GISLAINE CRISTINA PADOVANI

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"EVALUATION OF THE SURFACE OF RESTORATIVE MATERIALS SUBMITTED TO DEGRADATION BY BIOFILM, *IN SITU*"

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"AVALIAÇÃO DA SUPERFÍCIE DE MATERIAIS RESTAURADORES SUBMETIDOS À DEGRADAÇÃO POR BIOFILME, *IN SITU*"

Tese de Doutorado apresentada ao programa de Pós-Graduação em Materiais Dentários da Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do Titulo de doutora em Materiais Dentários.

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RESUMO

Os objetivos desta tese composta por dois capítulos foram: **Capítulo 1**- avaliar as características estruturais e a viabilidade do biofilme, *in situ*, acumulado após 07 dias sobre diferentes materiais restauradores e **Capítulo 2** - avaliar as características de superfície de diferentes materiais restauradores após 07 dias à biodegradação *in situ*. No capítulo 1 - quinze discos de cada material (IPS E.Max; Filtek Supreme; Vitremer; Ketac Molar Easymix; Amalgam GS-80) foram confeccionados em uma matriz metálica de 4,0mm X 1,5mm. O polimento foi realizado com o auxílio de discos Sof-Lex para os corpos-de-prova de Filtek Supreme, Vitremer e Ketac Molar Easymix; o Amalgam GS-80 foi polido com o kit de polimento para amálgama de prata e os corpos-de-prova de IPS E.Max foram jateados por óxido de alumínio, seguidos por ponta diamantada, taça de borracha e glazer. Para a realização da biodegradação *in situ*, foram selecionados quinze voluntários com idades entre 21–30 anos. Um dispositivo palatino, contendo 5 poços (um material restaurador por poço) foi confeccionado para cada voluntário. Após 07 dias de biodegradação imagens do biofilme utilizando o Microscópio de Varredura Confocal a Laser (CLSM) foram realizadas. A análise quantitativa foi realizada com o auxílio do *software* COMSTAT avaliando parâmetros como: área, biomassa, espessura média, espessura máxima e coeficiente de rugosidade. Na análise qualitativa, as imagens CLSM foram visualmente observadas e descritas como: viabilidade celular - predominância de verde e vermelho e arquitetura do biofilme - predominância e distribuição de espaços e canais no biofilme. Os dados foram analisados estatisticamente utilizando o teste de Kolmogorov-Smirnov e Kruskal-Wallis ao nível de 5% de significância. Os resultados demonstraram não haver diferença estatística entre os materiais avaliados e os parâmetros selecionados. Porém, Filtek Supreme e IPS E.Max apresentaram visualmente maior quantidade de células viáveis em comparação aos outros materiais restauradores. Assim, conclui-se que: na análise quantitativa não houve influência significativa do material restaurador sob as características morfológicas do biofilme. Entretanto, a análise qualitativa demonstrou aparentemente variações na viabilidade bacteriana frente aos diferentes

materiais restauradores. No capítulo 2 – A metodologia empregada na confecção e preparo dos corpos-de-prova foi a mesma utilizada no capítulo 1, sendo os corpos-de-prova submetidos à leitura de dureza, rugosidade de superfície, análise de Microscopia Eletrônica de Varredura e análise de Energia Dispersiva de Raio-X antes e após a biodegradação. Os dados foram submetidos aos testes de Kolmogorov-Smirnov e Tukey-Kramer, nível de significância de 5%. Todos os materiais restauradores estéticos mostraram aumento significativo nos valores de rugosidade após biodegradação, confirmada pelas micrografias. Para a análise da dureza, Vitremer, Ketac Molar Easymix e Amalgam Gs-80 demostraram aumento significativo nos valores após a biodegradação. A composição química inicial do Amálgama GS-80 e IPS E.Max não foi alterada pela ação do biofilme *in situ*, enquanto Filtek Supreme, Vitremer e Ketac Molar Easymix apresentaram alterações em seus espectros, com liberação de fluoreto para os materiais ionoméricos e adsorção de íons (Ca^{2+}, Cl^{-}) na superfície desses materiais. Pode-se concluir que os efeitos do biofilme sobre as propriedades de superfície são material-dependente.

Palavras-chave: Biofilmes, Materiais dentários, Microscopia confocal, Propriedades de superfície.

ABSTRACT

The objectives of this Thesis, accomplished on two chapters, were: Chapter 1 – to evaluate the influence of different restorative materials on the morphology of biofilm, in situ, and Chapter 2 – to evaluate the influence of biofilm on the surface characteristics of different restoratives materials. **Chapter 1**- Fifteen discs of each material (IPS E.Max; Filtek Supreme; Vitremer; Ketac Molar Easymix; Amalgam GS-80) were prepared using a metallic mold of 4.0 mm X 1.5 mm. Polishing procedure was performed with sof-lex discs on composite resin, glass ionomer cement and cement-modified glass ionomer specimens; silver amalgam specimens were polished with a polishing kit for amalgam; ceramic specimens were polished with a aluminum oxide jet, followed by a diamond drill, rubber tip and glazer. To perform the in situ biodegradation, fifteen volunteers were selected (aged 21-30 years). A palatal device containing 5 wells (one restorative material per well) was prepared for each volunteer. After 07 days of biodegradation, a morphology analysis of the biofilm was performed using confocal laser scanning microscopy. The images generated by confocal microscopy were then converted into numerical data with the aid of the COMSTAT software, which evaluated parameters such as: area, biomass, average thickness, maximum thickness and roughness coefficient. Qualitative analysis was also performed to assess the architecture and bacterial viability. Data were submitted to Kolmogorov-Smirnov test and Kruskal-Wallis tests at 5% of significance. The results showed no statistical difference between the materials studied for the selected parameters, although the Filtek Supreme e IPS E.Max showed a higher amount of viable cells compared to other restorative materials. Thus, it was concluded that the quantitative analysis did not showed a significant influence of the restorative material on the structural characteristics of the biofilm. However, the qualitative analysis showed variations in bacterial viability on different restorative materials. In chapter 2 – The methodology employed in the manufacture and preparation of the specimens was the same used in Chapter 1. The specimens were submitted to the reading of hardness, roughness analysis, Scanning Electron Microscopy and Energy Dispersive X-Ray analysis before and after

biodegradation. The data were subjected to the Kolmogorov-Smirnov and Tukey-Kramer test, at a significance level of 5%. All esthetic restorative materials showed a significant increase in roughness values after biodegradation. For the analysis of hardness, Vitremer, Ketac Molar Easymix and Amalgam Gs-80 demonstrated significant increase in the values after biodegradation. The initial chemical composition of the Amalgam GS-80 and IPS E.Max was not change by the action of biofilms *in situ*, while Filtek Supreme, Ketac Molar Easymix and Vitremer showed changes in their spectra, with fluoride release from glass ionomer materials and incorporation ions (Ca^{2+}, Cl^{-}) on the surface of some materials analyzed (Filtek Supreme, Ketac Molar Easymix and Vitremer).Under the conditions of this study, one can conclude that the effects of biofilm on the surface properties are material-dependent.

Key-words: Biofilms, Dental materials, microscopy confocal, surface properties

SUMÁRIO

INTRODUÇÃO

Biofilmes são complexos ecossistemas microbiológicos organizados em estrutura tridimensional e embebidos em matriz de polissacarídeo, aderidos a uma superfície sólida, necessitando apenas de fonte regular de nutrientes fornecida através de canais internos, os quais penetram na matriz para também conduzir oxigênio, metabólitos, produtos de excreção e enzimas (Carpentier & Cerf, 1993; Surman, *et al*., 1996).

A agregação dos microrganismos em biofilmes favorece, entre outros propósitos, a sobrevivência e multiplicação, pois as bactérias presentes no biofilme oral expressam fenótipos diferentes das bactérias planctônicas (culturas de células livres), o que leva o biofilme a apresentar maiores vantagens metabólicas, patogenicidade mais severa e tolerância a fatores ambientais como variação do pH, oxigênio, radiação UV, desidratação e predadores (Bradshaw et al., 1998; Sedlacek & Walker, 2007; Welin-Neilands & Svensäter, 2007). Assim, a simbiose entre as bactérias, sinergismo, co-agregação e aumento da capacidade de colonizar o substrato, especialmente daqueles microorganismos que não aderem ao substrato por si só, são fatores essenciais para a sobrevida do biofilme (Özok et al., 2007).

A organização em comunidades alcançada pelo biofilme constitui numa maior resistência a agentes antimicrobianos (aproximadamente 1.000 vezes) comparada as bactérias planctônicas, possivelmente devido à maior restrição física imposta pela matriz extracelular à difusão de agentes antimicrobianos, associado ao crescimento lento do biofilme. Além disso, o biofilme pode reter enzimas, como a β-lactamase, que inativam e degradam antibióticos (Baker-Austin, 2000; Gilbert et al., 2002; Patel, 2005; Anderson & O'toole, 2008).

A dinâmica de formação do biofilme ocorre em etapas distintas: adesão, colonização, acúmulo, comunidade clímax e dispersão (Wood *et al.,* 2000). Inicialmente, observam-se os organismos denominados colonizadores primários, que se aderem à determinada superfície, geralmente contendo proteínas ou outros compostos orgânicos, como a película adquirida, encontrada na cavidade oral. As células aderidas passam a

desenvolver-se, originando microcolônias que sintetizam matriz exopolissacarídica (EPS), a qual passa a atuar como substrato para a aderência de microrganismos denominados colonizadores secundários. Estes colonizadores secundários podem se aderir diretamente aos primários ou promover a formação de coagregados com outros. As microcolônias que compõem o biofilme podem ser de uma ou várias espécies, dependendo das condições ambientais. Por exemplo, em locais de grande estresse mecânico, tais como a superfície dental, o biofilme é bastante compactado e estratificado, além de fortemente aderido à superfície dental e/ou de materiais restauradores, resistindo às forças cisalhantes geradas durante a mastigação (Wood *et al*., 2000; Kolenbrander *et al*., 2010).

A adesão do biofilme à superfície sólida parece estar relacionada a dois processos: bioquímico, no qual adesinas específicas das bactérias (203-KDa, 259-KDa, 70-KDa) se ligam em receptores localizados nas superfícies de sólidos ou de outras bactérias (amilases, mulcinas, glucans e proteínas ricas em prolina); e físico-químico baseado na energia de superfície, forças de Van der Waals, e interações eletrostáticas (Rickard *et al*., 2003; Teughels *et al*., 2006), sendo a água fundamental na adesão, atuando como uma camada que neutraliza as cargas eletrostáticas negativas tanto da superfície sólida quanto da bactéria (Teughels *et al*., 2006). A partir das características de cada material, diferentes qualidades de superfície para a adesão do biofilme podem ser obtidas. Assim, certas interações físico-químicas, tais como: hidrofobia da superfície, partícula de carga, rugosidade e constituição química dos materiais também têm sido estudadas como fatores que contribuem para mediar à adesão bacteriana durante a formação do biofilme (Sawhney & Berry, 2009).

O desenvolvimento do biofilme está intrinsecamente relacionado ao sistema *quorum-sensing*, no qual há sinalização célula-célula que permite que estas reajam às mudanças do meio, podendo sobreviver e desenvolver-se (Keller & Surette, 2006). Em comparação com as células planctônicas, a maioria das características atribuídas ao crescimento microbiano em biofilmes pode ser explicada pelo fenômeno de transferência (Stewart, 2003). Em culturas de células livres (planctônicas), o transporte de solutos do meio líquido (normalmente bem homogeneizado) para uma célula, ou vice-versa, é um processo relativamente rápido, não constituindo um passo limitante na totalidade dos

bioprocessos que ocorrem na célula (Keller & Surette, 2006). Agregados microbianos, no entanto, são ambientes densamente unidos onde o fluxo de líquido é limitado e se dá através de difusão nos "canais de água" presentes em toda massa do biofilme (de Beer *et al*., 1994). Além disso, este processo é agravado pelo fato do biofilme ser uma massa espessa, com maior distância para o transporte de solutos, tornando-se cineticamente mais lento em comparação com os bioprocessos dos microrganismos. Nestas situações, formam-se gradientes de solutos no biofilme, constituindo condição favorável para o desenvolvimento de micronichos e cárie dentária (Characklis *et al*., 1990).

Uma característica essencial dos materiais restauradores deveria ser a capacidade de resistir à degradação inerente da cavidade oral, durante um período de tempo. Biodegradação é definida como a desagregação gradual de um material mediada pela atividade biológica específica. Na boca, este é um processo complexo, incluindo a desintegração e dissolução na saliva e outros tipos de produtos químicos, a degradação física, tais como o desgaste e erosão provocada pela ingestão de alimentos, a atividade de mastigação e bacteriana (Oilo, 1992).

O biofilme apresenta habilidade em colonizar materiais restauradores e protéticos (Kawai & Urano, 2001; Steinberg & Eyal, 2002; Eick *et al*., 2004; de Fúcio *et al.,* 2008, 2009; Brentel *et al*., 2010), podendo degradar a superfície de tais materiais (Willershausen *et al*., 1999; de Fúcio *et al*., 2008). Assim, a diminuição da dureza (Yap *et al*., 2000; Barbosa *et al*., 2012) e o aumento da rugosidade de materiais restauradores resinosos (Yap *et al*., 2000; Turssi *et al*., 2002) tem sido demonstrados *in vitro*. A superfície das restaurações torna-se mais susceptível ao desgaste e, consequentemente, à perda de componentes, podendo resultar na alteração da forma anatômica e afetar o desempenho clínico destes materiais. Além disso, o aumento da rugosidade de superfície dos materiais restauradores está diretamente relacionado à retenção de biofilme. Bollen *et al*. (1997) demonstraram que o aumento na rugosidade de superfície promove rápida colonização e maturação do biofilme, aumentando a susceptibilidade ao manchamento e corrosão dos materiais restauradores, além de aumentar o risco de desenvolvimento de cárie secundária e doença periodontal (Bagheri *et al*., 2005).

Embora, pouco se saiba sobre a microbiologia das lesões secundárias de cárie, pode-se sugerir que a quantidade de biofilme e seu potencial cariogênico dependem, entre outros fatores, das características de superfície dos materiais restauradores (Moura *et al*., 2004). Assim, materiais resinosos tendem a acumular biofilme potencialmente mais cariogênico e com maiores espessuras que materiais ionoméricos ou amálgama (Ono *et al*., 2007; Souza *et al*., 2009). Os cimentos ionoméricos têm demonstrado em estudos *in vitro* o desenvolvimento de biofilmes menos espessos, associados a aspectos de erosão na superfície desse material, provavelmente devido a maior liberação de íons como flúor e alumínio, que poderia influenciar o metabolismo bacteriano (de Fúcio *et al.,* 2008; Pandit *et al*., 2011). O amálgama de prata apresenta discreta alteração de superfície devido à lixiviação de seus componentes, principalmente o zinco, que atuariam inibindo o desenvolvimento do biofilme (Morrier *et al*., 1998; Auschill *et al*., 2002**)**. Discreta alteração da superfície foi encontrada nas cerâmicas odontológicas quando submetidas à biodegradação, provavelmente devido à ausência de liberação de íons com propriedades antibacterianas em decorrência da estabilidade química dos materiais cerâmicos e superfície vítrea (Auschill *et al*., 2002; de Fúcio *et al*., 2009; Brentel *et al*., 2010).

A incorporação de substâncias antibacterianas em alguns materiais dentários tende a diminuir a patogenicidade do biofilme (Dummer & Harrison, 1982; Ørstavick, 1985; Wallman-Björklund *et al*., 1987; Svanberg *et al*., 1990; Lyttle & Bowden, 1993; Netuschil *et al*., 1996; Nicholson *et al*., 2000; Hayacibara *et al*., 2003; de Fúcio *et al*., 2008). A resistência à biodegradação parece estar basicamente relacionada à composição química do material; no caso dos materiais restauradores poliméricos, à presença dos monômeros que formarão a matriz resinosa (Finer & Santerre, 2004); aos elementos iônicos que irão compor a matriz dos materiais ionoméricos (Sales *et al*., 2003); aos tipos de ligações presentes entre os componentes.

As diferentes classes de materiais restauradores possuem a capacidade de acumular biofilmes com características diversas de: espessura, biovolume, arquitetura interna e viabilidade celular (Stoodley *et al*., 1999, de Fúcio *et al*., 2008); dinamicamente, propriedades como rugosidade, dureza, resistência ao desgaste, e características microestruturais inerentes a essa gama de materiais restauradores também podem sofrer

alterações em função da acidogenicidade do biofilme e da capacidade desse material em resistir a tal condição (Willershausen *et al*., 1999).

A maioria dos estudos que avalia a estrutura do biofilme em relação ao metabolismo e ecologia microbiana está restrito ao modelo mono-espécie *in vitro*, pois esses contêm informações fundamentais, sendo possível controlar o crescimento e o ambiente do biofilme empregando menor tempo e custo**.** No entanto, no modelo *in vitro* não há formação do biofilme com heterogeneidade genotípica ou espacial. Há apenas a evolução de bactérias longe do ambiente natural (Sissons, 1997). Além disso, há um comportamento distinto no desenvolvimento do biofilme, como variações no número de unidades formadoras de colônia e espessura desse biofilme, apresentado por diferentes materiais restauradores que limitam a situação clínica e os resultados (Hahn *et al*., 1993; Brambilla *et al*., 2005; Beyth *et al*., 2006).

A relevância de um trabalho *in situ* reside principalmente na similaridade com a situação clínica como a presença de forças de cisalhamento decorrentes de movimentos funcionais, agentes antibacterianos presentes na saliva, oscilações de temperatura, pH e nutrientes. Além disso, o biofilme dental é composto por mais de 500 espécies de bactérias, sendo que mais da metade ainda não foi cultivada (Foster *et al*., 2004). Estudos *in situ* apresentam também vantagens sobre os ensaios clínicos: estas vantagens incluem *design* experimental mais flexível, menores problemas éticos, logísticos, custo, tempo, menor variabilidade e facilidade na interpretação dos resultados, além da obtenção de um biofilme intacto preservando a complexa estrutura do mesmo (Paradella *et al*., 2008). Entretanto, os desafios do método *in situ* estão na simulação dos vários fatores presentes na cavidade oral e na necessidade da colaboração de voluntários.

Assim, compreender a estrutura do biofilme e a interação deste com o material restaurador, mais próximo da situação clínica, através de análises quantitativa e qualitativa, considerando que o biofilme produzido na cavidade oral apresenta-se em condições diferentes daquelas encontradas na produção de biofilmes *in vitro*, é fundamental.

Neste contexto, esta TESE[1] composta por dois capítulos, propôs: 1) avaliar as características estruturais e a viabilidade do biofilme, *in situ*, acumulado após 07 dias sobre diferentes materiais restauradores; 2) avaliar as características de superfície de diferentes materiais restauradores após 07 dias à biodegradação *in situ*. As hipóteses testadas neste trabalho são: 1) diferentes materiais afetam de forma distinta a estrutura e morfologia do biofilme; 2) o biofilme altera a superfície dos materiais restauradores em função do tipo de material.

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[1] Esta tese está baseada na resolução da CCPG/002/06, a qual dispõe a respeito do formato das teses de mestrado e de doutorado aprovados pela UNICAMP.

CAPÍTULO 1

BACTERIAL ACCUMULATION ON DENTAL RESTORATIVE MATERIALS *IN SITU* – CLSM/COMSTAT ANALYSIS

ABSTRACT

The aim of this study was to evaluate the influence of different restorative materials on the architecture of biofilm, *in situ*. Fifteen discs of each material analyzed (IPS E.Max; Filtek Supreme; Vitremer; Ketac Molar Easymix; Amalgam GS-80) were fabricated in a metallic mold (4.0 mm-diameter X 1.5 mm-depth). To evaluate the accumulation of biofilm *in situ*, fifteen volunteers were selected to use palatal devices during 7 days. An individual intraoral acrylic resin palatal device, containing five wells (one restorative material per well) was prepared for each volunteer. The cariogenic challenge was provided by the application of a 20% sucrose solution extraorally on the specimens, 10x/day. After 7 days, the specimens were carefully removed from device and visualized by Confocal Laser Scanning Microscopy (CLSM). CLSM-captured images were analyzed quantitatively using COMSTAT software, for the following parameters: area, bio-volume, mean thickness, maximum thickness and roughness coefficient of the biofilm. Images were also qualitatively analyzed, through a descriptive analysis. The CLSM images were visually observed and described as: Cell viability– predominance of green and red and Biofilm Architecture - predominance and distribution voids and canals in biofilm. The statistical analysis was performed by using the Kolmogorov-Smirnov and Kruskal-Wallis tests, with a significance level of 5%. The medians of the biofilm parameters analyzed showed no significant statistical difference regarding the different materials. However, in a visual qualitative analysis of the biofilm, it seemed a material-dependent relationship, where Vitremer, Ketac Molar Easymix and Amalgam GS-80 showed visually a prevalence of non-viable cells forming small clusters but distributed by the biofilm, and voids were presented in smaller proportion in the biofilm volume compared to the Filtek Supreme and IPS

E.Max. The present findings observed through the quantitative analysis showed that regardless of the restorative material, the biofilm accumulated was structurally similar. However, the qualitative analysis showed variations in bacterial viability and architecture depending on the restorative material.

Key-words: Dental Biofilm, restorative materials, laser microscopy confocal, surface properties

1. INTRODUCTION

Biofilm is formed by organisms tightly bound one to the other and to the solid substrate by means of an exopolymer matrix into which they are embedded. Such a state brings about profound changes in the behavior of bacteria, their relation to the host and their response to environmental conditions.[1] Marsh & Bradshaw (1995)[2] described the process of developing a mature biofilm as: the formation of a conditioning film (acquired pellicle) on the tooth surface; interaction between salivary bacteria and the acquired pellicle (non-specific, followed by primary colonizers); attachment of secondary colonizers to the primary colonizers; development of horizontal and vertical stratification; growth and formation of a climax community.

Bacteria within the biofilm do not exist as independent entities but rather as a coordinated, metabolically integrated microbial community.[3] This community life-style within the dental plaque provides enormous potential benefits to the participating organisms including: (i) a broader habitat range for growth (the metabolism of early colonizers alters the local environment, making conditions suitable for the attachment and growth of more fastidious species); (ii) an increased metabolic diversity and efficiency (molecules that are normally recalcitrant to catabolism by individual organisms can be broken down by the microbial consortia); and (iii) an enhanced resistance to an environmental stress, antimicrobial agents, and host defenses.[3,4]

Biofilm accumulates on all surfaces in the mouth, regardless as to whether these are natural[5], artificial[6] or dental materials.[7] Three main factors are discussed as the cause of

biofilm formation on dental restorative composites: (a) increasing surface roughness resulting from abrasion processes;[8] (b) the material chemistry, influencing its wettability or surface free energy;[9] (c) the eluates and biodegradation products of restorative materials.[10]

It can be suggested that the amount of biofilm and its cariogenic potential depend on the substrate.[11] Thus, the resinous materials tend to accumulate a more cariogenic biofilm with higher thickness than ionomeric materials and amalgams.[12] The glass ionomer cements have shown in *in vitro* studies to have a higher release of fluoride and aluminum ions, influencing on the bacterial metabolism and, hence, in the development of thinner biofilms which are associated with aspects of erosion on the material surface.[13] The silver amalgam has shown a small surface alteration due to the leaching of its components, especially zinc, which would act inhibiting the development of the biofilm.[14] A slight surface change was also found in dental ceramics when subjected to the biodegradation, probably due to the lack of release of ions with antibacterial properties as a consequence of the chemical stability of ceramics.[15,16] However there are few studies that have evaluated the behavior of the biofilm when exposed, *in situ*, to the surface of different restorative materials.[17-19]

Fúcio *et al*. (2008) showed that *in vitro* the different restorative materials have the capacity to accumulate biofilm with different characteristics.[20] However, *in vitro* studies represent a simplified clinical design, and *in situ* studies could clarify the relationships between the multispecies biofilm formation and the oral environment with all its adversities, such as: continuous changes in pH, nutrients, temperature, among others. In this context, the purpose of this study was to evaluate the influence of different restorative materials on the architecture of biofilm, *in situ*. The hypothesis tested was that biofilm accumulated on the surfaces of different restorative materials have significant modifications regarding to their structural characteristics.

2. MATERIALS AND METHOD

2. 1. Specimen preparation and storage groups

Fifteen specimens of each restorative material described in Table 1 were fabricated using metal mold with 4mm of diameter and 1.5mm of depth, according to the manufacturers' instructions, at the temperature of 23 ± 1°C and relative humidity of 50 ± 5%. Except ceramic specimens, all materials were covered with an acetate strip (Probem Ltda, Catanduva, São Paulo, Brazil) and pressed on a glass slide to compact the material. The composite resin and resin-modified glass ionomer cement were photoactivated for 40s by a curing light (Elipar Trilight, 3M ESPE, St. Paul, MN, USA), while the chemically-cured glass ionomer cement and the amalgam were allowed to set at room temperature for 15 min. After the setting reactions, the ionomeric specimens were superficially protected with petrolium gelly. For the ceramic, specimens were fabricated in a prosthetic laboratory by using the pressing process in an oven (Programat P500 - Ivoclar Vivadent, Schaan, Liechtenstein), simulating the clinical reality. All specimens were stored for 24h at 37ºC and 100% of relative humidity and polished according to the manufacturer's instructions, with corresponding polishing kits for direct restorative materials as follow: Filtek Supreme, Vitremer and Ketac Molar Easymix, it was polished by Sof-Lex disks (3M ESPE, St. Paul, MN, USA); Amalgam GS-80 was polished with polishing kit (KG Sorensen, Cotia, SP, Brazil) and, the IPS E.Max disks were ground flat with aluminum oxide Jet (50 µm - Bio-Art, São Carlos, SP, Brazil), followed by diamond drill (4138F - KG Sorensen, Barueri, SP, Brazil), rubber tip (KG Sorensen, Barueri, SP, Brazil), washed in an ultrasonic bath (Ultrasonic Cleaner, Model USC1400, UNIQUE Ind. e Com. Ltda., São Paulo SP 04709-111, Brazil) and glazed.

2. 2. Panellists and ethical aspects

The study design was approved by the Ethics Committee (protocol 136/2009). Fifteen healthy adults, aged 21–30 years, with normal salivary flow rates, able to comply with the experimental protocol, participated in this study. They were not admitted to the study if any of the following criteria were present: active caries lesions or gingivitis, use of

fixed or removable orthodontic devices, use of any antibiotics within the 2 months prior to study initiation.[17] All the volunteers agreed to participate and signed an informed written consent.

2. 3. *In situ* phase

For each volunteer, an individual intraoral acrylic resin palatal device was made in which five well (5 mm-diameter × 2.5 mm-depth) were generated on the left and right sides, and into each of them, one restorative material per well was placed (Figure 1).[14] In order to allow biofilm accumulation, and protect it from mechanical disturbance, a plastic mesh was fixed on the acrylic resin, leaving a 1 mm space between the surface of the restorative material and the plastic mesh, for the accumulation of the dental biofilm on the specimens.[21]

Before receiving the intraoral devices, oral and written instructions of the *in situ* protocol were given to the volunteers, who signed it. There were no restrictions to the volunteer's diet. The only recommendation was to remove the device during meals and before ingesting any beverages or food, keeping the oral devices moist in plastic boxes provided by the researchers. Volunteers were instructed to perform their usually oral hygiene with a standardized fluoride dentifrice (1100 mg F/g as NaF). The appliances were extraorally brushed except the specimens, and volunteers were asked to brush only the palatal region of the devices, to avoid disturbing the biofilm.

In order to provide a cariogenic challenge, the volunteers were instructed to take out the appliance from the mouth, to remove the excess of saliva with gauze and drip one drop of 20% sucrose solution extraorally on the specimens, 10x/day at predetermined times (8:00, 9:30, 11:00, 12.30, 14:00, 15:30, 17:00, 18.30, 20.00 and 21.30). The sucrose was gently dried after 5 min and the device was reinserted into the mouth.[22]

2. 4. Confocal laser scanning microscopy (CLSM)

On the 7th day, approximately 12h after the last application of the sucrose solution and dentifrice, the volunteers stopped wearing the intraoral devices. The plastic mesh was removed and the material disks with bioflm were carefully collected from device

and washed three times with a sterile saline solution to remove non-adhering cells and placed in a Petri dish. Live/Dead Baclight bacterial viability stain (L13152) (Molecular Probes, Eugene, OR, USA) was used in this study. It consists of a two nucleic acid-binding stains mixture: 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). Bacterial Viability Kit was mixed according to the manufacturer's instructions and one drop was carefully applied directly onto the biofilm surface.[23] After incubation in the dark for 15min,[24] a non-invasive confocal imaging of fully hydrated biofilms was accomplished by means of an inverted microscope with a confocal laser scanning unit (Leica, modelo TCS SP5AOBS, Zeiss, Germany). An excitation wavelength of 488nm was used, and all light emitted between 500 - 590nm and over 595-713nm was collected by different filters. The lense of 20x 0.7 were used for CLSM analysis. Five equidistant image stacks of serial optical sections (right, left, top, bottom and center) of the biofilm were performed per specimens, mapping almost the entire specimen. Moreover, the images generated by CLSM allowed a qualitative analysis of the biofilm, observing mainly the architecture and the bacterial viability.

2. 5. Quantitative and Qualitative Analysis

2.5.1. Image processing by COMSTAT

CLSM-captured images were analyzed quantitatively using COMSTAT software, development by Heydorn *et al*. (2000),[25] written on the Matlab platform (The MathWorks, Inc., MA, USA). Images collected as z-stacks by 3D reconstruction consisted of a series of images with 1-μm intervals in z-section from the substrate to the top of the biofilm. A fixed threshold value and connected volume filtration were used for all image stacks. 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, five features from COMSTAT were selected to quantify biofilm structures: area, bio-volume, mean thickness, maximum thickness and roughness coefficient.

2.5.2. Qualitative Analysis

The qualitative analysis was conducted based on the following criteria observed on CLSM images, visually and were described as: Cell viability– predominance of green and red and Biofilm Architecture - predominance and distribution voids and canals in biofilm. All images obtained from all volunteers and all materials, using CLSM were analyzed and only descriptive analyzes were done. Representative images from all groups were selected. (Figure 2)

2. 6. Statistical analysis

The data of quantitative analysis were analyzed using the Kolmogorov–Smirnov test at a 5% level of significance to assess the normality of distribution. Because the data had a non-parametric distribution, Kruskal–Wallis tests at a 5% level of significance were used, via a SAS statistical software (North Carolina, USA).

3. RESULTS

Medians of different variables evaluated from the biofilm accumulated on the surface of the tested restorative materials are shown in Table 2. The median values of all parameters analyzed showed no statistically significant difference among the materials studied (Table 2).

Figure 2 shows the structure of the 7-day-old biofilm accumulated on the studied materials. It can be seen that apparently there was a difference in distribution of viable cells and non-viable cells, and in the architecture (voids and canals) of the biofilms formed on different restorative materials. Filtek Supreme and IPS E.Max present visually predominance of well defined viable cells and canals, whereas for Vitremer, Ketac Molar Easymix and Amalgam GS-80, there is a prevalence of non-viable cells forming small clusters but distributed throughout the area of the biofilm, and voids are in smaller proportion in the biofilm volume.

4. DISCUSSION

The present study used results from CLSM and COMSTAT with the aim of evaluating qualitatively and quantitatively the biofilm accumulated *in situ* on different restorative materials and under cariogenic condition. The Confocal Laser Scanning Microscope (CLSM) has been launched as a promising method for studying the architecture of biofilms in their natural hydrated state, with no requirement for dehydration, fixation, or staining. In addition, the optical sectioning properties of the CSLM mean that very thin optical sections can be taken at increasing depths through the biofilm, free from out-of-focus blurring. Such digitalized data can be re-assembled to provide tri-dimensional information.[26,27] COMSTAT was used for quantifying biofilm structures; its software incorporates ten features for quantitative characterization of three-dimensional biofilm images.[25]

The 7-day period was selected based on a pilot study (not shown here), in which no significant differences were found between 7 and 14 days for all parameters evaluated. Furthermore, Vale *et al*. (2007)[28] has showed that changes in the biofilm composition are observed within this period.

4. 1. Quantitative Analysis

This study did not find statistically significant difference between different biofilms provided by restorative materials. The parameters were analyzed because represent essential morphological characteristics from the formation and development of the biofilm. Thus, the area represents the region occupied by the biomass in each image of a stack, reflecting how efficiently the substrate is colonized by bacteria of the biofilm. The bio-volume represents the overall volume of the biofilm, being the value of the biomass volume divided by the substrate area ($\mu m^3/\mu m^2$). Mean biofilm thickness provides a measure of the spatial size of the biofilm and the maximum thickness over a given location, ignoring pores and voids inside the biofilm. The biofilm roughness describes the variability in the biofilm thickness and is an indicator of biofilm heterogeneity.[25]

For the results found in this study a suggested explication is that the biofilm after 7 days is in a stage community climax, in which there is a dynamic equilibrium with minor variations in composition and proportion of species.[29] Current ecological theories of competition are based heavily on the concept of competitive exclusion and the assumption that communities exist at competitive equilibrium.[29] Therefore, success or survival is based mainly on the physiological fitness of each competitor in a stable, uniform environment. Since a competitive equilibrium requires that the rates of change of all competitors to be zero, and since the physical environment, predation, and other factors are changing constantly, equilibrium rarely occurs in nature.[30] Thus, it is important to note that no quantitative difference was found in the biofilm architecture when it was accumulated on the surface of restorative materials that can release byproducts, especially in an acidic medium, and interfere with the accumulation of biofilm, such as fluoride, monomers and mercury, since the highest levels are released up to 14 days for ionomer materials, being the peak release on the 7th day, period used in this study[31,32] and 5 days for amalgam.[33]

Another factor that appears to be closely related is the medium in which the biofilm is inserted during the *in situ* studies, that is subjected to factors such as salivary flow, saliva buffer capacity, oral hygiene, bacterial variability, among others that may influence on adhesion and development of the biofilm. These factors were also confirmed by Hanning *et al*. (2012)[34] which observed a high inter-individual and intra-individual variability when investigated the efficacy of a new preparation in dental prophylaxis for oral biofilm management.[34] According to Zero (1995)[35] the major source of variation associated with *in situ* models should be of biological and not experimental origin. *In vitro* models are particularly well suited to experiments whose objective is to test a single process in isolation, where a more complex situation with many variables may confound the data.[36]

Transmission electron microscopy micrographs of specimens of composite resin, glass ionomer cements, silver amalgam, ceramic, titanium and unfilled resin did not show any evidence of anti-adherence or a biofilm reducing effect, and also showed no micro-morphological differences as compared to the early biofilm on dental enamel.[37] These findings may be ascribed to the presence of the pellicle layer, which possibly acts as

a diffusion barrier reducing the release of metallic cations, thereby apparently hiding any difference among the materials, in regard to surface properties and biocompatibility. Moreover, in the oral cavity, the large volume of saliva present can produce the liberation of ions below the minimum inhibitory concentration to cause significant quantitative alterations in the biofim.[38,39] Our findings corroborate those studies once the quantification of biofilm showed no statistical difference. However, qualitative analysis (architecture and viability) of the biofilm seems not be related with quantitative results.

4. 2. Qualitative Analysis

Differently from the quantitative analysis of the biofilm, the distribution and architecture along the x/y-axis of viable cells, non-viable cells, voids and canals in biofilms formed on different restorative materials were not regular. Visually, Filtek Supreme and IPS E.Max presented predominance of viable cells and well defined voids, whereas for Vitremer, Ketac Molar Easymix, Amalgan GS-80, there was a prevalence of non-viable cells forming small clusters but distributed by the biofilm. These voids were also reported by Wood *et al*. (2000)[40] 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 canals would be significant for the movement of material-damaging acids, bacterial toxins and other antigens within.

In addition, the chemical composition of the surface materials is important to the bacterial colonization, particularly when the surface has components that are either beneficial or detrimental to the adhered microflora. In composites resin, the degree of conversion is never complete, and approximately 5% to 10% of unpolymerized monomer can be extracted in water.[41] It has been suggested that especially the release of ethyleneglycol dimethylacrylate (EGDMA) and triethyleneglycol dimethacrylate (TEGDMA) from composite resins may enhance the viability of cariogenic bacteria[42] because such compounds appear to be utilized as a carbon source by anaerobic bacteria.[43] Takahashi *et al.* (2004)[44] found sparse vesicular-structured ethyleneglycol dimethylacrylate polymer surrounding bacterial cells and observed no difference in the number of colony-forming units compared to the control (no monomers), suggesting that the polymer can act

as a barrier to protect the cells. Therefore, these facts seem to explain the predominance of viable cells in the biofilm provided on Filtek Supreme composite resin.

Regarding the biofilm accumulated on ceramics, few studies were found in the literature.[15,41,45,46] Probably, the chemical stability and biocompatibility of dental ceramics produce a negligible rate of component release from these materials.[47] Thus, ceramics would not present the antibacterial property attributed to releasing ions, having no influence on the biofilm development. Indeed, Auschill *et al*. (2002)[14] and Eick *et al*. (2004)[48] verified high vitality values (from 34% to 86%) in biofilms accumulated on ceramics. However, these researches found very thin (1-6 μm) and weightless biofilm on these materials, coating just a reduced surface of the specimens. They affirmed that the smooth surface texture of ceramics does not support bacterial colonization. Note that this supports the idea, that thicker biofilms are less viable than thinner ones, due to a hampered supply of nutrients to a thick biofilm. Klapper (2004)[49] showed that a forest of mushrooms in the biofilm effectively creates a mushy layer-medium that slows the microbial nutrient transport to the bottom surface. Therefore, below this layer, limited nutrient substrate is available to biofilm cells, leading to the higher frequency of non-viable microorganisms in deeper biofilm regions. This could explain partially the difference between viable and non-viable cells found by the present study, mainly for thicker biofilms.

It has also been reported that ionomeric materials and resin-modified glass ionomer cement have the ability to neutralize acid solutions,[50] even in the long term[51,52] and to affect the bacterial metabolism.[53] Fluoride can act in multiple ways on the bacterial growth, such as: F-/HF can bind directly to many enzymes to modulate metabolism. Fluoride is also able to form complexes with metals, can mimic phosphate, with either positive or negative effects on a variety of enzymes and regulatory phosphatases. The fluoride action that appears to be the most important for glycolytic inhibition derives from its weak acid properties and the capacity of HF to act as a trans-membrane proton conductor.[51] Moreover, the aluminum released by ionomeric materials (Vitremer) may enhance the biological effects of fluoride[54] and consequently, to affect the viability of microorganisms from the biofilm.[55] Thereby, glass-ionomer cement indeed collects a thin

biofilm with a low viability (2% to 3%), possibly as a result of the fluoride release,[14] corroborating with the analyzed images.

It is evident from in vitro studies that silver amalgams have antibacterial properties.[56] In agreement with the results of Bundy *et al.* (1980)[57] Hg and Cu ions of the amalgam had the two best inhibitory activities over the experimental period (24 h). Five-day-old oral biofilms accumulated *in vivo* on gold and amalgam surfaces are known to be thick, fully covering the substrate surfaces, but, in contrast, were found to be barely viable (less than 8%).[14] For comparison, the viability of oral biofilms on enamel *in vivo* was between approximately 41% and 56%.[58] The low viability of oral biofilms on amalgam surfaces is probably due to the release of toxic compounds from the alloy amalgam, which consists of approximately 50% Hg and 35% Ag that slowly diffuse from the amalgam into the biofilm. There is no sufficient evidence that Ag is released from the amalgam and oxidized, not being it able to induce an antimicrobial effect. In this way, antimicrobial effects of amalgam should be attributed to Hg.[59]

Dental biofilms exist in a dynamic equilibrium with host defenses and are generally compatible with the integrity of the tissues they colonize.[60] A strong correlation is evident between the compositional and metabolic changes of the dental biofilms. The transition from an oral health to a disease state, including dental caries and periodontal disease,[61] depend on several parameters related to the host (caries risk, salivary flow, saliva buffering capacity), diet and biofilm (shifts in the composition of the predominant species induced by mechanisms of synergy/antagonism among the microorganisms as well as by nutrients and atmospheric gradients). To evaluate these individual characteristics suiting the restorative material to the development of a less pathogenic biofilm would be the most correct strategy apparently during restorative treatment.

Thus, the hypothesis that the biofilm accumulated on surfaces of different restorative materials would present different characteristics (quantitative and qualitative) after 7 days of development *in situ* has partially accepted. Qualitative analysis showed a material-dependence on cell viability and architecture of the biofilm.

5. CONCLUSION

It could be concluded by the quantitative analysis that regardless of the restorative material, the biofilm developed *in situ* was structurally similar for all restorative materials. The qualitative analysis showed that variations in bacterial viability are depending on the restorative material. Filtek Supreme and IPS E.Max visually presented predominance of well defined viable cells and canals, whereas for Vitremer, Ketac Molar Easymix and Amalgam GS-80, there is a prevalence of non-viable cells forming small clusters but distributed by the area of the biofilm; and voids are in smaller proportion in the biofilm volume.

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Table 1 – Materials used in this study

Materials	Classification	Contents (Manufacturer info)
IPS E. Max (Ivoclar Vivadent, Schaan, Liechtenstein)	Glass ceramic	Powder: 97% SiO_2, Al_2O_3, P_2O_5, K_2O, Na_2O, CaO, F, 3% TiO_2 and pigments Liquid: water, alcohol, chloride
Filtek Supreme (3M ESPE, St. Paul, MN, USA)	Composite resin	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)	Glass ionomer cement	Powder: fluorosilicate glass, strontium and lantanium Liquid: polycarbonic and tartaric acids and water
Amalgam GS-80 (SDI, Victoria, AUS)	Silver amalgam	Powder: 40% Ag, 31.3% Sn, 28.7% Cu Liquid: Mercury

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

Table 2 - Median (minimum – maximum value) of different variables depending on the materials studied.

Variables / Materials	Area (μm^2)	Bio-volume ($\mu m^3/\mu m^2$)	Mean Thickness (μm)	Maximum Thickness (μm)	Roughness Coeficient
Filtek Supreme	0.060 (0.02-0.15)	42.56 (30.33- 64.43)	43.02 (30.03-75.67)	50.82 (31.11-110.09)	0.10 (0-0.68)
Vitremer	0.061 (0.03-0.17)	47.33 (26.58- 55.12)	52.34 (32.76 – 93.87)	58.98 (33.13-98.58)	0.08 (0-0.51)
Ketac Molar Easymix	0.052 (0.04-0.14)	44.66 (30.62 -63.29)	50.73 (29.83-71.65)	57.77 (29.9-82.62)	0.07 (0-0.36)
Amalgam GS-80	0.071 (0.04 -0.15)	44.73 (27.58-84.47)	50.06 (29.19-105.59)	54.54 (30.91-113.52)	0.14 (0.01-0.3)
IPS E.Max	0.073 (0.04 -0.25)	42.91 (22.23-86.65)	46.88 (26.47-104.11)	51.11 (29.09-119.79)	0.14 (0.02-0.64)
p-value	0.3395	0.8380	0.9705	0.9795	0.2698

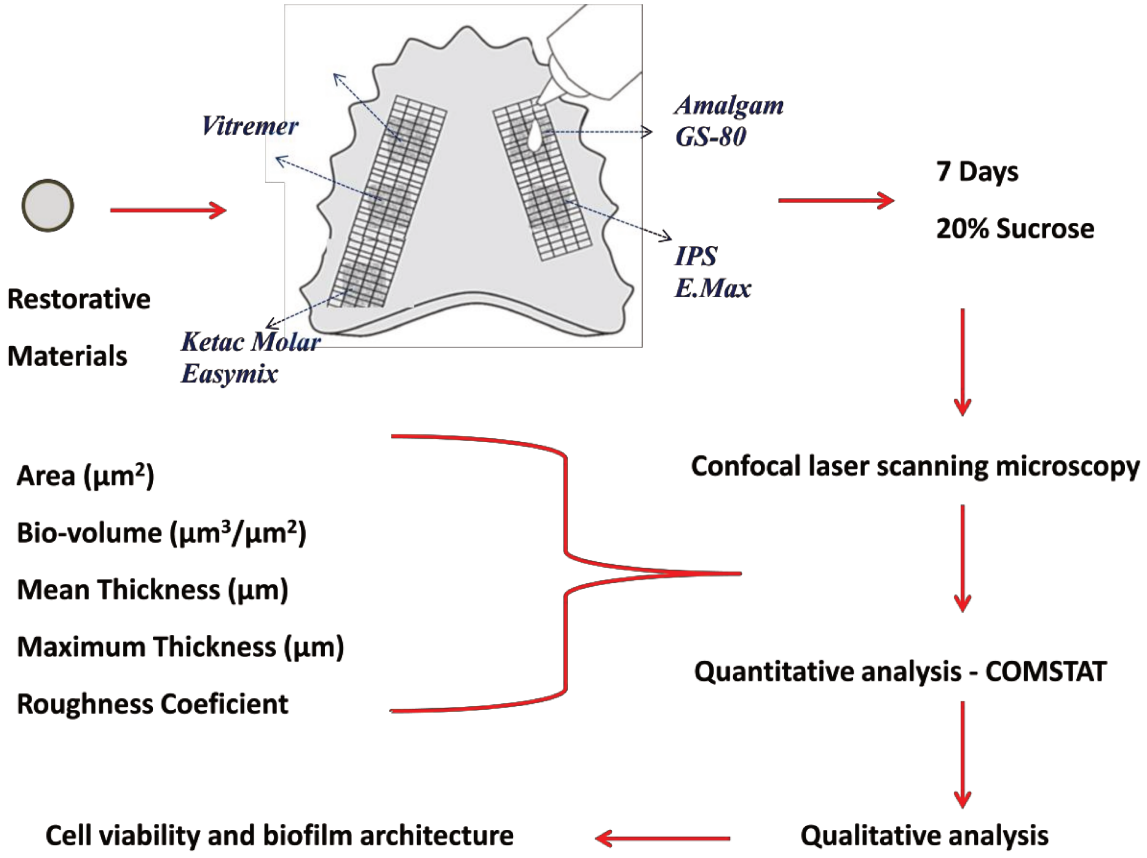

Figure 1 - Experimental design

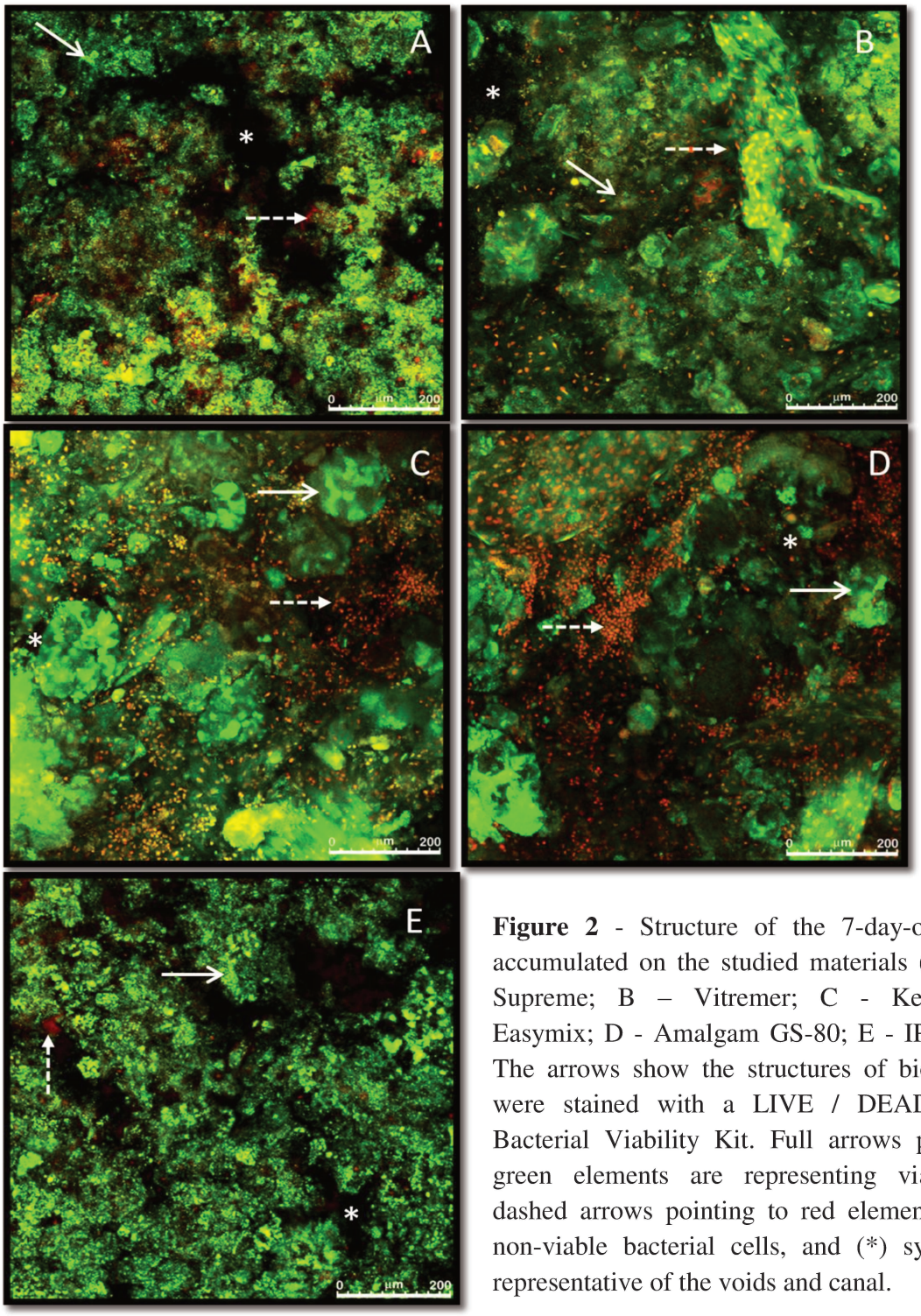

Figure 2 - Structure of the 7-day-old biofilm accumulated on the studied materials (A - Filtek Supreme; B – Vitremer; C - Ketac Molar Easymix; D - Amalgam GS-80; E - IPS E.Max). The arrows show the structures of biofilms that were stained with a LIVE / DEAD Baclight Bacterial Viability Kit. Full arrows pointing to green elements are representing viable cells, dashed arrows pointing to red elements indicate non-viable bacterial cells, and (*) symbols are representative of the voids and canal.

CAPÍTULO 2

IN SITU BIODEGRADATION OF RESTORATIVE MATERIALS*

ABSTRACT

This study aimed to evaluate the surface characteristics of restorative materials (roughness-Ra, Vickers hardness-VHN, chemical changes by energy dispersive spectroscopy-EDX and scanning electron microscopy (SEM) submitted to *in situ* biodegradation. Fifteen discs of each material [IPS E.Max(EM); Filtek Supreme(FS); Vitremer(VI); Ketac molar EasyMix(KM); Amalgam GS-80(AM)] were fabricated in a metallic mold (4.0 mm x 1.5 mm). Ra, VHN, SEM and EDX were initially evaluated. Fifteen healthy volunteers used during 7 days a palatal device containing one of each restorative material. After the interaction with the biofilm, Ra, VHN, SEM and EDX were once again performed. Data obtained from Ra and VHN were submitted to Kolmogorov-Smirnov and Tukey-Kramer tests ($p<0.05$). All esthetic restorative materials showed a significant increase on Ra values after biodegradation. Before biodegradation VHN were significant different between materials studied, as follow: EM>AM>FS>KM>VI. It was observed a significant VHN increase for AM, KM and VI, compared with VHN values before biodegradation. After biodegradation VHN values were significant different between materials studied, with the follow sequence: EM>AM>FS=KM>VI. SEM indicated a biodegradation on the surface of all materials studied showing porosities, cracks and roughness. FS after biodegradation showed Cl, K and Ca on surface, while for VI and KM, F didn`t show up on surface after biodegradation. EM and AM similar composition remained after biodegradation. It was concluded that the dental biofilm accumulated on different restorative materials in situ is material dependent. Overall, all materials changed after biodegradation in situ. The ionomer

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thus, all esthetic restorative materials showed increased roughness, confirmed by microscopy SEM, while materials and silver amalgam showed significantly higher hardness. The initial chemical composition of the composite resin and ionomer materials evaluated was significantly altered by the action of the biofilm *in situ.*

Clinical Significance: To understand the surface characteristics of restorative materials submitted to in situ biodegradation is an essential issue for the selection of appropriate restorative materials for each clinical situation and for the success of the restorative procedure.

Key-words: Dental Biofilm, restorative materials, *in situ*, surface properties.

1. INTRODUCTION

Biofilms form not only on dental hard and soft tissues, as the major cause of caries and periodontal diseases but also on the great diversity of restorative biomaterial surfaces used in the oral cavity.[1] However, the adhesion and the aggregation of microorganisms are different among materials with different composition and surface properties.[2,3] Still, the acid metabolites produced by a cariogenic biofilm can cause surface damages to most restorative materials, such as corrosion, roughness, softening, known as biodegradation.[4,5] 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.[6] Then, restorative materials should be preferentially resistant to that adverse condition in order to present a satisfactory performance.

There is limited knowledge about the influence of cariogenic biofilms on the surface of restorative materials. Long-term *in vitro* studies showed an increase of roughness values and morphology damages for resin composite, polyacid-modified composite,[7] and ionomeric materials.[5] An *in situ* study found lower hardness values for Vitremer after 14 days of biofilm accumulation.[8] Regarding metallic materials, as gold and amalgam, thick biofilms are observed covering its surface *in vivo*, but, in contrast, their cells were found to

be barely viable,[9] probably causing less deterioration on their properties. Conversely, biofilms on ceramic biomaterials were relatively thin, but highly viable (from 34% to 86%)[9]. Although dental ceramic is considered the most inert of all dental materials used for restorations,[10] surface degradation after interaction with biofilms *in situ* bring about news perspectives, since it reflects the complex environment and bioma observed in the oral conditions[8].

It is known that no *in vitro* test is capable of reproducing the complex degradation process. So, 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.[11] Nervertheless, 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. In this context, the *in situ* model is a recognized experimental design that has been successfully used to evaluate cariogenic dental biofilm formation.[12] There are few studies about the biofilm influence on surface characteristics of restorative materials *in situ*.[8,13,14] Then, the aim of this study was to evaluate the effect of *in situ* biodegradation on surface characteristics of restorative materials. The hypothesis tested was that restorative materials subjected to seven days of biofilm interaction have significant modifications regarding to their surface roughness, hardness and microstructure.

2. MATERIALS AND METHOD

2.1. Specimen preparation and storage groups

Fifteen specimens of each restorative material described in Table 1 were fabricated according to the manufacturers' instructions, using metal rings (4mm diameter; 1.5mm depth), temperature of 23 ± 1°C and relative humidity of 50 ± 5%.

The specimens, with the exception of the ceramic, were covered with an acetate strip (Probem Ltda, Catanduva, São Paulo, Brazil) and pressed on a glass slide to compact the material. The resin composite and resin-modified glass ionomer cement were photoactivated during 40s at the upper and lower surface of the matrix by a curing light

(Elipar Trilight, 3M ESPE, St. Paul, MN, USA), with an intensity of up to 750 mW/cm^2 checked by a curing light meter (Hilux Dental Curing Light Meter, Benliglu Dental Inc., Turkey). The chemically-cured glass ionomer cement and the amalgam were allowed to set at room temperature for 15 min. After the setting reactions, the ionomeric specimens' were superficially protected with petrolium gelly. For the ceramic, specimens were fabricated in a prosthetic laboratory by using the pressing process in an oven (Programat P500 - Ivoclar Vivadent, Schaan, Liechtenstein), simulating the clinical reality.

All the restorative materials were stored in an environment at 37°C with 100% of relative humidity for 24 h. Afterwards, each surface specimen's were polished according to the manufactures instructions. For Filtek Supreme, Vitremer and Ketac Molar were used Sof-Lex disks (3M ESPE, St. Paul, MN, USA), the silver amalgam was polished with polishing kit (KG Sorensen, Cotia, SP, Brazil) and the ceramic disks were ground flat with aluminum oxide Jet (50 µm - Bio-Art, São Carlos, SP, Brazil), followed by diamond drill (4138F - KG Sorensen, Barueri, SP, Brazil), rubber tip (KG Sorensen, Barueri, SP, Brazil), washed in an ultrasonic bath (Ultrasonic Cleaner, Model USC1400, UNIQUE Ind. e Com. Ltda., São Paulo SP 04709-111, Brazil) and glazed.

2.2. Surface roughness measurements

After finishing and polishing procedures, all the specimens were washed through sonication for 10min, dried and fitted to a surface roughness-measuring instrument (Surfcorder SE1700; Kosaka Corp., Tokyo, Japan). Prior to the hardness assessment, roughness analysis was performed to avoid interference in the results of the latter. Moreover, the specimens were divided in the middle, being the left side used for the roughness analysis and the right for hardness. To record roughness measurements, the needle moved at a constant speed of 0.5 mm/second with a load of 0.7 mN. The cut-off value was set at 0.25 mm to maximize filtration of surface waviness. Ra values for each specimen were taken across the diameter over a standard length of 0.25 mm. 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 (45°). A calibration was done periodically to check the performance of the surface roughness-measuring instrument.

2.3. Surface hardness

Hardness tests were carried out with a hardness tester (Shimatzu, Tokyo, Japan) by using a Vickers indenter and a load of 200 g for the composite resin, glass ionomer cements, silver amalgam and 500 g for the ceramic, with a dwelling time of 15 s. Five readings were taken for each specimen, and the mean VHN was calculated before and after the cariogenic challenge.

2.4. Surface morphology assessment and Energy-dispersive X-ray analisys

Three additional representative specimens of each group, before and after the *in situ* biofilm experiment, were rinsed, dried and mounted on a holder using double-sided adhesive carbon tape. Carbon was sputtered on the samples previously to the analysis. Energy-dispersive X-ray analysis was performed before and after the biodegradation. EDX measurements were calibrated before for stub, by a certified engineer, using the standard samples of Cr2O3, titanium, silica, and CaSiO3, as described by Statham (2004).[15] After this, the same specimens covered by carbon were also examined with a JEOL scanning electron microscope Model JSM 5600 LV (Tokyo, Japan), operating at 1,000x of magnification.

2.5. Panellists and ethical aspects

Fifteen healthy adults participated of the study (ages of 21–30 years). The volunteers were selected according to the following inclusion criteria: good general and oral health, normal salivary flow rate, absence of antibiotic use during 2 months before the experiment, absence of prosthesis or orthodontic devices, no signs of gingivitis and caries and ability to comply with the study.[13] Visual oral examinations were carried out by an experienced dentist. All the volunteers agreed to participate and signed an informed written consent. The study design was approved by the Ethics Committee (protocol 136/2009).

2.6. In situ phase

The volunteers were molded with alginate (Jeltrate - Dentsply, Petrópolis, RJ) and type III gypsum models was obtained. An individual intraoral acrylic resin palatal device, containing five wells each 2.5 mm-depth (one restorative material per well) was prepared for each volunteer[9], as observed in Figure 1. A plastic mesh was fixed on two sides of the appliance, leaving a 1 mm-space for the accumulation of the dental biofilm on the specimens. Before receiving the intraoral devices, oral and written instructions of the *in situ* protocol were given to the volunteers, to assure their adequation to the study. There were no restrictions to the volunteer's diet. The only recommendation was to remove the device during meals and before ingesting any beverages or food, keeping the oral devices moist in plastic boxes provided by the researchers. Volunteers were instructed to perform oral hygiene, three times/day with a standardized fluoride dentifrice (1100 mg F/g as NaF) and only the palatal region of the appliances were extraorally brushed, to avoid disturbing the biofilm. The cariogenic challenge was provided by the application of a 20% sucrose solution extra-orally on the specimens, 10x/day. The volunteers removed the devices from the mouth, the excess of saliva was cleaned with gauze and one drop of the solution was dripped onto each specimen at 8.00, 9.30, 11.00, 12.30, 14.00, 15.30, 17.00, 18.30, 20.00 and 21.30 hours.[16] The sucrose was gently dried after 5 min and the device was reinserted into the mouth. After 07 days, the specimens were carefully removed from device, washed in an ultrasonic during 10 minutes and final measurements of the roughness, hardness and surface morphology were performed.

2.7. Statistical analysis

The measurements were analyzed by using the Kolmogorov– Smirnov test at a 5% level of significance to assess the normality of distribution. A methodology of mixed models for repeated measures and Tukey-Kramer statistical tests at a 5% level of significance were used with a PROC MIXED SAS statistical software (North Carolina, USA).

3. RESULTS

The mean and standard deviations of surface roughness and hardness values of each material, before and after biodegradation *in situ,* are displayed in Tables 2 and 3, respectively.

All esthetic restorative materials studied showed a significant increase of surface roughness values after the biofilm/material interaction. Only the Amalgam GS-80 showed no statistical difference between the periods analyzed (before x after degradation). Comparing the materials studied before cariogenic challenge, the Amalgam GS-80 presented higher roughness values than other materials, followed by Vitremer, Ketac Molar Easymix and IPS E.Max. Filtek Supreme showed the lowest Ra values. However, after biodegradation, Amalgam GS-80 and IPS E.Max had higher values than Ketac Molar Easymix, while Filtek Supreme and Vitremer presented intermediate values and no statitical difference with other materials.

Regarding surface hardness values (VHN), it was observed that before cariogenic challenge, VHN were statistically different between materials studied, with the following sequence: IPS E.Max > Amalgam GS-80 > Filtek Supreme > Ketac Molar Easymix > Vitremer. Vitremer, Ketac Molar Easy mix and Amalgam GS-80 presented significant differences between experimental periods, with higher values after degradation than before. Filtek Supreme and IPS E.Max did not show difference on similar comparation. After biodegradation, it was found VHN as follow: IPS E.Max > Amalgam GS-80 > Filtek Supreme = Ketac Molar Easymix > Vitremer.

By EDX analysis presented in figure 2, the initial chemical composition of the Amalgam GS-80 and IPS E.Max evaluated was not altered by the action of the biofilm *in situ*. Thus, Amalgam GS-80 showed the presence of Hg, Sn, Ag, Si, Cu, while the IPS E. Max had in its composition Si, Al, K, Na, among others. However, Filtek Supreme, Vitremer and Ketac Molar Easymix did present alterations in their spectra. EDX results of Filtek Supreme revealed that Si had the highest weight percentage, followed by P and C, at the baseline and 7 days after the biodegradation. However, there was the adsorption of ions on the materials surface possibly originated from saliva. Vitremer and Ketac Molar

Easymix presented predominantly Al, Si and Ca, in the same amount before and after biodegradation. However, F had a decrease in its peak after seven days. Furthermore, there was also the incorporation of ions such as Ca^{2+} and Cl^{-} on the surface of these analysed materials.

The scanning electron micrographs in Figure. 3 showed details of the surface morphology of the studied materials, which are presented in rows (materials) and columns (periods). Regarding resin-based materials (Filtek Supreme and Vitremer), it was observed filler particles exposed by polishment procedures before biodegradation. Nevertheless, the cariogenic challenge produced an irregular coating surface, with the displacement of some filler in the organic matrix. Ketac Molar Easymix also presented exposed fillers and cracks on this surface before biofilm interaction, and after *in situ* experiment, the ionomeric material surface showed cracks and biodegraded areas with displacement of filler, as observed by arrows. For the Amalgam GS-80 studied, it was observed a subtle corroded aspect after surface degradation, while IPS E.Max showed increased amount of surface cracks associated with an increase in the size of the nodules when compared before biodegradation.

4. DISCUSSION

The success of restorative procedures depends on all its stages, since treatment planning and patient's adequacy until clinical steps and subsequent proservation and maintenance of restoration performed. Still, it would be important to select carefully a restorative material able to withstand the functional force and chemical environment of the oral cavity. Fundamentally, the factors known to cause surface damages to restorative materials include low pH due to cariogenic biofilm, consumption of acidic drinks or foodstuffs, and action of enzymes, which can soften the outermost layers and roughen restorative materials. [5,8,17-21]

Most conditions of oral cavity could be simulated by an *in situ* study, such as saliva properties (salivary flow, buffer capacity, clearance, minerals and protein content, enzymes), biofilm accumulation (diversity of species, microorganism selection, succession,

nutrient availability and competition), temperature fluctuations, aqueous environment, among others. However, little information is available regarding to its surface degradation after interaction with biofilms, *in situ*. Among the available studies, *in situ,* a focus was given either only on the evaluation of the biofilm characteristics or of the restorative material.[8,13,14] In this context, the present study allowed that dental biofilms were formed on different restorative materials using *in situ* model in order to analyze the consequences of this bio-interaction for materials'surface, under frequent cariogenic challenge.

Different groups of restorative materials (amalgam, composite resin, glass ionomers and ceramic) were selected due to studies that suggest that the biofilm accumulated and the biodegradation intensity is influenced by the surface upon it is developing, which is directly related to their physical and chemical properties. [8,14,22,23] Furthermore, the selected materials are representant of different classes of restorative materials used in Odontology, as well as of the classes stablished in Materials Science (i.e. metals, polymers, ceramics and composites).[24]

Firstly, all materials were handled according their manufacturer's recommendations, including photoactivation, setting time and polishment procedures. The polishing was carried out once this procedure improves the aesthetic characteristics and the durability of the restoration, decreases the porosity of the surface, decreases the surface staining and also improves its mechanical properties.[25] In the same way, the polishing removes the organic matrix of different restorative materials and exposes fillers particles, fact observed by SEM micrographs before biodegradation, it showed some scratches on composites and amalgam surfaces created by finishing/ polishing instruments, and the exposition of fillers particles of direct restorative materials studied (Figure 3).

According to roughness values, our findings found that all esthetic materials studied showed an increase of surface roughness values after biofilm activity. The acid atack by bacterial metabolism can cause the biodegradation through different ways for restorative materials. For Filtek Supreme, there is a release of TEGDMA and UDMA monomers from the resin matrix when it is in contact with salivary enzymes and bacterial acids[26].The Vitremer present hydroxyethyl methacrylate (HEMA), a highly hydrophilic cosolvent and main component released from the organic phase, when submitted to a

cariogenic challenge.[27] Glass ionomer cements withstand a complex process of absorption, disintegration and outward transportation of ions, with an erosive loss of matrix components and leaching of glass particles, increased in acid medium.[6] It is possible that loss of components from two Vitremer matrixes (polyacrylate–inorganic and polymer–organic) and from organic matrix of Filtek Supreme leads to changes in surface roughness.

For the dental ceramics, the increased initial roughness may be related to the characteristics of the polishing and not necessarily to the biodegradation, this result was not expected because the ceramics is considered the most inert of all dental materials used for restorative.[28]Thus, the *autoglazer* appears to have a more resistent surface to biodegradation than the *overglazer*, utilized in this work. Thus, Fahmy *et al*. (2009)[29] observed a crack length significantly smaller for the *autoglazed* group, while Atay *et al*. (2008)[30] showed greater color stability also for specimens with the *autoglazer* and Zaki *et al*. (2009)[31] showed that bleaching agents did not affect significantly the surface roughness of the *autoglazed* group. In this way, the vitreous ceramic of the glazer possibly suffered biodegradation due to the increase of roughness. Besides, Chang *et al*. (2011)[32] observed an increase in particle grit sizes for ceramics, which form nodules, thus corroborating with our SEM micrographs. This event possibly occurs due to the poor thermal conductivity of porcelain associated with the formation of large temperature spikes at the point of contact between the diamond bur and the porcelain.

However, the roughness of the amalgam did not alter significantly after the biodegradation, possibly due to some factors related to this material characteristics: the optimization of amalgam alloys by Innes & Youdelis (1963)[33] and further by Asgar (1974)[34] which produced amalgams of high copper content (> 6%) and rich in spherical copper particles that improved the corrosion resistence; the polishment that leads to a substantial increase in the corrosion resistance, once it removes the tin-mercury alloy (gamma-2 phase) and decreases the concentration of electrolytic cells[35] and finally, the formation of a passive layer on the surface that also contributes to the improvement of the corrosion resistence.[36] Considering the latter aspect, studies on the mercury liberation from dental amalgams suggest the formation of a passivation layer composed by an oxides film on the material surface, interfering with the dissolution process of the metal components

and diminishing substantially their lixiviation.[36-39] Furthermore, it is important to note that the period of 7 days is relatively short to promote a process of considerable corrosion on silver amalgams.

Regarding hardness analysis, it was observed that amalgam and ionomeric materials presented an increase of their values after biodegradation experiment, related probably to a post-hardening process over setting time. For Vitremer and Ketac Molar Easymix, this process could be explained by the slow rate of acid-base reaction forming the polyacrylate salts and the free-radical polymerization reaction, which continues after light-irradiation of Vitremer specimens.[40,41] A maturation over time also could be occur with amalgam, during trituration process, the mercury dissolves the surface of alloy particles and a plastic mass is formed by the setting and hardening of the amalgam. The amalgam crystallization can continue for several days,[28] according to ours results.

In a different way, the rapid setting reaction of resin composite is initiated by light exposure and most parts of the conversion process end immediately after photoactivation, leading to reduced post-irradiation polymerization.[42,43] Moreover, the presence of Bis-EMA and TEGDMA in the matrix composition possibly contributed to the stability of the hardness value of the Filtek Supreme. TEGDMA can decrease the surface softening caused by acids and increase the degree of polymerization of resin-based materials[44] and the Bis-EMA showed a lower amount of released products and a higher stability.[45] In the sintering process of ceramics, the compacted particles suffer a coalescence phenomenon that lead to the increase of the solid structure density. Consequently, the hardness of these materials undergoes a significant increase as the mechanical integrity of the body is favored. However, after sintered, the hardness of these materials tends to suffer little alteration after the setting reaction,[29,46,47] corroborating with our results.

The initial chemical composition of the Amalgam GS-80 and IPS E.Max evaluated was not significantly altered by the action of the biofilm *in situ*, while Filtek Supreme, Vitremer and Ketac Molar Easymix did present alterations in their spectra after biodegradation, according to the EDX results. Thus, it is observed that the fluoride release from Ketac Molar Easymix and Vitremer, after 7 days, occurs possibly due to their intrinsic characteristics, resulting in dissolution and diffusion processes mainly in acid medium.[20,48]

Parallely, after the *in situ* experiment there is the adsorption of ions such as Ca^{2+} and Cl^- on the surface of some analysed materials (Filtek Supreme, Vitremer and Ketac Molar Easymix), probably from saliva.

In the oral environment, an established or mature biofilm can accumulate at stagnant sites, as interproximal surfaces, gingival crevice and pits and fissures, beyond levels compatible with oral health,[49] developing of disease conditions such as secondary caries and demineralisation process of marginal enamel and dentin.[50] Then, it would be important to assess the patient individually ie, regarding their salivary flow, the caries risk, buffer capacity of saliva, diet, oral hygiene, among others, and then select carefully the restorative material for intraoral sites where biofilm would be protected against dynamic shear forces from saliva, tongue and toothbrushing, stimulating its accumulation and maturation.

Besides, the hypothesis that restorative materials subjected to biofilm interaction have a significant difference on surface roughness, hardness and microstructure after 7 days has to be partially accepted, since there was material-dependence among characteristics analyzed.

5. CONCLUSIONS

In conclusion, within their limits, the present findings showed that the influence of dental biofilm accumulated on different restorative materials *in situ* is a material-dependent event. Overall, all materials changed after biodegradation *in situ.* Thus, all esthetic restorative materials showed increased roughness, confirmed by microscopy SEM, while the ionomer materials and silver amalgam showed significantly higher hardness. The initial chemical composition of the composite resin and ionomer materials evaluated was significantly altered by the action of the biofilm *in situ.*

6. ACKNOWLEDGEMENTS

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Table 1 – Materials used in this study

Materials	Classification	Contents (Manufacturer info)
IPS E. Max (Ivoclar Vivadent, Schaan, Liechtenstein)	Glass ceramic	Powder: 97% SiO_2, Al_2O_3, P_2O_5, K_2O, Na_2O, CaO, F, 3% TiO_2 and pigments Liquid: water, alcohol, chloride
Filtek Supreme (3M ESPE, St. Paul, MN, USA)	Composite resin	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)	Glass ionomer cement	Powder: fluorosilicate glass, strontium and lantanium Liquid: polycarbonic and tartaric acids and water
Amalgam GS-80 (SDI, Victoria, AUS)	Silver amalgam	Powder: 40% Ag, 31.3% Sn, 28.7% Cu Liquid: Mercury

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

Table 2 – Surface roughness means (standard deviation), in µm.

Groups	Biodegradation	
	Baseline	**7 days**
Filtek Supreme	0.34 (0.07) Bc	1.74 (1.51) Aab
Vitremer	0.62 (0.17) Bb	1.87 (0.99) Aab
Ketac Molar Easymix	0.57 (0.17) Bb	1.37 (0.83) Ab
Amalgam GS-80	1.70 (0.66) Aa	2.81 (1.13) Aa
IPS E. Max	0.86 (0.45) Bb	2.40 (2.19) Aa

Means followed by different letters (capital-case letters in each row and lower-case letters in each column) differ significantly ($p \leq 0.05$). Kolmogorov– Smirnov and Tukey-Kramer statistical tests

Table 3 – Surface hardness means (standard deviation), in VHN.

Groups	Biodegradation	
	Baseline	**7 days**
Filtek Supreme	105.47 (2.09)Ac	101.27 (4.93)Ac
Vitremer	62.57 (6.00)Be	73.73 (7.25)Ad
Ketac Molar Easymix	81.59 (3.53)Bd	105.03 (5.95)Ac
Amalgam GS-80	129.45 (5.92)Bb	161.39 (27.13)Ab
IPS E. Max	581.05 (37.24)Aa	577.69 (21.41)Aa

Means followed by different letters (capital-case letters in each row and lower-case letters in each column) differ significantly ($p \leq 0.05$). Kolmogorov– Smirnov and Tukey-Kramer statistical tests.

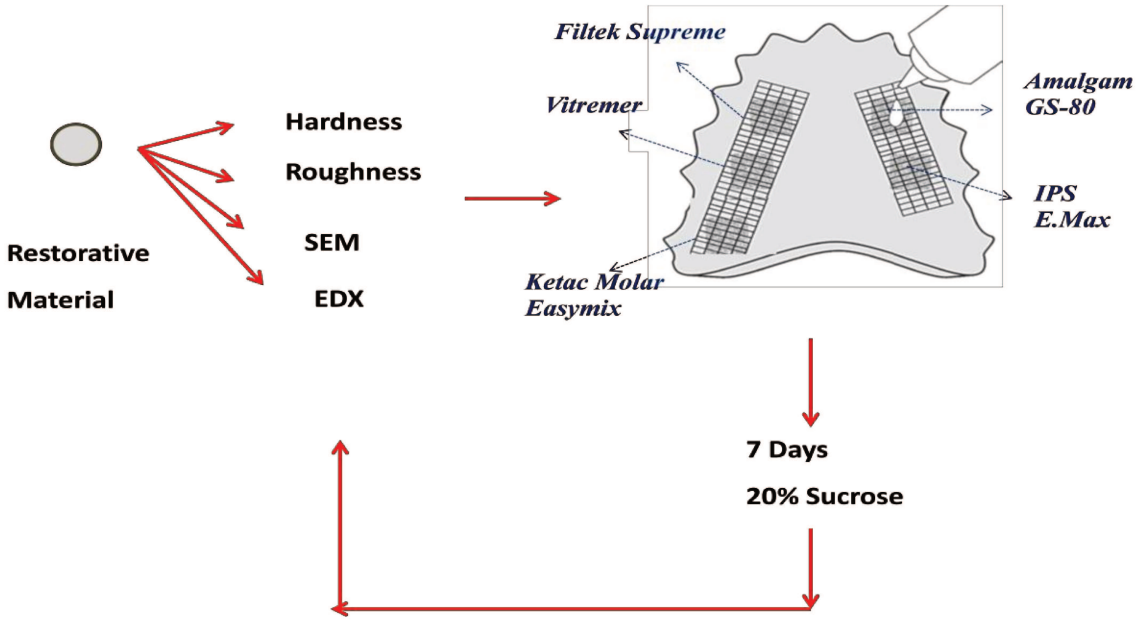

Figure 1 - Experimental design.

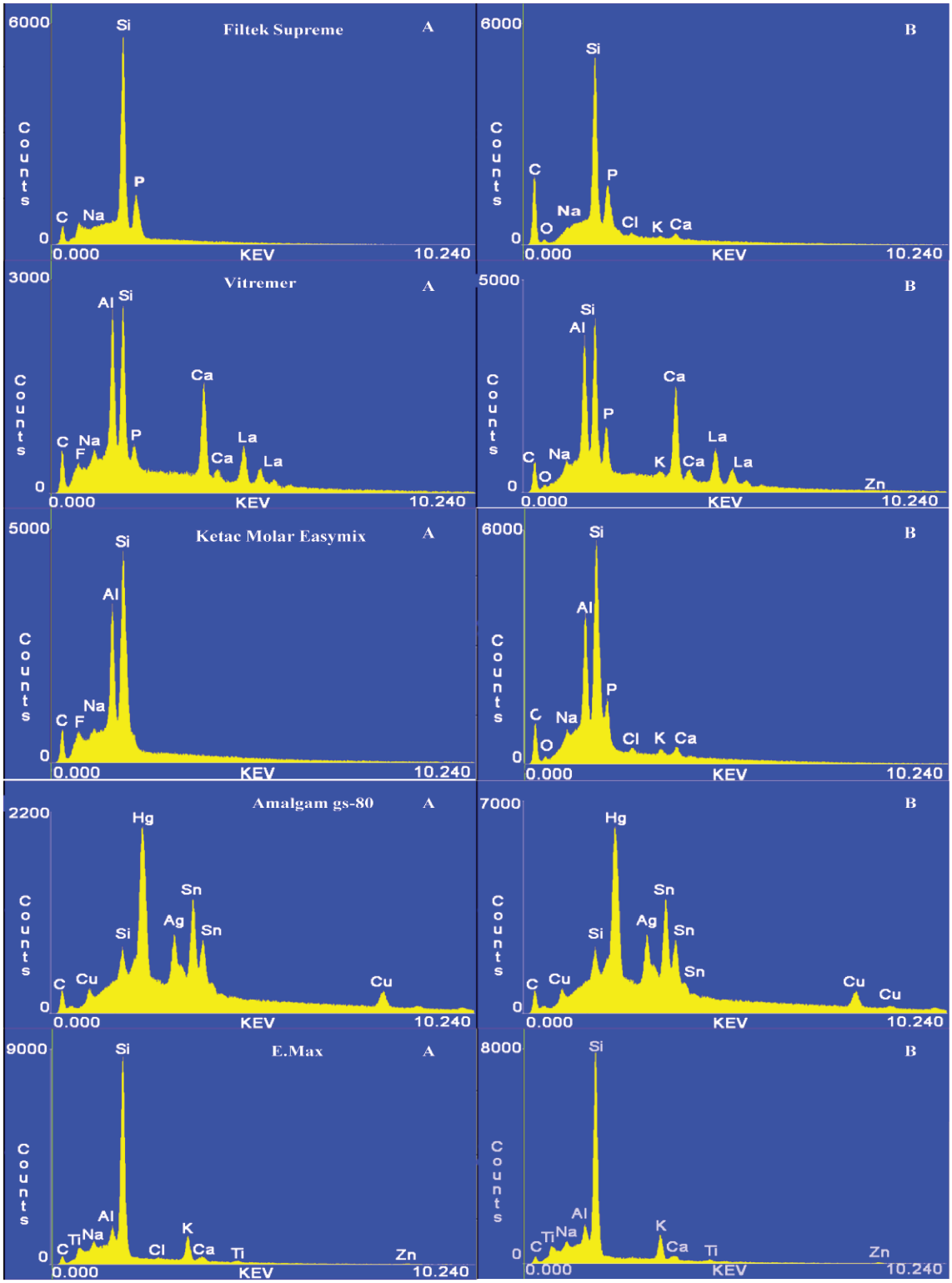

Figure 2 - EDX images of different restorative materials compared (A) before and (B) after biodegradation.

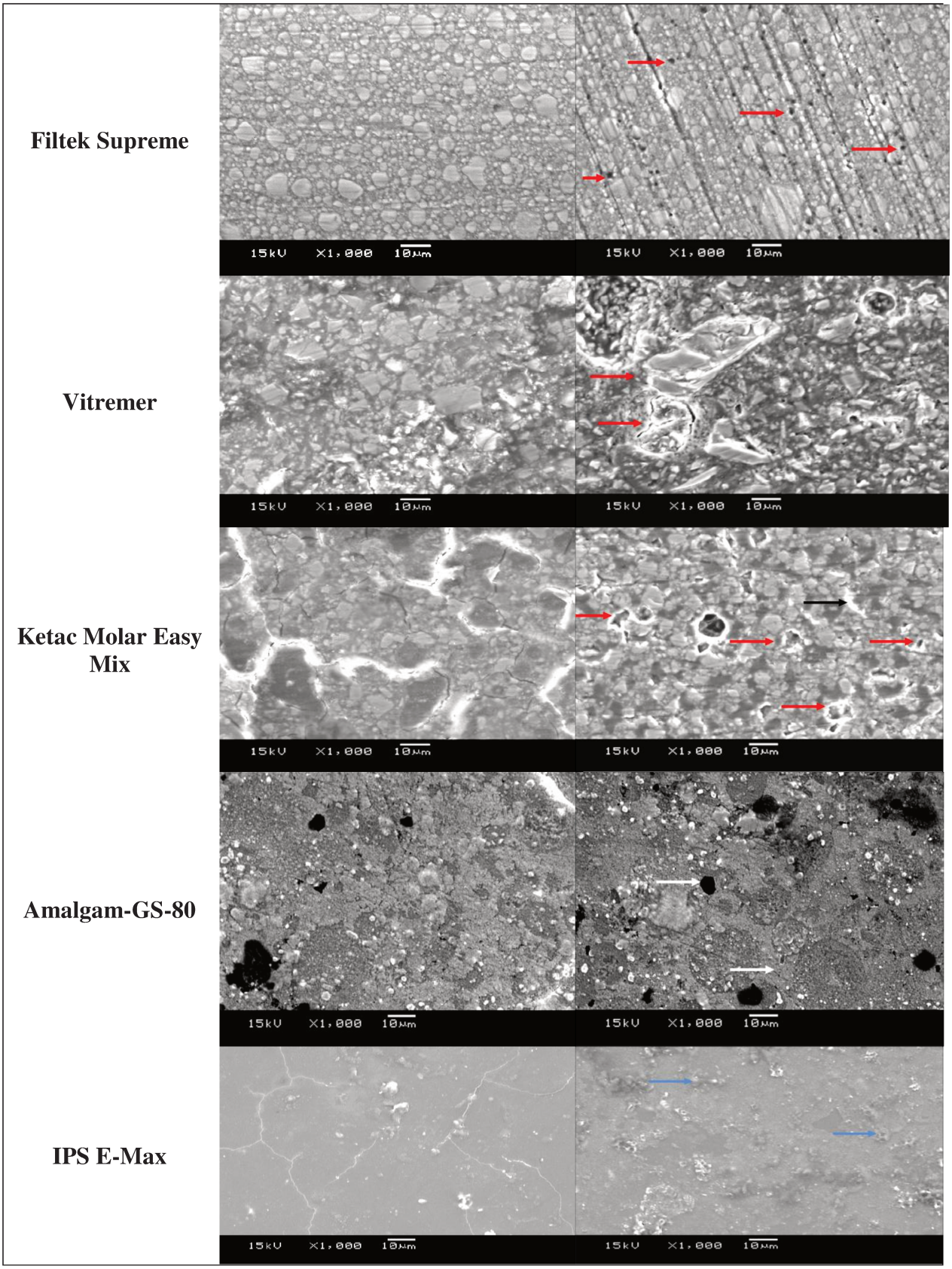

Figure 3 - SEM images of different restorative materials: Left - before and Right - after biodegradation (x1000). Red arrows show filler particles removed from the organic matrix; Black arrows show cracks; amalgam White arrows show various phases; Blue arrows show surface nodules.

CONSIDERAÇÕES GERAIS

A estrutura dentária e as restaurações estão constantemente sujeitas aos desafios térmicos, mecânicos e químicos na cavidade bucal. A associação destes fatores está diretamente relacionada à longevidade/estabilidade das restaurações, bem como a preservação da estrutura dentária (Oilo, 1992).

A degradação química pode ser causada por desafios ácidos, incluindo aqueles produzidos pelo biofilme cariogênico (Asmussen, 1984), dieta ácida (Yap *et al.*, 2002) e enzimas salivares (de Gee *et al.*, 1996).

As bactérias que compõem o biofilme não existem como unidades independentes, mas sim como uma comunidade microbiana integrada metabolicamente e com funções claramente coordenadas (Marsh & Bradshaw, 1999; Marsh & Bowden, 2000). O ambiente heterogêneo gerado no interior dos biofilmes promove diversidade genotípica e fenotípica, proporcionando uma forma de "Seguro biológico" que pode salvaguardar a "comunidade microbiana" (Boles *et al*., 2004). Essa diversidade parece intervir em inúmeras propriedades fundamentais das células, incluindo a motilidade, nutrição, secreção de produtos, desatachamento, e a formação de biofilme.

O biofilme apresenta habilidade de adesão em uma gama de superfícies. Na cavidade bucal, este se acumula nos tecidos moles, mucosa, dentes, materiais restauradores, implantes, entre outros (Carlén *et al*., 2001). A formação de biofilme sobre uma superfície dental é um fenômeno complexo e diferentes fatores-chave parecem estar envolvidos (Guggenheim *et al*., 2001). Em sequência temporal, há a adsorção de proteínas da saliva do hospedeiro sobre diferentes superfícies na cavidade oral. O próximo passo do processo de formação de biofilme envolve a adesão das células microbianas, quando as bactérias começam a ancorar. Nesta fase, ocorre também a colonização de novos sítios do hospedeiro, tal como descrito por Hannig, (1999).

A influência da superfície do material nas características estruturais do biofilme não se encontra bem definida, mas estudos sugerem que vários materiais restauradores podem ter atividade antibacteriana ou podem induzir o crescimento bacteriano (Quirynen &

Bollen, 1995; Teughels *et al*., 2006; Khalichi *et al*., 2009, Barbosa *et al.*, 2012). Assim, para o desenvolvimento deste estudo, foram selecionados representantes dos materiais restauradores utilizados frequentemente na prática clínica (Filtek Supreme, Vitremer, Ketac Molar Easymix, Amalgam GS-80, IPS E.Max), além de representantes de todas as classes de materiais classicamente dividida pela ciência dos materiais em: polímeros, cerâmicas, metais e compósitos (Caçister & Rethwisch, 2012).

Em contrapartida, as alterações de superfície em materiais dentários são rotineiramente detectadas durante a prática clínica, favorecendo o desenvolvimento de cárie adjacente às restaurações (Cenci *et al*., 2008; Cenci *et al*., 2009). As frequentes trocas de restaurações biodegradadas evidencia a necessidade de se avaliar o comportamento quanto à deposição superficial de biofilme e dinamicamente avaliar a arquitetura do biofilme quando em contato com a superfície dos diferentes materiais. Para isso, foi desenvolvido o experimento *in situ.*

Os estudos *in situ* possibilitam a reprodução das condições da cavidade bucal (Zero, 1995). O modelo *in situ* utilizado neste estudo está embasado na exposição extra-oralmente a um desafio cariogênico com solução de sacarose. Enquanto tal protocolo, objetiva gerar diminuição dos riscos para o paciente e aumentar o controle do experimento (Liao *et al*., 2007; Magalhães *et al*., 2007 Tenuta *et al*., 2003; Aires *et al*., 2006; Hara *et al*., 2003).

O Microscópio de Varredura Confocal a Laser (CLSM) lançado como um método promissor para o estudo da arquitetura de biofilmes em seu estado natural hidratado, sem exigência de fixação, desidratação, ou manchas foi o método utilizado neste estudo. Além disso, as propriedades ópticas de seccionamento do CSLM permite que secções ópticas ultra finas possam mapear o biofilme . A utilização de sondas fluorescentes específicas associadas ao modo de reflexão, proporciona também a possibilidade de identificar componentes da matriz específicas, bem como as bactérias (Wagner *et al*., 1994) no interior do biofilme (de Beer *et. al*., 1994).

O período de 7 dias foi selecionado devido resultados encontrados em estudo piloto (dados não publicados) no qual, não obseravram diferença estatisticamente significante entre 7 e 14 dias para os parâmentros avaliados. Além disso, Vale *et al*. (2007)

demonstraram que a desmineralização do esmalte ocorre após 7 dias de acúmulo do biofilme, sendo porém, observadas anteriormente alterações significantes na composição do biofilme, ressaltando assim, o bjetivo deste trabalho de avaliar a arquitetura biofilme.

Diante da complexidade que envolve a interface biofilme/material restaurador e dos importantes resultados dessa interação, muitos estudos (Fucio *et al*., 2008; Souza *et al*., 2009; Barbosa *et al.,* 2012) estão tentando elucidar características fundamentais desses meios para melhorar a compreensão do que rotineiramente ocorre na cavidade oral; porém, poucos estudos conseguem de forma fidedigna se aproximar da realidade, provavelmente devido às inúmeras variáveis de difícil reprodutividade em um trabalho científico e que podem interferir nessa resposta.

No capítulo 1, observaram-se algumas das características estruturais do biofilme *in situ*, quando em contato com a superfície de diferentes materiais restauradores, considerando que, a arquitetura do biofilme tende a ser um fator importante na modulação tanto da fisiologia microbiana quanto na determinação da ecologia do sítio (Wood *et al*., 2000). Assim, entender as características estruturais do biofilme acarretaria em compreender a formação de um biofilme com diferentes patogenicidades, entender a relação de homeostasia com a cavidade oral, e a prevalência do biofilme com alguns sítios específicos.

Os resultados encontrados nesta tese demonstraram não haver diferença quantitativa significativa na estrutura do biofilme quando em contato com diferentes materiais restauradores. Diversos fatores parecem estar relacionados a esta arquitetura quase que inalterada do biofilme, tais como: biofilme em estágio de comunidade clímax na qual, ocorre um equilíbrio dinâmico com variações menores na composição e proporção das espécies (Costerton *et al*., 1999); habilidade de alguns materiais restauradores em favorecer o desenvolvimento do biofilme, como verificado por Khalichi *et al*. (2009); Barbosa *et al.* (2012); estudos *in situ*, estão sujeitos a variáveis individuais e intrínsecas que podem interferir no desenvolvimento e maturação do biofilme (Higham *et al*., 2005) e a magnífica capacidade de adaptação do biofilme ao meio, envolvendo habilidades estruturais e funcionais de resistência a antibióticos e metais, que segundo Baker-Austin (2006) inclui: a redução da permeabilidade da membrana; inativação e modificação de

metal e antibiótico; e, alteração de um componente celular para diminuir a sua sensibilidade aos agentes externos. Diante de uma comunidade dinâmica e extremamente eficiente, penetrar e desestruturar esta arquitetura magnífica torna-se um desafio motivador.

Na análise dos capítulos 1 e 2, um fator relevante a ser observado é o comportamento similar da resina composta e do cimento de ionômero de vidro modificado, no qual houve o aumento da rugosidade, provavelmente devido à lixiviação de íons e de monômeros como: TEGDMA e HEMA (Kawai & Takaoka, 2002 Sideridou *et al*., 2003) e a predominância de células viáveis sendo pertinente ressaltar que, monômeros e íons podem modular de diferentes formas o desenvolvimento e metabolismo do biofilme cariogênico. Monômeros como TEGDMA (e seu derivado TEG), EGDMA e HEMA mostraram estimular o crescimento e a expressão gênica relacionada a enzimas produtoras de polissacarídeos extracelulares de bactérias cariogênicas em biofilmes e pH ácido (Schmalz *et al*., 2004; Khalichi *et al*., 2009).

Ainda considerando os capítulos 1 e 2, o cimento de ionômero de vidro, cimento de ionômero de vidro modificado e o amálgama de prata demonstraram predominância de células não viáveis na massa do biofilme. Klapper *et al.,* (2004) mostraram que a "floresta de cogumelos" no biofilme gera efetivamente uma camada gelatinosa que retarda o transporte de nutrientes da superfície para regiões mais profundas do biofilme, levando a maior frequência de microrganismos não viáveis nessas regiões. Todos os materiais restauradores estéticos apresentaram aumento da rugosidade, sendo que nos cimentos ionoméricos ocorre um complexo processo de absorção, desintegração e liberação de íons/monômeros (Oilo, 1992), ainda potencializado pelo baixo pH mantido pelos microrganismos acidogênicos (Czarnecka *et al*., 2002), na resina composta há a lixiviação dos monômeros HEMA (Kawai & Takaoka, 2002) e na cerâmica o desgaste superficial realizado com ponta diamantada, modificou a textura superficial, alterando sua energia superficial. Porém, o aumento da rugosidade não determinou maior acúmulo de biofilme, evidenciando que não é apenas a textura do material restaurador que pode ter influência no desempenho, mas também a composição. Este comportamento apresentado por materiais cerâmicos é justificado por Jensen (1990), que considera a baixa energia superficial deste material a causa da alteração na adsorção bacteriana.

No capítulo 2, Vitremer, Ketac Molar Easymix e Amalgan GS-80 apresentaram aumento da dureza inicial após contato com o biofilme, provavelmente devido à reação de presa desses materiais continuarem a ocorrer após alguns dias da presa inicial (Anusavice, 2003).

Finalmente, quando se considera a eleição de um material restaurador em situações clínicas, o cirurgião-dentista deve avaliar inúmeros aspectos: idade, higiene oral, dieta, risco à cárie do paciente, quantidade de tecido dentário remanescente, adaptação marginal, propriedades mecânicas e estéticas do material restaurador. Isso viabilizará a utilização de procedimentos preventivos de acordo com as necessidades individuais de cada paciente e a indicação do material restaurador, de acordo com o perfil de formação do biofilme, sendo este fator determinante no sucesso ou o fracasso do procedimento restaurador.

Os ensaios realizados no capítulo 1 suportam e complementam os resultados do capítulo 2, permitindo relacionar de maneira dinâmica diferentes materiais odontológicos ao biofilme *in situ*. Assim, estes estudos mostraram a importância do conhecimento das características intrínsecas dos materiais restauradores pelos cirurgiões-dentistas e a correta escolha destes em um tratamento restaurador. O desafio atual seria conhecer esta estrutura complexa, organizada, com altíssima capacidade de adaptação ao meio, como o biofilme. Depois de alcançado esse desafio, o cirurgião-dentista será capaz de modular o biofilme aos materiais restauradores, partindo de uma premissa adversa à praticada nos dias atuais.

CONCLUSÃO

Baseando - se nos resultados obtidos deste estudo, conclui-se que:

1. Pela análise quantitativa não houve interação significativa do material restaurador sob as características estruturais do biofilme. Entretanto, a análise qualitativa demonstrou variações na viabilidade bacteriana frente aos diferentes materiais restauradores. Assim, a resina composta e a cerâmica apresentaram predominância de células viáveis e canais definidos no biofilme.

2. Os efeitos do biofilme sobre as propriedades de superfície e microestrutura dos materiais apresentaram-se como material-dependente. Assim, todos os materiais restauradores estéticos apresentaram aumento da rugosidade, enquanto que os materiais ionoméricos e amálgama de prata apresentaram aumento significante da dureza.

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[1] 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

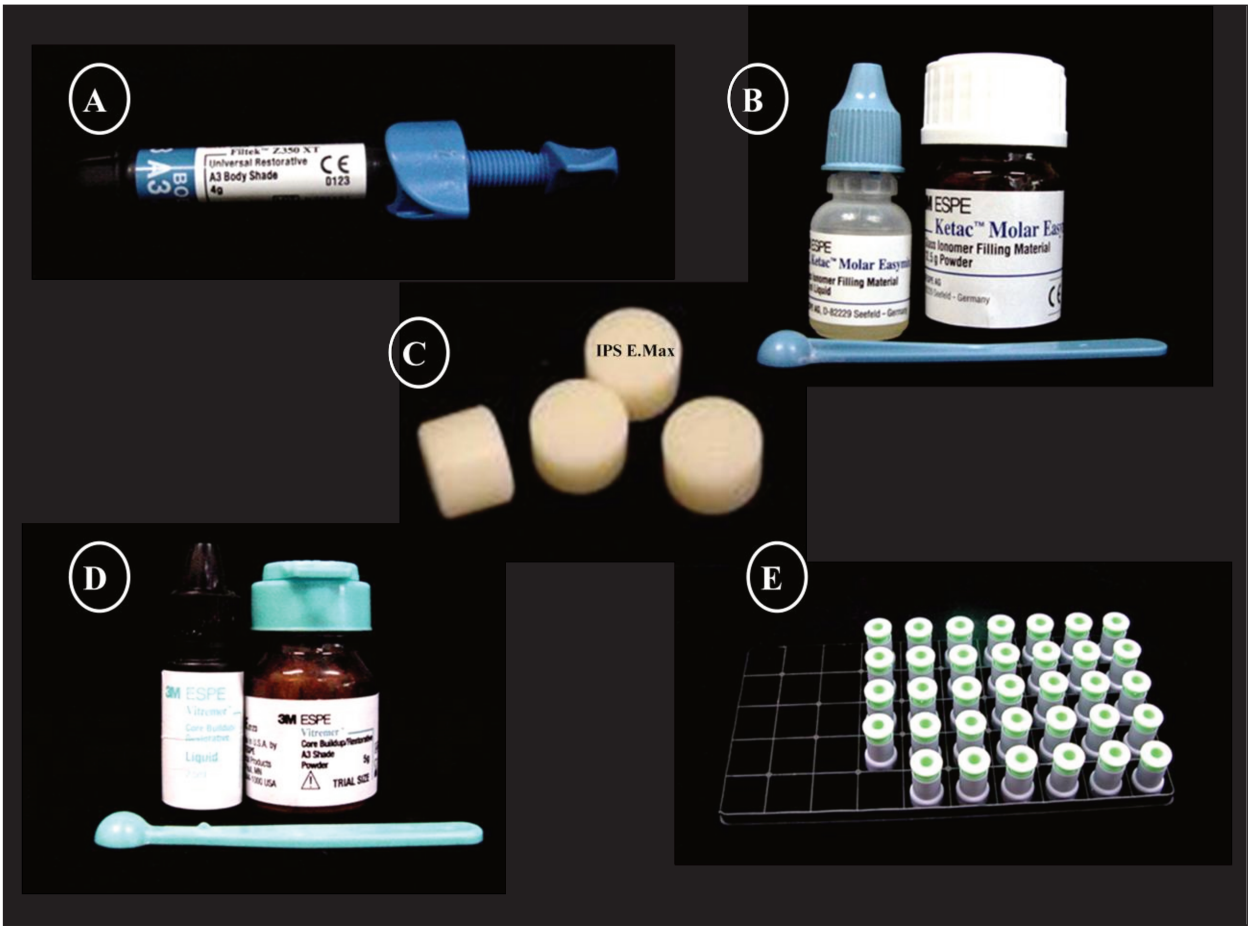

FIGURA 1 – *Materiais utilizados para confecção dos corpos-de-prova.*

A. Filtek Supreme (3M ESPE, St. Paul, MN, USA) – resina composta.

B. Ketac Molar Easymix (3M ESPE, St. Paul, MN, USA) – cimento de ionômero de vidro convencional.

C. IPS E.Max (Ivoclar Vivadent, Schaan, Liechtenstein) – cerâmica.

D. Vitremer (3M ESPE, St. Paul, MN, USA) – cimento de ionômero de vidro modificado por resina.

E. Amalgam GS-80 (SDI Limited, Dublin, Irlanda) – amálgama de prata.

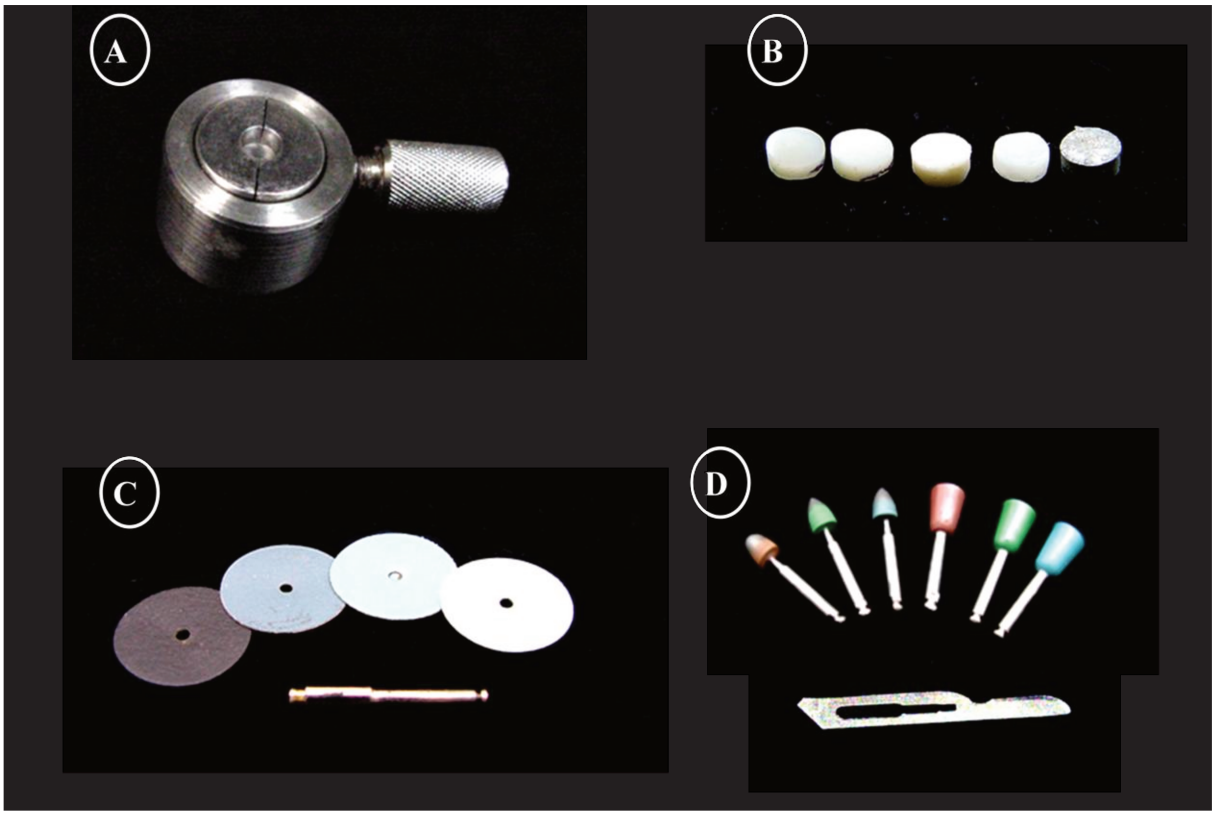

FIGURA 2 – *Confecção dos corpos-de-prova e materiais de acabamento e polimento.*

A. Matriz metálica para confecção dos corpos-de-prova.

B. Corpos-de-prova confeccionados (B1 – Filtek Supreme; B2 – Vitremer; B3 – Ketac Molar Easymix; B4 – IPS E.Max; B5 – Amalgam GS-80).

C. Kit de polimento Sof-lex (3M ESPE, St. Paul, MN, USA).

D. Kit de acabamento e polimento de amálgama (KG Sorensen, Cotia, SP, Brazil), Lâmina de bisturi nº 15 (Solidor, São Paulo, SP, Brasil).

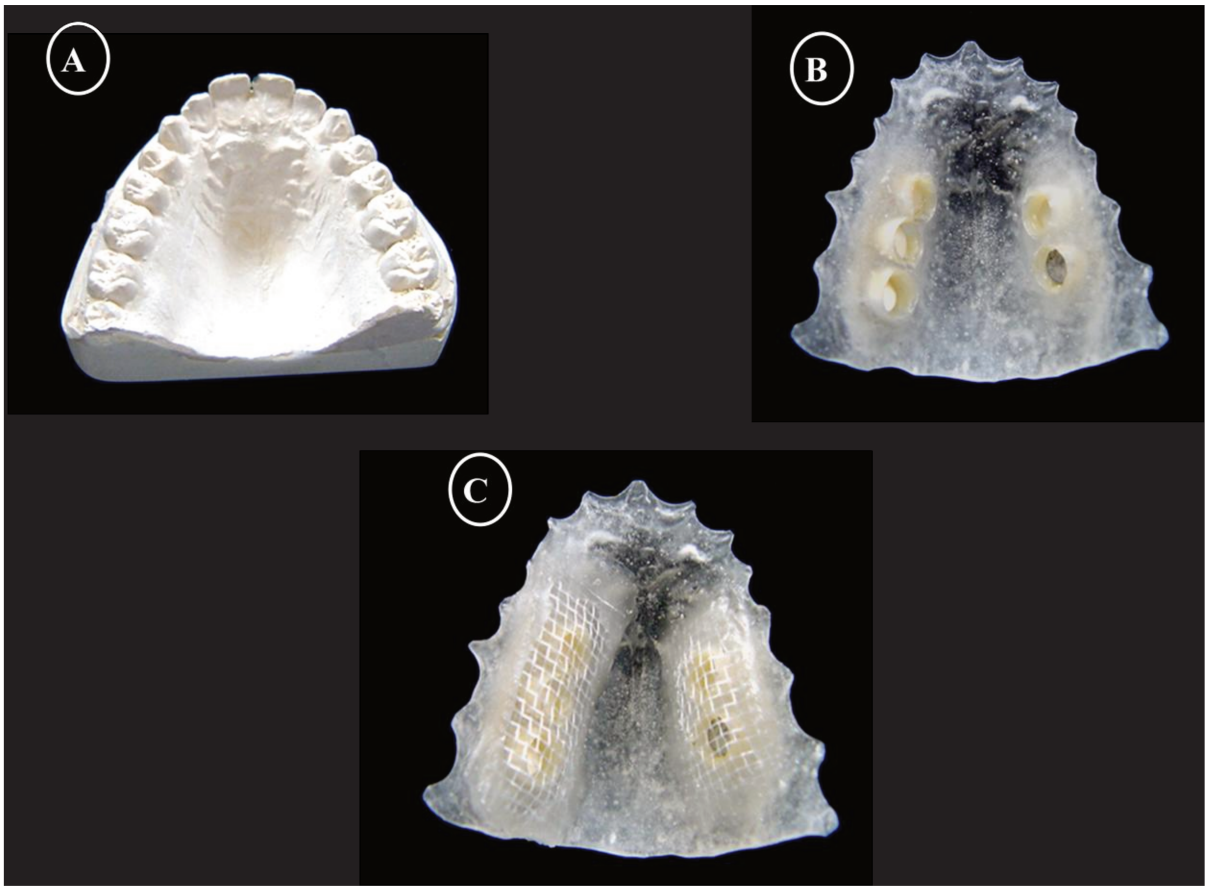

FIGURA 3 – *Materiais utilizados para confecção dos dispositivos intraoral.*

A. Modelo de gesso pedra.

B. aparelho de resina acrílica com corpos-de-prova fixados com cera pegajosa.

C. Aparelho de resina acrílica finalizado.

FIGURA 4 – *Fase de biodegradação in situ.*

A. Aparelho de resina acrílica adaptado à cavidade bucal do voluntário.

B. Porta-aparelho com gaze umedecida.

C. Creme dental e escova de dente padronizado.

D. Corpo-de-prova com biofilme aderido em sua superfície. O asterisco está demonstrando o biofilme e a seta está localizada no corpo-de-prova.

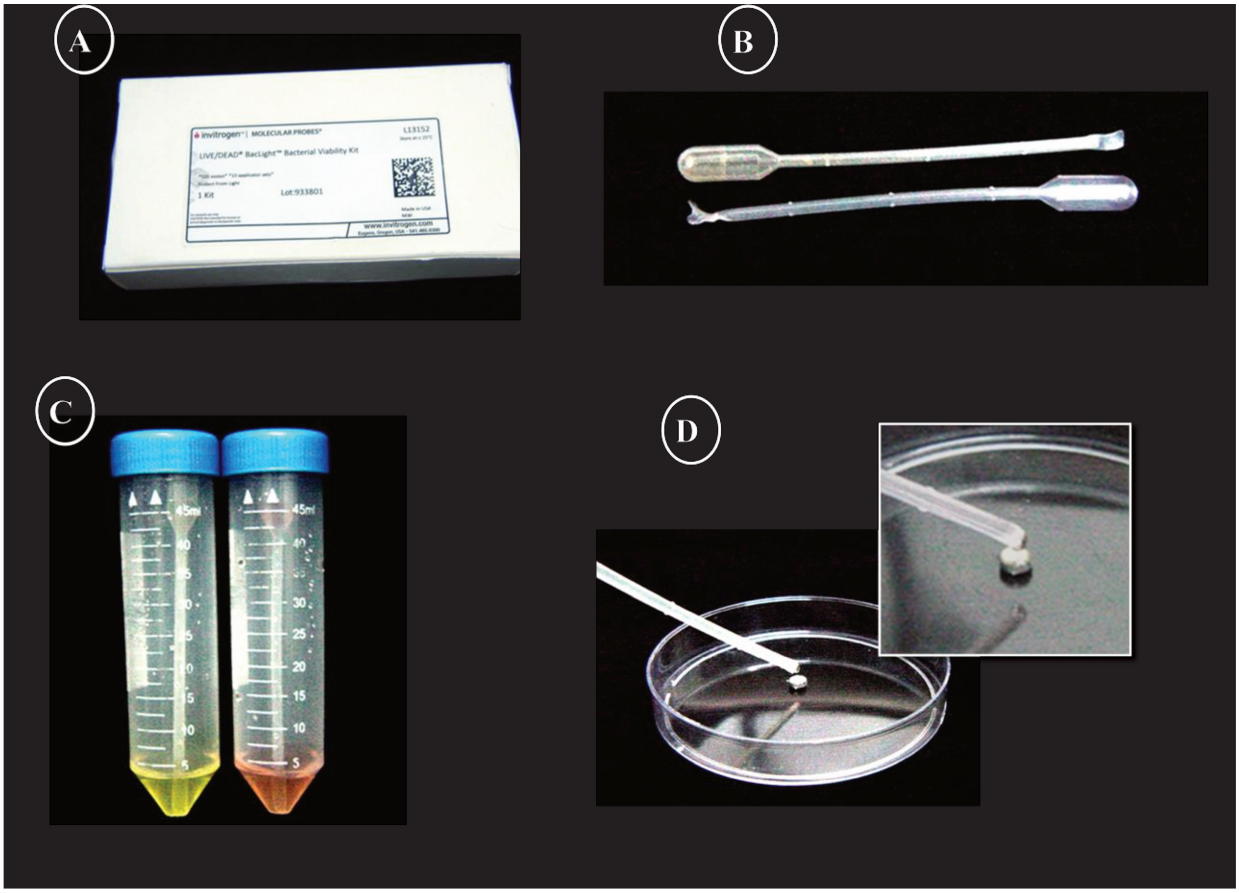

FIGURA 5 – *Materiais utilizados na realização da análise de microscopia confocal de varredura*

A. Kit de Viabilidade Bacteriana (LIVE/DEAD).

B. Corantes SYTO 9 e Propídio de iodeto.

C. Corantes diluídos em solução fisiológica.

D. Aplicação dos corantes sobre o corpo-de-prova.

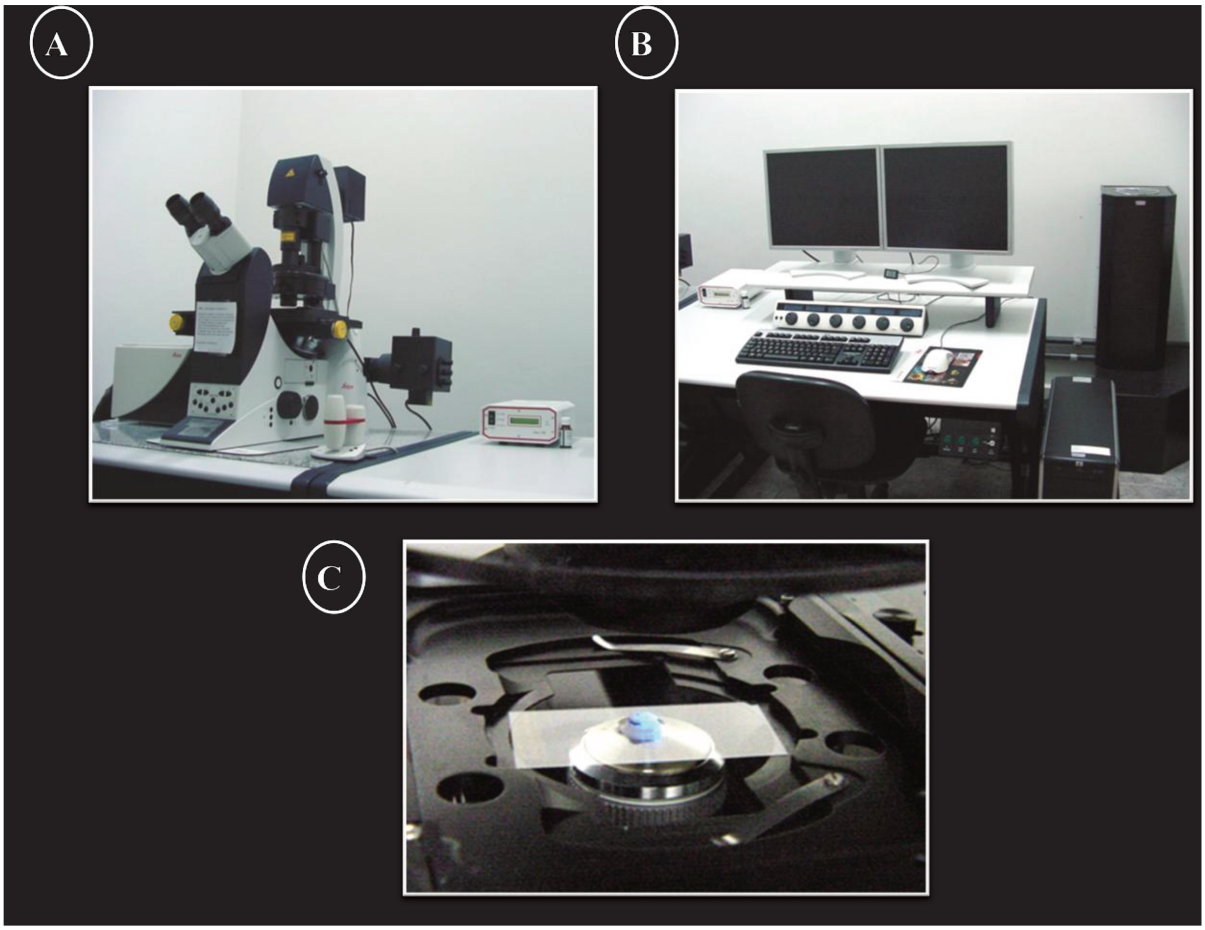

FIGURA 6 – *Microscópio de varredura confocal a laser utilizado para a realização das análises quantitativa e qualitativa.*

A. Parte óptica do microscópio de varredura confocal (LSM 510 META, Zeiss, Germany).
B. Hardware e software do microscópio de varredura confocal (LSM 510 META, Zeiss, Germany).
C. Corpo-de-prova fixado na lamínula e a varredura do laser de diodo.

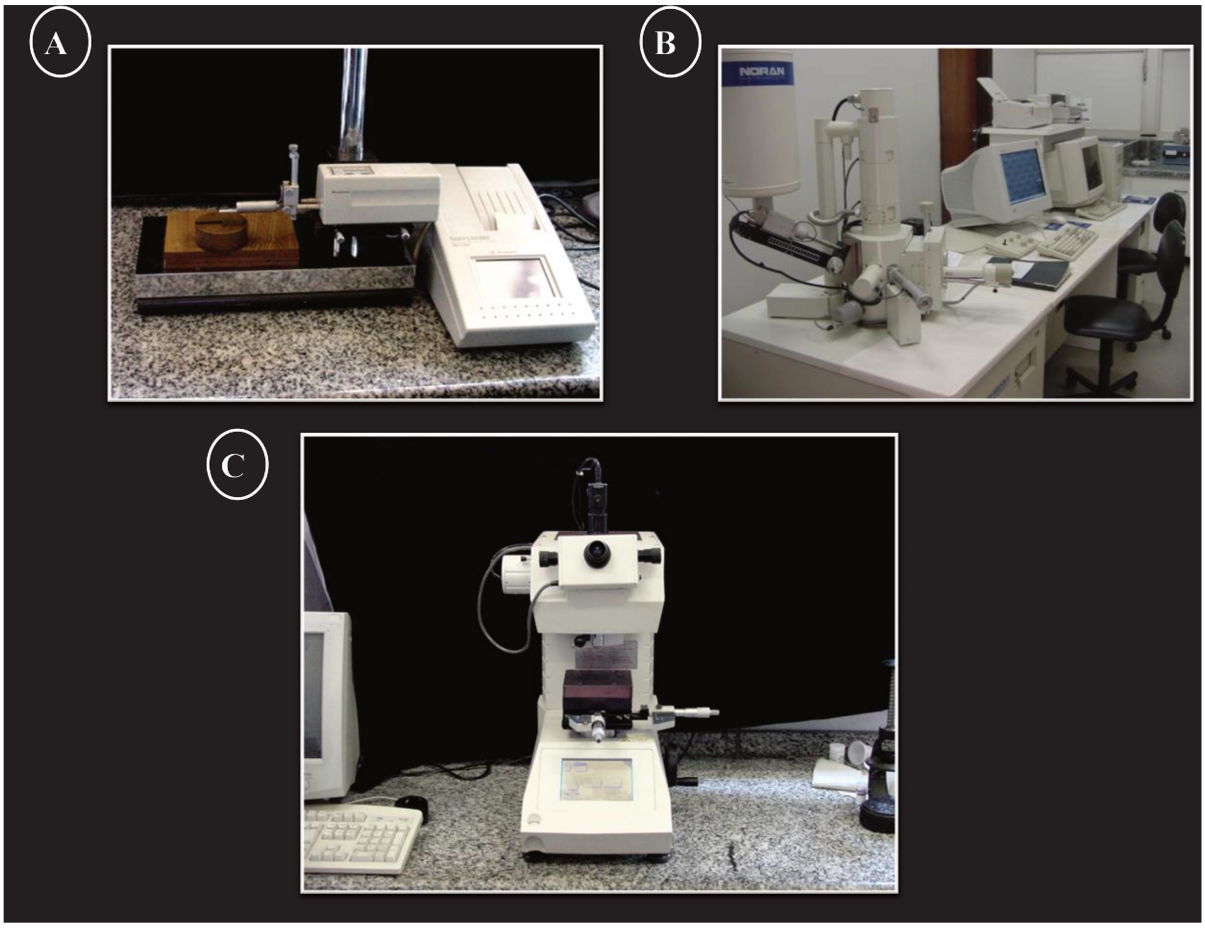

FIGURA 7 – *Equipamentos utilizados para a realização das análises quantitativa e qualitativa.*

A. Rugosímetro (Surfcorder SE1700, Kosaka Corp., Tókio, Japão).

B. Microscópio Eletrônico de Varredura (Balzers-SCD 050 Sputter Coater, Liechtenstein).

C. Durômetro (Shimatzu, Tokio, Japão).

ANEXO 1 – *Certificado de aprovação no Comitê de Ética*

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Avaliação, in situ, da interface entre materiais restauradores e biofilme dental"**, protocolo nº 136/2009, dos pesquisadores Gislaine Cristina Padovani e Regina Maria Puppin Rontani, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 25/11/2009.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Evaluation, in situ, of the interface between restouratives materials and dental biofilm"**, register number 136/2009, of Gislaine Cristina Padovani and Regina Maria Puppin Rontani, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at .

Prof. Dr. Pablo Agustin Vargas
Secretário
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

ANEXO 2 – *Certificado de submissão.*