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Dysbiotic changes of periodontal pathogens in patients wearing conventional and self-ligating orthodontic appliances

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Objective: This study aimed to analyse dysbiotic changes of periodontal pathogenic bacteria and their relationship with different types of fixed orthodontic appliances in a population located in Northwestern Mexico.
Methods: Three groups of patients were identified: a control group without orthodontic appliances (C), a conventional-ligating appliance group (CLA), and a self-ligating appliance group (SLA). Periodontal biofilm samples were collected for DNA extraction to identify the presence and load of *Treponema denticola*, *Prevotella intermedia*, and *Fusobacterium nucleatum*, using a quantitative real-time PCR technique.
Results: A total of 92 patients were included. The results showed that *F. nucleatum* was present in all groups including the control patients (C 96%, CLA 100%, and SLA 67%, respectively). Female participants displayed a higher frequency of periodontal pathogens than males, but males were more affected by *F. nucleatum*. In addition, the presence of *T. denticola* and *P. intermedia* was time-dependent, being more frequent in patients in treatment for longer than 12 months whereas CLA showed 74% and 78% of positive samples and SLA showed 78% and 89%, respectively. *F. nucleatum* was present in 100% of CLA samples before and after 12 months of treatment and its load was higher in the SLA group after 12 months.
Conclusions: Dysbiotic changes that could affect the periodontal tissues were seen in patients wearing orthodontic appliances. The frequency of *F. nucleatum* was significantly higher in CLA and noted with a greater load in SLA. In addition, female participants showed a higher frequency of periodontal pathogens while male subjects were more affected by *F. nucleatum*. As expected, treatment for longer than 12 months correlated with a higher frequency of all periodontal pathogens. The results support the concept that dysbiosis leading to periodontal disease can be caused by the rise of a dominant species, instead of the appearance of a new species.
(Aust Orthod J 2022; 39: 61 - 71. DOI: 10.2478/aoj-2023-0006)

Received for publication: November, 2022
accepted: January, 2023.

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Introduction

Malocclusion is a common dental condition that may be corrected by orthodontic treatment involving fixed or removable appliances.[1] Preserving the integrity of the periodontal tissues is a priority during treatment, and has led to the definition of specific hygiene protocols for orthodontic patients.[2] The principle of orthodontic treatment is based on corrective tooth movement, which is an occlusal reconstruction process involving alveolar bone remodelling and changes in bone morphology whose objectives are to correct function and establish dental and facial aesthetics.[3,4]

Fixed appliances may offer conventional-ligation (CLA) or self-ligation (SLA) mechanisms. The main consideration of CLA is to achieve better positional crown control in addition to improving root position following the closure of spaces. To retain an arch wire within a bracket slot, either stainless steel ligatures which can vary in size (0.009 to 0.014 inches) or elastic modules (circular shaped elastomers that can be deformed to engage bracket tiewings and maintain the arch wire), may be used.[5] As an alternative to CLA, two types of SLA have been developed: an active closing clip that exerts pressure on the arch wire, which in turn improves rotation control, and a passive bracket with a closing mechanism that transforms an open slot into a tube.[6]

The biggest challenge of orthodontic therapy is to complete treatment with the least impact on the oral tissues (Figure 1). Periodontal disease is a chronic inflammatory process, initiated by a dysbiotic bacterial biofilm[7] that leads to the destruction of investing tissue. Even though periodontal disease has been linked to a defined microbial biofilm composition, the effect of polymicrobial disruption on host homeostasis to periodontitis progression remains poorly understood.[8] Recently, metagenomic studies have estimated that the number of bacteria found in the periodontal tissues is up to 600 species.[9]

Fixed orthodontic appliance materials affect the adhesion of bacteria and the accumulation of biofilm as a result of the roughness of the devices, the free energy of the surfaces, and other physicochemical properties of the biomaterials which influence the retentive capacity of the biofilm.[10] In addition to

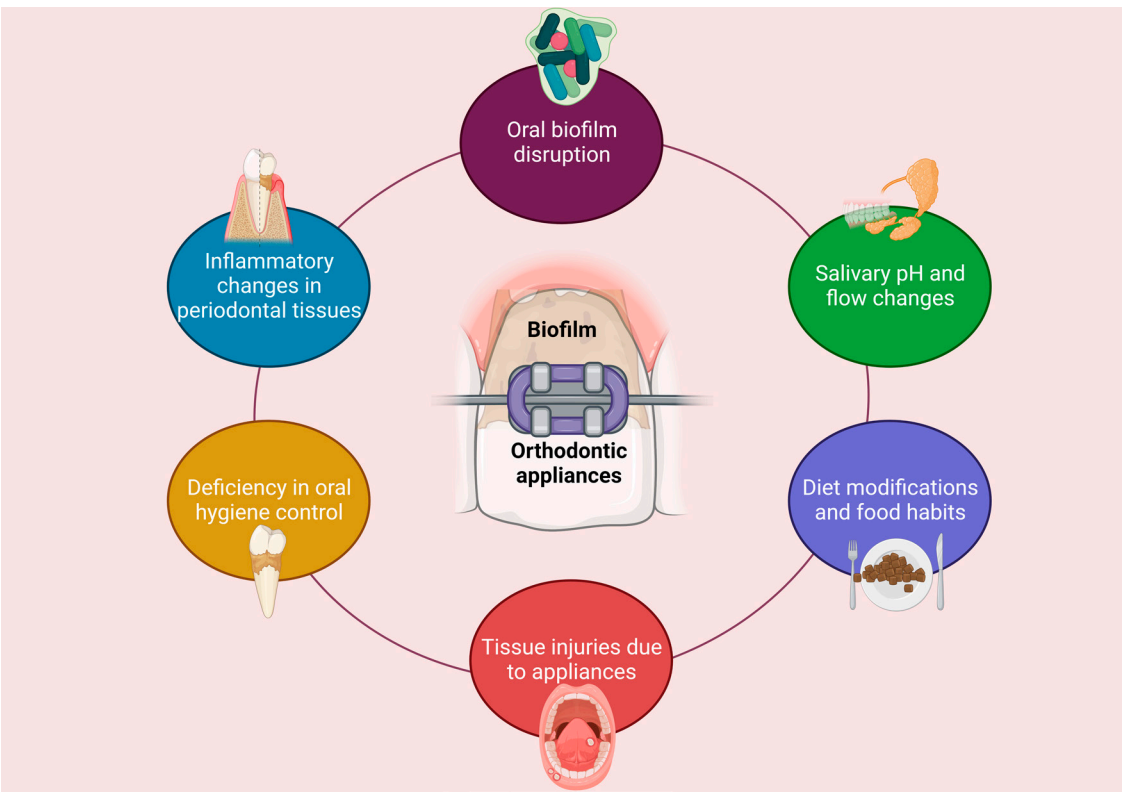

Figure 1. Orthodontic appliances impact oral health since patients must change their eating habits. Braces create oral hygiene difficulty and produce changes in salivary pH and disruption of the oral biofilm leading to inflammatory changes, plus damage to the periodontal tissues.

Table I. Primers and qPCR conditions to measure bacterial load.

Specific initiators	Amplified products (pb)	Bacteria
FTd 5′-TAATACCGAATGTGCTCATTTACAT-3′	316	*Treponema denticola*
RTd 5′-TCAAAGAAGCATTCCCTCTTCTTCTTA-3;		
FFn 5′-AGAGTTTGATCCTGGCTCAG-3′	408	*Fusobacterium nucleatum*
RFn 5′-GTCATCGTGCACACAGAATTGCTG-3		
FPi 5′-TTTGTTGGGGAGTAAAGCGGG-3′	576	*Prevotella intermedia*
RPi 5′-TCAACATCTCTGTATCCTGCGT-3′		

enamel decalcification that leads to incipient caries, periodontal damage can also occur[11] with associated local and systemic effects identified as root resorption, psychological disorders, gastrointestinal complications, allergic reactions, infective endocarditis, and chronic fatigue syndrome.[12]

Knowing which microbial species colonises orthodontic appliances is important to plan strategies and to implement specific preventive control measures during treatment.[13] Few studies have investigated periodontal pathogens when different types of fixed appliances have been placed. Previous research has determined that the microorganisms of Socransky′s 'red' complex are considered more pathogenic compared to the 'orange' complex regarding the aetiology of periodontal disease.[14] It is therefore, important to assess the changes in the frequency and load of Socransky′s red and orange complexes that colonise orthodontic appliances and to know the periodontal risk.[15] The present study aimed to explore dysbiotic changes in periodontal pathogenic bacteria associated with different types of fixed orthodontic appliances in a Northwestern Mexican population.

Materials and methods

Experimental design

Biofilm samples were collected from systemically healthy patients of both genders, who provided signed written informed consent. The study was approved by the ethics committee of the Autonomous University of Sinaloa and complied with the principles of the Declaration of Helsinki. The type of sampling was non-probabilistic. DNA concentrations up to 100 ng or greater were selected using spectrophotometry (nanodrop) and corroborated with electrophoresis tests. In total, 92 biofilm samples from patients were collected which subsequently formed three groups: patients without orthodontic appliances (N:31, group C) patients with conventional-ligating appliances (N:30, group CLA), and patients with self-ligating appliances (N:31, group SLA). The exclusion criteria were patients with an active diagnosis of COVID-19, who had received antibiotic treatment within the previous two weeks, those diagnosed with convulsive diseases, those with injuries or oral pathologies that were not the product of orthodontic treatment

Table II. qPCR conditions to measure bacterial load.

	Treponema denticola	Fusobacterium nucleatum	Prevotella intermedia	Cycles	Reading melt curve
Desn. initial	96°C-5 m	96°C-5 m	96°C-5 m	30	80°C
Desn. nat	95°C-30 s	95°C-30 s	95°C-30 s		
Align	55°C-45 s	56°C-45 s	56°C-45 s		
Ext	72°C-45 s	72°C-45 s	72°C-45 s		
Ext final	72°C-10 m	72°C-10 m	72°C-10 m		
Conservation	4°C	4°C	4°C		

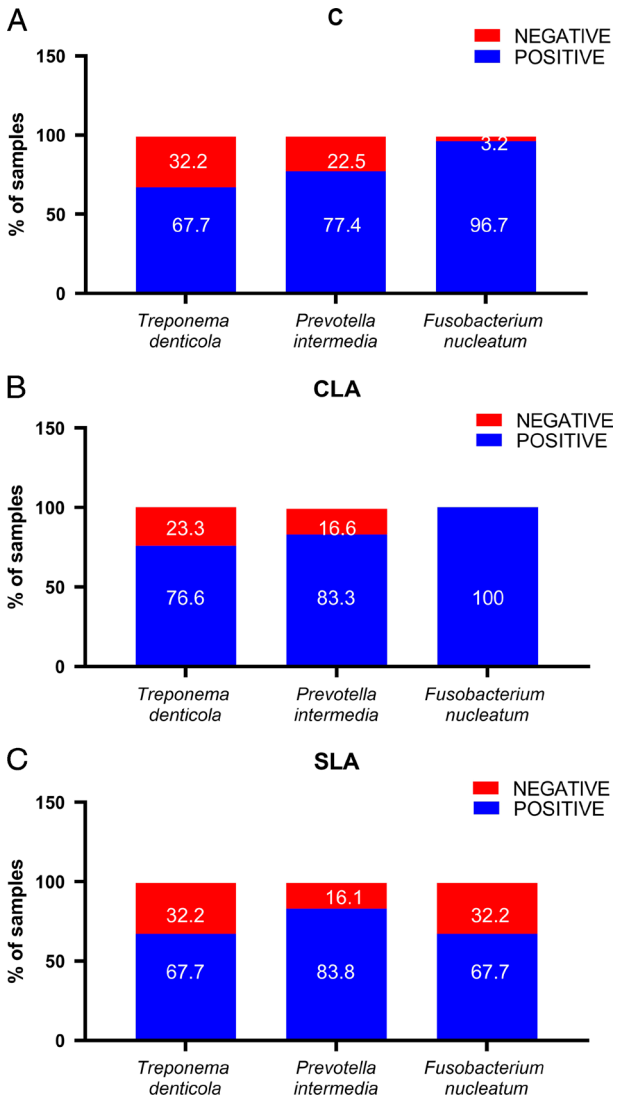

Figure 2. A, Presence of bacteria in C group. B, The bacterial presence of *F. nucleatum* is significantly higher in the CLA group displaying 100% of positivity when compared to SLA. C, Bacterial presence is lower in the SLA group.

(trauma, lacerations, surgeries), those with appliances other than conventional or self-ligating, pregnant patients, or those who, during the previous 2 hours, had carried out oral hygiene, ingested food or administered mouthwash. The clinical characteristics of the patients selected for biofilm sampling were those undergoing orthodontic treatment without extractions, those with mild or moderate crowding whose periodontal status was clinically healthy at the beginning of the treatment and at the time the sample was taken, patients without bleeding at the time of sample collection and who did not require any type of surgical procedure, and patients with a class I molar, class II and III molar relationship of no more than 4 mm.

Biofilm sample collection

The patients refrained from eating, drinking, brushing their teeth, and chewing gum for at least 2 hr before the sampling procedure. Sample collection was performed between 8:30 am and 10:30 am before any oral examination or manipulation, thus avoiding disrupting the oral microbiota.[16] Biofilm samples were taken from the areas adjacent to the fixed orthodontic appliances on the lower incisors using a CK6 instrument. The biofilm samples were placed in microtubes containing 500 µL of phosphate-buffered saline (PBS) 1X. The samples were kept at 4°C during transportation and stored at -80°C until DNA extraction.

DNA extraction

Genomic DNA was obtained from the biofilm samples using the Puregene Gentra Systems kit (DNA Isolation Kit, Minneapolis, MN, USA), according to the manufacturer's instructions and following their protocol.

Quantitative PCR

The presence and load of periodontal pathogenic bacteria were evaluated by quantitative polymerase chain reaction (qPCR). Sequences of the primers and the conditions applied for real-time PCR are shown in Table I. All species-specific primers targeted the variable regions of the 16S ribosomal RNA gene of the three strains. The qPCR conditions are listed in Table II. All assays were performed using SoFast EvaGreen Supermix (Bio-Rad) in a real-time PCR system (QuantStudio 12K Flex Real-Time PCR System, Thermo Fisher Scientific, Waltham, MA, USA) and the load was determined using the Ct threshold method, assigning 100% of the bacterial load to the C group.[14]

Statistical analysis

For a comparison of frequencies between groups, a chi-squared analysis was applied; for bacterial load analysis, a one-way ANOVA was followed by a Dunnet test to compare the conventional and self-ligating groups with the control group. *P*-values <0.05 were considered significant. All statistical analyses were performed using the software Graphpad Prism 8 (GraphPad Software, San Diego, CA, USA).

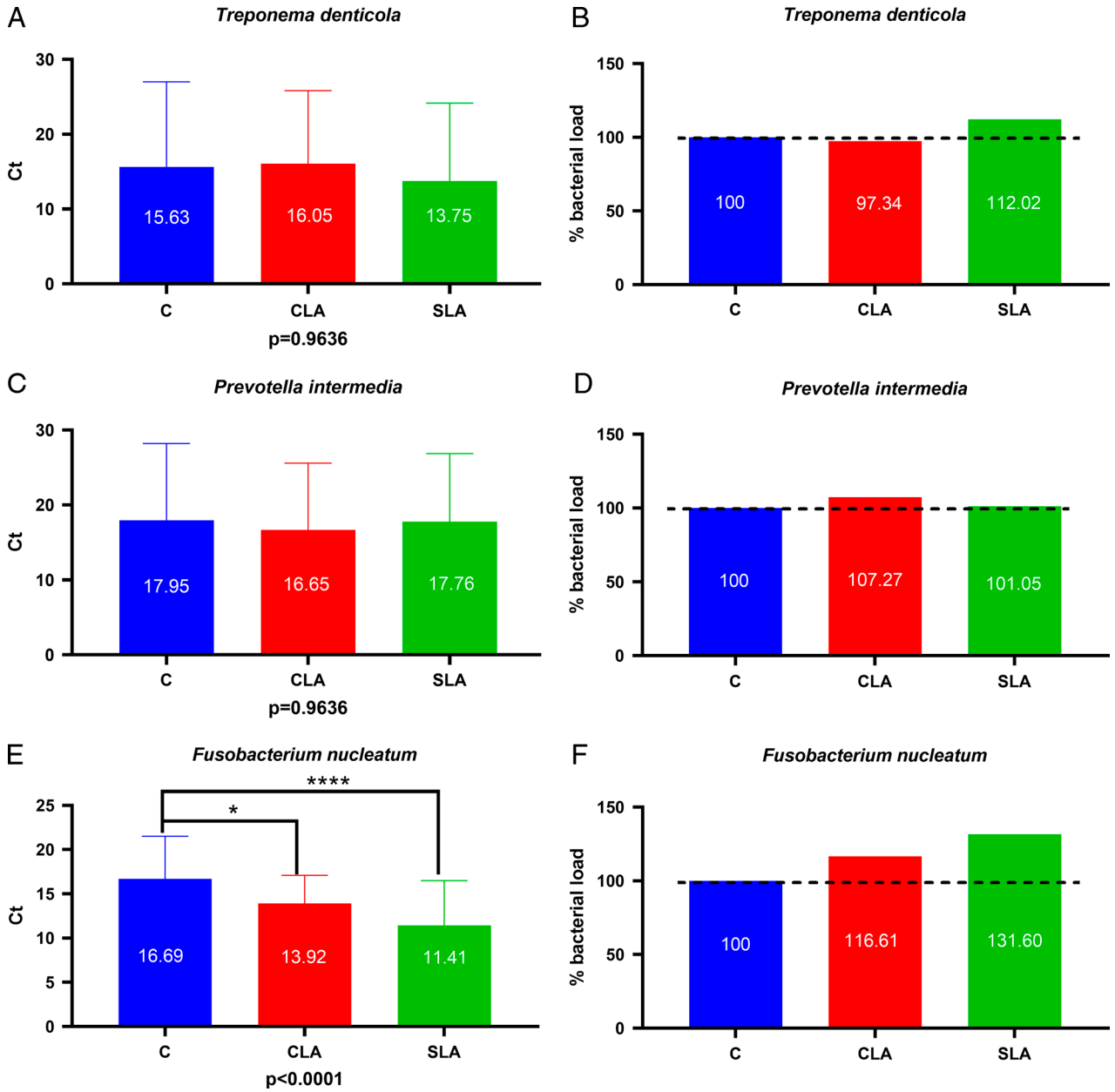

Figure 3. Percentages of bacterial load in the groups related to Ct, for *T. dentícola* (A, B), *P. intermedia* (C, D) and *F. nucleatum* (E,F). *F. nucleatum* showed statistical significance for CLA and SLA groups when compared to C group. Stars indicate statistical significance.

Results

Dysbiotic changes were seen in patients wearing orthodontic appliances: F. nucleatum frequency was significantly higher in CLA and with a greater load in SLA

The presence of periodontal pathogens was observed in all groups, but *F. nucleatum* was identified as the bacteria of highest frequency in all groups (C 96.7%, CLA 100%, and SLA 67.7%, respectively). It was noted that all samples of the CLA group were positive for *F. nucleatum* (Figure 2). *T. denticola* was the bacteria of lowest frequency, however, it was high in the CLA group (76.6%). *P. intermedia* was present in 77.4%, 83.3% and 83.8% of the samples of the C, CLA and SLA groups, respectively.

The percentages of bacterial load obtained using the Ct threshold method, are summarised in Figure 3. Although the differences in *T. denticola* were not significant, it was possible to observe a load difference

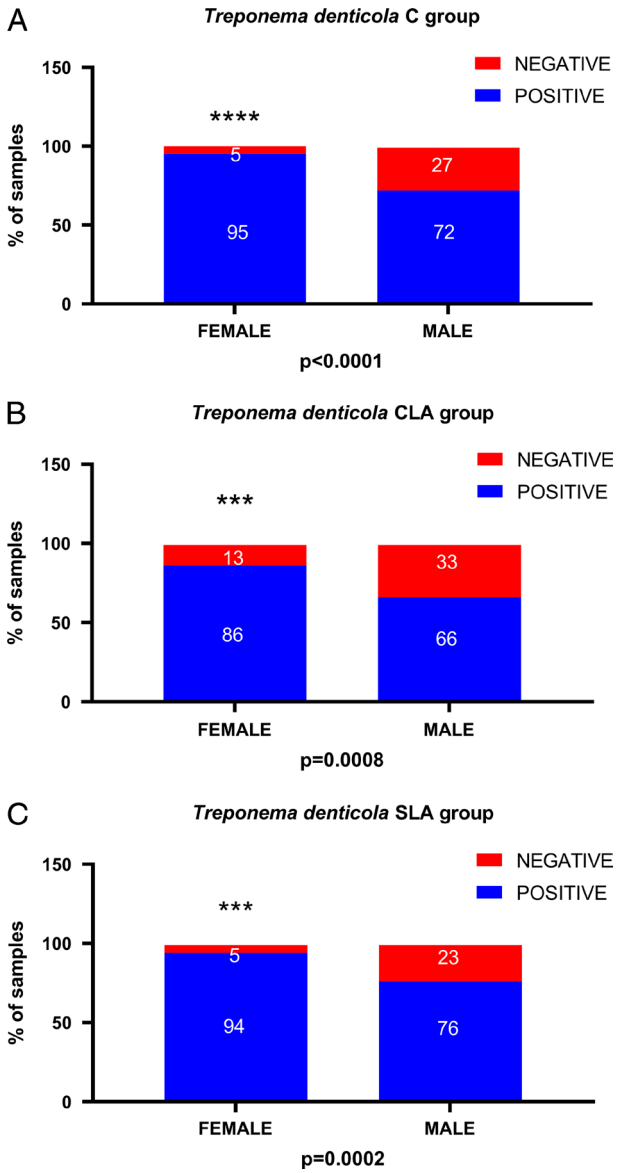

Figure 4. *T. denticola* was found with higher positive percentages in the female gender when compared to the male gender (female *vs* male) for all groups. Nevertheless, the higher percentage was found in the C group.

between the groups, in which the SLA group showed 12.02% greater load than C and 14.68% greater load than the CLA group. *P. intermedia* in the CLA group showed a 7.27% greater load than the C group and 6.21% above the SLA group. *F. nucleatum* load showed significant differences between the groups. SLA had a higher load, with 31.60% above the C group and 14.99% above the CLA group. These results show that *F. nucleatum* frequency was high in the CLA group, but its load was higher in the SLA group.

Female participants showed higher frequencies of periodontal pathogens while male subjects were more affected by F. nucleatum

T. denticola was found more frequently in females than males for all groups tested: C (95% *vs* 72%; p=0.0001), CLA (86% *vs* 66%; p=0.0008), and SLA (94% *vs* 76%; p=0.0002), respectively (Figure 4). In addition, the female higher percentage was found in the C group. For *P. intermedia* (Figure 5), the results for female *vs* male were C (65% *vs* 100%; p=0.0001), CLA (100% *vs* 73%; p=0.0001), and SLA (84% *vs*

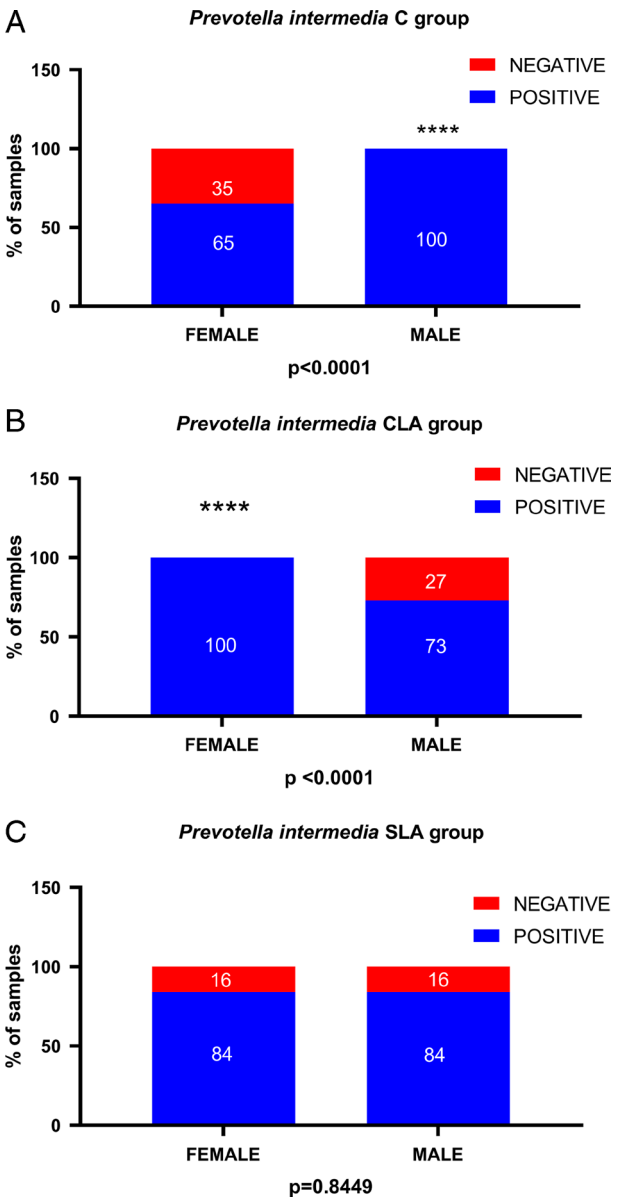

Figure 5. In *P. intermedia*, C group showed 100% of positivity in males. However, the CLA group had 100% of positivity for the female gender, meanwhile, SLA showed no significant differences between genders.

84%; p=0.8449), respectively. Group C showed a 100% frequency in male subjects, while group CLA had a 100% frequency in females and SLA showed the same frequency between the genders. The *F. nucleatum* results for female *vs* male were C (95% *vs* 100%; p=0.0235), CLA (100% *vs* 100%; p=0.9999), and SLA (77% *vs* 94%; p=0.1510), respectively (Figure 6). All groups wearing appliances showed almost 100% positivity for *F. nucleatum* in the male group (Figure 4).

Increased treatment time correlated with a higher frequency of periodontal pathogens

The CLA and SLA groups were subdivided into those with <12 months and >12 months of treatment.

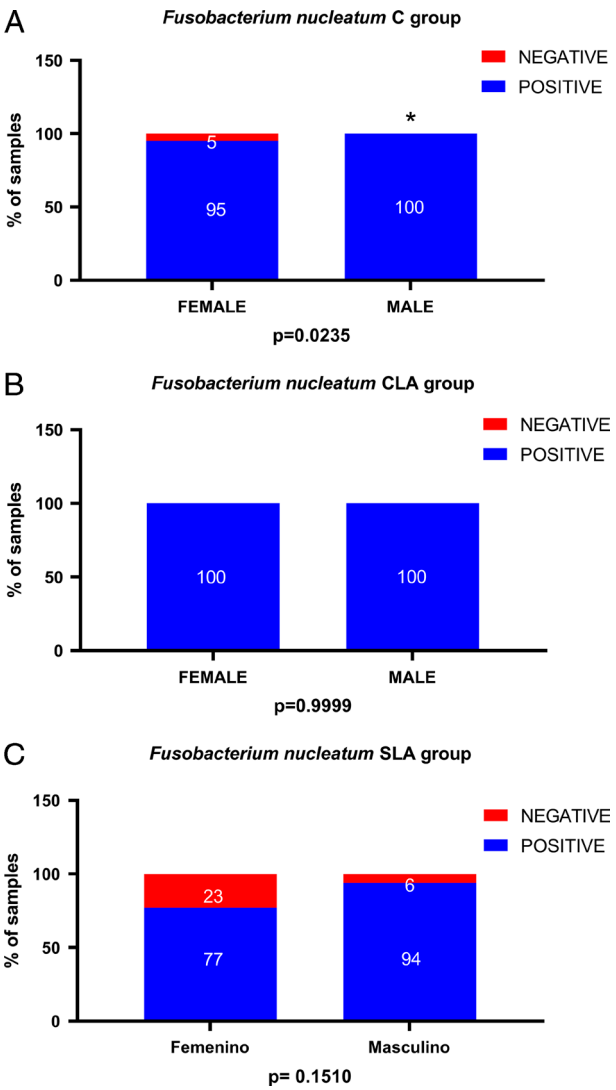

Figure 6. In the case of *F. nucleatum*, all groups showed almost 100% of positivity in the male group. *Statistical significance.

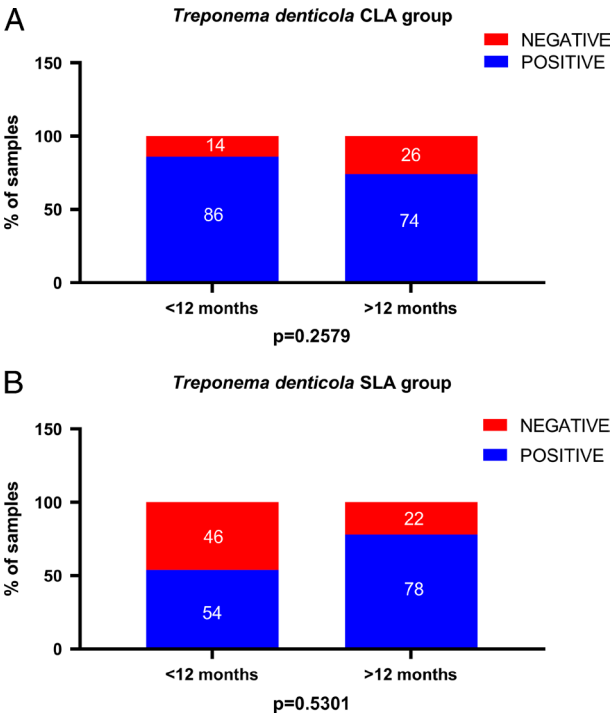

Figure 7. Differences between groups according to time of treatment. *T. denticola* has greater presence in CLA and SLA after 12 months of treatment.

The frequency of *T. denticola* (Figure 7) trended high in CLA (17 positive samples (74%)) and SLA (14 positives (78%)) groups after 12 months of treatment (the difference between the groups was not significant). The frequency of *P. intermedia* was significantly high in both the CLA (18 positives (78%)) and SLA (16 positives (89%)) groups after >12 months of treatment (Figure 8). Of note, *F. nucleatum* was found in 100% of CLA samples independently of treatment time and was more frequent in the SLA group after 12 months of treatment (Figure 9).

Discussion

The present results confirm that dysbiotic changes are seen in patients wearing CLA and SLA. A dysbiotic microbiome is present when the diversity and proportions of normal species are disturbed.[17] In this regard, dysbiosis generated by fixed orthodontic appliances is responsible for enamel demineralisation but also periodontal disease.[18] The current results revealed that all tested bacteria showed a higher load in SLA when compared with the C group. *P. intermedia* and *F. nucleatum* in those wearing CLA, showed a higher load when compared to the C group. These results support the concept that

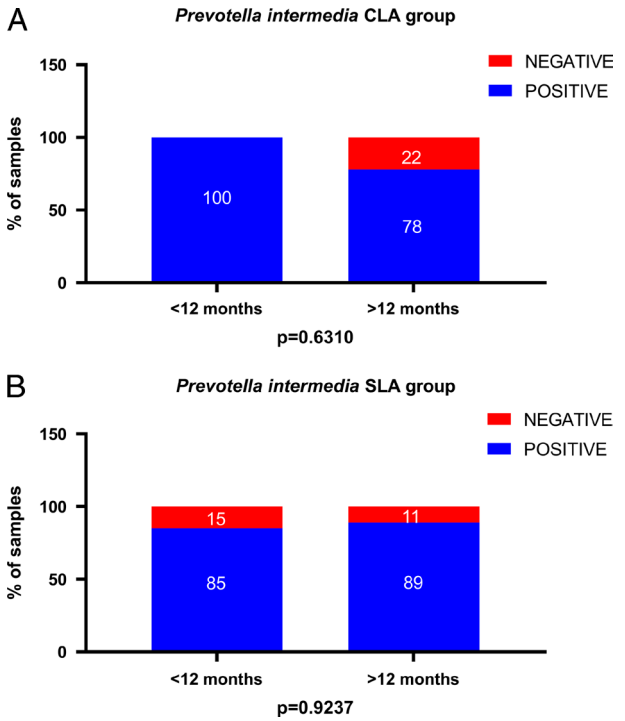

Figure 8. *P. intermedia* is higher after 12 months in CLA and SLA.

dysbiosis leading to periodontal disease can be caused by the rise of a dominant bacterial species, instead of the appearance of a new species.[8] All changes in oral bacterial composition accompanying dysbiosis and periodontal damage, have been related to other diseases including colo-rectal disease, cardiovascular diseases, Alzheimer's disease, rheumatoid arthritis, meningitis, lung, brain, liver or splenic abscesses, appendicitis, pneumonia, and diabetes.[9,19,20]

It is known that after the placement of fixed appliances, an initial biofilm is formed on material surfaces, often resulting in a worsening of the periodontal status. After an initial period of change, the host-microorganism balance can be restored.[21] However, the appliance material surface may be altered over time because of contact with food and drink, abrasive oral hygiene measures, or corrosive processes.[22] Since the attachment of appliances to the teeth makes it difficult to mechanically remove debris, the environment favours the formation of a biofilm.[10,23] Clinically, the bacterial side effects of the appliances become apparent as plaque-associated gingivitis, an increase in pocket probe depth, and bleeding on probing.[24] A recent study has explored the presence of bacteria in saliva and while salivary bacterial levels are helpful to indicate risk factors for the development of disease, *in situ* levels should also help to differentiate contamination patterns associated with the different appliance designs.[25]

The present study focused on metal braces for both CLA and SLA, to minimise the intrinsic variability associated with other materials such as ceramics. Previous studies have reported a greater accumulation of biofilm in CLA metal braces compared to aesthetic material braces.[10] A likely reason for a greater accumulation of biofilm on metal braces compared to aesthetic braces might relate to the nature of the ceramic surface, although aesthetic braces are usually larger and therefore have a greater surface area for biofilm adherence. However, ceramic surfaces are smoother, which makes it difficult for the biofilm to adhere and therefore facilitates mechanical removal.[26]

More recently, Gujar *et al.* investigated the infection levels of Socransky's orange and red bacterial complexes in patients wearing orthodontic appliances which included those with CLA. The findings showed that *T. denticola* had a higher frequency than other bacteria types in CLA patients.[14] In agreement, the present study also found a higher frequency of *T. denticola* (C 67.7%; CLA 76.6%; SLA 67.7%) in

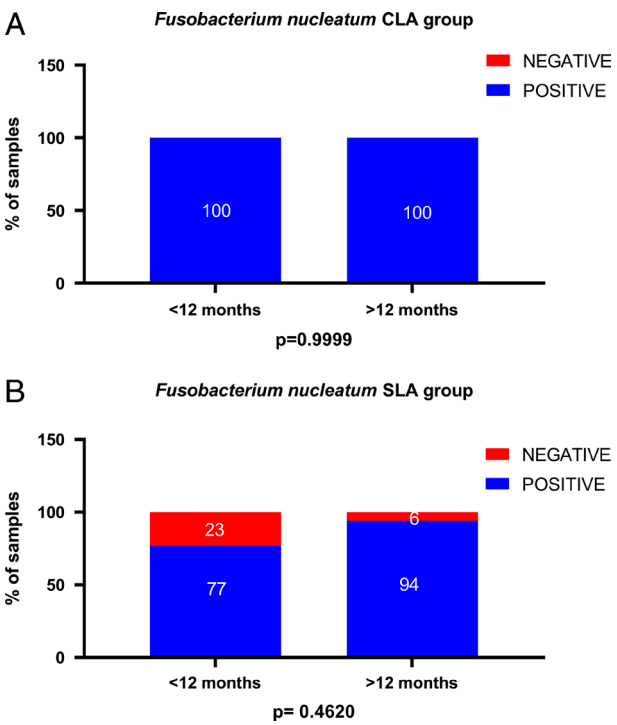

Figure 9. *F. nucleatum* is present in 100% of CLA samples before and after 12 months of treatment and is higher in the SLA group after 12 months.

the CLA group. *T. denticola* has been associated with the red complex (along with *Bacteroides forsythum* and *Porphyromonas gingivalis*),[14] and implicated in severe forms of periodontal disease.[27–31] The present results agreed with those of Pejda *et al*. who showed more positivity of red bacterial complexes on metal CLA appliances compared to metal SLA braces.[24] Although the load of *T. denticola* in the present study was not the highest, its presence was an important finding considering its known virulence which sets it apart from other bacteria.[32] In this regard, *T. denticola,* above threshold values of around 10–15% of the microbial biomass removed from the pocket, was a predictor of imminent attachment loss in a longitudinal prospective clinical trial.[33] It may be speculated that the current finding of *T. denticola* also assumes the presence of *P. gingivalis*, as a recognised pathobiont and synergistic partner that normally co-localise *in vivo*.[34–36] In addition, the bacteria can produce biofilms with increased biomass when grown together *in vitro* and have increased pathogenicity during co-infection in animal models of disease.[33] More importantly, the levels of this dysbiotic signature of the biofilm could be used as a biomarker/predictor of site disease activity and imminent progression.[37]

While *P. gingivalis* was not specifically identified, *F. nucleatum* and *P. intermedia,* two members of the orange complex (along with *Parvimonas micra, Campylobacter rectus, and Eubacterium nodatum)*, were studied.[38] The results indicated a significant presence of *F. nucleatum* in CLA as previously reported,[39] and also a higher load in SLA. The findings agreed with those of Bergamo *et al*. in which the species related to the red and orange complexes, including *F. nucleatum,* showed the highest *in situ* levels on self-ligating appliances 30 and 60 days after placement.[25] Clinically, this is important since, in conjunction with *Actinobacillus actinomycetemcomitans* (serotypes a and b), *F. nucleatum, P. gingivalis, P. intermedia, T. forsythia,* and *T. denticola* have been associated with the progress and severity of periodontal disease.[40] In this regard, it has been reported that SLA braces cause more changes in microbial, plaque, and bleeding indices than conventional braces over time.[41]

While females displayed a greater positivity for periodontal pathogens than males, except for *F. nucleatum*, Zorina *et al*. previously showed that females and males had equal periodontal pathogen colonisation; however, females had a higher risk of developing chronic periodontitis. The present results indicated that *P. intermedia* was present in 100% of the female samples in the CLA group. Nevertheless in males, the complex of *T. denticola* and *P. intermedia* has been correlated with the onset of chronic periodontitis.[42] The present results indicate that, in the case of *F. nucleatum,* the presence of fixed metal appliances does not significantly change the percentage of positivity between the groups. However, it must be considered that an alteration in the oral microbiota can promote inflammatory reactions in the periodontal tissues and the different types of appliances interfere with bacterial adherence.

The present study is limited by the lack of follow-up of patients after sample collection and by the clinical variables present over time to corroborate that dysbiotic changes lead to periodontal disease. Further studies need to be conducted to support this hypothesis and must consider different types of appliances, their design, orthodontics treatment, environmental, and cultural factors, related to diet, smoking, and oral hygiene.

Conclusions

The periodontal pathogens identified as *F. nucleatum*, *T. denticola,* and *P. intermedia* are found in healthy periodontal tissues; however, the present study showed that dysbiotic changes may be seen in patients wearing orthodontic appliances. *F. nucleatum* frequency was significantly higher in CLA and with a greater load in SLA which could affect the periodontal tissues. In addition, female participants showed higher frequencies of periodontal pathogens while male subjects were more affected by *F. nucleatum.* As expected, treatment lasting longer than 12 months correlated with a higher frequency of all periodontal pathogens. The results support the concept that dysbiosis, which leads to periodontal disease, can be caused by the rise of a dominant bacterial species, instead of the appearance of a new species. The evidence emerging from the present study should guide clinicians in modulating and enhancing patient motivation to prevent dysbiosis, thus reducing the risk of periodontal disease.

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Conflict of interest

The authors declare that there is no conflict of interest.

Authors' contributions

MB, RRP, and MAM conceived, designed, and participated in the acquisition, analysis, and interpretation of data for the work; JCQ, MAGL, JALG, and MAF participated in the acquisition, analysis, and interpretation of data; MB, RRP, AAH, CVM, YCS, and MAM participated in the drafting the work, revising it critically for important intellectual content; all authors contributed and approved of the final version to be published.

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