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ANÁLISE DOS EFEITOS DA INTERAÇÃO ENTRE S mutans E MATERIAIS RESTAURADORES ESTÉTICOS:

CARACTERÍSTICAS DO BIOFILME EM MICROSCÓPIO CONFOCAL DE VARREDURA A LASER E PROPRIEDADES DE SUPERFÍCIE DOS MATERIAIS APÓS 30 DIAS

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Orientador: Profª. Drª. Regina Maria Puppin Rontani
Co-orientadora: Profª. Drª. Renata de Oliveira Mattos Graner

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RESUMO

O desenvolvimento do biofilme de *Streptococcus mutans* sobre materiais restauradores e a biodegradação destes substratos em função dos metabólitos bacterianos podem ser influenciados pelas propriedades e características do material. A partir de uma revisão sistemática em que se verificou a carência de estudos a respeito dos efeitos do biofilme na superfície de materiais restauradores, foi proposto investigar algumas características quantitativas e qualitativas do biofilme após 30 dias de interação com materiais restauradores, além de analisar propriedades e microestrutura da superfície dos materiais que sofreram tal interação. Para cada material testado (cerâmica - C, resina composta nanoparticulada – RC e cimentos de ionômero de vidro modificado por resina - CIVMR e convencional - CIVC), foram confeccionados 25 discos sob condições assépticas, para distribuição em 3 grupos de estocagem: 1) 100% de umidade relativa a 37ºC (n=5); 2) meio de cultura a 37ºC (BHI + 1% sacarose) (n=5); 3) biofilme de *Streptococcus mutans* e meio de cultura a 37ºC (n=15). Valores de dureza do grupo 1 (valores imediatos) foram obtidos previamente à estocagem, a fim de se verificar alterações ao longo do tempo quando estocados em umidade relativa apenas. Após 30 dias de estocagem, os discos do grupo 3 foram levados para observação do biofilme corado e hidratado em microscopia de varredura confocal a laser (CLSM). As imagens obtidas auxiliaram na obtenção de valores de espessura de biofilme, bio-volume, coeficiente de rugosidade e superfície/volume, além de análises qualitativas quanto à distribuição de células viáveis/não viáveis e arquitetura do biofilme. Em seguida, todos os discos foram lavados em ultrassom e avaliados quanto à rugosidade, dureza e microestrutura de superfície. Os biofilmes apresentaram predominância de células mortas próximo à superfície dos discos e presença de canais e aglomerados celulares para todos os tipos de materiais. Dentre as propriedades do biofilme quantificadas, a espessura foi a única que mostrou diferença estatisticamente significante entre os materiais, sendo que C e RC desenvolveram biofilmes mais espessos que

CIVMR e CIVC. Não houve diferença estatisticamente significante quanto à rugosidade e dureza entre os grupos de medição imediata, 1, 2 e 3 para os discos de C e RC. Entretanto, o grupo 3 de RC apresentou microscopicamente biodegradação na superfície. CIVMR e CIVC tiveram valores de dureza aumentados quando comparados os valores imediatos ao grupo 1. Entretanto, o grupo 3 do CIVMR apresentou valores estatisticamente reduzidos de dureza e acrescidos de rugosidade de superfície. Já o CIVC apresentou maiores valores de rugosidade para o grupo 3 e nenhuma diferença estatística entre os três grupos de estocagem quanto à dureza. As micrografias do CIVMR e do CIVC também evidenciaram a biodegradação ocorrida na superfície destes materiais. Dentro das condições deste estudo, conclui-se que houve influência dos materiais sobre o desenvolvimento do biofilme e influência do biofilme sobre as propriedades e características microestruturais de superfície dos materiais testados, sendo material-dependente.

Palavras-chave: Biofilme dental, Materiais restauradores, Microscopia de varredura confocal a laser, Espessura, Propriedades de superfície.

ABSTRACT

Streptococcus mutans biofilm development on restorative materials and biodegradation of those materials due to bacterial acids are influenced by material properties and characteristics. Since a systematic review found a deficiency concerning studies related to effects of biofilm on the surface of restorative materials, the proposition for this investigation was to analyze some quantitative and qualitative biofilm characteristics after 30-days interaction with restorative materials. In addition, it was investigated changes on the surface properties and microstructure of materials after 30-days interaction. Twenty-five disks of each material tested (ceramic - C, nanofill composite - NC, resin-modified glass ionomer - RMGIC and conventional glass ionomer cement - CGIC) were made, at aseptic conditions, and distributed in 3 storage groups: 1) 100% relative humidity at 37ºC (n=5); 2) growth medium at 37ºC (BHI + 1% sucrose) (n=5); 3) *Streptococcus mutans* biofilm and growth medium at 37ºC (n=15). Vickers hardness values from group 1 were obtained previously storage, in order to observe aging by relative humidity. After 30 days storage, disks were stained, kept hydrated and observed by confocal laser scanning microscopy, whose images supported to acquire values concerning biofilm thickness, bio-volume, roughness coefficient and surface to volume ratio. Qualitative analyses related to viable / non-viable cells distribution and biofilm architecture also were realized. Subsequently, all disks were ultrasonically washed and analyzed to surface roughness, hardness and microstructure. Biofilms presented a progression more viable cells in superficial regions of the biofilm to proportionally more nonviable cells in the deeper regions of the biofilms, near the disk. Besides, cellular aggregates and fluid-filled channels were observed in biofilm developed on all materials. Concerning biofilm quantitative properties, thickness was the unique with difference statistically significant among materials. C and NC accumulated thicker biofilms than RMGIC and CGIC. There was no difference statistically significant among immediate and storage groups related to C and NC surface roughness and hardness. However,

group 3 of NC showed surface biodegradation microscopically. Group 1 of RMGIC and CGIC presented higher hardness values than immediate values. Nevertheless, hardness values from RMGIC group 3 decreased compared groups 1 and 2, while surface roughness values of group 3 increased statistically. Group 3 of CGIC showed higher roughness values than other groups and no difference statistically significant among three storage groups concerning hardness values. RMGIC and CGIC micrographs also demonstrated biodegradation on the surface materials. Within this study conditions, it was concluded that there was influence of restorative materials on biofilm development and influence of biofilm on the surface properties and microstructure characteristics of materials tested, being material - dependent.

Key Words: Dental biofilm, Restorative materials, Confocal laser scanning microscopy (CLSM), Thickness, Surface properties.

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INTRODUÇÃO

Biofilmes podem ser definidos como comunidades cooperativas e estruturadas de microrganismos, aderidas a superfícies ou interfaces e umas às outras, geralmente embebidas em uma matriz extracelular autoproduzida (Wood *et al*., 2000; Biofilmes, 2004). Esta estrutura forma-se particularmente de maneira muito rápida em quaisquer sistemas fluidos naturais em que uma fonte regular de nutrientes é fornecida aos microrganismos através de canais, os quais penetram na matriz para também conduzir oxigênio, metabólitos, produtos de excreção e enzimas. Desta forma, os biofilmes possuem profundas implicações para a natureza, medicina e indústria.

O biofilme dentário, estrutura anteriormente conhecida por placa bacteriana, encontra-se permanentemente na cavidade bucal, adjacente aos tecidos dentários e aos variados materiais utilizados nos tratamentos reabilitadores. Entretanto, na ausência de adequada higiene bucal, o biofilme pode acumular-se em áreas de retenção em níveis não compatíveis com a saúde bucal e promover cárie dentária e doenças periodontais (Marsh and Bradshaw, 1995). Pesquisas têm mostrado a habilidade de *Streptococcus mutans* em colonizar materiais restauradores e protéticos (Benderli *et al*., 1997; Kawai & Urano, 2001; Steinberg & Eyal, 2002; Eick *et al*., 2004), podendo biodegradar a superfície de tais materiais (Willershausen *et al*., 1999) e facilitar a ocorrência de cárie secundária, principal razão para a substituição de restaurações (Mjör & Moorhead, 2000). Portanto, seria interessante o uso de materiais odontológicos com propriedades antibacterianas, como a liberação de íons (Svanberg *et al*., 1990; Boeckh *et al*. 2002; Hayacibara *et al*., 2003), a fim de que os efeitos deletérios exercidos pelo biofilme às restaurações e próteses fossem minimizados. Entretanto, certas propriedades de superfície dos materiais, como a rugosidade de superfície, poderiam também influenciar em maior ou menor grau o acúmulo de biofilme sobre o respectivo substrato (Quirynen & Bollen, 1995). Portanto, materiais

restauradores poderiam acumular biofilmes com características diferentes, como espessura, arquitetura interna, viabilidade celular, entre outras.

Com o objetivo de estudar biofilmes em seu estado natural e hidratado, a microscopia de varredura confocal a laser vem sendo utilizada recentemente na Odontologia. Esta técnica microscópica proporciona a análise de biofilmes sem o preparo dos espécimes, dispensando procedimentos como desidratação, embebição e fixação. Desta maneira, a arquitetura e organização interna do biofilme são mantidas, além de outras características, como espessura e viabilidade celular (Wood *et al.*, 2000). Outra vantagem do microscópio confocal para o estudo do biofilme é sua capacidade de captar imagens em diferentes profundidades de foco, facilitando a visualização de espécimes irregulares, densos e espessos de maneira tridimensional (Kubínova & Janácek, 2001). Poucos estudos analisaram o biofilme dentário sob estas condições, principalmente após interação com materiais restauradores.

Ao mesmo tempo em que ocorre a influência dos materiais odontológicos sobre as características do biofilme, tais substratos sofrem um processo chamado biodegradação. Os metabólitos ácidos produzidos pelas bactérias, presentes no biofilme, produzem uma queda no pH do ambiente bucal, favorecendo alterações prejudiciais à superfície de restaurações e próteses (Asmussen, 1984; Yap *et al.*, 2001; Turssi *et al.*, 2002; Yap *et al.*, 2005). Consequentemente, a durabilidade clínica destes materiais fica comprometida. Propriedades como rugosidade, dureza, resistência ao desgaste, e características como microestrutura podem ser alteradas em função da acidogenicidade do biofilme e da capacidade do material em resistir a tal condição (Willershausen *et al.*, 1999).

O objetivo deste estudo[1] foi avaliar alterações nas propriedades e na microestrutura da superfície de materiais restauradores após interação com biofilme de *Streptococcus mutans* por meio de revisão sistemática e estudo *in*

[1] Este trabalho foi realizado no formato alternativo, com base na deliberação da Comissão Central de Pós-Graduação (CCPG) da Universidade Estadual de Campinas – UNICAMP, nº 001/98.

vitro, além de investigar características quantitativas e qualitativas do biofilme acumulado por 30 dias sobre os mesmos materiais *in vitro*.

CAPÍTULO 1

Dental biofilm: effects on esthetic restorative material surfaces: a systematic review[2]

[2] Manuscrito enviado para publicação no periódico Biomaterials.

ABSTRACT

Purpose: to make a systematic review of the effects of dental biofilm on restorative material surfaces, analyzed directly or indirectly by means of exposure to acid lactic. **Methods:** The authors searched Bireme, Medline, ISI, Cochrane Library, Scopus, Lilacs and the Internet for articles from 1980-2006. The main descriptors were “dental biofilm”, “dental plaque”, or “lactic acid” and “restorative materials” (ceramic-C; composite resin-CR; polyacid-modified composite resin-PMCR; resin-modified glass ionomer cement-RMGIC; conventional glass ionomer cement-CGIC). The inclusion criteria were studies that either did or did not identify damage on the surface of restorative materials from their interaction with *Streptococcus mutans* biofilm or lactic acid. Reviews, articles about effects of antibacterial properties of materials on dental biofilm, other acid solutions, bond strength, non-restorative materials and medical articles were excluded. **Results:** Only five studies were classified as grade A; only one surface aspect (microstructural) was classified as strong evidence. **Conclusions:** From the studies selected, it was not possible to verify strong evidence to biofilm effects on material surface properties as roughness, microhardness, wear/abrasion resistance, color and gloss. Only microsctrutural changes on the surface of PMCR and CGIC obtained strong evidence.

Key Words: review literature, biofilm, lactic acid, dental restorative materials, biodegradation.

INTRODUCTION

The need for tooth-colored restorations has increased recently, while metal-colored restorations such as, amalgam and metal casting restorations, have become unpopular from an aesthetic point of view. However, dentistry professionals underestimate the importance of preventive procedures such as, hygiene and diet guidance. Consequently, the longevity of restorations could be compromised and replacements required in short-term, due to secondary caries and biodegradation of restorative [1].

Biodegradation is defined as the gradual breakdown of a material mediated by specific biological activity. In the mouth, this is a complex process, including disintegration and dissolution in saliva and other types of chemical/physical degradation such as wear and erosion caused by food, chewing and bacterial activity. No *in vitro* test is capable of reproducing this complex process [2]. Nevertheless, many studies choose lactic acid as being representative of dental biofilm, because this is the most important metabolic product from *Streptococcus mutans* in the biofilm exposed to sucrose [3]. It is probable that concentration, pH and effective contact of this acid solution *in vitro* would differ from oral conditions, thus over-estimating degradation values.

The development of dental caries and biodegradation requires adhesion and colonization of odontopathogens. The caries-associated *Streptococcus mutans* can colonize all solid surfaces in the mouth, tooth substrate and restorative materials. Material surface physicochemical properties like surface free energy, hydrophobicity and surface roughness have an influence on bacterial adhesion [4,5]. Different restorative material characteristics really have the ability to produce different biofilms, as many studies already have shown, altering bacterial colonization number, weight and viability of biofilm, among other factors [6-10]. However, few researches in literature have studied the adverse effects of the biofilm / material interaction on material surfaces, known as biodegradation. It is extremely important to search scientific data about the damage done by biofilm on

materials to confirm the clinical importance of selecting an adequate restorative material for high caries-risk patients and to guide them on how to perform efficient dental biofilm control. The purpose of this article was to make a systematic review of the effects of dental biofilm on the surfaces of restorative materials, analyzed directly or indirectly by means of exposure to lactic acid.

METHODS

Question addressed by this review

What are the effects of the interaction between *Streptococcus mutans* biofilm and dental esthetic restorative materials (ceramic, composite resin, polyacid-modified composite resin, resin-modified glass ionomer cement, conventional glass ionomer cement) on the surfaces of these materials?

Literature search

The authors searched Bireme, Medline, ISI, Cochrane Library, Scopus, Lilacs and the Internet for articles from January 1980 to the end of May 2006. The search was supplemented by manual searching of reference lists from each relevant paper identified.

The main search terms were “dental biofilm”, “dental plaque” OR “lactic acid” AND (“composite resin” OR “polyacid-modified composite resin” OR “glass ionomer cement” OR “ceramic”). A total of 37 records were originally identified about biofilm / materials and 56 records about lactic acid / materials. Filters were then used to allow only subject papers to be connected, which resulted in 27 articles about lactic acid and 26 about biofilm. These were printed as abstracts, or full-text articles, if the abstract was missing. In a second step, two examiners selected relevant records independently and the articles that were considered of interest for the project were ordered in full-text versions. The search was limited to

Experimental Studies *in vivo*, *in vitro* and *in situ*. Only original papers were considered. Interim reports, abstracts, letters, short communications, and chapters in textbooks were discarded. Articles in English, Portuguese, Spanish and Swedish were accepted. During the evaluation process, reference lists were searched by hand. Further details of the search strategies used are available from the corresponding author.

Inclusion and exclusion criteria

After appraisal, studies were included only if they showed the effects of dental biofilm or lactic acid on the surfaces of restorative materials. The main characteristics / properties included were surface roughness, surface hardness, wear / abrasion resistance, color and gloss changes, and microstructural aspects. However, other properties were included, which could have a relationship with those previously cited, such as releasing ions and pH changes. Moreover, studies about biofilm must have studied *Streptococcus mutans* in mono or multi-species biofilms. *Streptococcus mutans* have characteristic properties that make this species the most important one for the development of dental caries and biodegradation of restorations, the reason for its value in this project. Among its characteristics, the fermentation of sugars and production of lactate and extracellular polysaccharides from sucrose fermentation could be listed. These abilities make it possible for *Streptococcus mutans* to adhere to different substrates in the mouth, as the pioneer colonizer, in addition to its prevalence in lower pH environments, such as cariogenic dental plaque [11].

Studies about the interaction between dental biofilm or lactic acid and luting cement or bonding materials were excluded. Bond and compressive strength were also not included.

Evaluation of scientific papers and levels of evidence

The papers that met the inclusion criteria were subjected to critical appraisal, carried out independently by at least two members of the project group (Suzana Beatriz Portugal de Fúcio, Fabíola Galbiatti de Carvalho). Data were extracted using a pilot-tested form and each paper was assessed with a score from A to C, according to predetermined criteria for methodology and performance, as defined in Table 1. In the case of disagreement between the examiners, the paper was re-evaluated and discussed by the entire group until consensus was reached. If, for some reason, a selected paper was found to be irrelevant for the research question, the article was excluded. A total of 16 papers were selected. Based on the evaluated literature, the final level of evidence was judged according to the protocol of the Swedish Council on Technology Assessment in Health Care [12], as described in Table 2.

RESULTS

Out of the 53 articles that were critically assessed, 16 studies found by this search were included in the project critical appraisal, on the grounds that they had studied surface or related properties of restorative materials and *Streptococcus mutans* or lactic acid biofilm (Table 3). Five articles classified as grade A [13-17] described the effects of dental biofilm or lactic acid on the surfaces of restorative materials. Among these effects, studies about surface properties (such as hardness, roughness, abrasion resistance, color, gloss) and visual analysis of microstructural damage (electron images) were searched. Lactic acid solution must have been used under similar conditions to those of pH from *Streptococcus mutans* biofilm (minimum of 4.0) [18]. In grade B [19-24] six articles verified effects on materials related to changes in surface characteristics found in grade A, such as releasing ions or pH changes, under the same conditions as in

grade A. Grade C was composed of five researches about properties found in A and B, however the pH of lactic acid could be below 4.0 [25-29].

Articles from grades A, B and C were included for evaluating evidence levels of properties/characteristics studied under acidic conditions (not considering biofilm or lactic acid). The following results were found: strong evidence of microstructural changes (erosion) on the surface of polyacid-modified composite resins and conventional glass ionomer cements; moderate evidence of increased fluoride release by resin-modified glass ionomer cements; and limited evidence of no fluoride release by composite resins; of pH changes by resin-modified and conventional glass ionomer cements; of increased fluoride and aluminum release by conventional glass ionomer cements; of increased aluminum release by resin-modified glass ionomer cements. Other properties were classified as in inconclusive level.

The main reason for excluding 37 of the articles about biofilm was that only biofilm characteristics were analyzed after its interaction with dental materials, such as the number of colony forming units count [30-43], wet and dry weights of biofilm [44], biofilm adherence tests [45,46], vital staining of biofilm / bacteria to obtain percentage of vital and dead bacteria and biofilm height [6,47], turbidimetric measurements of the suspension [8,9,48,49], electron microscopy observations of bacteria/biofilm [50-52], quantification of carbohydrates and proteins and pH measurement [53]. Furthermore, investigations that tested antibacterial properties of dental materials through growth-inhibition zone were excluded because of absence of biofilm development on material surfaces and absence of direct contact between the bacterial cell wall and the studied materials in this test [54-60].

Other studies were excluded since they concerned bonding / luting materials and metal alloys [61,62] and analyses of materials only before their contact with biofilm [63].

DISCUSSION

The present review was the first to systematically search and critically appraise the substantial literature related to the impact of dental biofilm or lactic acid on the surfaces of restorative materials. Systematic reviews are important tools for studying the relationship of dental biofilm accumulation and its metabolic products with damage to restorations, as the relationship biofilm / lactic acid and tooth structure have been well established. It is also important at this review will contribute towards the development of interest to understand the consequences of these interactions (biofilm, lactic acid and materials), since the influence of materials on biofilm development was observed in most of researches found in the literature.

The outcomes of the five studies included in grade A lead to different conclusions about different restorative materials. Only one study promoted the interaction of composite resin (CR) and biofilm [15] and one about CR and lactic acid [13]. The first showed no visible defects or very little difference compared with the control group (without bacterial contact) through Scanning Electron Microscopy (SEM) and an increase in surface roughness values after 35 days of CR exposure to biofilm *in vitro*, probably because of resin-matrix degradation and filler remaining intact. The second verified a decrease in abrasion resistance of macrofiller composite resin after six months of storage in lactic acid solution (pH 4.25). Studies about polyacid-modified composite resin (PMCR) showed clear defects in the surface microstructure and an increase in surface roughness values after biofilm interaction [15]. In addition, the lactic acid / PMCR interaction provided increased release of fluoride (F), aluminum (Al), strontium (Sr) and barium (Ba) and development of many voids on the surface of PMCRs (microstructure by SEM) after 7 days at pH 4.1 [17]. In addition, there was loss of surface material adjacent to cavity walls (30 days at pH 4.7) [16]. On the surface of conventional glass ionomer cements (CGIC), the following could be noted: higher levels of surface loss than PMRC after 30 days [16], and increased surface roughness, decreased

gloss, changed color, decreased hardness, increased toothbrush wear and severe erosion found by SEM, after thermal cycling test in lactic acid solution with pH 4.0 [14]. As regards resin-modified glass ionomer cements (RMGIC), there was increased release of Al, F, Sr and Ba, many small voids on the surface of this material and decreased particle size (SEM) after 7 days in lactic acid solution [17]. No study about the interaction with ceramics was found.

Considering the studies included in grade B, material-dependant results about the studied interaction could also be seen. A single research *in situ* showed no changes in fluoride release from CR after 14 days of biofilm deposition on its surface [19]. Furthermore, no F release by CR was detected *in vitro* after 3 hours in lactic acid (pH 4.3) and 7 days in contact with *Streptococcus mutans* biofilm [23]. Moreover, no Al was released and there was no pH change in biofilm during 6 hours [21] or 7 days of biofilm / CR interaction [23]. Controversially, Itota *et al.* [24] showed an increase in F release (pH 4.0; 10 weeks) from a type of CR with fluoro-alumino-silicate glass filler in its composition as F source, which was probably disintegrated by lactic acid. Hayacibara *et al*. [23] also showed the ability of PMCR, CGIC and RMGIC to inhibit pH drop and increase F and Al release when they were in contact with biofilm for 7 days.

Furthermore, in articles from Grade B, fluoride release from CGIC and RMGIC was confirmed *in situ* during 14 days of biofilm deposition [19] and *in vitro* during 7 days [21], 14 days [20] and 10 weeks [24] after exposure to lactic acid. Other ions, such as sodium (Na), calcium (Ca), and Al, were also obtained from CGIC in higher amounts under acidic conditions (pH 4.0) [20,23]. In addition, it was verified that CGIC and RMGIC increased the initial pH (4.0) of an acid solution in 10 minutes [22] or over the period of 10 weeks [24]. However, Francci *et al*. [21] did not detect any influence of these materials on pH levels after interaction with biofilm for 6 hours. This research could not be compared directly with others cited before, because different materials and methodologies were used. Compared with the Hayacibara *et al*. study [23], exclusively testing biofilm effects, 6 hours of exposure was probably a short period to identify changes in biofilm pH, presuming

that the effect would have occurred dependent on release of ions from materials. No study about interaction with ceramics was found for grade B either.

In grade C, only one study was found about CR and PMCR. Gömeç *et al*. [29] used lactic acid with pH 3.61 (simulating yoghurt) to store CR and PMCR for 7 days and found a decrease in hardness values. As regards RMGIC, these authors showed an increase in hardness values. The authors explained this as being due to absorption of some inorganic ions or deposition of stable complexes on the cement surface, thus forming a thin layer with low solubility. The development of a gel layer and porosities, without the lost of glass filler, on the surface of RMGIC was also demonstrated after 45 days of storage at pH 3.5 [26]. In addition, the same pH promoted clear lines and cracks starting at the filler particles or bubbles on RMGIC exposed for approximately 20 months, observed by confocal fluorescence microscopy [28]. Researches related to CGIC showed erosion development (holes at subsurface) through eroded depth and cryo-SEM images at pH 2.74 for 24h [27] and production of a gelatinous layer and porosities on the surface, but missing particles at pH 3.5 for 45 days [26]. While Gömeç *et al.* [29] demonstrated an increase in hardness values for CGIC, Mc Kinney *et al.* [25] observed a decrease in hardness values, wear resistance and particle dissolution at pH 2.67 for 3 weeks. This controversy about the hardness property could be explained by different materials and periods used in the two studies. Gömeç *et al.* [29] studied high-viscosity cement developed for Atraumatic Restorative Treatment (Fuji IX GP) and measured hardness values after 7 days of storage. It is probable that, within this period, the material continued its acid-based setting reaction, and consequently increased in hardness. However, in 3 weeks, hardness could be sensitive to degradation, showing reduced values [25]. Moreover, the higher resistance of ART cement, from higher powder: liquid ratio, could be an advantage over the cement used by Mc Kinney *et al*. (Fuji II).

Tooth-colored filling materials are increasingly requested by patients and used by dentists. Among the possible alternatives to metal restorations there are dental ceramics, composite resins, polyacid-modified composite resins and

glass ionomer cements. However, all these materials have some limitations to their durability when exposed to the adverse conditions in the mouth, resulting in different processes of degradation of each material. Biodegradation of glass ionomer cements is a complex process of absorption, disintegration and outward transportation of ions. As regards composite resins, incomplete conversion may leave unreacted monomers that might dissolve from the material in a wet environment; reactive sites are also susceptible to hydrolization or oxidation [2]. Ceramics could be dependant on glass resistance because their corrosion probably is represented by inward diffusion of water molecules which react with non-bridging oxygen atoms to form hydroxyl ions that diffuse out with the alkali ions to maintain electrical neutrality [64]. However, few studies have been conducted to measure the effects of biological adversity from mouth on these restorative materials.

This systematic review showed a small number of studies that explored the damage by biofilm to the surface of restorative materials. The greatest attention to the antibacterial properties of materials was obvious, that is, the effects of this interaction only on biofilm. The release of ions is an antibacterial characteristic of materials considered by authors. However, this review considered this property, as it could promote the surface degradation of materials.

It was also evident that the majority of researches studied glass ionomer cements, mainly with regard to influence on pH media and fluoride releasing. Other surface properties were tested on these materials by some investigations. Nevertheless, the different methodologies used in those studies made it impossible to confirm the analyzed data. Considering the inclusion criteria used in this review, few authors studied CR and PMCR. Furthermore, no study has investigated the biodegradation of dental ceramics.

CONCLUSIONS

From the studies selected, it was not possible to verify strong evidence to biofilm effects on surface properties as roughness, microhardness, wear/abrasion resistance, color and gloss of restorative materials studied. Only microsctrutural changes on the surface of PMCR and CGIC obtained strong evidence.

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Table 1 - Criteria for grading of assessed articles

GRADES	CRITERIA
A	*Streptococcus mutans* biofilm (mono or multi-species) Lactic acid with pH from 4.0 Restorative materials: ceramic, composite resin, polyacid-modified composite resin and glass ionomer cements (resin-modified and conventional) Analysis of surface properties: microhardness, roughness, wear/abrasion resistance, color, gloss and microstructural changes Bias or confounders taken in account Independent outcome assessment Statistical analysis
B	*Streptococcus mutans* biofilm (mono or multi-species) Lactic acid with pH from 4.0 Restorative materials: ceramic, composite resin, polyacid-modified composite resin and glass ionomer cements (resin-modified and conventional) Analysis of properties related to surface indirectly: ions release, pH changes Bias or confounders taken into account Independent outcome assessment
C	*Streptococcus mutans* biofilm (mono or multi-species) Lactic acid with pH below 4.0 Restorative materials: ceramic, composite resin, polyacid-modified composite resin and glass ionomer cements (resin-modified and conventional) No independent outcome assessment Potentially significant bias/confounders that could distort the results not considered

Table 2 - Definitions of evidence level*

EVIDENCE LEVEL	CRITERIA
STRONG	At least two studies with high level of evidence (grade A) or a good systematic review
MODERATE	One study with high level of evidence (grade A) and at least two studies with a moderate level of evidence (grade B)
LIMITED	At least two studies with a moderate level of evidence (grade B)
INCONCLUSIVE	Fewer than two studies with a moderate level of evidence (grade B)

*Modified from Britton (2000).

Table 3 - Results of references appraised

First author	Year	Biofilm	Lactic Acid	pH	Maximum Period	Materials	Method	Results of interaction	Grade
Dérand	1980		X	4.25	6 months	CR (macro x microfiller)	*In vitro*	Decreased abrasion resistance (macro)	A
Hotta	1995		X	4.0	Thermal cycling (50000)	CGIC (glazed, polished, none)	*In vitro*	Increased roughness values, color-change, decreased gloss, hardness, abrasion resistance, severe erosion (except to glazed)	A
Willershausen	1999	X			35 days	CR, PMCR	*In vitro*	Defects (PMCR), increased roughness values	A
Smales	2000		X	4.7	1 month	CGIC, PMCR	*In vitro*	Erosion (all); higher to GIC (Fuji IX)	A
Sales	2003		X	4.1	7 days	RMGIC, PMCR	*In vitro*	Increased F, Al, Sr, Ba release; voids without particles or smaller particles	A
Forss	1991	X			14 days	CGIC, CR	*In situ*	Increased F release from CGIC	B
Forss	1993		X	4.0	14 days	CGIC	*In vitro*	Increased F, Na, Ca, Al release	B
Francci	1999		X	4.3	3 hours	CR, RMGIC, CGIC	*In vitro*	Increased F release (RMGIC and CGIC)	B
		X		4.3	6 hours	CR, RMGIC, CGIC	*In vitro*	No pH changes (all materials)	
Nicholson	2000		X	4.5	10 minutes	CGIC, RMGIC	*In vitro*	Increased pH values	B
Hayacibara	2003	X			7 days	CGIC, RMGIC, PMCR, CR	*In vitro*	Inhibition pH drop, increased F and Al release (except CR)	B
Itota	2005		X	4.0	10 weeks	CR, RMGIC	*In vitro*	Increased F release (RMGIC and CR) and higher pH values	B
McKinney	1987		X	2.67	3 weeks	CGIC	*In vitro*	Decreased wear resistance, particles dissolution, decreased hardness values	C
Fano	2001		X	3.5	1 year	RMGIC	*In vitro*	Gel layer production, porosity open, particles absents (except to Vitremer)	C
Nomoto	2003		X	2.74	24 hours	CGIC	*In vitro*	Erosion and holes at subsurface	C
Fano	2004		X	3.5	20 months	RMGIC	*In vitro*	Clear lines, cracks	C
Gömeç	2004		X	3.61	7 days	CGIC, RMGIC, PMCR, CR	*In vitro*	Increased (GIC/RMGIC) and decreased (PMCR/CR) hardness values	C

CR = composite resin; PMCR = polyacid-modified composite resin; RMGIC = resin-modified glass ionomer cement; CGIC = conventional glass ionomer cement; F = fluoride; Al = aluminum; Sr = strontium; Ba = barium; Na = sodium; Ca = calcium

CAPÍTULO 2:

Qualitative and quantitative analyses of biofilms accumulated on esthetic restorative materials *in vitro*: confocal laser scanning microscopy and COMSTAT analyses

ABSTRACT

Purpose: to make a qualitative and quantitative assessment of the arrangement of microorganisms in biofilm developed on the surface of different material groups (ceramic - C, composite resin - CR, conventional – CGIC and resin-modified glass ionomer cements - RMGIC). **Methods:** *Streptococcus mutans* UA159 were used to develop a biofilm adherent to the surfaces of selected material disks by 30 days. The specimens were stained and analyzed by confocal laser scanning microscopy. Among biofilm properties, mean thickness, total bio-volume, roughness coefficient and surface to volume ratio were investigated, as well characteristics about distribution viable/nonviable cells by biofilm and its architecture. **Results:** There was statistically significant difference among restorative materials tested only for mean biofilm thickness value. C and CR accumulated the thickest biofilms. Qualitative analysis showed cellular aggregates and fluid-filled channels penetrating considerable depth of biofilm. In addition, images demonstrated a progression of more viable cells in superficial regions of the biofilm to proportionally more nonviable cells in the deeper regions of the biofilms near the disk. **Conclusion:** The restorative materials had an influence on 30-day-old biofilm only with regard to thickness values. Other biofilm characteristics analyzed did not show statistically significant difference among material substrates.

Key Words: biofilm, dental restorative materials, confocal microscopy, image analysis.

INTRODUCTION

Dental biofilm is the diverse microbial community found on the tooth surface, embedded in a matrix of polymers of bacterial and salivary origin. Although dental biofilm forms naturally on teeth, in the absence of adequate oral hygiene, it can accumulate at stagnant sites beyond levels compatible with oral health, and dental caries or periodontal disease can occur (Marsh and Bradshaw, 1995). Studies have shown the ability of mutans streptococci to colonize artificial substrates (Benderli *et al*., 1997; Kawai & Urano, 2001; Steinberg & Eyal, 2002; Eick *et al*., 2004), which could lead to development of dental material surface biodegradation (Willershausen *et al*., 1999) and secondary caries, the main reason for restoration replacement (Mjör & Moorhead, 2000). Consequently, there is increasing interest in using dental materials that might have antibacterial properties, such as the property to release ions, with the purpose of acting against oral microorganisms (Svanberg *et al*., 1990; Boeckh *et al*., 2002; Hayacibara *et al*., 2003). However, in additional to chemical composition, the physical characteristics of dental materials have an immense importance in bacterial adhesion and biofilm colonization. Surface roughness is considered an essential factor that determines the amount of biofilm accumulation (Quirynen & Bollen, 1995). Therefore, different restorative materials might present biofilms developing on their surfaces, with different characteristics, such as their architecture, viable/nonviable cell distribution, thickness, etc.

Biofilms can be studied in their natural hydrated state by confocal laser scanning microscopy (CLSM). This microscopy does not require specimens to be dehydrated, fixated or stained, offering a greater possibility to analyze living, fresh, or more easily prepared specimens (Wood *et al*., 2000). In addition, perfect registered stacks of serial optical sections can be obtained of thick microbiological samples. Digital images of such stacks represent suitable data for quantitative measurements as well as for computer 3D reconstructions that can be made by confocal software (Kubínova & Janácek, 2001).

The purpose of this study was to make a qualitative and quantitative assessment of some of the characteristics of the biofilms accumulated for 30 days on the surfaces of different restorative materials, and to make a comparison among the materials. The hypothesis tested was that biofilms developed on the surfaces of different restorative materials would present different characteristics (quantitative and qualitative) after 30 days of development *in vitro*.

MATERIALS AND METHOD

Specimen preparation

Table 1 shows the four groups of restorative materials tested: ceramic, nanofiller resin-based composite, conventional and resin-modified glass ionomer cements. Fifteen disks of each material (10mm diameter, 2mm thick) were prepared according to the manufacturer's instructions, using metal rings as molds, with sterile techniques (laminar flux chamber and sterilized instruments). The composite and glass ionomer cement disks were finished with slight pressure of acetate strips (Probem Ltda, Catanduva, São Paulo, Brazil) and glass plate only, without polishing techniques to avoid surface contamination. The ceramic disks were ground with granulation sandpaper of up to 1000μm, washed for 5min in ultrasonic bath and autoglazed.

Bacteria

The microorganisms used in this study were *Streptococcus mutans* UA159 to produce mono-organism biofilm, obtained from the culture collection of the Department of Microbiology and Immunology, Dental School of Piracicaba, Campinas State University. To prepare the inoculum, *S. mutans* was first grown on *Mitis salivarius* agar plates. The plates were incubated at 37ºC for 48h in a 5% supplemented CO_2 environment. Subsequently, single colonies were inoculated

into 5mL of brain-heart infusion (BHI) broth (Becton Dickinson and Company, Sparks, MD21152, USA) supplemented with yeast extract (Himedia Laboratories PVT Ltd., Mumbai, India) and incubated at 37ºC overnight.

30-days-biofilm development

The tests were performed on 24-well polystyrene plates (Multidish 24-well Nunclon). The investigated surface of each material disk was exposed under static conditions to 80µL of bacterial suspension in BHI broth of known concentration, to form an early biofilm on the test surface. The suspensions were adjusted to an optical density (OD) of 0.25 at 550nm (approximately 3.0 x 10^{10}CFU/mL). After two hours at room temperature, the non-adhering cells were removed by washing three times with sterile 0.9% NaCl solution (Montanaro *et al*., 2004). This exposure time was chosen, because complete biofilm formation in the oral cavity normally occurs in 2-4h (Marsh and Bradshaw, 1995). Next, a single disk of the materials listed in Table 1 was placed in each well, and two mL of sterile fresh BHI broth with addition of 1% (w/v) sucrose (Synth, LabSynth, São Paulo, Brazil) were added to the wells (Kreth *et al*., 2004). The plates were incubated for 30 days at 37ºC in a 5% supplemented CO_2 environment (Brambilla *et al*., 2005). Medium was renewed at 48h intervals by aspiring the spent medium from the wells and adding two mL of fresh medium (BHI + 1% sucrose). Following medium replacement, the plates were returned to incubation conditions. Contamination in the media was verified everyday using Gram staining and by plating samples.

Confocal laser scanning microscopy (CLSM)

After 30 days of incubation, the material disks were washed three times with sterile saline solution to remove non-adhering cells and placed in a Petri dish, biofilms upwards. LIVE/DEAD® Baclight™ Bacterial Viability Kit (Molecular Probes, Eugene, OR, USA) was mixed according to the manufacturer's instructions and

one drop was carefully applied directly onto the biofilm surface under investigation, without disturbing the samples. This stain package is composed of a mixture of two nucleic acid-binding stains: SYTO 9 and propidium iodide (PI). SYTO 9 stains all cells green, while PI penetrates cells whose cell membranes have been damaged, staining them red (Molecular Probes, 2004). After incubation in the dark for 15min (Boulos *et al*., 1999), non-invasive confocal imaging of fully hydrated biofilms was accomplished by means of an inverted microscope with a confocal laser scanning unit (LSM 510 META, Zeiss, Germany). An excitation wavelength of 488nm was used, and all light emitted between 500 - 550nm and over 560nm was collected by different filters. Lenses of 4x/0.13 were used for thickness measurements, 10x/0.3 for COMSTAT analysis, architecture and viability visualization. The resulting sets of confocal optical sections were collected by microscopy software as stacks of images. 3-D images were obtained from disks perpendicular and parallel to the cover slip.

Mean biofilm thickness value

The thickness mean (μm) provides a measurement of the spatial size of the biofilm, and is the commonest variable used in biofilm literature (Zaura-Arite *et al*., 2001; Hope & Wilson, 2003; Auschill *et al*., 2005; Cense *et al*., 2006). Previous results (unpublished) verified that thick and dense 30-day-old biofilms were not been completely crossed by a laser beam, making it impossible to locate the disks correctly under biofilm, in order to sum up precisely the number of sections until the upper biofilm cells. For thickness analysis, this study used a metal cube device fixed to a cover slip with double-faced adhesive tape, and the disks were fixed in this device perpendicular to the slip. This method enabled the disk/biofilm assembly to be visualized at a profile view, and with confocal software, five measurements of the same image of the biofilm were obtained at equidistant intervals (from disk surface to the end of the biofilm visualized) and the mean biofilm thickness value *per* image was calculated.

Image processing by COMSTAT

CLSM images were analyzed by the computer program COMSTAT (Heydorn *et al.*, 2000). The program requires setting a manual threshold value for all image stacks before quantitative characterization of three-dimensional biofilm images (the parallel to slip ones). This value was fixed by one operator by comparing the original gray-scale picture with the converted black-and-white picture to determine biomass (white) or background (black) pixels. Next, three features from COMSTAT were selected to quantify biofilm structures: bio-volume, roughness coefficient and surface to volume ratio.

Bio-volume represents the overall volume of the biofilm, the value of biomass volume being divided by the substratum area ($\mu m^3/\mu m^2$). Biofilm roughness describes the variability in the biofilm thickness, and is an indicator of biofilm heterogeneity (dimensionless). Surface to volume ratio reflects what fraction of the biofilm is in fact exposed to the nutrient flow and thus may indicate how the biofilm adapts to the environment (Heydorn *et al.*, 2000).

Statistical analysis

The dependent variables were restorative materials and biofilm properties (mean of thickness, bio-volume, roughness coefficient and surface to volume ratio). First the data were evaluated to check the equality of variances and normal distributions of errors. Next, a one-way variance analysis (ANOVA) model determined the significance of variables. The Tukey test was chosen for evaluating the significance of all pairwise comparisons with a significance limit of 5%.

RESULTS

Fluorescent staining of bacteria, Confocal Laser Scanning Microscopy (CLSM), and image analysis provided an effective method for visualizing and quantifying the bacterial arrangement within dental biofilms.

Biofilm visualization

To the naked eye, biofilms were qualitatively different, depending on the material-substrate. Figure 1-A showed that ceramic (a) and composite resin (b) presented biofilms that were thicker, with more irregular top surfaces, and with evidently larger numbers and sizes of micro-colonies on their surfaces. Glass ionomer cements (c and d) exhibited biofilms that were thinner and more homogeneous, uniform, with smaller sizes and lower numbers of visible micro-colonies than seen on the above-mentioned materials. This difference between material groups, such as the structure and thickness described, was detected by CLSM in profile view (disks perpendicular to slip), in figure 1-B. The CLSM images also helped to identify the formation of some mushroom shaped structures, with the majority of the biomass being in the upper areas of the biofilm.

Fig. 2 shows the vertical structure (3-D) of a 30-day-old biofilm developed on the studied materials. There was an uneven distribution of vital and dead microorganisms and voids in a very single biofilm layer. While some vital bacteria were dispersed in the dead biofilm mass (leading to a yellowish or orange color), a layer of nonviable cells in the deeper regions of the biofilms (adjacent to disks) was clear in all materials. Images demonstrated a progression of nonviable cells from the superficial to deeper regions of biofilms.

Biofilm thickness mean and COMSTAT features

The mean biofilm thickness value, bio-volume, roughness coefficient and surface to volume ratio of biofilms developed on the surface of tested materials are shown in Table 2. The mean biofilm thickness value of Empress 2 (760.2 ± 151.8μm) and Filtek Supreme (796.4 ± 233.2μm) showed statistically significant difference from mean thickness value of Vitremer (464.8 4 ± 195.0μm) and Ketac Molar Easymix (477.0 ± 193.8μm). Other biofilm properties tested did not present any difference among material groups.

DISCUSSION

The biological interaction between restorative materials and oral microflora is probably the main factor that determines the prognosis of the restorative treatment. Surface properties of restorative materials play a major role in bacterial adhesion and colonization of the restorations. The effects of restorative materials on oral microorganisms and biofilm formation have already been demonstrated in numerous other studies (Auschill *et al*., 2002; Eick *et al*., 2004; Montanaro *et al*., 2004; Brambilla *et al*., 2005; Beyth *et al*., 2006). However, these studies investigated biofilm a few hours up to a maximum of 10 days old. Willershausen *et al*. (1999) exposed materials over a period of up to 35 days, but only determined bacterial glucose consumption and lactate production. The present study produced interaction during 30 days between aesthetic restorative materials and *Streptococcus mutans* in order to observe the effects of these substrates on bacterial metabolism and proliferation, not only on initial adhesion and colonization. The selected period for this study could evidence biofilms with characteristics that differed among materials. However no similar studies were found in the literature, testing dental materials as substrate and 30-day-old biofilm. Moreover, other studies about biofilm thickness developed on dental materials observed by CLSM microscopy (Netushil *et al*., 1998; Auschill *et al*., 2001)

prepared the specimens by drying and fixing them. This led to biofilm shrinkage, and required extrapolation to estimate the natural thickness of the biofilms and surface topography. The present study measured biofilm thickness in a natural and hydrated state.

The present study found a progression of nonviable cells from superficial to deeper regions of the biofilms. Hope *et al*. (2002), by means of intensity profiles obtained by CLSM, showed the same situation. They promoted a 10-day-old biofilm with a constant-depth film fermenter (CDFF). Vital biofilm bacteria could be located in the upper areas due to having direct access to the nutrients from the exposed medium. Auschill *et al*. (2001) found dead cellular material covering vital microorganisms, relating this structure to increased resistance of biofilms to oral chemotherapeutics. However, they investigated five-day-old biofilm developed *in situ*, while the present study promoted advantageous static and nutrient conditions for biofilm development *in vitro*. Consequently, the biofilms studied by Auschill *et al*. were exposed to antibacterial factors from the salivary environment, which could have lead to the biofilm structure visualized by the authors. Furthermore, Auschill's research partially dried the biofilms before mounting them in a fixative containing toluene/paraloid resin. These specimen preparations could reduce the quality of data as regards spatial microorganism distribution.

Along the *xy*-axis the distribution of the microorganisms was not regular. Non-stained (black) bubble-like structures were present within the biofilm structure. These voids were also reported by Wood *et al*. (2000) and may have been filled with biological substances such as, exopolysaccharides and glycoproteins, which are not stainable by the fluorescent stain used. The presence of these channels would be significant for the movement of material-damaging acids, bacterial toxins and other antigens within the biofilm. In addition, the present investigation demonstrated mushroom-shaped structures in profile images of biofilm, similar to those found by Wood *et al*. (2000).

Sucrose provides the formation of stable biofilms by *S. mutans*. One percent of sucrose is sufficient to produce a thick, confluent biofilm, which was composed of dense microcolonies with small, water-channel-like void areas (Kreth *et al*., 2004). *S. mutans* biofilm developed with 1% sucrose in 16 hours by Kreth's research, in the absence of shear stress, presented an average biovolume of 16.71μm³/μm². This dimension is dependant on different sucrose concentrations. Probably, as the present study promoted the same nutrient conditions for all materials, the biovolume values did not differ statistically among different disks. These analogous values could also be limited by restricted penetration/reflection of CLSM laser light, which cannot completely cross the layers of 30-day-old biofilms, leading to a partial bio-volume analysis (only upper surface). In addition, biofilms with different mean thickness values could have different inner architectures, equalizing the influence of different substrates. A thicker and more porous biofilm could demonstrate a bio-volume similar to that of thinner and denser biofilms. However, density and porosity characteristics were not numerically determined by this study.

The same growth conditions probably promoted similar results with regard to the roughness coefficient and surface to volume ratio. Beyenal & Lewandowski (2002) argue that biofilm structure changes to maximize nutrient transport, making it possible that an increased interface between the biofilm-growth medium was developed to improve the biofilm nutrient intake. Moreover, all materials equally exposed to 1%-sucrose every 48 hours generated similar quantitative parameters. However, the basic mechanical model reported by Klapper (2004) showed that a forest of biofilm mushrooms effectively creates a mushy biofilm–medium layer that slows nutrient transport to the microbial floor. Therefore, below this layer, limited nutrient substrate is available to biofilm cells, leading to higher frequency of non-viable microorganisms in deeper biofilm regions. This could explain the spatial distribution of viable/nonviable cells found by the present study.

The chemical composition of the surface materials is important to bacterial colonization, particularly when the surface has components that are either beneficial or detrimental to the adhered microflora. As regards biofilm thickness values, there were higher statistically significant values for composite resin and ceramic than for glass ionomer cements. The bacterial growth-inhibitory potential of glass-ionomers is demonstrated by many studies (Forss *et al*., 1991; Herrera *et al*., 2000; Ertugrul *et al*., 2003; Duque *et al*., 2005) and attributed mainly to the release of fluoride (Loyola-Rodriguez *et al*., 1994). It has also been reported that resin-modified and conventional glass ionomer cements (RMGIC and CGIC, respectively) neutralize acid solutions (Nicholson *et al*., 2000), even in the long-term (Itota *et al*., 2005) and inhibit a drop in biofilm pH (Hayacibara *et al*., 2003). The dissolution of soluble products from materials leads to formation of a salt between the cement ions and acid medium. It is possible that a synergistic effect of fluoride releasing and neutralizing ability occurs, influencing biofilm development on glass ionomer cement surfaces. Fluoride affects bacterial growth and metabolism, through inhibition of ATPase and intracellular acidification (Hamilton, 1990), and consequently affects the acid production of microorganisms from biofilm.

In this study, high biofilm thickness values for composite resin disks were observed. Kawai & Tsuchitani (2000) verified that Bis-GMA, TEGDMA and UDMA promoted water-insoluble glucan synthesis by glucosyltransferase from *Streptococcus sobrinus*, contributing to biofilm accumulation. In addition, Takahashi *et al.* (2004) found sparse vesicular-structured polymer surrounding bacterial cells and no difference in numbers of colony-forming units for the control (no monomers), suggesting that the polymer can act as a barrier to protect the cells. Khalichi *et al*. (2004) demonstrated that TEGDMA hydrolyzed by-products (TEG) can stimulate *S mutans* growth in a pH representative of cariogenic state. Therefore, hydrolytic degradation of composite resin releases elutable compounds present on the surface (Bis-GMA, UDMA and TEGDMA), justifying the high thickness biofilm values found in CLSM images.

As regards the thick biofilm accumulated on ceramics, few studies were found in the literature. Probably, the chemical stability and biocompatibility of dental ceramics produce a negligible rate of component release from these materials (Anusavice, 1992). Thus, ceramics would not present the antibacterial property of releasing ions, having no influence on biofilm development. Indeed, Auschill *et al*. (2002) and Eick *et al*. (2004) verified really high vitality values in biofilm accumulated on ceramics. However, these researches found very thin and weightless biofilm on this material, coating a reduced surface of the specimens. They affirmed that the smooth surface texture of ceramics does not encourage bacterial colonization. Nevertheless, Auschill *et al*. developed biofilm *in situ* (5 days) and Eick *et al*. in continuous flux (48h). The absence of detachment forces (growth static conditions) of the present study possibly contributed to build up very thick biofilms on ceramic disks, in spite of their smooth surfaces, since it did not disturb and/or remove accumulated biofilm. Moreover, the short period of biofilm accumulation from the researches cited did not allow a high percentage of viable cells present on ceramic surface to promote thicker biofilms.

Thus, the hypothesis that 30-day-old biofilms accumulated on different restorative materials have different characteristics has to be partially accepted, since only mean biofilm thickness values showed statistically significant difference among the materials tested.

CONCLUSIONS

Within the limits of the present investigation, it could be concluded that *Streptococcus mutans* biofilms were influenced by different restorative materials only with regard to their thickness values. The highest thickness means were found for ceramic and composite resin after 30-days-interaction.

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Table 1 - Materials used in this study

Materials	Classification	Contents
IPS Empress 2 (Ivoclar Vivadent, Schaan, Liechtenstein)	Glass ceramic	Powder: 97% SiO_2, Al_2O_3, P_2O_5, K_2O, Na_2O, CaO, F and 3% TiO_2 and pigments; Liquid: water, alcohol, chloride
Filtek Supreme (3M ESPE, St. Paul, MN, USA)	Resin-based composite	Bis-GMA, Bis-EMA, UDMA, TEGDMA Zirconia/silica cluster filler and a nonagglomerated silica filler
Vitremer (3M ESPE, St. Paul, MN, USA)	Resin-modified glass ionomer	Powder: fluoroaluminosilicate glass; redox system Liquid: aqueous solution of a modified polyalkenoic acid, HEMA
Ketac Molar Easymix (3M ESPE, St. Paul, MN, USA)	Conventional glass ionomer	Powder: fluorosilicate glass, strontium and lantanium; Liquid: polycarbonic and tartaric acids and water

Bis-GMA = bisphenol glycidyl methacrylate; UDMA = urethane dimethacrylate; TEGDMA = triethylene glycol dimethacrylate; Bis-EMA = ethoxylated pisphenol-A dimethacrylate; HEMA = 2-hydroxyethyl methacrylate

Table 2 - Mean and standard deviation of thickness (in parentheses), bio-volume, roughness coefficient and surface area / volume of biofilms developed on the surface of different restorative materials

MATERIALS n = 15	MEAN THICKNESS µm	BIO-VOLUME $\mu m^3 / \mu m^2$	ROUGHNESS COEFICIENT dimensionless	SURFACE AREA/VOLUME 1 / µm
Empress 2	760.2 (151.8) a	79.8 (23.3) a	0.23 (0.1) a	0.07 (0.02) a
Filtek Supreme	796.4 (233.2) a	93.6 (58.3) a	0.27 (0.2) a	0.08 (0.03) a
Vitremer	464.8 (195.0) b	72.9 (24.1) a	0.22 (0.1) a	0.06 (0.03) a
Ketac Molar Easymix	477.0 (193.8) b	77.1 (38.9) a	0.25 (0.3) a	0.07 (0.05) a

Groups denoted by the same letter represent no significant difference ($p<0.05$).

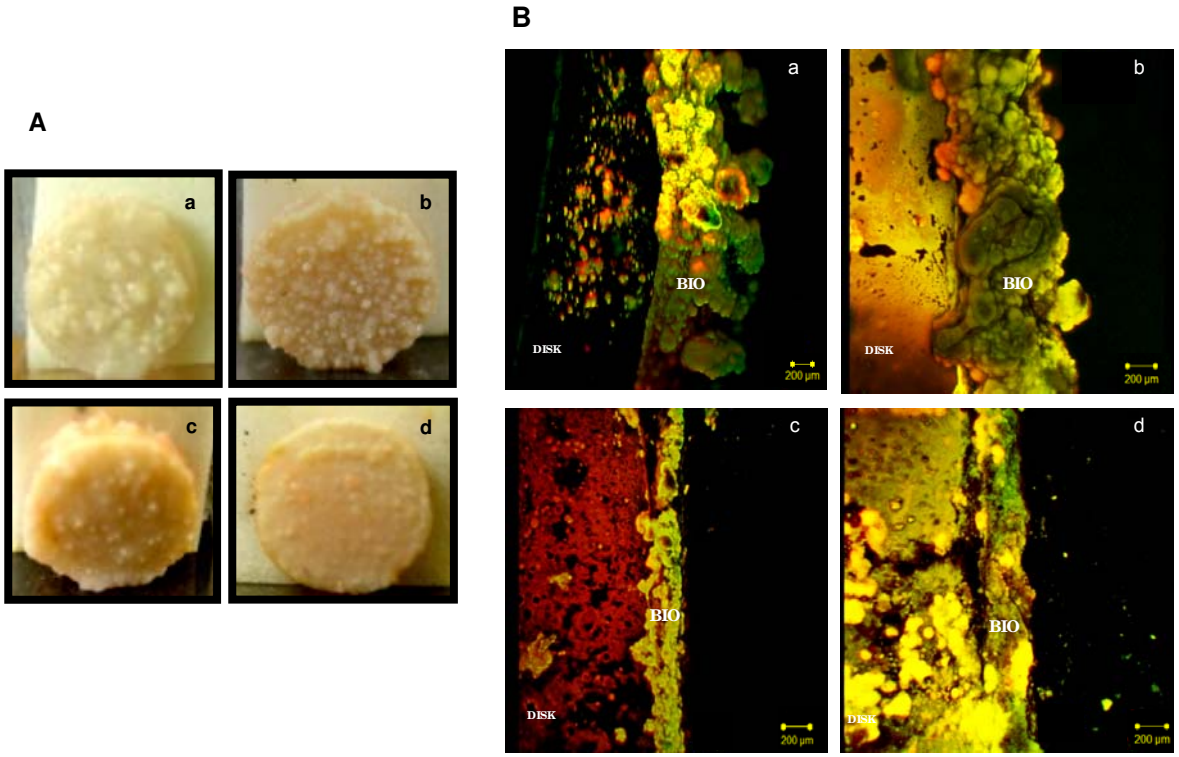

Figure 1 - A. Naked-eye visualization of material disks with 30-day-old biofilm deposited on surfaces (a. Empress 2; b. Filtek Supreme; c. Vitremer; d. Ketac Molar Easymix); B. Profile view of 30-days material disk / biofilm at CLSM (original magnification 4x/0.13).

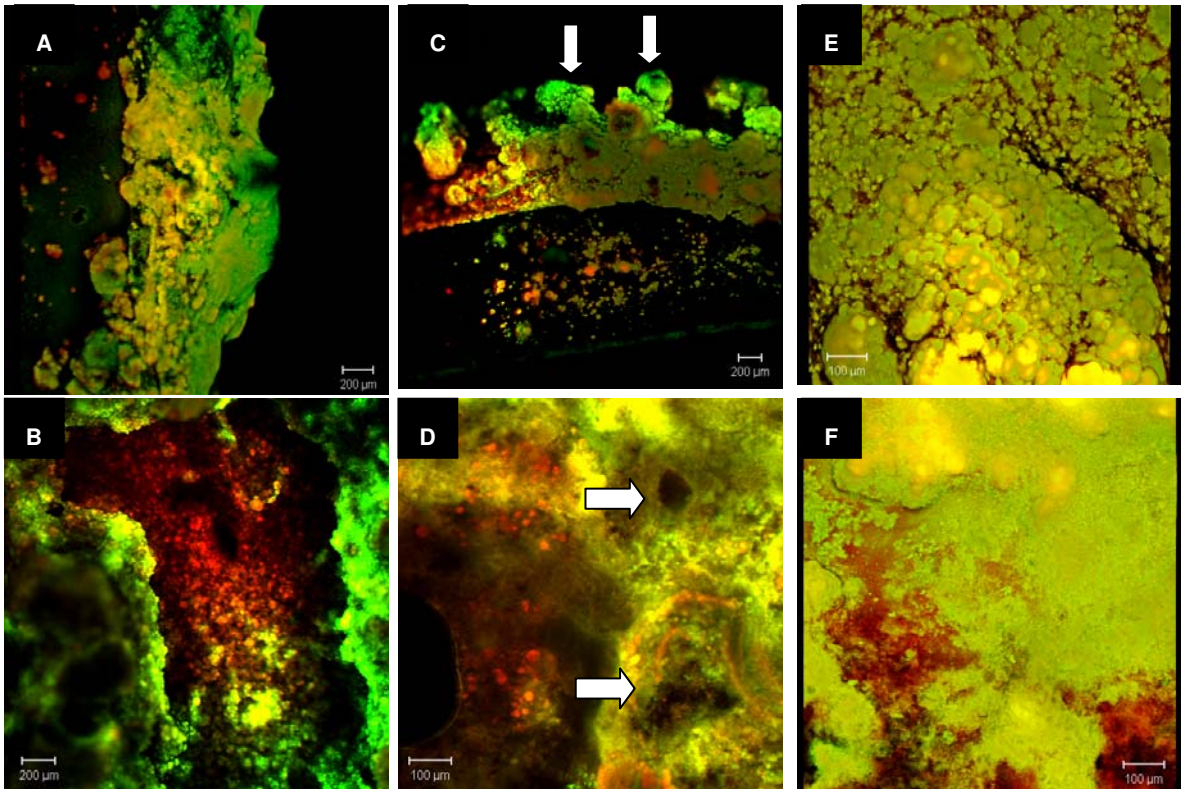

Figure 2 - A. Profile view of biofilm: yellow areas (dead cells) deeper and green areas (alive cells) superficial in biofilm; B. Frontal view: superficial layer with green cells and deeper red cells layer; C. Profile view: mushroom-shaped structure on biofilm surface (arrows); D. Frontal view: channels and voids present on biofilm surface (arrows); E. Frontal view: superficial area of biofilm accumulated on composite disk; F. Frontal view: superficial areas of biofilm accumulated on glass ionomer cement disk.

CAPÍTULO 3

The influence of 30-day-old *streptococcus mutans* biofilm on the surface of esthetic restorative materials – an *in vitro* study[3]

[3] Manuscrito formatado para publicação no periódico Dental Materials.

ABSTRACT

Purpose: to evaluate the effects of *S mutans* biofilm/restorative materials interaction on surface roughness, hardness and microstructure of materials tested compared with storage at 100% relative humidity or in a growth medium. **Methods:** Empress 2 (E2), Filtek Supreme (FS), Vitremer (V) and Ketac Molar Easymix (KM) were tested. Twenty five disks of each material were made and divided into three storage groups: 1) 100% relative humidity (n=5), 2) growth medium (BHI and 1% sucrose) (n=5) and 3) *Streptococcus mutans* biofilm-growth medium (n=15). Before storage, hardness measurements were immediately obtained from group 1 specimens. After 30 days of storage, the specimens were cleaned in order to obtain the surface roughness and hardness values, besides microstructure analysis by scanning electron microscopy (SEM). **Results:** E2 and FS did not present statistically significant difference among storage groups with regard to surface roughness and hardness, and between immediate hardness measurements and group 1. However, group 3 specimens of V and KM showed statistically significant higher surface roughness means than other groups. Group 1 specimens of V and KM also showed higher hardness values than the immediate measurements. Group 3 specimens of V presented decreased hardness values compared with other groups. SEM images showed an increase in surface degradation from group 1 to group 3 for FS, V and KM. **Conclusion:** Thirty-day-old biofilm promoted a negative effect on the surface microstructure of FS, V and KM, on the surface roughness of V and KM and on the hardness of V.

Key Words: biofilm, *Streptococcus mutans,* biodegradation, surface analysis, dental restorative materials, surface roughness, microstructure, nanocomposite, glass ionomer, ceramic.

INTRODUCTION

The ability of restorative dental materials to withstand the functional force and exposure to various substances in the mouth is an important requirement for their clinical performance for a considerable period of time. Some investigations have demonstrated surface damage to restorative materials caused by the chemical environment of the oral cavity [1,2,3,4]. Fundamentally, the factors known to cause these deleterious effects include low pH due to cariogenic biofilm [5,6], consumption of acidic drinks or foodstuffs [2,4,7,8], and action of enzymes [9], which can soften the outermost layers and roughen restorative materials.

Dental biofilms harboring cariogenic bacteria (caries-associated microorganisms) are among the virulent factors associated with the progression of tooth decay and periodontal diseases. Mutans streptococci are among bacteria proliferating in the dental biofilm. Their virulence is mainly due to their high adhesion capability, acidogenicity and aciduric properties [10]. These *Streptococcus mutans* characteristics could be responsible for surface damage to restorations, since this microorganism can be found on any hard surface in the oral cavity, such as enamel, implants, orthodontic appliances or restorative materials [11,12]. Nevertheless, little is actually known about the effects of accumulated dental biofilm on the surface properties and microstructure of restorative materials.

Therefore, the aim of this study was to test the hypothesis that esthetic restorative materials subjected to *Streptococcus mutans* biofilm interaction for 30 days differ in surface roughness, hardness and microstructure from those stored in 100% relative humidity or growth medium for the same period.

MATERIALS AND METHOD

In this study, four tooth-colored materials were used: Empress 2 (E2), Filtek Supreme (FS), Vitremer (V) and Ketac Molar Easymix (KM). Group

classification, composition properties and manufacturers of restorative materials tested are listed in Table1.

Specimen preparation and Storage groups

Twenty-five specimens of each aesthetic restorative material were fabricated using metal rings (10mm diameter and 2mm depth) according to the manufacturers` instructions, under aseptic conditions (laminar flux chamber and sterilized instruments) Glass ionomer cements (V, KM) and composite resin (FS) disks were covered with an acetate strip (Probem Ltda, Catanduva, São Paulo, Brazil) and pressed flat with a microscopic glass slide to compact the material and prevent void and bubble formation. FS and V were polymerized according to manufacturers` instructions with a curing light (Elipar Trilight, 3M ESPE, St. Paul, MN, USA) through the glass and the strip on top of the specimens. The intensity of the light-curing unit was checked before each sample run, using a curing light meter (Hilux Dental Curing Light Meter, Benliglu Dental Inc., Turkey). KM was allowed to set for 5min. Following light (V, FS) or chemical curing (KM), the specimens were not polished to avoid surface contamination. The ceramic disks were ground flat with up to 1000 μm granulation sandpaper, washed for 5min in an ultrasonic bath (Ultrasonic Cleaner, Model USC1400, UNIQUE Ind. e Com. Ltda., São Paulo SP 04709-111, Brazil) and autoglazed (without addition of an overglaze). Five specimens from each of the four aesthetic restorative materials were assigned to group 1, five to group 2 and fifteen for group 3. Group 1 specimens were measured for hardness immediately after they were manipulated and then maintained at 100% relative humidity (RH) and 37ºC; group 2 were stored in Brain Heart Infusion (BHI) broth (Becton Dickinson and Company, Sparks, MD21152, USA) supplemented with 1% (w/v) sucrose (Synth, LabSynth, São Paulo, Brazil), that is, the bacterial growth medium used in this study, but without microorganism inoculation; group 3 were stored in this growth medium after early *Streptococcus mutans* biofilm development on the surfaces of the disks. All these

storage conditions were maintained for 30 days in order to analyze changes in the surfaces of the materials afterwards.

Bacteria

The microorganisms used in this study were *Streptococcus mutans* UA159 to produce mono-organism biofilm. To prepare the inoculum, *S. mutans* was first grown on *Mitis salivarius* agar plates. The plates were incubated at 37ºC for 48h in a 5% supplemented CO_2 environment. Subsequently, single colonies were inoculated into 5mL of brain-heart infusion (BHI) broth (Becton Dickinson and Company, Sparks, MD21152, USA) supplemented with yeast extract (Himedia Laboratories PVT Ltd., Mumbai, India) and incubated at 37ºC overnight.

Biofilm development on group 3 specimens

The tests were performed on 24-well polystyrene plates (Multidish 24-well Nunclon). The investigated surface of each material disk from group 3 was exposed under static conditions to 80µL of bacterial suspension in BHI broth of known concentration to form an early biofilm on the test surface. The suspensions were adjusted to an optical density (OD) of 0.25 at 550nm (approximately 3.0 x 10^{10}CFU/mL). After two hours at room temperature, the non-adhering cells were removed by washing three times with sterile 0.9% NaCl solution [13]. Then, a single disk of the materials listed in Table 1 was placed in each well and 2mL of sterile fresh BHI broth with addition of 1% (w/v) sucrose (Synth, LabSynth, São Paulo, Brazil) were added to the wells [14]. The plates were incubated for 30 days at 37ºC in a 5% supplemented CO_2 environment. Medium was renewed at 48-hour intervals. Contamination in the media was verified everyday using Gram staining and by plating samples.

Surface roughness measurements

After 30 days of storage, specimens from groups 1, 2 and 3 were ultrasonically washed in distilled water for 10min, dried and fitted to the surface roughness-measuring instrument (Surfcorder SE1700; Kosaka Corp., Tokyo, Japan). The mean surface roughness values (Ra, μm) of the specimens were obtained from three successive measurements of the center of each disk, in different directions. A calibration block was used periodically to check the performance of the surface roughness-measuring instrument [15].

Surface hardness

Hardness tests were carried out with a hardness tester (Shimatzu, Tokyo, Japan) using a Vickers indenter and a load of 200g for composite resin and glass ionomer cements and 500g for ceramic, with a dwell time of 15 seconds. The Vickers Hardness Number (VHN) corresponding to each indentation was calculated by measuring the dimensions of the indentations made using the following formula: VHN = 0.1854 x P / D2, where P is the load applied (N), D is the average diagonal length of an indentation (mm) [16].

Five readings were taken for each specimen, and the mean VHN was calculated. For each material tested, a mean value of the results of all the specimens was calculated to characterize each group (1, 2 and 3), besides immediate measurement obtained from group 1 before storage.

Surface morphology assessment

Surface morphology changes were assessed by Scanning Electron Microscopy (SEM - Model Jeol JSM 5600 LV, Tokyo, Japan), including 300x and 1000x magnification.

Statistical analysis

The mean and standard deviations of Ra and VHN were determined. Data were analyzed using one-way ANOVA (analysis of variance) for calculating the differences among storage conditions. The Tukey test ($p \leq 0.05$) was used to detect specific differences within each material group.

RESULTS

The mean and standard deviations of surface roughness and hardness of each material under different storage conditions are displayed in Tables 2 and 3, respectively. E2 and FS did not present statistically significant difference as regards surface roughness and hardness under different storage conditions (groups 1, 2 and 3). Furthermore, the hardness values obtained immediately after specimen preparation did not differ from the values of the same material specimens kept in 100% RH at 37ºC for 30 days. With regard to surface roughness and hardness values, Vitremer (V) showed no statistically significant difference between groups without biofilm (1 and 2), but there was difference between these groups (1, 2) and group 3. Group 3 presented the roughest surface and least hardness. There was also a difference between the immediate measurements and 30-day 100% RH measurements as regards surface hardness of V and Ketac Molar Easymix (KM). With regard to surface roughness in KM, there was no difference between groups 1 and 2, but these groups (1, 2) were statistically different from group 3. Specimens from group 3 were the roughest among KM groups. No difference was found among groups 1, 2 and 3 with regard to hardness values.

The SEM-pictures in Figure 1 showed a detail of the surface structure of materials studied, distributed in rows and columns. In the surface of E2, no changes due to storage conditions could be found. G1 showed few a irregularities due to specimen confection, G3 showed residual biofilm on the surface (not

completely removed by ultrasonic cleaning) and higher magnification showed a Vickers indentation on the smooth ceramic surface. With regard to FS, although no statistically significant difference was detected among groups, SEM images demonstrated an increase in surface degradation from G1 to G3. V and KM micrographs also showed an increase in surface porosity from G1 to G3 and cracking in some areas.

DISCUSSION

The esthetic and longevity of tooth-colored restoratives are highly dependent on their surface characteristics and chemical composition. Residual surface roughness of restorations encourages biofilm accumulation, which may result in gingival inflammation, superficial staining, and secondary caries. The surface roughness of the studied materials is determined by finishing and polishing techniques, but could be affected by biological and chemical degradation in the oral environment. Although restoratives materials that are cured against a matrix are not without surface imperfections, they present the smoothest surfaces possible [15,17,18,19]. This method of surface finish was used to eliminate the influence of finishing techniques on the surface roughness results and to avoid contamination of the specimen surfaces. Nevertheless, since materials are allowed to cure against an acetate matrix, an increase or decrease in their surface hardness is material-dependent [18].

Biological and chemical softening of restorative materials may also result in decreased physical-mechanical properties, such as hardness. Hardness is considered an important property when comparing restorative materials. As the restorative materials studied were not exposed to any mechanical forces, the differences in hardness observed for the various materials after storage may be attributed to their chemical composition and the effects of biofilm on the different chemical components. Furthermore, when the immediate hardness values of group 1 specimens are compared to 100% RH storage specimens, variations in

hardness might be the result of the maturity status of a material and its setting reaction [20].

The present study did not find statistically significant differences among surface roughness and hardness measurements obtained from different storage conditions for ceramic Empress 2. Micrographs confirmed these results. This was really expected for dental ceramic, since this material is considered the most inert of all dental materials used for restorations [21]. However, little information is available regarding ceramic chemical durability, mainly biological durability. Some studies promoted interaction between biofilm and ceramic [11,12,22], but verified only biofilm characteristics after the interaction. Researches concerning changes in the surface of dental ceramic were found when interacting this material with bleaching agents and acidulated phosphate fluoride [23,24,25]. When stored in these products, there was a significant increase in surface roughness values for the ceramic studied. It is believed that ceramic degradation occurs by the acid attack on the silicon-oxygen network. Nevertheless, no study was found that promoted the biofilm/ceramic interaction, and that did or did not show damage to the ceramic surface, in order to compare the results with the present study.

Composite surface roughness and hardness values were not significantly affected by liquid medium and by *Streptococcus mutans* biofilm, nor did aging influence the hardness values found immediately after specimen preparation, or compared with group 1. The low susceptibility of Filtek Supreme to the detrimental effects of biofilm may be contributed in part to the presence of Bis-EMA and TEGDMA in its matrix composition. TEGDMA can decrease surface softening by acids and increase the degree of polymerization of resin-based materials [1]. In addition, experimental composites containing the ethoxylated version of Bis-GMA (Bis-EMA) showed a lower amount of released products (degradation products and residual monomers after 32 days of storage in esterase) and higher stability. This is due to decreased flexibility and the elimination of the hydroxyl groups from the Bis-GMA monomer to Bis-EMA, thus increasing the hydrophobicity of the monomer [9]. Hence, the reduction in water uptake may be

partially responsible for the biochemical stability of composites that are composed of this monomer, such as Filtek Supreme. Yap *et al.* [2,4] showed that hardness, surface roughness and shear strength of a Bis-EMA-based composite was not affected by food-simulating liquids, including lactic acid, after one week. Varying pH levels also had no influence on surface roughness of Bis-EMA-based composite [26]. Filtek SEM images showed an increase in surface degradation from group 1 to 3. At higher magnification, it is clear that a superficial layer was partially removed by storage conditions, probably as a result of resin-rich or oxygen-inhibition layer degradation [27,28], and spheroidal agglomerated particles became visible. Mitra *et al.* [29] observed Filtek Supreme by SEM after toothbrushing abrasion, and found defects smaller than the wavelength of light, since only nanosized particles were plucked away. Thus, the biodegradation visualized by SEM in the present study could have no influence on the surface roughness results due to nanoparticles dimensions. A single research was found in the literature that verified increased Ra after interaction between a composite and biofilm for 35 days [6]. Although similar to the present study as regards the experimental period and seeking damage to the material surface, different results were found, probably because that investigation studied a non-nanofilled composite and disinfected the specimens by immersion in a 70% ethanol solution for 24 hours after polishing procedures. This could have promoted an initial chemical degradation of the composites, since ethanol increases the surface roughness and decreases the hardness of many types of composites [2]. The present study preferred to avoid contaminating the specimen surfaces with polishing instruments, since this method of disinfection could influence the results of the investigation.

The relationship among values found for Vitremer was different from that of the materials discussed above. With regard to surface roughness and hardness, statistically significant difference from groups 1, 2 to group 3 was verified. In addition, there was difference among the immediate and 30-days storage in 100% RH (group 1) hardness measurements, indicating that a post-hardening process

occurred. Probably, this difference could be explained by the the slow rate of acid-base reaction forming the polysalt matrix and the free-radical polymerization reaction, which continues after light-irradiation [30]. Vitremer presented a significant increase in hardness between 1 and 15 days, remaining stable until 180 days, since it was stored in distilled water [20].

The difference among storage groups showed an increased surface roughness (confirmed by SEM images) and decreased hardness for specimens stored in growth medium together with biofilm (group 3). The post-hardening reaction in group 2 specimens perhaps overcame the possible plasticizing effect of the liquid medium (BHI) and promoted the absence of difference between the hardness values of groups 1 and 2. Nevertheless, specimens from group 3 were exposed to acid production by the *Streptococcus mutans* biofilm, mainly lactic acid [31], during the 30 experimental days, which probably promoted a pH drop in the medium and biofilm. The susceptibility to degradation of RMGIC, particularly in an acidic environment, could be favored by the insufficiently coherent entanglement of the cross-linked polyacrylate network and the polymer chain of the RMGIC [30]. Therefore, after acid attack (by bacterial metabolism or exposure to lactic acid), this material showed micromorphological damage [32,33], increased surface roughness [3,34] and increased levels of fluoride and aluminum release [35]. In addition to releasing ions, Vitremer composition presents hydroxyethyl methacrylate (HEMA), a highly hydrophilic cosolvent and main component released at the organic phase from RMGIC [36]. HEMA, TEGDMA, and additive decomposition products eluted from Vitremer after 7 days immersion in different media [37]. It is possible that loss of components from two Vitremer matrixes (polyacrylate-inorganic and polymer-organic) leads to changes in surface roughness and hardness. Micrographs showed extensive cracking that could have been due to dehydration occurring during the time the specimens were kept in the desiccator before being sputter-coated. Moreover, the corroded aspect of the surface observed, was probably because of the dissolution of the resin-modified glass ionomer cement matrix in acidic solutions, peripheral to the glass particles,

leading to surface matrix wash-off and corrosion leaving the silica cores more exposed.

Ketac Molar Easymix presented difference among hardness values between the immediate and 30-days storage in 100% RH (group 1) measurements, indicating that a post-hardening process also occurred. These results reflect the hardening phase of the setting reaction of glass-ionomers. This occurs after the gelation phase and involves the continued formation of aluminum salt bridges and increase in the ratio of bound to unbound water that can be removed by desiccation [38]. Yap *et al.* [39] verified glass ionomer maturation at 1 week, since stable hardness values were found, and Ellakuria *et al.* [20] at 15 days for Ketac Molar maturation.

The difference found among storage groups showed an increased surface roughness for group 3 specimens (visualized by SEM micrographs), while hardness values did not differ among storage conditions. Biodegradation of glass ionomer cements (GIC) is a complex process of absorption, disintegration and outward transportation of ions. Glass particles, ions, and some of the organic materials can be found in the solvent [40]. In lactic acid solutions, GIC was capable of increasing the pH of the immersion medium towards neutral. This buffering ability is related to erosive loss of GICs, since insoluble glass-lactate is formed [41]. The increased amount of fluoride released from glass ionomer cements into an acid solution is caused by an additional leaching of glass particles, leading to erosion of the surface layer of the cement as well [35,42]. Thus, it was expected that surface roughness values would be affected by *Streptococcus mutans* acid production. Moreover, the insoluble salts produced by erosion products from GIC and an acid medium could be deposited on the surface layer, leading to the formation of a thin layer with low solubility in GIC specimens [43]. As hardness values were not affected by biofilm conditions, it is probable that a resistant-layer had been deposited on Ketac Molar, overcoming the harmful effects of low pH on that property. Microscopically, the specimen surfaces presented increased porosity from group 1 to 3. Specimens stored with biofilm showed an extremely damaged

surface, with severe superficial matrix degradation, revealing protruded particles. This aspect certainly justifies the high surface roughness values found for group 3 specimens. Cracks from preparation for SEM were also detected.

The composite and, particularly the glass ionomer cements exposed to *Streptococcus mutans* biofilm for 30-day periods may increase the risk of clinical failure. The increased roughness of the glass ionomer specimens shown by Ra values and SEM micrographs strongly suggest that the life of the restoration could be severely reduced. The decreased hardness of resin-modified glass ionomer cement after its interaction with biofilm would contribute to accelerate the biodegradation process of this material in the mouth.

Thus, the hypothesis that 30-day-old biofilms accumulated on esthetic restorative materials promote different surface roughness values, hardness values and microstructure on the material surfaces compared with other storage conditions has to be partially accepted, since there was material-dependence among characteristics analyzed.

CONCLUSIONS

Within the limits of this study, it may be concluded that:

1. Surface roughness was influenced by biofilm/material interaction only for Vitremer and Ketac Molar Easymix.
2. Surface hardness was affected by time for Vitremer and Ketac Molar Easymix specimens and by biofilm/material interaction for Vitremer.
3. Microstructure analysis showed an increasing surface degradation from group 1 to group 3 for Filtek Supreme, Vitremer and Ketac Molar Easymix.
4. Empress 2 surface was not affected by biofilm/material interaction.
5. The effects of *Streptococcus mutans* biofilm on the surface properties and microstructure are material dependent.

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Table 1 - Materials used in this study

Materials	Classification	Contents
IPS Empress 2 (Ivoclar Vivadent, Schaan, Liechtenstein)	Glass ceramic	Powder: 97% SiO_2, Al_2O_3, P_2O_5, K_2O, Na_2O, CaO, F and 3% TiO_2 and pigments; Liquid: water, alcohol, chloride
Filtek Supreme (3M ESPE, St. Paul, MN, USA)	Resin-based composite	Bis-GMA, Bis-EMA, UDMA, TEGDMA Zirconia/silica cluster filler and a nonagglomerated silica filler
Vitremer (3M ESPE, St. Paul, MN, USA)	Resin-modified glass ionomer	Powder: fluoroaluminosilicate glass; redox system Liquid: aqueous solution of a modified polyalkenoic acid, HEMA
Ketac Molar Easymix (3M ESPE, St. Paul, MN, USA)	Conventional glass ionomer	Powder: fluorosilicate glass, strontium and lantanium; Liquid: polycarbonic and tartaric acids and water

Bis-GMA = bisphenol glycidyl methacrylate; UDMA = urethane dimethacrylate; TEGDMA = triethylene glycol dimethacrylate; Bis-EMA = ethoxylated pisphenol-A dimethacrylate; HEMA = 2-hydroxyethyl methacrylate

Table 2 - Mean and standard deviation of surface roughness (μm) of restorative material specimens from Groups 1 (100% relative humidity), 2 (growth medium) and 3 (*Streptococcus mutans* biofilm + growth medium) after 30 days of storage

MATERIALS	GROUP 1	GROUP 2	GROUP 3
Empress 2	0.091 ± 0.014 A	0.117 ± 0.045 A	0.106 ± 0.028 A
Filtek Supreme	0.224 ± 0.157 A	0.454 ± 0.157 A	0.541 ± 0.314 A
Vitremer	0.297 ± 0.245 A	0.485 ± 0.349 A	1.109 ± 0.532 B
Ketac Molar Easymix	0.419 ± 0.051 A	1.725 ± 0.492 A	2.695 ± 0.683 B

Groups denoted by the same letter represent no significant difference ($p<0.05$) among storage groups.

Table 3 - Mean and standard deviation of Vickers hardness number (VHN) of restorative material specimens: immediate measurements and after 30 days of storage (Groups: 1 - 100% relative humidity; 2 - growth medium; 3 - *Streptococcus mutans* biofilm + growth medium).

MATERIALS	IMMEDIATE	GROUP 1	GROUP 2	GROUP 3
Empress 2	514.40 ± 8.09 A	463.79 ± 69.47 A	500.55 ± 42.40 A	515.68 ± 24.64 A
Filtek Supreme	69.84 ± 2.37 A	70.74 ± 6.64 A	71.06 ± 13.60 A	70.15 ± 4.86 A
Vitremer	34.54 ± 7.63 A	53.51 ± 13.45 B	38.35 ± 2.78 B	26.55 ± 7.86 C
Ketac Molar Easymix	42.48 ± 2.38 A	69.95 ± 9.07 B	60.40 ± 11.36 B	54.55 ± 10.85 B

Groups denoted by the same letter in parentheses represent no significant difference ($p<0.05$).

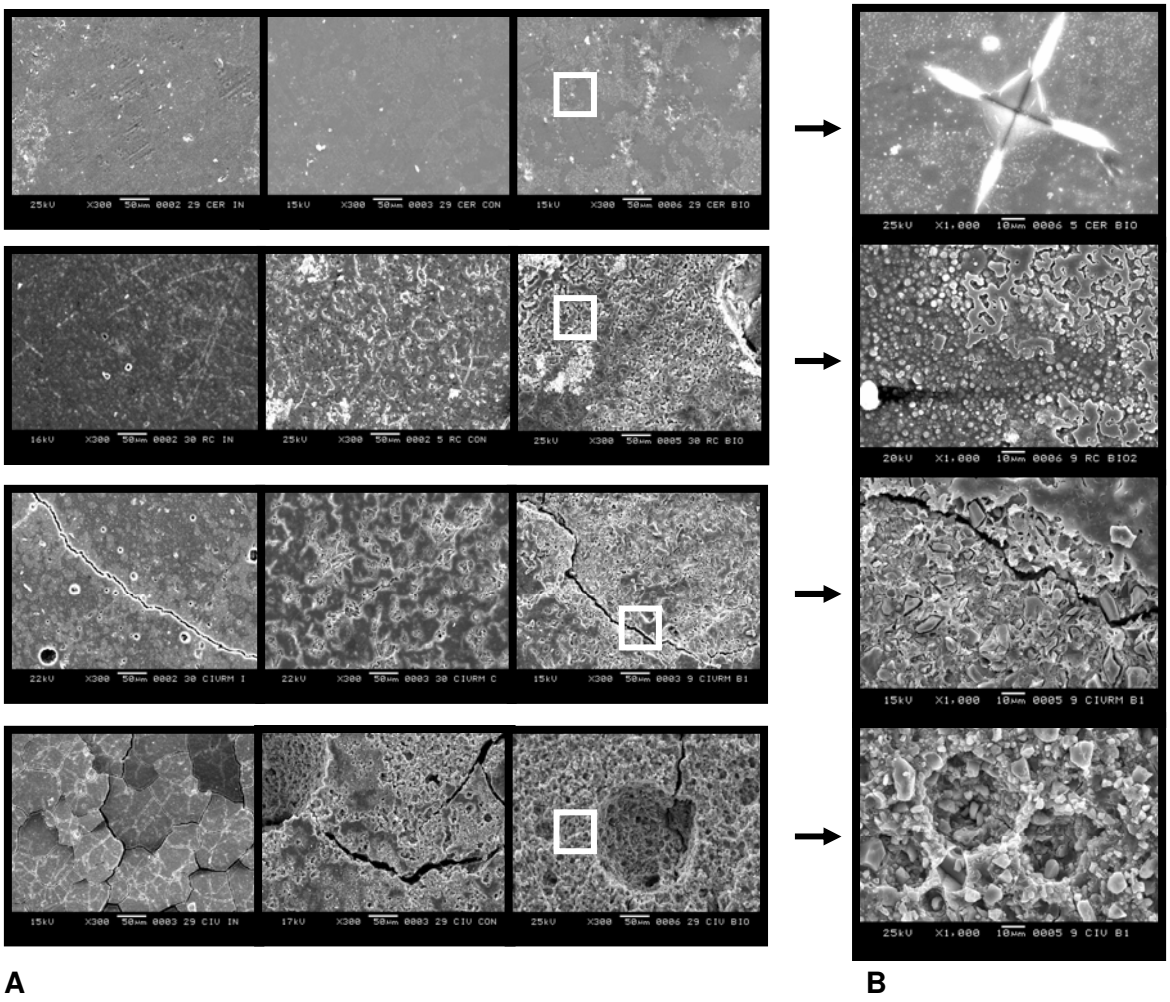

Figure 1 - Scanning Electron Microscopy images from surface of the restorative materials studied. Each row represents a different material: first row = Empress 2; second row = Filtek Supreme; third row = Vitremer; fourth row = Ketac Molar Easymix. A -The columns represent storage conditions (original magnification x300): first column = group 1; second column = group 2; third column = group 3. B – White square of each restorative material from group 3 at a original magnification x1000.

CONSIDERAÇÕES GERAIS

Os materiais restauradores odontológicos são submetidos constantemente a desafios mecânicos e químicos quando em função intra-bucal, que poderão conduzir as restaurações à biodegradação. Este processo significa a deterioração gradual dos materiais mediada por processos biológicos, químicos e mecânicos complexos, como a interação com saliva, alimentos, bebidas e microrganismos bucais. Tal deterioração pode se manifestar através da alteração em propriedades de superfície, como rugosidade (Willershausen *et al.*, 1999; Turssi *et al.*, 2003), dureza (Yap *et al.*, 2001; Gömeç *et al.*, 2004) e resistência ao desgaste (Hotta *et al.*, 1995), e alterações na microestrutura da superfície do material em contato com as substâncias degradantes (Turssi *et al.*, 2002). Logo, o material restaurador com maior habilidade em resistir à biodegradação conduzirá a um desempenho clínico satisfatório e maior longevidade das restaurações. A resistência à biodegradação está basicamente relacionada à composição química do material, isto é, no caso dos materiais restauradores poliméricos, dos monômeros que formarão a matriz dos materiais resinosos (Kawai & Takaoka, 2002; Finer & Santerre, 2004), os elementos iônicos que irão compor a matriz dos materiais ionoméricos (Nicholson, 1998; Sales *et al.*, 2003), os tipos de ligações presentes entre os componentes, entre outros. A partir destas características específicas a cada material, diferentes qualidades de superfície para a restauração podem ser obtidas.

O objetivo desta tese foi avaliar os efeitos da interação de 30 dias entre materiais restauradores estéticos e o biofilme de *Streptococcus mutans* sobre características do biofilme e propriedades dos materiais. Pôde-se observar que os diferentes materiais testados influenciaram o desenvolvimento dos biofilmes depositados sobre eles, enquanto a superfície dos materiais em contato com o biofilme também sofreram alterações em propriedades e microestrutura.

Através de revisão sistemática realizada no capítulo 1, observou-se na literatura analisada a escassez de artigos que tenham como objetivo o estudo das

implicações do biofilme sobre materiais restauradores. Dentre os estudos encontrados, verificou-se que a interação com biofilme ou ácido láctico pode prejudicar certas propriedades de superfície dos materiais restauradores, de maneira material-dependente. Os cimentos ionoméricos apresentam aumentada liberação de íons e aspectos de erosão na superfície após tais interações, enquanto resinas compostas sofrem aumento na rugosidade e diminuição na dureza de superfície. Já as resinas modificadas por poliácidos apresentam todas as alterações citadas. Nenhum artigo foi encontrado sobre possíveis efeitos em cerâmicas odontológicas. Infelizmente, os efeitos do biofilme/ácido láctico foram analisados com metodologias bem distintas pelos artigos encontrados, impossibilitando a classificação da maior parte das alterações citadas em um forte nível de evidência (segundo os critérios de classificação especificados na tabela 2 do capítulo 1).

A partir da dificuldade em encontrar sólidas conclusões no capítulo 1 quanto aos resultados da interação biofilme/materiais restauradores, os capítulos 2 e 3 foram desenvolvidos com este objetivo. Enquanto no capítulo 2 verificou-se as características do biofilme desenvolvido sobre os materiais após 30 dias, o capítulo 3 buscou detectar os efeitos nas propriedades dos materiais estudados.

No capítulo 2, observou-se a influência das características dos materiais sobre a espessura dos biofilmes de *Streptococcus mutans* depositados por 30 dias sobre discos de materiais. Diferentes composições e, conseqüentemente, propriedades poderiam explicar as maiores espessuras de biofilmes acumulados sobre Empress 2 e Filtek Supreme que sobre Vitremer e Ketac Molar Easymix. A ausência de liberação de íons com propriedades antibacterianas devido à estabilidade química dos materiais cerâmicos e a lixiviação de monômeros residuais capazes de estimular o crescimento bacteriano das resinas poderiam ter promovido biofilmes mais espessos. Em contrapartida, a liberação de íons, como flúor e alumínio, pelos cimentos ionoméricos poderiam evidenciar a influência no metabolismo bacteriano e, conseqüentemente, no desenvolvimento dos biofilmes menos espessos.

Deve-se considerar que as condições deste estudo diferem imensamente do ambiente bucal, uma vez que forças de cisalhamento intra-bucais provindas de movimentos funcionais, fatores salivares antibacterianos e oscilações de temperatura, pH e nutrientes não foram simuladas pelo experimento. Entretanto, mantendo-se as mesmas condições de crescimento para todos os espécimes testados, pôde-se isolar o fator **material restaurador** e determinar sua influência na espessura do biofilme acumulado. Já para as outras características analisadas do biofilme, é provável que a igualdade de condições experimentais tenha promovido resultados similares.

A importância da composição química dos materiais restauradores foi reafirmada no capítulo 3. Empress 2 e Filltek Supreme foram capazes de resistir à biodegradação, mantendo suas propriedades de superfície e microestrutura mesmo após a interação de 30 dias com biofilmes de *Streptococcus mutans* bastante espessos (capítulo 2). Independentemente dos metabólitos ácidos produzidos pelos microrganismos acumulados sobre tais materiais, as características físicas e químicas de superfície dos materiais prevaleceram em relação à ausência de biodegradação. A superfície vítrea e inerte da cerâmica e a presença de monômeros hidrófobos e nanopartículas na resina estudada poderiam ser responsáveis pelos resultados encontrados neste estudo. Já os cimentos ionoméricos não apresentaram o mesmo comportamento. Vitremer e Ketac Molar Easymix apresentaram aumento da rugosidade provocado pela interação com o biofilme, evidenciado microscopicamente através da degradação da matriz e protrusão de grandes partículas. A lixiviação de íons e de monômeros hidrófilos (HEMA) também levou à redução nos valores de dureza de superfície do Vitremer submetido ao contato com microrganismos.

Em resumo, esta tese contribuiu para o estudo do comportamento de materiais restauradores estéticos submetidos à interação com biofilmes de *Streptococcus mutans* por 30 dias, enfatizando dois aspectos importantes: os efeitos sobre o biofilme e deste sobre a superfície dos materiais. Os estudos apresentados nos capítulos 2 e 3 permitiram verificar diferentes comportamentos

dos materiais relacionados de maneira mais intensa com a composição destes que com as características do biofilme acumulado. Assim, estes estudos mostraram a importância da adequada seleção do material restaurador em um tratamento reabilitador, especialmente para pacientes que apresentam alto risco à cárie devido a grandes acúmulos de biofilme cariogênico e insatisfatória higiene dentária.

CONCLUSÕES

Baseado nos resultados desta tese pôde-se concluir que:

Na literatura selecionada pela revisão sistemática, verificou-se a carência de estudos que analisassem os efeitos do biofilme em propriedades de superfície e microestrutura dos materiais restauradores, impossibilitando a classificação em forte evidência da maior parte dos efeitos encontrados sobre os materiais.

O biofilme de *Streptococcus mutans* sofreu influência do material restaurador ao qual estava aderido por 30 dias apenas quanto à espessura. Biofilmes mais espessos foram encontrados depositados sobre espécimes de cerâmica e resina composta nanoparticulada. Outras características do biofilme estudadas mostraram-se semelhantes para todos os materiais.

A alteração na rugosidade de superfície, dureza e microestrutura em função da interação com o biofilme por 30 dias foi material-dependente. A rugosidade de superfície aumentou para Vitremer e Ketac Molar Easymix, enquanto a dureza diminuiu apenas para o Vitremer. Filtek Supreme, Vitremer e Ketac Molar Easymix apresentaram evidências de biodegradação microscopicamente.

A biodegradação de materiais restauradores decorrente da interação com o biofilme dentário teve como fator determinante o substrato, isto é, o material restaurador.

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[4] De acordo com a norma da FOP / UNICAMP, baseada na norma do International Committee of Medical Journal Editors – Grupo de Vancouver. Abreviatura dos periódicos em conformidade com o MEDLINE.

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APÊNDICE 1

PRANCHAS DE FIGURAS

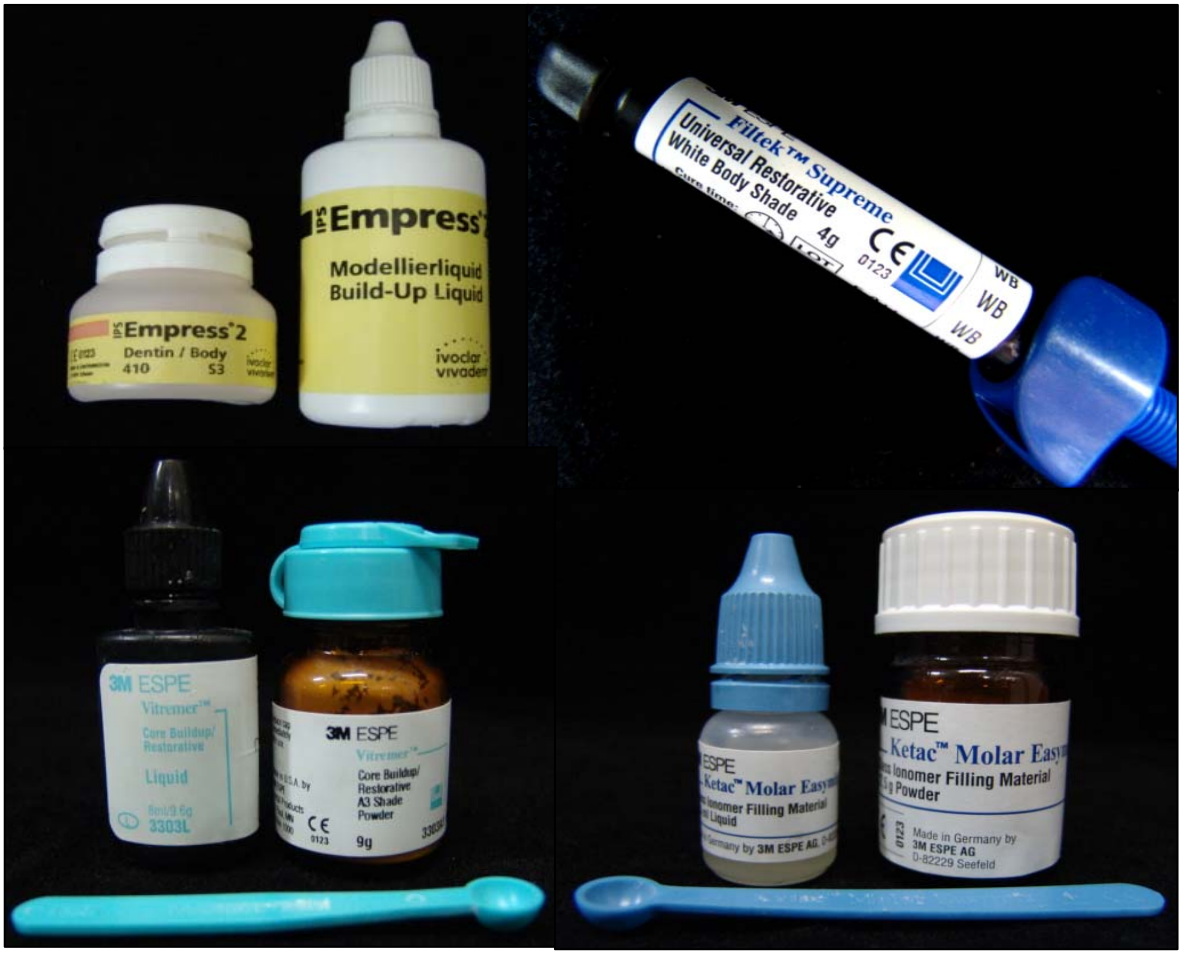

Figura 1 – ilustrações dos Capítulos 2 e 3, materiais utilizados para confecção dos espécimes

A. IPS Empress 2 (Ivoclar Vivadent, Schaan, Liechtenstein) – cerâmica
B. Filtek Supreme (3M ESPE, St. Paul, MN, USA) – resina composta
C. Vitremer (3M ESPE, St. Paul, MN, USA) – cimento de ionômero de vidro modificado por resina
D. Ketac Molar Easymix (3M ESPE, St. Paul, MN, USA) – cimento de ionômero de vidro convencional
E. Espécimes confeccionados na ordem de materiais descrita acima, da esquerda para a direita (discos de 10mm de diâmetro e 2mm de espessura)

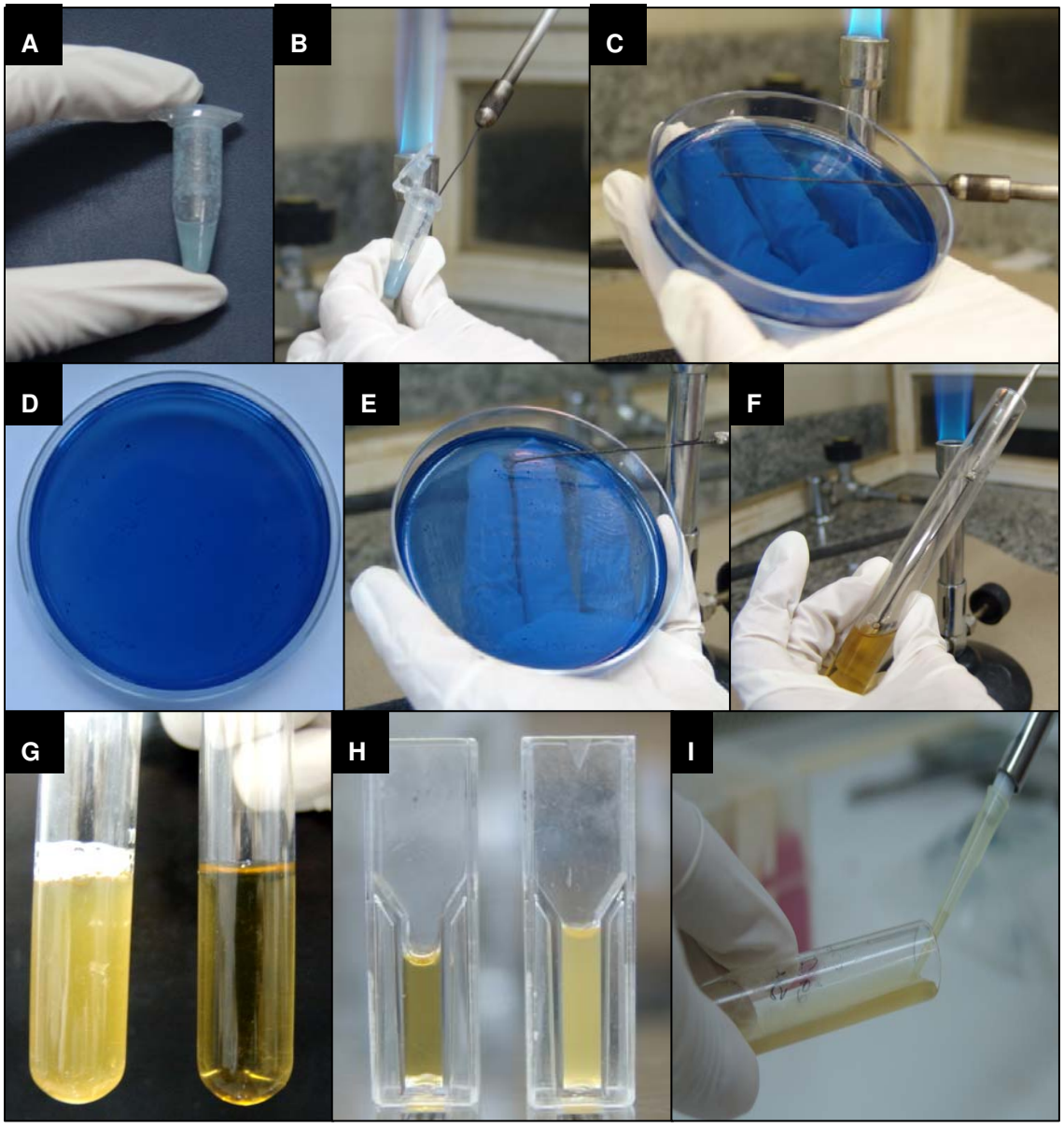

Figura 2 – ilustrações dos Capítulos 2 e 3, desenvolvimento do biofilme sobre os espécimes

A. Cepa de *Streptococcus mutans* UA159 congelada em eppendorf
B. Retirada de inóculo com alça
C. Semeadura do inóculo em placa com *Mitis salivarius* ágar
D. Colônias crescidas após incubação a 37°C em atmosfera suplementada com 5% de gás carbônico por 48 horas
E. Coleta de algumas colônias da placa

F. Inoculação das colônias em BHI (Brain-Heart Infusion) caldo
G. Tubos de ensaio com meios de cultura de turbidez diferentes: o da esquerda após incubação do meio com *Streptococcus mutans overnight* a 37°C em atmosfera suplementada com 5% de gás carbônico, e o da direita apenas meio de cultura
H. Cubetas para utilização em espectrofotômetro: o da esquerda com alíquota para a determinação do *blank* e o da direita para a determinação da densidade óptica do meio inoculado
I. Obtenção de inóculo com quantidade calculada do meio incubado
J. Obtenção de quantidade calculada de solução salina
L. União das porções de inóculo e salina para obtenção de meio com densidade óptica 0,25
M. Pipetagem de 80µL do meio citado no item acima sobre a superfície a ser estudada do disco de material restaurador para incubação por 2 horas e adesão de um biofilme inicial

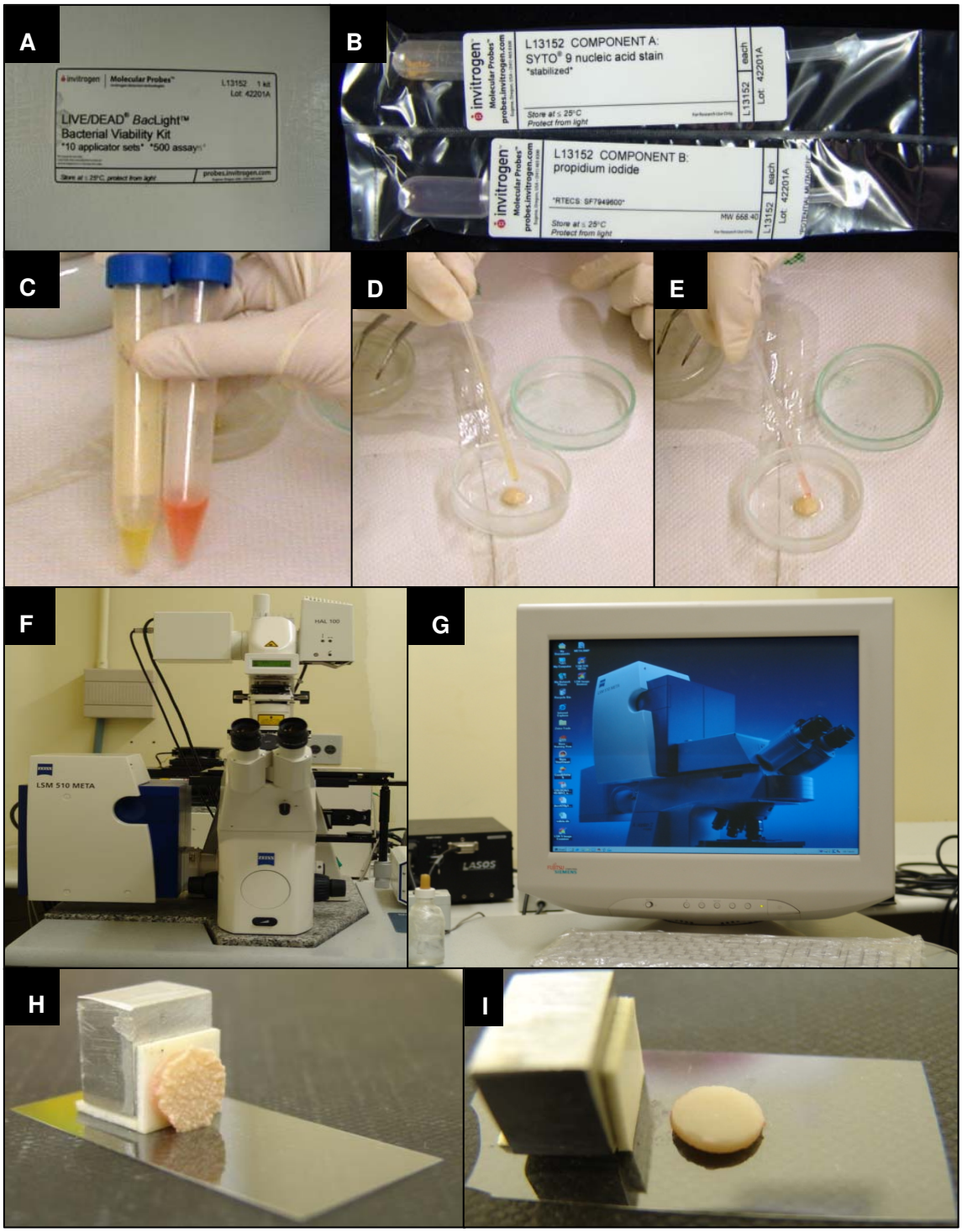

Figura 3 – ilustrações do Capítulo 2, procedimentos realizados para a visualização do biofilme em microscopia confocal de varredura a laser após 30 dias de acúmulo sobre os discos

A. Kit de corante LIVE/DEAD® BacLight™ Bacterial Viability (Molecular Probes, Eugene, OR, EUA)
B. Ampolas do kit contendo de maneira pré-dosada os corantes SYTO 9 (cima) e iodeto de propídio (baixo)
C. Tubos Falcon contendo os corantes diluídos em água destilada (esquerda: SYTO 9; direita: iodeto de propídio)
D. Aplicação de uma gota do corante SYTO 9 sobre o biofilme
E. Aplicação de uma gota do corante iodeto de propídio sobre o mesmo biofilme
F. Microscópio Confocal de Varredura a Laser (LSM 510 META, Zeiss, Alemanha)
G. Software do microscópio acoplado
H. Disco coberto por biofilme posicionado em lamínula de maneira que vistas de perfil pudessem ser obtidas pelo microscópio
I. Disco com superfície coberta pelo biofilme posicionada em contato com a lamínula para obtenção de imagens vistas frontalmente

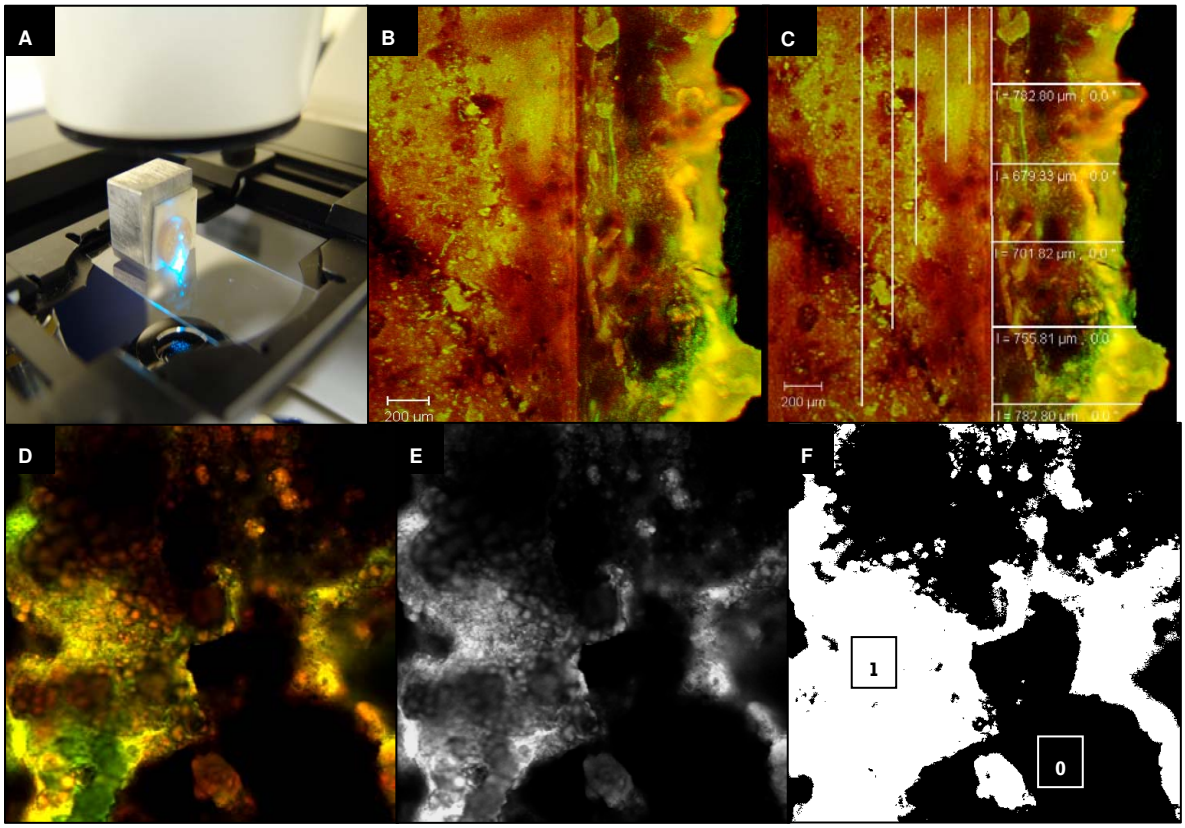

Figura 4 – ilustrações do Capítulo 2, procedimentos realizados para a análise dos biofilmes após 30 dias de acúmulo sobre os discos

A. Lamínula contendo conjunto disco/biofilme adaptado ao microscópio e laser FITC ligado para a obtenção de imagens de perfil
B. Vista perfil do conjunto disco/biofilme num aumento de 4x/0.13

C. Utilização do software do próprio microscópio para a obtenção de 5 medidas eqüidistantes da espessura do biofilme visualizado (B)
D. Vista frontal do biofilme num aumento de 10x/0.3
E. Adaptação da imagem original (D) em escala de cinza para a realização do *threshold* pelo software COMSTAT
F. Adaptação da imagem com threshold determinado (E) em preto e branco para a determinação de valores de biomassa (branco – 1) e *background* (preto – 0) pelo software COMSTAT

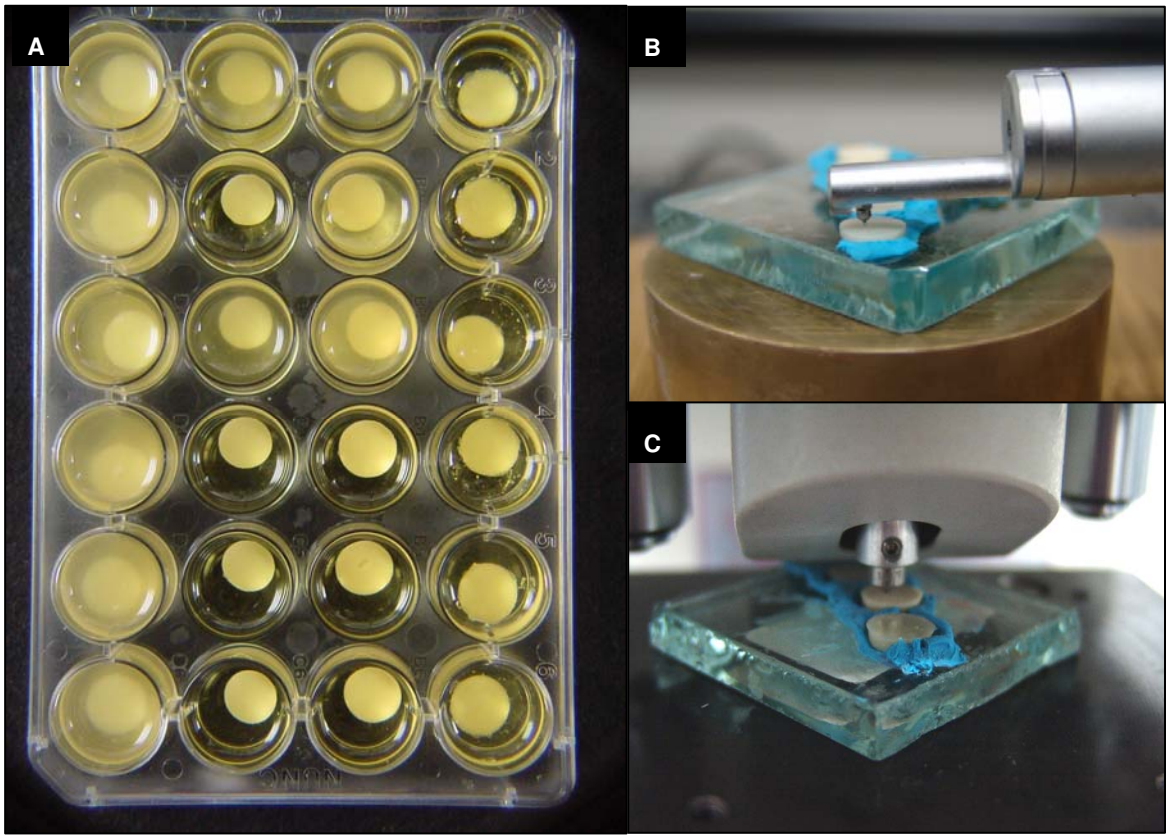

Figura 5 – ilustrações do Capítulo 3, análise dos discos de materiais.

A. Placa de cultura de 24 poços (Multidish 24-well Nunclon) nos quais os discos do grupo 2 (armazenamento apenas em meio de cultura) e grupo 3 (meio d cultura e biofilme) foram mantidos por 30 dias;
B. Agulha do rugosímetro (Surfcorder SE 1700, Japão) posicionada para leitura na superfície do espécime;
C. Ponta do microdurômetro (Shimatzu, Japão) posicionada para realização da indentação sobre a superfície em estudo.

APÊNDICE 2

Análise Estatística Capítulo 2

Delineamento experimental: Espessura Média do biofilme

Transformação das observações segundo função exponencial

Tabela 1 – Análise de Variância dos dados de espessura média

Causa de variação	G.L.	S.Q.	Q.M.	Valor de F	Pr>F
Material	3	575,6220412	191,8740137	16,83	<0,0001
Resíduo	46	524,513761	11,402473		
Total corrigido	56	1453,644473			

R-Quadrado	Coef. Var.	Raiz QMR	Média de t_espes
0,639173	13,82104	3,376755	24,43199

Tabela 2 – Teste de Tukey para médias de espessura para o fator material

Grupo	Repetições	Médias originais	5%
IPS Empress 2	13	27,442	A
Filtek Supreme	15	27,948	A
Vitremer	15	21,447	B
Ketac Molar Easymix	14	21,092	B

Médias seguidas por letras distintas diferem entre si ao nível de significância indicado.

Delineamento experimental: Bio-volume do biofilme

Transformação das observações segundo função exponencial

Tabela 3 – Análise de Variância dos dados de bio-volume

Causa de variação	**G.L.**	**S.Q.**	**Q.M.**	**Valor de F**	**Pr>F**
Material	3	0,39184109	0,13061370	0,75	0,5266
Resíduo	44	7,63296498	0,17347648		
Total corrigido	54	11,34773575			

R-Quadrado	Coef. Var.	Raiz QMR	Média de t_biov
0,327358	9,703898	0,416505	4,292142

Tabela 4 – Teste de Tukey para médias de bio-volume para o fator material

Grupo	**Repetições**	**Médias originais**	**5%**
IPS Empress 2	13	4,3381	A
Filtek Supreme	14	4,4030	A
Vitremer	15	4,2339	A
Ketac Molar Easymix	13	4,1941	A

Médias seguidas por letras distintas diferem entre si ao nível de significância indicado.

Delineamento experimental: Coeficiente de Rugosidade do biofilme

Transformação das observações segundo função exponencial

Tabela 5 – Análise de Variância dos dados de coeficiente de rugosidade

Causa de variação	G.L.	S.Q.	Q.M.	Valor de F	Pr>F
Material	3	0,01990084	0,00663361	0,22	0,8818
Resíduo	44	1,32509318	0,03011575		
Total corrigido	54	2,10410139			

R-Quadrado	Coef. Var.	Raiz QMR	Média de t_coefru
0,370233	38,28405	0,173539	0,453293

Tabela 6 – Teste de Tukey para médias de coeficiente de rugosidade para o fator material

Grupo	Repetições	Médias originais	5%
IPS Empress 2	13	0,47372	A
Filtek Supreme	14	0,47689	A
Vitremer	15	0,44282	A
Ketac Molar Easymix	13	0,41954	A

Médias seguidas por letras distintas diferem entre si ao nível de significância indicado.

Delineamento experimental: Razão superfície / volume do biofilme

Transformação das observações segundo função exponencial

Tabela 7 – Análise de Variância dos dados de superfície / volume

Causa de variação	G.L.	S.Q.	Q.M.	Valor de F	Pr>F
Material	3	0,69441494	0,23147165	1,26	0,3011
Resíduo	44	8,10790481	0,18427056		
Total corrigido	54	11,66444307			

R-Quadrado	Coef. Var.	Raiz QMR	Média de t_supvol
0,304904	-15,93329	0,429267	-2,694154

Tabela 8 – Teste de Tukey para médias de superfície / volume para o fator material

Grupo	Repetições	Médias originais	5%
IPS Empress 2	13	-2,5645	A
Filtek Supreme	14	-2,5781	A
Vitremer	15	-2,8072	A
Ketac Molar Easymix	13	-2,8183	A

Médias seguidas por letras distintas diferem entre si ao nível de significância indicado.

Análise Estatística Capítulo 3

Delineamento experimental: Dureza dos materiais

Transformação das observações segundo função exponencial

Tabela 9 – Análise de Variância para a variável dureza dos materiais em diferentes condições de meio (grupo 1 = umidade relativa; grupo 2 = meio de cultura; grupo 3 = meio e biofilme)

Causa de variação	G.L.	S.Q.	Q.M.	Valor de F	Pr>F
Meio	2	4,006795	2,003397	3,53	0,0338
Resíduo	82	46,519721	0,567314		
Total corrigido	100	4577,908926			

R-Quadrado	Coef. Var.	Raiz QMR	Média de t_dureza
0,989838	6,736459	0,753202	11,18098

Tabela 10 – Teste de Tukey para médias de dureza de IPS Empress 2 para o fator condição do meio

Grupo	Repetições	Médias originais	5%
1	8	463,79	A
2	16	500,55	A
3	40	515,68	A

Médias seguidas por letras distintas diferem entre si ao nível de significância indicado.

Tabela 11 – Teste de Tukey para médias de dureza de Filtek Supreme para o fator condição do meio

Grupo	Repetições	Médias originais	5%
1	8	70,7420	A
2	8	71,0560	A
3	40	70,1453	A

Médias seguidas por letras distintas diferem entre si ao nível de significância indicado.

Tabela 12 – Teste de Tukey para médias de dureza de Vitremer para o fator condição do meio

Grupo	Repetições	Médias originais	5%
1	8	53,5080	A
2	8	38,3480	A
3	32	26,5460	B

Médias seguidas por letras distintas diferem entre si ao nível de significância indicado.

Tabela 13 – Teste de Tukey para médias de dureza de Ketac Molar Easymix para o fator condição do meio

Grupo	Repetições	Médias originais	5%
1	8	69,9520	A
2	8	60,3900	A
3	32	54,5527	A

Médias seguidas por letras distintas diferem entre si ao nível de significância indicado.

Tabela 14 – Teste t pareado para médias de dureza de IPS Empress 2 para o fator tempo

Grupo	Repetições	Médias originais	5%
Imediato	5	514,4000000	A
30 dias	5	463,7920000	A

Médias seguidas por letras distintas diferem entre si ao nível de significância indicado.

Tabela 15 – Teste t pareado para médias de dureza de Filtek Supreme para o fator tempo

Grupo	Repetições	Médias originais	5%
Imediato	5	69,8440000	A
30 dias	5	70,7420000	A

Médias seguidas por letras distintas diferem entre si ao nível de significância indicado.

Tabela 16 – Teste t pareado para médias de dureza de Vitremer para o fator tempo

Grupo	Repetições	Médias originais	5%
Imediato	5	34,5440000	A
30 dias	5	53,5080000	B

Médias seguidas por letras distintas diferem entre si ao nível de significância indicado.

Tabela 17 – Teste t pareado para médias de dureza de Ketac Molar Easymix para o fator tempo

Grupo	Repetições	Médias originais	5%
Imediato	5	42,4800000	A
30 dias	5	69,9520000	B

Médias seguidas por letras distintas diferem entre si ao nível de significância indicado.

Delineamento experimental: Rugosidade de Superfície dos materiais

Transformação das observações segundo função exponencial

Tabela 18 – Análise de Variância para a variável rugosidade de superfície dos materiais em diferentes condições de meio (grupo 1 = umidade relativa; grupo 2 = meio de cultura; grupo 3 = meio e biofilme)

Causa de variação	**G.L.**	**S.Q.**	**Q.M.**	**Valor de F**	**Pr>F**
Meio	2	12,2346879	6,1173440	26,37	<0,0001
Resíduo	82	19,0213247	0,2319674		
Total corrigido	100	151,9339407			

R-Quadrado	Coef. Var.	Raiz QMR	Média de t_rugos
0,874805	-60,60957	0,481630	-0,794643

Tabela 19 – Teste de Tukey para médias de rugosidade de IPS Empress 2 para o fator condição do meio

Grupo	**Repetições**	**Médias originais**	**5%**
1	8	0,0914	A
2	16	0,1175	A
3	40	0,1060	A

Médias seguidas por letras distintas diferem entre si ao nível de significância indicado.

Tabela 20 – Teste de Tukey para médias de rugosidade de Filtek Supreme para o fator condição do meio

Grupo	Repetições	Médias originais	5%
1	8	0,2242	A
2	8	0,4543	A
3	40	0,5409	A

Médias seguidas por letras distintas diferem entre si ao nível de significância indicado.

Tabela 21 – Teste de Tukey para médias de rugosidade de Vitremer para o fator condição do meio

Grupo	Repetições	Médias originais	5%
1	8	0,2971	A
2	8	0,4854	A
3	32	1,1089	B

Médias seguidas por letras distintas diferem entre si ao nível de significância indicado.

Tabela 22 – Teste de Tukey para médias de rugosidade de Ketac Molar Easymix para o fator condição do meio

Grupo	Repetições	Médias originais	5%
1	8	0,4190	A
2	8	1,7246	A
3	32	2,6952	B

ANEXOS

De: Biomaterials <biomaterials@online.be>
Enviado: sexta-feira, 5 de janeiro de 2007 16:03:07
Para: <nana_beatriz@hotmail.com>
Assunto: Submission Confirmation for Dental biofilm: effects on esthetic restorative material surfaces: a systematic review

Dear Ms Fúcio,

I am writing to acknowledge receipt of your submission entitled "Dental biofilm: effects on esthetic restorative material surfaces: a systematic review" which you have submitted to the journal Biomaterials. We are very pleased that you have the confidence in the journal to submit your work to us.

You will be able to check on the progress of your paper by logging on to Editorial Manager as the corresponding author. The URL is http://biomat.edmgr.com/.

Your manuscript will be given a reference number shortly.

Thank you for submitting your work to this journal.

Yours sincerely,

Peggy O'Donnell
Managing Editor
Biomaterials

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Piracicaba, 18 de Dezembro de 2006.

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