments, biosynthesis of secondary metabolites, were significantly abundant in PPP. Interestingly, in a recent study, Gan et al revealed that chronic apical periodontitis accelerates atherosclerosis via gut microbiota dysbiosis and altered metabolites such as disruptions in lipid metabolism and primary bile acid synthesis, leading to elevated levels of taurochenodeoxycholic acid, taurocholic acid, and tauroursodeoxycholic acid to contribute to atherosclerosis development in apoE-/- mice on a high-fat diet.[55] Importantly, PPP-enriched 3-Hydroxy-L-phenylalanine and L-Phenylalanine, as well as PPP-decreased N-Acetyl-D-Glucosamine have been reported as biomarkers for periodontitis in previous studies.[56] These findings indicate that PPP exhibits a high capability for energy metabolism and possibility of atherosclerosis development compared to HCs.

Finally, we have investigated the relationship and connections between the oral microbiota and metabolites, as microbial structure determines function. The oral microbiome is shaped throughout life by various factors including host genetics, maternal transmission, dietary habits, oral hygiene practice, medications, and systemic factors.[57] Our study delved into the oral microbiota and metabolites of pregnant women with periodontitis, where hormonal levels and oral hygiene were identified as key influencers of the oral microbiota. Given that both PPP participants and HCs were from the same city in Northwest China with similar oral hygiene care practices, our study's credibility is enhanced. Correlation

analysis revealed a significant positive correlation between PPP-enriched metabolites and PPP-abundant species; a similar trend was observed in HCs. Furthermore, the predicted functional modules enriched in PPP showed a significant positive association with PPP-enriched metabolites. These findings underscore the consistency of changes in oral microbial composition, function, and metabolites during pregnancy-related periodontitis.

In the future, there is still potential for improvement: 1) The diagnosis in this study was based on clinical examination including BI, CI-S, AL, PD, and visible gingival redness and swelling; we will try to improve the poor patient compliance to obtain the oral X-rays from the PPP; in the next study, efforts will be made to enhance patient compliance for obtaining oral X-rays from the PPP to ensure accurate assessment of bone loss since dental radiographs are considered safe during pregnancy; 2) Our study represents the first study of oral microbiome and metabolism in pregnant women with and without periodontitis, although most of the oral pathogens and some of the functional pathways revealed in our study were consistent with those observed in non-pregnant patients with periodontitis (details were compared and depicted in the second, third, and fourth paragraphs of the discussion section), there was limited information available on oral microbial metabolites. Therefore, we plan to expand the sample size to encompass a larger cohort of pregnant patients with periodontitis from diverse regions in China, such as Lanzhou, Xi'an, Xi'ning, Guangzhou etc, to ensure the generalizability of our findings across different geographical areas since the maternal periodontal disease is a highly prevalent condition that has been studied extensively in relation to adverse pregnancy outcomes. 3) The underlying mechanisms by which oral pathogens contribute to the onset and exacerbation of periodontitis during pregnancy need to be elucidated through in vivo (Wistar rat were divided into several groups: male, female, and gestational rats; oral pathogens and LPS were injected into the gingiva of the second mandibular molar of rats; then the rats were sacrificed on the 3rd, 7th, and 10th day of modeling, and the jaws were analyzed for histology and immunocytochemistry).

Conclusions

In conclusion, our findings have revealed significant alterations in the oral microbial composition and function, as well as metabolic profile in pregnant women with and without periodontitis. Our results not only offer a more convenient, less harmful and robust auxiliary method for detecting periodontitis during pregnancy due to the ease of sample collection compared to conventional detection methods, but also provide further insights into mechanism investigation and intervention strategies targeting periodontitis during pregnancy. Additionally, the study contributes to identifying molecular signatures for drug development and targeted therapies, ultimately contributing to personalized and precision medicine.

Data Sharing Statement

The data that support the findings of this study are openly available at the China National GeneBank Database (CNGBdb) at https://db.cngb.org/, reference number CNP0004326.

Ethics Approval Statement

This study involves the use of human subjects and has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). The study was approved by the Medical Research Ethics Review Committee of General Hospital of Ningxia Medical University (No. 2020-682) on August 26, 2020.

Patient Consent Statement

Informed consent and questionnaire related to oral hygiene were obtained from each subject.

Acknowledgments

We give our sincere thanks to the volunteers involved for this study. Also, we thank Wuhan Metware Biotechnology Co., Ltd. and BGI-Shenzhen for their metabolomic detection and shotgun metagenomic sequencing in this study.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work. The submitting author has been authorised by all co-authors to submit the research article.

Funding

This work was supported by the Ningxia Natural Science Foundation (Grant No. 2020AAC03356); Western Clinical and Research Fund Project of Chinese Stomatological Association (Grant No. CSA-W2023-04); Natural Science Foundation of Gansu Province (Grant No. 22JR11RA058); Cuiying Scientific Training Program for Undergraduates of Lanzhou University Second Hospital (Grant No. CYXZ2023-05).

Disclosure

The authors declare no conflict of interest.

References

1. Jang H, Patoine A, T WT., et al. Oral microflora and pregnancy: a systematic review and meta-analysis. *Sci Rep*. 2021;11(1):16870. doi:10.1038/s41598-021-96495-1
2. Saadaoui M, Singh P, Al KS. Oral microbiome and pregnancy: a bidirectional relationship. *J Reprod Immunol*. 2021;145:103293. doi:10.1016/j.jri.2021.103293
3. Li W, Liang H, Lin X, et al. A catalog of bacterial reference genomes from cultivated human oral bacteria. *NPJ Biofilms Microbiomes*. 2023;9(1):45. doi:10.1038/s41522-023-00414-3
4. Yang P, Ma G, Lu T, et al. The influence of the oral microbiota in full-term pregnant women on immune regulation during pregnancy. *J Reprod Immunol*. 2024;165:104298. doi:10.1016/j.jri.2024.104298
5. Hajishengallis G, Chavakis T. Local and systemic mechanisms linking periodontal disease and inflammatory comorbidities. *Nat Rev Immunol*. 2021;21(7):426–440. doi:10.1038/s41577-020-00488-6
6. Tettamanti L, Lauritano D, Nardone M, et al. Pregnancy and periodontal disease: does exist a two-way relationship? *Oral Implantol*. 2017;10(2):112–118. doi:10.11138/orl/2017.10.2.112
7. M CC, J KP, B WK, et al. The oral microbiome and adverse pregnancy outcomes. *Int J Womens Health*. 2017;9:551–559. doi:10.2147/IJWH.S142730
8. Puertas A, Magan-Fernandez A, Blanc V, et al. Association of periodontitis with preterm birth and low birth weight: a comprehensive review. *J Matern Fetal Neonatal Med*. 2018;31(5):597–602. doi:10.1080/14767058.2017.1293023
9. Moore S, Ide M, Y CP, et al. A prospective study to investigate the relationship between periodontal disease and adverse pregnancy outcome. *Br Dent J*. 2004;197(5):251–258,247. doi:10.1038/sj.bdj.4811620
10. Bourgeois D, Inquimbert C, Ottolenghi L, et al. Periodontal Pathogens as Risk Factors of Cardiovascular Diseases, Diabetes, Rheumatoid Arthritis, Cancer, and Chronic Obstructive Pulmonary Disease-Is There Cause for Consideration? *Microorganisms*. 2019;7(10):424. doi:10.3390/microorganisms7100424
11. Curtis MA, Diaz PI, Van Dyke TE, Mariano RJ. The role of the microbiota in periodontal disease. *Periodontal*. 2020;83(1):14–25. doi:10.1111/prd.12296
12. Abusleme L, K DA, Dutzan N, et al. The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *ISME J*. 2013;7(5):1016–1025. doi:10.1038/ismej.2012.174
13. Griffen A L, J BC, H CJ, et al. Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. *ISME J*. 2012;6(6):1176–1185. doi:10.1038/ismej.2011.191
14. Zaura E, W BB, Prodan A, et al. On the ecosystemic network of saliva in healthy young adults. *ISME J*. 2017;11(5):1218–1231. doi:10.1038/ismej.2016.199
15. Li Y, Qian F, Cheng X, et al. Dysbiosis of Oral Microbiota and Metabolite Profiles Associated with Type 2 Diabetes Mellitus. *Microbiol Spectr*. 2023;11(1):e379622.
16. Barbour A, Elebyary O, Fine N, et al. Metabolites of the oral microbiome: important mediators of multikingdom interactions. *FEMS Microbiol Rev*. 2022;46(1). doi:10.1093/femsre/fuab039
17. D LA, B SJ, G WW, et al. Microbial beta-glucuronidases drive human periodontal disease etiology. *Sci Adv*. 2023;9(18):g3390. doi:10.1126/sciadv.adg3390
18. C AG, R DW, Jenderseck R S, et al. Relationship between the percentage of subgingival spirochetes and the severity of periodontal disease. *J Periodontal*. 1982;53(9):550–556. doi:10.1902/jop.1982.53.9.550
19. Lu H, Zou P, Zhang Y, et al. The sampling strategy of oral microbiome. *Imeta*. 2022;1(2):e23. doi:10.1002/imt2.23
20. Rosenbaum J, Usyk M, Chen Z, et al. Evaluation of Oral Cavity DNA Extraction Methods on Bacterial and Fungal Microbiota. *Sci Rep*. 2019;9(1):1531. doi:10.1038/s41598-018-38049-6
21. C AG, Flores-Vergara M A, Krasynanski S, et al. A modified protocol for rapid DNA isolation from plant tissues using cetyltrimethylammonium bromide. *Nat Protoc*. 2006;1(5):2320–2325. doi:10.1038/nprot.2006.384

22. Fang C, Zhong H, Lin Y, et al. Assessment of the cPAS-based BGISEQ-500 platform for metagenomic sequencing. *Gigascience*. 2018;7(3):1–8. doi:10.1093/gigascience/gix133
23. Beghini F, J ML, Blanco-Miguez A, et al. Integrating taxonomic, functional, and strain-level profiling of diverse microbial communities with bioBakery 3. *Elife*. 2021;10.
24. Blanco-Miguez A, Beghini F, Cumbo F, et al. Extending and improving metagenomic taxonomic profiling with uncharacterized species using MetaPhlAn 4. *Nat Biotechnol*. 2023;41(11):1633–1644. doi:10.1038/s41587-023-01688-w
25. T WT, Sohn M, Manning S, et al. Metagenomic analysis examines oral microbiome changes and interplay with immune response following prenatal total oral rehabilitation. *J Transl Med*. 2023;21(1):172. doi:10.1186/s12967-023-03997-9
26. Manzoor M, Leskela J, Pietiainen M, et al. Shotgun metagenomic analysis of the oral microbiome in gingivitis: a nested case-control study. *J Oral Microbiol*. 2024;16(1):2330867. doi:10.1080/20002297.2024.2330867
27. Weiser JN, Ferreira DM, Paton JC. Streptococcus pneumoniae: transmission, colonization and invasion. *Nat Rev Microbiol*. 2018;16(6):355–367. doi:10.1038/s41579-018-0001-8
28. Wilson M, Martin R, T WS, et al. Clinical and laboratory features of Streptococcus salivarius meningitis: a case report and literature review. *Clin Med Res*. 2012;10(1):15–25. doi:10.3121/cmr.2011.1001
29. Mitchell J. Streptococcus mitis: walking the line between commensalism and pathogenesis. *Mol Oral Microbiol*. 2011;26(2):89–98. doi:10.1111/j.2041-1014.2010.00601.x
30. D SA, Sezer S, Ozdemir N F, et al. Streptococcus vestibularis bacteremia following dental extraction in a patient on long-term hemodialysis: a case report. *NDT Plus*. 2008;1(4):276–277. doi:10.1093/ndtplus/sfn071
31. Wang Q, Chen X, Hu H, et al. Structural changes in the oral microbiome of the adolescent patients with moderate or severe dental fluorosis. *Sci Rep*. 2021;11(1):2897. doi:10.1038/s41598-021-82709-z
32. Hajishengallis G, Lamont RJ. Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. *Mol Oral Microbiol*. 2012;27(6):409–419. doi:10.1111/j.2041-1014.2012.00663.x
33. S SS, Haffajee A D, A CM, et al. Microbial complexes in subgingival plaque. *J Clin Periodontal*. 1998;25(2):134–144. doi:10.1111/j.1600-051X.1998.tb02419.x
34. Zeng H, Chan Y, Gao W, et al. Diversity of Treponema denticola and Other Oral Treponeme Lineages in Subjects with Periodontitis and Gingivitis. *Microbiol Spectr*. 2021;9(2):e70121. doi:10.1128/Spectrum.00701-21
35. Desai AK, Bonura EM. Multi-valvular infective endocarditis from Gemella morbillorum. *BMJ Case Rep*. 2021;14(7).
36. Hill GB, Ayers OM, Kohan AP. Characteristics and sites of infection of Eubacterium nodatum, Eubacterium timidum, Eubacterium brachy, and other asaccharolytic eubacteria. *J Clin Microbiol*. 1987;25(8):1540–1545. doi:10.1128/jcm.25.8.1540-1545.1987
37. B LK, Pasquill K, C PE, et al. Streptococcus anginosus group bloodstream infections in the western interior of British Columbia, Canada. *Infect Dis (Lond)*. 2018;50(6):423–428. doi:10.1080/23744235.2017.1416163
38. Ye C, Xia Z, Tang J, et al. Unculturable and culturable periodontal-related bacteria are associated with periodontal inflammation during pregnancy and with preterm low birth weight delivery. *Sci Rep*. 2020;10(1):15807.
39. Rams TE, Sautter JD, van Winkelhoff AJ. Antibiotic Resistance of Human Periodontal Pathogen Parvimonas micra Over 10 Years. *Antibiotics*. 2020;9(10).
40. Macuch PJ, Tanner AC. Campylobacter species in health, gingivitis, and periodontitis. *J Dent Res*. 2000;79(2):785–792. doi:10.1177/00220345000790021301
41. Idate U, Bhat K, Kotrashetti V, et al. Molecular identification of Capnocytophaga species from the oral cavity of patients with chronic periodontitis and healthy individuals. *J Oral Maxillofac Pathol*. 2020;24(2):397. doi:10.4103/jomfp.JOMFP_33_20
42. Morgan MA, Goldstein EJ. Bulleidia extructa: an underappreciated anaerobic pathogen. *Anaerobe*. 2021;69:102339. doi:10.1016/j.anaerobe.2021.102339
43. Ko Y, M LE, C PJ, et al. Salivary microbiota in periodontal health and disease and their changes following nonsurgical periodontal treatment. *J Periodontal Implant Sci*. 2020;50(3):171–182. doi:10.5051/jpis.2020.50.3.171
44. E RT, Hawley CE, J WE, et al. Centipeda periodontii in human periodontitis. *Odontology*. 2015;103(3):286–291. doi:10.1007/s10266-014-0166-1
45. Cho H, Ren Z, Divaris K, et al. Selenomonas sputigena acts as a pathobiont mediating spatial structure and biofilm virulence in early childhood caries. *Nat Commun*. 2023;14(1):2919. doi:10.1038/s41467-023-38346-3
46. Chen CK, Wilson ME. Eikenella corrodens in human oral and non-oral infections: a review. *J Periodontal*. 1992;63(12):941–953. doi:10.1902/jop.1992.63.12.941
47. Larsen JM. The immune response to Prevotella bacteria in chronic inflammatory disease. *Immunology*. 2017;151(4):363–374. doi:10.1111/imm.12760
48. Bao K, Bostanci N, Thurnheer T, et al. Proteomic shifts in multi-species oral biofilms caused by Anaeroglobus geminatus. *Sci Rep*. 2017;7(1):4409. doi:10.1038/s41598-017-04594-9
49. Hernandez M, Mayer M, Santi-Rocca J. Editorial: the Human Microbiota in Periodontitis. *Front Cell Infect Microbiol*. 2022;12:952205. doi:10.3389/fcimb.2022.952205
50. Bourgeois D, Inquimbert C, Ottolenghi L, et al. Periodontal Pathogens as Risk Factors of Cardiovascular Diseases, Diabetes, Rheumatoid Arthritis, Cancer, and Chronic Obstructive Pulmonary Disease-Is There Cause for Consideration? *Microorganisms*. 2019;7(10):424.
51. Cai Z, Lin S, Hu S, et al. Structure and Function of Oral Microbial Community in Periodontitis Based on Integrated Data. *Front Cell Infect Microbiol*. 2021;11:663756. doi:10.3389/fcimb.2021.663756
52. Tanner AC. Anaerobic culture to detect periodontal and caries pathogens. *J Oral Biosci*. 2015;57(1):18–26. doi:10.1016/j.job.2014.08.001
53. Naginyte M, Do T, Meade J, et al. Enrichment of periodontal pathogens from the biofilms of healthy adults. *Sci Rep*. 2019;9(1):5491. doi:10.1038/s41598-019-41882-y
54. Shi M, Wei Y, Nie Y, et al. Alterations and correlations in microbial community and metabolome characteristics in generalized aggressive periodontitis. *Front Microbiol*. 2020;11:573196. doi:10.3389/fmicb.2020.573196
55. Gan G, Lin S, Luo Y, et al. Unveiling the oral-gut connection: chronic apical periodontitis accelerates atherosclerosis via gut microbiota dysbiosis and altered metabolites in apoE−/− Mice on a high-fat diet. *Int J Oral Sci*. 2024;16(39). doi:10.1038/s41368-024-00301-3

56. Huang Y, Zhou P, Liu S, et al. Metabolome and microbiome of chronic periapical periodontitis in permanent anterior teeth: a pilot study. *BMC Oral Health*. 2021;21(1):599. doi:10.1186/s12903-021-01972-8
57. Sedghi L, DiMassa V, Harrington A, et al. The oral microbiome: role of key organisms and complex networks in oral health and disease. *Periodontal*. 2021;87(1):107–131. doi:10.1111/prd.12393