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Original Article

Longitudinal Analysis of Bacterial Colonization on Clear Orthodontic Retainers Using 16S rRNA Sequencing

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ABSTRACT

This study aimed to investigate the temporal changes in bacterial communities on clear orthodontic retainers over a 14-day period. Saliva and plaque samples were collected from the surfaces of clear retainers worn by five healthy volunteers who had not previously used any other orthodontic appliances. Participants were instructed to wear the retainers continuously for 14 days, removing them only for meals and cleaning with a soft-bristle toothbrush. Microbial composition was analyzed using Illumina MiSeq sequencing of the bacterial 16S rRNA gene. Bioinformatic analyses were conducted via the QIIME pipeline to assess both intra- and intergroup microbial diversity. Bacterial communities on the retainers and in saliva exhibited notable changes over the 14-day period. At the phylum level, Firmicutes increased significantly on the retainers by 1.26-fold ($p = 0.0194$) at 7 days and 1.34-fold ($p = 0.0123$) at 14 days compared to saliva. Conversely, Campylobacteriota decreased 1.80-fold ($p = 0.05$) on retainers at 7 days relative to saliva. At the genus level, several bacterial taxa showed a significant increase in relative abundance on the retainers after 14 days. The presence of a clear orthodontic retainer may promote changes in oral microbial communities that could contribute to enamel alterations or periodontal tissue damage, particularly following two weeks of use.

Keywords: Orthodontic appliance, Essix C+ retainer, 16S rRNA gene sequencing, Oral microbiome, Orthodontic materials

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Introduction

The oral cavity hosts a highly diverse microbiota, comprising over 700 bacterial species. Each individual possesses a "core" microbiome, which is generally consistent across individuals, alongside a "variable" microbiome, which can include both commensal and potentially pathogenic bacteria [1, 2]. Orthodontic treatment has been associated with an increased risk of dental caries and gingival inflammation. This is largely due to challenges in maintaining oral hygiene, leading to the accumulation of plaque and bacterial biofilms on teeth and around the gingival sulcus. While the normal oral microbiota maintains oral health, excessive plaque buildup can shift the microbial balance, promoting the proliferation of pathogenic bacteria that may damage periodontal tissues and elevate caries risk [3-5]. Given that nearly 90% of oral microbes exist in biofilm form, understanding biofilm dynamics is essential [6, 7].

Environmental changes, such as the placement of orthodontic brackets or other appliances, can alter the oral microbial community, promoting the growth of pathogenic bacteria [8, 9]. Orthodontic appliances facilitate bacterial retention, which increases biofilm accumulation and can contribute to enamel demineralization, gingival inflammation, and periodontal disease. Common pathogenic species associated with these effects include *Streptococcus mutans*, *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, *Fusobacterium nucleatum*, and *Aggregatibacter actinomycetemcomitans*. *S. mutans*, in particular, has been detected in areas adjacent to orthodontic appliances and is implicated in caries development, enamel decalcification, and post-orthodontic white-spot lesions and gingivitis [10-16]. A systematic review reported that the abundance of these bacteria increases during active orthodontic treatment, with a notable decline observed within three months after appliance removal [17, 18]. Subgingival pathogenic

bacteria have been shown to rise within one month of treatment initiation and remain elevated for up to three months post-treatment [19].

Several studies have examined bacterial adherence to orthodontic brackets. Pellissari *et al*. evaluated biofilm formation around fixed brackets, straight arch wires, and modular elastomeric ties, finding that individuals without appliances primarily harbored commensal and enamel-associated microorganisms, while appliance wearers showed a higher prevalence of pathogenic Gram-negative bacteria [20, 21]. Papaioannou *et al*. investigated bacterial adherence on stainless steel, ceramic, and plastic brackets, concluding that bracket material had minimal influence on microbial colonization [22, 23]. Consistently, stainless steel bracket placement has been linked to increased biofilm accumulation on teeth and heightened periodontal colonization [24, 25].

In recent years, removable aligner therapy has gained popularity due to its aesthetic appeal, comfort, and facilitation of oral hygiene. Invisalign, a widely used aligner system, provides patients with a series of removable trays, typically replaced every one to two weeks [26-29]. Studies have reported that bacterial abundance in genera such as *Firmicutes*, *Lactobacillales*, *Bacteroides*, *Granulicatella*, *Porphyromonas*, *Prevotella*, *Haemophilus*, *Acinetobacter*, and *Streptococcus* significantly increases on uncleaned retainers within 24 hours [30, 31]. Other investigations observed elevated levels of *Campylobacter rectus*, *F. nucleatum*, *Prevotella melaninogenica*, *P. intermedia*, *Fusobacterium periodonticum*, *T. denticola*, *T. forsythia*, and *P. gingivalis* associated with clear aligner use [32, 33]. Additionally, *S. mutans* has been shown to increase in patients using removable appliances compared to untreated controls [34, 35]. Guo *et al*. reported that *Firmicutes*, *Actinobacteria*, and *Tenericutes* were more abundant during aligner therapy [36, 37]. A 2022 study found that *Streptococcus* and *Granulicatella* were significantly enriched on clear aligner trays relative to tooth-associated plaque, whereas *Actinomyces*, *Corynebacterium*, and *Selenomas* decreased, indicating that aligners serve as a unique microbial niche and potential reservoir for pathogenic bacteria [38].

Many studies have investigated biofilm accumulation on conventional fixed orthodontic appliances and compared it to removable appliances. While several studies have focused on the composition of biofilms on traditional orthodontic devices, research on bacterial colonization of clear aligners and retainers—and the corresponding changes in salivary microbiota over time—is limited. One study examined bacterial shifts over a 24-hour period; however, to our knowledge, no studies have evaluated the bacterial composition at multiple time points (0, 7, and 14 days) on both clear retainers and in saliva. Essix C+ is a widely used thermoplastic material for fabricating clear aligners and retainers. Unlike aligners, which actively move teeth, retainers are passive devices designed to maintain tooth position. In this study, we aimed to characterize the plaque formation on clear orthodontic retainers and the changes in salivary bacterial composition in patients wearing retainers over a 14-day period. Based on prior evidence and our preliminary observations, we hypothesized that the bacterial composition on the retainers and in saliva would shift between day 7 and day 14. This prospective in vivo study investigates these microbial changes using 16S rRNA gene sequencing.

Materials and Methods

This pilot study was conducted as a prospective clinical investigation of bacterial colonization on orthodontic clear retainers at defined time points. Five adult participants were recruited, and written informed consent was obtained from each prior to study initiation. Ethical approval was granted by the Rutgers University Institutional Review Board (IRB protocol # Pro2018001627). Inclusion criteria were: adults aged 25–65 with well-controlled periodontal health, presence of all permanent teeth from first molar to first molar, and no previous use of retainers. Exclusion criteria included poor systemic health, prior retainer use, tobacco use, professional dental cleaning within two weeks, poor oral hygiene, need for prophylaxis, history of heart murmur or rheumatic fever, pregnancy, current or recent (within one month) antibiotic therapy, and chronic use of NSAIDs or corticosteroids.

On day 1 (T0), participants underwent a periodontal assessment and completed a health and oral hygiene questionnaire to confirm eligibility. Prophylaxis was performed, and participants received oral hygiene instructions, including the provision of a toothbrush and toothpaste, with instructions to brush twice daily and floss once daily. Use of any additional oral hygiene products was prohibited during the 14-day study period. Participants were instructed to wear the clear retainer continuously, removing it only for eating and cleaning with a soft-bristle toothbrush and warm water.

Alginate impressions were taken at T0, poured in mounting stone, and used to fabricate clear Essix C+ retainers (Dentsply Sirona, Charlotte, NC, USA) with a thickness of 0.020” (0.5 mm). Samples of unstimulated saliva and retainer plaque were collected at T0 (day 1), T1 (day 7), and T2 (day 14). Retainers were assumed to be sterile at T0. All impressions, retainer fabrication, and sample collection were performed by a single investigator to ensure consistency.

DNA extraction, library construction, and sequencing

Genomic DNA was isolated from saliva and plaque samples collected from clear retainers using the QiaAmp DNA kit (Qiagen, Hilden, Germany). DNA concentration and purity were assessed with a Nanodrop ND-1000 spectrophotometer (Industriestrasse, Sursee, Switzerland) at 260 nm. Library preparation and sequencing were

carried out at the Rutgers Genomic Center (NJMS, Newark, NJ, USA). DNA quantification was additionally performed using a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA). Approximately 20 ng of genomic DNA was used to generate amplicons targeting the V3–V4 hypervariable regions of the bacterial 16S rRNA gene. Forward primers included the sequence 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTACGGGNGGCWGCAG, while reverse primers contained 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACA GGACTACHVGGGTATCTAATCC. The first PCR products were subsequently used as templates in a second PCR round incorporating Illumina NexteraXT index primers (Illumina, San Diego, CA, USA). Prepared libraries were sequenced on an Illumina MiSeq system using a paired-end 2 × 300 bp protocol, with image acquisition and base calling handled by the embedded MiSeq Control Software.

Bioinformatics analysis

Raw sequencing data were assessed for quality using FASTQC (v0.11.9) and trimmed to remove adapters and low-quality sequences with cutadapt (v3.2). Amplicon sequence variants (ASVs) were inferred and classified taxonomically using DADA2 (v1.20.0) with the SILVA rRNA database (release 138) as reference. Alpha and beta diversity metrics were calculated using QIIME2 (v2021.2.0), and rarefaction analyses were performed to normalize sequencing depth across samples. Differential abundance testing between groups was performed using DESeq2 (v1.26.0), with statistical significance determined at an FDR-adjusted p-value < 0.05.

Microbial culture and enumeration

To quantify viable bacteria, retainer surfaces were rinsed in phosphate-buffered saline (PBS; ThermoFisher Scientific, Waltham, MA, USA) to remove loosely attached cells. Biofilm was then dislodged from the retainer by vortexing. Both saliva and retainer biofilm samples were serially diluted, plated on blood agar, and incubated under anaerobic conditions at 37 °C for 72 hours. Colony-forming units (CFUs) were counted to assess bacterial abundance.

Results and Discussion

Bacterial CFUs were determined in saliva and on retainers at days 0, 7, and 14. As depicted in **Figure 1**, saliva samples consistently contained higher bacterial counts than retainer samples at both day 7 and day 14 ($p < 0.001$). Comparisons of retainer samples between days 7 and 14 revealed a significant increase in CFUs over time ($p = 0.0263$), demonstrating progressive microbial colonization on the retainer surface throughout the study period.

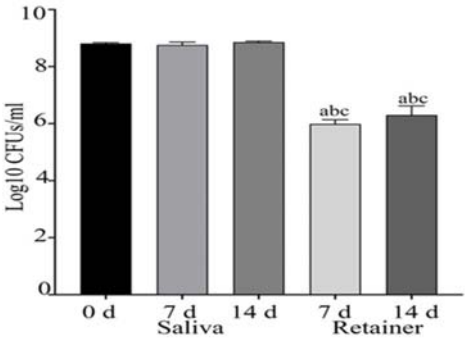


Figure 1. Colony-forming units (CFUs) were measured from saliva and from plaque collected on the retainer surface at 7 and 14 days of wear. Bacterial counts in saliva at days 0, 7, and 14 were significantly higher than those on the retainer surface at corresponding time points ($p < 0.0001$). Statistical comparisons are noted as follows: a, versus saliva at day 0; b, versus saliva at day 7; c, versus saliva at day 14.

Microbial diversity and community richness of saliva and retainer samples

To evaluate microbial richness and evenness within individual samples, alpha diversity metrics were calculated for both salivary and retainer-associated plaque communities at 7 and 14 days. Richness estimators, including Chao1 and ACE (Abundance-based Coverage Estimator), were applied to assess the number of taxa present in saliva, while the Shannon index quantified overall microbial diversity in both saliva and retainer samples. Across all indices, there were no statistically significant changes in alpha diversity over the 14-day period **(Table 1)**.

Beta diversity, which captures differences in microbial community composition between samples, was examined using Principal Coordinates Analysis (PCoA) based on the Jaccard index. This analysis indicated that bacterial communities associated with the retainer differed from those in saliva. Nevertheless, temporal changes within saliva and retainer samples were minimal, suggesting that the microbial structure remained relatively stable over the 14-day period **(Figure 2)**.

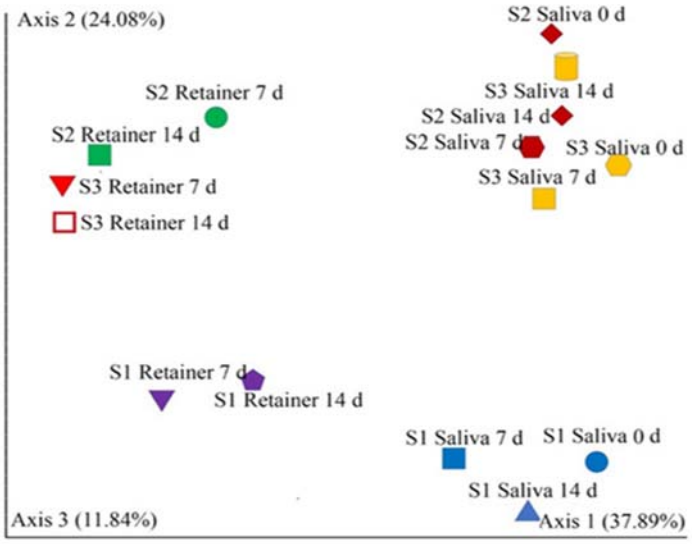


Figure 2. Beta diversity analysis of the oral microbiome following retainer placement. Principal Coordinates Analysis (PCoA) based on the Jaccard index was used to assess differences in microbial composition across all samples. No significant variation in the overall structure of the oral microbiota was observed between saliva and retainer-associated samples. However, the sample distribution was statistically distinct according to Permutational Analysis of Variance (PERMANOVA, $p < 0.05$). Saliva samples clustered predominantly on the right side of the primary horizontal axis, while retainer samples were grouped on the left side. Symbols represent sample types: round, saliva at 7 days; cylinder, saliva at 14 days; cone, retainer at 7 days; diamond, retainer at 14 days.

Table 1. Alpha diversity comparisons between saliva and retainer at 7 and 14 days.

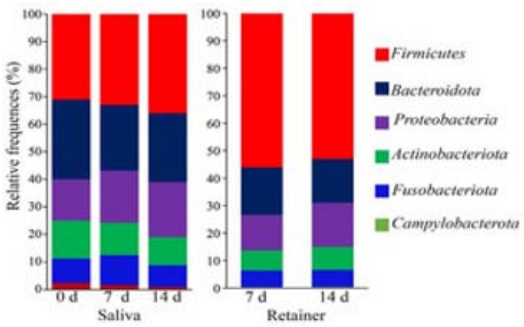
Alpha Diversity	Saliva 0 d	Saliva 7 d	Saliva 14 d	Retainer 7 d	Retainer 14 d
Shannon	5.28 ± 0.79	5.52 ± 0.29	5.62 ± 0.17	4.56 ± 0.55	4.47 ± 1.27
Observed features	132.67 ± 82.95	156.33 ± 22.6	164.67 ± 15.50	145.33 ± 55.87	146.00 ± 57.17
Faith_pd	12.84 ± 3.10	13.45 ± 1.40	13.70 ± 1.32	12.13 ± 2.55	14.76 ± 2.10
Evenness	0.79 ± 0.01	0.76 ± 0.02	0.76 ± 0.2	0.64 ± 0.05	0.62 ± 0.14

Comparison of alpha diversity indices

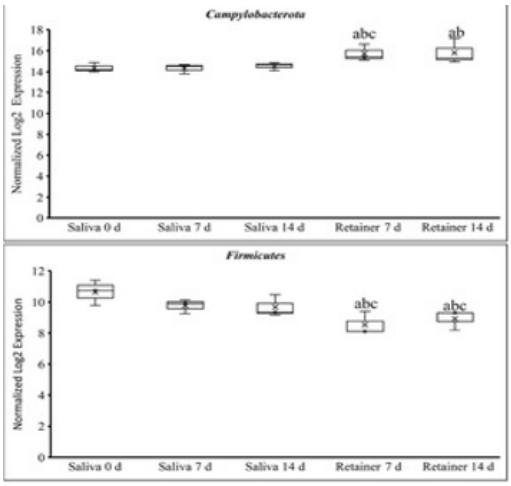
No statistically significant differences in alpha diversity were observed among the groups. Statistical analyses were conducted using either ordinary one-way ANOVA or the Kruskal–Wallis test, as appropriate. Data are presented as mean ± SEM.

Comparison of microbial abundance and composition in saliva and retainer plaque

To further investigate shifts in microbial community structure, we analyzed the relative abundance of bacteria at both phylum and genus levels. At the phylum level, the six most prevalent phyla—Actinobacteria, Fusobacteria, Firmicutes, Bacteroidetes, Proteobacteria, and Campylobacterota—were consistently observed in both saliva and retainer samples **(Figure 3a)**. Comparative analysis of phylum-level abundances revealed that Firmicutes increased significantly on the retainer, with a 1.33-fold rise at day 7 ($p = 0.0021$) and a 1.5-fold increase at day 14 ($p = 0.0063$) relative to saliva at day 0 **(Figure 3b)**. When comparing saliva and retainer at day 7, Firmicutes on the retainer were elevated by 1.26-fold ($p = 0.0193$), while at day 14, retainer-associated Firmicutes increased by 1.34-fold compared to salivary levels. In contrast, Campylobacterota displayed reduced abundance on the retainer, with 1.80-fold ($p = 0.0008$) and 1.5-fold ($p = 0.0051$) decreases at days 7 and 14, respectively, relative to saliva at day 0. The remaining phyla were similarly distributed between saliva and retainer samples. Overall, most bacterial taxa detected in saliva were also present on the retainer throughout the 14-day period.

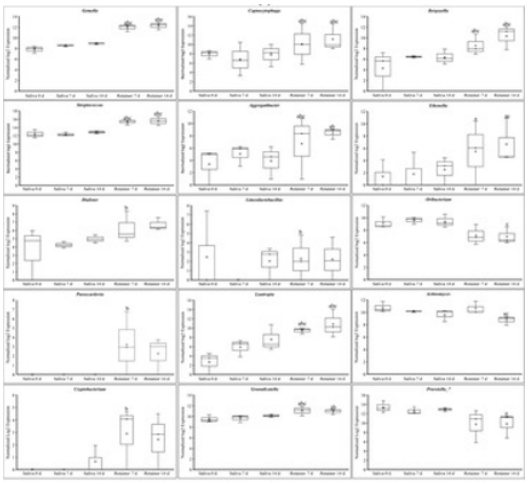


a)



b)

Figure 3. Phylum-Level Analysis of Oral Microbiota. a) Relative abundances of the dominant bacterial phyla in saliva and on the retainer, with each color representing a specific phylum. b) Normalized log2 fold changes in phylum-level microbiota on the retainer compared with saliva at 0, 7, and 14 days. Comparisons: a = saliva at day 0; b = saliva at day 7; c = saliva at day 14.

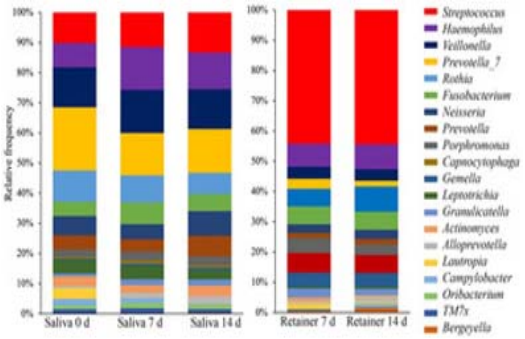


b)

Figure 4. Genus-Level Microbial Analysis. a) Relative abundances of the predominant bacterial genera in saliva and on the clear retainer, with each color representing a specific genus. b) Normalized log2 fold changes in bacterial genera between saliva and retainer at 7 and 14 days. Comparisons: a = saliva at day 0; b = saliva at day 7; c = saliva at day 14.

Genus-level comparison of bacterial abundance between saliva at day 0 and retainer at day 7

To investigate microbial shifts in greater detail, we analyzed the genus-level composition. The relative abundances of the predominant genera are illustrated in **Figure 4a**. Comparison between saliva at baseline (day 0) and retainer plaque at day 7 revealed significant increases in several genera on the retainer: *Gemella* (3.93-fold, $p < 0.0001$), *Streptococcus* (2.61-fold, $p = 0.001$), *Capnocytophaga* (3.90-fold, $p = 0.009$), *Bergeyella* (2.90-fold, $p = 0.016$), *Granulicatella* (1.60-fold, $p = 0.021$), *Lautropia* (4.80-fold, $p = 0.0004$), *Eikenella* (3.51-fold, $p = 0.053$), *Aggregatibacter* (3.80-fold, $p = 0.016$), and *Actinobacillus* (3.50-fold, $p = 0.016$). Conversely, *Campylobacter* decreased by 1.90-fold ($p = 0.016$). Similar patterns were observed when comparing baseline saliva to retainer plaque at day 14 **(Figure 4b)**, indicating a consistent enrichment of specific genera on the retainer over time.



a)

Comparison of bacterial abundance between baseline saliva and retainer at day 14

The relative abundance of bacterial genera was evaluated to investigate changes in the microbial community over time. When comparing saliva at baseline (day 0) to retainer plaque at day 14, significant increases were observed for *Gemella* (4.3-fold; $p < 0.0001$), *Streptococcus* (3.10-fold; $p < 0.0001$), *Capnocytophaga* (3.90-fold; $p = 0.01$), *Bergeyella* (4.5-fold; $p = 0.0001$), *Granulicatella* (1.40-fold; $p = 0.051$), *Lautropia* (7.4-fold; $p < 0.0001$), *Eikenella* (3.90-fold; $p = 0.037$), and *Actinobacillus* (2.90-fold; $p = 0.03$). In contrast, *Actinomyces* (2.00-fold; $p = 0.013$), *Prevotella-7* (2.61-fold; $p = 0.028$), and *Aggregatibacter* (3.1-fold; $p = 0.048$) showed significant decreases **(Figure 4b)**.

Comparison between saliva and retainer at day 7

At day 7, retainer-associated plaque demonstrated higher relative abundances of several genera compared to saliva, including *Gemella* (3.4-fold; $p < 0.0001$), *Streptococcus* (2.87-fold; $p = 0.0002$), *Dialister* (2.62-fold; $p = 0.008$), *Parascardovia* (4.23-fold; $p = 0.024$), *Cryptobacterium* (3.40-fold; $p = 0.038$), *Capnocytophaga* (3.28-fold; $p = 0.030$), *Bergeyella* (2.64-fold; $p = 0.024$), *Granulicatella* (1.44-fold; $p = 0.036$), *Limosilactobacillus* (2.33-fold; $p = 0.042$), *Lautropia* (2.63-fold; $p = 0.047$), and *Aggregatibacter* (3.12-fold; $p = 0.043$) **(Figure 4b)**.

Comparison between saliva and retainer at day 14

By day 14, similar trends persisted, with the retainer showing elevated abundances of *Gemella* (3.40-fold; $p < 0.0001$), *Streptococcus* (2.86-fold; $p < 0.0001$), *Bergeyella* (3.93-fold; $p = 0.0008$), *Capnocytophaga*

(3.35-fold; p = 0.026), *Lautropia* (2.97-fold; p = 0.030), and *Eikenella* (3.70-fold; p = 0.042). Conversely, *Actinomyces* (1.92-fold; p = 0.015) and *Oribacterium* (2.1-fold; p = 0.038) were less abundant on the retainer **(Figure 4b)**. Notably, several genera were exclusive to either saliva or the retainer at both 7 and 14 days, indicating distinct microbial niches.

After 7 days, genera such as *Cutibacterium, Bifidobacterium, Campylobacter, Bulleidia, Dubosiella, Mogibacterium, Shuttleworthia, Bradyrhizobium, Sphingomonas, Neisseria,* and *Haemophilus* were present in saliva but absent on the retainer, whereas *Slackia, Prevotella, Tannerella, UCG8, Solobacterium, Isobaculum, Lactobacillus UCG11, Veillonella, Propionigenium,* and *Leptotrichia* were observed on the retainer but not in saliva. At day 14, *Tannerella, Bulleidia, Lactobacillus, Lactococcus, UCG11, Mogibacterium,* and *Bradyrhizobium* were exclusive to the retainer, while *Slackia, Dubosiella, Mycoplasma, Butyrivibrio,* and *Streptobacillus* were only found in saliva.

This study examined temporal changes in the oral microbiome on clear thermoplastic retainers and in saliva over a 14-day period. Our results indicate that the microbial community composition evolves continuously, with notable shifts between day 7 and day 14. Certain genera were detected exclusively on the retainer, while others were only present in saliva, consistent with prior findings by Yan *et al*. [31]. The distinct microbial patterns likely result from the retainer enclosing the teeth, creating a localized environment distinct from saliva, unlike fixed orthodontic brackets, which remain in continuous contact with salivary microbiota.

Common pathogenic bacteria were detected at higher frequencies in both saliva and on the retainer at 7 and 14 days. These microorganisms have been associated in the literature with caries development, enamel demineralization, gingivitis, and periodontal disease [10-12, 39-41]. *Streptococcus* spp., belonging to the Firmicutes phylum, was the most abundant genus on the clear retainer at both time points. This aligns with Yan *et al*., who reported a significant increase in Firmicutes on the inner surface of clear aligners within the first 24 hours of use [31, 42]. Similarly, a systematic review by Lucchese *et al*. reported elevated levels of *S. mutans* on removable orthodontic appliances, highlighting the potential for clear aligners to act as reservoirs for cariogenic bacteria and increase the wearer's caries risk [39].

In the present study, *Granulicatella* showed an approximately 2-log increase on the retainer at day 7 relative to saliva. Previous reports have described decreased *Granulicatella* in saliva [43], whereas other studies detected *Granulicatella elegans* at higher levels in plaque from orthodontic patients with white-spot lesions [10]. *Parascardovia*, commonly associated with dental caries, was also significantly enriched on the retainer at day 7 [10]. *Gemella*, a facultatively anaerobic Gram-positive cocci linked to bacteremia and infective endocarditis, was similarly elevated on the retainer at both time points [31].

Consistent with Yan *et al*. [31], *Actinomyces* abundance decreased with prolonged retainer use. While some studies report variable trends for *Actinomyces* depending on the orthodontic appliance and timing, our results suggest that the retainer environment is less favorable for its proliferation [43].

Kado *et al*. [43] examined supragingival plaque and saliva in fixed orthodontic patients at baseline, 6 months, and appliance removal, identifying *Proteobacteria, Firmicutes, Bacteroidota, Fusobacteriota,* and *Actinobacteriota* as the predominant phyla. Notably, *Firmicutes* decreased over treatment, *Proteobacteria* fluctuated, and *Bacteroidota* and *Fusobacteriota* increased toward appliance removal. TM7 and Spirochetes were also present in lower numbers [43]. Our study observed the same predominant phyla in both saliva and retainer samples, with Spirochetes at comparatively low levels, suggesting that the microbial composition at 7 and 14 days persisted through retainer use. These results imply that prolonged retainer wear could increase caries risk and periodontal susceptibility [43].

Actinobacteria levels remained relatively stable from 7 to 14 days, slightly higher in saliva than on the retainer, consistent with their role as stable environmental microbiota [31, 43]. *Campylobacter* was more abundant in saliva than on the retainer, consistent with prior observations that this facultative anaerobe can contribute to periodontal pathogenesis [43].

Genera associated with gingivitis, including *Solobacterium, Parvimonas,* and *Selenomonas*, were detected on the retainer at both 7 and 14 days [43-45], along with periodontitis-associated bacteria such as *Fusobacterium* and *Tannerella* [46, 47]. *F. nucleatum* serves as a bridge between early and late colonizers in periodontal biofilms [46, 47], while *Tannerella* was enriched on the retainer, supporting its potential pathogenic role following orthodontic appliance placement [31].

From day 7 to day 14, the diversity of bacteria present exclusively in saliva significantly decreased. At day 7, twelve genera were identified in saliva that were absent on the retainer, whereas at day 14, only five genera were detected in saliva but not on the retainer. Of these five, three were the same as those observed at day 7. Camelo-Castillo *et al*. [48] reported that a decline in microbial diversity is associated with less stable and less healthy communities. This observation aligns with the results of the present study, which showed that prolonged retainer use from 7 to 14 days led to a reduction in microbial diversity. These findings suggest that extended retainer use may promote a less favorable oral microbial environment and indicate that saliva's microbial composition can be influenced by clear retainer usage, contrary to the findings of Zhao *et al*. [49].

The alpha diversity results provide insight into microbial richness and evenness in each sample, reflecting the stability and health of the microecological environment [31]. Beta diversity analyses indicated differences in microbial composition among the groups, showing that the community structure in both saliva and on the retainer shifted between day 7 and day 14. Some species decreased in abundance while others increased, highlighting that the duration of retainer usage influences microbial colonization on the inner surface of the clear retainer. Factors contributing to bacterial accumulation on the retainer include microgrooves, wear or abrasion of the material, and plaque buildup on the teeth or gingival margin. Understanding these microbial dynamics is critical for determining optimal retainer cleaning intervals and evaluating retainer longevity to prevent potential adverse effects.

Limitations of the study

Several limitations should be acknowledged. Although any intraoral appliance can alter bacterial communities, additional factors may have influenced bacterial composition in saliva and on the retainer, including patient-specific caries risk and tooth anatomy. These variables were not controlled in this study and may affect biofilm formation and bacterial accumulation. Another limitation is the small sample size, comprising only five participants, which restricts the generalizability of the findings; larger-scale studies are warranted. Furthermore, oral hygiene practices varied among participants despite standardized instructions to brush twice daily and floss once daily, potentially contributing to inter-individual differences in bacterial composition over the 14-day period. Future studies should implement more strictly controlled oral hygiene protocols to maintain consistent retainer and oral environments.

This study represents the first in vivo investigation of bacterial composition on the inner surfaces of clear retainers at both 7 and 14 days. Further research should examine changes in oral microflora on clear retainers from day 0 to day 14, rather than focusing only on day 7 to 14. Subsequent studies could also evaluate whether the teeth develop a similar biofilm to the retainer and assess interventions or products that reduce retainer-associated biofilm formation in vivo. While many bacterial genera were shared between saliva and the retainer at day 14, notable compositional changes occurred between day 7 and day 14, potentially due to the enclosed environment created by the retainer. Importantly, persistent bacteria from day 7 to day 14 included several pathogenic genera associated with gingivitis, dental caries, periodontitis, and enamel demineralization.

Significance of the study

Several factors contribute to bacterial accumulation on the inner surfaces of clear retainers, including microgrooves in the retainer material, worn or abraded areas, and plaque deposits on teeth or along the gingival margin. In this study, Firmicutes, Bacteroidota, Proteobacteria, Actinobacteriota, and Fusobacteriota were identified at the highest frequencies in samples from both saliva and the retainer. These bacterial taxa are early colonizers that can persist throughout orthodontic treatment and have been reported in previous studies to remain detectable up to the conclusion of therapy. The presence of these early colonizers can disrupt the oral microbial balance, contributing to dysbiosis and increasing the risk of dental caries and periodontal disease in patients undergoing long-term orthodontic treatment. However, this dysbiosis is expected to reverse following the removal of the clear retainer.

Understanding microbial changes on the inner surface of clear retainers is crucial for establishing optimal cleaning intervals and assessing the retainer's longevity relative to potential adverse effects on the host. Orthodontists should consistently monitor patients' periodontal health throughout treatment, regardless of age or general health. Establishing clear guidelines for retainer cleaning frequency is essential to provide both patients and clinicians with effective oral hygiene protocols. The microbial insights from this study can help define the maximum safe usage period of clear retainers before adverse microbial effects may occur.

Conclusion

Over the 14-day period, microbial diversity decreased in saliva, and the clear retainer harbored a range of bacteria associated with dental caries, gingivitis, periodontitis, and enamel demineralization. Plaque biofilm development was time-dependent, showing increased abundance and diversity from day 7 to day 14. By day 14, the biofilm had become more pathogenic, highlighting the importance of monitoring retainer usage and implementing effective cleaning practices to maintain oral health during orthodontic treatment.

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Conflict of Interest: None

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Ethics Statement: None

References

1. Deo PN, Deshmukh R. Oral microbiome: Unveiling the fundamentals. J Oral Maxillofac Pathol. 2019;23(1):122–8.
2. Ragimov RM, Zakaev CT, Abdullaeva NM, Esiev RK, Pushkin SV, Nauruzova DM, et al. Analysis of effectiveness of the use of multifunctional biopolymers of chitosan and alginate in dentistry. J

Adv Pharm Educ Res. 2022;12(3):21-7. doi:10.51847/yWRLcwYTDC

3. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. J Clin Microbiol. 2005;43(11):5721–32.
4. Mahfouz ME, Abdulaziz M, Alotaibi S, Alamri A, Basfar A, Alsubhi A, et al. Depression among first generation medical students and non-first-generation medical students in the West of Saudi Arabia. World J Environ Biosci. 2022;11(4):42-7. doi:10.51847/VjiWxmL1Vk
5. Hasimun P, Mulyani Y, Zakaria H, Setiawan AR. Centella asiatica effect in a high fat and fructose diet-induced model of metabolic disorder in rats. J Biochem Technol. 2021;12(1):1-5. doi:10.51847/GzeDdExlEH
6. Spratt DA, Pratten J. Biofilms and the oral cavity. Rev Environ Sci Biotechnol. 2003;2(2):109–20.
7. Ashurko I, Esayan A, Magdalyanova M, Tarasenko S. Current concepts of surgical methods to increase mucosal thickness during dental implantation. J Adv Pharm Educ Res. 2021;11(3):37-41. doi:10.51847/bY9xiuZKJf
8. Kitada K, de Toledo A, Oho T. Increase in detectable opportunistic bacteria in the oral cavity of orthodontic patients. Int J Dent Hyg. 2009;7(2):121–5.
9. Phan NH. Cultural values and corporate tax avoidance: an empirical evidence from Vietnam. J Organ Behav Res. 2021;6(2):18-30. doi:10.51847/fzBCy1LSWm
10. Tanner ACR, Sonis AL, Holgerson PL, Starr JR, Nunez Y, Kressirer CA, et al. White-spot lesions and gingivitis microbiotas in orthodontic patients. J Dent Res. 2012;91(9):853–8.
11. Tufekci E, Dixon JS, Gunsolley JC, Lindauer SJ. Prevalence of white spot lesions during orthodontic treatment with fixed appliances. Angle Orthod. 2011;81(2):206–10.
12. van der Veen MH, Attin R, Schwestka-Polly R, Wiechmann D. Caries outcomes after orthodontic treatment with fixed appliances: Do lingual brackets make a difference? Eur J Oral Sci. 2010;118(3):298–303.
13. Chapman JA, Roberts WE, Eckert GJ, Kula KS, Gonzalez-Cabezas C. Risk factors for incidence and severity of white spot lesions during treatment with fixed orthodontic appliances. Am J Orthod Dentofacial Orthop. 2010;138(2):188–94.
14. Rego RO, Oliveira CA, dos Santos-Pinto A, Jordan SF, Zambon JJ, Cirelli JA, Haraszthy VI. Clinical and microbiological studies of children and adolescents receiving orthodontic treatment. Am J Dent. 2010;23(5):317–23.
15. Ajwa N, AlRashoud R, AlWehaibi J, AlMazyad A. Orthodontic clear aligner vs fixed appliances' influence on oral microbiota and salivary parameters': a systematic review. Ann Dent Spec. 2021;9(4):16-22. doi:10.51847/b0IvlxNZ6C
16. Singh GP, Attavar SH, Kavuri S. Application of cone-beam computed tomography in diagnosis and treatment of multiple canals– a case report. Ann Dent Spec. 2022;10(2):15-8. doi:10.51847/vgeNZYRIRH
17. Gayirbegov DS, Mandzhiev DB. The biological justification of manganese requirements in fat-tailed ewes in arid zone conditions. J Biochem Technol. 2021;12(2):19-23. doi:10.51847/eriI3YuWkw
18. Guardado PV, Reyes AL, Rojas MdL, Romero CC, Delgadillo RH, Sanchez-Najera RI, et al. Tannerella forsythia, an orthodontic point of view. Int J Appl Dent Sci. 2021;7(4):242–5.
19. Guo R, Lin Y, Zheng Y, Li W. The microbial changes in subgingival plaques of orthodontic patients: A systematic review and meta-analysis of clinical trials. BMC Oral Health. 2017;17:90.
20. Cora H, Mikail EH, Karabulut A. Strategic dynamics of Turkey-Azerbaijan relations: an analysis of organizational and management practices during the soviet era. Ann Organ Cult Leadersh Extern Engagem J. 2021;2:1-14. doi:10.51847/XAMFo7XpdA
21. Pellissari BA, Sabino GSP, de Souza Lima RN, Motta RHL, Suzuki SS, Garcez AS, Basting RT, Barbosa JA, Martins Montalli VA. Antimicrobial resistance of bacterial strains in patients undergoing orthodontic treatment with and without fixed appliances. Angle Orthod. 2021;91(5):672–9.
22. Papaioannou W, Panagopoulos A, Koletsi-Kounari H, Kontou E, Makou M. Adhesion of Porphyromonas gingivalis and biofilm formation on different types of orthodontic brackets. Int J Dent. 2012;2012(1):471380.
23. Çelik İ, Keser G, Pekiner FN. Evaluating dental students' awareness and knowledge of malignant melanoma. Asian J Curr Res Clin Cancer. 2021;1(2):1-9. doi:10.51847/shBb7BFc7B
24. Li Y, Stewart CA, Finer Y. Comprehensive review of infection control and management in dental implants. Ann Pharm Pract Pharmacother. 2022;2:6-9. doi:10.51847/cU4ZAzCGqo
25. Levrini L, Mangano A, Montanari P, Margherini S, Caprioglio A, Abbate GM. Periodontal health status in patients treated with the Invisalign® system and fixed orthodontic appliances: A 3 months clinical and microbiological evaluation. Eur J Dent. 2015;9(3):404–10.
26. Dalaie K, Fatemi SM, Ghaffari S. Dynamic mechanical and thermal properties of clear aligners after thermoforming and aging. Prog Orthod. 2021;22(1):15.
27. Adeleke OA. Development and enhancement of liquisolid compact containing rifampicin and quercetin: an in-vitro and in-vivo investigation.

Pharm Sci Drug Des. 2022;2:14-25. doi:10.51847/lw1PmMAVuw

28. Zibi RDN, Tala VRS, Mbopi PY, Bayaga NH, Tcheuffa GMN, Ngoupayo J. Comparative evaluation of antiplasmodial and cytotoxic activities of alkaloid extracts from Coffea arabica and Coffea canephora. Spec J Pharmacogn Phytochem Biotechnol. 2022;2:20-6. doi:10.51847/nuTu3O3VU6
29. Janužis G, Razukevičius D, Latakas D, Pečkus R. A retrospective evaluation of anatomical structure perforations associated with dental implants using cone beam computed tomography. J Curr Res Oral Surg. 2022;2:6-15. doi:10.51847/TbDYX3TXry
30. Ahmed S, Algarni T, Alshareef M, Alhussain A, Alrashidi K, Alahmari S. Occurrence of oral mucosal lesions in patients attending a private university dental hospital in Riyadh, Saudi Arabia. Turk J Dent Hyg. 2022;2:5-10. doi:10.51847/PKMHv3LlbK
31. Yan D, Liu Y, Che XX, Mi SC, Jiao Y, Guo LJ, et al. Changes in the microbiome of the inner surface of clear aligners after different usage periods. Curr Microbiol. 2021;78(2):566–575.
32. Gujar AN, Al-Hazmi A, Raj AT, Patil S. Microbial profile in different orthodontic appliances by checkerboard DNA-DNA hybridization: An in-vivo study. Am J Orthod Dentofacial Orthop. 2020;157(1):49–58.
33. Puzovic M, Morrissey H, Ball PA. Intravenous treatment in home and outpatient settings: a comprehensive literature review. Int J Soc Psychol Asp Healthc. 2022;2:8-22. doi:10.51847/JMyEiUBztI
34. Shcherbin DV, Safonova AN, Polyakova LA, Egorov DE, Filimonova SI, Kazakova VM. Impact of study load on the visual sensory system functionality in students. Ann Pharm Educ Saf Public Health Advocacy. 2022;2:1-6. doi:10.51847/RdGp1sE08i
35. Batoni G, Pardini M, Giannotti A, Ota F, Giuca MR, Gabriele M, et al. Effect of removable orthodontic appliances on oral colonisation by mutans streptococci in children. Eur J Oral Sci. 2001;109(5):388–92.
36. Guo R, Zheng Y, Liu H, Li X, Jia L, Li W. Profiling of subgingival plaque biofilm microbiota in female adult patients with clear aligners: A three-month prospective study. PeerJ. 2018;6:e4207.
37. Iqbal B, Dey I, Mushtaq I. Exploring erdheim-chester disease: a histopathological insight into a rare disorder. J Med Sci Interdiscip Res. 2022;2(2):26-30. doi:10.51847/S7n4idY6zO
38. Shokeen B, Viloria E, Duong E, Rizvi M, Murillo G, Mullen J, et al. The impact of fixed orthodontic appliances and clear aligners on the oral microbiome and the association with clinical parameters: A longitudinal comparative study. Am J Orthod Dentofacial Orthop. 2022;161(4):e475–85.
39. Lucchese A, Bondemark L, Marcolina M, Manuelli M. Changes in oral microbiota due to orthodontic appliances: A systematic review. J Oral Microbiol. 2018;10(1):1476645.
40. Abdelkader H, Bergeron S. Exploring professionals' views on the ethical considerations of clinically provided safer injection education for people who inject drugs. Asian J Ethics Health Med. 2022;2:1-9. doi:10.51847/4rEkDE06Lw
41. Roberts-Wolfe D, Sacchet MD, Hastings E, Roth H, Britton W. Study the effectiveness of memory specialization training on rumination and emotional processing in cancer patients. J Integr Nurs Palliat Care. 2021;2:1-7. doi:10.51847/acLC4GKpv7
42. Ahmad S, Khan TM, Ayub F, Mubarak N, Khaliel AM, Elhanish AAS, et al. Meta-analysis of urinary tract infections among patients with chronic kidney disease. Bull Pioneer Res Med Clin Sci. 2022;2(2):7-27. doi:10.51847/KZV1W3ahoN
43. Kado I, Hisatsune J, Tsuruda K, Tanimoto K, Sugai M. The impact of fixed orthodontic appliances on oral microbiome dynamics in Japanese patients. Sci Rep. 2020;10:21989.
44. Diaz PI, Hoare A, Hong BY. Subgingival microbiome shifts and community dynamics in periodontal diseases. J Calif Dent Assoc. 2016;44(9):421–35.
45. Hong BY, Araujo MVF, Strausbaugh LD, Terzi E, Ioannidou E, Diaz PI. Microbiome profiles in periodontitis in relation to host and disease characteristics. PLoS ONE. 2015;10(3):e0121857.
46. Bolstad AI, Jensen HB, Bakken V. Taxonomy, biology, and periodontal aspects of Fusobacterium nucleatum. Clin Microbiol Rev. 1996;9(1):55–71.
47. Kolenbrander PE, London J. Adhere today, here tomorrow—Oral bacterial adherence. J Bacteriol. 1993;175(11):3247–52.
48. Camelo-Castillo A, Novoa L, Balsa-Castro C, Blanco J, Mira A, Tomas I. Relationship between periodontitis-associated subgingival microbiota and clinical inflammation by 16S pyrosequencing. J Clin Periodontol. 2015;42(11):1074–82.
49. Zhao R, Huang RH, Long H, Li Y, Gao MY, Lai WL. The dynamics of the oral microbiome and oral health among patients receiving clear aligner orthodontic treatment. Oral Dis. 2020;26(2):473–83.