JOURNAL OF ORAL MICROBIOLOGY
2023, VOL. 15, 2229693
https://doi.org/10.1080/20002297.2023.2229693

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AC-MICROBIOME MODULATORS AND ORAL HEALTH

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Exploration of the interplay between spatially distinct microbial habitats through comparative analysis

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ABSTRACT

Objectives: The oral microbiome is closely associated with systemic diseases, indicating the presence of bacteremia and inflammatory mediators in the systemic circulation. Our research aims to investigate the relationship between the oral microbiome and other microbial habitats.

Methods: We analyzed 180 specimens from 36 patients, including saliva, buccal swab, plaque, stool, and blood samples from a healthy group (Non_PD, $n = 18$) and a periodontitis group (PD, $n = 18$). The final analysis included 147 specimens, with varying sample sizes for each group. Metagenomic analysis was performed using prokaryotic 16S rRNA on the MiSeq platform (Illumina).

Results: PD saliva showed significant richness differences (P's < 0.05), similar to plaque. Buccal swabs had slight variations. Microbial network analysis revealed altered microbial interactions in the PD group, with decreased interactions in saliva and buccal swabs, and increased interactions in plaque. In our analysis of nine specimens where all paired habitat samples could be analyzed, microorganisms linked to oral periodontitis were found in sterile blood samples, resembling the oral cavity's composition.

Conclusions: Microbiome differences should consider overall microbial-environment interactions, alongside diversity and richness. Our data cautiously suggest that disease-related changes in the salivary microbiome may be reflected in blood specimens through the oral-blood axis.

ARTICLE HISTORY
Received 10 April 2023
Revised 18 June 2023
Accepted 20 June 2023

KEYWORDS
Microbiome; habitat; saliva; blood; plaque; genomics; periodontitis

Introduction

Our bodies unknowingly engage in cohabiting relationships with trillions of microbes. The human oral cavity alone harbors at least 600 prevalent species belonging to 13 well-known phyla, as documented in the Human Oral Microbiome Database [1]. These microorganisms constitute approximately 2% of an adult's body mass (1.5 kg) [2], equivalent to the size of the human brain or liver [3]. Different microbes occupy various habitats within the human body, creating complex and finely-tuned adaptive ecosystems that are specific to each body habitat [4]. While the majority of these organisms are commensal, some are mutualistic, and a few can be pathogenic [5]. Each individual maintains a distinct and stable microbiome, including both microbes and the microbial environment, even in a healthy state [5,6].

Among the diverse human microbiomes, the oral cavity stands as one of the most crucial, housing thousands of species and populations [1,7]. Numerous studies have focused on various types of oral microbial specimens. The potential of saliva as a readily accessible source of oral microbial specimens was initially suggested in the 1960s when the presence of salivary RNA was detected [8]. Salivary nucleic acid contents exhibit comparable quality to those obtained from blood [9]. Since most components of saliva originate from the bloodstream, the composition of saliva closely reflects that of blood. Small molecules from systemic circulation infiltrate into saliva, with plasma ultrafiltrate and saliva sharing similar electrolyte compositions [10].

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/20002297.2023.2229693.

The oral microbiome not only influences oral health and diseases but also has implications for systemic conditions, including diabetes mellitus, cardiovascular diseases, and cancers [11,12]. The relationship between the oral microbiome and systemic diseases was proposed as early as the 1890s [13]. Bacteremia and/or inflammatory mediators present in the systemic circulation can manifest in the oral microbiome [14], suggesting a reciprocal relationship between systemic conditions and the oral microbiome. However, despite much speculation, our understanding of the connection between the oral and systemic microbiomes remains limited.

To elucidate the relationship between the oral microbiome and systemic diseases, it is crucial to first establish the link between the oral and systemic microbiomes. Periodontitis, a prevalent chronic disease characterized by the destruction of tooth-supporting structures, periodontal ligaments, and alveolar bone, profoundly impacts both quality of life and overall health [11,15,16]. The oral microbiome plays a pivotal role in the pathophysiology of periodontitis. Pathogenic microbes such as *Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia, Treponema denticola, Fusobacterium nucleatum*, and *Campylobacter* species have been identified at periodontitis sites and are associated with disease exacerbation [17,18]. These pathogens produce various toxins that directly affect periodontal tissue and stimulate the host immune response, further aggravating tissue damage.

The advancement of high-throughput molecular techniques [19,20] has presented new opportunities to enhance the utilization of specimens from diverse microbial habitats and has sparked extensive research in the field of microbiome studies. Large-scale cohort studies investigating the microbiome across various body habitats, such as the Metagenomes of the Human Intestinal Tract (MetaHIT) project [21] and the Human Microbiome Project (HMP) [22], were conducted more than a decade ago. The diversity of the human microbiome across different habitats has been recognized for many years through population-scale studies [23–25]. However, despite the identification of consistent differences between body habitats, a previous study that compared the microbiomes of four habitats on a daily basis for up to 15 months in two individuals reported substantial temporal variability [26]. Therefore, the selection of specimens from representative habitats that can best reflect the characteristics and variability of individual microbiomes, while also being stable over time and readily obtainable, is critical for microbiome research. In this study, we compared pairs of specimens from different microbial habitats obtained from healthy oral dentition and periodontitis patients to ascertain the relationships between these habitats. By examining patients without systemic disease, we aimed to identify microbiome changes in different habitats in response to periodontal disease. Through this comparison, we can gain a better understanding of the relationships between the oral and systemic microbiomes.

Materials and methods

Subjects and sample collection

This study utilized genomic DNA (gDNA) specimens obtained from the Periodontal Human Specimen Storage Registry at Seoul National University Bundang Hospital. The specimens were collected between 2015 and 2019, and their use for secondary research purposes was approved by the provider within the designated storage period. The registry specimens consisted of five spatially distinct specimens: saliva, buccal swabs, plaque from the oral cavity, stool, and blood. The collection followed a standardized protocol used in the HMP [23,27]. An experienced periodontist recorded the collection and group classification of oral microbial specimens. The collection of oral cavity specimens followed a specific order: buccal swabs, saliva, and plaque. Blood samples were collected on the same day as saliva collection. Patients were instructed to fast for 8 hours and abstain from oral hygiene for at least 2 hours before the collection of oral specimens. The buccal swab samples were collected from the inner buccal mucosa of the right and left cheek using cotton swabs provided with the buccal swab kit. Saliva was collected for 20 minutes without stimulation. All periodontal probing depths were pre-recorded, and subgingival plaque was taken from the two deepest pockets during sampling. The gDNA was isolated from each specimen using commercial kits following the manufacturer's instructions (Supp. Text 1). Extracted DNA was prepared for sequencing according to the protocol of the HMP consortium [28].

Out of the 92 patients from whom specimens were obtained during the specified period, we selected 36 patients based on probing pocket depth as a clinical measure of periodontal disease. An experienced periodontist measured an average of 157 pocket depths in each patient, ranging from a minimum of 61 to a maximum of 168 pocket depths. The selected specimens were divided into two groups based on Matuliene et al. [29]: the healthy group (Non_PD) with probing depth <3 mm in all periodontal regions, and the periodontitis group (PD) with probing depth >6 mm at least in one site. The Non_PD group showed the absence of pocket depths greater than 5 mm, while the PD group exhibited an average of 68.3 pockets with depths greater than 5 mm. The study protocol received approval from the Institutional Review Board of Seoul National University Bundang Hospital (approval number: B-1810–499–301).

16S rRNA gene sequencing

Metagenomics analysis was performed by analyzing the variable V3 and V4 regions (519F-806 R) of prokaryotic 16S rRNA among nine variable regions interspersed between conserved regions. The sequencing of the target region of the 16S rRNA gene followed the Illumina 16S Metagenomic Sequencing protocol (Illumina, San Diego, CA). Prior to sequencing, the DNA quality was assessed using PicoGreen reagent and Nanodrop (Agilent Technologies, Santa Clara, CA).

For PCR amplification, 10 ng of input gDNA aliquots were used as per the protocol. The specimens were amplified using forward (519F: 5′-CCTACGGGNGGCWGCAG-3′) and reverse primers (806 R: 5′-GACTACHVGGGTATCTAATCC -3′) to generate a library of the 16S rRNA gene. The final purified product was quantified using KAPA Library Quantification kits for Illumina Sequencing platforms, following the manufacturer's protocol (Kapa Biosystems, Woburn, MA). The quality of the purified product was assessed using a LabChip GX HT DNA High Sensitivity Kit (PerkinElmer, Waltham, MA). The normalized and pooled libraries were subsequently sequenced on the MiSeq platform (Illumina).

Pre-analysis curation of sequencing data

The sequencing data underwent processing using a data curation pipeline implemented in Mothur (version 1.39.5) [30] and QIIME (version 1.9.1) [31]. Several pre-analytical filtering criteria were applied to the successfully sequenced and trimmed data. The following five criteria were used: exclusion of low-quality reads based on length (<400 bp or > 500 bp), removal of ambiguous reads containing increased N, elimination of sequences with mismatched primer sequences, filtering out sequences with Phred score base < 20, and removal of chimeric sequences. Additionally, any sequences that did not align against the appropriate subset of the SILVA database [32] and Greengenes [33] were discarded, along with chimeric sequences. To define operational taxonomic units (OTUs), the UCLUST algorithm with a specified OTU similarity threshold of 97% was meticulously employed within the QIIME platform. The representative sequences of these OTUs were then subjected to taxonomic assignment using the RDP classifier [34] and the Greengenes database. Finally, an alignment was generated using the pre-aligned sequence, which is a Greengenes core set of the PyNAST algorithm.

Data analysis and statistical analysis

The 597 selected OTUs, obtained through clustering with a difference distance cutoff of 0.03, were compared using the Basic Local Alignment Search Tool (BLAST) to perform taxonomic assignments. The filtered data were then subjected to diversity and interaction analyses.

For diversity analysis, four alpha-diversity indices (OTU, Chao1, Shannon Index, Inverse Simpson Index) were calculated. Beta diversity was determined using principal coordinate analysis (PCoA) with Bray-Curtis dissimilarity distances, based on the genera detected using QIIME. Comparisons between paired specimens were conducted using the Wilcoxon signed-rank test and/or Mann – Whitney U-test with SPSS Statistics 25.0 (SPSS Inc., Chicago, IL).

In addition, linear discriminant analysis (LDA) and LDA with effective size (LEfSe) were also performed [35]. To predict microbiome-associated functional pathways, we utilized phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) analysis [36].

Network analysis was employed to investigate the interactions among microbes. For the microbiome network analysis, we collected OTUs with frequencies of at least 30% in each group. Subsequently, OTUs that were not present in each group were excluded to perform group-specific analyses. To infer microbial ecological networks from the OTU datasets, we utilized the statistical method SPIEC-EASI (SParse InversE Covariance Estimation for Ecological Association Inference) [37]. The network analysis was performed using the neighborhood and StARS (Stability Approach to Regularization Selection) selection method, with a minimum λ threshold set at 0.01 [38]. All the aforementioned steps were computed using the R package [SpiecEasi] [39].

Furthermore, to validate the environmental influences on the freedom of movement between microbes and habitats in each specimen, neutral model fitness tests based on Hubbell's neutral theory were conducted [40,41].

Results

Clinical characteristics

We conducted comprehensive microbiome analyses on five different habitat specimens from two groups: PD ($n = 18$) and Non_PD ($n = 18$). All subjects included in the study were healthy individuals without any medical issues. After excluding 33 specimens with insufficient DNA extraction out of the initial 180 specimens collected from 36 patients, a total of 147 specimens were included in the final analysis. The distribution of specimens in the final analysis was as follows: saliva ($n = 18$ in the Non_PD group, $n = 16$ in the PD group), plaque ($n = 18$ in both groups), buccal swabs ($n = 18$

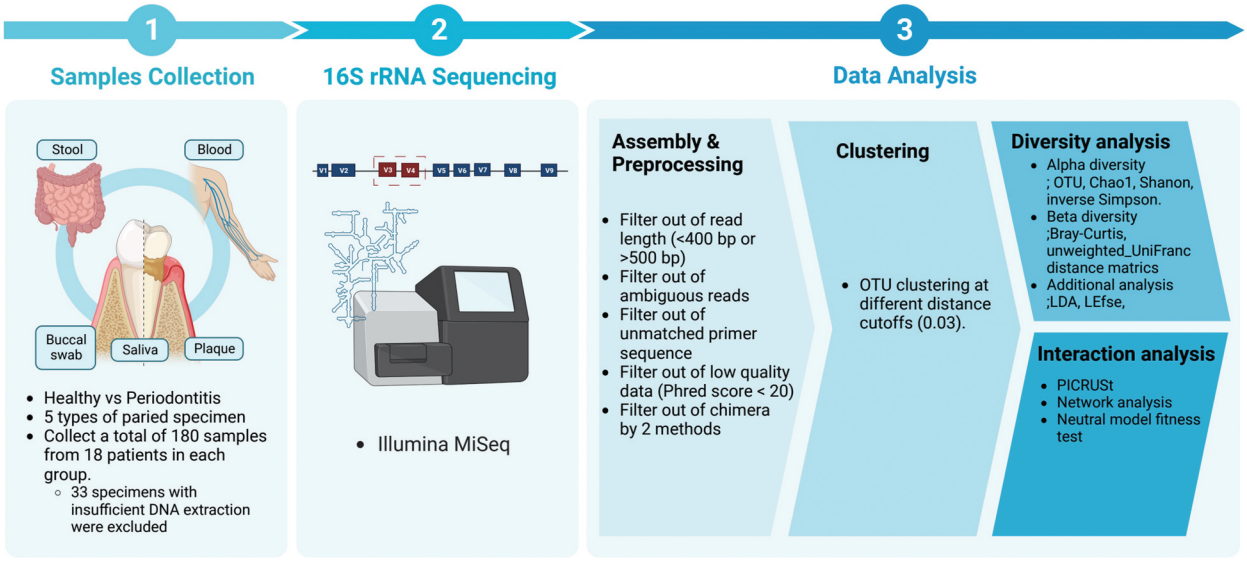

Figure 1. Overall workflow from sample collection to metagenomic data analysis in this study. Figures were created with BioRender [42].

in both groups), stool ($n = 18$ in the Non_PD group, $n = 14$ in the PD group), and blood ($n = 7$ in the Non_PD group, $n = 2$ in the PD group). Figure 1 provides an overview of the overall workflow employed in the study. The mean pocket depth was 2.6 mm in the PD group and 2.1 mm in the Non_PD group, with a statistically significant difference ($P < 0.001$). No significant differences were observed between the two groups in terms of body mass index (BMI) or smoking, which are factors that can influence the oral environment (Table 1).

Differences in the microbiome between PD and Non_PD groups

There were no significant differences in the alpha-diversity of species diversity within each specimen type, as estimated by OTU, Chao1, Shannon Index, and Inverse Simpson Index, between the PD and Non_PD groups, except for the saliva specimens (Table 2). Specifically, the PD group exhibited significantly higher values for OTU, Chao1, and the Shannon Index, which represent species richness, in their saliva specimens (all $P < 0.05$). Interestingly, all four indices of alpha-diversity demonstrated an increasing trend in the PD group, although no statistically significant differences were observed between specimens from other habitats, except for saliva.

Differences in the microbiome among oral specimens

At the phylum level, there were no differences in taxonomic composition in the oral cavity between the PD and Non_PD groups. Saliva and plaque exhibited similar compositions, although some differences in proportions were observed (Figure 2). However, buccal swabs showed distinct differences between the groups, with certain low-level microbes, such as Spirochaetes, Synergistetes, Tenericutes, and Verrucomicrobia, being absent in both PD and Non_PD buccal swabs. Given the unpredictable effect of inflammation, our subsequent analyses focused on

Table 1. Demographic characteristics of experimental group (periodontitis) and control group (healthy).

Characteristics		Experimental group (Periodontitis, $N = 18$)	Control group (Healthy, $N = 18$)	P-value
Age		52.4 ± 7.3 *	44.5 ± 14.6 *	0.051
Sex	Male	7 (38.9%)	4 (22.2%)	0.469
	Female	11 (61.1%)	14 (77.8%)	
Weight (Kg)		65.0 ± 11.3 *	60.3 ± 7.6 *	0.681
Height (cm)		161.9 ± 10.1 *	160.3 ± 7.3 *	0.205
BMI		24.7 ± 2.4 *	23.5 ± 2.2 *	0.094
Smoking	Yes	1 (5.6%)	0 (0.0%)	1.00
	No	17 (94.4%)	18 (100.0%)	
Mean Pocket Depth (mm)		2.6 ± 0.32*	2.1 ± 0.06*	<0.001
	No. of PPD < 5	2603	2946	
	No. of PPD ≥ 5	135	0	

Note: *Values are presented as mean ± standard deviation.
– Abbreviations: BMI, body mass index; PPD, probing pocket depth.

Table 2. Comparison of alpha-diversity of observed species for each habitat of specimen between the two groups using the mean ± standard deviation format.

Habitat of specimen		Experimental group	Control group	*P*-value
Plaque	No. of specimen	18	18	
	Alpha-diversity index			
	OTU	114.94 ± 29.37	111.72 ± 15.80	0.563
	Chao1	126.13 ± 33.14	121.37 ± 16.15	0.563
	Shannon	4.26 ± 0.73	4.44 ± 0.51	0.696
	Inverse Simpson	0.89 ± 0.09	0.90 ± 0.05	0.988
Saliva	No. of specimen	16	18	
	Alpha-diversity index			
	OTU	133.38 ± 19.74	110.06 ± 23.18	0.007
	Chao1	150.68 ± 24.73	128.88 ± 28.36	0.020
	Shannon	4.05 ± 0.22	3.56 ± 0.66	<0.005
	Inverse Simpson	0.88 ± 0.02	0.83 ± 0.10	0.055
Buccal swab	No. of specimen	18	18	
	Alpha-diversity index			
	OTU	110.56 ± 28.72	98.78 ± 23.92	0.279
	Chao1	125.63 ± 36.04	112.80 ± 24.38	0.279
	Shannon	3.40 ± 0.79	3.25 ± 0.87	0.963
	Inverse Simpson	0.79 ± 0.10	0.77 ± 0.14	0.913
Stool	No. of specimen	14	18	
	Alpha-diversity index			
	OTU	109.57 ± 46.55	88.50 ± 31.59	0.377
	Chao1	122.63 ± 55.74	102.01 ± 39.83	0.398
	Shannon	3.56 ± 1.17	3.23 ± 0.80	0.779
	Inverse Simpson	0.79 ± 0.14	0.79 ± 0.11	1.000
Blood	No. of specimen	2	7	
	Alpha-diversity index			
	OTU	93.50 ± 50.21	48.71 ± 48.51	0.333
	Chao1	95.17 ± 47.85	49.93 ± 48.47	0.333
	Shannon	4.26 ± 2.84	3.33 ± 0.98	0.889
	Inverse Simpson	0.79 ± 0.27	0.81 ± 0.10	1.000

Note: – Abbreviations: OTU, operational taxonomic unit.

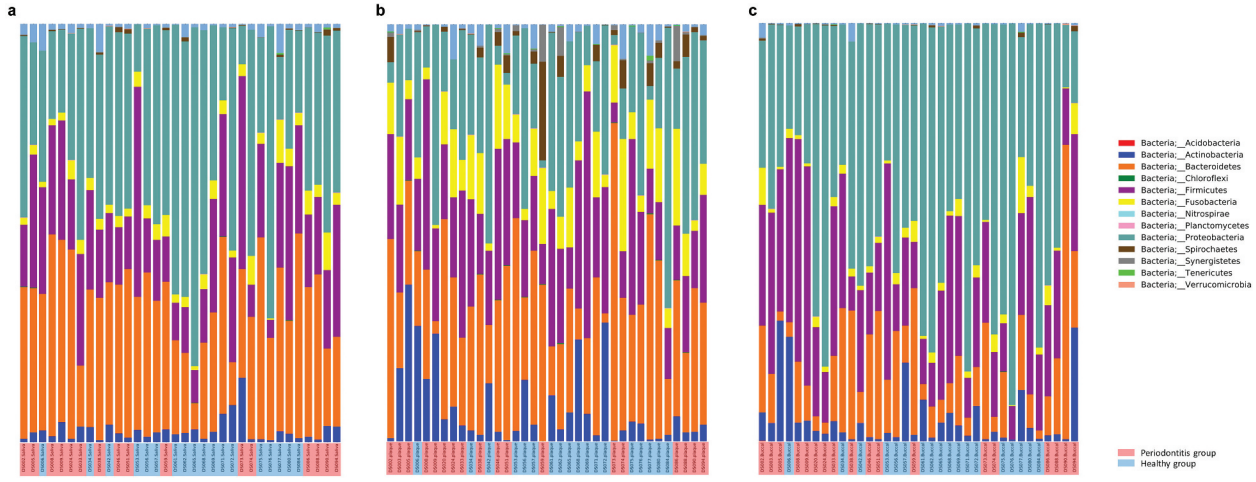

Figure 2. Taxonomic composition of specimens from each oral habitat at the phylum levels. (A)saliva, (B) plaque, and (C) buccal swabs of periodontitis and healthy groups. Specimen names are shown on the x-axis and OTU proportions are shown on the y-axis. The saliva and plaque showed slight differences in proportion but were similar in composition. However, buccal swabs were quite different from other oral specimens.

the PD group. Supplementary Figure S1 compares the alpha-diversity of specimens from different oral habitats, showing no significant differences in species richness ($P = 0.024$) or species diversity (Shannon Index, $P = 0.21$; Inverse Simpson Index, $P = 0.21$) between saliva and plaque. Additionally, as depicted in Supplementary Figure S2, the majority of bacterial communities present in saliva specimens were also found in plaque, resulting in a clustered data cloud in principal coordinate analysis. The heatmap displaying species ratios at the genus level according to oral habitat (Figure 3) revealed a closer association between saliva and plaque compared to buccal swabs. Furthermore, the cladograms generated from LEfSe analysis, focusing on microorganisms with LDA scores > 4, indicated substantial overlap between the microbes present in plaque and saliva, although certain species were exclusive to plaque (Figure 4).

In addition, we performed PICRUSt analysis based on the LEfSe data to predict microbiome-related

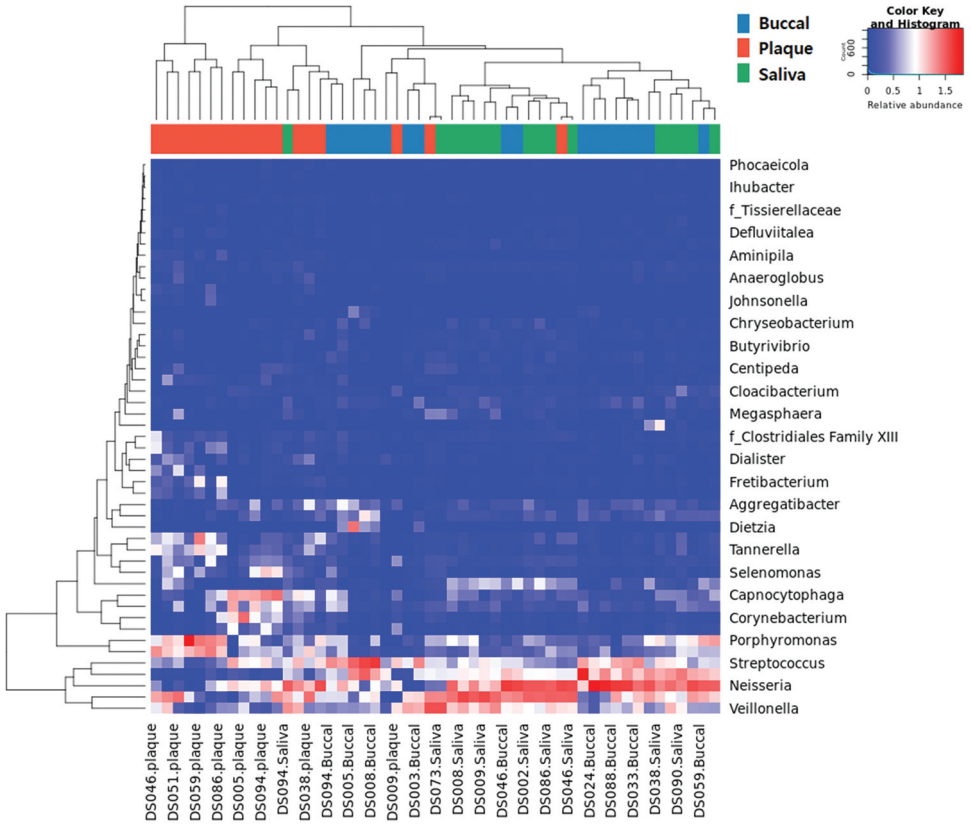

Figure 3. Heat map of genus-level species proportions of specimens of oral habitats in the periodontitis (PD) group. Cells are colored based on the standardized (scaled and mean centered) percentage of significant species for each data set. Data sets were hierarchically clustered based on Euclidean distances using the COMPLETE method.

functional pathways at the phylum level for each OTU. The enrichment of bacteria-related functional pathways in plaque showed differences between the PD and Non_PD groups (Figure 5). Notably, in saliva, significant differences in restriction enzyme-related pathway aberrations were observed only in the PD group with an LDA score > 2.

The enrichment of bacteria-related functional pathways in plaque differed between the PD and Non_PD groups (Figure 5). Notably, in saliva, significant differences in restriction enzyme-related pathway aberrations were observed only in the PD group with an LDA score > 2.

Changes in microbial interactions within oral specimens

The microbial network analysis of different oral specimens revealed altered microbial interactions between the PD and Non_PD groups. A total of 106 specimens were analyzed, including 18 saliva specimens in both groups, 18 plaque specimens in both groups, 18 buccal swabs in the Non_PD group, and 16 buccal swabs in the PD group. Only OTUs present in at least 30% of the samples were included in the analysis. Edge density (D-value) was calculated to compare the expected number of edges based on the number of nodes with the actual number of visible edges. A higher D-value indicates a greater number of edges and more interactions.

The PD group exhibited lower microbial interactions in saliva (D = 0.006 in the PD group, D = 0.0122 in the Non_PD group) and buccal swabs (D = 0.0063 in the PD group, D = 0.0159 in the Non_PD group), while plaque showed higher microbial interactions in the PD group compared to the Non_PD group (D = 0.0158 in the PD group, D = 0.0141 in the Non_PD group) (Figure 6).

Saliva specimens from the Non_PD group displayed interactions between various OTUs, including Bacteroidetes, Fusobacteria, Spirochaetes, Firmicutes, and Spirochetes. In contrast, the PD group showed interactions primarily between Bacteroidetes and Firmicutes as the main hubs. Common salivary microbial interactions between the groups were positive but showed an attenuated pattern in the PD group. *Aggregatibacter actinomycetemcomitans*, a species found in both PD and Non_PD groups, exhibited only positive interactions within the Non_PD group but showed a negative interaction with *Prevotella saliva* in the PD group.

In plaque specimens, the Non_PD group had Spirochaetes, Fusobacteria, Bacteroidetes, and Actinobacteria as the main hubs, while the PD group had Fusobacteria, Proteobacteria, Bacteroidetes, and Firmicutes as the main hubs. Interactions involving Fusobacterium in plaque specimens were positive in the Non_PD group but negative in the PD group.

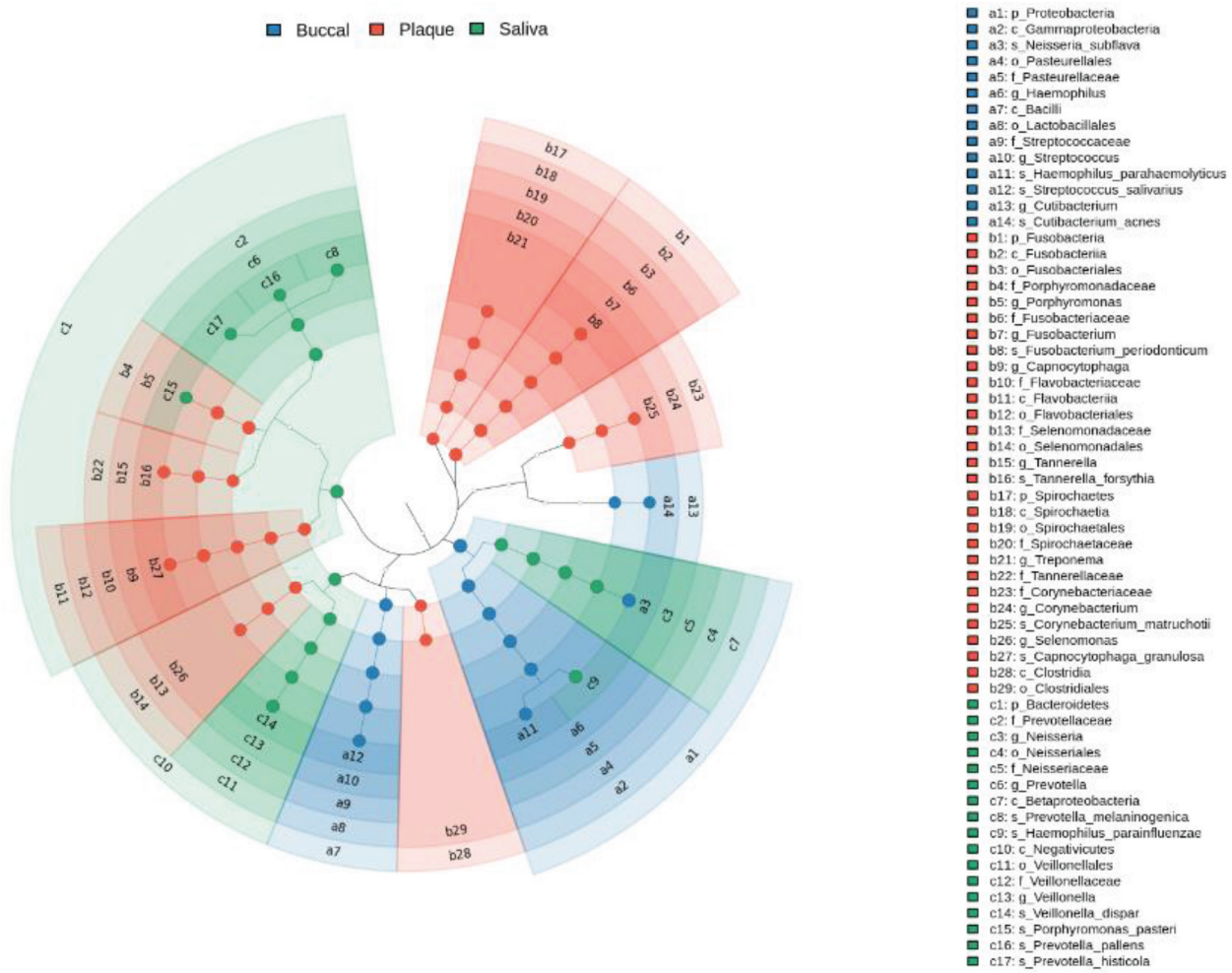

Figure 4. Microbiome characterization of specimens from the periodontitis (PD) group according to oral habitat by LEfSe analysis. Cladograms were derived from LEfSe analysis of differential microbial taxa for each specimen. The central-colored point denotes each colored specimen's root of the tree of bacteria and expanded to each ring representing the next lower taxonomic level from phylum to genus level. There were also microorganisms confined to plaque but many overlapped with those in saliva.

Buccal swab specimens exhibited similar microbial interactions to saliva specimens in the Non_PD group, whereas microbial interactions were largely lost in the PD group.

The effects of the environment on changes in microbial composition were analyzed using neutral model fitness tests based on Hubbell's neutral theory [40,41]. A neutral environment allows microorganisms to move freely, while a niche environment restricts their movement. The resilience of the environment was determined by the migration rate (m) and the coefficient of determination (R^2), with higher values indicating a more neutral environment. In the PD group, saliva represented the most neutral environment ($m = 0.192$, $R^2 = 0.815$ in the PD group; $m = 0.124$, $R^2 = 0.774$ in the Non_PD group), while plaque and buccal swabs represented niche environments (plaque: $m = 0.011$, $R^2 = 0.561$ in the PD group; $m = 0.017$, $R^2 = 0.619$ in the Non_PD group; buccal swabs: $m = 0.031$, $R^2 = 0.49$ in the PD group; $m = 0.065$, $R^2 = 0.675$ in the Non_PD group) (Supplementary Figure 3).

Relationships between microbial habitats: oral–blood axis?

The microbial compositions of specimens from different oral habitats (saliva, plaque, buccal swabs) as well as blood and stool were analyzed at the phylum to species levels. The aim was to investigate the relationships between these habitats and determine if the microbial compositions in blood were similar to those in the oral microbiome, particularly saliva and plaque. Specimens from the five habitats were collected simultaneously from each patient for comparison. However, due to the insufficient amount of DNA, only nine blood specimens could be analyzed, along with the corresponding paired habitat specimens from these patients. To assess the association of specific microbes with the progression of periodontitis disease, the count and ratio of taxonomic groups at the genus level were compared among the different habitats. Table 3 and Supplementary Table S1 present the results. The proportions of saliva and

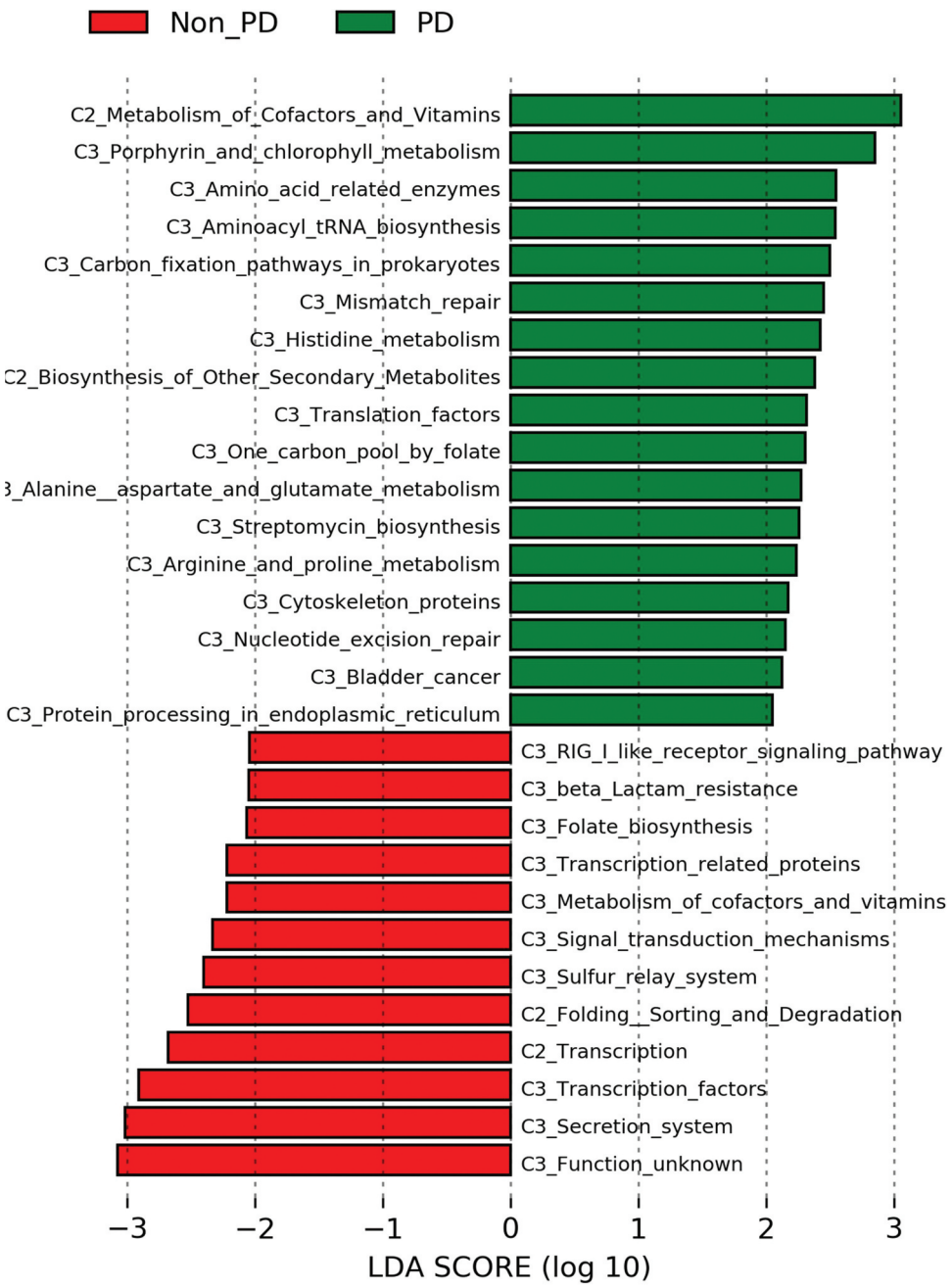

Figure 5. Prediction of microbiome-related functional pathways in plaque specimens based on 16S rRNA gene sequencing data. PICRUSt analysis based on the LEfSe data revealed differentially enriched bacterial functions associated with either the periodontitis (PD) or healthy (Non_pd) group.

plaque microbes in the oral habitat were found to be similar, while buccal swab specimens exhibited differences in microbial percentages and the absence of certain microorganisms. Increased levels of saliva or plaque microbes were detected in the blood specimens, whereas most of these microbes were not observed in stool specimens.

Discussion

To the best of our knowledge, this study represents the first comparative metagenomics analysis examining the relationship between the oral and systemic microbiomes using simultaneously collected specimens from both healthy individuals and periodontitis patients. Although there were no significant differences in microbial diversity within the oral microbiome, we observed significant variations in microbial interactions and/or the environment. Notably, microorganisms detected in oral specimens were also identified in the blood specimens of patients without systemic disease, suggesting a connection between the oral and systemic microbiomes.

We explored the relationships among different microbial habitats by analyzing specimens obtained

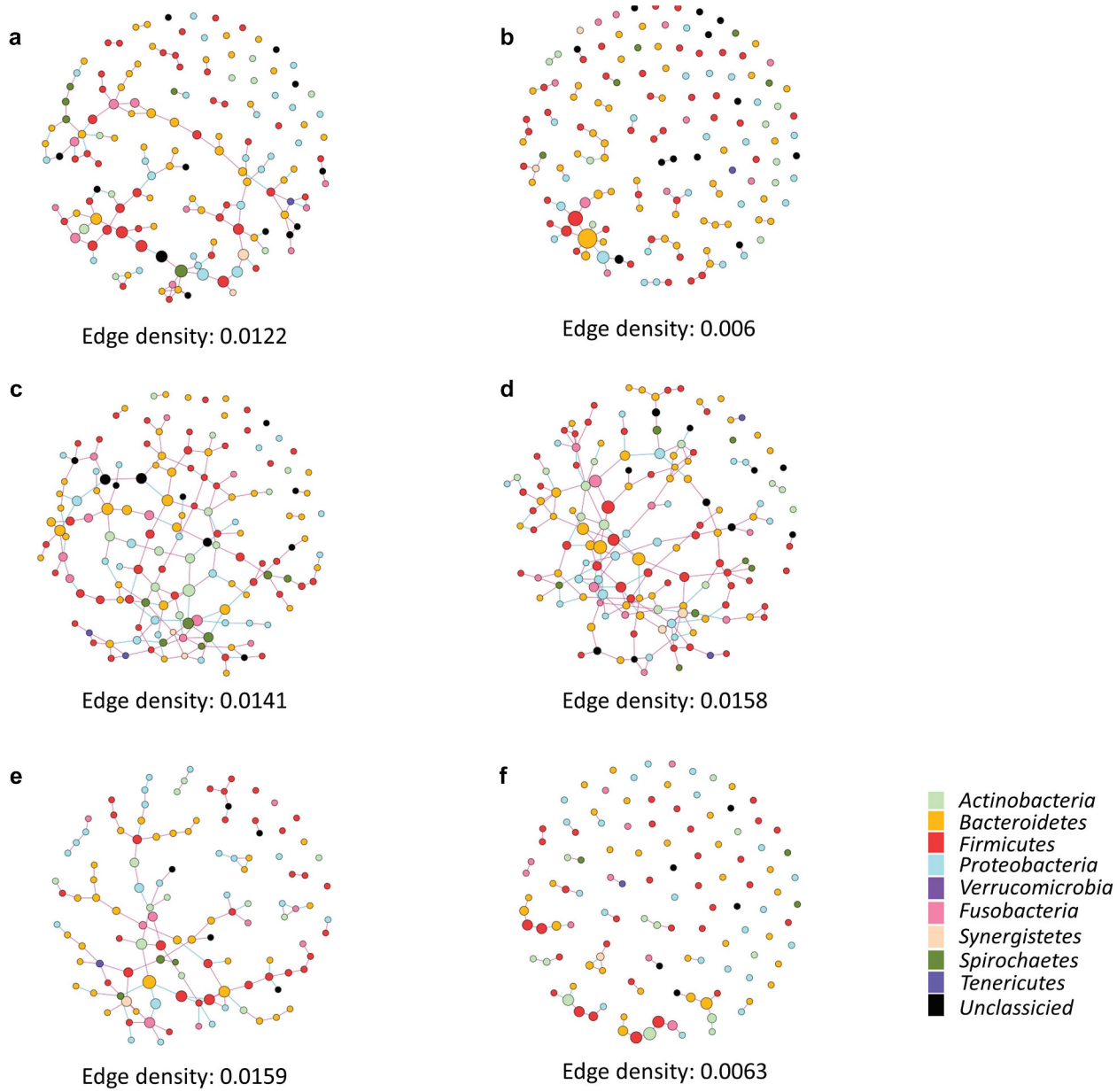

Figure 6. Microbial network analysis between healthy (Non_pd) and periodontitis (PD) groups in different oral specimens. (a) Saliva in the Non_PD group, (b) saliva in the PD group, (c) plaque in the Non_PD group, (d) plague in the PD group, (e) buccal swab in the Non_PD group, and (f) buccal swab in the PD group. Nodes represent OTUs. The color of the node indicates the phylum level of each OTU, and the size of node indicates the betweenness Z-score. Edges between nodes represent interactions between OTUs, with positive interactions shown in red and negative interactions shown in light blue. The edge density was calculated by comparing the expected values of edges based on the number of nodes to the value of the edges actually appearing; the PD groups had fewer interactions than the Non_PD group.

from various sites in periodontitis patients exhibiting alterations in the oral microbiome. While plaque is considered the primary specimen for assessing periodontitis pathogenesis [43], saliva has been shown to exhibit similar microbial patterns to plaque, reflecting changes in the oral microbial habitat. This finding is consistent with previous studies that have reported comparable plaque and salivary microbiomes in patients with periodontitis [44]. In contrast, buccal swab specimens exhibited some differences compared to plaque specimens. These differences are reasonable considering previous reports indicating that the buccal mucosa is characterized by species-specific colonization patterns [45]. The use of saliva offers several advantages, as it can be obtained noninvasively, is convenient for preservation and transport, and can be easily and repeatedly collected from individuals of different age groups, including young children and the elderly [46]. Moreover, saliva provides genomic DNA and nucleic acid contents of good quality, comparable to those obtained from blood, although the levels are approximately half that of blood [9].

Notably, as we explored the interactions among microbiomes and environments, we were able to assess changes in the microbiomes related to disease

Table 3. Most representative results of taxonomy counts (ratio) of periodontitis-associated microbes in paired specimens from five habitats.

Specimen No.	Genus	Saliva	Plaque	Buccal swabs	Blood	Stool
DS056	Campylobacter	21 (0.132)	37 (0.193)	83 (0.387)	261 (0.885)	-
	Fusobacterium	369 (2.317)	100 (0.522)	413 (1.982)	553 (1.875)	-
	Porphyromonas	1,753 (11.006)	2,578 (13.448)	1,375 (6.417)	1,973 (6.688)	-
	Prevotella	3,976 (24.964)	450 (2.347)	3,451 (16.107)	3,437 (11.651)	-
	Tannerella	1 (0.006)	20 (0.104)	2 (0.009)	1 (0.003)	-
DS061	Campylobacter	51 (0.235)	112 (0.637)	23 (0.151)	84 (0.350)	-
	Fusobacterium	384 (1.769)	661 (3.758)	71 (0.466)	295 (1.230)	-
	Porphyromonas	280 (1.290)	27 (0.154)	39 (0.256)	3 (0.013)	-
	Prevotella	3,839 (17.685)	987 (5.611)	416 (2.731)	537 (2.239)	3,539 (24.970)
	Tannerella	18 (0.083)	44 (0.250)	7 (0.046)	-	-
DS084	Campylobacter	16 (0.062)	18 (0.093)	12 (0.041)	-	-
	Fusobacterium	439 (1.700)	648 (3.348)	90 (0.307)	41 (0.557)	-
	Porphyromonas	2,936 (11.368)	690 (3.566)	306 (1.043)	485 (6.586)	1,198 (4.635)
	Prevotella	9,420 (36.475)	952 (4.919)	182 (0.620)	774 (10.511)	8,644 (33.443)
	Tannerella	26 (0.101)	265 (1.369)	18 (0.061)	-	-
DS090	Campylobacter	49 (0.295)	131 (0.631)	4 (0.017)	757 (2.462)	-
	Fusobacterium	974 (5.863)	437 (2.103)	48 (0.201)	5,126 (16.672)	-
	Porphyromonas	1,129 (6.796)	258 (1.242)	6 (0.025)	18,842 (61.281)	518 (5.812)
	Prevotella	926 (5.574)	6,087 (29.300)	48 (0.201)	1,771 (5.760)	6,083 (68.256)
	Tannerella	224 (1.348)	37 (0.178)	-	1,084 (3.526)	-

Note: The most representative case of paired habitat specimens in which migration of periodontitis-associated microbes between habitats can be inferred.

progression. Dysbiosis, which refers to a disturbance in the balanced ecosystem of the oral microbiome, can lead to the development of oral diseases and adverse health effects [47]. As periodontitis progresses, the diversity of plaque microbes changes, accompanied by alterations in the interactions among environmental flora. Plaque formation is influenced by the passive transport of bacteria through saliva, changes in the microbial environment surrounding solid surfaces (such as tooth surfaces), and interactions among microorganisms [48]. The structure of plaque results from a delicate balance between microbial adhesion, growth, and removal. As plaque matures, the dominant bacteria within it shift from one group to another [48], indicating potential changes in the diversity of the plaque microbiome over time. In saliva, which serves as a transport medium for microbes, microbial diversity increases, and certain microbial interactions become more dominant, while overall normal interactions among microbes decrease. Regarding environmental relationships, various microbes migrate from saliva to plaque, creating an actively migrating neutral environment in saliva compared to the niche environment of plaque, which is densely populated with pathogenic microbes.

In addition to the similarities in microbial composition and diversity across different habitat specimens, the results of functional pathway analyses using 16S rRNA metagenomics data revealed differences according to the progression of periodontitis. Saliva specimens, in particular, displayed differences in restriction enzyme functional pathways based on disease status. Restriction modification systems in bacteria play a crucial role not only in defense but also in maintaining population heterogeneity and facilitating adaptation to changing environmental conditions. They are also essential for the colonization of host tissues by pathogenic bacteria [49]. The 16S rRNA analysis, which was confined to the V3 and V4 regions, failed to identify restriction enzyme aberrations, which is significant in light of the pivotal role of such enzymes in bacterial evolution and ecology.

Changes in the composition of the microbiome significantly contribute to disease morbidity. Recently, there has been increasing interest in comprehensively interpreting ecological covariates to understand the microbiome-disease relationship [18,50,51]. In this regard, the analysis of ecological covariates beyond the microbiome components, through network analysis, holds significant value. Our network analysis revealed disparities between the PD and Non_PD groups in terms of microbial interactions involving *A. actinomycetemcomitans*. The strong association between *A. actinomycetemcomitans* and the development of periodontitis has been well established [45,52]. The results of our network analysis indicate variations in microbial interactions based on the extent of inflammation, suggesting a pivotal role in determining disease progression.

We were able to identify microorganisms related to periodontitis in the oral cavity through analysis of sterile blood specimens. The microbiome of blood has been a subject of significant discussion. In the past, the presence of microbes in blood components such as red blood cells and white blood cells has been confirmed [53], and there have been arguments suggesting that the presence of microbes in the bloodstream is not associated with pathogenicity [54]. However, a recent large-scale study conducted on healthy individuals has provided undeniable evidence of microbial presence in the blood, although the exact origin of these microbes remains uncertain [55]. Nonetheless, these findings strongly indicate the

existence of a blood microbiome. Furthermore, our study's findings are significant as they demonstrate that the composition and proportion of microorganisms identified in the blood are similar to those found in the oral cavity. This suggests the possibility of migration between these two habitats. Saliva contains a diverse range of biological components, including DNA, RNA, proteins, microorganisms, and metabolites. On average, an adult secretes and swallows approximately 1 to 1.5 liters of saliva per day [56]. In an experimental study using a mouse model, live bacteria stained with carboxyfluorescein diacetate succinimidyl ester were found in the cecum after oral injection of periodontitis-related microbes from saliva, in an amount proportional to the daily intake of an adult human [57]. The excessive influx of inflammatory microbes can influence the manifestation of systemic disease through intestinal dysbiosis [47]. For instance, dysbiosis through the oral-gut-liver axis has been suggested to contribute to the development of cirrhosis [58]. The structural characteristics of the human body, with an entrance and an exit and different points of contact with the systemic circulation, allow for the identification of microorganisms that have entered the digestive system through significant amounts of saliva. Therefore, we propose the possibility of microbial migration to a systemic habitat (i.e. blood) via a potential oral-blood axis. Previous studies have demonstrated connections between systemic inflammation and the oral microbiome based on interactions between oral diseases and systemic diseases, such as diabetes, cardiovascular diseases (e.g. atherosclerosis), and cancers [11–15,59,60]. These findings further support the existence of an oral-blood axis.

This study had some limitations. First, the number of specimens included in the analyses was small, and the microbiome data from blood specimens were not satisfactory due to the limited volumes of blood obtained. Additionally, prediction and analysis of gene function in the microbiome were lacking, as the sequence data were analyzed only for the V3 and V4 regions of 16S rRNA. Functional analysis of the microbiome would require analysis using data obtained through shotgun sequencing. Moreover, as periodontitis is a heterogeneous condition with no specific cause, clear differences in the microbiome compared to the healthy group were not observed. Therefore, further follow-up studies focusing on diseases with well-established causal relationships are necessary.

In summary, we compared pairs of specimens from different microbial habitats obtained from healthy subjects and periodontitis patients to determine the relationship between the oral and systemic microbiomes. To understand differences in the microbiome, it is crucial to investigate not only diversity and species richness but also changes in the interactions among microbes and the tissue environment. Our data suggest that changes in the salivary microbiome in response to disease state are also reflected in blood specimens via the oral-blood axis.

Acknowledgment

The authors would like to thank all subjects who provided specimens for this study.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by research fund No. 2021R1A2C1006466 and No.2023R1A2C002783 from the National Research Foundation of Korea and grant No. 06–2021–0228 from the SNUBH Research Fund.

Author contributions

H.Kim contributed to the minor data analysis, and interpretation, drafted the manuscript, and critically revised the manuscript; J.Hong contributed to the data acquisition and critically revised manuscript; P.Yun contributed to the data acquisition and revised the manuscript.; K Hwang contributed to the data acquisition and revised the manuscript.; K Kim contributed to data acquisition and revised the manuscript.; H.Lee contributed to the conception, design of the study, data acquisition, and critically revised the manuscript; K.U.Park contributed to the conception, and design of the study, data acquisition, and interpretation, and critically revised the manuscript. All authors gave final approval and agreed to be accountable for all aspects of the work.

Data availability statement

The raw microbiome sequencing data have been deposited in the NCBI Sequence Read Archive database with accession No. PRJNA937013 http://www.ncbi.nlm.nih.gov/bioproject/937013.

Ethical approval

The study protocol was approved by the Institutional Review Board Seoul National University Bundang Hospital, with number B-1810-499-301.

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