Adhesion and Initial Colonization of *Streptococcus mutans* is Influenced by Time and Composition of Different Composites

La Adhesión y la Colonización Inicial de *Streptococcus mutans* están Asociadas al Tiempo y la Composición de Diferentes Compuestos

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ABSTRACT: The aim of this study was to evaluate the adhesion and initial colonization of *S. mutans* on the surface of novel composite resins during three experimental periods. Biofilms were formed on specimens of Enamel Plus HRI (Hri), FiltekTM Silorane (Si), Tetric EvoCeram® Bulk Fill (Te), KaloreTM (K), and FiltekTM Z250 (Z) (n=4/experimental period). After 4, 8 and 24 hours, the specimens were assessed for bacterial colony forming unit (CFU/mm^2) levels and scanned by electron microscopy. All material surfaces showed a similar susceptibility to bacterial adhesion at 4 hours (p>0.05). The amount of microorganisms in the formed biofilms increased at 8 hours for all groups (p<0.05) and decreased at 24 hours only for Te and Z groups (p<0.05). After 24 h, the K group showed higher microorganism counts compared to the other groups. All composite resins evaluated were susceptible to adhesion by streptococci. However, bacteria attachment was not sufficient to maintain *S. mutans* colonization on Tetric EvoCeram® Bulk Fill and FiltekTM Z250 composites. Composite K presented the highest mean values for bacterial adhesion. Also, it was observed that the composition of resins could interfere with colonization mechanisms.

KEY WORDS: composite resins; biofilm, *streptococcus mutans*, electron microscopy.

INTRODUCTION

Composite resins are esthetic materials widely used in restorative dentistry. Over the past decades, there has been continuous development in attempts to improve the properties of composite resins. Most of these resins are methacrylate-based composites (Weinmann *et al*., 2005). However, methacrylate-based composites' increased polymerization shrinkage correspond to a critical disadvantage as this could lead to marginal gaps between the material and the cavity walls (Goldberg, 2008), resulting in biofilm accumulation and occurrence of caries adjacent to the restoration (Buergers *et al*., 2009).

In order to overcome this limitation of methacrylate-based composites, a composite resin that exhibits "silorane" (association of siloxanes and oxiranes) in its composition was developed (Gao *et al*., 2012; Gregor *et al*., 2013). Another monomer developed in an attempt to reduce polymerization shrinkage is the high molecular weight urethane dimethacrylate-based monomer named DX-511. This monomer can be found in the KaloreTM composite (Boaro *et al*., 2013). However, there are no studies in the literature evaluating the bacterial adhesion of this type of composite resin to date. Thus, considering that microorganisms have a higher affinity for resin composites in comparison to the enamel and other restorative materials, such as ceramic and metals

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(Delaviz *et al*., 2014), studies involving microorganisms' attachment to resins surfaces are relevant.

Caries development at the restoration margins is a considerable reason for composite restoration failure (Bernardo *et al*., 2007). Recurrent decay is linked to failure of the bond between the tooth and composite and to increased levels of the cariogenic bacterium, *Streptococcus mutans*, localized around the perimeter of these materials (Filoche *et al*., 2010; Spencer *et al*., 2014). Thus, considering that dental caries is preceded by the attachment of indigenous oralbacteria, evaluation of bacterial adhesion or colonization on resin-based composites could be highly desirable to guide material selection.

In this sense, *Streptococcus mutans* plays an important role in the pathogenesis of the caries disease (Bowen, 1999). Its role as a pioneer organism in biofilm formation supports the subsequent colonization of the surface by other bacterial species (Spencer *et al*.), providing a cascade of events leading to the demineralization process. In order to prevent bacterial adhesion and, consequently, control dental caries, some authors have studied chemical modifications of resin composition (Ferracane, 2011). Despite improved physical and mechanical properties, there is no consensus in the literature regarding the influence of the surface properties on bacterial adhesion (Buergers *et al*.; Claro-Pereira *et al*., 2011).

Thus, in order to predict biological adhesion of resin-based materials, the aim of this study was to evaluate susceptibility of dental composites to *S. mutans* adhesion and initial biofilm formation. The null hypothesis was that there would be no differences between the dental restorative materials studied regarding the adherence susceptibility of cariogenic *S. mutans*.

MATERIAL AND METHOD

Experimental design. Biofilms were formed on specimens of composite resin and were collected at 4, 8, and 24 hours (n = 4/experimental period). After the formation of biofilm during the experimental periods, the specimens were assessed for bacterial colony forming unit (CFU/mm^2) levels (n = 3/experimental period) and analyzed by scanning electron microscopy (n = 1/experimental period).

Preparation of specimens. Five different micro- or nanohybrid composites were selected for the study (n=4). Table I shows the materials evaluated and their manufacturers. Cylindrical specimens (3.0 mm in diameter and 2.0 mm th ickness) of the test materials were prepared according to the manufacturers' specifications, using a prefabricated circular Teflon mold. After the insertion of composites at the upper face, a cellulose acetate strip was used to provide composite regularization. The disk-shape specimens were light-cured (SDI Radii plus, SDI, Bayswater, Australia; 1500 mW/cm^2) for 40 s and finally removed from the plate. Disk samples were measured (surface area of 32.85 $\pm$ 0.45 cm^2) and sterilized by gamma radiation for 30 minutes (RAD Source RS-2000, 160kV, 25mA, 130Gy).

Initial biofilms and microbiological analysis. Initial biofilms of *S. mutans* UA159 were formed on disk surfaces in batch cultures for 4, 8, or 24 hours (n=3). The biofilms were grown in ultrafiltered (10 kDa molecular-weight cut-off) buffered tryptone yeast-extract broth containing 1 % (w/v) sucrose. At selected time points (4-, 8- and 24-hour-old initial biofilm), the disks were dip-rinsed in sterile saline solution (to remove excess culture medium or bacteria that was not attached) and transferred to tubes containing 0.9 % NaCl. The biofilms were removed by sonication using 15 s pulses at an output of 20 % amplitude (Fischer Scientific, CL-334) in order to homogenize biofilms for microbiological analysis.

An aliquot of 100 µL of the sonicated biofilm suspension was diluted in 0.9 % NaCl and serial dilutions were inoculated in duplicate by the drop-counting technique in BHI (Brain Heart Infusion) agar plates. The plates were incubated in 10 % CO$_2$ at 37 oC for 24 hours. The colony-forming units (CFU) were counted and the results expressed in CFU/mm^2.

Scanning Electron Microscopy (SEM). The structure of the biofilms was qualitatively examined by SEM (n=1). Briefly, samples were fixed in modified Karnovsky's solution (2.5 % glutaraldehyde, 4 % paraformaldehyde, in 0.1 M sodium cacodylate, pH=7.2-7.4) and post-fixed in 2 % osmium tetroxide solution for 2 hours, washed, and subjected to dehydration in increasing concentrations of alcohol (30 %, 50 %, 70 %, 80 %, 90 %, 95 %, and 100 %). Next, samples were dried in a Critical Point Dryer (BAL-TEC) with liquid CO$_2$, mounted on a copper support, and metallized with gold-pale in DENTON VACCUM DESK II20. The electron micrographs were obtained using a scanning electron microscope (JEOL JSM-5410, USA), 3.5 nm, at 30 kV.

Table I. Trade mark, manufacturer, and composition of the composite resins evaluated in the study.

Composite Resins	Manufacturer	Composition
Enamel Plus HRI (Hri)	Micerium SpA	- Matrix: Methacrylate-based chemistry (Bis-GMA, UDMA, Butanediol dimethacrylate) - Nano-hybrid composite - Content of filler (80% weight): Glass filler with high refractive index and surface treated nano zirconium oxide particles.
Filtek™ Silorane (Si)	3M ESPE	- Matrix: Silorane resin-based chemistry - Micro-hybrid composite - Content of filler (76% weight): Quartz particles and radiopaque Yttrium Fluoride
Tetric EvoCeram® Bulk Fill (Te)	Ivoclar Vivadent	- Matrix: Methacrylate-based chemistry (Bis-GMA, Bis-EMA and UDMA) - Nano-hybrid composite - Content of filler (80% by weight): barium aluminum silicate glass, ytterbium fluoride and spherical mixed oxide
Kalore™ (K)	GC Dental Products	- Matrix: UDMA, dimethacrylate co-monomers and DX-511 monomer - Nano-hybrid composite - Content of filler (82% weight): Fluoroaluminosilicate glass, High Density Radiopaque (HDR) prepolymerized fillers and silicon dioxide
Filtek™ Z250 (Z)	3M ESPE	- Matrix: Methacrylate-based chemistry (Bis-EMA, UDMA, Bis-GMA) - Micro-hybrid composite - Content of filler (82% by weight): Zirconia/Silica

Statistical analysis. The analysis were performed with SAS software (SAS Institute Inc. The SAS System, release 9.3, 2012, SAS Institute Inc., Cary, NC, USA) with a significance level of 0.05. Data were analyzed by analysis of variance (ANOVA) followed by Holm-Tukey test.

RESULTS

There were performed two statistical comparisons. First, when analyzed the influence of time on the composites behavior, all composites showed means lower than $1x10^4$ CFU/mm² at 4 h. From 4 to 8 hours, all composites showed significantly increased values of CFU/mm² ($p<0.05$). Finally, at 24 hours, two behaviors were observed: the composites Hri, K, and Si did not exhibit significant variation in means of CFUs ($p>0.05$), while Te and Z exhibited a significant reduction in means of CFUs ($p<0.05$). Figure 1 illustrates the time-dependent influence on biofilm formation on the composites surfaces represented by uppercase letters in Table II.

Once completed the analysis of the influence of time on the composites behavior, there were performed the comparisons between the composites, independently, in each time, as highlighted in lowercase letters in Table II. At 4 hours, there was no statistical difference between the composites. At 8 and 24 h, the K composite presented the highest means of CFUs ($p<0.05$) (Fig. 2).

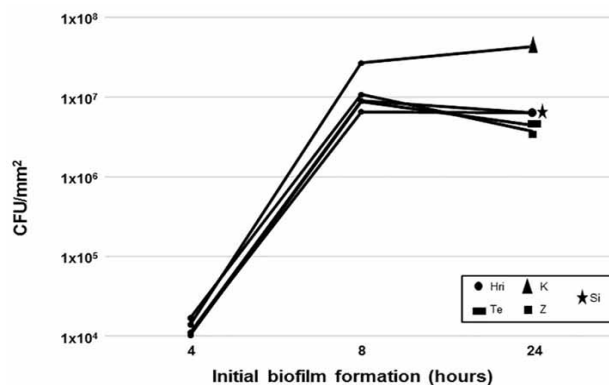Figure 3 shows representative SEM images after qualitative analysis from specimens at the

Fig. 1. Means of colony forming units (CFU/mm2) according to composites and biofilm formation (hours).
Hri: Enamel Plus HRI; K: KaloreTM; Si: FiltekTM Silorane; Te: Tetric EvoCeram® Bulk Fill; Z: FiltekTM Z250.
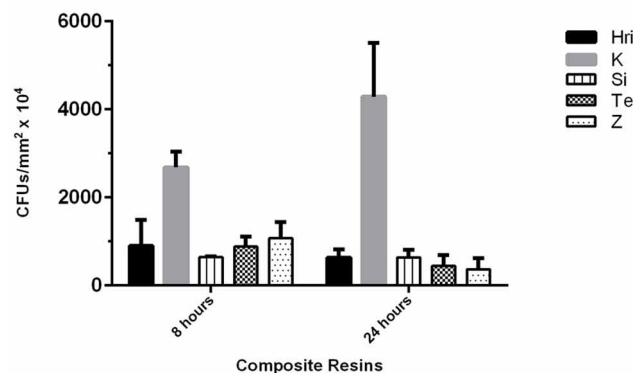

Fig. 2. Mean (standard deviation) upper limit of the confidence interval (95 %) of CFU/mm2 of composite resins at 8 and 24 hours. Hri: Enamel Plus HRI; K: KaloreTM; Si: FiltekTM Silorane; Te: Tetric EvoCeram® Bulk Fill; Z: FiltekTM Z250.

Table II. Microbiological analysis of initial biofilms formed on different composites resins for 4, 8, or 24 hours (Mean ± SD; n = 3)

Composite	Initial biofilm formation (CFU/mm^2 x 10^4)		
	4 h	8 h	24 h
Hri	1.09 ± 0.22 Ba	897.06 ± 593.37 Ab	632.51 ± 183.94 Ab
K	1.40 ± 0.29 Ba	2680.40 ± 359.44 Aa	4286.31 ± 1228.30 Aa
Si	1.10 ± 0.11 Ba	649.81 ± 13.49 Ab	639.14 ± 172.67 Ab
Te	1.03 ± 0.16 Ca	883.74 ± 223.01 Ab	445.89 ± 244.0 Bb
Z	1.69 ± 0.50 Ca	1072.87 ± 366.49 Aab	369.20 ± 255.01 Bb

Note: Within columns, lowercase letters represent comparisons among composites within each time of evaluation (equal letters indicate no significant difference [p>0.05]). Within lines, uppercase letters represent comparisons among times of evaluation for each composite (different letters indicate significant difference [p<0.05]). Hri: Enamel Plus HRI; K: KaloreTM; Si: FiltekTM Silorane; Te: Tetric EvoCeram® Bulk Fill; Z: FiltekTM Z250.
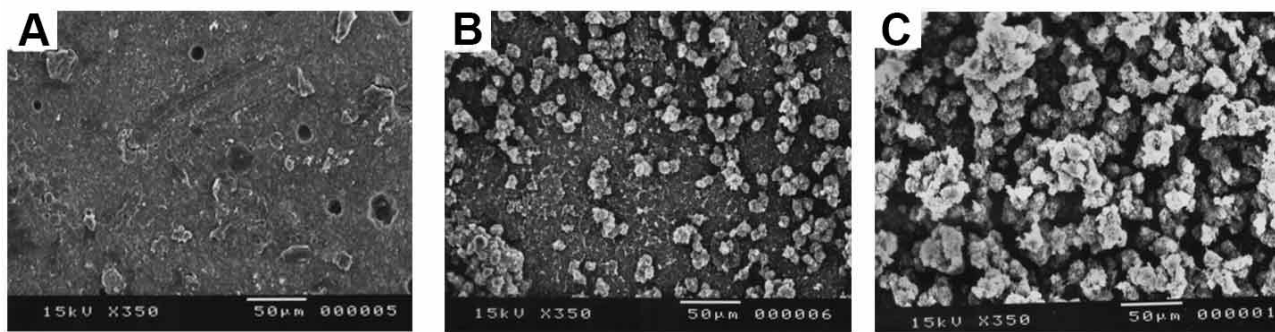

Fig. 3. Representative scanning electron micrographs showing progressive increase in the bacterial adhesion to the composite resins surfaces at 4 hours of incubation with *S. mutans* (A), 8 hours (B) and 24 hours (C).

different times (4, 8, and 24 hours), showing a progressive increase in the amount of *S. mutans* on the surface of the composite resins according to time. No clear differences in the pattern of adhesion between the different composites were observed.

DISCUSSION

The reproduction of all in vivo conditions of the oral cavity for the analysis of biofilm formation is a very challenging process. For example, the use of intraoral splints for evaluation of different experimental materials is complex, time-consuming, and it's difficult to find a standardized methodology (Hahnel *et al.*, 2014). Our model evaluated the adhesion of *S. mutans* at acid pH, since that we used sucrose as a substrate in culture medium (Gregor *et al.*). However, pellicle acquired and other conditions that mediate in vivo bacterial adhesion were not contemplated. Nevertheless, the test conditions used allowed us to evaluate the adhesive properties of the restorative materials in a medium appropriated for bacterial growth. This condition allowed us to observe similar

bacterial adhesiveness at first 4 hours of initial colonization for all groups, suggesting that initial colonization is not dependent of specimens surface (Barsotti *et al.*, 1989).

The method for sterilization of the samples used in this study was by gamma irradiation. This is the method of choice, once it sterilizes without high temperature and pressure, or neither chemical products or gases. This method also does not affect the antibacterial properties of resin dental materials (Carvalho *et al.*, 2009).

According to the manufacturers, all the composite resins evaluated in this study present improved polishing ability due to their composition of micro or nano particles. Specimens evaluated in this study were prepared with a Teflon matrix and a cellulose acetate strip to promote less bubble inclusion and regularization of the upper face. Pereira *et al.* (2011) evaluated the *S. mutans* adhesion on the surface of three composite resins following different finishing and polishing techniques. The authors showed that the group that received a matrix strip in contact with the resin surface and no additional finishing or polishing

promoted the lowest bacterial adhesion on the surface of the micro-hybrid and nanofilled composites in the absence of human saliva.

In our study, it was observed that *S. mutans*' biofilm adhesion to composite resin surfaces is influenced by the dental material's composition and the period of time evaluated. In agreement with Buergers *et al*., the results of the present study showed that the evaluation of the scanning electron micrographs confirmed that *S. mutans* could adhere to the surface of different composite resins. According to our qualitative analysis, the evaluation of the scanning electron micrographs revealed no detectable differences in the pattern of adhesion between the groups evaluated during the different experimental periods.

Our results suggest that for Hri, K, and Si composites the bacterial adhesion increased with time. Some factors could affect bacterial adhesion, such as microstructure of the material including monomer composition, size, shape, and distribution of inorganic filler particles and physico-chemical characteristics like the roughness and hydrophobicity of the surface of the biomaterials (An & Friedman, 1998; Teughels *et al*., 2006).

A high hydrophobicity may lead to lower absorption of hydrophilic molecules and improve the material's resistance to water and water-soluble species (Weinmann *et al*.). Schweikl *et al*. (2013) reported that the highest amounts of *S. gordonii* were detected on the hydrophilic surface of a glass ionomer cement, whereas a relatively small number of cells were found on the hydrophobic surfaces of a silorane-based composite (Filtek Silorane). Similarly, Buergers *et al*. found a lower quantity of adhering streptococci on the Filtek Silorane and methacrylate-based composites, such as Filtek Z250 and Tetric EvoCeram, which could be a result from their increased hydrophobicity. Since that colonization involves fundamental electrostatic, van der Waals, and hydrophobic interactions this is a complex process that remains poorly understood (An & Friedman).

Our findings were in agreement with Claro-Pereira *et al*. who found no statistically significant differences between FiltekTM Silorane and a methacrylate-based composite regarding bacterial adhesion. Therefore, these findings indicate that parameters other than hydrophobicity, like functionality of the surface, including charged groups, may be more relevant for the adhesion of microorganisms.

As already mentioned, the monomer could play an important role in relation to the bacterial adhesion. In our study, in contrast to Hri, K and Si results, duration of adhesion was not the same for Te and Z groups. Matrices with Bis-GMA and TEGDMA tend to suffer more water sorption, however the amount of inorganic component present in the composite is inversely proportional to this phenomenon (Toledano *et al*., 2003). In the present study, the lower mean of CFU/mm^2 presented by the composite resins Te and Z after 24 hours of incubation can be due the fact that both composites present the same matrix composition (BIS-GMA, BIS-EMA and UDMA).

The K composite presents monomers with a molecular weight almost double that of the Bis-GMA, which affords its low shrinkage (Yamasaki *et al*., 2013). However, for 8 and 24 hours of colonization, Kalore specimens significantly increased bacterial counting suggesting that polymerization shrinkage was not a factor to prevent bacterial adhesion. Furthermore, that composite presents a different monomer (DX-511 or Dupont monomer) which has an unknown influence on bacterial adhesion to biomaterials surfaces. Another specificity of the Kalore composite is that it has pre-polymerized particles, which increases the filler content (69 % vol). Unfortunately, there are no studies in the literature evaluating the microbial adhesion to Kalore composite, that could allow for comparison with the results showed in the present study.

Further studies are necessary to determine not only the amount of bacterial adhesion to composite resins but also the surface properties like hydrophobicity, surface free energy, and electrical charge.

CONCLUSIONS

It was possible to conclude that:

- *S. mutans* biofilm adhesion to composite resin surfaces was influenced by the composition of resin composites in a time-dependent manner;
- All composite resins evaluated were susceptible to adhesion by *streptococci*. However, bacteria attachment was not sufficient to maintain *S. mutans* colonization on Tetric EvoCeram® Bulk Fill and FiltekTM Z250 composites; and
- Kalore composite presented the higher mean values for bacterial adhesion at 8 and 24 hours.

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RESUMEN: El objetivo de este estudio fue evaluar la adhesión y la colonización inicial de *S. mutans* en la superficie de nuevas resinas compuestas durante tres períodos experimentales. Las biopelículas se formaron en muestras de Enamel Plus HRI (Hri), FiltekTM Silorane (Si), Tetric EvoCeram® Bulk Fill (Te), KaloreTM (K) y FiltekTM Z250 (Z) (n = 4 / período experimental). Después de 4, 8 y 24 horas, las muestras se evaluaron para determinar los niveles de la unidad formadora de colonias bacterianas (UFC / mm^2) y se escanearon por microscopía electrónica. Todas las superficies del material mostraron una susceptibilidad similar a la adhesión bacteriana a las 4 horas (p> 0,05). La cantidad de microorganismos en las biopelículas formadas aumentó a las 8 horas para todos los grupos (p <0,05) y disminuyó a las 24 horas solo para los grupos Te y Z (p <0,05). Después de 24 horas, el grupo K mostró mayores recuentos de microorganismos en comparación con los otros grupos. Todas las resinas compuestas evaluadas fueron susceptibles a la adhesión por estreptococos. Sin embargo, la unión de bacterias no fue suficiente para mantener la colonización de *S. mutans* en los compuestos Tetric EvoCeram® Bulk Fill y FiltekTM Z250. El compuesto K presentó los valores medios más altos para la adhesión bacteriana. Además, se observó que la composición de las resinas podría interferir con los mecanismos de colonización.

PALABRAS CLAVE: resinas compuestas, biofilm, *Streptococcus mutans*, microscopía electrónica.

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