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Original Article

Oral colonization of *Candida albicans* and *Streptococcus mutans* in children with or without fixed orthodontic appliances: A pilot study

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Abstract *Background/purpose:* Adolescents undergoing fixed orthodontic therapy have an increased risk of oral diseases due to additional plaque accumulation sites. However, the effect of fixed orthodontics appliances (FOAs) on the colonization of *Candida albicans* (*Ca*) and *Streptococcus mutans* (*Sm*), two synergistic oral pathogens, is largely unknown and was, therefore, the primary objective of this pilot investigation.
Material and methods: Sixteen children aged 10–15 years were enrolled, nine in the FOA and seven in the control groups. Saliva and occlusal plaque were collected, and the *Ca* and *Sm* levels were quantified with a quantitative real-time polymerase chain reaction (qPCR) assay.
Results: A trend of higher *Ca* levels was observed in the saliva and occlusal plaque of the FOA group, while the control group contained higher levels of *Sm*. Furthermore, for *Sm* levels, a positive correlation between saliva and occlusal plaque was shown in both the FOA and control groups; in contrast, *Ca* levels were negatively correlated between these samples only in the FOA group. Between *Ca* and *Sm*, a positive correlation was observed in saliva and occlusal plaque in the control group; however, this relationship was disrupted in the FOA group.

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Conclusion: Our preliminary study demonstrated that the presence of FOAs disturbs the colonization of *Ca* and *Sm* within the oral cavity. This perturbation might increase orthodontic patients' risk for *Ca*- and *Sm*-related diseases.

Introduction

A significant portion of the pediatric population at the adolescent stage suffers malocclusion and jaw problems as part of their growth and development. Malocclusions are one of the most common oral health problems.[1] These conditions are often managed with orthodontic treatment, which includes fixed and removable orthodontic appliances.[2] One of the most common fixed orthodontic treatments is the application of fixed orthodontic appliances (FOAs).[3]

The introduction of FOAs in the oral cavity greatly affects oral hygiene by increasing food retention, which leads to plaque accumulation.[4] This complex oral biofilm is composed of a network of bacteria, fungi, and other microorganisms. Studies have demonstrated that FOAs can elevate the colonization of fungi such as *Candida* spp. and gram-positive bacteria, including *Streptococcus* spp. and *Lactobacillus* spp. in the oral cavity. This heightened level of pathogens might be responsible for complications associated with orthodontic treatment, such as caries and gingivitis.[5–8]

Among these microorganisms, *Candida albicans* is a common fungal opportunistic pathogen present in the oral cavity of 62% of preschool children and 71% of school children.[9] Previous studies demonstrated that *C. albicans* has the ability to colonize the dentin and enamel and functions as a reservoir for the spread of the microorganism.[10,11] Of the bacterial species, *Streptococcus mutans* has a well-established role in the etiology of caries.[12]

The co-occurrence of *C. albicans* and *S. mutans* has been described in children with dental caries.[13,14] Recently, more emphasis has been placed on the cross-kingdom interactions between *C. albicans* and *S. mutans* in the oral cavity. The cross-talk between the two species has been suggested to mutually enhance their colonization.[15,16] In the presence of an orthodontic appliance, this could set a stage for children to be more prone to caries development. Previously, some studies have demonstrated the presence of *S. mutans* and *C. albicans* on removable orthodontic appliances,[17] but little is known about their colonization in the presence of the FOAs. This pilot study aimed to investigate the impact of FOAs on *C. albicans* and *S. mutans* levels and their correlation in saliva and occlusal plaque in children.

Material and methods

Ethics

This study was conducted in accordance with the Declaration of Helsinki, reviewed and approved by the Institutional Review Board (#16—000282). Written informed consent was obtained from parents or legal guardians of all the subjects that participated in the study.

Subjects recruitment

Sixteen participants were recruited from the pediatric patient population of the University of California, Los Angeles (UCLA) Children's Dental Centre. The enrolled subjects included 9 subjects with fixed orthodontic appliances (FOAs) and 7 without FOAs (control). Participants were eligible based on the following inclusion criteria: healthy children (ASA physical status, class I) aged 10—15 years old, who were not taking any medication, and no antibiotic use within the last 6 months. The FOAs had an additional requirement of metal fixed orthodontic appliances (FOAs), while the control group must have no FOAs or removable appliances. Participants were excluded from the study if they had generalized rampant caries, periodontitis, halitosis, open sores or ulcerations, chronic systemic diseases, reduced saliva production, or any other medical conditions that could influence the oral microbiome. All participants were asked to avoid eating or drinking for at least 1 h before sample collection and received routine oral hygiene care such as tooth brushing instructions, professional teeth cleaning, and topical fluoride application.

Questionnaire and oral examination

Each participant completed a questionnaire, which provided demographic information, oral hygiene habits, and dental treatment history. Oral clinical evaluation and radiographic exams were performed by trained clinical examiners at the UCLA School of Dentistry. The participant's dental caries status was recorded using decayed, missing, and filled tooth (DMFT) criteria proposed by the World Health Organization.[18]

Oral sample collection

All participants were asked to withhold their teeth cleaning and avoid eating and drinking at least 1 h before sample collection. Two oral samples were collected from each individual: saliva and occlusal plaque. Participants were instructed to rinse with water for the saliva collection to remove all saliva from the mouth. Then, 2—3 mL of unstimulated saliva was collected by drooling/spitting directly into a 50 mL Falcon conical sterile tube (Fisher Scientific, Pittsburg PA, USA) kept on ice during the collection. The posterior occlusal plaque was collected using one Pikster™

(Erskine oral care, Marian del Rey, CA, USA) per each quadrant, and all 4 quadrants were pooled (four-quadrant sample) into a sterile 1.5 mL micro-centrifuge tube (Eppendorf, AG, Hamburg, Germany) containing glycerol to a final concentration of 25%. All the collected samples, either saliva or plaque, were frozen immediately and stored at −80 °C.

Extraction of genomic DNA

Total genomic DNA was extracted from our participant's oral samples (saliva and occlusal plaque) using the Epicenter MasterPure™ DNA purification kit (Lucigen Corporation, Middleton, WI, USA) following the manufacturer's instructions with modifications.[19] The extracted DNA was stored at −20 °C until further use.

Real-time quantitative polymerase chain reaction (qPCR) and sensitivity test

The fungi and *C. albicans* levels in the saliva and occlusal plaque samples were assessed by real-time quantitative polymerase chain reaction (qPCR), using the fungi-specific (ITS1, ITS2)[20,21] and the *C. albicans*-specific (SAP)[22,23] primers (Table 1). In parallel, we also performed qPCR to assess the total bacteria and *S. mutans* levels, using the universal 16S RNA gene primers for bacteria[24] and *S. mutans* specific glucosyltransferases (gtfB)[25] gene primers (Table 1).

The qPCR reactions were performed using Bio-Rad iCycler Thermal Cycler with iQ5 Multicolour Real-Time PCR Detection System (BioRad iQ 5 RTPCR QPCR, BioRad, Hercules, CA, USA). Amplification was carried out as followed: the cycling condition was 95 °C for 10 min, followed by 40 cycles of denaturation for 30 s at 95 °C, then primers annealing at 60 °C for fungi or 58 °C for *C. albicans*-specific primers for 30 s, and extension at 72 °C for 30 s. For bacterial universal and *S. mutans* specific primers, we use similar amplification conditions as described above, except for the annealing temperature, which was 64 °C for both primers. All amplifications and detections were carried out in a BioRad iQ5 qPCR 96-well reaction plate with optical caps (BioRad) and performed three times. In addition, the iQ5 Optical System Software generated quantification cycle values (Cq) which were analyzed along with melting point data. Additional information on the analysis is in Appendix 1.

Statistical analyses

Statistical analysis with one-way ANOVA followed by Kruskal–Wallis multiple comparisons test was performed using GraphPad (GraphPad Prism version 8.0.0, GraphPad Software, San Diego, California USA), and difference significance were defined as follows: $^{*}P \leq 0.01$, $**P \leq 0.001$ and $***P \leq 0.0001$.

Results

Study subjects

The 16 pediatric participants enrolled in this pilot study included 9 subjects with FOAs and 7 control individuals. The participants' age, gender, ethnicity, DMFT index, oral hygiene parameters, and duration of orthodontic treatment are summarized in Table 2. In brief, the gender distribution was 12 (75%) females and 4 (25%) males, the age ranged from 10 to 15 years with an average of 12.4 (±1.5) and ethnicity included 81.3% Hispanic and 18.7% non-Hispanic, and the caries experience was 0.2 (±0.4) DMFT score. Overall, there were no significant differences between the FOA and control groups for all of the parameters mentioned above. In addition, subjects with FOAs had an average of 15.4 (±5.8) months in treatment duration, ranging from 10 to 27 months.

Levels of *C. albicans* and fungi in saliva and occlusal plaque

The introduction of FOAs in the oral cavity may impact the maintenance of oral hygiene, plaque accumulation, and microbial colonization. We quantified the levels of *C. albicans* and total fungi in saliva and occlusal plaque samples collected from subjects with or without FOAs via qPCR. Overall, the fungi levels in all samples were low. However, the control group had a significantly higher fungi load in the

Table 1 Oligonucleotide primer sequences used in this study.

Target	Primer name	Sequence (5′-3′)	AT (°C)	Amplicon size (bp)
Fungi[20,21]	ITS1-F	CTTGGTCATTTAGAGGAAGTAA	60	Vary in different species
	ITS2-R	GCTGCGTTCTTCATCGATGC		
Candida albicans[22,23]	SAP-F	CTGCTGATATTACTGTTGGTTC	58	263
	SAP-R	CCACCAATACCAACG GTATC		
Bacteria[24]	Eub338	ACTCCTACGGGAGGCAGCAG	64	200
	Eub518	ATTACCGCGGCTGCTGG		
Streptococcus mutans[25]	*gtf* B–F	CTACACTTTCGGGTGGCTTG	64	250
	gtf B-R	GAAGCTTTTCACCATTAGAAGCTG		

AT- Annealing temperature; AL- Amplicon length.
ITS - Internal Transcribed Spacer Region; SAP - Secreted Aspartyl Proteinase.
gtf B- codes glucosyltransferase B.

Table 2 Demographic and oral hygiene information obtained from the study questionnaire.

Group Characteristic	Control		FOAs		Total	
	(n=7)	%	(n=9)	%	(n=16)	%
Gender						
Female	4	57.1	8	88.9	12	75.0
Male	3	42.9	1	11.1	4	25.0
Ethnicity						
Hispanic	5	71.4	8	88.9	13	81.3
Non-Hispanic	2	28.6	1	11.1	3	18.7
Age (years)						
	11.9 (± 1.3)		12.9 (± 1.6)		12.4 (±1.5)	
DMFT index						
	0.3 (± 0.5)		0.1 (± 0.3)		0.2 (± 0.4)	
Orthodontic appliances period (months)						
			15.4 (±5.8)			
Professional dental cleaning frequency						
None	0	0.0	0	0.0	0	0.0
Once a year	1	14.3	1	11.1	2	12.5
More than once a year	6	85.7	8	88.9	14	87.5
Tooth brushing frequency						
Not brushing	0	0.0	0	0.0	0	0.0
Once a day	0	0.0	0	0.0	0	0.0
More than once per day	7	100.0	9	100.0	16	100.0
Flossing frequency						
Not flossing	1	14.3	2	22.2	3	18.8
Less than once per day	2	28.6	4	44.4	6	37.5
Once per day	3	42.9	3	33.3	6	37.5
More than once per day	1	14.3	0	0.0	1	6.3
Mouthrinse use frequency						
Not using mouthrinse	2	28.6	6	66.7	8	50.0
Less than once per day	2	28.6	0	0.0	2	12.5
Once per day	0	0.0	3	33.3	3	18.8
More than once per day	3	42.9	0	0.0	3	18.8

Control — **without** Fixed Orthodontic Appliances; **FOA** — **with** Fixed Orthodontic Appliances.

occlusal plaque samples (Fig. 1A). Higher levels of *C. albicans* in the saliva and occlusal plaque of the FOAs were apparent but not statistically significant (Fig. 1B).

Levels of *S. mutans* and bacteria in saliva and occlusal plaque

In parallel to fungi and *C. albicans* levels, we also quantified the *S. mutans* levels in the saliva and occlusal plaque. A significantly higher level of total bacteria was detected in the FOA group of both saliva and occlusal plaque samples (Fig. 2A). Moreover, independent of the study group, the occlusal plaque site displayed higher levels of total bacteria when compared to the saliva samples. While there were no statistically significant differences in either sample type, we observed a trend of higher *S. mutans* levels in the occlusal plaque in the control group (Fig. 2B).

Correlation between levels of *C. albicans* and *S. mutans*

We investigated the possible correlations between the different microorganism's levels (Fungi, bacteria, *C. albicans*, and *S. mutans)* in saliva and occlusal plaque using linear regression analysis. In occlusal plaque and saliva, *C. albicans* levels were positively correlated in the control group (R^2=0.94; p = 0.0003), while in the FOA group, the correlation was negative (R^2=0.24; p = 018) (Fig. 3A). However, the linear correlation between the occlusal plaque and the saliva *S. mutans* levels was positive in both the control (R^2 = 0.94; p = 0.0003) and the FOA groups (R^2 = 0.67; p = 0.007) (Fig. 3B).

In addition, when comparing *C. albicans* and *S. mutans* levels, positive correlations were observed in saliva (R^2 = 0.78; p = 0.008) and occlusal plaque (R^2 = 0.77;

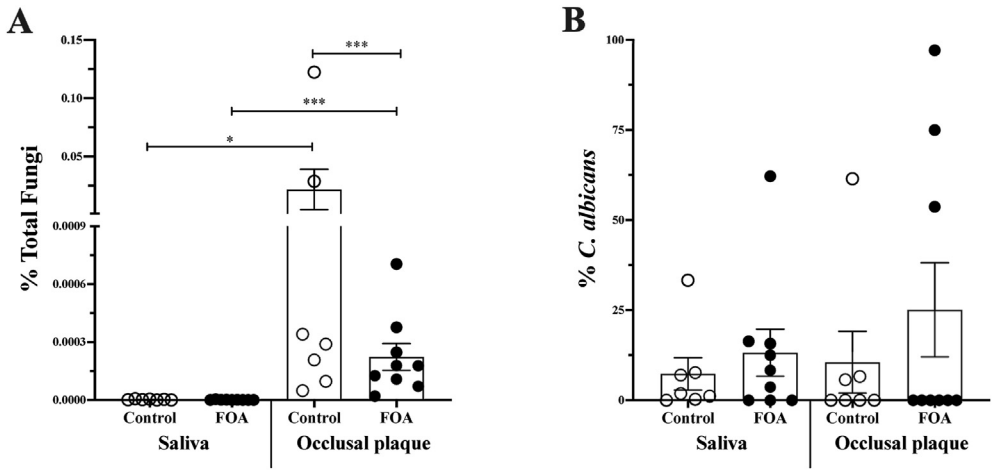

Figure 1 *Candida albicans* and fungi levels quantification in saliva and occlusal plaque. Fungi-specific **(ITS1, ITS2)** and the *C. albicans*-specific **(SAP)** primers were used to evaluate the levels of fungi and *C. albicans* using qPCR. Percentage of total fungi **(A),** and ***C. albicans*** **(B)** within the total fungi, in the saliva and occlusal plaque collected from subjects with **(FOA,●)** and without fixed orthodontic appliances **(Control,○).** The groups significant differences were analyzed with one-way ANOVA followed by Kruskal–Wallis multiple comparisons test; *P <0.01, **P < 0.001, ***P < 0.0001.

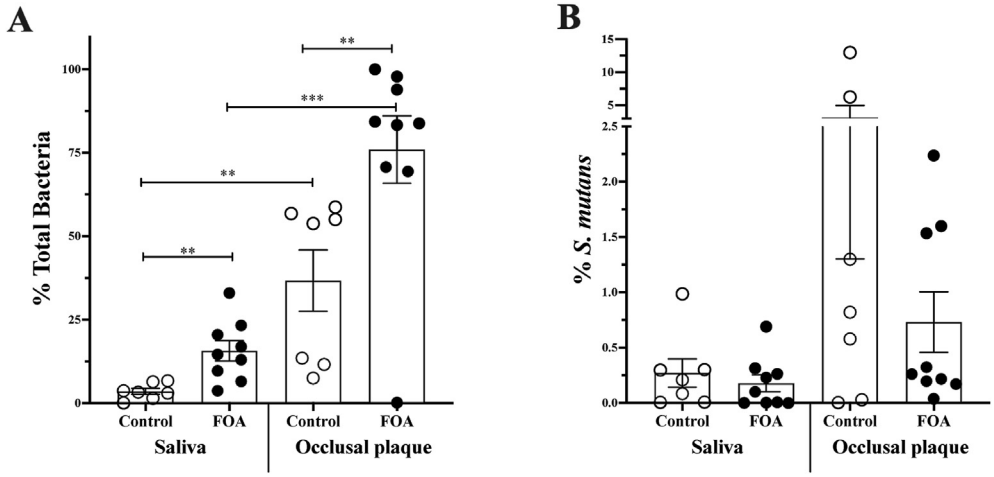

Figure 2 *Streptococcus mutans* and total bacteria levels quantification in saliva and occlusal plaque. Universal bacterial specific **(Eub1, Eub2)** and the *S. mutans* specific (***gtfB*, glucosyltransferase B**) primers were used to evaluate the level of total bacteria and *S. mutans* in using qPCR. Percentage of total bacteria **(A)** and *S. mutans* **(B)**, within the total bacteria, in the saliva and occlusal plaque collected from subjects with **(FOA,●)** and without fixed orthodontic appliances **(Control,○).** The groups differences significance was analyzed with one-way ANOVA followed by Kruskal–Wallis multiple comparisons test; *P < 0.01, **P < 0.001, ***P < 0.0001.

p = 0.009) in the control group (Fig. 4A), but not in FOA subjects (Fig. 4B).

Discussion

Malocclusion is a prevalent problem in the pediatric population, which most commonly has been managed with FOAs. The introduction of FOAs affects oral hygiene maintenance, stimulating microbial accumulation and promoting plaque formation. Thus, disturbance of the normal oral microflora environment by long-term use of fixed orthodontic appliances may increase the vulnerability of the patients to oral disease. For example, imbalances in bacterium–fungus interactions are known to influence the transition from a healthy to a diseased state.[26] Particularly in dental caries, high levels of *S. mutans* and *C. albicans* have been detected and are known to function synergistically in the plaque biofilms of children.[14,27,28] However, little is known about their levels and interactions in individuals with FOAs. To explore this important question, this pilot clinical study investigated the effect of FOAs on *C. albicans* and *S. mutans* levels and their correlation in saliva and occlusal plaque in children.

The oral cavity consists of complex microbial ecosystems, including teeth, tongue, and saliva. Saliva has immense

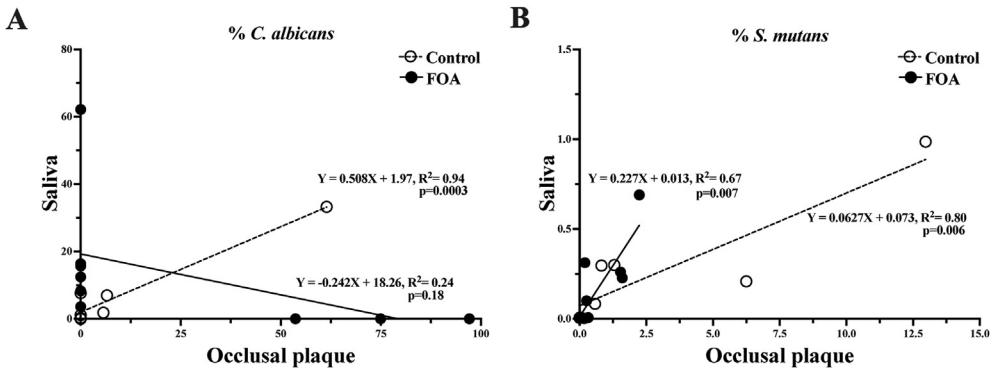

Figure 3 Correlation between saliva and occlusal plaque *C. albicans* and *S. mutans* levels in FOA and control groups. The graphics represent the linear regression analysis: correlation between saliva and the occlusal plaque *C. albicans* **(A)** and *S. mutans* **(B)** levels on the subjects with **(FOA,●)** and without fixed orthodontic appliances **(Control,○)**.

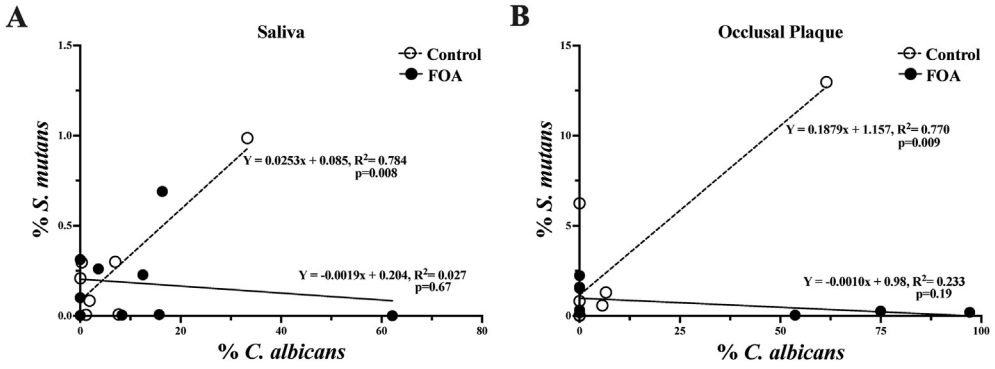

Figure 4 Correlation between *S. mutans* and *C. albicans* levels in the FOA and control groups. The graphics represent the linear regression analysis between *S. mutans* and *C. albicans* levels in saliva **(A)** and occlusal plaque **(B)** of the subjects with **(FOA,●)** and without fixed orthodontic appliances **(Control,○)**.

potential as a diagnostic fluid for the identification and monitoring of diseases. The relative ease of collection of saliva further strengthens large-scale diagnostic purposes.[29] Importantly, saliva reflects the composition of the oral microbiome, mirroring the local supragingival and subgingival microbiota, and the overall oral health.[30]

In the oral cavity, *S. mutans* is known to be most prevalent in the occlusal surfaces of the teeth, while it is unclear how this affects the colonization of its partner, *C. albicans*.[31] Previously, it has been shown that fungi comprise a small portion of the salivary oral microbiome.[32] They are often found in the oral cavity of healthy individuals, with *C. albicans* being the most predominant species (approximately 60%—70%).[33] Several *Candida* spp. have been isolated from caries in both children and adults with a prevalence ranging from 66% to 97% in pediatric populations and 31%—56% in adult populations.[10]

The use of quantitative real-time PCR (qPCR) with species-specific primers provides an accurate and sensitive method for detecting and quantifying individual species. This method has been shown to have a high sensitivity for the quantification of *S. mutans* and *C. albicans*.[13,34,35] Therefore, our study applied the qPCR detection method for *C. albicans* and *S. mutans* quantification in saliva and occlusal plaque samples. Consistent with previous studies,[32,36] the overall levels of fungi detected in all samples were generally low (Fig. 1A). Both the control and FOA groups displayed high levels of total fungi in the occlusal plaque (Fig. 1A). However, the *C. albicans* levels were higher in occlusal plaque only in the FOA group compared to saliva (Fig. 1B). For total bacteria, significantly higher abundance was observed in the occlusal plaque of both groups (Fig. 2A). This pattern could be explained by tooth surface architecture. Pits and fissures within occlusal surfaces provide a special topography, making plaque removal very difficult and harboring higher microbial loads.[37]

Comparing between groups, the FOA subjects had significant lower total fungi levels in occlusal plaque than the control (Fig. 1A). Within the fungi, we detected higher levels of *C. albicans*, although not statistically significant, in saliva and occlusal plaque in the FOA group compare to the control (Fig. 1B). The increase in *C. albicans* level in the presence of the fixed orthodontic appliance is in accordance with previous studies both *in vivo* and *in vitro* systems.[38,39] However, a previous investigation revealed a lack of significant changes in salivary *C. albicans* in the presence of the FOA even though the study used a different method of detection.[40]

In the presence of the FOA, lower *S. mutans* levels were observed in both sample types. This was more pronounced

in the occlusal plaque than saliva; however, it was not statistically significant (Fig. 2B). Taken together, we observed the trend that the FOA group had higher *C. albicans* and low *S. mutans* levels in saliva and occlusal plaque. However, the levels of *S. mutans* and *C. albicans* may not always correspond to their synergistic relationship. A previous study demonstrated a positive correlation between *C. albicans* and *S. mutans* in saliva but a negative relationship in dental plaque.[13] However, the correlation between *C. albicans* and *S. mutans* levels in the presence of the FOA is not well understood.

In our study, the *C. albicans* levels in saliva and occlusal plaque were positively correlated in the control group, while a negative correlation was observed in the FOA group (Fig. 3A). The presence of the FOAs may contribute to the negative correlation as it acts as a retentive surface and may serve as a reservoir for fungal accumulation. This may prevent the release of the fungi to saliva or localization on the occlusal surfaces.[2]

In terms of *S. mutans*, we observed a positive correlation between the occlusal plaque and the saliva levels in both the control and the FOA groups (Fig. 3B). These results are in agreement with previous studies that demonstrated the preferential localization of *S. mutans* for occlusal surfaces.[31] Ultimately, we compared *C. albicans* and *S. mutans* relationship and found positive correlations for their levels in saliva and occlusal plaque in the control group, but not the FOA subjects (Fig. 4).

The ratio of *S. mutans* and *C. albicans* may contribute to the health and disease state. When the FOAs are inserted in the oral cavity, disruption of the balance occurs. This may favor the colonization of *C. albicans* and other partner bacteria, which can be explored in future studies. Although our pilot study was cross-sectional in nature, we demonstrated that the FOAs disrupt the existing relationship between *C. albicans* and *S. mutans* species that may impact the future oral health of orthodontic patients. Future studies should consider expanding the investigation into a longitudinal study with a larger sample size.

In conclusion, the presence of FOAs perturbed the correlation between *C. albicans* and *S. mutans*. Specifically, there was a significant increase in the overall bacterial load and a trend of higher *C. albicans* accumulation on the occlusal surfaces of the FOA group, which might increase the risk of orthodontic patients for fungal and bacterial related diseases. Therefore, it is essential to explore further the impact of FOAs treatment on the individual's oral microbiome and the association with oral health and diseases.

Declaration of competing interest

All authors declare no conflict of interests relevant to this article.

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Appendix 1

Supplementary material and methods

Extraction of genomic DNA

Total genomic DNA was extracted from our participant's oral samples (saliva and occlusal plaque) using the Epicenter MasterPure™ DNA purification kit (Lucigen, USA) following the manufacturer's instructions with modifications. Briefly, prior to the kit purification protocol, samples were subjected to mechanical grinding with glass beads followed by lysozyme treatment for 2 h at 37 °C. The quantity and quality of DNA were measured with NanoDrop 2000 (Thermo Fisher Scientific, USA). The extracted DNA was stored at −20 °C until further use.

Real -time quantitative polymerase chain reaction (qPCR) and sensitivity test

The fungi and *C. albicans* levels in the saliva and occlusal plaque samples were assessed by real-time quantitative polymerase chain reaction (qPCR), using the fungi-specific (ITS1, ITS2) and the *C. albicans*-specific (SAP) primers (Table 1). To assess the total bacteria and *S. mutans* levels, using the universal 16S RNA gene primers for bacteria and *S. mutans* specific glucosyltransferases (gtfB) gene primers (Table 1). Briefly, the reaction mix consisted of 0.5 μM of each primer with 1X SYBR Green Master Mix (BioRad) and template DNA. For template DNA, 100 ng of saliva and 15 ng of occlusal plaque DNA was used in a reaction volume of 20 uL, while 100 ng of DNA was used for fungal (*C. albicans* SN152) and bacterial (*S. mutans* UA140) genomic DNA.

To generate standard curves for real-time PCR, a tenfold serial dilution of reference species (*C. albicans* SN152, or *S. mutans* UA140) DNA, initial 10 ng, was used as templates and the threshold at the different dilution points averaged. Standard curves were generated with linear relationships with universal primers. The fungi-specific (ITS1, ITS2) and the *C. albicans*-specific (SAP) primers (Table 1) were verified using genomic DNA from different fungi and *C. albicans* (SN152) reference strains. Additionally, specificity of the universal 16S rRNA-specific and *S. mutans*-specific primers (glucosyltransferase B, *gftB*) (Table 1) was confirmed using genomic DNA from common oral bacterial species and *S. mutans* (U140) reference strains. Moreover, the respective melting peaks were used to assess the specificity of the amplicon.

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