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Microbial Profiling of Saliva, Oral Rinse, Subgingival Plaque and GCF Reveals Site-Specific Dysbiosis in Periodontitis: A Within-Subject Comparison of 150 Participants

Ran Lee[1] | Jin-Young Park[1] | Yuan Park[1] | Kyu Kim[2] | Jong-Won Ha[2] | Jung-Seok Lee[1]

[1]Department of Periodontology, Research Institute for Periodontal Regeneration, Yonsei University College of Dentistry, Seoul, South Korea | [2]Division of Cardiology, Severance Cardiovascular Hospital, Yonsei University College of Medicine, Seoul, South Korea

Correspondence: Jung-Seok Lee (cooldds@yuhs.ac)

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ABSTRACT

Aim: This study aimed to compare the microbial communities across four oral sample types—saliva, oral rinse, subgingival plaque and gingival crevicular fluid (GCF)—and to identify disease-associated microbiota in periodontitis.

Methods: Oral samples were collected from 150 adults, each providing four types of samples in the same visit. Saliva (5 mL) and oral rinse (10 mL, 30-s swish) were collected prior to clinical examination. Subgingival plaque was sampled using a curette from the two deepest pockets, followed by GCF collection via 20-s insertion of gingival retraction cords at the same sites. All samples underwent 16S rRNA (V3–V4) sequencing. Site-specific microbial profiles were evaluated across all participants. For disease comparisons, only individuals with clear periodontal status (periodontally healthy, $n = 41$; stage III/IV periodontitis, $n = 43$) were included, excluding stage I/II cases ($n = 66$).

Results: Saliva and oral rinse formed one microbial cluster; plaque and GCF formed another. Alpha diversity was found increased in disease, except in GCF. Beta diversity showed the most distinct disease-related shift in GCF. Red complex pathogens and GCF-specific differentially abundant taxa were markedly enriched in periodontitis.

Conclusions: GCF yielded the clearest microbial differentiation between health and periodontitis, supporting its diagnostic utility.

1 | Introduction

Advancements in 16S ribosomal RNA sequencing have enabled the identification of uncultivable and previously unknown microbial species. As a result, there has been a surge in oral sampling research aimed at gaining a deeper understanding of the oral microbiome associated with health and disease. However, variability in the sample collection methods across studies has hindered comparability and reproducibility. Among various sample types, subgingival plaque—the co-aggregation of bacteria in the form of a biofilm—has traditionally been the most widely used and extensively studied as the primary aetiologic agent in periodontitis (Ng et al. 2021; Socransky et al. 2013; Teles et al. 2013). Gingival crevicular

Ran Lee and Jin-Young Park contributed equally to this work.

fluid (GCF) has also been extensively investigated for its inflammatory biomarkers and their potential prognostic value (Barros et al. 2016; Fatima et al. 2021). Meanwhile, saliva and oral rinse have been used because of their ease of collection and their potential to reflect both the oral and, to some extent, gut microbiota following ingestion (Belstrøm 2020; Fan et al. 2018).

Periodontitis is characterised by a complex interaction between the subgingival biofilm and the host immunity, resulting in the loss of periodontal attachment (Tonetti et al. 2018). Although the role of bacteria in the aetiology of periodontitis has been debated for decades, the trending concept is that a polymicrobial, synergistic, and dysbiotic microbiota expresses specific gene combinations that converge to form a pathologic community (Hajishengallis and Lamont 2012). The focus of periodontal microbiology research, therefore, has expanded beyond the conventionally defined infectious species—such as those in the red complex—to a vast catalogue of microorganisms in the state of health and disease (Scannapieco and Dongari-Bagtzoglou 2021). In addition, based on the evolving concept of periodontal pathogenesis, increasing attention is being directed towards the relationship between periodontitis-associated systemic comorbidities and the oral microbiome, including the role of surrogate markers of inflammation (Hajishengallis 2022).

Despite these advances, selecting an appropriate sampling strategy remains a significant challenge in multidisciplinary research. While subgingival plaque and GCF represent site-specific microenvironments associated with periodontal pockets, saliva and oral rinse are non-site-specific and reflect the cumulative microbial composition of the entire oral cavity. Previous studies have investigated individual sample types or compared limited pairs, such as saliva versus subgingival plaque (Belstrøm, Constancias, Markvart, et al. 2021; Belstrøm, Constancias, Drautz-Moses, et al. 2021) or saliva versus oral rinse (Takeshita et al. 2016). However, comprehensive within-subject comparisons across all four major sample types—particularly in a large cohort—remain absent in the literature. This represents a critical gap, as microbiome data are increasingly being used not only for research but also for diagnostic applications, including salivary-based disease screening, despite limited validation of these sample types for periodontal diagnosis.

Therefore, the aim of this study was to compare microbial communities derived from saliva, oral rinse, subgingival plaque and GCF collected from the same subjects in relation to the periodontal status.

2 | Materials and Methods

2.1 | Study Design

This retrospective study analysed 600 samples obtained from 150 subjects who were previously enrolled in three independent prospective cohorts conducted at the Yonsei University Health System, Severance Hospital (approval nos. 4-2022-0533, 4-2021-0947) and Yonsei University Dental Hospital (approval no. 2–2022-0053). Although each cohort study was originally designed for a different research purpose, the samples were collected using identical protocols and were thus eligible for inclusion in the present study.

The study complied with the Declaration of Helsinki, and the protocol was approved by the Institutional Review Board of Yonsei University Dental Hospital (approval no. 2-2022-0053).

2.2 | Subjects

All participants were ≥ 18 years of age and had at least 18 remaining teeth. Exclusion criteria included current pregnancy or lactation, uncontrolled diabetes mellitus, autoimmune disorders such as rheumatoid arthritis or lupus, chronic use of corticosteroids or immunosuppressants and recent use (within 3 months) of antibiotics or oral probiotics. These criteria were applied uniformly across all three contributing cohorts.

For each patient, a panoramic radiograph was taken and a full-mouth periodontal examination was performed by periodontists at the Department of Periodontology, including assessments of probing pocket depth (PD), bleeding on probing (BoP), gingival recession (GR) and gingival enlargement (ENL) across the full dental arch, thereby facilitating the calculation of clinical attachment level (CAL). All periodontal examinations and diagnoses were performed by two board-certified periodontists at Yonsei University Dental Hospital. A calibration meeting was held prior to study initiation and follow-up meetings during the study. Subjects were periodontally classified according to the 2017 Workshop on Periodontal Diseases and Conditions (Tonetti et al. 2018). All 150 participants were initially included in site-specific microbial profiling to evaluate differences among the four oral sample types, regardless of periodontal status. For comparative analyses between health and disease, only participants with clear periodontal status—either periodontally healthy or stage III/IV periodontitis—were selected. Subjects with stage I or II periodontitis were excluded to minimise ambiguity in disease classification and to enhance contrast between groups.

For subgroup analysis, healthy individuals (healthy group, $n = 41$) were compared with subjects diagnosed with stage III or IV periodontitis (periodontitis group, $n = 43$). A total of eight sample types were analysed: healthy saliva (HS), healthy oral rinse (HO), healthy subgingival plaque (HP), healthy GCF (HG), periodontitis saliva (PS), periodontitis oral rinse (PO), periodontitis subgingival plaque (PP) and periodontitis GCF (PG).

2.3 | Sample Collection

Four types of oral samples were collected from each subject: saliva, oral rinse, subgingival plaque and gingival crevicular fluid (GCF), resulting in 600 samples.

All patients were instructed to fast for at least 3 h and abstain from oral hygiene procedures prior to the collection of oral samples.

1. For saliva samples, a non-stimulated saliva sample of 5 mL was collected from the subjects into a conical tube

containing RNAlater Stabilisation Solution (Thermo Fisher Scientific, Waltham, USA).

2. For oral rinse samples, subjects were instructed to vigorously swish 10 mL of distilled water in their mouth for 30 s, then rinse and spit into a conical tube containing RNAlater Stabilisation Solution.
3. Subgingival plaque samples were collected from the two teeth with the deepest pocket probing depth in the entire oral cavity. Before sample collection, the supra-gingival plaque was carefully removed, and the site was isolated with cotton rolls and gently dried to remove moisture. The subgingival plaque was then carefully collected using a Gracey curette. Samples contaminated with blood were excluded. The two plaque samples per subject were pooled into a microcentrifuge tube containing RNAlater.
4. GCF samples were collected using a sterilised gingival retraction cord (Ultrapak #000) inserted into the gingival sulcus of the same two teeth from which subgingival plaque was obtained. After isolating the site and drying it with cotton rolls, the cord was gently placed for 20 s and then carefully removed. Both cords were pooled into a single microcentrifuge tube without RNAlater.

All samples were immediately stored at −80°C until DNA extraction.

Samples were collected in a fixed order (saliva, oral rinse, clinical examination, subgingival plaque and then GCF) to minimise cross-contamination.

2.4 | Microbial DNA Isolation, Amplification and Sequencing

Total DNA extraction, 16S rRNA gene amplification, library preparation and sequencing were performed using standardised protocols. Detailed procedures—including primer design, PCR conditions, purification and sequencing quality control—are described in Appendix A.

2.5 | Bioinformatic Processing and Statistical Analysis

Microbiome data analysis and visualisation were conducted using Python (version 3.7.12) and R (version 4.2.2). Alpha diversity was assessed using the Shannon index, and group differences were evaluated using the Wilcoxon rank-sum test. Beta diversity was calculated using Bray–Curtis distances, and group differences were tested using PERMANOVA (Anderson 2001), which was performed using the adonis2 function in the *vegan* package (999 permutations), with subject ID set as a stratification variable to account for repeated measures and smoking status included as a covariate. Differential taxon abundance between periodontal health and periodontitis was analysed using MaAsLin2 (Mallick et al. 2021), with smoking status also included as a covariate. In addition, sparse partial least squares discriminant analysis (sPLS-DA) was employed to evaluate the classification performance of each sample type in discriminating periodontitis from periodontal health. All *p*-values were adjusted for multiple comparisons using the Benjamini–Hochberg method (Benjamini and Hochberg 1995).

3 | Results

3.1 | Demographic Characteristics

One-hundred and fifty subjects were included in this study (Table 1). Smokers were more frequent in the periodontitis group compared to the periodontal health group ($p = 0.034$). All clinical parameters, including PD, CAL and BoP, differed significantly between the two groups ($p < 0.001$).

TABLE 1 | Demographic characteristics and clinical parameters of subjects.

			Subgroup analysis		
		Total	**Periodontal health**	**Stage III, IV periodontitis**	***p*-value**
Age	*n*	150	41	43	
	Mean (SD)	56.93 (11.28)	55.46 (14.71)	52.67 (8.48)	0.288
	Min–max	29–78	29–78	29–66	
Gender					
Female	*n* (%)	55 (36.7)	19 (46.3)	13 (30.2)	0.129
Male	*n* (%)	95 (63.3)	22 (53.7)	30 (69.8)	
Smoking					
Yes	*n* (%)	29 (19.3)	4 (9.8)	12 (27.9)	0.034
No	*n* (%)	121 (80.7)	37 (90.2)	31 (72.1)	
PD (mm)	Mean (SD)	3.03 (0.86)	2.36 (0.38)	4.1 (0.73)	< 0.001
CAL (mm)	Mean (SD)	3.49 (1.23)	2.58 (0.49)	4.95 (1.2)	< 0.001
BoP (%)	Mean (SD)	33.36 (27.73)	10.36 (8.66)	60.6 (24.43)	< 0.001

3.2 | Taxonomic Profiles Across Sample Types

Over 95% of sequences belonged to seven predominant phyla: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, *Saccharibacteria_TM7* and *Spirochaetes*. Saliva and oral rinse samples exhibited higher relative abundances of *Firmicutes* and *Proteobacteria*, whereas subgingival plaque and GCF showed higher levels of *Bacteroidetes, Fusobacteria* and *Spirochaetes* (Figure 1A, Table S1).

At the genus level, *Streptococcus* was the most dominant taxon in saliva and oral rinse, followed by *Veillonella*, *Prevotella*, *Neisseria* and *Haemophilus*. In contrast, subgingival plaque and GCF exhibited a more even distribution, with higher relative abundances of *Porphyromonas*, *Fusobacterium*, *Treponema*, *Capnocytophaga*, *Corynebacterium* and *Leptotrichia* (Figure 1B, Table S2).

3.3 | Diversity

Alpha diversity (Shannon index) was significantly higher in subgingival plaque compared to all other sample types ($p < 0.001$), whereas no significant differences were observed among GCF, oral rinse and saliva ($p > 0.05$) (Figure 1C).

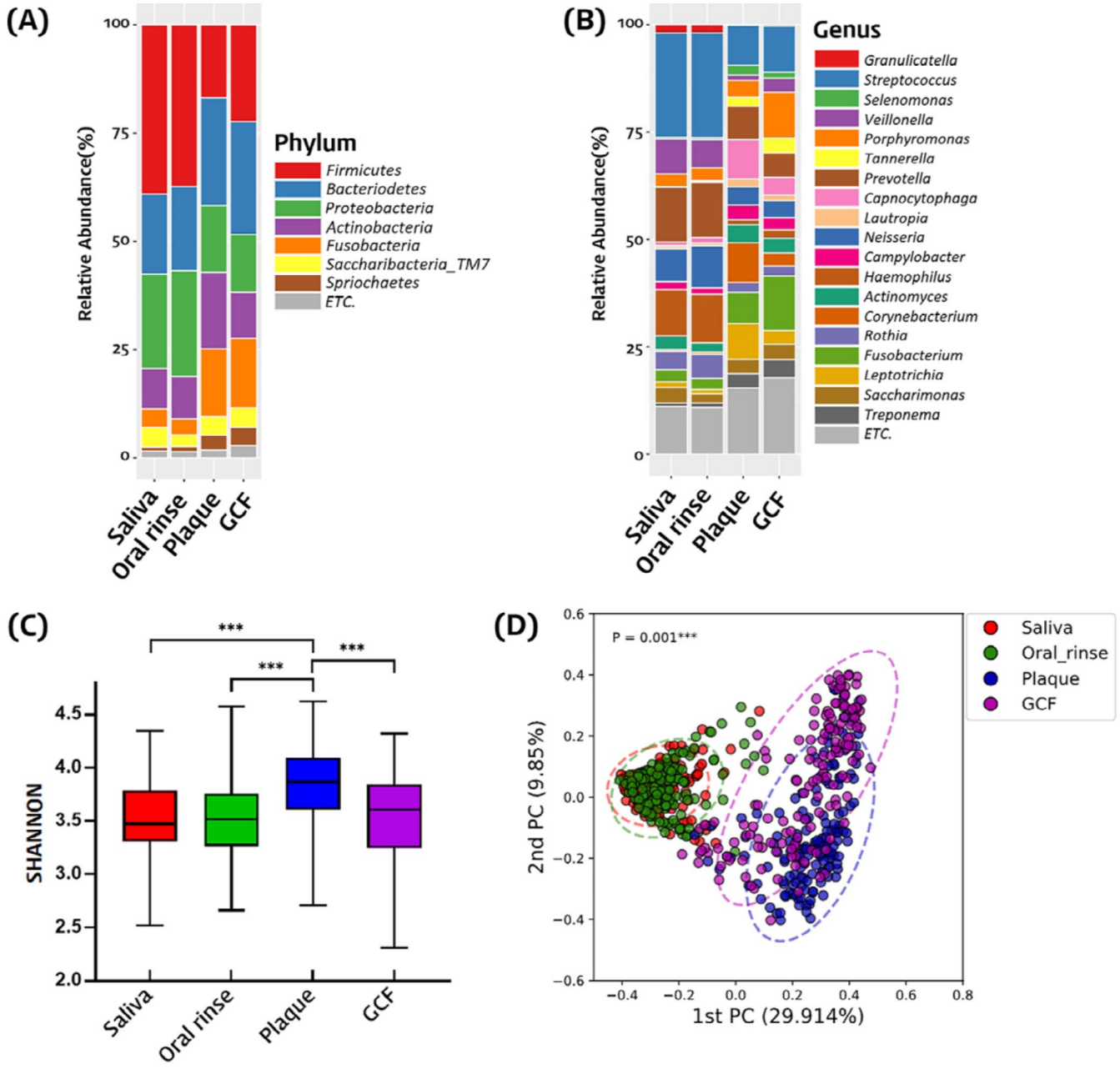

FIGURE 1 | Microbial community composition and diversity across four oral sampling methods in 150 subjects.A relative abundance bar plot showing the relative bacterial proportions at the phylum (A) and genus (B) level between four sampling methods. (C) Box plots showing the alpha-diversity estimation scores between four sampling methods which were calculated using the Shannon index alpha-diversity indices. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. (D) All 600 samples were clustered, and beta diversity was calculated using principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity.

Beta diversity assessed using principal coordinates analysis (PCoA) based on Bray–Curtis dissimilarity revealed two distinct clusters: one comprising saliva and oral rinse, and the other comprising subgingival plaque and GCF (Figure 1D). Saliva and oral rinse samples appeared tightly clustered, while subgingival plaque and GCF were more widely dispersed, indicating greater compositional variability.

PERMANOVA results confirmed that saliva and oral rinse were the most similar sample types (pseudo-$F = 4.593$, $R^2 = 0.015$, $p = 0.001$), followed by subgingival plaque and GCF (pseudo-$F = 21.415$, $R^2 = 0.066$, $p = 0.001$). In contrast, the greatest dissimilarity was observed between saliva and subgingival plaque (pseudo-$F = 126.523$, $R^2 = 0.297$, $p = 0.001$) (Tables S3, S4).

3.4 | Microbial Differences Between Periodontal Health and Periodontitis

3.4.1 | Microbial Profile

At the phylum level, the relative abundances of *Firmicutes*, *Proteobacteria* and *Actinobacteria* were higher in the healthy group compared to the periodontitis group. In contrast, *Bacteroidetes*, *Saccharibacteria_TM7*, *Fusobacteria* and *Spirochaetes* were more abundant in the periodontitis group (Figure 2A).

At the genus level, the healthy group exhibited higher relative abundances of *Streptococcus*, *Actinomyces* and *Rothia*, whereas *Porphyromonas*, *Tannerella*, *Fusobacterium* and *Treponema* were more predominant in the periodontitis group (Figure 2B).

3.4.2 | Alpha Diversity

Alpha diversity (Shannon index) was significantly higher in the periodontitis group compared to the healthy group for saliva ($p = 0.003$), oral rinse ($p = 0.005$) and subgingival plaque ($p < 0.001$); however, for GCF, the difference was not statistically significant ($p = 0.206$) (Figure 2C).

Within the healthy group, there were no significant differences in alpha diversity; however, within the periodontitis group, subgingival plaque showed greater alpha diversity than other sites (all $p < 0.05$) (Figure 2C).

3.4.3 | Beta Diversity

In health, saliva and oral rinse samples showed no statistically significant difference in microbial composition (pseudo-$F = 0.748$, $R^2 = 0.009$, $p = 0.731$). In contrast, the same comparison in the periodontitis group yielded a statistically significant difference (pseudo-$F = 2.216$, $R^2 = 0.025$, $p = 0.015$). All other pairwise comparisons among sampling methods showed significant differences ($p < 0.001$). Additionally, microbial compositions were significantly different between the periodontally healthy group and the periodontitis group ($p < 0.001$) (Table S5).

Differences in microbial composition for each sample type between health and periodontitis was assessed using the Bray–Curtis dissimilarity index (Figure 3B). Differences in microbial composition were the highest in GCF (0.74 ± 0.06), followed by subgingival plaque (0.69 ± 0.08), oral rinse (0.58 ± 0.06) and saliva (0.58 ± 0.05). Moreover, the density distribution of within-group distances showed that GCF values were skewed towards the higher end of the index, suggesting more pronounced microbial changes in periodontitis (Figure 3C).

3.5 | Relative Abundance of Red Complex Species

The relative abundance of red complex bacteria (*Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia*) varied by sample type and periodontal status (Figure 4). In both subgingival plaque and GCF, all three species were significantly more abundant in the periodontitis group than in the healthy

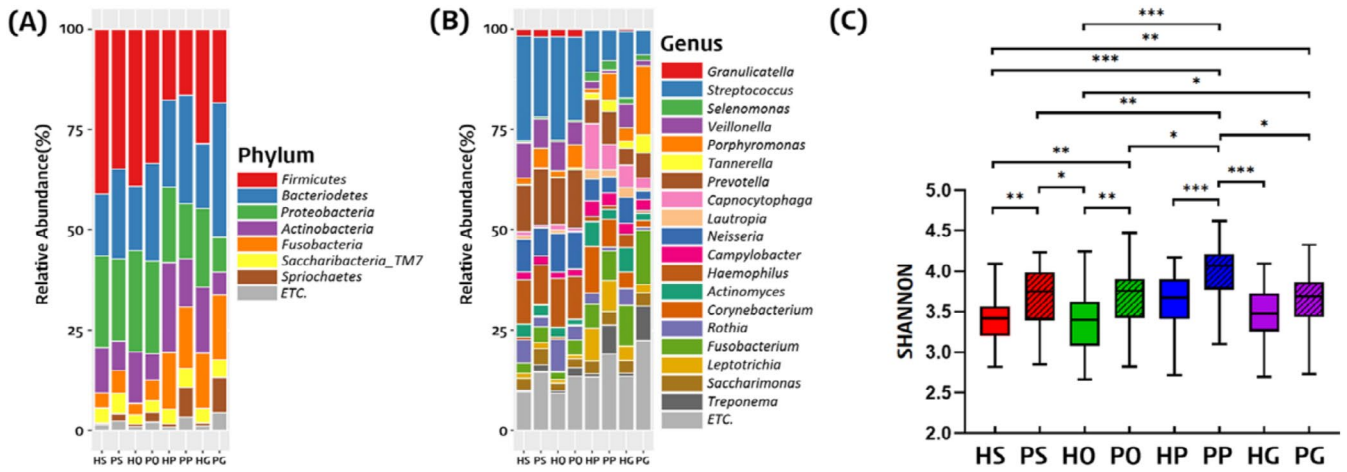

FIGURE 2 | Comparison of the microbial communities among periodontal health (HS, HO, HP, HG) and stages III–IV periodontitis (PS, PO, PP, PG). (A) A relative abundance bar plot showing the relative bacterial proportions at the phylum level between each group. (B) A relative abundance bar plot showing the relative bacterial proportions at the genus level between each group. (C) Box plots showing the alpha-diversity estimation scores between each group, which were calculated using the Shannon index alpha-diversity indices. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

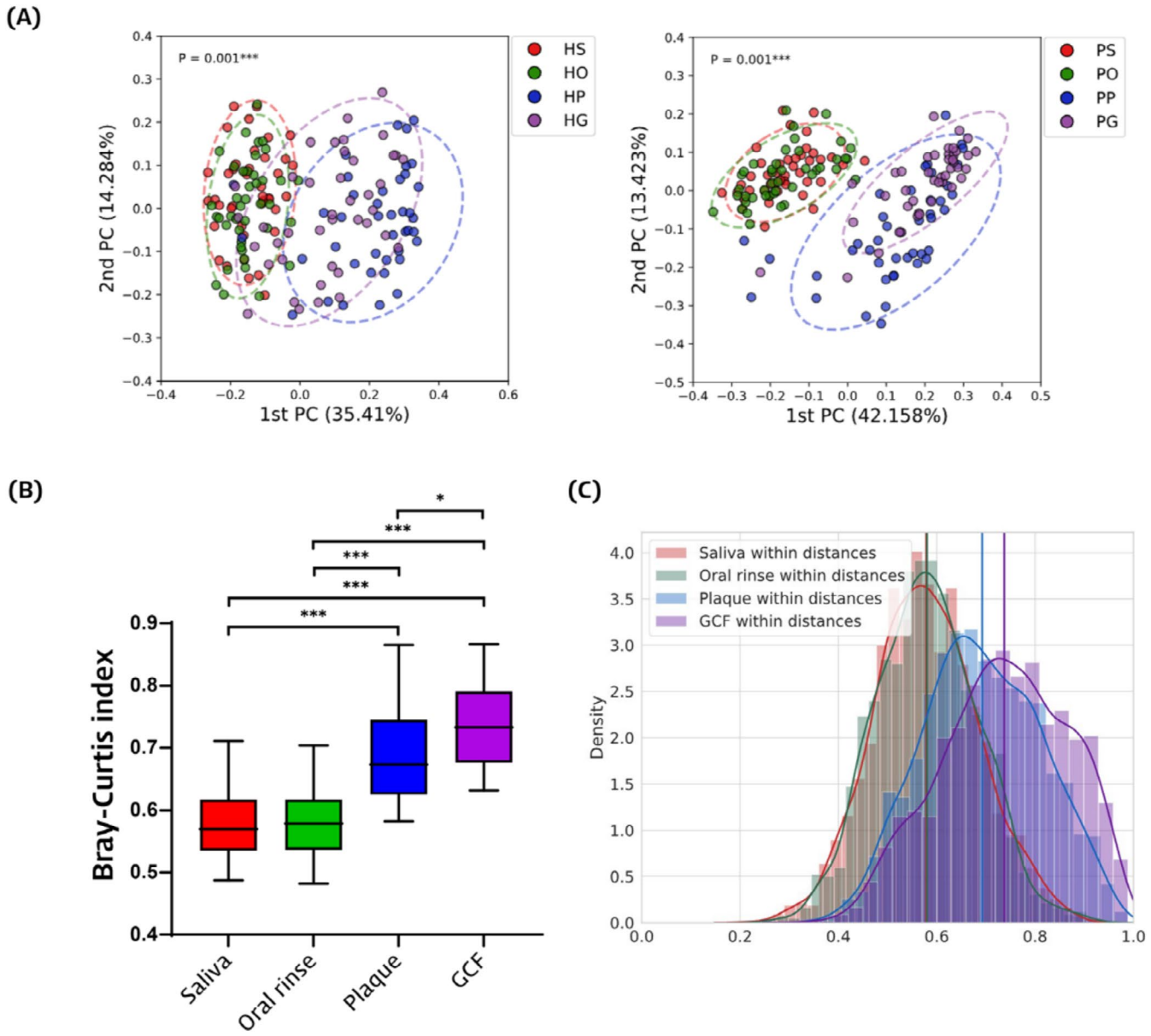

FIGURE 3 | Comparison of microbial community structure and within-sample variability between periodontal health and stages III–IV periodontitis across four oral sample types. (A) Periodontal health (HS, HO, HP, HG) and stages III–IV periodontitis (PS, PO, PP, PG) were visualised in a principal coordinate analysis (PCoA) plot, using Bray–Curtis distance for each representative group. Ellipses with 95% confidence intervals were drawn to represent the bacterial community profile. (B) The Bray–Curtis index between periodontal health and stages III–IV periodontitis is represented for each sample type. GCF shows the largest microbial difference between periodontal health and stages III-IV periodontitis. (C) The density distribution of within-group distances (Bray–Curtis dissimilarity) for microbial communities in four different types of oral samples: saliva, oral rinse, plaque and GCF. The *x*-axis represents the Bray–Curtis dissimilarity index, which ranges from 0 (indicating identical microbial communities) to 1 (indicating completely dissimilar communities), while the *y*-axis represents the density of these distances within each sample type.

group ($p < 0.001$). While saliva and oral rinse showed lower overall levels of red complex species, significant differences by periodontal status remained evident ($p < 0.001$).

3.6 | Identification of Differentially Abundant Taxa

MaAsLin2 analysis revealed differentially abundant taxa between health and periodontitis across sample types: 31 in saliva, 47 in oral rinse, 52 in plaque and 81 in GCF. GCF had the highest number of unique taxa ($n = 22$), while saliva, oral rinse and plaque had 2, 5 and 4 unique species, respectively (Table S7). Twenty-one taxa were shared across all sample types (Figure 5A, Table S6); among them, 17 were enriched in periodontitis—including red complex species—and 4 in health (Figure 5B).

3.7 | Classification Performance of Sample Types Using Sparse Partial Least Squares Discriminant Analysis

To assess the diagnostic potential of each oral sample type for distinguishing periodontitis from periodontal health, we

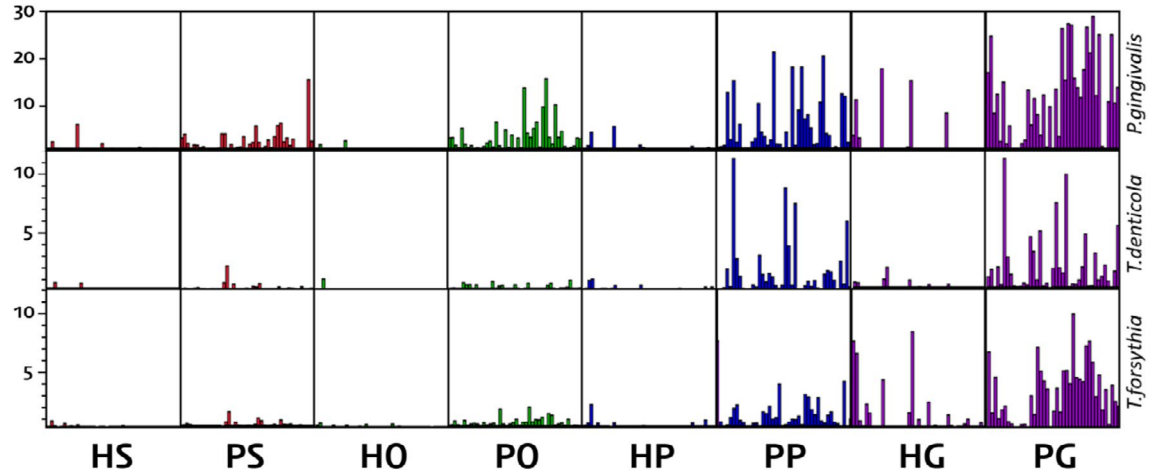

FIGURE 4 | Relative abundance of red complex bacteria across four oral sample types in subjects with periodontal health and periodontitis. Bar plots illustrate the relative abundances of red complex species, including *Porphyromonas gingivalis* (*P. gingivalis*), *Treponema denticola* (*T. denticola*) and *Tannerella forsythia* (*T. forsythia*). Each bar on the *x*-axis represents an individual subject, and the *y*-axis indicates relative abundance.

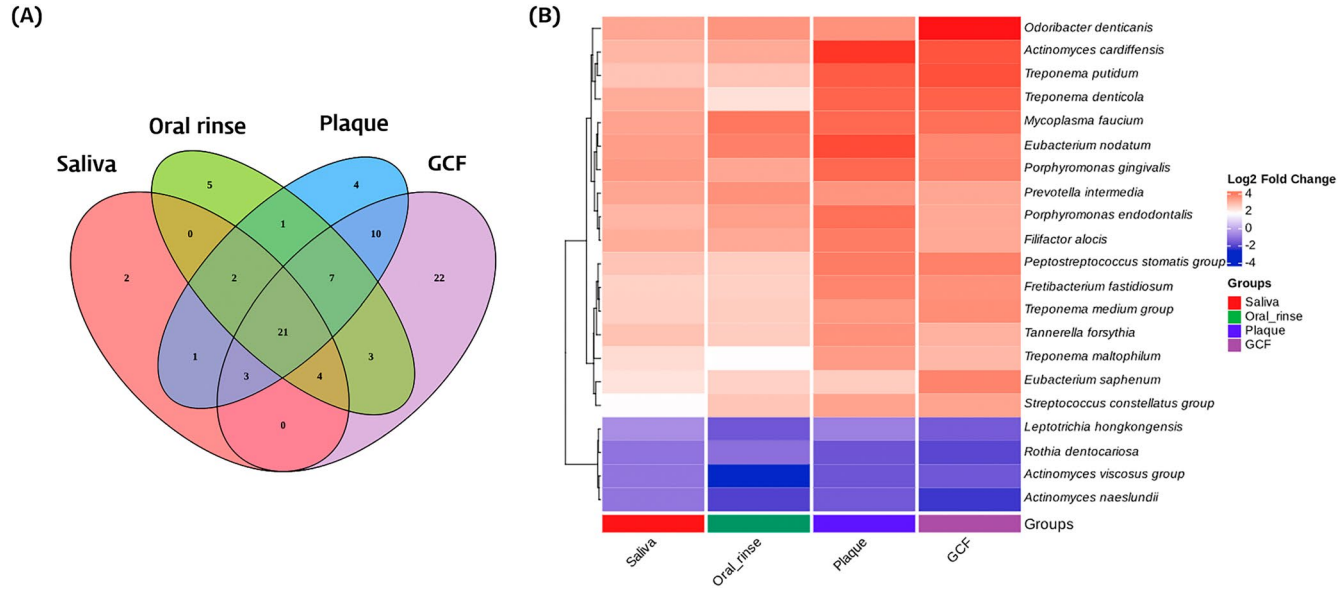

FIGURE 5 | Shared differentially abundant microbial taxa across four oral sample types in periodontitis. (A) Venn diagram illustrating the distribution of taxa that showed significant differential abundance between periodontal health and disease as identified by MaAsLin2 analysis. The four sample types—saliva, oral rinse, subgingival plaque and GCF—are represented by distinct colours. Each number denotes the count of differentially abundant taxa specific to or shared among the sample types. The highest number of sample type–specific differentially abundant taxa was observed in GCF (22 taxa), whereas subgingival plaque exhibited the greatest overlap with other sample types. The central region ($n = 21$) indicates taxa that were significantly differentially abundant in all four sample types. (B) Heatmap illustrating the $\log_2$ fold change values of 21 taxa that showed consistent differential abundance between periodontal health and disease across all four sample types. The colour gradient represents $\log_2$ fold change values, with red indicating enrichment in the periodontitis group and blue indicating enrichment in the periodontal health group. Differential abundance analysis was performed using MaAsLin2, with smoking status included as a covariate to control for its potential confounding effect.

performed sPLS-DA, which showed that GCF had the best diagnostic performance for distinguishing periodontitis from health (AUC = 0.992), followed by oral rinse (0.967), plaque (0.963) and saliva (0.923) (Figure 6). Key discriminative taxa with variable importance in projection (VIP) scores > 1.5 are presented in Figure S2.

4 | Discussion

This study demonstrated several key findings regarding the oral microbiome in health and periodontitis. First, microbial communities clustered into two major ecological niches—saliva and oral rinse forming one group, and subgingival plaque and

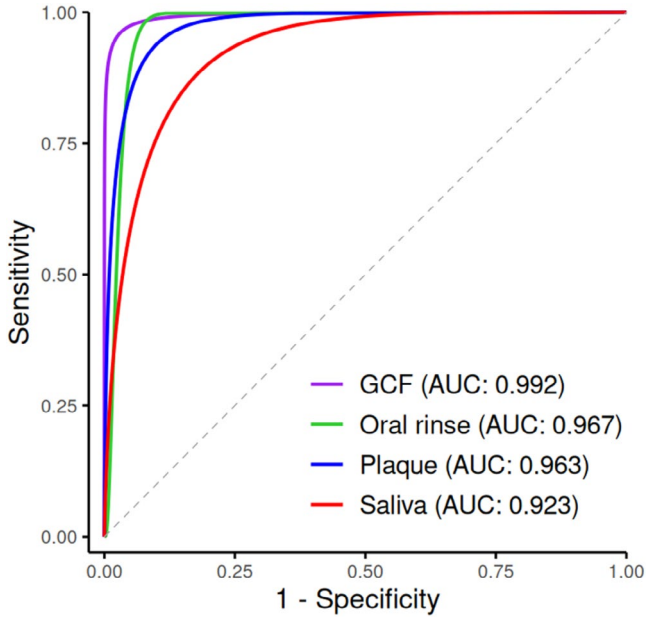

FIGURE 6 | Receiver operating characteristic (ROC) curves derived from sparse partial least squares discriminant analysis (sPLS-DA) models to evaluate the diagnostic performance of microbiome profiles across four oral sample types: saliva, oral rinse, subgingival plaque and gingival crevicular fluid (GCF). ROC curves were constructed using sPLS-DA to assess the discriminatory power of microbiome compositions from GCF, oral rinse, subgingival plaque, and saliva in distinguishing periodontitis from periodontal health.

gingival crevicular fluid (GCF) forming another. Second, periodontitis was associated with significantly increased alpha diversity in most sample types, except GCF. Third, beta diversity analyses revealed distinct microbial community shifts in periodontitis, with GCF exhibiting the most pronounced changes. Lastly, GCF not only exhibited the highest relative abundance of red complex pathogens but also showed the greatest number of differentially abundant taxa distinguishing periodontal health from stage III/IV periodontitis.

The niche-specific clustering observed in this study highlights fundamental ecological distinctions within the oral cavity. Saliva and oral rinse samples primarily represent planktonic or loosely attached microbial communities derived from mucosal surfaces, tongue and supragingival plaque (Caselli et al. 2020; Segata et al. 2012). In contrast, subgingival plaque and GCF originate from the periodontal pocket and harbour biofilm-associated anaerobes closely linked to disease (Ng et al. 2021; Teles et al. 2010). In particular, GCF—collected through gingival cord insertion—likely reflects microbial communities embedded in the periodontal pocket microenvironment, thereby capturing local physiological and pathological states (Barros et al. 2016). In this study, we opted for gingival retraction cords over paper strips because of the former's higher absorption efficiency in deep pockets and prior validation in microbial studies (Lee et al. 2020). Although the cord-based technique may raise concerns about potential overlap with plaque-associated biofilm, our findings revealed that GCF and subgingival plaque harboured distinct microbial profiles, as demonstrated by beta diversity metrics and PERMANOVA analysis (pseudo-$F = 21.415$, $p < 0.001$, $R^2 = 0.066$). These results suggest that the observed differences are not solely attributable to methodological factors but reflect genuine ecological divergence between the two niches. The distinct clustering supports recent evidence that the sampling site has a profound impact on the oral microbiome composition and interpretation (Caselli et al. 2020; Li et al. 2022; Liu et al. 2020).

Unlike the decreased alpha diversity typically associated with dysbiosis in systemic inflammatory conditions such as inflammatory bowel disease or metabolic disorders (Loh and Blaut 2012; Scannapieco and Dongari-Bagtzoglou 2021), our study found that periodontitis was associated with increased microbial diversity in saliva, oral rinse and subgingival plaque samples. Although the GCF group showed a similar trend, the difference was not statistically significant. These findings are consistent with previous reports suggesting that periodontitis-related dysbiosis may involve microbial enrichment and community restructuring rather than diversity loss (Abusleme et al. 2021; Curtis et al. 2020; Griffen et al. 2012). Therefore, this pattern may represent a distinct periodontal dysbiosis model in which alpha diversity is maintained or elevated through the emergence of pathobionts and altered microbial networks.

Nevertheless, discrepancies in alpha diversity across studies warrant careful interpretation. Some studies have reported comparable diversity between health and periodontitis (Kirst et al. 2015; Relvas et al. 2021), possibly due to variability in study populations, disease definitions and sampling protocols. The current study addressed this by focusing on well-defined cohorts at two extremes of the periodontal spectrum—healthy individuals and patients with advanced stage III/IV periodontitis. Furthermore, previous reports that observed minimal differences in alpha diversity often used GCF as the sampling source (Kirst et al. 2015), which aligns with our observation that GCF diversity remained relatively stable across health and disease. This may reflect the spatial and volumetric limitations of the gingival crevice, which, despite inflammation, may not support the same biomass expansion seen in other oral niches.

To complement alpha diversity, the simplified subgingival microbial dysbiosis index (SMDI) (Chen et al. 2022) confirmed significantly higher dysbiosis scores in periodontitis. GCF showed the largest health–disease separation (Figure S3), reinforcing its diagnostic value. This aligns with the sPLS-DA and MaAsLin2 results, which consistently highlighted GCF as the most informative matrix.

Importantly, while diversity metrics provide useful insights, beta diversity and community composition offer a more nuanced view of dysbiosis. Our results showed that GCF exhibited the most substantial compositional shift between health and disease, as evidenced by the highest Bray–Curtis dissimilarity scores and clear group separation in PCoA. This highlights the gingival crevice as a key site of microbial–host interaction and suggests that microbial remodelling in GCF reflects disease-related ecological imbalance. The broader right-skewed distribution of intra-group dissimilarity in periodontitis patients further implies increased inter-individual variability and ecological instability.

These compositional shifts do not necessarily entail a loss of diversity but rather signify functional and structural reorganisation of the microbial community. According to emerging ecological models, periodontitis-associated dysbiosis represents a shift from a stable, symbiotic microbiota to a pathologic, polymicrobial community characterised by the expansion of virulent species and disrupted homeostasis (Kumar 2021). The observed increase in alpha diversity in periodontitis may therefore result from cooperative pathogenicity, where disease arises not from single species but from synergistic interactions among metabolically complementary microbes (Scannapieco and Dongari-Bagtzoglou 2021).

Among the four sample types, GCF stood out as the most informative matrix for detecting periodontal pathogens. All three red complex species—*P. gingivalis*, *T. denticola* and *T. forsythia*—were detected at significantly higher abundances in GCF than in other samples, underscoring its value in disease stratification. Additionally, GCF exhibited the greatest number of differentially abundant taxa unique to this sample type, with 22 taxa showing significant differences between health and periodontitis only in GCF, including *Selenomonas infelix* and *Treponema lecithinolyticum* (Hiranmayi et al. 2017; Vashishta et al. 2019). These findings reinforce the potential of GCF as a diagnostic substrate for microbial surveillance and the identification of disease-associated taxa (Barros et al. 2016; Teles et al. 2010). The distinct microbial profiles between GCF and subgingival plaque likely reflect the inherent differences in sampling strategies. While plaque sampling targets the adherent biofilm, GCF collected via cord captures the fluid milieu enriched with planktonic bacteria, host-derived components and potentially invasive species residing within the sulcular epithelium. This may provide a complementary perspective on the microbial dynamics relevant to disease activity.

Although smoking is known to impact the oral microbiome, no significant group-level differences were observed between smokers and non-smokers in our cohorts. This may be due to limited power or subtle functional effects not detectable by 16S rRNA profiling. Regarding GCF sampling, although plaque contamination cannot be fully excluded, distinct clustering and PERMANOVA results support that GCF harbours unique microbial communities. Its fluidic nature enables the capture of planktonic and invasive species, reinforcing its complementary role to plaque sampling in disease profiling.

This study's strengths include its large sample size, within-subject comparisons across four sampling sites and robust cohort classification. However, some limitations should be noted. First, taxonomic assignments were based on 97% sequence similarity of 16S rRNA reads, which may not provide definitive species-level resolution. While this threshold is commonly used, it can lead to over- or under-classification of taxa, and some of the species-level findings—particularly differentially abundant taxa—should be interpreted with caution. Future studies employing whole meta-genome sequencing or species-specific validation are needed to corroborate these results and improve taxonomic accuracy. Second, our cohort excluded early-stage disease, potentially overlooking microbial shifts in initial disease progression. Future research should explore a broader range of periodontal conditions and employ high-resolution sequencing technologies to refine microbial diagnostics.

5 | Conclusion

In conclusion, distinct dysbiotic patterns of the oral microbiome in periodontitis were clearly delineated across different sampling sites. Among the tested modalities, GCF provided the most discriminative microbial signatures, reflecting disease-associated alterations with the highest precision. While oral rinse, saliva and dental plaque also offer practical, non-invasive sampling options, GCF should be considered the preferred specimen for accurate microbial profiling in periodontal research and potential diagnostic applications.

Author Contributions

R. Lee, J.-Y. Park, J.-S. Lee contributed to the study design. R. Lee, K. Kim contributed to the data analysis and interpretation. R. Lee, J.-Y. Park, made the first draft of the manuscript. Y. Park, K. Kim, J.-W. Ha, and J.-S. Lee critically revised the manuscript. All authors gave their final approval and agreed to be accountable for all aspects of the work.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Mean relative abundances (%) of the seven most dominant bacterial phyla detected in saliva, oral rinse, subgingival plaque and gingival crevicular fluid (GCF) samples. **Table S2:** Mean relative abundances (%) of the 19 most dominant bacterial genera detected in saliva, oral rinse, subgingival plaque and gingival crevicular fluid (GCF) samples. **Table S3:** PERMANOVA and PERMDISP results for the effect of smoking status on microbial community composition and dispersion across four oral sample types. **Table S4:** PERMANOVA results of bacterial communities among the four sampling methods. **Table S5:** PERMANOVA results of pairwise comparisons among the four oral sampling methods in periodontal health and periodontitis. **Table S6:** Differential abundance of bacterial taxa across oral sample types identified by MaAsLin2 analysis. **Table S7:** GCF-specific differentially abundant taxa between periodontal health and stage III–IV periodontitis identified by MaAsLin2. **Figure S1:** Principal coordinates analysis (PCoA) plots illustrating the effect of smoking status on microbial community composition across four oral sample types: saliva, oral rinse, subgingival plaque and gingival crevicular fluid (GCF). **Figure S2:** Sparse partial least squares discriminant analysis (sPLS-DA) loading plots showing discriminative microbial taxa across four oral sample types (saliva, oral rinse, subgingival plaque, gingival crevicular fluid [GCF]). **Figure S3:** Simplified subgingival microbial dysbiosis index (SMDI) scores across sample types in periodontal health and periodontitis.

Appendix A

Materials and Methods

Pre-Treatment and DNA Extraction

Prior to DNA extraction, all samples underwent a standardised pre-treatment protocol as follows. DNA was subsequently extracted from the homogenised samples using the FastDNA Spin Kit for Soil (MP Biomedicals, CA, USA), following the manufacturer's instructions.

Saliva Samples

A total of 800 μL of saliva sample preserved in RNAlater was transferred into a lysing matrix E tube, to which 200 μL of sodium phosphate buffer and 122 μL of MT buffer were added. The mixture was homogenised using the FastPrep instrument at 6.0 m/s for 40 s and was used for DNA extraction.

Oral Rinse Samples

From the preserved oral rinse, 800 μL of sample was added to a lysing matrix E tube along with 200 μL of sodium phosphate buffer and 122 μL of MT buffer. The sample was homogenised at 6.0 m/s for 40 s and subsequently used for DNA extraction.

Subgingival Plaque Samples

Approximately 800 μL of the plaque sample was transferred into a lysing matrix E tube containing 200 μL of sodium phosphate buffer and 122 μL of MT buffer. After homogenisation at 6.0 m/s for 40 s, the sample was used for DNA extraction.

Gingival Crevicular Fluid (GCF) Samples

Two retraction cords containing GCF were inserted into a lysing matrix E tube to which 978 μL of sodium phosphate buffer and 122 μL of MT buffer were added. The tube was homogenised at 6.0 m/s for 40 s and used for DNA extraction.

Library Preparation and Quality Control

Following DNA extraction, the V3–V4 regions of the 16S rRNA gene were amplified using fusion primers designed to include P5/P7 graft binding sequences, i5/i7 indices, Nextera consensus sequences, Illumina sequencing adaptors and the target-specific primers 341F and 805R. The full sequences of the fusion primers were as follows:

- **341F:** 5′-AATGATACGGCGACCACCGAGATCTACAC-XXXXXXXX-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG-3′
- **805R:** 5′-CAAGCAGAAGACGGCATACGAGAT-XXXXXXXX-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-GACTACHVGGGTATCTAATCC-3′

PCR amplification was performed under the following conditions: initial denaturation at 95°C for 3 min; 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s; a final elongation step at 72°C for 5 min.

The PCR products were confirmed by 1% agarose gel electrophoresis and visualised using a Gel Doc system (Bio-Rad, Hercules, CA, USA). Amplified products were purified using magnetic bead–based cleanup (CleanPCR, CleanNA, Netherlands), and short non-target fragments were removed using the ProNex size-selective purification system (Promega, Southampton, UK). The concentration and purity of the pooled amplicons were measured using the QuantiFluor dsDNA system (Promega, USA) with a fluorometer. The quality and size distribution of the amplicons were further assessed using a Bioanalyzer 2100 (Agilent Technologies, CA, USA) with a DNA 7500 chip.

Libraries were excluded from sequencing if the PCR product concentration was below 20 ng/μL, the total volume was less than 20 μL or if the agarose gel showed no distinct band or a smeared band. Final sequencing was conducted by CJ Bioscience (Seoul, Korea) using the Illumina MiSeq platform in accordance with the manufacturer's protocol.