scientific reports

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Resin composite aggregated S-PRG particles are not superior to non-S-PRG under microcosm biofilm

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This study assessed the effect of composite resins, aggregated or not with S-PRG particles, and the use of toothpaste in controlling demineralization and bacterial growth. Human molars were distributed into 3 groups: control (CT) – sound teeth, Beautifil Bulk Restorative System (aggregated with S-PRG) (BB), Filtek One Bulk Fill (without S-PRG) (FB). Teeth destined for groups BB and FB previously received Class I preparations (4 × 4 × 4 mm), followed by single-increment restorations. All teeth were sectioned mesiodistally, with all specimens subjected to cariogenic challenge for 5 days, including microcosm biofilm formation. Half of each tooth was exposed to toothpaste (CTF, BBF, FBF). The loss of microhardness was assessed considering the initial microhardness as 100% on enamel, dentin, and composite resin substrates. Colony Forming Units (CFU/mL) were counted in 3 media. Data analysis used one-way ANOVA, Tukey HSD test, and paired t-test ($\alpha = 0.05$). Toothpaste significantly reduced CFU/mL for total bacteria and genus Streptococcus ($p < 0.05$), with no significant difference for Streptococcus mutans. Enamel microhardness was positively affected by toothpaste. Both restorative systems controlled enamel demineralization, with FB and FBF outperforming BB and BBF. There was minor degradation of both composite resins, between 10% and 22%. Toothpaste effectively reduced microorganisms, irrespective of the composite resin. Regarding demineralization control, both restorative systems, with and without S-PRG particles, were effective on enamel.

Keywords Demineralization, Resin composite, Toothpaste, Dental caries, Secondary caries

Direct restoration is a procedure that is part of the dentist's daily routine. Among the materials used, composite resin is the first choice for the vast majority of clinicians. This is due to the fact that this restorative material allows for minimal intervention due to its ability to adhere to the tooth structure and also provides reinforcement in regions where the preparation is weakened, which avoids unnecessary wear and tear on healthy tissue[1]. Furthermore, aesthetic and mechanical properties meet patient satisfaction[2]. However, replacement rates for restorations are still very high[1,3], being higher than those for primary restorations[1].

The relative low longevity of dental restorations increases the health costs related to a new intervention[2]. Especially in posterior teeth, the most used composite resins are microhybrid, nanohybrid and nanoparticle, with no difference between them with regard to clinical performance[4]. In addition to the material, the bulk fill technique has been widely used in posterior teeth[5], presenting itself as a simpler procedure without negatively impacting clinical behavior, when compared to the incremental technique[5,6]. Thus, regardless of the classification of composite resin and technique used, the main reason for restoration failure is secondary caries[1,3].

Secondary caries is a new lesion that occurs adjacent to a pre-existing restoration[7]. Composite resin has the inherent characteristic of polymerization contraction, which, added to mechanical and thermal loads in the oral environment, in which the restoration is subjected, can lead to the emergence of gaps at the tooth/restoration interface, facilitating the accumulation of biofilm and the development of carious lesion[2]. The roughness of the composite resin together with other surface properties can also favor bacterial colonization[8,9]. However, among the different etiological factors of caries, the risk of caries presented by the patient directly impacts the longevity of the restoration, that is, patients with a high risk of caries are more likely to develop carious lesions. This is

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justified due to the fact that these patients have health problems that can reduce protective effects, such as saliva and difficulty in performing/adhering to adequate oral hygiene[3,10].

Thus, the use of restorative materials with principles of controlling biofilm and/or stimulating remineralization are adjuvants to be considered in the control of secondary caries, facilitating the longevity of restorations[11,12]. Some companies have launched restorative materials on the market that interact with the tooth structure in order to prevent the development of carious lesions[13], such as composite resin aggregated with Surface Pre particles Reacted Glass ionomer (S-PRG), also called gionomer[14]. S-PRG is a glass particle surrounded by a silica gel, which releases strontium (Sr^{+2}), borate (BO_3^{-3}), fluoride (F^-), sodium (Na^+), silicate (SiO_3^{-2}) and aluminum (Al^{+3}). The release of these ions is related to antimicrobial effects[15–18], preventing demineralization of the tooth structure[19,20]. Among the commercially available resins with S-PRG particles is Beautifil Bulk Restorative (Shofu, Japan). However, most previous studies were designed with experimental resins and different concentrations of S-PRG[16,17,20], short biofilm formation time[18]or single-species biofilm[15,20]. Despite all the complexity of the oral environment, in vitro studies should seek to mimic this as much as possible as, in this way, they allow a better understanding of the material's mechanism[11].

Therefore, this in vitro study involves commercially available resins, a human tooth substrate in enamel and dentin and a microcosm biofilm model from human saliva. The objective was to assess the effect of composite resins, aggregated or not with S-PRG particles, and the use of toothpaste in controlling demineralization and bacterial growth. The null hypotheses to be tested were: (1) there would be no difference between composite resins, with and without S-PRG particles, in the control of biofilm and demineralization of the tooth structure; (2) there will be no difference detected when using the toothpaste with the restorative systems.

Methods

Ethical considerations

Human teeth and human saliva were used, with approval from the Human Research Ethics Committee – Federal University of Mato Grosso do Sul (MS/Brazil) (protocol: 3.678.506, CAAE: 21527119.6.0000.0021), in accordance with the code of ethics of the World Medical Association - Declaration of Helsinki.

Sample preparation

Healthy human third molars ($n=30$) were randomly divided into 3 groups – CT, BB and FB, as shown in Table 1. Previously, all teeth were kept in Chloramine T for a maximum of 3 months before carrying out the restorative procedure and underwent by standardization process with regularization of the cusps 1 mm from the central groove using a polisher metallographic (Teclago PL01 Lagoa Vargem Grande Paulista, SP, Brazil) and #600 silicon carbide sandpaper (3 M, Sumaré, SP, Brazil).

In teeth that used composite resin, Class I (O) cavity preparations were performed manually with a cylindrical diamond tip with a rounded end (n° 3145 and 3145FF, KG Sorensen, Cotia, SP, Brazil) at high speed (Kavo Kerr, Brea, CA, USA), with the dimensions of 4×4×4 mm (mesio-distal distance, bucco-lingual distance, depth) checked with a periodontal probe. The diamond tips were replaced every 5 preparations. The prepared teeth were placed in an ultrasonic bath for 10 min (Schuster, L100, Santa Maria, RS, Brazil). An adhesive system, as indicated by the specific commercial brand, for composite resin groups without S-PRG particles – Filtek One Bulk Fill (FB/FBF) was used with Scotchbond Universal (3 M Oral Care, St Paul, MN, USA) and for the Beautifil Bulk Restorative resin (with S-PRG particles), the FL Bond II self-etching system (Shofu Inc., Kyoto, Japan). Before application, selective conditioning on the enamel was carried out with 37% phosphoric acid (Condac, FGM, SC, Brazil) for 15 s, followed by rinsing with a dental syringe for 20 s and air-dried surface. Table 2 lists the materials used in the study, their chemical composition, and a complete description of the adhesion protocols. After the adhesive procedure, the composite resin was inserted in a single increment of 4 mm and photoactivated for 40 s (Bluephase Ivoclar Vivadent, Barueri, SP, Brazil) with an irradiance of 1000 mW/cm^2. After every five restorations, the irradiance was checked with a radiometer (Ecel, Ribeirão Preto, SP, Brazil).

After 48 h of storage in distilled water at 37 °C, the restored teeth were sectioned in the mesio-distal direction using a precision metallographic cutter (Buehler, Lake Bluff, Illinois, USA) with a diamond disc (Buehler, Lake Bluff, Illinois, USA) under constant irrigation. Each half obtained was polished with a sequence of silicon carbide sandpaper (#1000, #1200 and #2000, 3 M, Sumaré, SP), 12 mm diameter felt disc (TDV dental Ltda, Pomerode, SC, Brazil) and 0.5 µm diamond paste (Ultradent Products Inc., Indaiatuba, SP, Brazil). After each sanding, the halves passed through an ultrasonic bath for 5 min and at the end of polishing for 10 min in order to remove debris. Half of each specimen was protected with nail polish with the aim of having a control area

Groups	Description
CT	Control sound teeth without the use of toothpaste
CTF	Control sound teeth with the use of toothpaste
BB	Resin composite S-PRG (Beautifil Bulk Restorative/Adhesive system FL Bond II) without the use of toothpaste
BBF	Resin composite S-PRG (Beautifil Bulk Restorative/Adhesive system FL Bond II) with the use of toothpaste
FB	Resin composite (Filtek One Bulk Fill/ Scotchbond Universal) without the use of toothpaste
FBF	Resina composta (Filtek One Bulk Fill/Scotchbond Universal) with the use of toothpaste

Table 1. Description of the groups, with the respective materials evaluated in the study. *The experiment was carried out in three distinct phases, biological triplicates ($n=3$ each), with a final $n=9$.

Brand	Type	Manufacter	Composition	Application protocol
FL-Bond II	Two-step self- etching adhesive system with S-PRG	Shofu Inc, Kyoto, Japan	Primer: water, ethanol, carboxylic acid monomer, phosphoric acid monomer, and initiator Adhesive: S-PRG filler based on fluoro-boro- aluminosilicate glass, UDMA, TEGDMA, 2-HEMA, initiator	1. Acid etchant was applied for 15 s on enamel 2. Rinse for 20 s/air dried surface 3. Apply primer for 20 s (one drop) 4. Solvent evaporation for 20 s 5. Apply bonding agent, do not air dry 6. Light cure for 20s
Beautifil Bulk Restorative	Bulk Fill resin composite with S-PRG	Shofu Inc, Kyoto, Japan	Bis-GMA, UDMA, Bis-MPEPP, TEGDMA, S-PRG filler based on fluoroboroaluminosilicate glass, polymerization initiator, pigments and others.	Single increment (4 mm) and light-cured for 40 s
Scotchbond Universal	Universal adhesive system	3 M Oral Care, Minesota, USA	Methacryloyloxydecyl dihydrogen phosphate, phosphate monomer, dimethacrylate resins, hydroxyethyl methacrylate, methacrylate-modified polyalkenoic acid copolymer, filler, ethanol, water, silane, camphorquinone.	1. Acid etchant was applied for 15s on enamel 2. Rinse for 20 s/air dried surface 3. Apply adhesive for 20 s (two layers) 4. Solvent evaporation for 20 s 5. Light cure for 20 s
Filtek One Bulk Fill	Bulk Fill resin composite	3 M Oral Care, Minesota, USA	Organic Matrix: AUDMA, UDMA, and 1,12-dodecane-DMA Fillers: non- agglomerated/non- aggregated 20 nm silica filler, a non- agglomerated/non- aggregated 4 to 11 nm zirconia filler, aggregated zirconia/ silica cluster filler (comprised of 20 nm silica to 4 to 11 nm zirconia particles), and ytterbium trifluoride filler of 100 nm particles; 76.5 wt 58.4 vol% Other components: camphorquinone.	Single increment (4 mm) and light-cured for 40 s
Colgate Total 12 (clean mint)	Toothpaste	Colgate-Palmolive, São Paulo, Brazil	Glycerin, agua, hydrated silica, sodium lauryl sulfate, arginine, aroma, zinc oxide, cellulose gum, benzyl alcohol, poloxamer 407, zinc citrate, tetrasodium pyrophosphate, xanthan gum, cocamidopropyl betaine, sodium fluoride (1450 ppm), sodium saccharin, phosphoric acid, sucrolose.	Ratio of 1:3 (toothpaste: deionized water) for 2 min. Only CTF, BBF and FBF groups.
Abbreviations: 2-HEMA, 2-hydroxyethylmethacrylate;10-MDP, 10-methacryloyloxydecyl dihydrogen phosphate; bis-GMA, bisphenol A-glycidyl methacrylate; bis-MPEPP, bisphenol A polyethoxy methacrylate; S-PRG, surface-reaction PRG; TEGDMA, triethylene glycol dimethacrylate; UDMA, urethane dimethacrylate, AUDMA: aromatic urethane dimethacrylate; Bis-MPEPP- bisphenol A polyethoxy methacrylate.				

Table 2. Brand, type, manufacturer, chemical compositions of the materials used in this study, and application protocol.

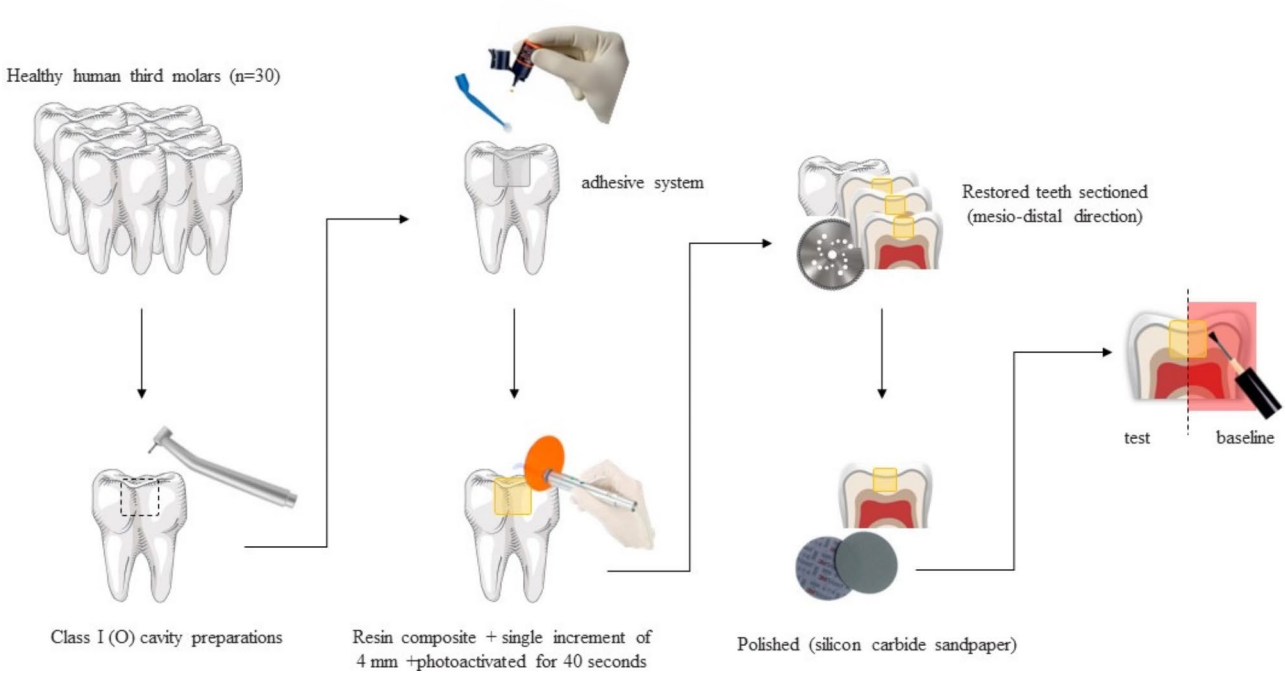


Fig. 1. Schematic diagram of the tooth preparation methodology for carrying out the tests.

(baseline) and a test area in the same specimen (Fig. 1). The samples were sterilized using ethylene oxide (90% ETO/10% CO_2) for 2 h under pressure $-15 \pm 0.1 KgF/cm^3$. All samples were exposed to the inoculum to form microcosm biofilm, with only one half of each tooth also being exposed to fluoride toothpaste, forming the groups as described in Table 1.

Saliva collection

The inclusion criteria for human saliva to be used as inoculum were: children between 9 and 12 years old with caries activity and without periodontal disease; exclusion: volunteers who use or have used antibiotics in the last 3 months prior to collection.

The collection was carried out in the morning, and on that day the volunteers did not brush their teeth and were left to eat food for at least 2 h. Saliva was stimulated by chewing a sterile rubber material with a standard size of 1 cm in length for 10 min. The volunteers' saliva pool was homogenized, diluted in glycerol in a proportion of 70% saliva/30% glycerol[21], fractionated into 1 mL aliquots and stored at − 20 °C. This saliva was used as a microcosm for biofilm formation.

Microcosm biofilm formation

To construct the microcosm biofilm formation model using human saliva, modifications were made to a previously described model[21,22] and its feasibility was verified in a previous pilot study. Aliquots of saliva were thawed, and part was used for biofilm formation and another part was analyzed for cell viability by counting colony-forming units in the Brain Heart Infusion (BHI), Mitis Salivarius (MS) and Mitis Salivarius Bacitracin Sucrose (MSBS).

To form the composite medium, the saliva was sterilized by the filtration process with 0.25 M dithiothreitol (DTT), centrifugation, filtration with 0.22 µm filters and stored at − 20 °C[23]. On the first day of the experiment, this saliva was thawed and mixed with the Mueller- Hinton (MH) culture medium, in a proportion of 60:40%, respectively.

Each tooth sample (sound or restored) was aseptically inserted into a well of a 24-well plate and 1.5 mL of inoculum composed of saliva and compound medium (sterile saliva and MH) in a ratio of 1:50, added and kept in microaerophilia, 5% CO_2 and 37 °C, for 8 h. Next, the medium was removed, the samples were washed with phosphate-buffered saline (PBS) for 5 s and a new medium (MH/saliva) composed of 0.2% sucrose was added to the well (1.5 mL/well). The plate was incubated at 5% CO_2 and 37 °C for 16 h to complete the first day[21].

After the cariogenic challenge, the samples were washed with PBS and the groups with fluoride supplementation (CTF, BBF and TBF) were exposed to 1 mL of a toothpaste solution (Colgate Total 12 Clear Mint - Colgate-Palmolive Industrial Ltda, São Bernardo do Campo, SP, Brazil), in a ratio of 1:3 (toothpaste: deionized water)[22,24], for 2 min. The samples were washed again and the new medium was added to well. The application of the toothpaste as well as the change of the medium occurred daily. The cariogenic challenge lasted five days, including microcosm biofilm formation.

Figure 2 summarizes the experiment protocol.

Microbial biofilm quantification

After the fifth day of the experiment, total microbial quantification was carried out on BHI agar (Brain Heart Infusion) and differentiation of oral *streptococcus colonies* using MS media (Mitis Salivarius) and MSBS (Mitis Salivarius Bacitracina Sucrose)[21]. Each specimen was placed in 5 mL of PBS and agitated using a vortex (Biomixer QL-901, Biomex biotechnology, Ribeirão Preto, SP, Brazil) for 1 min and an ultrasonic bath for 8 min. This suspension was diluted in saline solution serially from 10^{-1} to 10^{-8} and the quantification of colony-forming units per 1 mL (CFU/mL) was performed using the drop plating technique. The different species of *Streptococcus* bacteria had their colonies morphologically differentiated using a binolucar stereoscope with 20x magnification (ST30 2 L, Coleman, Santo André, SP, Brazil). Bacteria were counted using a manual colony counter (CP602, SPlabor, Presidente Prudente, SP, Brazil).

Microhardness test

The microhardness meter (Shimadzu HMV, São Paulo, SP, Brazil) with Knoop tip used a static force of 25 g for 10 s in enamel and composite resin[25]and 10 g for 15 s in dentin[26]. The strength and application time were also established in a pilot study that verified the possibility of correct reading in both the control area and the test area (demineralized). Six measurements were taken, with distances of 100 µm between them, on each substrate - enamel, dentin and composite resin; 3 in each area/substrate – control area (baseline) and test area, as shown in Fig. 3. For each sample, the average of the three indentations in each substrate/area was used to calculate mineral loss. Thus, in the baseline area a value for initial surface microhardness (MDSi) was obtained and in the test area a value for final surface microhardness (MDSf). To analyze mineral loss, the relative surface microhardness (rMDS) was measured for each substrate (enamel, dentin and composite resin) using the formula: rMDS = (MDSf / MDSi)x 100, where the MDSi value was considered 100%[27].

Scanning Electron Microscopy (SEM)

Qualitative analysis of the samples was carried out with a low vacuum scanning electron microscope (SEM) (Hitachi TM 3000, São Paulo, SP, Brazil), at an acceleration voltage of 20 kV on one sample from each group ($n = 1$). Prior to SEM analysis, no sample preparation was undertaken. Images were obtained at 30x magnification to visualize the 3 substrates in the same area and 1500x magnifications on each substrate, enamel, dentin, and composite resin. For this analysis, 3 teeth were used ($n = 1$).

Micro-CT imaging and measurements

Micro-CT measurements were performed using a SkyScan-Bruker 1173 model scanner. The voltage used in the X-ray tube was 50 kV with a current of 160 µA, and a 1 mm aluminum filter was used. Each projection was obtained by averaging 3 projections with an exposure time of 800 ms for each. The sample was rotated 180° with an angular step of 0.35°. The image resolution was 6 micrometers. The projections were reconstructed using the

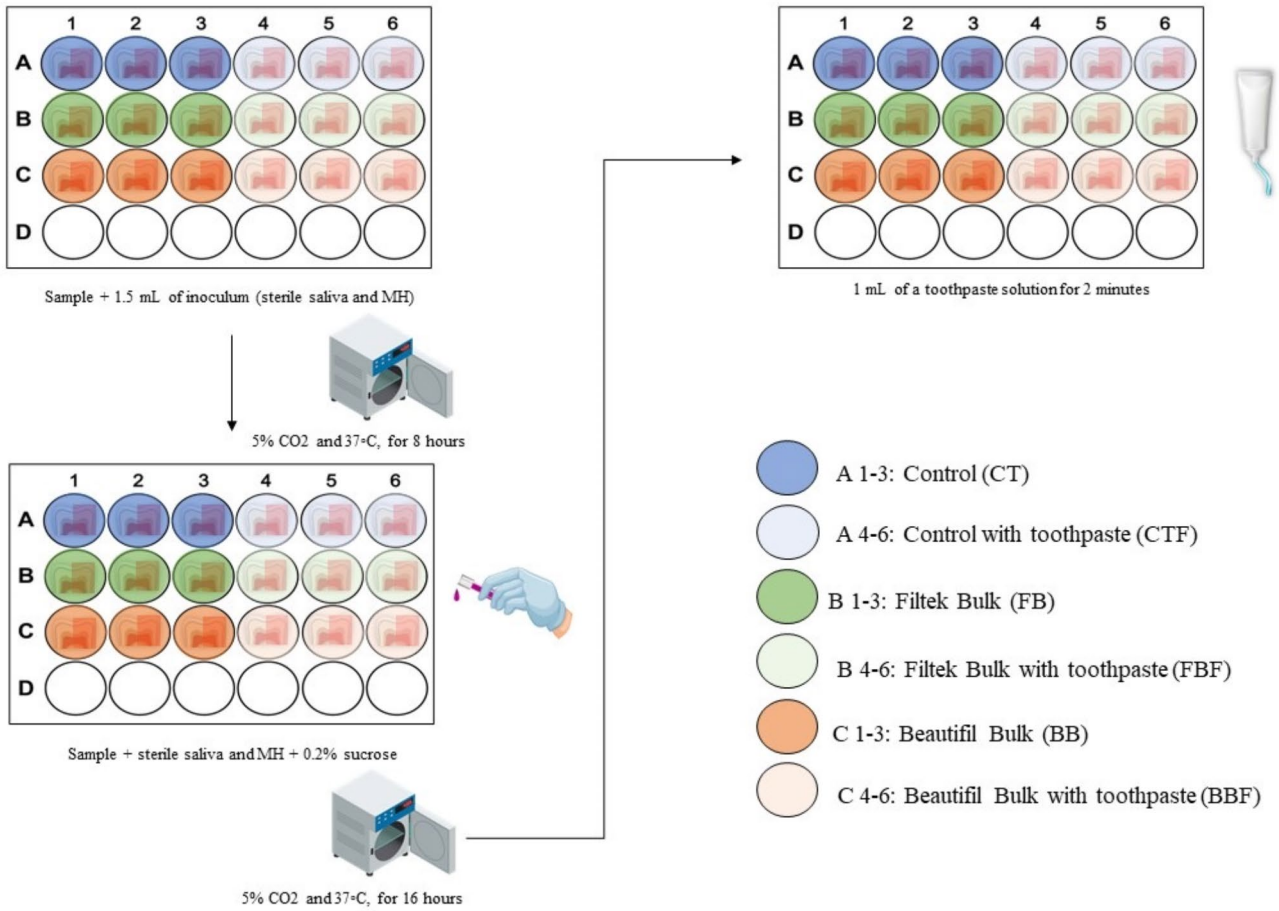


Fig. 2. Representative images of the experimental protocol.

NRecon software and analyzed in the Data Viewer software, both provided by the scanner manufacturer. One representative sample from each group was used.

Data analysis

The experiment was carried out in three distinct phases, biological triplicates ($n=3$ each), with a final $n=9$ (Table 1) to reduce possible systematic errors and ensure the reproducibility of the results. The data obtained were subjected to the normality (Kolmogorov-Smirnov) and homogeneity (Bartlett) test, and were subsequently analyzed by one-way ANOVA, followed by Tukey's post-test. Data regarding the use of toothpaste were also analyzed using paired t-tests. All tests used a p value <0.05, as statistically significant. Statistical analysis was conducted with GraphPad Prism 5.0 (GraphPad Software).

Results

Microbial biofilm quantification

In view of the restorative materials tested, bacterial growth was observed on all substrates evaluated, enamel, dentin and composite resin, regardless of the restorative system. However, the use of toothpaste had a significant impact on reducing the growth of total bacteria (BHI medium) and in the selective medium for oral bacteria of the genus *Streptococcus*, showing a difference compared to the groups without using toothpaste, as shown in Fig. 4a and b. When comparing the restorative materials used (BB and FB), no statistically significant differences were observed between the groups ($p>0.05$) in terms of the growth of total microorganisms, oral *Streptococcus*, and *Streptococcus mutans*, as illustrated in Fig. 4a and b, and 4c.

Assessment of relative microhardness

Table 3 shows the results of the microhardness test, according to the groups and substrates analyzed. All groups demonstrated a reduction in microhardness under the action of the cariogenic biofilm, since relative microhardness is related to the percentage of initial microhardness in each substrate evaluated. The toothpaste was effective on the enamel substrate, without the use of restorative material, where CTF > CT, showing no difference between the BB and BBF groups, as well as FB and FBF. Still in enamel, the restorative material influenced the highest relative microhardness, since all groups with restoration differed from the control (CT), without restoration ($p<0.05$). On the dentin substrate, the toothpaste alone was not effective in controlling

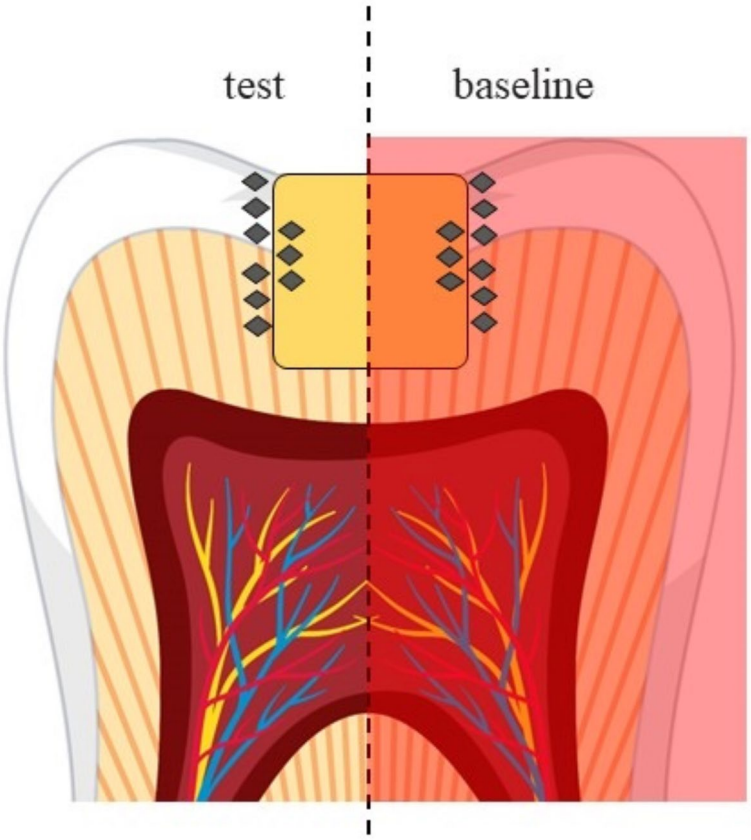


Fig. 3. Schematic representation of the indentations. Surface microhardness measurements carried out on the composite resin substrate and 40 µm from the adhesive interface on enamel and dentin substrates in both protected (baseline) and unprotected (test) areas.

demineralization, as no difference was detected between CT and CTF, BB and BBF, FB and FBF. However, when associated with the restorative material, it demonstrated greater microhardness in dentin, FBF > CT ($p < 0.05$). The composite resin suffered a small change in microhardness when subjected to cariogenic biofilm, with the final microhardness being 80–90% of the initial microhardness. The toothpaste also did not change the microhardness property of the composite resin ($p > 0.05$).

Scanning Electron Microscopy (SEM)

The SEM images in the overview (30X magnification - Fig. 5) demonstrate the difference, in the same tooth, between the area that was exposed to the biofilm (test) and the area that was protected (baseline), with the biofilm formed in the experiment being clear. A reduction in biofilm was observed in the groups that used the toothpaste in the experiment (CTF, BBF and FBF) when compared to the groups that did not use it (CT, BB and FB), which confirms the results obtained in the quantification of biofilm in the BHI medium.

On the enamel substrate, magnification of 1500X, a prismatic effect of the enamel is noted in the test area of all groups when compared to the baseline area, which indicates changes similar to those observed in carious lesions. It is also possible to observe biofilm adhered to the demineralized enamel (black arrows), mainly in the CT, BB and FB groups. Biofilm deposition is even clearer on the dentin substrate, where it is possible to see practically all of the dentin covered by *coccus* - shaped bacteria. In both enamel and dentin, in the groups that used fluoride toothpaste (CTF, BBF and FBF) a deposit of crystals suggestive of calcium fluoride (white stars) was noticed.

On the composite resin substrate, less biofilm adhesion is observed compared to the dentin substrate, especially. The difference in composition with types and formats of inorganic fillers is clear when comparing the groups that used the Beautifil Bulk Restorative composite resin and the groups using the Filtek composite resin One Bulk Fill.

Micro-CT imaging and measurements

The representative micro-CT images (Fig. 6) demonstrate the differences between the area subjected to the cariogenic challenge and the control area (baseline), highlighting the different depths of the carious lesions formed in the enamel and dentin substrates. Table 4 presents the average depths of carious lesions in the tooth structure, suggesting a protective effect of the restorative material on the enamel and of the toothpaste on both substrates.

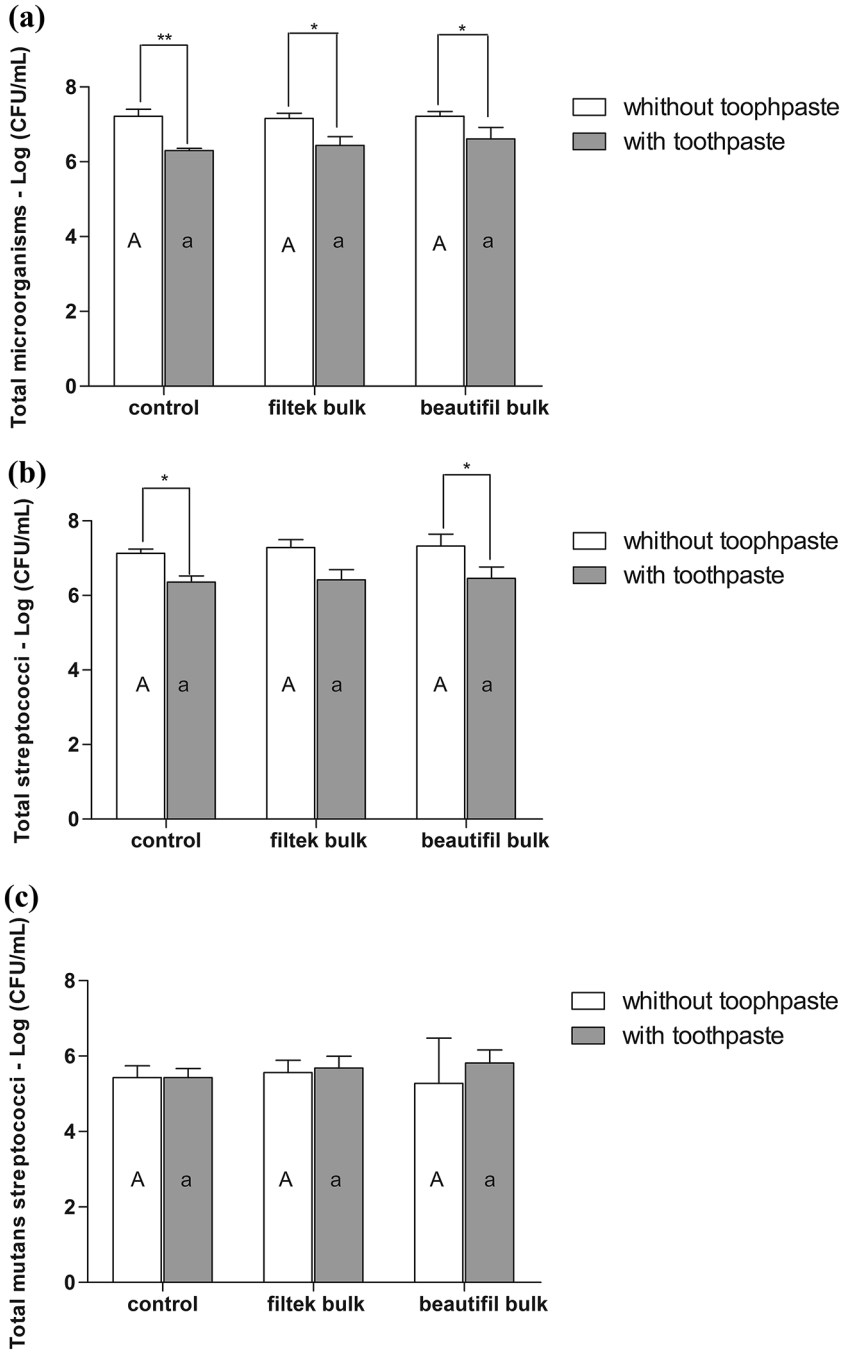


Fig. 4. Microbial quantification graphs in BHI (**a** Total microorganisms), MS (**b** Total *Streptococcus*) and MSBS media (**c***Streptococcus Mutans)*, according to the groups evaluated. Columns represent mean and standard deviation ($n = 9$), when connected with the asterisk (*) they are statistically different (paired t-test, $p < 0.05$) and (**) $p < 0.01$. Capital letters compare groups without using toothpaste, while lowercase letters compare groups using toothpaste (Tukey test). Groups identified with different letters show a statistical difference ($p < 0.05$).

Discussion

Ion-releasing restorative materials and their interaction with tooth structure represent a growing topic in dental research and clinical practice. In this study, in addition to investigating composite resins, the use of a toothpaste containing fluorine and arginine on enamel, dentin and composite resin substrates was also evaluated. Our results detected a difference in the quantification of biofilm and demineralization in enamel between the restorative systems evaluated, as well as the effect of toothpaste on biofilm formation in BHI and MS media, which leads to the rejection of the first and second hypotheses. The detection of microorganisms of various species, the observed loss of microhardness in the analyzed substrates indicate the effectiveness of the experimental model,

Group	Enamel	Dentin	Resin Composite
CT	35.5 ± 8.2^{c}	47.2 ± 14.1^{b}	–
CTF	$53.2 \pm 14.8^{a, b}$	$54.7 \pm 13.8^{a, b}$	–
BB	45.5 ± 8.6^{b}	$60.7 \pm 17.5^{a, b}$	$78.6 \pm 11.8^{a, b}$
BBF	48.1 ± 13.0^{b}	$59.5 \pm 13.4^{a, b}$	82.4 ± 7.6^{b}
FB	66.0 ± 7.9^{a}	$61.4 \pm 10.2^{a, b}$	90.1 ± 5.7^{a}
FBF	68.8 ± 18.0^{a}	69.2 ± 13.0^{a}	$87.9 \pm 5.3^{a, b}$

Table 3. Relative surface microhardness (% rMDS) according to the groups and substrates evaluated. *Different letters indicate statistical differences between lines in the same column (same substrate) ($p < 0.05$).

which aims to continuously expose the microcosm biofilm to sucrose for five days, as demonstrated in previous studies[21,22].

Brain Heart Infusion medium, a significant reduction was observed in all groups that used the toothpaste (Figs. 4a and 6). The oral microbiota of caries-active individuals is diverse, with a predominance of firmicutes, such as *Streptococcus* and *Lactobacillus*. However, it is also important to highlight the considerable presence of other bacteria, with *Actinomyces* being the most abundant[28,29]. These bacteria, as demonstrated previously, are sensitive to fluoride[30]. In addition to fluorine, arginine stimulates the buffering capacity, which contributes to the reduction of total microorganisms and influences the functional profile of the biofilm[29,31]. A previous study on the composition and activity of oral biofilm revealed that oral *Streptococcus*are the species most affected by exposure to fluoride[31]. This explains the lower amount of total *Streptococcus* with the use of toothpaste in MS medium in the present study.

When the same restorative material is evaluated, without and with application of toothpaste in MS medium, a difference was observed between BB and BBF, with the latter showing a lower amount of oral *Streptococcus*.This can be attributed to the release of fluoride ions from the composite resin with S-PRG particles[17], which, when combined with the toothpaste, increased the fluoride concentration, enhancing the reduction of microorganisms of this species. It is important to mention that *Streptococcus mutans* are more resistant to the action of fluoride, with their direct susceptibility depending on its concentration to reduce the effects of virulence. A previous study demonstrated that the volume and thickness of the *S. mutans* biofilm did not decrease, even under different fluoride concentrations[32]. This justifies the lack of action of the toothpaste on *Streptococcus mutans*, as represented in the middle MSBS graph (Fig. 4c). Furthermore, it is important to note that biofilm from caries-active individuals demonstrates greater resistance to the effects of toothpaste compared to biofilm from caries-free individuals[29]. In the present study, the biofilm was formed using saliva from individuals aged 9 to 12 years with mixed dentition and active caries. This approach more accurately reproduces the polymicrobial nature of dental caries[33]. Previous research indicates that mixed and permanent dentition have a greater abundance of cariogenic microorganisms in the biofilm of individuals with active caries than those without caries[34]. This increase in specific microbial load may have contributed to the observed resistance to the action of the toothpaste when the *Streptococcus mutans* medium was analyzed.

The images obtained by SEM revealed that the dental enamel in the area subjected to the cariogenic biofilm presented a prismatic appearance in contrast to the baseline area (Fig. 5). The lesion formed is visible in Fig. 6. The evaluation of microhardness showed significant differences between the groups in relation to this substrate. When analyzing the impact of the toothpaste, greater microhardness was observed in the control group that received fluoride + arginine (CTF) compared to the group that did not receive this treatment (CT). As mentioned previously, toothpaste played a role in reducing biofilm, which has a direct effect on reducing acidity and acid exposure time, which in turn is associated with less mineral loss. It is important to highlight that toothpaste that combines 1.5% arginine and fluoride has been shown to be more effective in preventing carious lesions compared to toothpastes that contain only fluoride[35]. This can be attributed to the prebiotic action of arginine, which neutralizes the pH of the biofilm, contributing to the reduction of demineralization[29].

On the other hand, in the groups that used composite resin, the toothpaste did not have an effect on controlling demineralization in enamel. However, the restorative system, composed of the composite resin and the adhesive system, demonstrated effectiveness, as they differed from the control group (CT). The S-PRG filler itself and S-PRG filler-containing materials interact with the tooth structure, controlling caries lesions, due to the release of multiple ions, as previously reported[14]. Despite not preventing the penetration of bacteria into the adhesive interface[20], this resin can modulate the metabolic activity of *S. Mutans*, especially in glycolysis, evidenced by the lower production of lactic and formic acid in the presence of ions F^{-} and Bo_3^{-3} [14,36]. These ions also contribute to rapid pH neutralization[36,37]. These factors contribute to reducing demineralization in the tooth structure, according to the results found in the present study. Sr^{+2}, synergistically with F^{-}, contributes to the remineralization process[37]. A previous study indicated higher Ca and P content in enamel adjacent to the restoration with experimental resin containing 70% by weight of S-PRG particles, similar to commercial Beautifil Bulk Restorative composite resin, demonstrating the ability of this resin to induce remineralization[20].

Products with S-PRG particles are considered bioactive by the manufacturer. Unlike the resinous system composed of Filtek resin, One Bulk Fill and Scotchbond Universal adhesive. The latter demonstrated a positive effect in controlling demineralization in enamel, superior to that shown by the BB and BBF groups. This result differs from a previous study, which investigated the combination of FL Bond II (FL) and Scotchbond Universal (SBU) adhesive systems with Beautifil II (BEF) and Estelite (EST) composite resins, with FL/BEF being the best

combination to contain demineralization, as reported by the authors. Although SBU/BEF has shown similar efficacy to FL/BEF, at enamel lesion depth (μCT), under biofilm challenge and cyclic pH[19]. The Scotchbond Universal adhesive system contains the monomer 10- *methacryloxydecyl phosphate*(10-MDP) in its composition, which establishes chemical bonds with the calcium in the tooth structure, providing effective and stable bonding strength with enamel and dentin. Furthermore, it promotes the formation of an acid resistance zone at the adhesive interface[38], thus substantiating the results obtained with this system.

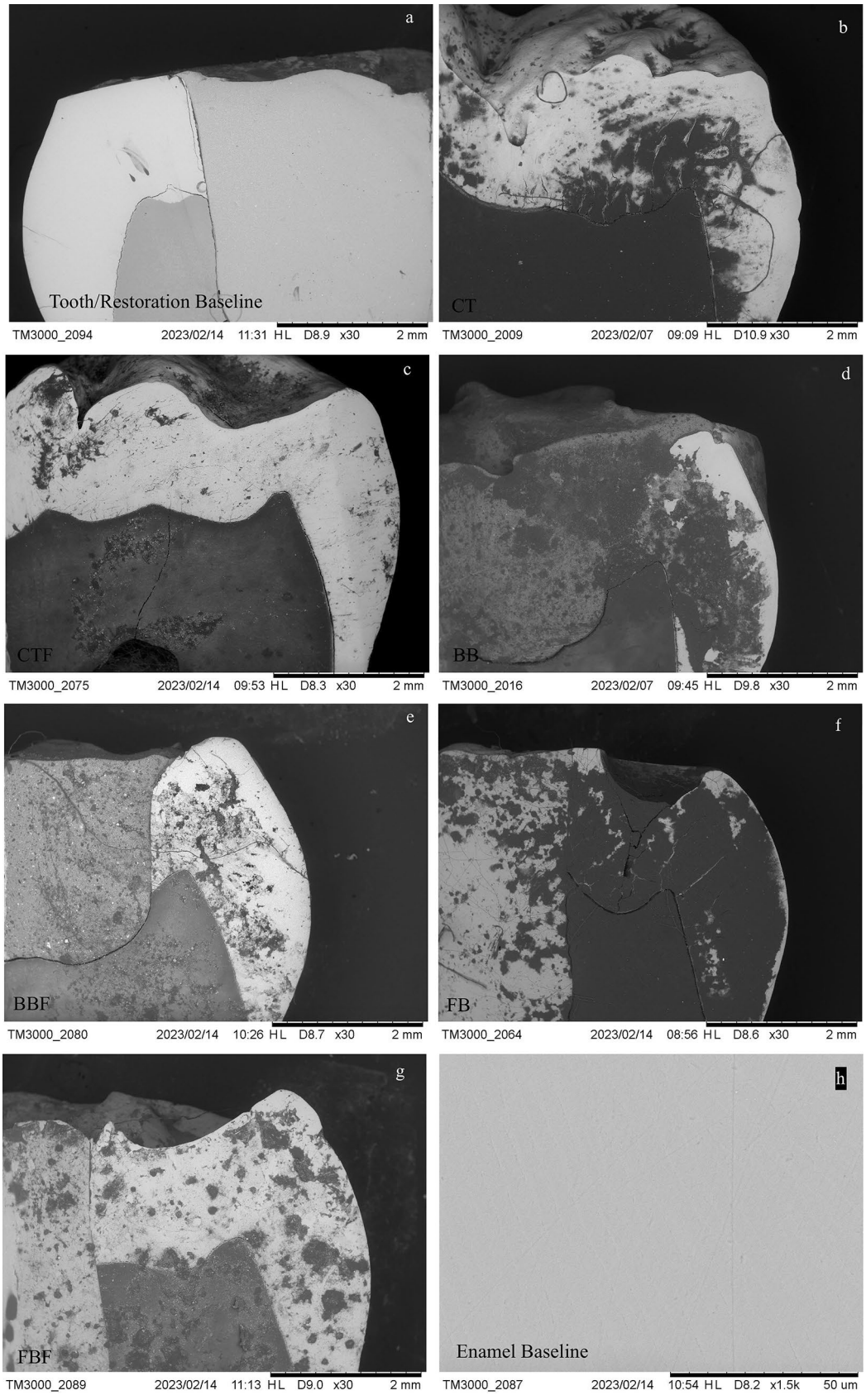
In dentin, the higher organic content and complexity of the tissue make the control of the demineralization and remineralization process more complicated. In the dentin substrate, microhardness analysis revealed a significant difference only between the FBF and CT groups, in relation to demineralization. The 10-MDP monomer, present in the Scotchbond Universal adhesive system, chemically interacts with collagen fibrils in dentin[39,40]. In this process, there is a superficial dissolution of hydroxyapatite around the collagen fibrils, followed by the deposition of MDP-Ca salts, which are insoluble and contribute to the protection of these structures[41]. In addition to the salts, part of the 10-MDP monomer remains in the adhesive layer and contributes to reducing acidity and increasing hydrophobicity[40]. The formation of an acid-resistant layer provided by the 10-MDP monomer and the benefits of the previously discussed toothpaste contributed to the differential results obtained by the FBF group.

Previous studies have demonstrated the effect of composite resin with S-PRG particles in controlling carious lesions in dentin[19,20], which contrasts with the results obtained in the present study. S-PRG particles exhibited a substantial reduction in metalloproteinase (MMP) activity, but did not influence cathepsins, demonstrating partial protection of enzymatic activity in the carious process[42]. These enzymes are related to the degradation of the dentin matrix in processes such as dental caries[43]. In this case, S-PRG particles were placed in direct contact with dentin and the concentration was not dependent on ion leaching from a resinous material. This result suggests that the inhibition of MMPs is correlated with the concentration of ions and also with their contact time with the dentin matrix. Furthermore, an acid resistance zone was not detected when the FL Bond II adhesive (with S-PRG particles) was used, while the groups that used Scotchbond Universal demonstrated a lower depth of carious lesion in dentin under cyclic pH[19]. Therefore, methodological differences between in vitro studies must be considered, with an emphasis on microcosm biofilm, since biofilms with multiple species are more resistant and challenging[44]. This suggests that the amount of ions released from the composite resin with S-PRG did not have an effective concentration to control demineralization in dentin. It is important to highlight that in the study by Zhou et al., 2021[20] the composite resins used were experimental and the S-PRG particles were not silanized, a relevant distinction from our study, which employed a commercial composite resin containing silanized S-PRG particles. Silanization of particles prolongs the time needed for ions to leach, as well as for recharge[19].

Both composite resins evaluated, Beautifil Bulk Restorative and Filtek One Bulk Fill demonstrated degradation when subjected to cariogenic challenge, as observed in the relative surface microhardness, which comprises 80 to 90% of the initial microhardness. Although, biofilm formation on composite resin is not entirely avoidable[45], in the SEM images from our study, it is possible to notice a smaller accumulation of biofilm on the composite resins when compared, for example, to the dentin substrate, regardless of the restorative material used. This similarity is mainly attributed to the surface smoothness obtained by polishing the specimens. Different material surface properties influence bacterial adhesion, such as wettability and surface energy. However, roughness can be considered the most relevant, as the smoother the surface, the lower the microbial adhesion will be[8,9]. The biodegradation of composite resin occurs through acid and enzymes produced in the oral microbiome[8]. Consequently, this degradation makes the composite resin rougher, providing greater bacterial adhesion. This circle between biofilm formation-degradation-increased roughness reduces the longevity of the restoration[8].

The complex environment allows for a better understanding of the antibacterial effects of materials[11]. In this context, this in vitro study sought to simulate the situation of individuals who are at high risk of dental caries. Thus, the microcosm biofilm was formed from the saliva of individuals with active caries, the substrates used were obtained from extracted human teeth and the use of toothpaste was instituted. The results of our study ensured adequate biofilm formation, visible to the naked eye, including the formation of non- cavitated white spot. Although previous studies confirm the release of ions from products with S-PRG particles[16,17,20,37,44], we did not detect superiority of the FL Bond II/ Beautifil Bulk Restorative restorative system over the Scotchbond Universal/ Filtek One Bulk Fill, when subjected to a challenging cariogenic environment. Although in vitro studies allow greater reproducibility and the ability to isolate factors to be observed, the design does not allow the oral environment to be copied with complete fidelity, which is a limitation of the study. Something to take into consideration is the removal/disintegration of biofilm that occurs during brushing, which was not reproduced in this in vitro study. Greater biofilm maturation tends to reduce the effects of antibacterial additives, as it allows microorganisms greater adaptation time[11]. On the other hand, only two clinical studies have been carried out comparing a composite resin with S-PRG particles and a conventional one[46,47]. While one evaluated resins composite in primary teeth, being favorable to conventional resin[47]. The other study evaluated resins composite in permanent teeth and the absence of secondary caries using Beautifil II LS composite resin[46], both with two years of follow-up. However, no study included patients with high caries activity, not representing an inhospitable environment. Thus, the present study allows a better understanding of what happens in places where biofilm is difficult to remove, such as in Class II restorations of individuals with difficulty adhering to frequent and correct use of dental floss. Future studies involving different times of biofilm formation and more frequent use of toothpaste should be carried out.

In conclusion, the toothpaste helps to reduce microorganisms, regardless of the composite resin used. Regarding the control of demineralization of the tooth structure, both restorative systems, with and without S-PRG particles, were effective on human enamel, regardless of the use of toothpaste. Therefore, there is no



superiority of the composite resin with S-PR G particles over a conventional bulk resin, when subjected to cariogenic challenge in biofilm microcosms.

◀**Fig. 5**. Micrographs obtained by SEM with magnification of 30X (**a**-**g**) tooth / restoration) and 1500X (enamel (**h**-**n**), dentin (**o**-**u**) and composite resin (**v**-**z**)), in the protected (baseline) and unprotected (test) areas according to the groups evaluated: CT - control with healthy teeth, without the use of toothpaste; CTF - control with healthy teeth, using toothpaste; BB - S-PRG composite resin (Beautifil Bulk Restorative/FL Bond II adhesive system) without the use of toothpaste; BBF - S-PRG composite resin (Beautifil Bulk Restorative/FL Bond II adhesive system) using toothpaste; FB - composite resin (Filtek One Bulk Fill/ Scotchbond Universal) without using toothpaste; FBF - composite resin (Filtek One Bulk Fill/ Scotchbond Universal) with the use of toothpaste. Black arrows indicate biofilm on the substrate and white stars indicate the residual presence of toothpaste, which may be related to the formation of calcium fluoride

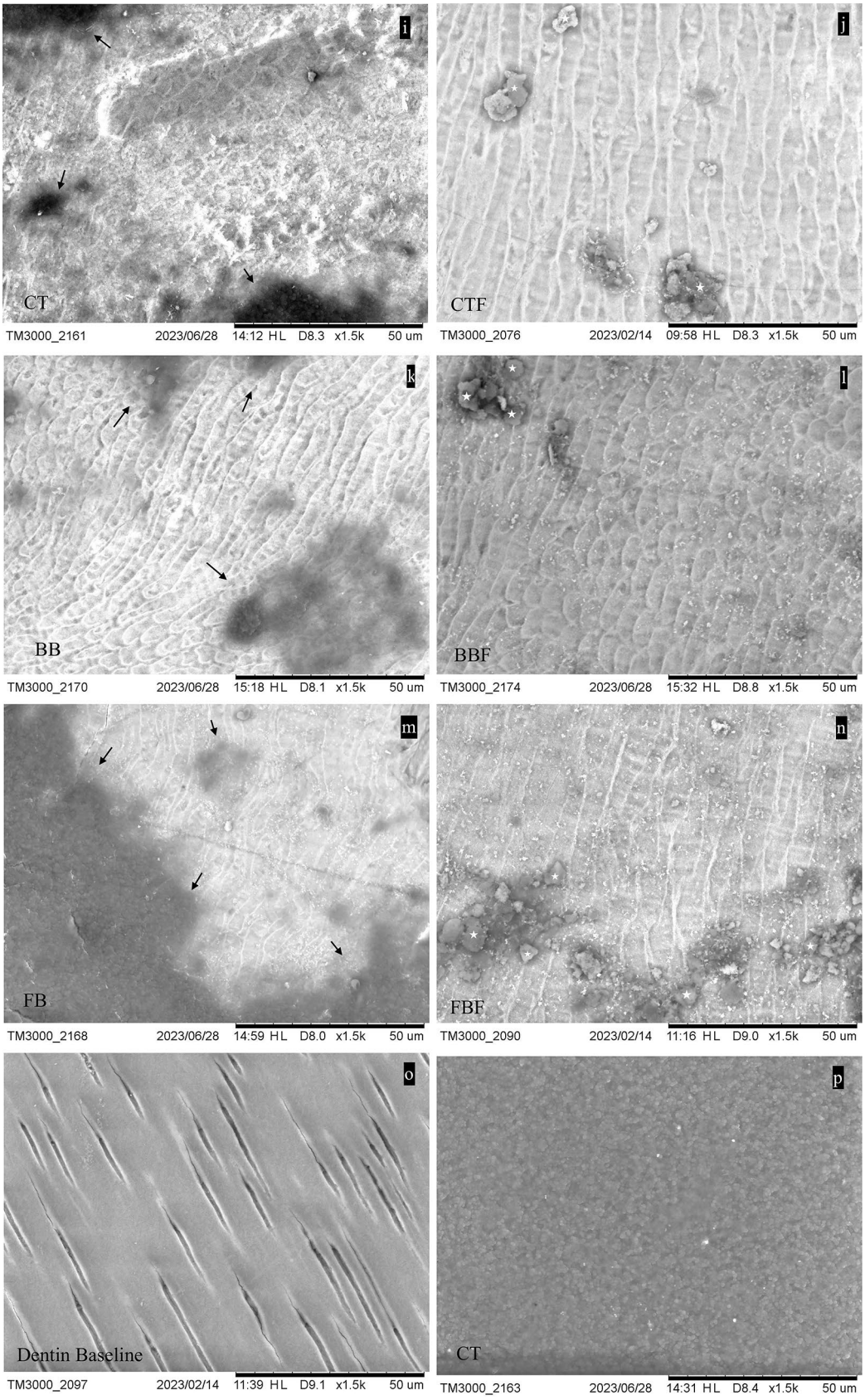


Figure 5. (continued)

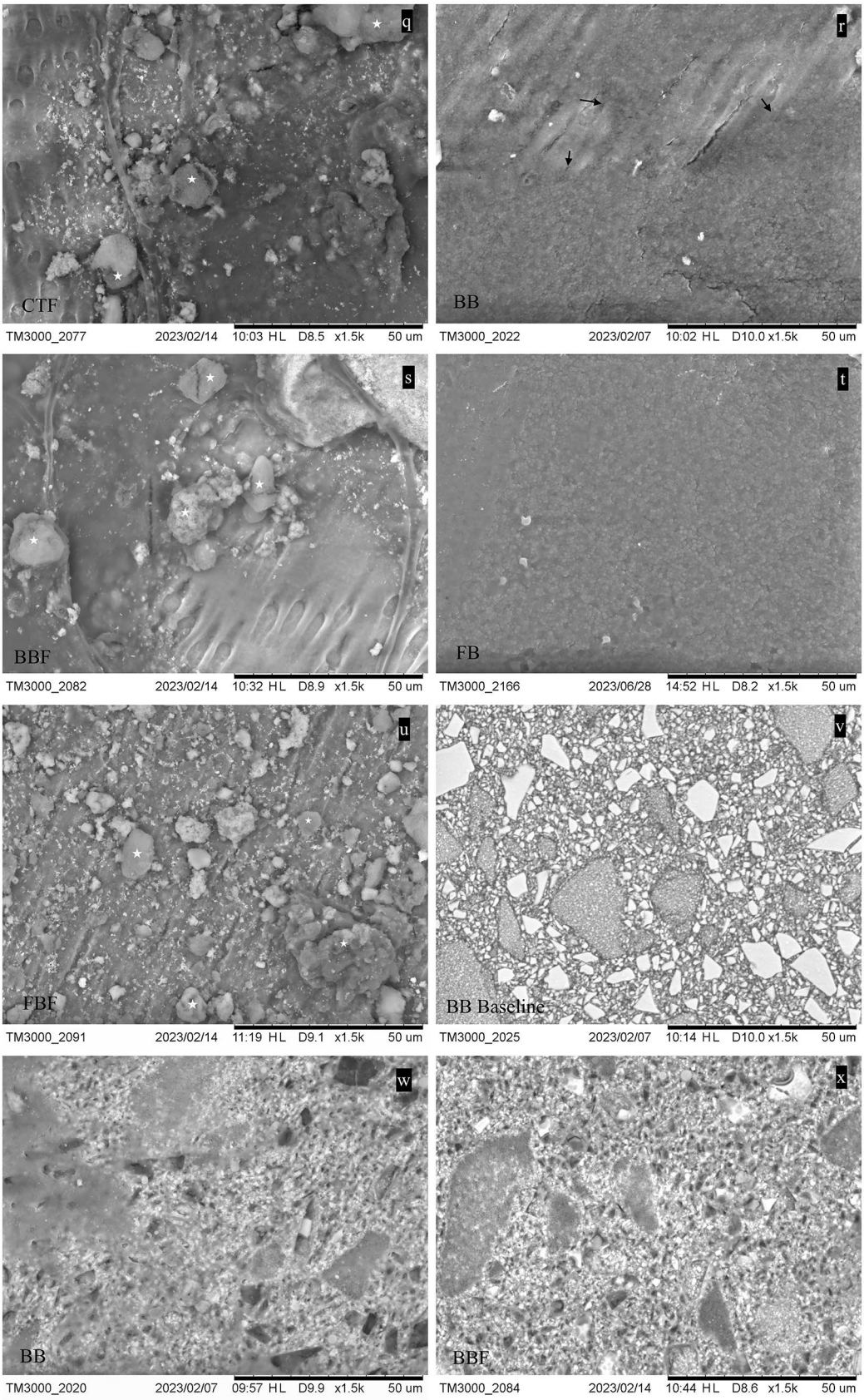


Figure 5. (continued)

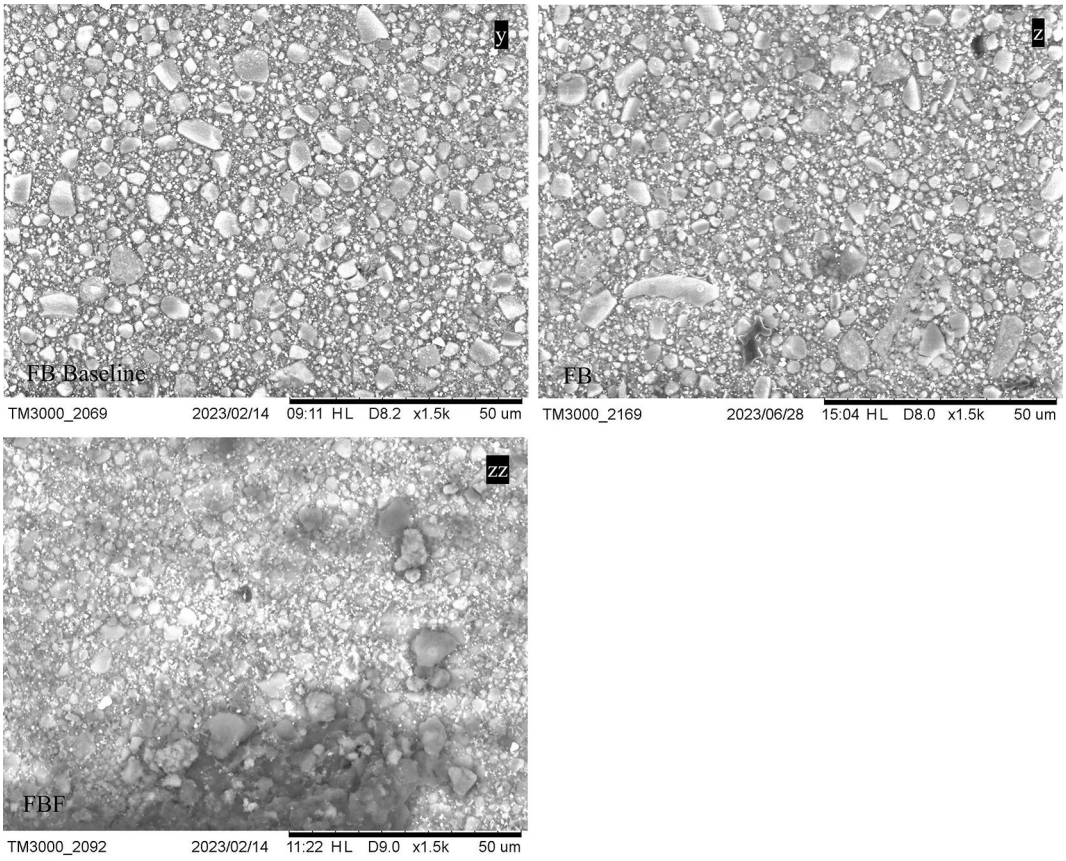


Figure 5. (continued)

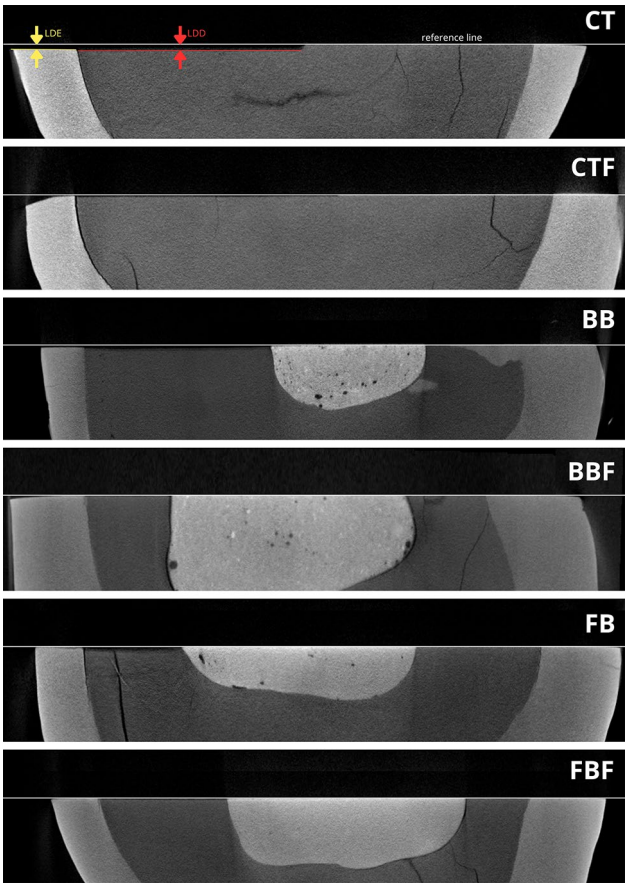


Fig. 6. Representative micro-CT images of a sample from each group. LDE - Lesion depth in enamel; LDD - Lesion depth in dentin

Group	LDE	LDD
CT	82.0 ± 6.4	104.1 ± 6.6
CTF	23.4 ± 5.2	27.1 ± 3.8
BB	14.2 ± 1.5	98.1 ± 4.9
BBF	12.4 ± 4	20.2 ± 3.0
FB	< 6	84.8 ± 6.0
FBF	< 6	12.4 ± 1.7

Table 4. Lesion depth in enamel (LDE) and dentin (LDD) in the evaluated groups (μm). *Mean and standard deviation of 3 measurements (μm) in each sample ($n = 1$).

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Received: 17 March 2024; Accepted: 30 October 2024
Published online: 16 January 2025

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Author contributions
Conceived and designed the study: A.F. & M.C.S.M. Conducted the study: A.F., M.C.S.M. & V.A.A.B. Analyzed the data: M.C.S.M., E.I.J., A.C.A. Interpreted the data: all authors. Wrote the manuscript: A.F. Read, revised, and agreed to be accountable for the manuscript: all authors.

Funding
No funding was obtained for this study.

Declarations

Competing interests
The authors declare no competing interests.

Ethics approval
All procedures performed in this study involving human teeth were in accordance with the ethical standards of the institutional and national human research committee (CEP/UFMS - Federal University of Mato Grosso do Sul (MS/Brazil); protocol: 3.678.506, CAAE: 21527119.6.0000.0021) and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed consent
Informed consent was obtained from all individual participants included in the study.

Additional information
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