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| RESEARCH ARTICLE

Effect Of Fixed Brackets and Invisalign on Oral Total Bacterial Load and Profiles of Porphyromonas Gingivalis, Streptococcus Mutans, and Streptococcus Sobrinus: A Qpcr Study

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| ABSTRACT
Orthodontic treatments, particularly fixed brackets and invisalign clear aligners, are known to alter the oral microbiome, potentially influencing the prevalence of oral pathogens. This study aimed to determine the impact of these orthodontic appliances on the distribution of *Porphyromonas gingivalis*, *Streptococcus mutans*, and *Streptococcus sobrinus* in saliva samples using absolute quantitative real-time PCR. This cross-sectional study was conducted in Erbil-Iraq from February 2025 to April 2025. Ninety subjects were divided into three groups for this analysis: thirty subjects with metallic fixed orthodontic appliances, thirty subjects with Invisalign aligners, and thirty as a control group without any orthodontic appliances. Unstimulated salivary samples were collected, and then bacterial DNA was extracted and target bacterial pathogens were quantified using absolute quantitative Real-Time PCR. The total bacterial load was significantly higher in the fixed bracket group ($5.4 \times 10^5 \pm 3.5 \times 10^5$ CFU/µl) compared to the controls (p=0.002). *Streptococcus mutans* was present in all groups, with significantly higher levels in fixed bracket users ($3.6 \times 10^4 \pm 2.3 \times 10^4$ CFU/µl) compared to clear aligner users ($7.5 \times 10^3 \pm 6.9 \times 10^3$ CFU/µl) and controls ($1 \times 10^3 \pm 3.2 \times 10^3$ CFU/µl). The prevalence of *Porphyromonas gingivalis* was higher in fixed bracket users (100%) compared to clear aligner users (93.3%) and controls (90%). There were no significant differences in the levels of *Porphyromonas gingivalis*, *Streptococcus sobrinus*, or total bacterial load between the clear aligner and control group (p>0.05). Fixed brackets contribute to higher bacterial loads, particularly *Streptococcus mutans*. In contrast, Invisalign clear aligners have less impact on oral pathogens, potentially offering advantages for maintaining oral hygiene during orthodontic treatment.

| KEYWORDS

Bacterial load, Clear Aligners, Erbil city, Fixed Brackets, Oral pathogens, Saliva.

| ARTICLE INFORMATION

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1. Introduction

The oral microbiome is a rich ecosystem that contains approximately 1,000 bacterial species, along with fungus, viruses, and protozoa. They play an important role in both oral and systemic health (1). Microbial biofilms formed by oral microorganisms are central to the pathogenesis of dental caries and periodontitis (2, 3). Disruption of this ecological balance of the oral microbial community is known as dysbiosis, which is linked to systemic conditions (4, 5). Orthodontic treatments such as fixed brackets and clear aligners, are known to affect the balance of oral microbiomes and periodontal health (6). Fixed appliances can affect oral hygiene which leading to more plaque buildup and a higher risk of periodontal problems (7, 8). While, clear aligners are often considered as a more hygienic alternative; however, their impact on the oral microbiota requires further investigation (9). The most common oral pathogens linked to periodontal diseases and dental caries are *Porphyromonas gingivalis, Streptococcus mutans,* and *Streptococcus sobrinus* (10), and (11). Several methods have been developed to identify and quantify oral pathogenic microorganisms. For instance, traditional bacterial culture is considered the gold standard method. However,

bacterial culture is highly sensitive and requires skill, selective media, and strict quality control procedures (2). On other hand, quantitative real-time PCR has proving highly effective in identifying and measuring bacterial DNA in oral samples (12, 13). Saliva analysis for bacterial quantification offers valuable insights into microbial changes induced by different orthodontic appliances (14). While, existing literature presents mixed findings regarding the impact of clear aligners on oral health. Some studies reported no significant changes of bacterial concentration during orthodontic treatments especially in clear aligner cases and other reported the prevalence reduce of periodontopathogen bacteria during orthodontic therapy (15-17). Other studies have reported that clear aligners offer advantages over fixed appliances, leading to better periodontal outcomes due to improved oral hygiene during orthodontic treatment (9, 18).

Therefore, this study aimed to compare the distribution of *P. gingivalis*, *S. mutans*, and *S. sobrinus* in the saliva of patients with fixed brackets and invisalign clear aligners. An absolute quantitative real-time PCR was used to determine microbial changes associated with these orthodontic treatments, thereby informing clinical practices and enhancing patient oral health outcomes.

2. Materials and Methods

2.1 Study Design

This cross-sectional study was conducted in Erbil, Iraq, from February to April 2025. This study included ninety participants divided into three groups: thirty subjects without orthodontic appliances as a control group, thirty patients with fixed metal brackets, and thirty patients with invisalign aligners. All participants who had been wearing orthodontic appliances for at least six months were included, and their average age ranged from 16 to 40 years old. Exclusion criteria included active caries, antibiotic usage within the last three months, smoking, periodontal or systemic disorders, prosthetic dental devices, and craniofacial deformities.

2.2 Saliva sample collection and bacterial DNA extraction

Unstimulated whole saliva was obtained following standardized method (19). briefly, volunteers accumulated saliva in the mouth for 5 minutes and then the accumulated fluid was spat into tubes. Participants fasted overnight and avoided morning oral hygiene, except water intake. The samples were obtained between 10:00 AM and 12:00 PM. Supernatants and pellets were separated and frozen in 1 mL aliquots at −80°C for future use.

2.3 Bacterial Genomic DNA Extraction

The genomic DNA purification kit (Thermo Fisher, USA) was used to extract bacterial genomic DNA from saliva samples in accordance with the manufacturer's instructions. The first stage involved mixing 400 µL of Lysis Solution with 200 µL of saliva and incubating for five minutes at 65°C. The aqueous phase containing DNA was moved to a different tube following the addition of 600 µL of chloroform and centrifugation. DNA was precipitated using the Precipitation Solution and then washed with cold 70% ethanol. The DNA pellet was resuspended in 100 µL of sterile, deionized water. A nanodrop spectrophotometer was used to test DNA purity (20). The isolated DNA was kept at -20°C until used for PCR (21). For qPCR, each sample was adjusted to 10 ng (2 µl) of DNA.

2.4 Microbial Analysis Using qPCR

The quantitative real-time PCR (qPCR) method was used to investigate the microbial distribution of the saliva samples. *Streptococcus mutans*, *Streptococcus sobrinus*, and *Porphyromonas gingivalis* specific primers were used, as shown in Table (1). We used the Bio-Rad iQ5 equipment (Bio-Rad, USA) to perform real-time PCR. A final volume of 25 µl was created by adding distilled water, 10 µl of 2x iQ SYBR Green Supermix (Bio-Rad), 1 µl of each primer (100 pmol), and 2 µl of purified DNA from the samples to the reaction mixtures. The PCR conditions optimized based on protocols from previous studies and the real-time PCR machine was set up for 40 amplification cycles (20, 22). A scatter plot was used for the construction of the standard curve. For each target bacteria a standard curve was constructed from serial dilution of known pure bacterial cultures as shown in Figure (1 - 3), the concentration adjusted to 1µl of DNA which corresponded to a defined number of CFU per 1 µl of saliva sample. The cycle threshold (Ct) values were used to determine bacterial quantities, and the results were expressed as (CFU/µl). The results were analyzed to compare the bacterial distribution between the three groups, and significant differences in bacterial quantities were determined. SPSS version 25 was used for the statistical analysis (IBM Corp., Armonk, NY, USA). Data were analyzed using descriptive statistics with a significance level set at $p < 0.05$. Missing values were not imputed.

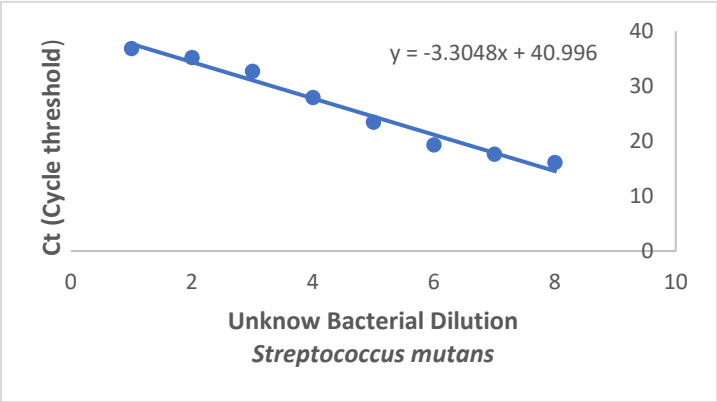


Figure 1: Standard curve for *Streptococcus mutans* absolute quantification by real-time PCR

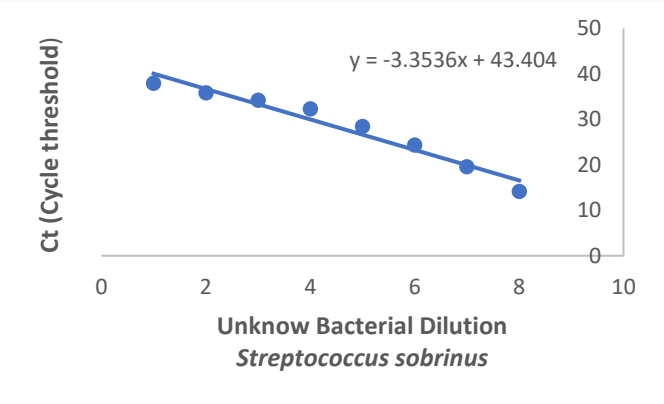


Figure 2: Standard curve for *Streptococcus sobrinus* absolute quantification by real-time PCR

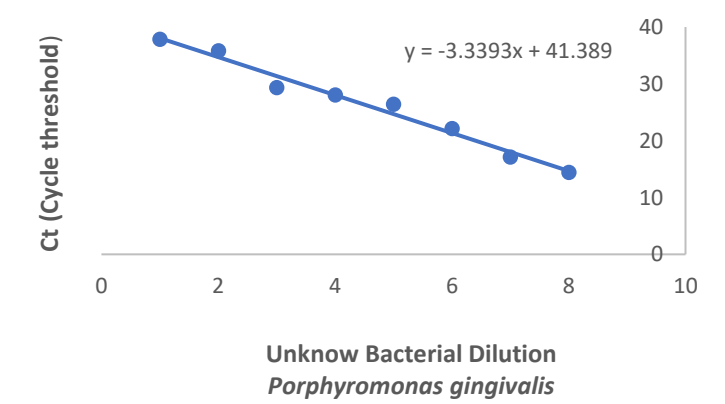


Figure 3: Standard curve for *Porphyromonas gingivalis* absolute quantification by real-time PCR

2.5 Ethical Considerations

The Ethics Committee of Hawler Medical University College of Dentistry granted ethical approval, and prior to enrollment, written informed consent was obtained from all participants.

Table 1: Used primers in this study

Gene	Primer	Primer Sequence (5-3)	Amplicon size (bp)	References
16S rRNA Universal	F1	TGGAGCATGTGGTTTAATTCGA	160 bp	(20, 23).
	R1	TGCGGGACTTAACCCAACA		
gtfB, Streptococcus mutans	F2	CTACACTTTCGGGTGGCTTG	261 bp	(21, 24).
	R2	GAAGCTTTTCACCATTAGAAGCTG		
gtfU, Streptococcus sobrinus	F3	AAAACATTGGGTTACGATTGCG	156 bp	(13, 24).
	R3	CGTCATTGGTAGTAGCCTGA		
16SrDNA, Porphyromonas gingivalis	F4	AGGCAGCTTGCCATACTGCG	404 bp	(22, 25).
	R4	ACTGTTAGCAACTACCGATGT		

3. Results

The study analyzed salivary bacterial loads among 90 participants divided into three groups: fixed brackets (n=30), Invisalign clear aligners (n=30), and controls (n=30), as shown in Table (2). Demographic data revealed no significant differences in gender distribution ($p > 0.05$).

Table 2: Demographic characteristics of participants: gender and age distribution

Charcateristics		Fixed Brackets n=30, %	Clear Aligners n=30, %	Control n=30, %	P value
Gender	Male	16 (53.3%)	11 (36.7%)	15 (50%)	0.796* 0.297**
	Female	14 (46.7%)	19 (63.3%)	15 (50%)	
Age *Mean ± SD*		28.86 ± 5.1	25.1 ± 5.7	27.56 ± 6.27	0.3820* 0.1172**

*= fixed brackets vs control, **= clear aligners vs control

Microbiological quantitative real-time PCR based analysis revealed distinct patterns of bacterial colonization across groups, Figure (4). *Streptococcus mutans* was universally detected (100%) in all groups. For *Streptococcus sobrinus*, detection rates were similarly high in fixed brackets (86.7%), clear aligners (83.3%), and controls (86.7%). In contrast, *Porphyromonas gingivalis* exhibited striking differences: it was detected in 100% of fixed bracket users, compared to 93.3% of clear aligner users and 90% of controls. These results suggest that while *S. mutans* and *S. sobrinus* colonization is largely unaffected by appliance type, fixed orthodontic appliances may selectively promote *P. gingivalis* proliferation, potentially exacerbating periodontal risks compared to removable aligners.

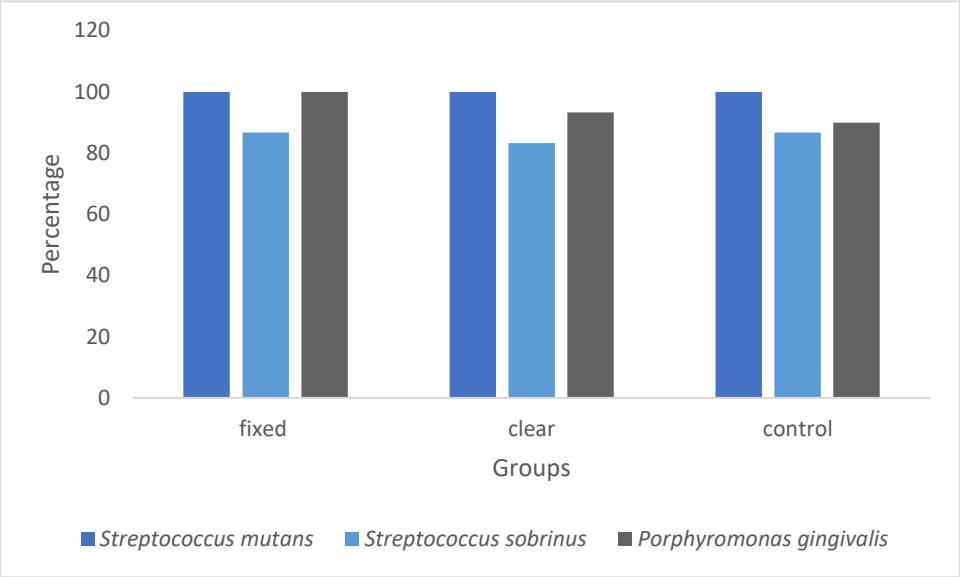


Figure 4: Frequency of bacterial detection according to groups

The study compared bacterial loads (CFU/µl) among patients with fixed orthodontic brackets, clear aligners, and controls (no appliances), Table (3). Total bacterial load was highest in the fixed brackets group ($5.4 \times 10^5 \pm 3.5 \times 10^5$ CFU/µl), followed by clear aligners ($3.8 \times 10^5 \pm 1.5 \times 10^5$ CFU/µl) and controls ($3.2 \times 10^5 \pm 1.3 \times 10^5$ CFU/µl), with a statistically significant difference between fixed brackets and controls (P = 0.002). However, clear aligners showed no significant differences from controls (P = 0.103). *Streptococcus mutans* levels were markedly elevated in fixed brackets ($3.6 \times 10^4 \pm 2.3 \times 10^4$ CFU/µl) and clear aligners ($7.5 \times 10^3 \pm 6.9 \times 10^3$ CFU/µl) groups compared to controls ($1 \times 10^3 \pm 3.2 \times 10^3$ CFU/µl), with highly significant differences (P < 0.0001 for both comparisons). In contrast, *Streptococcus sobrinus* showed no significant differences across groups (P> 0.05). Similarly, *Porphyromonas gingivalis* levels were higher in both groups but showed no significant differences compared to controls (P> 0.05).

Table 3: Comparison of Bacterial Loads (CFU/µl) Among Patients with Fixed Brackets, Clear Aligners, and Controls

Bacteria	Fixed Brackets	Clear Aligners	Control	P Value
Total bacterial load *Mean ± SD (CFU/ µl)*	$5.4 * 10^5 \pm 3.5 * 10^5$	$3.8 * 10^5 \pm 1.5 * 10^5$	$3.2 * 10^5 \pm 1.3 * 10^5$	0.002* 0.103**
Streptococcus mutans Mean ± SD (CFU/ µl)	$3.6 * 10^4 \pm 2.3 * 10^4$	$7.5 * 10^3 \pm 6.9 * 10^3$	$1 * 10^3 \pm 3.2 * 10^3$	< 0.0001* < 0.0001**
Streptococcus sobrinus Mean ± SD (CFU/ µl)	$5.6 * 10^2 \pm 4.4 * 10^2$	$6.2 * 10^2 \pm 9.6 * 10^2$	$4.6 * 10^2 \pm 4.9 * 10^2$	0.409* 0.419**
Porphyromonas gingivalis Mean ± SD (CFU/ µl)	$1.6 * 10^2 \pm 7 * 10$	$1.6 * 10^2 \pm 1.3 * 10^2$	$1.4 * 10^2 \pm 6 * 10$	0.239* 0.447**

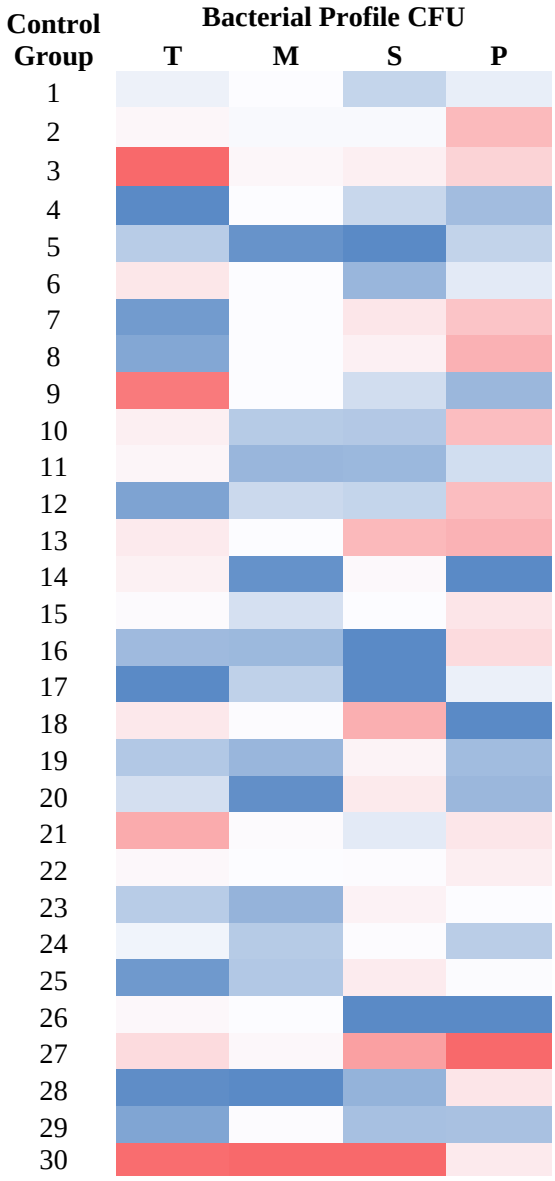
*= fixed brackets vs control, **= clear aligners vs control

Correlation analysis assessed the relationships between total salivary bacterial load and specific bacterial species in all groups, including controls, Table (4). The heatmaps display participitants specific data, comparing total bacterial loads and individual target pathogens Figure (5 - 7). The columns of the heatmap are standardized from the heatmap for comparison between patients. The intensity of the red color indicates higher bacterial distribution, while the intensity of the blue color represents lower bacterial distribution. A statistically significant moderate positive correlation was identified between total bacterial load

and *Streptococcus mutans* (r = 0.564, p < 0.001). While, total bacterial number, *Porphyromonas gingivalis* (r= 0.187, p= 0.078), and *Streptococcus sobrinus* (r = 0.206, p = 0.051) showed no significant correlation.

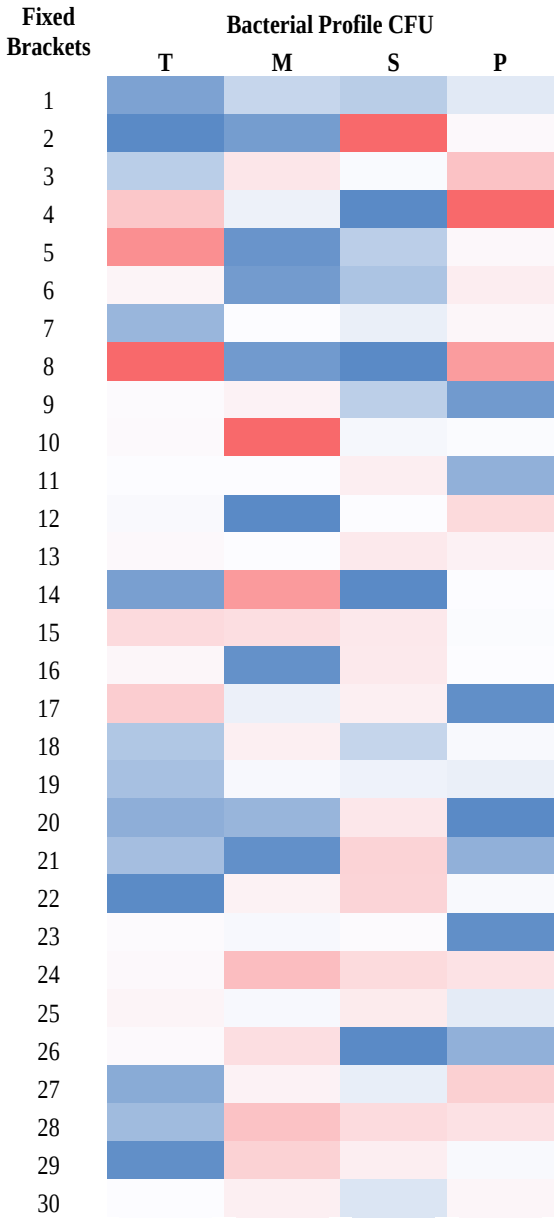
Table 4: Correlation between total salivary bacterial load and others

Variable	Correlation Coefficient	p-value	Interpretation
Streptococcus mutans	0.564	0.000	significant moderate positive correlation
Streptococcus sobrinus	0.187	0.078	non significant weak positive correlation,
Porphyromonas gingivalis	0.206	0.051	non significant weak positive correlation,



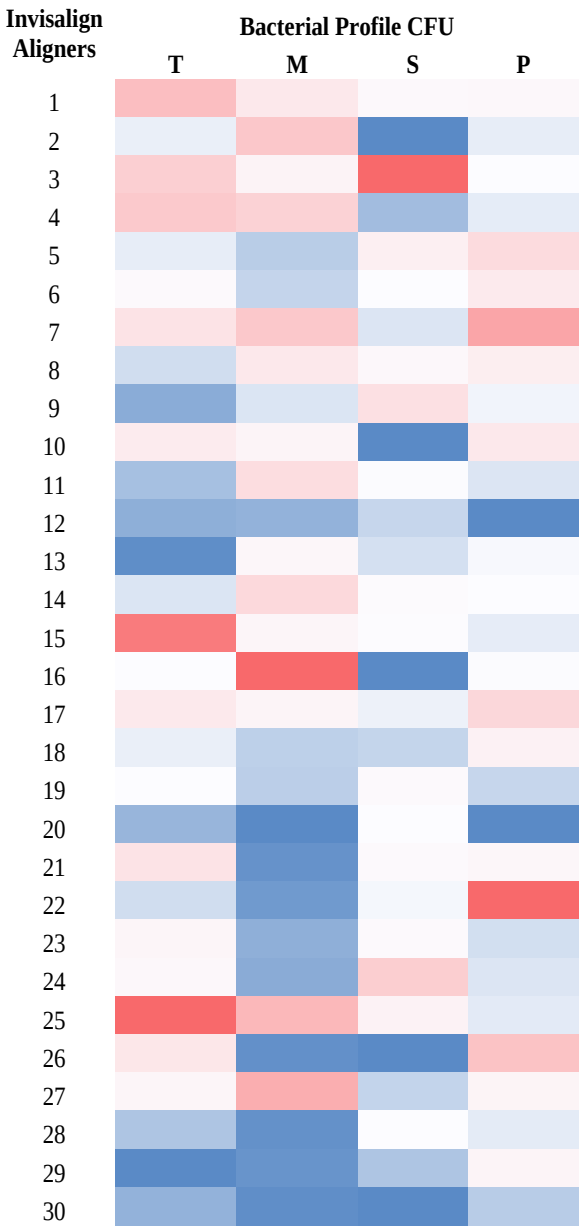
T: *Total bacterial load*, M: *Streptococcus mutans*, S: *Streptococcus sobrinus*, P: *Porphyromonas gingivalis*

Figure 5: Heatmap of total bacterial load, *Streptococcus mutans*, *Streptococcus sobrinus* and *Porphyromonas gingivalis*, among control group, TBTOOL



T: *Total bacterial load*, M: *Streptococcus mutans*, S: *Streptococcus sobrinus*, P: *Porphyromonas gingivalis*

Figure 6: Heatmap of total bacterial load, *Streptococcus mutans*, *Streptococcus sobrinus* and *Porphyromonas gingivalis*, among patients with Fixed Brackets, TBTOOL



T: *Total bacterial load*, M: *Streptococcus mutans*, S: *Streptococcus sobrinus*, P: *Porphyromonas gingivalis*

Figure 7: Heatmap of total bacterial load, *Streptococcus mutans*, *Streptococcus sobrinus* and *Porphyromonas gingivalis*, among patients with Invisalign Aligners, TBTOOL

3. Discussion

The oral cavity functions as an open microbial ecosystem, continuously receiving and expelling nutrients and microorganisms which have important roles in systemic health (26, 27). This environment maintains a dynamic equilibrium, with microbiota composition shaped by exogenous factors (orthodontic appliances) and host-related endogenous influences (28, 29). Orthodontic treatment influences oral health by changing the microecological balance, impacting host immune responses, and modifying oral hygiene habits (30).

This study evaluated the effects of fixed orthodontic braces versus Invisalign aligners on salivary microbial profiles, specifically quantifying total bacterial load, *Streptococcus mutans, Porphyromonas gingivalis*, and *Streptococcus sobrinus* through absolute qPCR. The results underscore the importance of patient awareness regarding biofilm-related risks during orthodontic therapy, emphasizing the need for preventive measures to mitigate complications and ensure favorable treatment outcomes (18).

Quantitative real-time PCR analysis revealed an increase in total bacterial load after six months of orthodontic treatments. Total bacterial load was highest in the fixed brackets group ($5.4 \times 10^5 \pm 3.5 \times 10^5$ CFU/µl), followed by clear aligners ($3.8 \times 10^5 \pm 1.5 \times 10^5$ CFU/µl) and controls ($3.2 \times 10^5 \pm 1.3 \times 10^5$ CFU/µl), significant difference observed (P = 0.002) between fixed brackets and control group. In comparison, clear aligners demonstrated no notable variations relative to untreated controls (*P* = 0.103). In alignment with these findings, a prior prospective investigation assessed the impact of fixed orthodontic devices on microbial communities and oral health via 16S rRNA gene sequencing analysis in 45 participants aged 10–35 years. This study identified a marked rise in salivary anaerobic and pathogenic bacterial populations, which could adversely affect oral health, alongside documented structural shifts in the oral microbiota (31). Further corroborating this evidence, a longitudinal prospective investigation involving 24 adolescents (mean age 14.6 ± 1.0 years) tracked microbiological and clinical parameters from appliance placement to three months after treatment, similarly reported that fixed appliances significantly altered microbial composition and clinical periodontal indicators, with certain treatment-induced changes persisting irreversibly even after appliance removal (32). These findings align with prior research involving 25 patients aged 20–35 years undergoing invisalign clear aligner therapy, which demonstrated no significant alteration in overall microbial biodiversity during the initial six-month treatment period (33). This stability may be attributed to behavioral adaptations, such as increased toothbrushing frequency and reduced sugar consumption, both of which are commonly associated with removable appliance protocols.

Streptococcus mutans was consistently found (100%) in all groups in the present study. Furthermore, both the fixed appliance ($3.6 \times 10^4 \pm 2.3 \times 10^4$ CFU/µL) and clear aligner ($7.5 \times 10^3 \pm 6.9 \times 10^3$ CFU/µL) groups exhibited significantly higher levels of *Streptococcus mutans* than the controls ($1 \times 10^3 \pm 3.2 \times 10^3$ CFU/µL), exhibiting highly significant differences (P < 0.0001). These results are consistent with a longitudinal study of 69 participants, ages 6 to 17, which found that over the first 6 months of orthodontic appliance therapy using conventional culture medium, there were statistically significant increases in salivary S. mutans (p < 0.05) (34). Over all, the concentration of *Streptococcus mutans* was lower in patients with clear aligner ($7.5 \times 10^3 \pm 6.9 \times 10^3$ CFU/µL) compare to fixed appliance ($3.6 \times 10^4 \pm 2.3 \times 10^4$ CFU/µL). This aligns with prior findings demonstrating that clear aligner therapy facilitates superior oral hygiene maintenance relative to fixed multibracket appliances among 80 adult subjects , only 8% of clear aligner patients exhibited high *S. mutans* levels after six months of treatment, contrasting sharply with approximately 40% of fixed multibracket appliances patients (14).

According to the results, *Streptococcus sobrinus* exhibited comparably high prevalence in fixed appliance (86.7%), clear aligner (83.3%), and control (86.7%) groups. In contrast, Porphyromonas gingivalis detection rates varied markedly: 100% in fixed appliance users, 93.3% in clear aligner users, and 90% in controls. Both *Streptococcus sobrinus*, and *Porphyromonas gingivalis* levels showed no significant diffrences in both groups compared to controls (P > 0.05). This contrasts with a prior real-time PCR study reporting divergent prevalence rates of *Streptococcus sobrinus* 12% in fixed appliance patients versus 90% in non-orthodontic controls (12). These findings align with a prior PCR-based study reporting *P. gingivalis* in 92.0% of healthy individuals and 100% of patients undergoing periodontal treatments (35). These results agree with a prior study reporting negligible differences in *P. gingivalis* levels between two groups, clear aligner 14 subjects and fixed appliance 13 subjects, during the first six months (36).

Notably, the universal detection of *P. gingivalis* in fixed appliance users mirrors levels observed in treated periodontitis patients, suggesting that fixed orthodontic systems may foster a microbial environment akin to active periodontal disease. Conversely, the lower P. gingivalis prevalence in clear aligner users (93.3%) and controls (90%) parallels baseline periodontal health profiles, underscoring the potential benefits of removable appliances in mitigating dysbiosis.

To determine the normal distribution of data, the Shapiro-Wilk and Kolmogorov-Smirnov tests were used. Every variable had statistically significant variations from normality (p < 0.001 for both tests), including total bacterial count, gtfB, gtfU, and p. For instance, non-normal distributions were indicated by the Shapiro-Wilk statistic of 0.663 (p < 0.001) and the Kolmogorov-Smirnov statistic of 0.204 (p < 0.001) for the total bacterial count. Similarly, normality assumptions were disrupted by gtfB (K-S: 0.238, p < 0.001; S-W: 0.730, p < 0.001), gtfU (K-S: 0.387, p < 0.001; S-W: 0.248, p < 0.001), and p (K-S: 0.393, p < 0.001; S-W: 0.337, p < 0.001). Given the non-parametric nature of the data, Spearman's rank-order correlation was employed to evaluate relationships between variables. A statistically significant moderate positive correlation was identified between total bacterial load and Streptococcus mutans (r = 0.564, p < 0.001). Although several studies have explored the relationship between *Streptococcus mutans* and dental caries (37-39), the specific correlation between total bacterial load and *S. mutans* in the context of salivary levels has not been widely reported in the existing literature.

4. Conclusion

In this study, *Porphyromonas gingivalis*, *Streptococcus mutans*, and *Streptococcus sobrinus* were the main oral bacterial pathogens that were investigated among patients with fixed orthodontic brackets and clear aligners. The findings of this study revealed that fixed appliances increased the bacterial load in the oral cavity, particularly *Streptococcus mutans*, which is linked to a higher risk of dental caries. In patients using clear aligners, no significant differences were observed in total bacterial loads, *Porphyromonas*

gingivalis, or *Streptococcus sobrinus* compared to the control group, suggesting a potential advantage for maintaining oral health during orthodontic treatment.

5. Limitations

The cross-sectional design limits long-term observations, only a few microbial taxa were studied, and the small participant cohort may affect generalizability. Confounding factors like hygiene, diet, and treatment adherence were not controlled. Larger, more controlled studies are needed to validate these findings.

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