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ORIGINAL ARTICLE

Longitudinal changes in subgingival biofilm composition following periodontal treatment

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Abstract

Background: Current periodontal treatment involves instrumentation using hand and/or ultrasonic instruments, which are used either alone or in combination based on patient and clinician preference, with comparable clinical outcomes. This study sought to investigate early and later changes in the subgingival biofilm following periodontal treatment, to identify whether these changes were associated with treatment outcomes, and to investigate whether the biofilm responded differently to hand compared with ultrasonic instruments.

Methods: This was a secondary-outcome analysis of a randomized-controlled trial. Thirty-eight periodontitis patients received full-mouth subgingival instrumentation using hand ($n = 20$) or ultrasonic instrumentation ($n = 18$). Subgingival plaque was sampled at baseline and 1, 7, and 90 days following treatment. Bacterial DNA was analyzed using 16S rRNA sequencing. Periodontal clinical parameters were evaluated before and after treatment.

Results: Biofilm composition was comparable in both (hand and ultrasonics) treatment groups at all time points (all genera and species; p[adjusted] > 0.05). Large-scale changes were observed within groups across time points. At days 1 and 7, taxonomic diversity and dysbiosis were reduced, with an increase in

health-associated genera including *Streptococcus* and *Rothia* equating to 30% to 40% of the relative abundance. When reassessed at day 90 a subset of samples reformed a microbiome more comparable with baseline, which was independent of instrumentation choice and residual disease.

Conclusions: Hand and ultrasonic instruments induced comparable impacts on the subgingival plaque microbiome. There were marked early changes in the subgingival biofilm composition, although there was limited evidence that community shifts associated with treatment outcomes.

KEYWORDS
biofilm, microbiome, periodontitis, treatment

1 | INTRODUCTION

Periodontitis is characterized by an inflammatory response to dysbiotic subgingival plaque biofilm communities resulting in destruction of the supporting alveolar bone and periodontal ligament, ultimately causing tooth loss in susceptible patients. Periodontal treatment involves a series of steps aimed at holistically managing patients and their periodontitis.[1,2] This treatment includes professional mechanical plaque removal (PMPR) and subgingival instrumentation.[2,3] Whilst periodontal treatment delivers beneficial clinical outcomes, the risk of recurrent disease remains high among treated patients.[4]

Currently, both hand and ultrasonic instruments are used in isolation or combination for PMPR/subgingival instrumentation. Despite different mechanisms of action, instrumentation choice is largely guided by patient and clinician preference. Previous studies have demonstrated similar clinical improvement following each instrumentation technique.[5,6] Whether these clinical improvements arise via any differential impact of each instrument type on the biofilm is not known, although recent in vitro reports suggest that the impact of the ultrasonic instrument may extend beyond the point of contact between instrument and biofilm.[7,8]

Current evidence suggests that periodontitis arises via dysbiosis of the entire subgingival plaque community,[9,10] promoting gingival inflammation. In turn, inflammation drives further community dysbiosis.[11,12] Cross-sectional analysis of the microbiome demonstrates increases in taxonomic diversity, richness, and the abundance of obligate anaerobes in periodontitis compared with periodontal health,[13,14] with several species consistently elevated in periodontitis.[15]

Periodontal treatment has been demonstrated to induce widespread microbial shifts in the subgingival plaque, commensurate with the formation of a microbiota more comparable with periodontal health.[16–18] However, the majority of periodontal treatment studies either do not report instruments used or report use of a "blended" approach with respect to instrumentation technique, employing both hand and ultrasonic scalers. Additionally, these studies have evaluated single follow-up time points between 1 and 5 months.[16–18] As such, the immediate impact of instrumentation on the subgingival plaque microbiome, and the steps involved in the subsequent microbial community recovery, are seldom investigated. This led to the hypothesis that the immediate impact of ultrasonic instrumentation in vivo may be different to that of hand instrumentation.

This study was a secondary analysis from a randomized-controlled trial (RCT),[6] and sought to define whether the use of hand (HI) or ultrasonic instruments (UI) has differing impacts on the subgingival plaque microbiota. As such, we investigated the microbial impacts of each instrumentation technique at a range of short-term follow-up time points (day 1, day 7, and day 90), to establish whether such findings may translate to compositional shifts in vivo. Additionally, this study aimed to characterize how changes in subgingival biofilm communities following mechanical disruption relate to clinical outcomes.

2 | METHODS

2.1 | Patient recruitment

The current study presents a secondary outcome from a single-center RCT performed at Glasgow Dental Hospital with patient demographics, clinical, and primary outcome data (systemic inflammation) published previously.[6] The sample size calculation was based on the study's previously published primary outcome: serum C-reactive protein (CRP) levels at day 1 post-treatment (described in detail[6]),

which required $n = 34$ (17 in each group) to detect a minimum difference of at least 1 standard deviation between CRP levels at day 1 between hand and ultrasonic instrumentation. The current analysis of the subgingival plaque microbiome was a secondary outcome of this study and is thus exploratory in nature. Prior to patient recruitment, the study received ethical approval (Health and Social Care Research Ethics Committee A: 18/NI/0059) and was registered with ClinicalTrials.gov (ID: NCT03501316). The Declaration of Helsinki was adhered to throughout the trial.

Patient recruitment, inclusion, and exclusion criteria were described previously.[6] All patients were systemically healthy, aged between 32 and 65 years old, and referred to Glasgow Dental Hospital for treatment of periodontitis. Periodontitis was defined as probing depths of ≥5 mm on two or more teeth at non-adjacent sites with cumulative probing depths of ≥40 mm.[19,20] Cumulative probing depth was calculated by examining six sites on each tooth, as previously described.[6,21–23] In brief, the deepest site on each tooth was recorded and if this value was ≥5 mm, it contributed to the cumulative total. Each tooth with probing depths of ≥5 only contributed a single value (deepest site) toward this cumulative total.

2.2 | Treatment and study design

All interventions were performed by an experienced dental hygienist (D.M.) and a specialist trainee in restorative dentistry and periodontics (M.P.). Both practitioners were calibrated by pocket chart completion on the first twelve patients, and a kappa score was calculated (0.66). All patients received Step 1 and Step 2 of treatment. The subgingival instrumentation in Step 2 was completed within 24 hours. Thirty-eight patients completed the study, with 20 patients receiving treatment exclusively with HI and 18 patients receiving treatment exclusively with UI ($n = 38$ in total). For HI, this $n = 20$ included one patient who was not included in the primary outcome analysis[6] due to an elevated baseline serum CRP level (11.97 mg/L); this patient's baseline microbiota did not substantially differ from that of the remaining cohort at baseline. Treatment was performed as previously described[6] using Gracey and Universal curettes and hoes[*] for HI, or Cavitron Ultrasonic inserts[†] for UI. Patients attended a baseline visit, at which Step 1 was completed (referred to as "baseline"), a Step 2 treatment visit, and then a day 1 post-subgingival instrumentation visit (referred to as "day 1"), a day 7 post-subgingival instrumentation visit (referred to as "day 7"), and a day 90 post-subgingival instrumentation visit (referred to as "day 90") (see Figure S1 in the online *Journal of Periodontology*). Periodontal clinical parameters were recorded at baseline and day 90.

2.3 | Subgingival plaque site selection and sampling

This was a single-site analysis, with sampling performed by an experienced, trained, dental hygienist (D.M.) and a specialist trainee in restorative dentistry (M.P.). At baseline, a single "deep" site in each patient (i.e., pocket depth of ≥5 mm) was selected (generally the deepest pocket in the quadrant on a tooth that was not deemed of hopeless prognosis), and subgingival plaque was collected from the same site at 1, 7, and 90 days following treatment. To collect samples the supragingival plaque was initially removed, and subgingival plaque was harvested using a curette. Samples were placed immediately into 500 μL sterile phosphate buffered saline[‡] and centrifuged (13,500 RPM for 10 min) to obtain bacterial pellets. The supernatant was discarded, and bacterial pellets stored at −80°C until further analysis.

2.4 | Bacterial DNA extraction

DNA was extracted from subgingival plaque samples using the MagNA Pure LC DNA isolation kit III for bacteria and fungi,[§] performed on the MagNA Pure LC 2.0 instrument.[§] An additional lysis step with an enzymatic cocktail (including lysozyme, mutanolysin, and lysostaphin) was included in all extractions exactly as described previously.[24] Following extraction, DNA was concentrated using Vivacon 500 tubes[**] and final concentrations measured on a Qubit TM3 fluorometer.[††]

2.5 | rRNA sequencing

For 16S sequencing, the Metagenomic sequencing Library Preparation protocol developed by Illumina was followed (Part #15044223, Rev A). An Illumina amplicon library was prepared using the Illumina_16S_341F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and Illumina_16S_805R (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC)

[*] LM Dental, Parainen, Finland.
[†] Dentsply Sirona, North Carolina, USA.
[‡] Sigma–Aldrich, Gillingham, UK.
[§] Roche Diagnostics, Mannheim, Germany.
[**] Sartorious, Goettingen, Germany.
[††] Thermofisher, Massachusetts, USA.

primers, which amplify the V3–V4 hypervariable regions. Following amplification, DNA was sequenced on an Illumina MiSeq using the 2 × 300 bp paired-ends protocol. All sequencing data are deposited in the NCBI Sequencing Read Archive under BioProject PRJNA736618.

2.6 | Taxonomic classification

Taxonomic classification was performed as previously described.[18,24–26] Prior to taxonomic classification, sequences were pre-processed to remove low quality bases at the end of reads. An amplicon sequence variant (ASV) table was obtained from paired-end FASTQ files using the DADA2 pipeline in R.[27] Chimeras and singletons were removed and the final ASVs were compared against the *Homo sapiens* genome (GRCh38.p13) to remove host reads using Bowtie2.[28] Singletons were removed and taxonomic classification was achieved by comparison to the non-redundant SILVA reference database,[29] using the naïve Bayesian classifier method. ASVs with genus-level classification but without exact species matching were assigned using the BLASTN tool against the SILVA database with a 97% similarity threshold.[30] During analysis, two samples from the HI group (1 at day 7, 1 at day 90) had <5000 total reads, and were not included in any statistical analysis based on rarefaction curves ($n = 19$ for HI at day 7 and day 90).

2.7 | The subgingival microbial dysbiosis index (SMDI)

Post hoc, the subgingival microbial dysbiosis index (SMDI) was calculated for each sample as described previously.[31] Briefly, the sequencing data were reanalyzed using the BLASTN-based species-level taxonomy assignment algorithm as previously described,[32,33] and the resultant species read counts were normalized by centered log-ratio (CLR) transformation. The abundances of 49 predefined normobiotic and dysbiotic species were used to calculate the SMDI as the mean CLR abundance of dysbiotic species minus the mean CLR abundance of normobiotic species.

2.8 | Statistical analysis

Clinical characteristics were assessed using general linear models (GLMs) in SPSS.[‡‡] The baseline level of each variable, smoking status, and age were included as covariates, and results refer to fully adjusted models. Analysis of the subgingival plaque microbiome was performed using R programming language (v3.4+) or MicrobiomeAnalyst software.[34] For the latter, default filtering was applied (prevalence = 20%, interquartile range = 10%).

For α-diversity indices (observed species, Shannon, abundance-based coverage estimator [ACE]), values were calculated at species level rarefying to 14,000 reads using the Vegan library in R.[35] Within-group and between-group differences in α-diversity were assessed using Wilcoxon signed-rank and Mann–Whitney tests, respectively, as data followed a non-normal distribution in histograms.[§§] Principal coordinates analysis (PCoA) and corresponding analysis of similarity (ANOSIM) were performed using MicrobiomeAnalyst software, which functions through the phyloseq package in R.[36]

Longitudinal univariate analysis of genus and species was conducted using Wilcoxon signed-rank tests (wilcox.test function) in R. For this analysis, a 50% prevalence filter was applied to discern consistent differences across all patients. Additionally, genera and species were only included in the analysis if they had an average abundance superior to five times the smallest percentage above zero.[18,26] Excluding clinical data analysis, all *p*-values were adjusted for multiple comparisons using the Benjamini–Hochberg false discovery rate (FDR) of 5%. After calculation, SMDI values were evaluated using histograms and found to display normal distribution. Therefore, longitudinal and between-group analysis was performed using paired and unpaired *t*-tests where appropriate, with smoking categories compared using one-way analysis of variance (ANOVA) with Tukey's post hoc test.[§§]

3 | RESULTS

3.1 | Clinical treatment response

Thirty-eight patients were recruited and completed the study. According to the 2017 classification, 45% of the patients had generalized stage 4 grade C periodontitis, 42% had generalized stage 3 grade B periodontitis, and 13% had generalized stage 3 grade C periodontitis.[37] Patient demographics have been published previously.[6] The site-specific and whole-mouth clinical variables demonstrated substantial improvement at day 90 following treatment with no differences between the UI and HI groups across any clinical parameter (see Table S1 in the online *Journal of Periodontology*). Both treatment groups showed similar probing pocket depth (PPD) in sampled sites (7.67 for UI vs. 7.65 mm for HI) at baseline. The PPD significantly

[‡‡] IBM, New York, USA.

[§§] GraphPad PRISM, California, USA.

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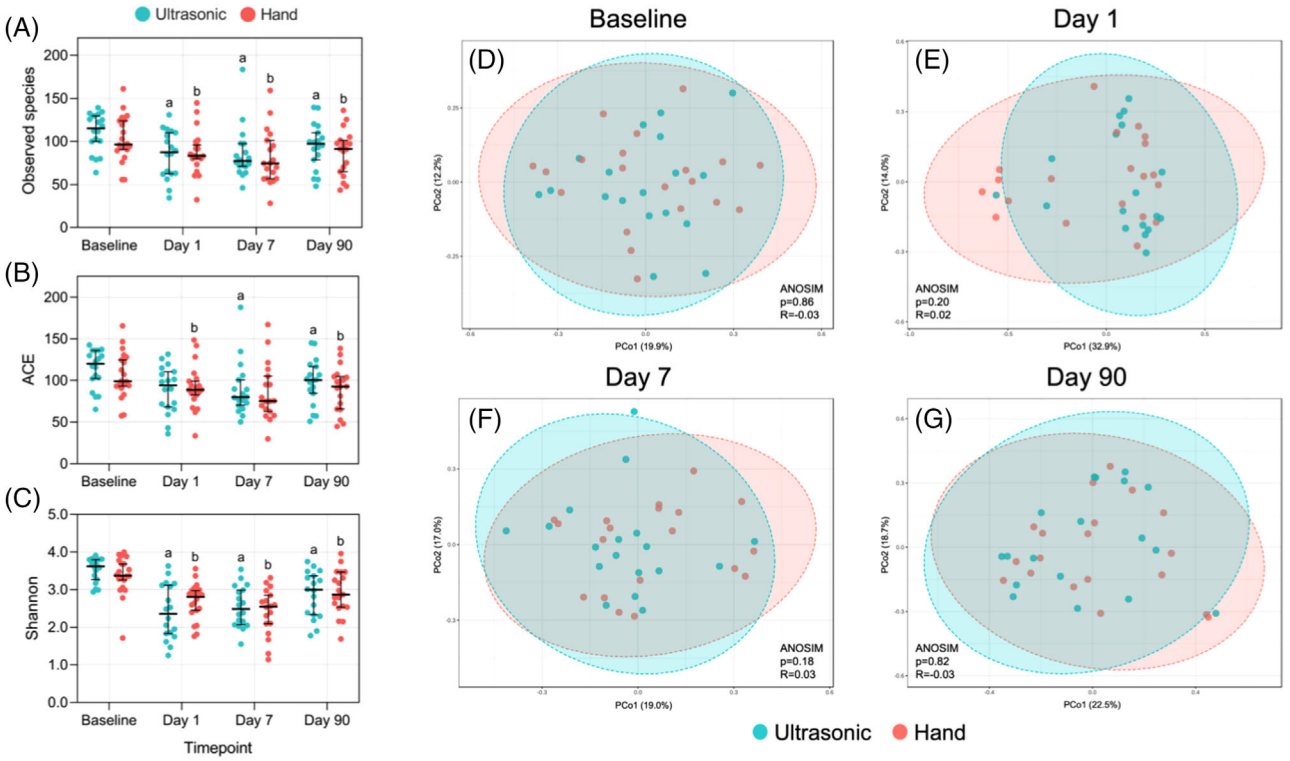

FIGURE 1 Alpha- and beta-diversity of subgingival plaque samples. The number of observed species (A), ACE (B), and Shannon (C) indexes between ultrasonic (blue) and hand (red) treatment groups are shown; error bars display medians and 95% confidence intervals. Longitudinal statistics are Wilcoxon signed-rank tests where "a" represents significant difference compared with baseline in the ultrasonic group, and "b" represents significant difference compared with baseline in the hand group. No differences were observed between groups (Mann–Whitney *U* test). Bray–Curtis-based PCoA was also performed at the species level across ultrasonic and hand-treated sites at baseline (D), day 1 (E), day 7 (F), and day 90 (G). Ellipses represent 95% confidence intervals. Between-group statistics refer to the ANOSIM function, all $p > 0.05$. For baseline and day 1, $n = 38$. For day 7 and day 90, $n = 37$. ACE, abundance-based coverage estimator; ANOSIM, analysis of similarity; PCoA, principal coordinate analysis

reduced following treatment regardless of instrumentation choice ($p < 0.001$ for both, paired *t*-test). Clinical improvement within these sites was comparable between instrumentation groups ($p = 0.94$, GLM).

3.2 | Comparing α- and β-diversity between treatment groups

Rarefaction curves suggested a reliable estimate of taxonomic diversity was feasible when rarefying to 14,000 reads per sample (see Figure S2 in the online *Journal of Periodontology*). The observed species, Shannon, and ACE indices were calculated to evaluate the diversity of subgingival plaque samples (Figure 1A–C). There were significant reductions in the number of observed species and Shannon index within both the UI and HI groups as early as day 1 posttreatment (both $p < 0.05$, Figure 1A,B). These reductions were maintained until day 90, with no significant differences between treatment groups at any time point ($p > 0.1$). Similar longitudinal results were found for the number of observed species and ACE index (Figure 1C). There were no significant differences in the number of observed species or ACE index between groups at any time point.

This was consistent with β-diversity analysis, using Bray–Curtis-based PCoA at each time point (Figure 1D–G). Similar to the comparison of relative abundances, species-level PCoA at each time point suggested no differences between HI and UI at baseline ($p = 0.86$, Figure 1D), day 1 ($p = 0.20$, Figure 1E), day 7 ($p = 0.18$, Figure 1F), or day 90 ($p = 0.82$, Figure 1G).

3.3 | Compositional shifts following hand and ultrasonic scaling

Alterations in the subgingival plaque composition were assessed between groups at each time point. The top 20 most abundant genera are displayed (Figure 2), equating to >70% of the total composition at all time points. The baseline microbiota were similar in the HI and UI groups,

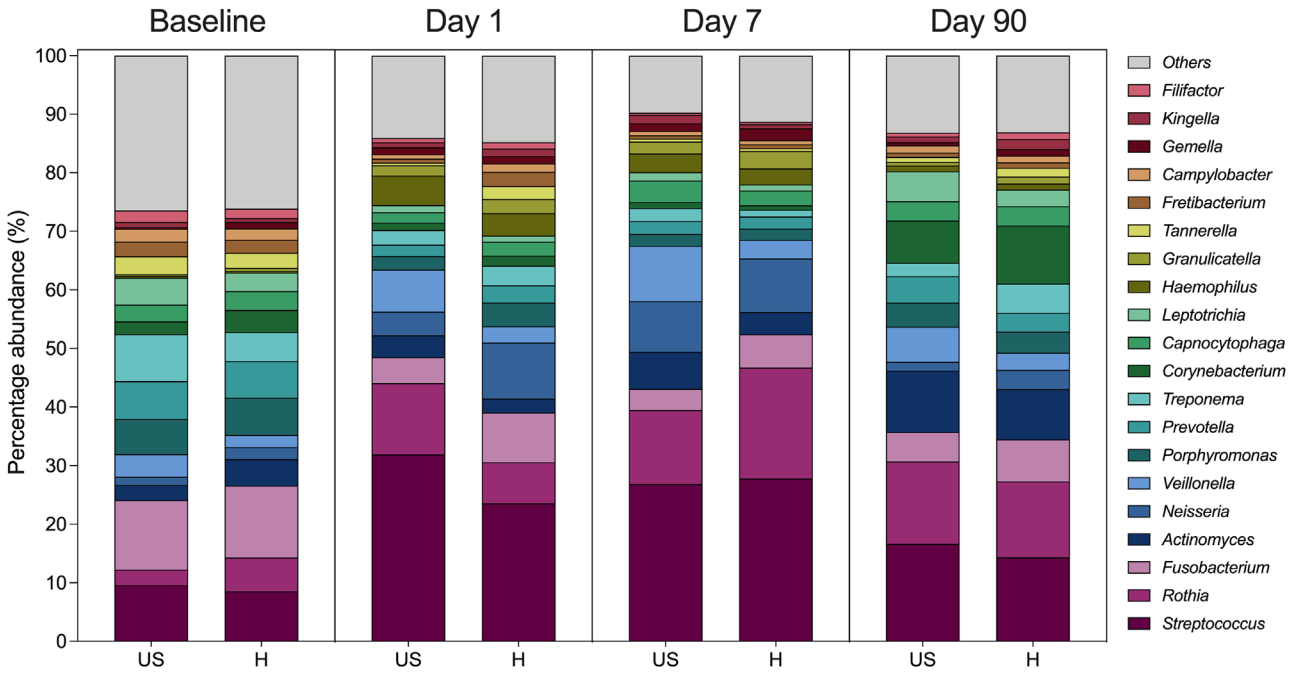

FIGURE 2 Genus composition of subgingival plaque samples. Graphs display the average abundance of genera within each group at baseline, day 1, day 7, and day 90. The top 20 most abundant genera are displayed, with all others grouped together (gray bars). No differentially abundant genera were identified between the US and H treatment groups at any of the time points (Mann–Whitney *U* tests adjusted for multiple comparisons using the FDR [5%] approach). For baseline and day 1, $n = 38$. For day 7 and day 90, $n = 37$. FDR, false discovery rate; H, hand; US, ultrasonic

with no detectable alterations in the abundance of any genera or species (all $p[adj] > 0.05$). There were longitudinal large-scale compositional changes within each group. There were no significant differences in the abundance of any genera or species between groups at day 1, 7, or 90 following treatment. The SMDI, which estimates dysbiosis in the overall subgingival plaque composition,[31] reduced at all posttreatment time points compared with baseline. There were no differences in SMDI between groups at any time point (Figure 3).

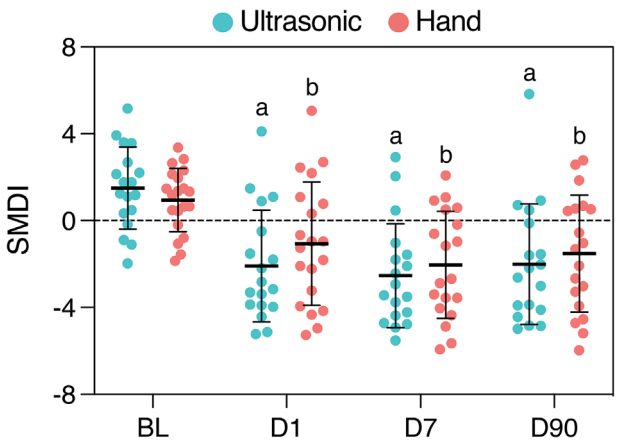

FIGURE 3 Comparisons of the subgingival microbial dysbiosis index between ultrasonic and hand groups at each time point. Individual sites are represented in each graph; error bars display means and standard deviations. Longitudinal statistics are paired *t*-tests where "a" represents significant difference compared with baseline in the ultrasonic group, and "b" represents significant difference compared with baseline in the hand group. No differences were observed between groups (unpaired *t*-test). All statistics were corrected for multiple comparisons using the false discovery rate (FDR, 5%) approach. For baseline and day 1, $n = 38$. For day 7 and day 90, $n = 37$. BL, baseline; D, day; SMDI, subgingival microbial dysbiosis index

3.4 | Longitudinal alterations following subgingival instrumentation

There were significant longitudinal differences in alpha diversity and SMDI, with no detectable differences between groups. Therefore, longitudinal analysis was performed across the entire cohort (UI and HI combined) to characterize the changes of the subgingival plaque microbiota following subgingival instrumentation. This analysis revealed consistent shifts in the abundance of 3 genera following treatment. Of these, 22 genera significantly differed at all time points compared with baseline (Figure 4A), demonstrating that differences were observed rapidly and

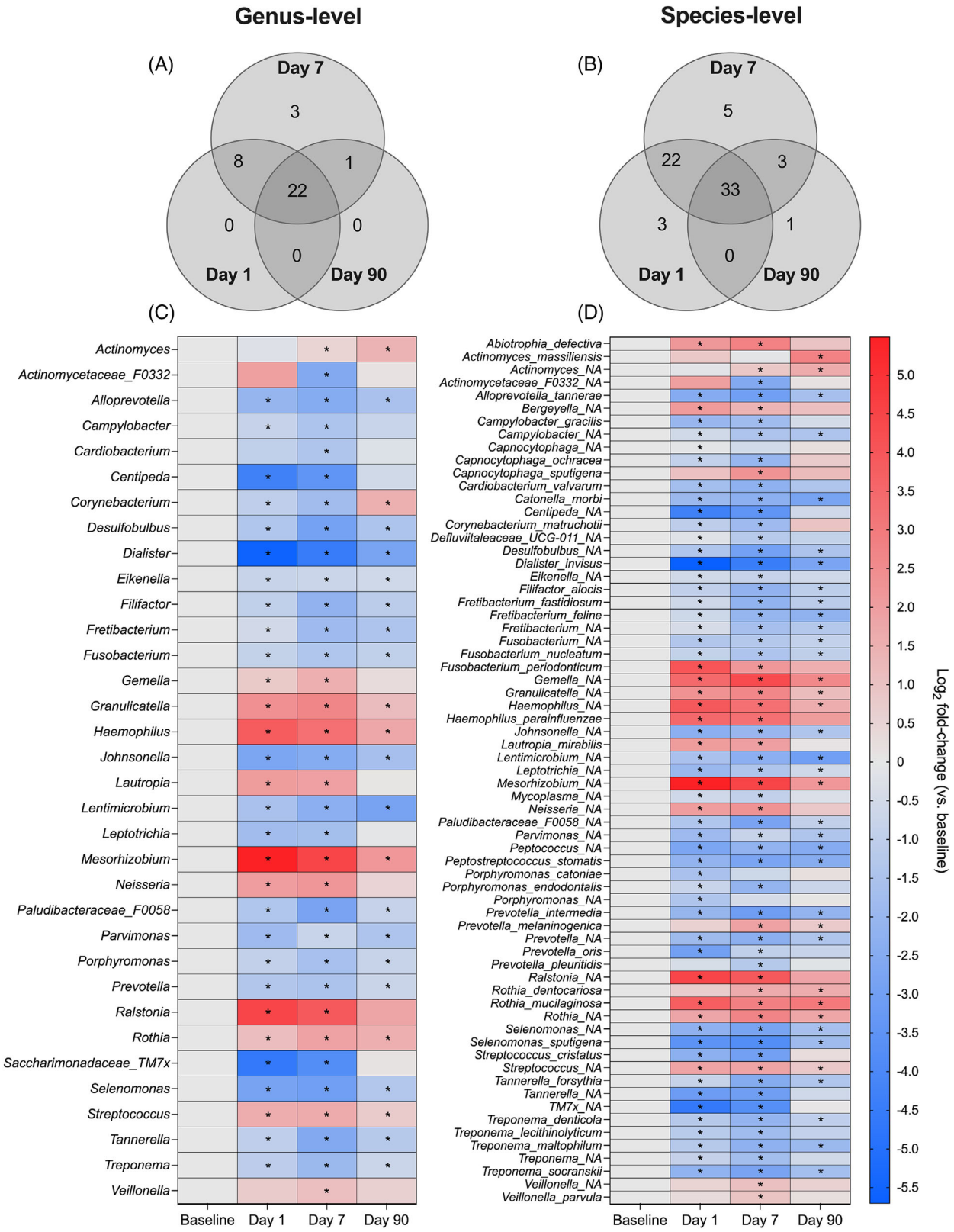

FIGURE 4 Genus-level and species-level differential abundance following full-mouth debridement. Venn diagrams display the overlap in abundant genera (A) and species (B) which significantly differed at each time point. Heatmaps display genera (C) and species (D) which significantly differed compared with baseline. Data are represented as $\log_2$ fold-change compared with baseline. Statistics refer to Wilcoxon signed-rank test with each time point versus baseline, adjusted for multiple comparisons using the false discovery rate (FDR, 5%) approach. *p[adjusted] < 0.05. For baseline and day 1, $n = 38$. For day 7 and day 90, $n = 37$. Only abundant genera (> 0.5% at any time point) and species (> 0.25% at any time point) were included. NA, not available

maintained throughout the study period. This included several disease-associated anaerobic genera such as *Porphyromonas*, *Tannerella*, *Treponema*, *Prevotella*, *Filifactor*, and *Selenomonas* (Figure 4C). Several other genera such as *Desulfobulbus*, *Dialister*, and *Parvimonas* also followed a similar pattern. In contrast, several "health-associated" genera, including *Streptococcus*, *Rothia*, *Haemophilus*, and *Granulicatella*, were significantly increased at all time points compared with baseline.[38,39]

An exception to this trend was *Corynebacterium*, which significantly reduced in abundance at days 1 and 7, but increased at day 90 compared with baseline. Other genera including *Campylobacter*, *Leptotrichia*, and *Neisseria* significantly differed only at early time points (day 1, 7) and returned to baseline levels by day 90, whilst one, *Actinomyces*, significantly differed only at days 7 and 90. No genus was differentially abundant only at days 1 or 90. Thus, the majority of alterations were either induced rapidly and maintained, or recovered gradually over the study period. Analogous shifts were observed at the species level with 67 species significantly differing in abundance across all time points (Figure 4B). Of these, 33 were significantly different at all time points, albeit many of these were unclassified (Figure 4D, items labeled as "*[species]_NA*").

3.5 | Longitudinal changes in the subgingival plaque microbiota

To visualize the reformation of the subgingival plaque microbiota, PCoA was performed across time points with each time point added sequentially (Figure 5A). As expected, the baseline samples clustered together on the right-hand side. At day 1, the majority of samples formed a distinct cluster compared with those at baseline, albeit a small degree of overlap remained in a subset of samples. Distinct clustering was maintained until day 7, with a high degree of overlap between the day 1 and 7 samples. At day 90, no clustering was observed, and sample compositions appeared highly spread across the preceding time points (Figure 5B). Comparative analysis of each time point separately with baseline demonstrated larger differences between baseline and day 1 (ANOSIM $R = 0.43$, $p < 0.001$) and day 7 ($R = 0.64$, $p < 0.001$) compared with day 90 ($R = 0.26$, $p < 0.001$). Overall, it appeared that a subset of day 90 samples had reformed as a composition similar to baseline, whilst others maintained a composition similar to days 1 and 7. Notably, when applying a color gradient to these plots, samples appeared clustered by SMDI values. This was consistent across all samples and specifically those at day 90 (see Figure S3A, B in the online *Journal of Periodontology*).

3.6 | Dysbiosis and disease severity

To investigate factors potentially associated with the day 90 microbiota, the SMDI was assessed in relation to clinical variables. Site PPD was considered as an indicator of the microenvironment from which subgingival plaque samples were harvested. Despite large heterogeneity in the PPD of sampled sites at day 90, there was no association between SMDI and site PPD (Figure 6A). Additional analysis was performed to address the potential influence of neighboring sites by using the whole-tooth PPD (average of 6 sites around sampled tooth), and similar results were obtained (Figure 6B).

Given previous links between gingival inflammation and biofilm dysbiosis,[11,12] it was hypothesized that the nature of the microbiome following treatment may instead be associated with the overall level of residual inflammation. To test this, the periodontal inflamed surface area (PISA) was employed as an estimate of total inflammatory burden.[40] However, this analysis also suggested no positive or negative association between the day SMDI and PISA (Figure 6C). For example, some samples contained a low SMDI despite patients displaying higher residual PISA, whilst the highest SMDI (5.84) was found in a patient with a day 90 PISA of only 60.66 mm^2—commensurate with the range observed in periodontal health.[41] Despite previous links between smoking and the subgingival microbiome,[42] we observed no clear association between the day 90 SMDI and smoking status (Figure 6D). Further exploratory analysis found no association between the day 90 SMDI and plaque index (Spearman $R = 0.15$, $p = 0.39$), bleeding on probing (Spearman $R = 0.03$, $p = 0.88$), or the proportion of sites ≥5 mm (Spearman $R = 0.17$, $p = 0.31$) at this time point (data not shown).

4 | DISCUSSION

These data compare the effects of hand and ultrasonic instrumentation on the subgingival microbial community. To the best of our knowledge this analysis of microbial community sequentially over time is the first to document the early and late-stage changes following periodontal treatment at these time points and to identify notable variation in community recovery. Earlier studies using targeted approaches such as DNA-DNA hybridization and PCR for quantification of certain disease-associated species have suggested comparable microbial outcomes following a range of instrumentation techniques[5,43,44]; our data indicate that such effects extend across the entire microbiome when comparing HI and UI. Both hand and ultrasonic scalers significantly reduced subgingival plaque dysbiosis and taxonomic diversity, which are both elevated in

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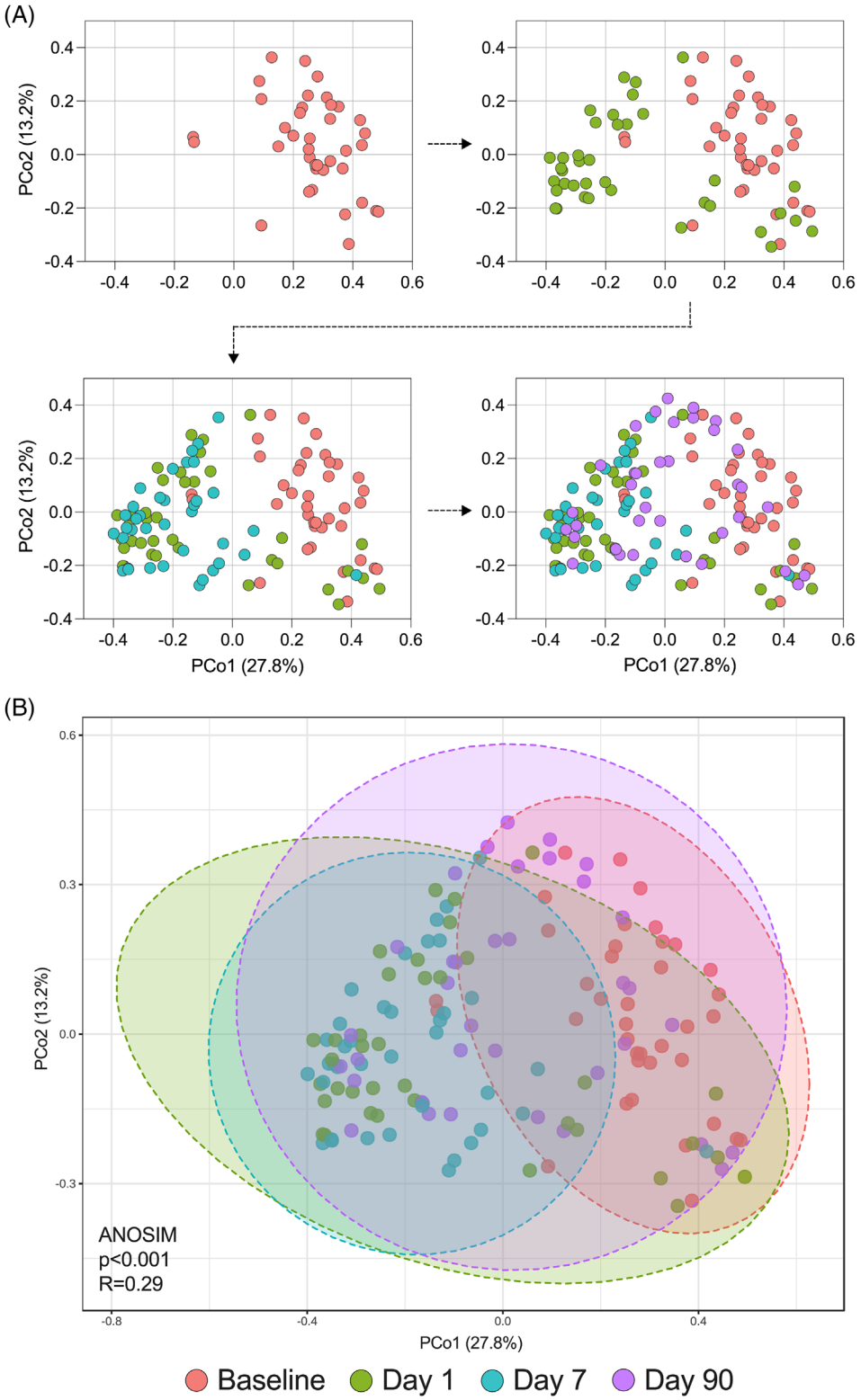

FIGURE 5 Compositional and community shifts following full-mouth debridement. (A) Species-level Bray–Curtis-based PCoA, with individual time points sequentially added on to the figure. (B) All samples combined with confidence intervals. Statistics refer to ANOSIM. For baseline and day 1, $n = 38$. For day 7 and day 90, $n = 37$. ANOSIM, analysis of similarity; PCoA, principal coordinate analysis

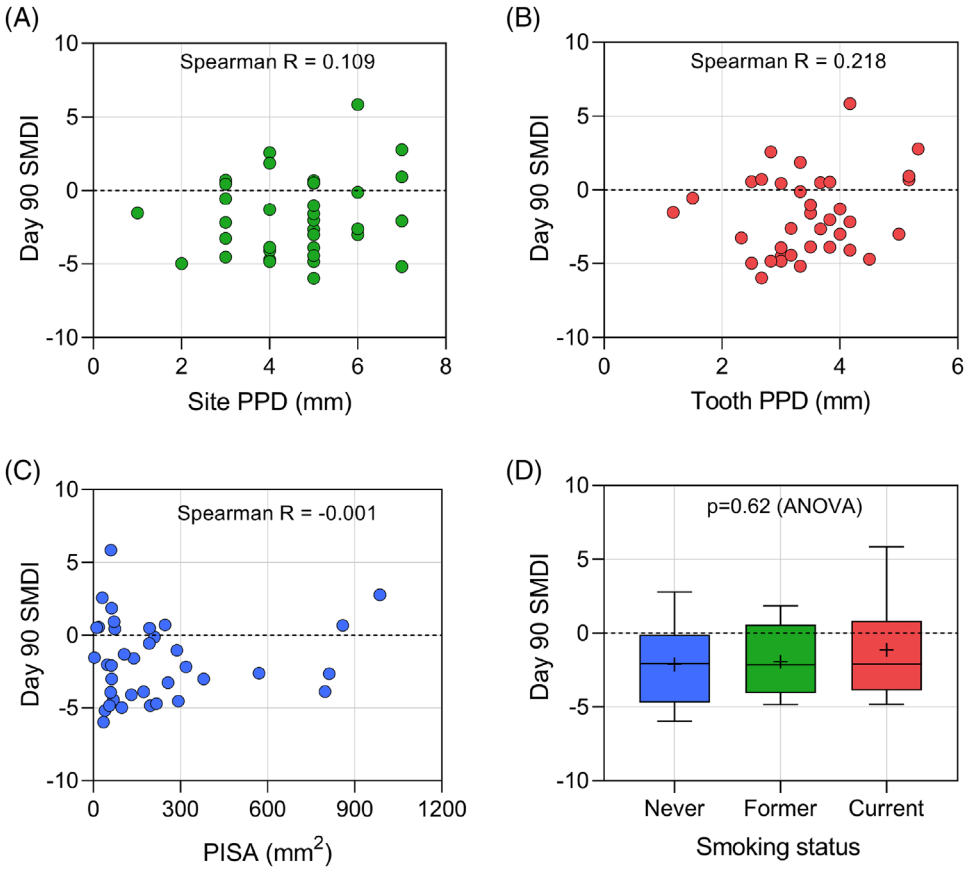

FIGURE 6 Investigating the day 90 SMDI. Scatterplots represent the day 90 SMDI against (A) site PPD, (B) tooth PPD, and (C) PISA at day 90. (D) Boxplot graphs represent the day 90 SMDI between smoking categories, where the horizontal line represents the median and "+" represents the mean of each group. Statistics refer to Spearman-Rho correlations (A–C) or one-way ANOVA with Tukey's post hoc test (D), $n = 37$. ANOVA, analysis of variance; PISA, periodontal inflamed surface area; PPD, pocket probing depth; SMDI, subgingival microbial dysbiosis index

diseased sites.[13] Whilst in vitro reports have demonstrated elevated levels of biofilm removal following ultrasonic versus hand instrumentation,[7,8] our data imply that the in vivo situation is likely to be more complex.

Assessing the entire cohort, longitudinal microbial alterations were comparable with previous studies investigating the subgingival plaque microbiota following periodontal treatment.[16,17] In the current study, *Streptococcus*, *Rothia*, and *Neisseria* were elevated at day 1 and day 7 compared with baseline, commensurate with the spatiotemporal model of oral bacterial colonization.[45] Interestingly, the rapid expansion of *Rothia* has also been previously documented at 2 and 6 week time points,[46] indicating that such alterations are likely maintained past the 7 day follow-up within the current study.

Overall most compositional alterations were either induced rapidly and maintained over the course of the study, or recovered between days 7 and 90. In regard to the latter, similar observations have been found in healthy subjects in a study that collected the subgingival plaque at 10 time points following mechanical debridement ranging from 0 hours (immediately after) to 3 months.[47] Despite differences in disease states of the respective cohorts, some similarities were observed in microbial shifts between the study by Wang et al.[47] and the current study. Notably, an increase in *Rothia*, *Streptococcus*, and *Neisseria*, along with reductions in *Campylobacter*, *Filifactor*, *Porphyromonas*, and *Tannerella* at days 1 and 7, were consistent among cohorts. In the study by Wang et al., the authors observed the largest compositional difference from baseline at 7 hours to 3 days—rather than 0 to 4 hours—with an almost entire recovery by 3 months. This community reformation may be expected in healthy subjects where the core microbiota is likely more stable; however, our current study also demonstrates a similar response within diseased sites.

Although the microbiota appeared relatively consistent among patients at day 1 and day 7, there was marked heterogeneity in the composition of day 90 samples with some samples resuming a high abundance of disease-associated species. Interestingly, there were no associations with this apparent recovery of a disease-associated community and

residual clinical disease, smoking status, or treatment group. This is supported by previous studies where a clear disease-associated microbial signature persisted in some patients despite widespread clinical improvement.[17,48] The significance of this microbial response in the absence of clinical disease is unknown, although it has been speculated that a high abundance of disease-associated species, even without clinical disease, may be prognostic of future disease progression.[14] Nonetheless, it is intriguing that such a differing microbial response is observed among a cohort of patients who all improved clinically, received similar treatment, and demonstrated a relatively comparable microbiota in preceding time points.

Several factors are known to alter the oral microbiome and may offer a partial explanation of these results. It has been reported that children who have parents suffering from periodontitis display an elevated abundance of disease-associated organisms,[49] supporting earlier work that cohabitation can influence the composition of different oral niches.[50] Additionally, a genetic susceptibility to the colonization of red-complex species has been reported by a genome-wide association study[51]—suggesting host genetic variables may be important in driving microbial composition.

In a similar sense, during development of the SMDI it was highlighted that a small subset of periodontitis samples contained relatively low levels of dysbiosis, whilst a small subset of healthy samples contained high levels of dysbiosis.[31] It was suggested that periodontitis samples with low dysbiosis may represent quiescent sites or patients hyperresponsive to dysbiosis, whereas healthy sites with high dysbiosis may represent a form of tolerance or sites at risk of disease progression.[31] Similarly, it could be proposed that individual patients may have a "dysbiosis threshold." We explored whether the change in the SMDI from baseline to day 90 associated with the change in clinical variables and found a weak association (Pearson $R = 0.40$) with the change in PPD, but not PISA—and larger long-term studies will be required to determine how SMDI relates to treatment outcomes.

A commonly discussed viewpoint is that the oral microbiome is naturally resilient to widespread compositional shifts.[12,52] The results in the current study represent the mechanical removal of a stable dysbiotic community and its regrowth (i.e., the growth of the remaining biofilm combined with the inoculation of microorganisms from supragingival plaque and saliva) at different time points. We show that there is a (partial) recovery of a health-associated composition, but the dysbiosis appears to return over time. Given that pockets were still present (although reduced) after treatment, it is likely that disease-associated organisms recolonize and establish themselves as biofilms grow and mature, contributing to the chronic nature of periodontitis.

It is worth noting that there are inevitable limitations with detailed analyses of patient samples. This was a secondary outcome and therefore the study was not a priori designed to evaluate longitudinal differences in microbial plaque. Additionally, sequencing 16S rRNA fragments is not quantitative as results are computed as relative abundances. Given the nature of the treatment performed, it is assumed that the total microbial load of day 1 and possibly day 7 samples will be less than that at baseline or day 90, and the results here merely reflect compositions at each point in time. Additionally, subspecies taxonomic resolution is unobtainable using a fragment of the 16S rRNA gene, and species level is largely provisional for closely related organisms. This is of importance in subgingival plaque where different species of *Fusobacterium* and subspecies of *F. nucleatum* are suggested to play differing roles in biofilm formation and architecture in vitro.[53] In relation to the current study, the relative abundance of *F. nucleatum* significantly reduced at days 1 and 7, whilst the abundance of *F. periodonticum* increased, which may warrant further investigation.

5 | CONCLUSION

This analysis revealed no differences in the subgingival plaque microbiota following HI or UI. Assessing longitudinal alterations, the composition was dominated by *Streptococcus*, *Actinomyces*, and *Rothia* species at day 1, whilst a small proportion of disease-associated anaerobes (*Fusobacterium*, *Porphyromonas*) persisted throughout treatment and recovery. By day 90, some sites maintained this *Streptococcus*, *Actinomyces*, *Rothia*-dominated composition, whilst others showed a composition more comparable with baseline. This response did not appear to be substantially associated with any clinical, behavioral, or demographic variables evaluated in this study. It is possible that factors including the environment, genetics, diet, and biofilm quantity may shape this longer-term microbial response. Understanding the implications of this variability in microbial response, and its drivers, could help guide and rationalize adjunctive periodontal treatments.

AUTHOR CONTRIBUTIONS

All authors have made substantial contributions to the conception, design, and/or analysis of this study. Marilyn Goulding, Gordon Ramage, Alex Mira, and Shauna Culshaw conceived the study, participated in study design, and supervised laboratory work and analysis. William Johnston, Michael Paterson, Krystyna Piela, and Shauna Culshaw performed clinical sample processing. Michael

Paterson, Paddy Watson, Krystyna Piela, and Shauna Culshaw performed clinical data acquisition and analysis. William Johnston, Bob T. Rosier, Miguel Carda-Diéguez, Gordon Ramage, Alex Mira, and Shauna Culshaw were responsible for 16S rRNA sequencing data acquisition, analysis, and manuscript preparation. Divyashri Baranyia, Tsute Chen, and Nezar Al-Hebshi performed bioinformatic data analysis and critically revised the manuscript. All authors have read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

William Johnston received grant funding from Dentsply Sirona, Pennsylvania, USA. At the time of this study, Marilyn Goulding was the Manager of Clinical Research, Global Clinical Affairs, Dentsply Sirona, Pennsylvania, USA. Shauna Culshaw received grant funding, personal fees and non-financial support from Dentsply Sirona, Pennsylvania, USA. Bob T. Rosier, Miguel Carda-Diéguez, Michael Paterson, Paddy Watson, Krystyna Piela, Gordon Ramage, Divyashri Baranyia, Tsute Chen, Nezar Al-Hebshi, and Alex Mira declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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