UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

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Antibacterial potential of restorative materials against cariogenic species

BAURU
2017

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Potencial antibacteriano de materiais restauradores contra espécies cariogênicas

Dissertação constituída por artigo apresentada a Faculdade de Odontologia de Bauru - Universidade de São Paulo para obtenção do título de Mestre em Ciências no Programa de Ciências Odontológicas Aplicadas, na área de concentração Dentística.

Orientador: Profª. Drª. Juliana Fraga Soares Bombonatti

Versão Corrigida

BAURU

2017

Oliveira, Naiara Araújo de

OL4a Antibacterial potential of restorative materials against cariogenic species/ Naiara Araújo de Oliveira – Bauru, 2017.

60 p. : il. ; 31cm

Dissertação (Mestrado) – Faculdade de Odontologia de Bauru. Universidade de São Paulo

Orientador: Prof. : Profª. Drª. Juliana Fraga Soares Bombonatti

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FOLHA DE APROVAÇÃO

Dedico este trabalho ....

... aos meus pais, ***Maria Solange*** *e* ***João Batista*** *e a minha avó* ***Vicentina Maria*** *(in memoriam), pelo amor, dedicação e apoio. Sei que sempre posso contar com vocês, meu alicerce e porto seguro. Esta conquista também é de vocês.*

... ao meu irmão ***Danilo*** *e a todos os* ***meus familiares*** *e* ***amigos*** *de Minas e os de Bauru por todos os momentos já vividos e que ainda iremos trilhar.*

Agradeço...

*... a **Deus** por minha vida e mais esta conquista, por sempre iluminar meu caminho.*

*... à minha Orientadora, **Profa. Dra. Juliana Fraga Soares Bombonatti** e minha coorientadora **Profa. Dra. Flaviana Bombarda de Andrade**, pela oportunidade e dedicação profissional. Obrigada por serem as grandes responsáveis por este trabalho, por todo o tempo, confiança e pela sabedoria transmitida.*

*Ao **Prof. Dr. Sérgio Kiyoshi Ishikiriama**, pela oportunidade, sempre pronto a ajudar e principalmente, pela confiança e sabedoria transmitida.*

*Ao **Hospital de Reabilitação de Anomalias Craniofaciais** (Centrinho) por permitir mais esta etapa em minha vida profissional e pelo apoio e compreensão dos meus colegas de trabalho: **Aparício, Esdra, Lilian e Nádia** e pelo incentivo da **Dra. Terumi.***

*À **Faculdade de Odontologia de Bauru,** Universidade de São Paulo.*

Aos professores do departamento de Dentística e Materiais Odontológico por todo ensinamento e apoio.

Aos funcionários dos departamentos de Dentística, Materiais Dentários, Endodontia, Laboratórios de Bioquímica e das Clínicas.

Aos meus colegas de mestrado **Angélica, Fabrícia, Fernanda, Giovanna, Isabela, Lígia, Lorena, Mauro** *e* **Natália,** *pelo companheirismo e amizade construída durante nosso convívio.*

A minha amiga e dupla **Isabela Furlaneto Leão** *pelo companheirismo, ajuda em todas as horas. Obrigada pela parceria ao longo desta jornada e por ser esta pessoa sempre positiva e de muita luz. Meu muito obrigada pela amizade construída, sentirei saudades, mas estou na torcida.*

A todos que de alguma forma contribuíram para a realização deste trabalho, o meu obrigada!

Agradecimentos institucionais

À ***Comissão de pós-graduação*** *da Faculdade de Odontologia de Bauru, pela competência e eficiência.*

Aos ***funcionários da Biblioteca e Documentação da FOB/USP*** *que sempre atenciosos colaboraram para a aquisição de toda a da informação necessária para tornar possível essa dissertação*

A ***Universidade de São Paulo*** *pelas condições de estudo e pesquisa proporcionadas.*

Ao ***Programa de Aperfeiçoamento em Ensino (PAE)*** *por permitir o começo do exercício da docência.*

Ao ***programa de pós-graduação em Odontologia da FOB/USP*** *e a Diretora e vice-diretor da Faculdade de Odontologia de Bauru,* ***Prof.ª Dr. ª Maria Aparecida de Andrade*** *e* ***Prof. Dr. Carlos Ferreira dos Santos****, pela gestão, coordenação e geração de oportunidades de aprendizado utilizando a infraestrutura desta Escola.*

A todos o meu muito obrigada!

“ Nada melhor do que um sonho, para se criar o futuro...”
Victor Hugo

ABSTRACT

ABSTRACT

Objective: To determine the antibacterial potential of five restorative materials against microorganisms associated with caries process: *Streptococcus mutans*, *Lactobacillus casei* and *Bifidobacterium dentium.* **Methods:** The antibacterial effect of five different restorative materials were evaluated in this study: Fuji IX Extra, Fuji II LC, Ion-Z, Beautifil II and Filtek Z250XT using two methodologies: agar diffusion and direct contact tests. Glass ionomer cements (GIC) were used immediately or 10 minutes after manipulation. For the agar test the mean inhibition zones in millimeter (mm) were measured, and for the direct contact test the number of colony forming units was evaluated by scores. **Results:** Fuji IX Extra presented the greatest mean inhibition zone (14,00 mm) for *L. casei*, and Ion-Z immediately after manipulation showed inhibition zones for all three bacteria tested. At the direct contact test, *S. mutans* was the most resistant bacteria with dense bacterial growth, and *B. dentium* demonstrated lower resistance against the materials tested. **Conclusions:** Considering the methodology used and result analysis, conventional Glass Ionomer Cements presented better results than resinous materials, and the finding varied according tested bacteria.

Key words: Glass Ionomer Cements. Antibacterial agents. Dental caries. *Bifidobacterium dentium. Streptococcus mutans. Lactobacillus casei*.

Resumo

RESUMO

Potencial antibacteriano de materiais restauradores contra espécies cariogênicas.

Objetivo: Determinar o potencial antibacteriano de cinco materiais restauradores contra microrganismos envolvidos com o processo da cárie: *Streptococcus mutans*, *Lactobacillus casei* e *Bifidobacterium dentium.* **Métodos:** Cinco materiais restauradores foram analisados neste estudo: Fuji IX Extra, Fuji II LC, Ion-Z, Beautifil II e Filtek Z250XT. Foram testados em relação ao efeito antibacteriano através de duas metodologias denominadas: teste de difusão em ágar e teste contato direto. Os Cimentos de Ionômeros de Vidro foram utilizados imediatamente ou 10 minutos após manipulação. Para o teste de difusão em ágar a média das zonas de inibição em milímetros (mm) foram calculadas e para o teste de contato direto, o número de unidade formadora de colônia foram avaliados por score. **Resultados:** Fuji IX Extra apresentou maior zona de inibição de 14 mm para o *L. casei* e o Ion-Z utilizado após a manipulação apresentou zonas de inibição para todas as bactérias testadas. No teste de contato direto, a bactéria mais resistente foi o *S. mutans* com denso crescimento bacteriano e *B. dentium* demonstrou uma menor resistência em relação aos materiais testados. **Conclusão:** Pode-se concluir que os Cimentos de Ionômeros de Vidro convencionais apresentaram melhores resultados do que os materiais resinosos, variando de acordo com a bactéria testada.

Palavras Chaves: Cimentos de Ionômeros de Vidro. Antibacterianos. Cárie Dental. *Bifidobacterium. Streptococcus mutans. Lactobacillus casei*.

SUMÁRIO

1 Introduction

1 INTRODUCTION

The development of dental materials with antibacterial properties is an important goal in Dentistry to minimize the occurrence of recurrent caries (TARASINGH et al, 2015; VERMEERSCH et al, 2005). Recurrent caries is the main reason to replace resin composite restorations (NAOUM et al, 2012), and can be associated with degradation of restorative materials, allowing the entrance of cariogenic bacteria (HOTAWANI et al, 2013) and decreasing restoration longevity. Consequently, the material should prevent the metabolism of residual bacteria and inhibit new bacteria at the tooth/restoration interface, presenting proper antibacterial activity (TARASINGH et al, 2015).

The glass ionomer cement (GIC) has the best ability to prevent caries onset and progress, with laboratory and clinical evidences (NAOUM et al, 2012). This potential is correlated with some properties like chemical adhesion to dental structure and capability to release fluoride (GARCIA-CONTRERAS et al, 2015). The ability to release fluoride ions of GICs, could help in remineralization of the initial caries lesions, preventing the lesion progress and providing protection at the tooth/restoration interface (NAOUM et al, 2011). In addition, the low initial pH of GIC while setting may contribute to the antibacterial activity (VERMEERSCH et al, 2005).

The search for new restorative materials that are stable, esthetic and present strength and durability, are biocompatible and have antibacterial potential is a team work between industry and research. Therefore, hybrid materials such as resin modified glass ionomer cement, compomer and giomer were developed to combine the properties of GIC that release fluoride and the resin composite esthetics (HOTAWANI et al, 2013).

The addition of hydroxyethyl-methacrylate (HEMA) or bisphenol glycidyl methacrylate (BisGMA) in a resin-modified glass ionomer cement improved the mechanical properties as compressive resistance, hardness, greater elasticity modulus, higher solubility resistance and resilience (GARCIA-CONTRERAS et al, 2015). However, some challenges remain such as esthetic deficiency and difficulties to work because of its sticky consistency.

Within this purpose, the giomer system was introduced as a direct composite resin. It is available in Beautifil II, a composite resin manufactured by Shofu. This is a universal nano-

hybrid composite, made from functional particles that have surface pre-reacted glass filler (S-PRG filler), which work as a charge in composite resin. In this particle, the acidic/base reaction occurs within the glass particles (glass fluoride - aluminum -borosilicate particles) and alkenoic acid in the presence of water before incorporation in the resin (NAOUM et al, 2011). This characteristic differs giomers from compomers because the glass ionomer hydrogel inside compomers are formed after the contact with water that occurs after polymerization. In this way, the utilization of giomer is more significant because the acidic/base reaction is truly associated with the mechanical properties of the composite (TARASINGH et al, 2015). Thus, it is expected to keep the antibacterial properties, because its performance could be similar to a GIC.

An important clinical relevance for GIC during and after setting, besides its mechanical properties, is the capability to maintain its antibacterial effect, which may help to decrease or eliminate the bacteria in cavity walls, reducing the bacterial cariogenicity; additionally, the bio-function effect depends on a combination of retention time and amount of fluoride released (IAMAZATO et al, 2003; HOSZEK and ERICSON, 2008).

S. mutans and *L. casei,* acidogenic-aciduric bacterial species, are recognized to be involved in caries process. *S. mutans* is considered an etiological agent for carious lesions and *L. casei* particularly involved in carious lesion progression (YADIKI et al, 2016; ANDRADE et al, 2014). Therefore, the presence of other microbial species such as *Scardovia wiggsiae* and *Bifidobacterium spp*., which are acid-resistant and are also associated with dental caries were reported (HENE et al, 2015).

No papers were found in the literature testing the antibacterial effect of Ion-Z, a new conventional GIC with zinc, against bacteria and few studies with Giomer system. Experiments testing the ability of restorative materials to act on *Bifidobacterium dentium*, were neither not found.

This study evaluated the antibacterial efficacy of different restorative materials on common microorganisms involved in the caries process: *Streptococcus mutans, Lactobacillus casei* and *Bifidobacterium dentium*.

2 ARTICLE

2 ARTICLE

The article presented in this Dissertation was written according to Journal of Dentistry (Annex A).

Antibacterial potential of restorative materials against cariogenic species

Abstract:

Objective: To determine the antibacterial potential of five restorative materials against microorganisms associated with caries process: *Streptococcus mutans*, *Lactobacillus casei* and *Bifidobacterium dentium.* **Methods:** The antibacterial effect of five different restorative materials were evaluated in this study: Fuji IX Extra, Fuji II LC, Ion-Z, Beautifil II and Filtek Z250XT using two methodologies: agar diffusion and direct contact tests. Glass ionomer cements (GIC) were used immediately or 10 minutes after manipulation. For the agar test the mean inhibition zones in millimeter (mm) were measured, and for the direct contact test the number of colony forming units was evaluated by scores. **Results:** Fuji IX Extra presented the greatest mean inhibition zone (14.00 mm) for *L. casei*, and Ion-Z immediately after manipulation showed inhibition zones for all three bacteria tested. At the direct contact test, *S. mutans* was the most resistant bacteria with dense bacterial growth, and *B. dentium* demonstrated lower resistance against the materials tested. **Conclusions:** Considering the methodology used and result analysis, conventional Glass Ionomer Cements presented better results than resinous materials, and the finding varied according tested bacteria.

Clinical Significance: Evaluate restorative materials behavior against cariogenic species involved in caries process, predict if they could inhibit their growth. In mouth, we have many factors involved in caries lesions process and in recurrent caries, so this *in vitro* investigation had that conventional Glass Ionomer Cement could have an efficacy to inhibit bacterial growth and the addition of some elements like zinc could contribute to its behavior.

Key words: Glass Ionomer Cements. Antibacterial agents. Dental caries. *Bifidobacterium dentium. Streptococcus mutans. Lactobacillus casei*.

1. Introduction

The development of restorative materials with antibacterial activity is fundamental for successful restorations and is clinically relevant, because they can minimize the consequence of recurrent caries lesions [1,2]. Recurrent caries is a complication which is the main reason to replace a restoration, limiting their lifetime [3,4]. To present proper antibacterial activity, the material should prevent the metabolic activity of residual bacteria in the cavity and inhibit new bacteria that may access the cavity through microleakage at the tooth/restoration interface [5].

Glass ionomer cement (GIC) has the best ability to prevent caries onset and progress, with laboratory and clinical evidences [3]. It is frequently used for restorations in patients with high caries risk because of its biocompatibility, cariostatic properties [6], fluoride release [7] and chemical adherence with dental structure [8].

As a consequence, researches have tried to find alternatives to upgrade the physical and antibacterial properties of GIC with some additives like chlorhexidine gluconate [9], EGCG (Epigallocatechin-3-gallate), a polyphenol present in green tea, hydroxyapatite and strontium [10], but in some cases these changes decreased the material's physical properties. A recent material available in Brazil is Ion-Z, a conventional GIC that present in its composition zinc oxide particles that can contribute to GIC abilities to inhibit bacterial growth in addition to its fluoride-leaching ability.

Hybrid materials such as resin-modified glass ionomer cement, compomer and giomer are postulated to combine the fluoride-releasing property of GIC and the esthetics of composite resin [11]. These materials demonstrated antibacterial inhibitory effect against *S. mutans* [5], and the addition of hydroxyethyl-methacrylate (HEMA) or bisphenol glycidyl methacrylate (BisGMA) improved the mechanical properties [7]. In addition to HEMA and BisGMA, the giomer, presented in Beautifil II, has functional particles that work as a charge inside the composite, namely surface pre-reacted glass fillers (S-PRG filler), which are able to release and recharge fluoride [1]. In this way, it is expected to keep the antibacterial properties, because its performance could be similar to a GIC.

An important clinical relevance for GIC during and after setting, besides it mechanical properties, is the capability to maintain its antibacterial effect, which may help to decrease or eliminate the bacteria in cavity walls, reducing the bacterial cariogenicity [12,13].

S. mutans and *L. casei,* acidogenic-aciduric bacterial species, are recognized to be involved in caries process. *S. mutans* is considered an etiological agent for carious lesions and

L. casei particularly involved in carious lesion progression [9,14]. Therefore, the presence of other microbial species such as *Scardovia wiggsiae* and *Bifidobacterium spp*., which are acid-resistant and are also associated with dental caries were reported [15]. Thus, this investigation evaluated the antibacterial efficacy of different restorative materials from common microorganisms involved in caries process: *Streptococcus mutans, Lactobacillus casei* and *Bifidobacterium dentium*.

2. Materials and methods:

2.1. Experimental design

This *in vitro* study had two study variables, restorative materials and setting time, by two methodologies. The tested materials were Fuji IX Extra, Fuji II LC, Ion-Z, Beautifil II and Filtek Z-250XT. The glass ionomer cements were evaluated immediately after manipulation and 10 minutes after manipulation. The methodologies used were agar diffusion and direct contact tests.

2.2. Materials and specimen preparation:

The materials used in this study are detailed in Table 1. The materials were prepared according to the proportion and manufacturer's instructions in aseptic conditions. GICs were analyzed immediately after manipulation (I) and 10 minutes after manipulation (M), as detailed below.

For the agar diffusion test, four wells (3-mm thickness and 5-mm diameter) were punched in agar plates and the five restorative materials were placed directly in contact with agar and inoculum. The GICs were prepared and applied immediately (I) inside wells or inside tubes with sterile instruments and Centrix dispenser. Fuji II LC (I) was cured inside for the agar test; for the direct contact test, it was cured immediately after manipulation and inserted in tubes.

The GICs tested after 10 minutes (M), with an initial setting, and the resin specimens were prepared in a sterilized bipartite matrix (3-mm thickness and 5-mm diameter), covered and pressed between two transparent glass plates to delimit the material's thickness, using digital pressure. The resins and Fuji II LC were cured with a light curing unit (DB 685, DABI ATLANTE, São Paulo, Brazil) at an intensity of 961mW/cm^2 for 20 seconds on both sides. Then, 10 minutes after preparation for GICs, the materials were inserted in the culture medium, in wells for the agar diffusion test and inside tubes with broth for the direct contact

test. Fuji II LC (M) specimens were maintained inside the matrix for 10 minutes for initial setting, then light cured and carried out on tests.

A 2% Chlorhexidine Gluconate solution was used as control of bacterial inhibition in the agar diffusion test and direct contact test.

2.3. Bacterial strains

S. mutans (ATCC 25175), *L. casei* (ATCC 334) and *B. dentium* (ATCC 27534) reference strains were used. Bacterial cultures were frozen to obtain stocks of each species. When necessary, the stocks were reactivated. The reactivated microorganisms *S. mutans* and *L. casei* were cultivated in BHI (Brain Heart Infusion) broth (Difco, BD, Sparks, MD, USA). For *B. dentium* growth, a mix of BHI, TSB (Tryptic Soy Broth) and Yeast Extract Granulated broth, (Difco, BD, Sparks, MD, USA) were used in anaerobic conditions. *S. mutans* and *L. casei* were incubated inside anaerobic jars with a candle flame to get a CO_2 environment. For *B. dentium,* besides the anaerobic jar, an Anaerobac sachet was used (Probac do Brazil LTDA, Santa Cecília, São Paulo, Brazil) to remove all oxygen. After initial proliferation, the bacteria were transferred to new medium for three consecutive days until their exponential multiplication. All bacteria were tested by Gram straining in the beginning and the end of the experiment, to secure the purity of the strains. The culture medium turbidity was read on a visible light spectrophotometer SF325NM (Bel Photonics, Brazil Ltda, Osasco, SP) with 540 nm wavelength to obtain the inoculum absorbance and adjusted to Mc Farland scale. The inoculum suspension was prepared in sterile saline and diluted to 10^5 and 10^3 CFU/mL (Colony Forming Units/mL) for agar diffusion test and direct contact test, respectively, according to pilot studies.

2.4. Agar diffusion test

BHI agar was used for agar diffusion test. About 18 mL of BHI agar was spread evenly to a thickness of 5 mm in the Petri dishes. After solidification, four wells (3-mm thickness and 5-mm diameter) were punched in agar plates where the materials were directly applied. Then, 100μL of bacteria inoculum were poured with micropipette over the agar surface and spread evenly using a plate spreader to ensure distribution of the bacteria inocula. All procedures were carried out under aseptic conditions in a laminar airflow chamber.

Then, the previously manufactured specimens were positioned inside agar wells. The Petri dishes were maintained under laminar airflow chamber for two hours to allow diffusion

of materials components, then the Petri dishes were incubated in an anaerobic jar at 37ºC for 48 hours. Sterile paper discs with 5-mm diameter soaked in 2% chlorhexidine gluconate solution was placed under agar and used as control.

The diameter of inhibition zones in millimeters (mm) around the material discs were measured after 48 hours. The results were expressed as mean diameters and standard deviation. This test was performed in triplicate for each restorative material.

2.5. Direct Contact Test

The inocula of the tested bacteria were diluted in sterile saline solution to 10^3 UFC/mL. After bacteria distribution inside tubes with broth, the specimens of restorative materials were added to each tube. The reaction mixture was incubated at 37ºC for 24 hours in anaerobic conditions. After incubation, it was possible to observe the media turbidity (bacterial growth), which was obtained by reading in a visible light spectrophotometer SF325NM (Bel Photonics, Brazil Ltda, Osasco, SP) with 540 nm wavelength. After reading, 50 μL of each tube were taken away and spread in agar plates to determine bacterial growth, by CFU/mL counting. The confluence of colonies was categorized in scores, which were visually observed according to the criteria given in Table 2, after 48 hours of incubation. The bacterial growth data were the total sum of both scores. The test was performed in triplicate for each restorative material and also for chlorhexidine gluconate solution, which was used as control.

Agar diffusion test is a popular test to evaluate antibacterial properties of restorative materials. However, it has some disadvantages like, it depends on the solubility and diffusion properties of both the test material and media. Although direct contact test in which the test material remains in direct contact with the bacteria, independent of the diffusion properties, may be more suitable for testing restorative materials and cements than agar diffusion test [16]

To validate the tests did in this study, for direct contact test, control tubes was used with the materials in absence of inoculum, demonstrated absence of bacterial growth and one tube was used with bacterial inoculum, without restorative material, presented a confluent growth of microorganisms. For agar diffusion test sterile paper discs imbibed with chlorhexidine was used as a control, showing a higher inhibition zones for the three bacteria tested.

2.6. Statistical analysis

To examine the inhibition zones, one-way ANOVA followed by Tukey's test for multiple comparisons were applied at a significance of 5% (p=0.05). Two-way ANOVA was applied to assess the inhibition zones of GIC (I) and (M), considering material and time. Direct contact data were submitted to non-parametric tests using Kruskal-Wallis followed by Dunn's test for multiple comparisons. The analyses were carried out on SPSS for Windows software, version 19.0 (IBM, Statistic, Chicago, USA), at a significance level of 0.05%.

3. Results

3.1. Agar diffusion test

The mean values and standard deviations of the growth inhibition zones of five restorative materials against three bacteria are shown in Table 3. Among the five tested materials, Ion-Z (I) demonstrated inhibition zones for all three tested bacteria. Chlorhexidine gluconate, as the control group, showed the major inhibition zones for all bacteria: *S. mutans* (16.66 mm), *L. casei* (28.50 mm) and *B. dentium* (32.16 mm). Some materials did not present inhibition zones, as shown in Table 3 and Figure 1.

For *S. mutans* just Fuji IX (I) and Ion-Z (I) presented inhibition mean zones of 3.00 and 10.66 respectively. *L. casei* had more difference between materials. Considering the GIC tested immediately, Fuji IX (I) and Ion-Z (I) had means of inhibition zones of 6.66 and 7.33, respectively. When evaluate all tested materials, Fuji IX Extra (M) showed the broadest inhibition zone of 14.0 mm.

B. dentium did not show difference among the materials. Fuji IX Extra (M), Ion-Z (I), Ion-Z (M) and Beautifil II had shown inhibition zones over this bacterium. Analyzing data between the three bacteria tested, the major inhibition zone was promoted by Fuji IX Extra (M) over *L. casei* (14.00).

Comparing GIC immediately or after 10 minutes of manipulation Table 4., *S. mutans* was inhibited by Fuji IX Extra (I) and Ion-Z (I) when the immediately material was used, with significant statistical difference. Also, when analyzing *L. casei,* Fuji IX Extra (M) had a better performance than GICs immediately. *B. dentium* was inhibited for GIC (I) and (M) without significant statistical difference.

3.2. Direct Contact Test

Only chlorhexidine demonstrated absence of bacterial growth Table 5. Comparing the three bacteria tested, *S. mutans* was the higher resistant bacterium against all five tested restorative materials.

The mean score for *S. mutans* was a dense bacterial growth on the entire agar surface for all restorative materials tested. Statistical differences among restorative materials were not observed for *L. casei*, which presented a score 2 of growth in all plates.

B. dentium showed differences of growing among restorative materials tested. Fuji II LC (I) was the material that allowed a higher bacterial growth. Most of the materials presented scores between 1.66 to 2.22 within the same statistical classification. Fuji IX Extra (M) promoted a slight increase in inhibition of this microorganism compared to the other materials and its performance was not statistically different from chlorhexidine.

4. Discussion

This *in vitro* study evaluated the potential of different restorative materials against bacteria involved in caries lesion process and investigating data literature were not found papers testing the antibacterial effect of Ion-Z, a new conventional GIC with zinc, against bacteria. Scarce studies analyzing Giomer system was found and experiments testing restorative materials against *B. dentium*, were neither not found, so we selected then. It is known that the levels of *S. mutans, Lactobacillus* and *Bifidobacteria* are positively correlated to caries experience in children and also in adults. *B. dentium* is the predominant *Bifidobacteria*, but there is little information about its colonization in dentition. Contrary of *S. mutans, B. dentium* does not colonize hard tissue surfaces but dentin exposed by caries lesions facilitate the attachment and proliferation of *B. dentium*, suggesting that they have a predisposition to colonize carious dentine [19,20]. Both *Lactobacillus* and *Bifidobacteria* prefer lower pH to produce acids, so *S. mutans* acid production, promotes a convenient environment to these species [21]. Therefore, these three bacteria were selected for tests in the present study because their cariogenic abilities.

S. mutans was the most resistant bacteria in the present study, presented a score 3 in direct contact test, showing a dense bacterial growth for all restorative materials tested, not even for GICs was different. However, in agar diffusion test, Fuji IX Extra (I) and Ion-Z (I) could inhibit *S. mutans* growth, demonstrated that the low initial pH and maybe a fluoride release of these materials could have a potential to inhibit its growth.

Fluoride is considered an anticariogenic agent that could inhibit bacterial growth and their metabolism with a reduction of acid manufacture [8,16]. The GIC eluate had inhibit the acid production of oral *Streptococci,* stopped the pH fall completely around pH 4.8-5.0 and special notability in decreased the rate of acid production at pH 5.5, suggesting that *Streptococcus* acid production could be inhibited in areas adjacent to GIC fillings at an acidic pH around the critical pH of tooth demineralization (pH of 5.5), leading in a reduction of *Streptococcus* cariogenicity [6]. In this way, is believed that GIC contribute to the inhibition of recurrent caries because contain a high percentage of fluoride (10-20% in the powder fraction), and part of this fluoride are released in the vicinity of the cement, that could diffuse to the surrounding tooth structure [13].

Comparing GICs in agar diffusion test in the present study, the conventional GICs also had a different scale of inhibition against bacteria tested, proving that they can work better against microorganisms involving in caries process than resinous materials. Among GICs, Ion-Z, available in Brazil shortly, demonstrated inhibition for the three bacteria tested, as well as (I) and (M), maybe because it has particles of zinc oxide, that could had contributed to inhibiting bacterial growth. Similar outcome was shown in a study [22], comparing antimicrobial properties of 14 different restorative materials, nine of which were GIC. The GIC materials which contain zinc oxide produced zones of inhibition larger than materials without zinc oxide.

Experimental resins composites with S-PRG filler in different concentrations (13.9% or greater) demonstrated inhibition of *S. mutans* growth on their surfaces caused by the release of ions from these fillers [23]. Beautifil II, a composite resin which present this S-PRG filler, designed as a fluoridated glass filler with a glass-ionomer matrix layer surrounded by a thicker hydrogel layer, that are incorporated in the resin matrix are postulated to be capable to release and recharge fluoride. Comparing three restorative materials: Beautifil II, F-2000 (Compomer) and Fuji II LC against *S. mutans,* Fuji II LC exhibited the highest mean inhibitory zone and Beautifil II were in the second place in inhibition. This is because Beautifil II behave more like a resin-modified GIC and has a cumulative fluoride release of about 20% of the original GIC [5]. These finds were contrarily of the present study, because Beautifil II cannot inhibit the growth of *S. mutans* and neither for *L. casei,* just for *B. dentium,* in agar diffusion test, Beautifil II presented inhibition zones and for direct contact test it had shown a moderate bacterial growth. This find can suggest that Beautifil II could have a different behavior of the composite resin Filtek Z250 XT tested, and maybe Beautifil II could

have a slight fluoride leaching ability. Filtek Z250 XT did not shown zones of inhibition, maybe because it was a material without bio-function.

B. dentium, in this study, demonstrated a lower resistance against the tested restorative materials in direct contact test, presented a mean score of slight and moderate bacterial growth, showing that these materials could have a potential to inhibit their proliferation, and this bacterium are more sensitive to materials behavior. Besides materials, Fuji IX Extra (M) demonstrated a better potential to inhibit this bacterium. In agar diffusion test for *B. dentium*, the materials that presented inhibition zones were Fuji IX Extra (M), Ion-Z (I) and (M) and Beautifil II.

Testing two GIC (Fuji II LC and Fuji IX Extra) with or without chlorhexidine, all the experimental materials showed antibacterial properties against *S. mutans,* and these properties decreased with time, but Fuji II LC with chlorhexidine presented the major value [9]. These results comparing materials are different for our, because we did not have antibacterial zones for Fuji II LC, a resin modified GIC in any of the bacteria tested and also comparing Fuji II LC and Fuji IX Extra, just Fuji IX Extra presented inhibition zone.

In agar diffusion test, for *L. casei,* Fuji IX Extra (M) presented the major inhibition zone (14.00) between restorative materials. Ion-Z (I) and (M) presented an intermediate potential to inhibit *L. casei*, but superior to the other restorative materials tested. For direct contact test, *L. casei* presented a mean score of 2, a moderate bacterial growth, for all materials tested without statistical difference, showing that materials could partially inhibit *L. casei*.

Nowadays, the attention is turning to a less removal of tooth structure, called the minimal intervention dentistry. In this conception, demineralized dentin was maintained thus, some active bacteria reside in the cavity, because this dentin had a potential to remineralize. This minimal intervention has some principles: remineralization of early lesions; reduction cariogenic bacteria; minimum surgical intervention; repair rather than replacement of defective restorations; disease control. GIC for this kind of minimal intervention is so important and could profit it, because release fluoride and other ions and has the potential of chemical adhesion to tooth structure. The adhesion between material and tooth will help preventing bacterial microleakage and the ions available in the cement: calcium, phosphate and fluoride, could help in remineralization [24]. Following this thought, the materials tested in this study, the conventional GICs presented a better performance against bacteria and Ion-Z

(I) was capable to act the three bacteria tested and could contribute to inhibit or decrease bacterial growth, , but other studies is necessary to clarify it behavior.

No restorative material could be completely powerful in preventing bacterial microleakage or recurrent caries [4,24]. Manufacturing bio-function restorative materials that would be able to maintain their physical properties and antibacterial activity after being placed in the cavity, it will be helpfully to eliminate the damaging effect caused by bacterial microleakage [12], preventing bacterial growth and caries lesions progression.

5. Conclusions

Considering the methodology used and result analysis, the conventional GICs presented a better antibacterial effect comparing to resinous materials, and the finding varied according tested bacteria. *S. mutans* was the most resistant bacteria in direct contact test, and in agar diffusion test just the conventional GICs (I) could inhibit its growth. *L. casei* presented a moderate bacterial growth, for all materials tested and conventional GICs I and M had presented inhibition zone. *B. dentium,* demonstrated a lower resistance in direct contact test and in agar diffusion test, the conventional GICs I and M and Beautifil II had presented inhibition zones.

Conflict of interest

There is no conflict of interests to be reported.

Acknowledgements

The authors wish to thanks Bauru School of Dentistry – Universityof São Paulo for the opportunity to did this investigation, and the Professor PhD Evandro Watanabe, Ribeirão Preto School of Dentistry / University of São Paulo for giving us *S. mutans* and *L. casei* strains. ,

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TABLES CAPTURE

Table 1 – Materials evaluated:

Materials	Classification	Composition
Fuji IX Extra (***Shofu)***	Conventional Glass Ionomer Cement	Fluoride - aluminum - silicate glass, potassium persulphate and ascorbic acid.
Fuji II LC (***Shofu)***	Modified glass ionomer cement	Fluoride - aluminum - silicate calcium glass particles, composite monomers and photo initiators.
Ion Z (***FGM)***	Conventional Glass Ionomer Cement	Glass of calcium-aluminum-zinc-fluoride-silicate,polycarboxilic acid, deionized water, titanium dioxide, iron oxide.
Beautifil II (***Shofu)***	Composite resin - (Giomer system)	Glass particle S-PRG[a], glass fluoride - aluminum -borosilicate particles, TEGDMA[b], BisGMA[c], particles' size 20-40 nm.
Filtek Z250 (***3M ESPE)***	Composite resin	BisGMA, UDMA[d], BisEMA[e] (zirconia/silica), particles' size 0,01 – 3,5µm.

[a] S-PRG: surface pré-reacted glass;
[b]TEGDMA: triethyleneglycoldimethacrylate.
[c]BisGMA: bisfenolglicidilmethacrylate
[d]UDMA: urethanedimethacrylate;
[e]BISEMA: 2,2-Bis[4-(2-methacryloxyethoxy)phenylpropane.

Table 2. Criteria to determining bacterial growth on the agar surface (Modified from Iamazato [17]).

Score	Status
3	Dense bacterial growth on the entire agar surface.
2	Moderate bacterial growth on the entire agar surface.
1	Slight bacterial growth on the entire agar surface.
0	No bacterial growth.

Table 3. Mean values of inhibition zones+/- S.D. (in mm)

MATERIALS	*S. mutans*	*L. casei*	*B. dentium*
Fuji IX Extra (M)	0.00±0.00 ^{A,a}	14.00±1.73 ^{A,b}	9.33±8.14 ^{A,a}
Fuji IX Extra (I)	3.00±5.19 ^{A,a}	6.66±1.15 ^{B,a}	0.00±0.00 ^{B,a}
Fuji II LC (M)	0.00±0.00 ^{A,a}	0.00±0.00 ^{C,a}	0.00±0.00 ^{B,a}
Fuji II LC (I)	0.00±0.00 ^{A,a}	0.00±0.00 ^{C,a}	0.00±0.00 ^{B,a}
Ion-Z (M)	0.00±0.00 ^{A,a}	2.00±3.46 ^{C,a,b}	7.33±1.52 ^{A,b}
Ion-Z (I)	10.66±4.61 ^{B,a}	7.33±1.15 ^{B,a}	6.33±5.68 ^{A,a}
Beautifil II	0.00±0.00 ^{A,a}	0.00±0.00 ^{C,a}	6.00±0.00 ^{A,a}
Filtek Z-250 XT	0.00±0.00 ^{A,a}	0.00±0.00 ^{C,a}	0.00±0.00 ^{B,a}
Chlorhexidine	16.66±0.57 ^{B,a}	28.5±0.50 ^{D,b}	32.16±1.25 ^{B,c}

ANOVA and Tukey's test;α=0.05; SD.: standard deviation. Same upper case letters indicate no statistical difference in the column. Same lower case letters indicate no statistical difference in the lines

Table 4. Mean values of inhibition zones+/- S.D. (in mm) comparing GIC immediately or after 10 minutes of manipulation.

MATERIALS	*S. mutans*	*L. casei*	*B. dentium*
Fuji IX Extra (M)	0.00±0.00 ^{A,a}	14.00±1.73 ^{A,b}	9.33±8.14 ^{A,a}
Fuji IX Extra (I)	3.00±5.19 ^{B,a}	6.66±1.15 ^{B,C,a}	0.00±0.00 ^{B,a}
Fuji II LC (M)	0.00±0.00 ^{A,a}	0.00±0.00 ^{D,a}	0.00±0.00 ^{B,a}
Fuji II LC (I)	0.00±0.00 ^{A,a}	0.00±0.00 ^{D,a}	0.00±0.00 ^{B,a}
Ion-Z (M)	0.00±0.00 ^{A,a}	2.00±3.46 $^{C, D,a,b}$	7.33±1.52 ^{A,b}
Ion-Z (I)	10.66±4.61 ^{B,a}	7.33±1.15 ^{B,a}	6.33±5.68 ^{A,a}

ANOVA two criteria. Same upper case letters indicate no statistical difference in the column. Same lower case letters indicate no statistical difference in the lines.

Table 5. Mean scores of bacteria growths under material

MATERIALS	*S. mutans*	*L. casei*	*B. dentium*
Fuji IX Extra	3.00±0.00 ^{A,a}	2.00±0.00 ^{A,b}	1.33±0.50 ^{A,C,b}
Fuji IX Extra (I)	3.00±0.00 ^{A,a}	2.00±0.00 ^{A,b}	1.66±0.50 ^{A,b}
Fuji II LC	3.00±0.00 ^{A,a}	2.00±0.00 ^{A,b}	2.00±0.00 ^{A,b}
Fuji II LC (I)	3.00±0.00 ^{A,a}	2.00±0.00 ^{A,b}	2.44±0.50 ^{B,b}
Ion-Z	3.00±0.00 ^{A,a}	2.00±0.50 ^{A,b}	2.00±0.00 ^{A,b}
Ion-Z (I)	3.00±0.00 ^{A,a}	1.89±0.60 ^{A,b}	1.77±0.60 ^{A,b}
Beautifil II	3.00±0.00 ^{A,a}	2.00±0.00 ^{A,b}	2.22±0.40 ^{A,b}
Filtek Z-250 XT	3.00±0.00 ^{A,a}	2.00±0.00 ^{A,b}	1.66±1.30 ^{A,b}
Chlorhexidine	0.00±0.00 ^{B,a}	0.00 ±0.00 ^{B,a}	0.00±0.00 ^{C,a}

Kruskal-Wallis and Dunn test; α=0.05. Same upper case letters indicate no statistical difference in the column. Same lower case letters indicate no statistical difference in the lines

FIGURE CAPTURE

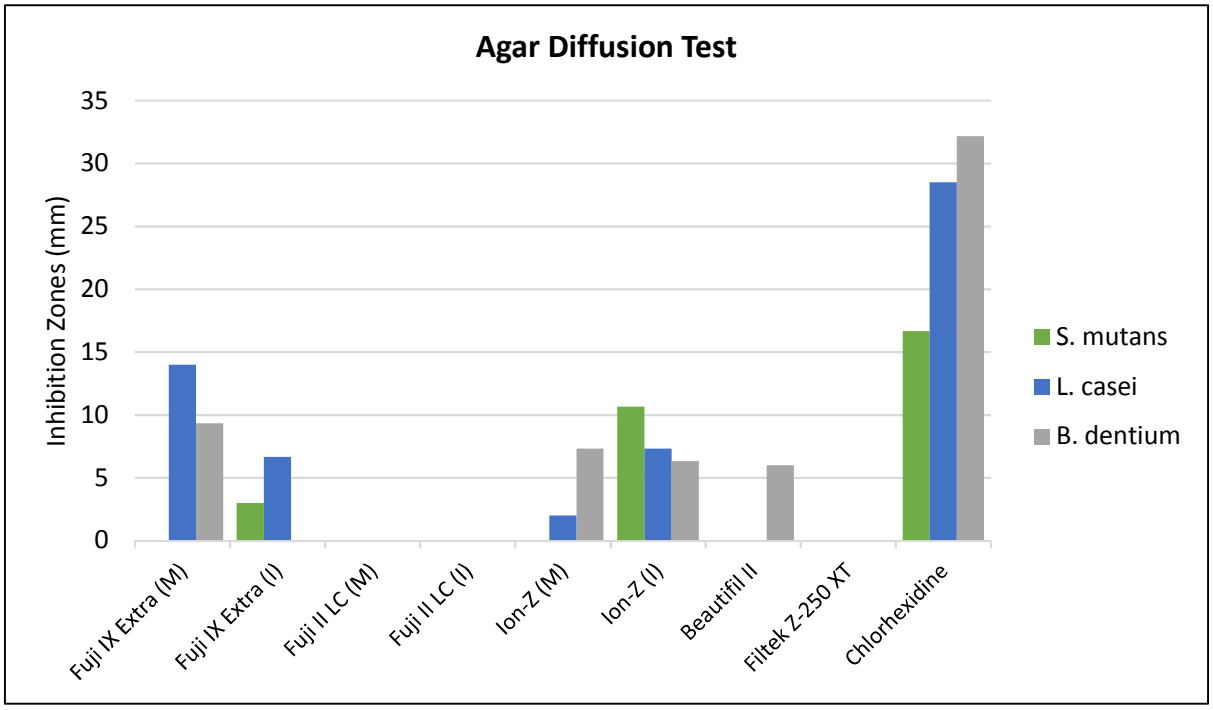

Figure 1. Inhibition zones mean (in mm)

3 Discussion

3 DISCUSSION

Caries are a complex and multifactorial diseases, in which environmental and behavioral risk factors interact, causing an imbalance in physiologic equilibrium between tooth mineral and biofilm fluid. The rate of oral biofilm formation and its structural composition vary substantially between individuals and could determine patient's caries risk (YADIKI, J.V. et al, 2016; KAUR, R. et al, 2013; FEJERSKOV, O., 2004).

S. mutans and *L. casei,* acidogenic-aciduric bacterial species, are recognized to be involved in caries process. *S. mutans* is considered an etiological agent for carious lesions and *L. casei* particularly involved in carious lesion progression (YADIKI, J.V. et al, 2016; ANDRADE, F.B. et al, 2014). Therefore, the presence of other microbial species such as *Scardovia wiggsiae* and *Bifidobacterium spp*., which are acid-resistant and are also associated with dental caries were reported (HENNE, K. et al, 2015).

The levels of *S. mutans, Lactobacillus* and *Bifidobacteria* are significantly correlated to caries experience in children and also in adults. *B. dentium* is the predominant *Bifidobacteria*, but there is little information about its colonization in dentition. Contrary of *S. mutans, B. dentium* does not colonize hard tissue surfaces but dentin exposed by caries lesions facilitate the attachment and proliferation of *B. dentium*, suggesting that they have a predisposition to colonize carious dentine (KAUR, R. et al., 2013; MANTZOURANI, M. et al, 2009). Both *Lactobacillus* and *Bifidobacteria* prefer lower pH to produce acids, so *S. mutans* acid production, promotes a convenient environment to these species (MATOS, B.M. et al, 2016). Therefore, these three bacteria were selected for tests in the present study because their cariogenic abilities.

Agar diffusion test is a popular test to evaluate antibacterial properties of restorative materials. However, it has some disadvantages like, it depends on the solubility and diffusion properties of both the test material and media. Although direct contact test in which the test material remains in direct contact with the bacteria, independent of the diffusion properties, may be more suitable for testing restorative materials and cements than agar diffusion test (FEROZ, S.M.A., BHOYAR, A., KHAN, S. 2016). However, in this study we did not have many difference comparing the tests for the three bacteria.

B. dentium, in this study, demonstrated a lower resistance against the tested restorative materials in direct contact test, presented a mean score of slight and moderate bacterial growth, showing that these materials could have a potential to inhibit their proliferation. Besides materials, Fuji IX Extra (M) demonstrated a better potential of inhibition without statistical difference in relation to chlorhexidine. For *B. dentium* in agar diffusion test, the materials that had presented inhibition zones were Fuji IX Extra (M), Ion-Z (I) and (M) and Beautifil II, and just for *B. dentium* Beautifil could presented inhibition zones.

Fluoride is considered an anticariogenic agent that could inhibit bacterial growth and their metabolism with a reduction of acid manufacture (FEROZ, S.M.A., BHOYAR, A., KHAN, S., 2016; SAKU, S.et al., 2010). The GIC eluate had inhibit the acid production of oral *Streptococci,* stopped the pH fall completely around pH 4.8-5.0 and special notability in decreased the rate of acid production at pH 5.5, suggesting that *Streptococcus* acid production could be inhibited in areas adjacent to GIC fillings at an acidic pH around the critical pH of tooth demineralization (pH of 5.5), leading in a reduction of *Streptococcus* cariogenicity (NAKAJO, K.et al, 2009). In this way, is believed that GIC contribute to the inhibition of recurrent caries because contain a high percentage of fluoride (10-20% in the powder fraction), and part of this fluoride are released in the vicinity of the cement, that could diffuse to the surrounding tooth structure (HOSZEK, A. and ERICSON, D., 2008).

Comparing GICs in the present study, they also had a different scale of inhibition against the three bacteria tested, proving that they work better against microorganisms involving in caries process than resin materials. In agar diffusion test, the GICs applied (I) presented more inhibition zones than the GIC (M), showing that the initial low pH the material, could contribute to the inhibition of bacterial growth. Among tested materials Ion-Z, a new GIC available in Brazil, demonstrated a higher inhibition for the bacteria tested, maybe because it has particles of zinc oxide, that could have contributed to inhibiting bacterial growth. Similar outcome was shown in a study (SCHERER, W., LIPPMAN, N., KAIM, J., 1989), comparing antimicrobial properties of 14 different restorative materials, nine of which were GIC. The GIC materials which contain zinc oxide produced zones of inhibition larger than that without zinc oxide. It should also be noted that, cations such as zinc, calcium, or magnesium, have a specific potential or efficacy for bacterial inhibition.

To validate the tests did in this study, for direct contact test, control tubes was used with the materials in absence of inoculum, demonstrated absence of bacterial growth and one

tube was used with bacterial inoculum, without restorative material, presented a confluent growth of microorganisms. For agar diffusion test paper imbibed with chlorhexidine was used as a control, showing a higher inhibition zones for the three bacteria tested.

S. mutans was the most resistant bacteria in the present study, presented a score 3 in direct contact test, showing a dense bacterial growth for all restorative materials tested, not even for GICs was different. However, in agar diffusion test, Fuji IX Extra (I) and Ion-Z (I) could inhibit *S. mutans* growth, demonstrated that the low initial pH and maybe a fluoride release of these materials could have a potential to inhibit its growth.

Experimental resins composites with S-PRG filler in different concentrations (13.9% or greater) demonstrated inhibition of *S. mutans* growth on their surfaces caused by the release of ions from these fillers (MIKI, S. et al, 2016). Beautifil, a composite resin which present this S-PRG filler, designed as a fluoridated glass filler with a glass-ionomer matrix layer surrounded by a thicker hydrogel layer, that are incorporated in the resin matrix are postulated to be capable to release and recharge fluoride. Comparing three restorative materials: Beautifil II, F-2000 (Compomer) and Fuji II LC against *S. mutans,* Fuji II LC exhibited the highest mean inhibitory zone and Beautifil II were in the second place in inhibition. This is because Beautifil behave more like a resin-modified GIC and has a cumulative fluoride release of about 20% of the original GIC (TARASINGH, P. et al, 2015).

In relation to dental plaque formation in three composite resin: Beautifil II, Filtek Z 250 and Clearfil AP-X. Beautifil II had a lower plaque formation on its surface, might be due to its fluoride releasing capability, exercising a change in the surrounding environment (SAKU, S.et al, 2010). This finds were contrarily of the present study, because Beautifil II just demonstrated inhibition for *B. dentium* in agar diffusion test and for direct contact test it had shown a moderate bacterial growth.

Testing two GIC (Fuji II LC and Fuji IX Extra) with or without chlorhexidine, all the experimental materials showed antibacterial properties against *S. mutans,* and these properties decreased with time, but Fuji II LC with chlorhexidine presented the major value (YADIKI, J.V. et al, 2016). This results comparing materials are different for the results in this study, because we did not have antibacterial zones for Fuji II LC, a resin modified GIC in any of the bacteria tested and also comparing Fuji II LC and Fuji IX Extra, just Fuji IX Extra presented inhibition zone.

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Nowadays, the attention is turning to a less removal of tooth structure, called the Minimal intervention dentistry, and maybe some active bacteria reside in the cavity. This minimal intervention has some principles: remineralization of early lesions; reduction cariogenic bacteria; minimum surgical intervention; repair rather than replacement of defective restorations; disease control. In this context, demineralized dentin was maintained in the cavity, because it had a potential to remineralize. GIC for this kind of minimal intervention is so important and could profit it, because release fluoride and other ions and has the potential of chemical adhesion to tooth structure. The adhesion between material and tooth will help preventing bacteria microleakage and the ions available in the cement: calcium, phosphate and fluoride, could help in remineralization (TYAS, M.J. et al, 2000). Following this thought, the materials tested in this study, Ion-Z (I) demonstrated a better performance and could contribute to inhibit or decrease bacterial growth, and the zinc oxide may help in remineralization of the dentin, but other studies is necessary to clarify it behavior.

No restorative material could be completely powerful in preventing bacterial microleakage or recurrent caries (ASKAR, H. et al, 2017; TYAS, M.J. et al, 2000). Manufacturing bio-function restorative materials that would be able to maintain their physical properties and antibacterial activity after being placed in the cavity, it will be helpfully to eliminate the damaging effect caused by bacterial microleakage (IMAZATO, S. et al, 2003), preventing bacterial growth and caries lesions progression.

4 Final Considerations

4 FINAL CONSIDERATIONS

Considering the methodology used and result analysis, all restorative materials presented a weak antibacterial potential, and the finding varied according tested bacteria. *S. mutans* was the most resistant bacteria, presented a dense bacterial growth for all restorative materials tested and in agar diffusion test, just Fuji IX Extra (I) and Ion-Z (I) could inhibit its growth. Fuji IX Extra (M) presented the major inhibition zone for *L. casei*, and in direct contact test *L. casei* presented a moderate bacterial growth, for all materials tested without statistical difference. *B. dentium,* demonstrated a lower resistance in direct contact test and in agar diffusion test, the restorative materials that had presented inhibition zones were Fuji IX (M), Ion-Z (I) and (M) and Beautifil II.

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ANNEXES

ANNEX A – Guidelines for Journal of Dentistry submissions:

Essential title page information • Title. Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible. • Author names and affiliations. Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lowercase superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author. • Corresponding author. Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author. • Present/permanent address. If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes. The title page should contain the following information: - Title of paper - Short title - Name(s), job titles and address(es) of author(s) (no academic degrees necessary) - Name, address, telephone, fax and e-mail of the corresponding author - Up to 6 keywords Spelling: International English. Authors are urged to write as concisely as possible.

The house style of Journal of Dentistry requires that articles should be arranged in the following order: Title, Abstract, Introduction, Materials and Methods, Results, Discussion, Conclusions, Acknowledgements, References, Tables, Figures. A cover letter should accompany the new manuscript submission, within which the authors should indicate the significance of the work being submitted in a statement no more than 100 words. A signed permission note (details below) must also be included. Abstract: should not exceed 250 words and should be presented under the following subheadings: Objectives, Methods; Results; Conclusions (For Reviews: Objectives; Data; Sources; Study selection; Conclusions). A 50 word 'Clinical Significance' statement should appear at the end of the abstract advising readers of the clinical importance and relevance of their work. These subheadings should appear in the text of the abstract. Please repeat the title of the article at the top of the abstract page. Introduction: must be presented in a structured format, covering the following subjects,

although not under subheadings: succinct statements of the issue in question, and the essence of existing knowledge and understanding pertinent to the issue.

In keeping with the house style of Journal of Dentistry, the final paragraph of the introduction should clearly state the aims and/or objective of the work being reported. Prospective authors may find the following form of words to be helpful: "The aim of this paper is to ..." Where appropriate, a hypothesis (e.g. null or a priori) should then be stated. Keywords: up to 6 keywords should be supplied. Abbreviations and acronyms: terms and names to be referred to in the form of abbreviations or acronyms must be given in full when first mentioned. Units: SI units should be used throughout. If non-SI units must be quoted, the SI equivalent must immediately follow in parentheses. The complete names of individual teeth must be given in the test. In tables and legends for illustrations individual teeth should be identified using the FDI two-digit system. Statistics Statistical methods should be described with enough detail to enable a knowledgeable reader with access to the original data to verify the reported results. When possible, findings should be quantified and appropriate measures of error or uncertainty (such as confidence intervals) given. Details about eligibility criteria for subjects, randomization and the number of observations should be included. The computer software and the statistical method(s) used should be specified with references to standard works when possible (with pages specified). See http://www.icmje.org/manuscript_1prepare.html for more detailed guidelines. Illustrations: should be submitted electronically using appropriate commercial software. Prospective authors should follow the relevant guidelines (available from: http://www.elsevier.com/ artworkinstructions). In addition, it is noted that while authors sometimes need to manipulate images for clarity, manipulation for purposes of deception or fraud will be seen as scientific ethical abuse and will be dealt with accordingly. For graphical images, journals published by Elsevier apply the following policy: no specific feature within an image may be enhanced, obscured, moved, removed, or introduced. Adjustments of brightness, contrast, or color balance are acceptable if and as long as they do not obscure or eliminate any information present in the original. Nonlinear adjustments (e.g. changes to gamma settings) must be disclosed in the figure legend. Abstract The Abstract should not exceed 250 words and should be presented under the following subheadings: Objectives, Methods; Results; Conclusions. A 50 word 'Clinical Significance' statement should appear at the end of the abstract advising readers of the clinical importance and relevance of their work. These subheadings should appear in the text of the abstract. Please repeat the title of the article at the top of the abstract page. For Review Articles the abstract should be presented under the following subheadings:

Objectives; Data; Sources; Study selection; Conclusions. Graphical abstract Although a graphical abstract is optional, its use is encouraged as it draws more attention to the online article. The graphical abstract should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. You can view Example Graphical Abstracts on our information site. Authors can make use of Elsevier's Illustration and Enhancement service to ensure the best presentation of their images and in accordance with all technical requirements: Illustration Service. Keywords Provide a maximum of 6 keywords, using British spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes. Formatting of funding sources List funding sources in this standard way to facilitate compliance to funder's requirements: Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa]. It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding. If no funding has been provided for the research, please include the following sentence: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. Artwork Image manipulation Whilst it is accepted that authors sometimes need to manipulate images for clarity, manipulation for purposes of deception or fraud will be seen as scientific ethical abuse and will be dealt with accordingly. For graphical images, this journal is applying the following policy: no specific feature within an image may be enhanced, obscured, moved, removed, or introduced. Adjustments of brightness, contrast, or color balance are acceptable if and as long as they do not obscure or eliminate any information present in the original. Nonlinear adjustments (e.g. changes to gamma settings) must be disclosed in the figure legend. Electronic artwork General points • Make sure you use uniform lettering and sizing of your original artwork. • Embed the used fonts if the application provides that option. • Aim to use the following fonts in your illustrations: Arial, Courier,

Times New Roman, Symbol, or use fonts that look similar. • Number the illustrations according to their sequence in the text.

Use a logical naming convention for your artwork files.

Provide captions to illustrations separately. Size the illustrations close to the desired dimensions of the published version.

Submit each illustration as a separate file. A detailed guide on electronic artwork is available. You are urged to visit this site; some excerpts from the detailed information are given here. Formats If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format. Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below): EPS (or PDF): Vector drawings, embed all used fonts. TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi. TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi. TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi. Please do not: • Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors; • Supply files that are too low in resolution; • Submit graphics that are disproportionately large for the content. Color artwork Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article. Please indicate your preference for color: in print or online only. Further information on the preparation of electronic artwork. Illustration services Elsevier's WebShop offers Illustration Services to authors preparing to submit a manuscript but concerned about the quality of the images accompanying their article. Elsevier's expert illustrators can produce scientific, technical and medical-style images, as well as a full range of charts, tables and graphs. Image 'polishing' is also available, where our illustrators take your image(s) and improve them to a professional standard. Please visit the website to find out more. Figure captions Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (not on

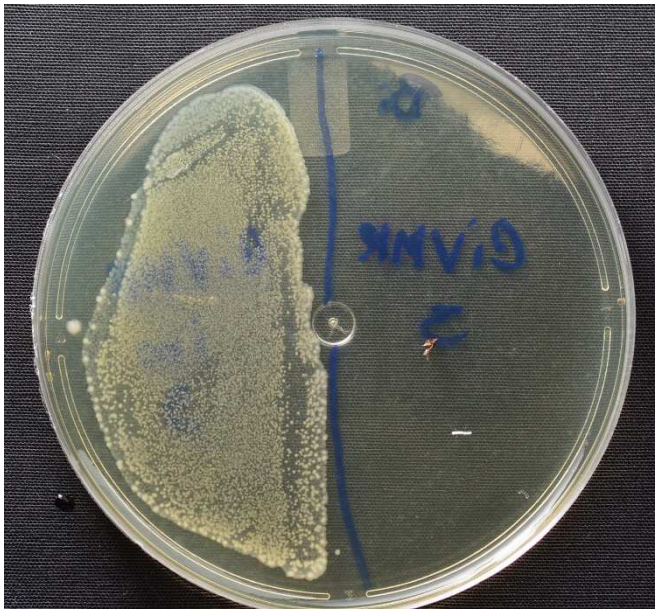
the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used. Tables Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules and shading in table cells. References Citation in text Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication. Reference links Increased discoverability of research and high quality peer review are ensured by online links to the sources cited. In order to allow us to create links to abstracting and indexing services, such as Scopus, CrossRef and PubMed, please ensure that data provided in the references are correct. Please note that incorrect surnames, journal/book titles, publication year and pagination may prevent link creation. When copying references, please be careful as they may already contain errors. Use of the DOI is encouraged. A DOI can be used to cite and link to electronic articles where an article is in-press and full citation details are not yet known, but the article is available online. A DOI is guaranteed never to change, so you can use it as a permanent link to any electronic article. An example of a citation using DOI for an article not yet in an issue is: VanDecar J.C., Russo R.M., James D.E., Ambeh W.B., Franke M. (2003). Aseismic continuation of the Lesser Antilles slab beneath northeastern Venezuela. Journal of Geophysical Research, http://dx.doi.org/10.1029/2001JB000884i. Please note the format of such citations should be in the same style as all other references in the paper. Web references As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list. Data references This journal encourages you to cite underlying or relevant datasets in your manuscript by citing them in your text and including a data reference in your Reference List. Data references should include the following elements: author name(s), dataset title, data

repository, version (where available), year, and global persistent identifier. Add [dataset] immediately before the reference so we can properly identify it as a data reference. The [dataset] identifier will not appear in your published article.

ANNEX B– Criteria used to determine and calibrated analyzer of bacterial growth score on the agar surface in direct contact test.

Score 0
(No bacteria growth)

Score 1
(Slight bacteria growth on the whole surface of the agar)



Score 2 (Moderate bacteria growth on the whole surface of the agar)

Score 3 (Dense bacteria growth on the whole surface of the agar)

ANNEX C - SOME FIGURES OF EXPERIMENT

Sterile bipartite matrix

Restorative tested materials inhibition zone for chlorhexidine and Fuji IX Extra.