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Analysis of biofilm formation by *Candida albicans* in different types of orthodontic fixed appliances and devices

Análise da formação de biofilme por *Candida albicans* em diferentes tipos de aparelhos e dispositivos ortodônticos fixos

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ABSTRACT

Objective: in this study, biofilm formation by *Candida albicans* in fixed orthodontic appliances was evaluated. **Material and Methods:** a total of 300 conventional metal brackets (MC), ceramic (CB), self-ligation (SLB), nickel-titanium (NiTi), and nickel-chromium (NiCr) wires, and ligatures types were organized into thirty groups (n=10). To induce biofilm formation, brackets, wires, and ligatures were joined, sterilized, placed in 24-well plates, contaminated with standardized suspensions of *C. albicans* (10^7 cells/mL), and incubated at 37 °C for 48 h with shaking. The biofilms formed were detached using an ultrasonic homogenizer, and suspensions were serially diluted and plated on Sabouraud dextrose agar to determine colony-forming units per mL. Scanning electron microscopy was performed before and after the biofilm formation. **Results:** lower amount of biofilm formation was observed in the MC group than in the CB and SLB groups ($p<0.0001$). SLB and CB showed similar biofilm formation rates ($p=0.855$). In general, the cross-sectional wires .018"x.025" showed higher biofilm formation when associated with the three types of brackets. When brackets, wires, and ligatures were associated, the sets with NiCr wires and SSL ligatures with MC brackets ($p=0.0008$) and CB ($p=0.0003$) showed higher biofilm formation. **Conclusion:** thus, brackets of MC with NiTi and NiCr wires showed lower biofilm formation, regardless of the ligature and cross-sectional or gauge of the wire and, MC and CB brackets with NiCr wires and SSL ligatures were more likely to accumulate biofilms.

KEYWORDS

Biofilms, *Candida albicans*, Orthodontic appliances, Orthodontic brackets, Scanning electron microscopy.

RESUMO

Objetivo: neste estudo, a formação de biofilme por *Candida albicans* em aparelhos ortodônticos fixos foi avaliada. **Material e Métodos:** um total de 300 bráquetes metálicos convencionais (MC), cerâmicos (CB), autoligados (SLB), com fios de níquel-titânio (NiTi) e níquel-cromo (NiCr) e tipos de ligaduras foram organizados em trinta grupos (n=10). Bráquetes, fios e ligaduras foram unidos, esterilizados, colocados em placas de 24 poços, contaminados com suspensões padronizadas de *C. albicans* (10^7 células/mL) e incubados a 37°C por 48 h para a formação de biofilmes. Os biofilmes formados foram rompidos por meio de um homogeneizador ultrassônico e suspensões foram diluídas e semeadas em ágar Sabouraud-dextrose para determinar as unidades formadoras de colônias por mL. A microscopia eletrônica de varredura foi realizada antes e após a formação do biofilme. **Resultados:** foi observada menor formação de biofilme no grupo MC em comparação aos grupos CB e SLB ($p<0,0001$). A formação de biofilme foi semelhante nos grupos SLB e CB ($p=0,855$). Em geral, os fios de seção

transversal .018"x.025" apresentaram maior formação de biofilme quando associados aos três tipos de bráquetes. Os conjuntos com fios de NiCr e ligaduras SSL com bráquetes MC (p=0,0008) e CB (p=0,0003) apresentaram maior formação de biofilme. **Conclusão:** bráquetes MC com fios de NiTi e NiCr apresentaram menor formação de biofilme, independente da ligadura e secção transversal ou bitola do fio e, braquetes MC e CB com fios de NiCr e ligaduras SSL foram mais propensos a acumular biofilmes.

PALAVRAS-CHAVE

Biofilmes, *Candida albicans*, Aparelhos ortodônticos fixos, Bráquetes ortodônticos, Microscopia eletrônica de varredura.

INTRODUCTION

Studies have shown that fixed orthodontic appliances are biofilm retainers that can hinder hygiene [1,2,3], forming sites for accumulation of food residues and aggregation of microorganisms [4]. Together, brackets, wires, bands, and ligatures can further aggravate these conditions [5]. Although modifications in these accessories are made to minimize biofilm retention, doubts remain regarding whether these modifications result in the greater or lesser accumulation of residues and microorganisms when compared with conventional and self-connected devices [6-10].

The permanent consolidation of biofilm can cause periodontal changes, white spots in the enamel, and even dental caries [5,11,12]. Many studies have reported that the main microorganisms involved in caries are *Streptococcus* spp. and *Lactobacillus* spp. However, it is known that caries are not caused by a limited number of specific bacteria, but by a change in the microbial population in which *Candida* spp. play an important role [12]. *Candida* is a collecting agent present in the oral cavity of approximately 30% to 35% of the adult population without evidence of infection [13]. Therefore, in certain individuals and in specific situations, they can assume filamentary form, producing oral diseases such as oral candidosis.

There is an indication of the cariogenic potential of *C. albicans* in enamel and dentin lesions in cooperation with *Streptococcus mutans* [14]. It was observed that glucans produced by certain bacteria, such as *S. mutans*, can increase the adhesive capacity of *C. albicans* [15] and that yeast can be used by *S. mutans* to support its adhesion and aid the cariogenic process [16]. *In vitro* studies suggest *C. albicans* has a high acidogenic potential and ability to cause mineral loss and, therefore, is adjunct to the process of establishment of dental caries [17,18]. In addition, it can increase the incidence of caries in rats when associated with microbiota with low cariogenic potential [19].

Perkowski et al. (2019) [20] evaluated oral microbiota as a risk factor for health complications in patients treated with removable or fixed devices compared with patients without a device and found the prevalence of *C. albicans* to be higher in patients with fixed apparatus, with a statistically significant difference in the presence of *C. albicans* in patients treated with appliances when compared to those not treated. They also found that the use of a fixed orthodontic appliance alters the state of the oral cavity, impacting the colonization of the biofilm by opportunistic/pathogenic strains and, consequently, increasing the risk of its dissemination to various tissues and organs, emphasizing that the oral cavity can act as a reservoir of microorganisms that can induce infections of clinical importance. Having acknowledged the participation of *C. albicans* biofilm in some pathogenic processes, the aim of this study was to evaluate the biofilm formation of *C. albicans* on the surface of orthodontic brackets of different materials, together with wires and ligatures.

MATERIAL AND METHODS

Production of biofilms

The sample consisted of 300 orthodontic rods of the upper central incisors of the straight wire technique, Roth prescription, with a .022" slot (Morelli®, Sorocaba, Brazil), and 300 segments of 1 cm wires fastened by elastomeric and metallic ligatures. Thirty groups (n=10) were created based on the type of bracket (conventional metallic/MC, self-ligating brackets/SLB, ceramic bracket/CB), wire metallic alloy (Nickel-Chromium/NiCr, Nickel-Titanium/NiTi), wire size and cross-section (.014", .018", .018" x .025") and ligatures types (025 mm-stainless steel ligature/SSL, elastomeric chain/EC) (Table I).

Biofilms were cultured in 24-well culture plates (TPP®, Trasadingen, Switzerland) containing different brackets, following the methodology described by Pereira et al. (2011) [21], with necessary modifications. Suspensions of strains of *C. albicans* (ATCC 18804) were adjusted to 10^7 cells/mL using a spectrophotometer (B582, Micronal, São Paulo, Brazil), and 2 mL BHI broth (Brain Heart Infusion, Difco, Detroit, USA) was added to each well. The plates were incubated with shaking at 75 rpm (Quimis, Diadema, Brazil) for 90 min at 37°C to facilitate initial adhesion. After the adhesion phase, the suspension was aspirated and each well was washed with 2 mL of phosphate-buffered saline (PBS) to remove non-adherent cells. Subsequently, 2 mL of BHI broth was added to each well to form a biofilm.

Table I - Research groups according to bracket type, arc metal alloy and ligature type

Group	Bracket	Archwire	Ligature
G1	MC	.014" NiTi	EC
G2	MC	.014" NiTi	SSL
G3	MC	.018" NiTi	EC
G4	MC	.018" NiTi	SSL
G5	MC	.014" NiCr	EC
G6	MC	.014" NiCr	SSL
G7	MC	.018" NiCr	EC
G8	MC	.018" NiCr	SSL
G9	MC	.018"x.025" NiTi	EC
G10	MC	.018"x.025" NiTi	SSL
G11	MC	.018"x.025" NiCr	EC
G12	MC	.018"x.025" NiCr	SSL
G13	CB	.014" NiTi	EC
G14	CB	.014" NiTi	SSL
G15	CB	.018" NiTi	EC
G16	CB	.018" NiTi	SSL
G17	CB	.014" NiCr	EC
G18	CB	.014" NiCr	SSL
G19	CB	.018" NiCr	EC
G20	CB	.018" NiCr	SSL
G21	CB	.018"x.025" NiTi	EC
G22	CB	.018"x.025" NiTi	SSL
G23	CB	.018"x.025" NiCr	EC
G24	CB	.018"x.025" NiCr	SSL
G25	SLB	.014" NiTi	No ligature
G26	SLB	.018" NiTi	No ligature
G27	SLB	.014" NiCr	No ligature
G28	SLB	.018" NiCr	No ligature
G29	SLB	.018"x.025" NiTi	No ligature
G30	SLB	.018"x.025" NiCr	No ligature

All the plates were incubated at 37°C for 48 h with shaking at 75 rpm. After the incubation period, the brackets were washed twice with PBS and the biofilms were detached using a Sonopuls HD 2200 ultrasonic homogenizer (Bandelin Electronic, Berlin, Germany) at 50 W for 30 s. The suspensions were serially diluted and plated on Sabouraud dextrose agar (Difco, Detroit, USA) to determine the colony-forming units per mL (CFU/mL).

Scanning Electron Microscopy (SEM)

After biofilms formation, the devices were fixed in 1 ml of 2.5% glutaraldehyde for 1 h. Specimens were then dehydrated serially using increasing concentrations of ethanol (10%, 25%, 50%, 75%, and 90%) for 20 min each, followed by immersion in 100% ethanol for 1 h. The plates were kept in an incubator at 37°C for 24 h to permit total drying of the specimens. After drying, the specimens were transferred to aluminum stubs and sputter coated with gold for 160 s at 40 mA (Vacuum Desk II; Denton Vacuum LLC, Moorestown, NJ, USA). The specimens were examined and imaged using a JSM-5600 scanning electron microscope (JEOL USA, Inc., Peabody, MA).

Statistical analysis

The CFU/mL (log_{10}) counts were analyzed by ANOVA and Tukey's test using the GraphPad Prism 6 program (GraphPad Software, Inc., San Diego, CA, USA), with a significance level of 5%.

RESULTS

The MC group showed a lower amount of biofilm formation in relation to the SLB and CB groups, which were very similar to each other (Figure 1).

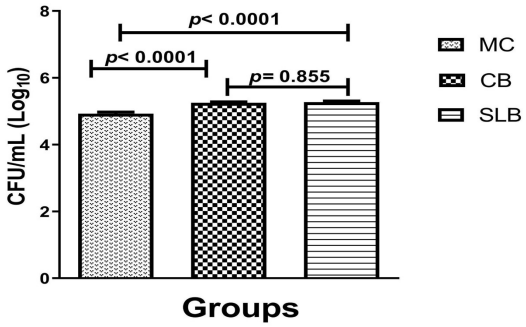

Figure 1 - Quantitative analysis of biofilm formation by CFU/mL count in different types of brackets: Means and standard deviations of *C. albicans* ATCC18804 CFU/mL (log) values in the following groups: conventional metallic/MC, self-ligating brackets/SLB, ceramic bracket/CB. ANOVA and Tukey's test, $p \leq 0.05$.

The amount of biofilm formed on different sets of brackets and wires is shown in Table II. In general, greater biofilm formation by *C. albicans* was observed when .018"x.025" rectangular wires were used together with brackets. For the MC and CB brackets, a greater accumulation of biofilm was observed when NiCr wires were used. It was impossible to identify a greater accumulation of biofilm, for the SLB brackets, when NiCr or NiTi alloys were used.

In the interaction between the types of ligatures, brackets, and wires, different amounts of CFU/mL were observed in the colonization of *C. albicans* biofilms. The combinations that showed highest biofilm accumulation were MC-NiCr-SSL and CB-NiCr with both EC and SSL. The sets that exhibited low biofilm formation were MC-NiCr-EC and CB-NiTi-SSL. There was a significant difference in biofilm formation between sets when the types of brackets, MC and CB were considered (Figure 2).

SEM analysis performed based on these results, showed that the bracket surfaces had recesses, sharp angles, grooves, and clips, as in SLB brackets, that facilitated biofilm retention (Figure 3). In Figure 3C, it is possible to observe the presence of yeasts and hyphae of *C. albicans*, mainly in the niches formed by the bracket clip.

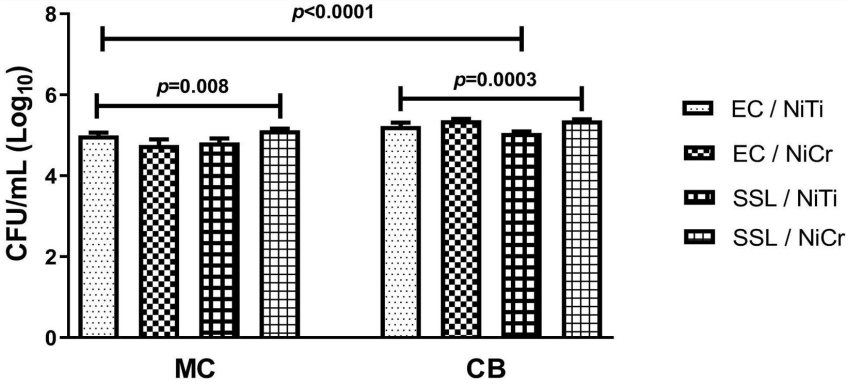

Figure 2 - Quantitative analysis of biofilm formation by CFU/mL count in different types of brackets, wires and ligatures: Means and standard deviations of *C. albicans* ATCC18804 CFU/mL (log) values in the following groups: conventional metallic/MC and ceramic bracket/CB with types of wires and ligatures. ANOVA and Tukey's test, $p \leq 0.05$.

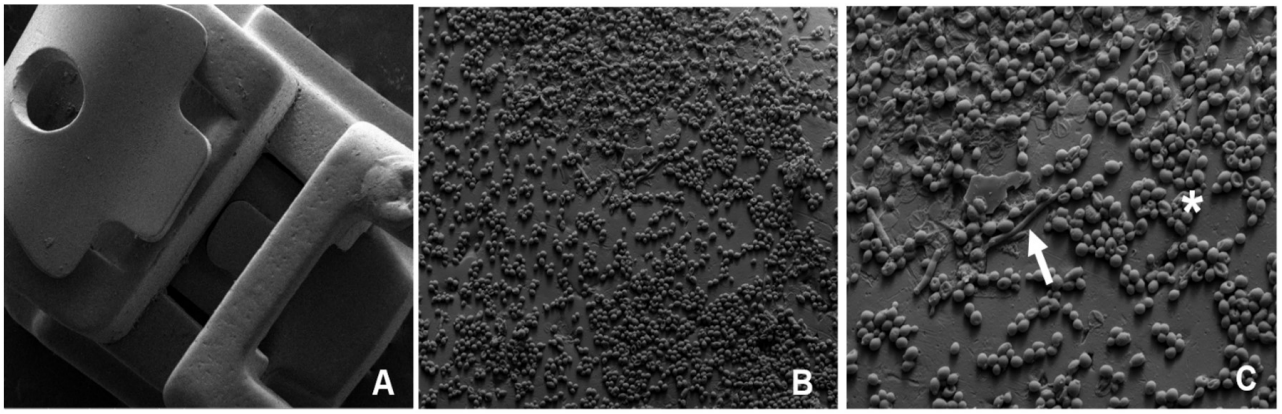

Figure 3 - Scanning electron microscopy images of biofilms formed by *C. albicans* on SLB brackets surfaces, as follows: (A) 80X; (B) 1500X; (C), 3000X magnifications. It is possible to observe the presence of yeasts (*), and hyphae (→) of *C. albicans*.

Table II – Means and standard deviations of CFU/mL (log_{10}) in the tests performed in different types of brackets and archwires

		CFU/mL (log_{10})			
		.014"	.018"	.018"x .025"	*p* value
NiCr	MC	5.076± 0.336	4.871 ± 0.201	5.278 ± 0.091	*p*< 0.0001
	CB	5.309 ± 0.148	5.487 ± 0.108	5.298 ± 0.129	
	SLB	5.166 ± 0.059	5.416 ± 0.078	5.534 ± 0.145	
NiTi	MC	5.300 ± 0.284	4.849 ± 0.308	4.680 ± 0.155	*p*< 0.0001
	CB	4.886 ± 0.212	5.237 ± 0.436	5.240 ± 0.043	
	SLB	5.310 ± 0.069	5.264 ± 0.126	5.332 ± 0.054	

DISCUSSION

Information about the formation of *C. albicans* biofilms in components of fixed orthodontic appliances, which hinders oral hygiene and increases plaque retention, is essential because this microorganism is present in oral microbiota and can participate in pathological processes such as caries [22,23]. This study simulated fixed orthodontic appliances to contribute to the understanding of the adhesion of *C. albicans* biofilms during orthodontic treatment, which contributes to the formation of more retentive areas.

The association between *Candida* species and the establishment of dental caries has been reported in several works [22-24]. Caries begin with the formation of biofilms by cariogenic microorganisms such as *S. mutans* and *C. albicans* [25]. The interaction between these microorganisms can be mutualistic, where both are favored, with a greater ability to adhere to surfaces when in association [16]. *In vitro* studies suggest that *C. albicans* has a high acidogenic potential and capacity to cause mineral loss and is therefore an adjunct in the process of establishing dental caries [18]. Furthermore, although the role of *C. albicans* in periodontal disease is not well established, it is considered an important pathogen in both the progression and persistence of this disease [26].

Brackets cause the maintenance of good oral hygiene difficult and create microbial shelters, resulting in biofilm build-up. The use of SLB became widespread, as it did not include elastic or metallic ligatures and was believed to be more hygienic [27,28]; however, this hypothesis was not proven in our study.

Pellegrini et al. (2009) [27] evaluated biofilm retention during treatment with conventional brackets in interaction with rubber band ligations and self-ligated brackets (In-Ovation R/Mini-Ovation, GAC) and concluded that patients with self-ligated brackets had lower biofilm rates than those who received conventional brackets. However, in this study, the levels of hygiene of the sample were not considered, and the results from Pellegrini et al. (2009) [27] study could be influenced by the location of collection, which was performed around the bracket and at the tooth and bracket interface as opposed to our methodology, which evaluated the formation of biofilms on the entire surface of the bracket by sonication. However, corroborating our results, the authors state that SLB retain more biofilm either because they have more retentive areas [29,30], larger dimensions and a more complex design [31], or because of the clip [6,32].

According to Van Gastel et al. (2007) [29], when analyzing the distribution of the biofilm through the brackets using SEM, irregularities in the interface between different parts of the self-ligating brackets were observed. This was confirmed in our results through the same analysis. Regardless of the type of bracket, the authors agree that the slot region closed by the clip retains the most biofilm, probably because it behaves like a closed tube [6,32].

In this study wires with a round cross-section (.014" or .018") did not differ significantly in terms of biofilm accumulation. The wire with a rectangular cross-section (.018"x.025") promoted greater accumulation of biofilm, probably because of the formation of more retentive niches due to the presence of sharper angles in its conformation. Particularly, in phases of orthodontic treatment, when it is necessary to use larger wires, according to our results, individuals might be more susceptible to biofilm formation and creation of retentive areas, which suggests that there is a correlation between biofilm removal and the use of auxiliary and professional methods to preserve the patient's oral health. Thus, the orthodontist needs to pay greater attention to these parameters.

There was no significant difference in the accumulation of biofilm between EC and SSL, corroborating the literature on the presence of *P. gingivalis* [1]. In some combinations, one type accumulated more biofilm; in other combinations, it accumulated less biofilm. More biofilm was always observed in combinations with SLB. When evaluating the retention of *S. mutans* biofilms around MC brackets with SSL, EC, and SLB brackets, combinations with EC showed greater biofilm accumulation [30]. Through real-time PCR, the authors observed that the accumulation of *S. mutans*, *S. sobrinus*, *L. casei*, and *L. acidophilus* in MC with SSL and SLB was similar [33]. Although no ligatures are used in self-ligated brackets, calcification of bacterial plaque in the cervical region may occur [32], making it difficult to open and close the clip, which can lead to its malfunction. It is important to adopt effective mechanisms of daily

oral hygiene by the patient and periodically by the professional to negate these issues [34].

In this study a significant increase in number of colonies of *S. mutans* and *C. albicans* in individuals with fixed orthodontic appliances and the effectiveness of removing this biofilm through proper brushing was observed. Thus, even if an individual is undergoing orthodontic treatment and accessories conducive to greater accumulation of microorganisms are used, such as self-ligating brackets and rectangular NiCr wires, professional prophylaxis and proper instruction in oral hygiene are essential for a significant reduction in bacterial plaque, and plaque control in orthodontic patients is extremely important for maintaining oral health and preventing periodontal disease [35,36]. As a relevant adjunct to more effective oral hygiene, professionals should guide their patients regarding the daily use of mouthwashes [37,38].

CONCLUSION

Thus, brackets of MC with NiTi and NiCr wires showed lower biofilm formation, regardless of the ligature and cross-sectional or gauge of the wire and, MC and CB brackets with NiCr wires and SSL ligatures were more likely to accumulate biofilms.

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Author's Contributions

EEF, DFLMO, RPJ, PAD: Conceptualization. EEF, DFLMO, RPJ, PAD: Hypothesis. EEF, DFLMO, RPJ, PAD: Experiments. EEF, DFLMO, RPJ, PAD, PPB: Writing. PPB: Data tabulation. PPB: Statistical. AOCJ, WO, SMR: Supervision.

Conflict of Interest

No conflicts of interest declared concerning the publication of this article.

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Regulatory Statement

The study was waived of ethical approval because it did not include patients or animals, only standard microbiological samples acquired for laboratory studies were used (Candida ATCC).

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