Short-term variation in the subgingival microbiota in two groups of patients treated with clear aligners and vestibular fixed appliances: A longitudinal study

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Short-term variation in the subgingival microbiota in two groups of patients treated with clear aligners and vestibular fixed appliances: a longitudinal study.

Running Title

Evaluation of the subgingival microbiota changes

Lombardo Luca[1], Palone Mario[2], Scapoli Luca[3], Siciliani Giuseppe[4], Carinci Francesco[5].

[1]Adjunct Professor, Postgraduate School of Orthodontics, University of Ferrara, Via Luigi Borsari 46, Ferrara 44121, Italy.

[2] Research Fellow, Postgraduate School of Orthodontics, University of Ferrara Via Luigi Borsari 46, Ferrara 44121, Italy.

[3] Assistant Professor, Department of Experimental, Diagnostic and Specialty Medicine, Alma Mater Studiorum, University of Bologna, Bologna, Italy.

[4] Chairman and Professor, Postgraduate School of Orthodontics, University of Ferrara, Via Luigi Borsari 46, Ferrara 44121, Italy.

[5] Full Professor, Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy.

Corresponding Author

Dr Mario Palone, Research Fellow, Postgraduate School of Orthodontics, University of Ferrara, Ferrara, Italy.

E-mail: mario.palone88@gmail.com

Telephone +39 3289523202

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Competing interests

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Author contributions

Conception of the study: Carinci Francesco and Lombardo Luca.

Data collection: Palone Mario.

Microbiological statistical analysis: Carinci Francesco and Scapoli Luca.

Drafting of the manuscript: Scapoli Luca and Palone Mario.

Critical revision of the manuscript for important intellectual content: Lombardo Luca and Carinci Francesco.

Approval of the final version of the manuscript to be published: Palone Mario, Carinci Francesco, Scapoli Luca, Siciliani Giuseppe, Lombardo Luca.

Abstract

Objective: To evaluate the subgingival microbiological changes during the first six months of therapy with clear aligners (CAs) and fixed appliances (FAs). The null hypothesis was that there would be no microbiological differences between the two.

Setting/Sample: Two groups of patients to be treated, respectively, with CAs (14 patients; 9 females and 5 males; mean age 21 years ± 0.25) and FAs (13 patients; 8 females and 5 males; mean 14 years ± 0.75), were consecutively recruited.

Materials and Methods: Subgingival microbiological samples were obtained at the right upper central incisor and right first molar at four different time-points: before appliance fitting (T0), and at 1 month (T1), 3 months (T3) and 6 months (T6) thereafter. Total bacterial load (TBL) and counts of the bacteria *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum, Campylobacter rectus*, *Treponema denticola* and *Tannerella forsythia* were determined using real time PCR.

Results: TBL did not vary in the CA group, while a significant increase was detected after 3 and 6 months of treatment in the FA group. Unlike red complex species, *C. rectus* and *F. nucleatum* were often detected: levels remained stable in the CA group but increased progressively in the FA group.

Conclusion: The type of orthodontic appliance influences the subgingival microbiota. TBL increased in the FA group but not in the CA group, although the levels of the individual periodontal pathogenic bacteria species did not significantly increase during the observation period.

DR MARIO PALONE (Orcid ID : 0000-0001-6198-3053)

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Key Words: Real time PCR; subgingival microbiota; periodontal pathogenic bacteria, clear aligner, fixed appliances.

Introduction

Nowadays, patients of all ages request orthodontic treatment for correcting malocclusion and achieving good aesthetics and function.[1] However, bulky fixed orthodontic equipment is inevitably and closely linked to the accumulation of plaque,[2] which is the main cause of damage to dental and periodontal tissues.

This involves increased plaque and bleeding index levels (PI and BI),[3] periodontal pocket development[4] and marginal alveolar bone loss if untreated,[5] as well as white spot development and caries,[6] and pathological changes in bacterial composition.[3] Quantitative and qualitative changes in the subgingival,[7] rather than supragingival, microbiota seem to be responsible for periodontal damage.[8] The increased proliferation of periodontal pathogenic bacterial species, favoured by the presence of orthodontic appliances, disrupts the balance with the host's defences, [9] which seems to be the main trigger for the clinical changes observed.[10] However, it is not clear whether these bacterial and clinical alterations are transitory [11,12] or permanent, although improvements in the gingival indices have been reported after the conclusion of orthodontic therapy.[13,14]

Pan et al. investigated the subgingival microbiota by real time polymerase chain reaction (real time PCR) in a group of patients treated with fixed appliances (FAs), analysing both the total bacterial load (TBL) and four periodontal pathogenic bacterial species, as compared with a control group. Three months from the end of treatment, they found partial reversibility of the subgingival microbiological situation to almost pre-treatment levels.[12] However, some studies have argued otherwise.[10,11]

Nonetheless, a systematic review of the literature showed that clear aligners (CAs) enable better control of oral hygiene than FAs, being removable and, therefore, facilitating correct execution of oral hygiene procedures.[15] In addition, several prospective studies have indicated that patients treated with CAs have better periodontal health indices than subjects treated with FAs,[16,17] but no significant differences in the oral microbiome composition.[10,18] Of note, these differences in periodontal health disappear when continuous reinforcement of home hygiene procedures is provided by a dental hygienist. [19]

To date, however, no study has simultaneously analysed the quantitative and qualitative effects of different orthodontic devices on the subgingival microbiota. Therefore, the aim of this prospective study was to analyse the longitudinal changes in the subgingival microbiota profile using real time PCR during the first six months of therapy in two groups of patients treated with different orthodontic appliances (FA vs. CA). The null hypothesis was that there would be no significant differences between the two groups in terms of the changes induced in the subgingival microbiota composition.

Materials and Methods

Patient sample and clinical records

Twenty-seven subjects were consecutively recruited for this prospective study by the University of _____ Department of Orthodontics. The study design was approved by the University of _____ Postgraduate School of Orthodontics Ethics Committee (1/2018).

Recruited subjects were divided into two study groups based on both clinical evaluation and diagnostic digital set-up procedure, carried out by a single expert orthodontist (__) calibrated on the Index of Orthodontic Treatment Need (IOTN). Each participant was assigned to a group according to both malocclusion complexity, evaluated by diagnostic records with IOTN grade and diagnostic digital set-up, and the personal preference expressed by the patient. Specifically, only patients with an IOTN grade ≥ 3 were considered for orthodontic treatment, and clear aligner therapy was not appropriate when it would need to perform any tooth movement considered unpredictable to achieve via clear aligners alone, such as: >15° derotation of rounded teeth, root movements, translational movements, extrusion >0.6mm and intrusion >1mm.[20] Hence, one group, comprising 13

patients (8 females and 5 males; average age 14 years ± 0.75), was treated using FA (Primo Brackets, Sweden & Martina, Due Carrare, Padua), and the other, composed of 14 patients (9 females and 5 males; average age 21 years ± 0.25), was treated using CA (F22 Aligner, Sweden & Martina, Due Carrare, Padua). All teeth, including second molars, were orthodontically treated.

For the purposes of this study, patients were selected based on the following inclusion criteria: 1) no systemic disease; 2) no periodontal treatment in the previous 9 months; 3) no use of antimicrobial or anti-inflammatory agents in the 6 months before or during the treatment observation period (under penalty of leaving the clinical trial); and 4) healthy periodontal status at enrolment, with periodontal probing depth (PPD) indices of less than 4 and <10% sites of bleeding on probing (BOP, %), considering all teeth.[21,22] Periodontal analysis was performed by a single expert periodontal clinician (__) calibrated in both BOP index and PPD measurements.

CAT (clear aligner therapy) was carried out via polyurethane F22 aligners (Sweden & Martina, Due Carrare, Padua) with vestibular grip points created using GRADIA DIRECT LoFlo flow composite (GC Orthodontics Europe GmbH, Harkortstraße, Breckerfeld, Germany). The FA treatment, on the other hand, was performed using metal brackets (Primo Brackets, Sweden & Martina, Due Carrare, Padua) that were indirectly bonded with GRADIA DIRECT LoFlo composite (GC Orthodontics Europe GmbH, Harkortstraße, Breckerfeld, Germany). Metal ligatures were used to tie the archwires.

Before sampling, supragingival biofilm was removed using a sterile curette, and sites were isolated using sterile cotton wads. At each site, a sample of the subgingival microbiota was taken using sterile paper probes with ISO 60 diameter tip and 2% taper (Dentsply Sirona, York, Pennsylvania, USA). Specifically, the paper probe was inserted about 1 mm inside the gingival sulcus and left for roughly 30 seconds (Fig. 1). Specimens were inserted into a sterile tube, stored immediately at 4 °C, and processed for DNA purification the following morning. [23]

Samples were taken at distovestibular and distopalatal sites at the maxillary right central incisor (1.1) and mesiovestibular and mesiopalatal sites at the maxillary right first molar (1.6) before appliance delivery (T0), and 1 month (T1), 3 months (T3) and 6 months (T6) thereafter. Mandibular teeth were excluded from microbial evaluation as accidental debonding more often affects the lower arch, and would have invalidated subgingival microbial sampling if it had occurred. However, to our knowledge, no previous studies have provided evidence of the arches having different microbiota compositions, so the results should be generalizable.

All subjects were adequately informed regarding home hygiene procedures, i.e., brushing teeth at least three times daily after main meals, with no use of mouthwash and no chlorhexidine or any antimicrobial agent. No particular dietary restrictions were suggested. Hygiene motivation and procedures were reinforced at each appointment and investigated verbally.

Quantification of periodontal bacteria

Bacterial profiling was performed by real time PCR quantification of genomic DNA molecules, which is directly related to the number of bacteria in the specimens. All subjects, sample sites and time-points were represented by an alphanumeric code, ensuring that the expert microbiologist who performed the procedures described was operating blind.

Specimens were processed to extract and purify DNA using a method that include two consecutive incubations with lysozyme and proteinase K, in order to ensure indiscriminate Gram-positive and -negative bacterial lysis. Once extracted, DNA was purified by QIAxtractor instrument using a dedicated QIAcube HT purification kit (Qiagen, Milan, Italy).

Quantitative PCR of 16S rRNA genes was performed via the hydrolysis probes method. The total bacterial load (TBL) and the 6 bacterial species of interest, namely *Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola, Fusobacterium nucleatum, Campylobacter rectus and Aggregatibacter actinomycetemcomitans*, were quantified in each specimen.[9]

Primers and probe oligonucleotides were designed based on a 16S rRNA gene sequence from the Human Oral Microbiome Database (HOMD 16S rRNA RefSeq Version 10.1), counting 845 entries. All sequences were aligned in order to find either a consensus sequence or less conserved spots. Three real time PCR runs were performed for each sample. A reaction quantified the total amount of bacteria using two degenerate primers and a single probe matching a highly conserved sequence (Biomers.net, Ulm, Germany). Highly specific primers and probes were used in two multiplex PCR reactions to detect each species. The first multiplex reaction detected and quantified the three red complex bacteria, i.e., *P. gingivalis*, *T. forsythia*, and *T. denticola*, while the second detected *F. nucleatum*, *C. rectus* and *A. actinomycetemcomitans*. PCR conditions were optimized to ensure sensitivity, specificity, and no inhibition in case of imbalanced target amounts. Absolute quantification assays were performed using the Viia7 thermal cycler (Applied Biosystems, Monza, Italy). The amplification profile was initiated by a 10-minute incubation period at 95°C to activate polymerase, followed by a two-step amplification of 15 s at 95°C and 60 s at 57°C for 40 cycles. Plasmids containing synthetic DNA target sequences (Eurofin MWG Operon, Ebersberg Germany) were used as standards for the quantitative analysis. Standard curves for each target were constructed in triplex reaction, using a mix of the same amounts of plasmids in serial dilutions ranging from 10 to 10000000 copies. All these experiments were performed with non-template controls to exclude reagent contamination.[23]

Statistical analysis

Data from quantitative real time PCR included an estimation of the total bacterial load and the respective amounts of the 6 bacterial species. Each patient was sampled at four time-points. A non-parametric statistical procedure was adopted because data was not normally distributed. Paired Wilcoxon signed-rank tests were performed to examine whether the bacterial load significantly changed between time points (T0-T1, T0-T3, T0-T6, T1-T3, T1-T6, and T3-T6). The null hypothesis was that the median difference between pairs of observations would be zero, while a positive or negative median would suggest that the patient's periodontal bacteria tended to vary over time. The level of significance was set at 0.05. Data distribution was summarized as median and interquartile range. The *P* values obtained in each test were displayed in tables; red font indicates that the sum of the positive difference in ranks was larger than that of the negative difference in ranks, meaning an increase in TBL over time, while green P values indicate a reduction in TBL.

Results

The study included 27 orthodontic subjects, 14 treated with CAs and 13 with FAs. Evaluations of the total bacterial load (TBL) and of the main periodontal pathogens at teeth 1.1 and 1.6 were carried out before and during the first 6 months of treatment. Two patients from the CA group dropped out before T6, but their data for the previous time-points was nonetheless included. TBL data for single teeth and in-patient means for both groups are plotted in Figure 2.

Formal statistical analysis of the data was performed by the non-parametric Wilcoxon signed-rank test for correlated samples, and results are reported in Table 1. Considering the CA group, there was no significant change in TBL across the various time-points at either investigated site. In the FA group, on the other hand, there was a slight, non-significant decrease in TBL at tooth 1.6 (P = 0.807) at 1 month of treatment, followed by a progressive and significant increase in TBL. Specifically, significant increases were detected at both teeth after 3 months (P = 0.016 at tooth 1.1; P = 0.039 at 1.6) and 6 months (P = 0.006 at 1.1; P = 0.016 at 1.6) of FA treatment with respect to T0 (Table 1). Moreover, there was a statistically significant increase in TBL at tooth 1.6 between T3 and T6 (P = 0.023).

In order to reduce random fluctuation and to focus on the effect of orthodontic treatment on microbial flora, data obtained from the two tooth sites were pooled; the resulting patient means were compared at different time-points (Table 2), and the data were plotted in Figure 2. With both orthodontic treatments, a decrease in TBL was apparent after the first month of treatment, although this was not statistically significant. After three months, however, the TBL displayed an increase that approached significance in the CA group (P = 0.084 vs. T0), while the difference was statistically significant in the FA group (P = 0.033 vs. T0). After six months of treatment, the TBL was no different

from pre-treatment values in the CA group (P = 1.000 vs. T0), while in the FA group, there was a further statistically significant increase in TBL (P = 0.011 vs. T0).

Six main periodontal pathogen species were also monitored. For simplicity, data are presented only as a mean of the two tooth sites sampled (Table 3 and 4). *A. actinomycetemcomitans* was never detected, while the red complex species (*P. gingivalis*, *T. forsythia* and *T. denticola*) were rarely detected. There were no variations in the amounts of any of these species between the different time-points in either of the groups investigated. The other two investigated species, on the other hand, were found in most of the specimens, but there were between-group differences. Specifically, *F. nucleatum* levels remained similar at the different time-points in the CA group, while significant increases were observed in the FA group at all time-points with respect to T0 (P = 0.019 vs. T1, P = 0.003 vs. T3 and P = 0.009 vs. T6). Similarly, the levels of *C. rectus* in the FA group significantly increased over time as compared to T0 (P = 0.013 for T1; P = 0.003 for T3; and P = 0.004 for T6). However, in the CA group, the levels of *C. rectus* at T6 were significantly higher than at T1 (P = 0.028), but not with respect to T0.

Discussion

The aim of this prospective study was to evaluate and compare changes in the subgingival microbiota during the first six months of therapy in two groups of patients treated with FAs or CAs. This observational period was chosen because the two procedures generally have different treatment times depending on case complexity, [20,24] but six months is a reasonable span of time during which both procedures are still in progress.

The difference in age between the two groups was due to the consecutive recruitment, and the greater demand for aesthetic appliances by older patients. According to Feres et al., however, subgingival microbiota composition seems not to be influenced by aging, and its composition remains fairly stable throughout life. Hence, this age difference could be considered negligible for the purposes of this study. [25]

The literature agrees that the use of FAs cause a worsening of the periodontal indices,[4] and qualitative and quantitative changes in the subgingival[26,27] and supragingival bacterial species.[28] This seems to be mainly due to the non-removable nature of FAs.[10] However, the same topic has been less investigated in CAs, especially from a microbiological perspective.[16] Indeed, analysis of the literature reveals that only Guo et al. have previously explored the effect that orthodontic aligners have on the subgingival microbiota profile; they found a non-pathological alteration in the subgingival plaque, with a decrease in microbiological diversity over the first three months of

therapy in a group of 10 female patients, and that pre-therapy levels of the periodontal pathogenic species remained stable.[17]

In addition to the paucity of studies in the literature, many of those that have been carried out have relied on culturing methods [3] or DNA probes [10] to assess changes in microflora. We, on the other hand, chose to use real time PCR as a method of microbiological analysis in this longitudinal study. Indeed, although a multiple-culture method for bacteria identification (culturomics) has recently been introduced, this still suffers from several drawbacks, such as its low ability to culture some species and the difficulty in identifying the so-called 'not yet culturable' microorganisms. [29] Therefore, the real time PCR method was preferred due to its quantitative and qualitative potential, and the fact that it is particularly suitable for the detection of anaerobic bacterial species that are difficult to culture. [30]

Despite these advantages, scrutiny of a recent meta-analysis conducted by Papageorgiou et al., reveals that of the 24 studies investigated, only 6 used real time PCR as a microbiological investigation method.[11] Furthermore, the majority of these investigated only FAs, and only one study compared FAs to removable-type devices, but these were not well specified.[31] Moreover, the majority of studies investigated only TBL and *P. gingivalis*, while we assessed levels of several periodontal pathogens known to be responsible for periodontal disease.[9]

As for the FA group, we detected a significant increase in TBL after three and six months of therapy with respect to the pre-treatment values. A comparison of the two groups, keeping the sampling sites separate, revealed a reduction of TBL at site 1.6 during the first month in both groups. This finding is likely linked to greater attention paid by patients to home oral hygiene during the initial phases of therapy, and to initial instructions provided by the orthodontist, who stressed its importance, especially in the less accessible posterior areas.[32]

Nonetheless, there was a subsequent significant increase in TBL in the FA group in the final observation phase (T3–T6). In contrast, the CA group displayed a decrease in TBL in the final phase (T3–T6). After pooling the data, the same trend was observed. This trend seems to be attributable to the fact that the aligners, being removable, can be cleaned and favour home hygiene procedures, confirming the concept already expressed in other studies.[15,17] It should also be taken into account that after 6 months of orthodontic treatment, there is an improvement in crowding, which could also significantly facilitate oral hygiene manoeuvres.[4]

Madariaga et al. did not find differences in gingival health between patients treated respectively with FA and CA when motivation was continuously reinforced by a specialist in dental hygiene, although they did not perform a microbiological evaluation. According to our findings, it could be speculated that different orthodontic appliances could exert different influences on the composition

and quantity of the subgingival microbiota due, presumably, to their different encumbrance and removability. That being said, these differences do not necessarily have a clinical implication on gingival health, which instead seems be guaranteed by good motivation for performing optimal home hygiene procedures.[19]

Although no evidence is provided in this regard, this may explain why the results of our study seem to contrast with the data obtained by Al-Jewair T et al., who reported a worsening of oral hygiene just after insertion of FA with an improvement beginning in the fifth month. [33] Nevertheless, our findings seem to be in agreement with those of other authors, who show good oral hygiene during the first months of therapy, with a subsequent worsening as orthodontic treatment goes on. [35]

Analysis of the individual periodontal pathogens excluded the presence of *A. actinomycetemcomitans* in the observation period in both groups; this periodontal pathogenic bacteria is found in patients with periodontitis stage 3/4, grade C (previously described as aggressive periodontitis),[35] and our finding is in line with that of Demling et al., who did not find this bacterium during the early stages of orthodontic treatment in healthy patients.[36] *P. gingivalis*, *T. forsythia* and *T. denticola* did not fluctuate significantly across the six-month observation period in either group, and this finding is in line with Liu et al.[26] However, our finding is in contrast with that reported by Kim et al., who found a significant increase of *T. forsythia* at 3 and 4 months from the beginning of FA treatment.[27]

Our study showed a statistically significant increase in *C. rectus* in the CA group from T1–T3 and from T3–T6, after an initial decrease during the first month of therapy; as regards *F. nucleatum*, we detected no statistically significant variations. In the FA group, on the other hand, there was a statistically significant increase with respect to pre-treatment values in both *F. nucleatum* and *C. rectus* at all measured time-points. Socransky et al. have shown that the bacterial species belonging to the orange complex create an optimal substrate for the subsequent colonization of periodontal pathogenic bacterial species, so it cannot be excluded that an identical study conducted over longer observation times could reveal a significant increase in bacteria belonging to the red complex.[37]

The limitations of this prospective study lie mainly in the small sample size and in the reduced observation time for clinical quantification of pathological changes in the subgingival microbiota. Indeed, generation of the conditions for the onset of periodontal disease is a long process, as it is based on a succession of various pathogenic species that create the conditions necessary for colonization by the most virulent bacterial species; this mechanism cannot be fully evaluated in six months of observation. Regarding the sample size calculation, statistical power analysis could not be formally performed because some of the parameters required, such as effect size and standard

deviation of effect size, were largely unpredictable. Indeed, no previous studies have reported data useful for this purpose.

Moreover, there was an age difference between the two groups, which appeared to be due to the consecutive recruitment of the 27 subjects analysed, and to fact that adult patients more frequently demanded aesthetic treatment. As mentioned, however, subgingival microbiota composition does not seem to be influenced by aging. [27]

Finally, it is important to consider that gingivitis and periodontitis are complex diseases in which microbial and other factors, such as host immunologic response, personal habits like alcohol or tobacco consumption, as well as genetic predisposition, play a role; in other words, this investigation focused on subgingival microbial status, which is one of the most important factors for periodontal diseases, but not the only one. Further investigations considering all risk factors should be performed on a larger sample in future.

Conclusions

- Clear aligners appear to be associated with non-statistically significant TBL fluctuation. As regards periodontal pathogenic species, there was a statistically significant increase in *C. rectus* alone over six months of therapy.
- Fixed appliances are associated with a statistically significant increase in TBL over the first six months of therapy; the periodontal pathogenic species that showed a statistically significant increase were *C. rectus* and *F. nucleatum*.
- The null hypothesis is rejected. A possible explanation of such findings could be due to FAs hampering home oral hygiene as compared to removable aligners, due to their encumbrance and non-removability.
- According to these preliminary findings, a further randomized controlled trial (RCT) including clinical periodontal evaluation should be performed to test the hypothesis that the two orthodontic techniques investigated require different professional hygiene recall frequencies or differentiated preventative home hygiene procedures.

List of abbreviations: CAs (clear aligners); FAs (fixed appliances), real time PCR (real-time polymerase chain reaction), PI (plaque index), GI (gingival index), BI (bleeding index), TBL (total bacterial load), IOTN (index of orthodontic treatment need), PPD (periodontal probing depth), BOP (bleeding on probing)

Conflict of interest statement

All authors declare they have no competing interests.

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Clear Aligner

Table 1

Tooth 1.1

Time points	N. Patients	Percentiles			Paired Wilcoxon signed rank tests		
		25th	50th (Mean)	75th	vs t1	vs t3	vs t6
t0	14	2,05E+04	4,76E+04	1,90E+05	0,363	0,074	0,754
t1	14	2,55E+04	6,15E+04	1,55E+05		0,140	0,480
t3	14	5,23E+04	2,19E+05	5,74E+05			0,583
t6	12	1,76E+04	7,89E+04	8,29E+05			

Tooth 1.6

Time points	N. Patients	Percentiles			Paired Wilcoxon signed rank tests		
		25th	50th (Mean)	75th	vs t1	vs t3	vs t6
t0	13	4,09E+04	7,35E+04	5,65E+05	0,109	0,074	1,000
t1	13	1,71E+04	7,01E+04	1,80E+05		0,056	0,117
t3	13	8,29E+04	2,02E+05	9,08E+05			0,754
t6	13	3,03E+04	3,88E+05	6,17E+05			

Fixed Appliances

Tooth 1.1

Time points	N. Patients	Percentiles			Paired Wilcoxon signed rank tests		
		25th	50th (Mean)	75th	vs t1	vs t3	vs t6
t0	13	2,6E+04	7,3E+04	1,1E+05	0,600	0,016*	0,006*
t1	13	2,4E+04	7,2E+04	2,2E+05		0,221	0,013*
t3	13	3,5E+04	1,2E+05	9,2E+05			0,249
t6	13	1,6E+05	8,6E+05	1,2E+06			

Tooth 1.6

Time points	N. Patients	Percentiles			Paired Wilcoxon signed rank tests		
		25th	50th (Mean)	75th	vs t1	vs t3	vs t6
t0	13	3,9E+04	6,6E+04	2,3E+05	0,807	0,039	0,016*
t1	13	3,5E+04	6,6E+04	5,9E+05		0,023*	0,023*
t3	13	7,4E+04	3,6E+05	2,1E+06			0,422
t6	13	1,7E+05	5,7E+05	6,4E+06			

Table 1: Total bacterial load observed at different time-points at teeth 1.1 and 1.6 in patients

Clear Aligners

Time points	N. Patients	Percentiles			Paired Wilcoxon signed rank tests		
		25th	50th (Mean)	75th	vs t1	vs t3	vs t6
t0	14	3,6E+04	8,3E+04	5,7E+05	0,300	0,084	1,000
t1	14	2,4E+04	9,3E+04	1,5E+05		0,096	0,117
t3	14	7,0E+04	2,5E+05	8,0E+05			0,583

treated with CA and FA. Data are reported as median and interquartile range. Differences between time-points were evaluated using paired Wilcoxon signed-rank tests; red indicates an increasing trend, while green indicates a diminishing trend ($p<0.05$*).

Table 2

t6	12	4,6E+04	3,0E+05	8,3E+05

Fixed Appliances

Time points	N. Patients	Percentiles			Paired Wilcoxon signed rank tests		
		25th	50th (Mean)	75th	vs t1	vs t3	vs t6
t0	13	4,1E+04	8,1E+04	1,6E+05	0,917	0,033*	0,011*
t1	13	3,9E+04	6,3E+04	4,1E+05		0,075	0,013*
t3	13	7,4E+04	1,9E+05	1,5E+06			0,196
t6	13	1,6E+05	9,2E+05	3,9E+06			

Table 2: Mean values of total bacterial load observed at different time-points in patients treated with CA and FA. Data are reported as median and interquartile range. Differences between time-points were evaluated using paired Wilcoxon signed-rank tests; red indicates an increasing trend, while green indicates a diminishing trend ($p<0.05$*).

Clear Aligners

Timepoints	N. Patients	Percentiles			Paired Wilcoxon signed rank tests		
		Actynomices Actinomicetemcomitans					
		25th	50th (Mean)	75th	vs t1	vs t3	vs t6
t0	14	0	0	0	1,000	1,000	1,000
t1	14	0	0	0		1,000	1,000
t3	14	0	0	0			1,000
t6	14	0	0	0			
		Campilobacter Rectus					
		25th	50th (Mean)	75th	vs t1	vs t3	vs t6
t0	14	0	46	508	0,799	0,155	0,099
t1	14	0	0	142		0,041*	0,034*
t3	14	0	173	1494			0,754
t6	12	114	760	3251			
		Fusobacterium Nucleatum					
		25th	50th (Mean)	75th	vs t1	vs t3	vs t6
t0	14	179	920	29172	0,382	0,363	0,530
t1	14	172	905	2352		0,096	0,071
t3	14	254	3605	18342			0,937
t6	12	778	7720	29812			
		Porphiromonas Gengivalis					
		25th	50th (Mean)	75th	vs t1	vs t3	vs t6
t0	14	0	0	0	0,317	0,180	0,109
t1	14	0	0	0		0,180	0,102
t3	14	0	0	0			0,715
t6	12	0	0	1601			

		Tannarella Forsythia					
		25th	50th (Mean)	75th	vs t1	vs t3	vs t6
t0	14	0	0	0	0,317	0,317	0,655
t1	14	0	0	0		0,317	0,317
t3	14	0	0	0			0,655
t6	12	0	0	0			

		Treponema Denticola					
		25th	50th (Mean)	75th	vs t1	vs t3	vs t6
t0	14	0	0	0	0,317	0,593	0,173
t1	14	0	0	0		0,109	0,028*
t3	14	0	0	26			0,575
t6	12	0	15	1007			

Table 3

Table 3: Mean values of individual periodontal pathogens observed at different time-points in patients treated with CA. Data are reported as median and interquartile range. Differences between time-points were evaluated using paired Wilcoxon signed-rank tests; red indicates an increasing trend, while green indicates a diminishing trend ($p<0.05$*).

Fixed Appliances

Timepoints	N. Patients	Percentiles			Paired Wilcoxon signed rank tests		
		Actynomices Actinomicetemcomitans					
		25th	50th (Mean)	75th	vs t1	vs t3	vs t6
t0	14	0	0	0	1	1	1
t1	14	0	0	0		1	1
t3	14	0	0	0			1
t6	14	0	0	0			
		Campilobacter Rectus					
		25th	50th (Mean)	75th	vs t1	vs t3	vs t6
t0	13	0	0	32	0,013*	0,003*	0,004*
t1	13	0	373	1220		0,06	0,034*
t3	13	36	1162	4846			0,53
t6	13	165	1893	6833			
		Fusobacterium Nucleatum					
		25th	50th (Mean)	75th	vs t1	vs t3	vs t6
t0	13	14	402	1104	0,019*	0,003*	0,009*
t1	13	298	2364	7003		0,152	0,013*
t3	13	2884	13657	144754			0,308
t6	13	1803	33068	752636			
		Porphiromonas Gengivalis					
		25th	50th (Mean)	75th	vs t1	vs t3	vs t6
t0	13	0	0	0	0,317	1,000	0,317
t1	13	0	0	0		0,317	0,655
t3	13	0	0	0			0,317
t6	13	0	0	0			
		Tannarella Forsythia					
		25th	50th (Mean)	75th	vs t1	vs t3	vs t6
t0	13	0	0	0	0,109	0,109	0,109
t1	13	0	0	14		1,000	0,893
t3	13	0	0	444			0,5
t6	13	0	0	79			
		Treponema Denticola					

		25th	50th (Mean)	75th	vs t1	vs t3	vs t6
t0	13	0	0	0	0,066	0,317	0,109
t1	13	0	0	77		0,066	0,715
t3	13	0	0	0			0,109
t6	13	0	0	29			

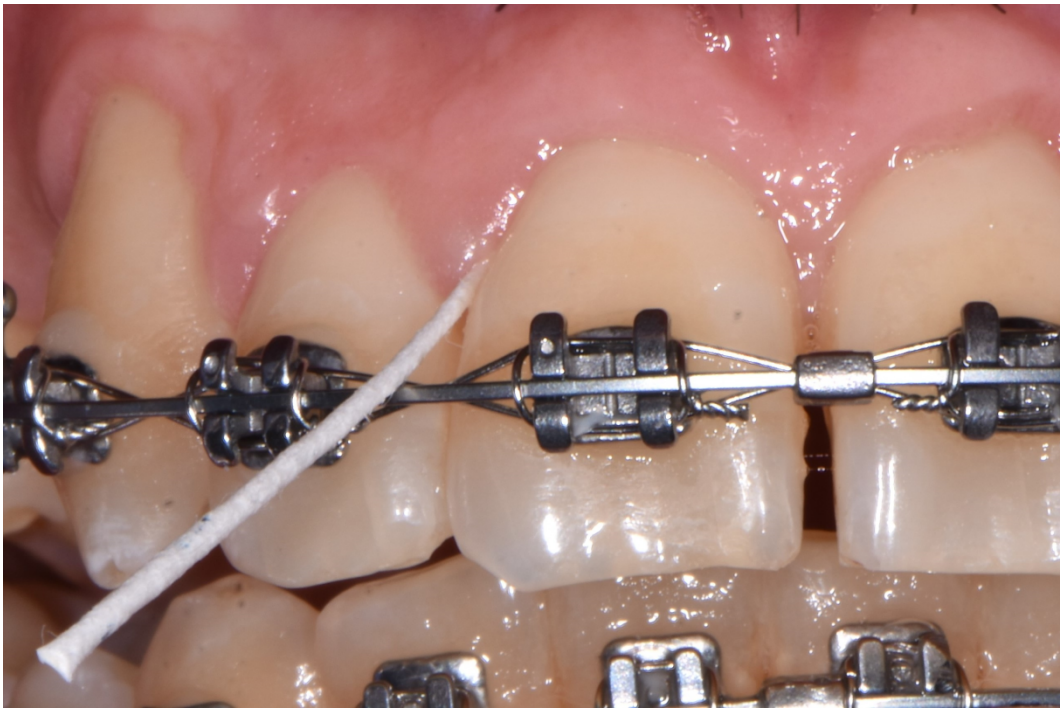
Table 4

Table 4: Mean values of individual periodontal pathogens observed at different time-points in patients treated with FA. Data are reported as median and interquartile range. Differences between time-points were evaluated using paired Wilcoxon signed-rank tests; red indicates an increasing trend, while green indicates a diminishing trend ($p<0.05$*).

Figure Legends

Figure 1: The subgingival plaque sampling procedure

Figure 2: Plots represent the logarithmic transformation of TBL measured in periodontal pockets of orthodontic patients at different time-points, i.e. before treatment (t0), after 1 month (t1), after 3 months (t3), and after 6 months (t6) of treatment for the CA group (A), FA Group (B), and after pooling data of both groups (C). The box is bounded on the top by the third quartile and on the bottom by the first quartile. The median divides the box. The whiskers indicate the maximum and minimum values observed.



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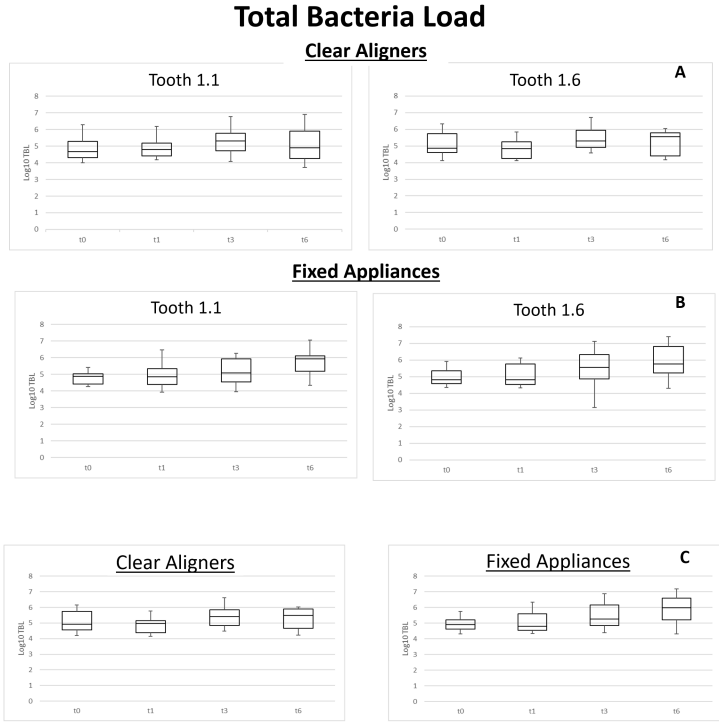

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