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Assessing the marginal seal of bioactive restorative materials in class II cavities with a bacterial penetration model

Sabina Noreen Wuersching *, Lisa Kohl, Reinhard Hickel, Falk Schwendicke, Maximilian Kollmuss

Department of Conservative Dentistry and Periodontology, LMU University Hospital, LMU Munich, Goethestrasse 70, Munich 80336, Germany

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ABSTRACT

Objectives: The aim of this study was to examine the marginal seal of novel bioactive restorative materials and the material-related properties associated with bacterial microleakage.
Methods: Class II cavities prepared into human extracted teeth were restored with: *Venus Diamond* (**VD**) + selective enamel etching (SEE)/self-etching universal adhesive (SEA), *ACTIVA BioACTIVE RESTORATIVE* (**AB**) + SEE/SEA, *Cention Forte* (**CF**) + Cention Primer, *Ketac Universal Aplicap* (**KU**), *EQUIA Forte HT* (**EF**) and *Surefil One* (**SO**) and exposed to a cariogenic multi-species bacterial suspension for 7 days. Bacterial microleakage was visualized with a modified gram staining protocol and bacterial penetration depths were microscopically determined after sectioning the teeth. Disc-shaped specimens (10 mm x 2 mm, n = 6) were used for assessing possible antimicrobial effects and the pH of the materials.
Results: Bacterial microleakage occurred in 14.7 % (VD), 7.1 % (AB), 2.9 % (CF), 47.6 % (KU), 34.0 % (EF) and 55.7 % (SO) of the examined margins. When bacterial penetration occurred, it was limited to the enamel in cavities restored with KU, EF and SO, but reached into dentin of VD, AB, and CF restorations. While SO led to bacterial growth arrest, all other materials only exhibited a weak antibacterial effect. CF immersed in water created an alkaline pH (~9), which remained high until the end of the measurement after 3 months.
Conclusions: Bacterial microleakage occurred less frequently when adhesive pretreatment was performed prior to restoration. CF showed promising results in terms of a tight marginal seal, which may be attributed to continuous ion release and local pH regulation.
Clinical significance: Establishing materials with an improved marginal seal is essential for ensuring longevity of direct restorations and preventing secondary caries development. Bioactive restorative materials, when used with complementary adhesives, show greater resilience to bacterial penetration compared to self-adhesive materials, making them a promising future alternative to nanohybrid composites.

1. Introduction

Secondary caries remains one of the most frequently reported reasons for failure of direct posterior restorations [1]. The presence of cariogenic biofilm and its propagation into marginal gaps is recognized as the main biological cause of secondary caries, however, the longevity of direct restorations is known to depend on further factors such as the operator's care during placement of the restoration, patient adherence, and material-related properties [2]. Today, nanohybrid composites are considered the gold standard for direct posterior restorations and have essentially replaced dental amalgam as a restorative material for class I and II cavities. This development is primarily attributed to their excellent esthetics and the fact that they are used with an adhesive system, which allows a defect-oriented, minimally invasive tooth preparation. In contrast, amalgam restorations have a high potential for weakening the tooth due to the extensive tooth preparation required for sufficient mechanical retention [3,4]. Nonetheless, composite restorations in posterior teeth are still not superior to amalgam in terms of longevity or resistance to the occurrence of secondary caries [5]. Polymerization shrinkage and the technique-sensitive application procedure can

Abbreviations: VD, *Venus Diamond*; AB, *ACTIVA BioACTIVE RESTORATIVE*; CF, *Cention Forte*; KU, *Ketac Universal Aplicap*; EF, *EQUIA Forte HT*; SO, *Surefil One*; SEE, selective enamel etching; SEA, self-etching universal adhesive; GIC, glass ionomer cement.

* Corresponding author.
E-mail address: sabina.wuersching@med.uni.-muenchen.de (S.N. Wuersching).

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contribute to a poor marginal seal, as it is crucial to maintain a dry field during adhesive placement, or else the restoration may fail to sufficiently adhere to the tooth [6]. Moreover, composite restorations are higher in cost and their application procedure is generally more time-consuming compared to amalgam. Hence, there is a need for amalgam alternatives, especially in cases where a quick and economic solution is required.

Glass ionomer cements (GICs) are a class of bioactive material composed of a calcium aluminum fluorosilicate glass powder with basic properties and polyacrylic acid, which together form a hard cement over a short setting time through an acid-base reaction [7]. GICs are thought to reduce the risk for caries formation by their ability to release biologically active ions, thereby restoring the remineralization equilibrium at the tooth-restoration interface, for example when there is an increased bacterial load [8]. Furthermore, GICs have the advantage that they are self-adhesive and do not call for an adhesive system. Unlike composites, which are placed into the cavity using an increment layering technique, GICs can be applied in bulk and thus provide a quick solution for posterior cavities [9]. However, the main problem with GICs is their low resistance to fracture and wear, which limits their use to smaller cavities that are supported by a sufficient amount of remaining hard tissue [10,11].

To overcome the disadvantages of composites and GICs, concepts to create “eclectic” restoratives emerged with the intention to incorporate favorable properties from both classes of materials, that is, the high flexural strength and good esthetics of composites paired with the ion releasing features and the simpler application procedure of GICs [10,12]. In recent years, there has been a vast development of novel classes of restorative materials with bioactive properties, including alkasites, composite hybrids and improved resin-modified GICs. All of these materials are characterized by their potential remineralization properties and their ease of application as they can be placed in bulk [13]. In addition to their ability to release biologically active ions for creating a tight marginal seal, the materials’ resistance to bacterial penetration at the tooth-restoration interface may also be attributed to intrinsic antimicrobial properties [14]. Furthermore, local pH regulation mechanisms in the presence of cariogenic bacteria play a role in altering biofilm pathogenicity and inhibiting the development of secondary caries [15].

Since the number of available restorative materials with bioactive properties is increasing, thorough in vitro investigations are necessary to evaluate their clinical applicability. Therefore, this study aimed to examine the marginal seal of bioactive restorative materials used for restoring class II cavities. In addition, we investigated potential antimicrobial properties and the pH environment they create, as these factors may affect bacterial colonization at the restoration margins. Given the advancements in glass filler technology, we hypothesize that novel restorative materials containing fillers with improved bioactivity provide superior restoration margins compared to the currently available nanohybrid composites and conventional GICs.

2. Materials and methods

2.1. Restoratives, bacterial strains, and growth media

2.1.1. Restorative materials

Six materials for direct posterior restorations were examined in this study and were divided into two groups. Group 1 comprised materials requiring an adhesive: the nanohybrid composite *Venus Diamond* (**VD**, Kulzer, Hanau, Germany) as the control material for the resin-modified GIC *ACTIVA BioACTIVE RESTORATIVE* (**AB**, Pulpdent, Watertown, MA, USA) and for the alkasite composite *Cention Forte* (**CF**, Ivoclar, Schaan, Liechtenstein). Group 2 included self-adhesive GIC-based materials: the conventional GIC *Ketac Universal Aplicap* (**KU**, Solventum, Saint Paul, MN, USA) as the control group for the glass hybrid *EQUIA Forte HT* (**EF**, GC Europe, Leuven, Belgium) and for the self-adhesive GIC-composite hybrid *Surefil One* (**SO**, Dentsply Sirona, York, PA, USA).

2.1.2. Bacterial strains and growth media

All bacterial strains were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *Actinomyces naeslundii* (DSM 17233), *Lactobacillus paracasei* (DSM 4905) and *Streptococcus mutans* (DSM 20523), *Streptococcus oralis* (DSM 20627) and *Streptococcus sanguinis* (DSM 20567) were used in this study. All strains were grown and maintained on Schaedler agar plates supplemented with Vitamin K_1 and 5 % Sheep Blood (Becton Dickinson, Franklin Lakes, NJ, USA). For growth in liquid media, the bacteria were cultured in Brain-Heart-Infusion broth (BHI, Becton Dickinson) supplemented with Hemin (5 μg/ml) and Vitamin K_1 (1 μg/ml). The bacteria were grown at a temperature of 37°C and a humidity level of 60 % in a CO_2 enriched atmosphere with 5.8 % CO_2.

2.2. Evaluation of the marginal tightness

2.2.1. Restoration of human extracted teeth

The use of human extracted teeth was approved by the local ethics committee (registration No. 21-0976KB). 72 molars free of caries or fillings were selected, cleaned and two class II cavities (occlusal-mesial, occlusal-distal) were prepared in each tooth. The teeth were sterilized at 121 °C for 15 min and then divided into 6 groups (n = 12 per group) for restoration with the different materials. The materials were applied to the cavities according to the manufacturers’ recommendation and under sterile working conditions to avoid contamination. A universal adhesive (Scotchbond Universal, Solventum) was applied to the cavities restored with VD and AB. *Cention Primer* was used as a cavity liner prior to placing fillings with CF. KU, EF and SO were used without an adhesive. Detailed information about each material and its application procedure is shown in Table 1. The restored teeth were stored in a sterile container for 24 h at 100 % humidity to allow setting of all materials containing a self-curing portion.

2.2.2. Incubation of restored teeth with bacterial suspension

Single colonies of *A. naeslundii, L. paracasei, S. mutans, S. oralis* and *S. sanguinis* were inoculated separately in 20 ml BHI and grown to their individual stationary phase. The bacterial suspensions were diluted with fresh BHI and adjusted to an optical density (OD) yielding approximately 10^5 bacteria [16]. Equal volumes of the five diluted suspensions were combined in a glass flask. The restored teeth were coated with two layers of nail varnish on all surfaces, leaving a margin of 1 mm around the restoration. The teeth were placed in a 50 ml falcon with their occlusal surface facedown, and 20 ml of the multi-species bacterial suspension were added to each falcon. The teeth were incubated for 7 days at 37°C (humidity level of 60 %, 5.8 % CO_2), and growth medium was changed every 48 h. The bacterial suspension was checked every two days by plating and culturing the discarded growth medium on agar plates.

2.2.3. Microscopical evaluation of the marginal sealing quality

After incubation, the teeth were removed from the bacterial suspension and washed twice with phosphate-buffered saline (PBS). The nail varnish was removed from the teeth and the biofilm grown along the margins of the restoration was visualized with a modified gram staining technique [17]. In brief, the teeth were submerged in a 1 % crystal violet staining reagent and washed off with distilled water to remove dye not bound to the biofilm. After being left to air-dry, the teeth were embedded in a cold polymerizing resin (Technovit 4004, Kulzer Technique) and cut through the center of the restoration along the vertical tooth axis with a diamond saw, providing two cross-sections of each tooth. The sections were attached to microscope slides and bacterial penetration along the margins was examined with a phase contrast microscope (Axiovert 40 C, Axiocam 305 color, Carl Zeiss, Oberkochen, Germany). The penetration depth [μm] was measured at four different

Table 1
Materials, composition, and application procedure. SEE = selective enamel etching, SEA = self-etching universal adhesive. * based on information by the manufacturer.

Material	Type	Application and curing	Composition*	Ion release*
Venus Diamond (**VD**, Kulzer GmbH) *LOT K010203*	Nanohybrid resin composite	SEE (30 s) + SEA, apply in 2 mm layers, light cure (1100 mW/cm^2, 20 s)	Ba-Al-B-F silicate glass, TCD-urethaneacrylate, silica, ≥ 2.5- < 5 % UDMA, ≥ 1-≤ 5 % TEGDMA, titanium dioxide, fluorescent pigments, metallic oxide pigments, organic pigments, aminobenzoicacidester, BHT, camphorquinone	
ACTIVA BioACTIVE RESTORATIVE (**AB**, Pulpdent) *LOT 210402*	Resin-modified glass ionomer cement	SEE (15 s) + SEA, apply in 4 mm layers, self-cure each layer (20–30 s), light cure up to 4 mm ("low intensity", 20 s)	44.6 % blend of diurethane and other methacrylates with modified polyacrylic acid, 6.7 % silica, amorphous, 0.75 % sodium fluoride	calcium, phosphate, and fluoride
Cention Forte (**CF**, Ivoclar) *LOT Z01DTR*	Alkasite	Apply *Cention Primer* (10 s), activate capsule, mix (15 s, 4000 rpm), apply in bulk, optional: light cure up to 4 mm (1100 mW/cm^2, 15 s)	Ca-fluorosilicate glass, Ba-Al silicate glass, copolymer, Ca-Ba-Al fluorosilicate glass, 25–50 % UDMA, 2.5 - < 10 % ytterbium trifluoride, 10–25 % aromatic aliphatic UDMA, DCP, PEG−400-DMA	hydroxide, calcium, and fluoride
Ketac™ Universal Aplicap™ (**KU**, Solventum) *LOT 8247292*	Glass ionomer cement	Activate capsule, mix (4000 rpm, 8 s), apply in bulk, self-cure 3 min 40 s	Oxide glass, water, copolymer of acrylic acid – maleic acid, tartaric acid, benzoic acid	fluoride
EQUIA Forte HT (**EF**, GC Europe) *LOT 210208 A*	Bulk fill glass hybrid	Activate capsule, mix (4000 rpm, 10 s), apply in bulk, self-cure 2 min 30 s	95 % fluoro aluminosilicate glass, 5 % polyacrylic acid powder, reinforced with silicate particles, 25-< 50 % polyacrylic acid, 5-< 10 % polybasic carboxylic acid, 5 -< 10 % tartaric acid	fluoride
Surefil One (**SO**, Dentsply Sirona) *LOT 2203000115*	Self-adhesive composite hybrid	Activate capsule, mix (4200–5000 rpm, 8 s), apply to cavity, self-cure (6 min) or light cure min. after 1 min 30 s (≥ 800 mW/cm^2, 20 s)	Al-P-Sr-Na-F silicate glass, water, highly dispersed silicon dioxide, acrylic acid, polycarboxylic acid, ytterbium fluoride, bifunctional acrylate, self cure initiator, 4-tert-Butyl-N,N-dimethylaniline, iron oxide pigments, barium sulfate pigment, manganese pigment, camphorquinone, stabilizer	fluoride

margins per cross-section: proximal mesial, proximal distal, occlusal mesial, occlusal distal (Zeiss AxioVision SE64 Rel. 4.9.1 Software). For each material, a total of 96 margins were examined. The mean depth was calculated among all margins with a measured penetration depth greater than 0 µm. In addition to documenting the absolute values, the penetration depths were categorized into subgroups indicating whether the colonized margins were restricted to the enamel, the enamel-dentin junction (EDJ) or reached into dentin.

2.3. Antimicrobial properties

A direct contact test was performed to assess the antimicrobial activity of the restorative materials. In brief, the restorative materials (n = 6) were applied in 2 mm layers to the bottom of the wells of a 48-well plate as described above. Overnight cultures of *A. naeslundii, L. paracasei, S. mutans, S. oralis* and *S. sanguinis* grown in BHI broth medium were diluted in fresh medium and adjusted to an optical density (OD) yielding approximately 10^5 bacteria. Equal volumes of the five diluted strains were combined and mixed. The diluted multi-species bacterial suspensions were added to the wells containing the materials at the well bottom and to empty wells for the control group. After 24 h and 48 h of incubation, the viable planktonic bacteria in each well were quantified using a plating and culture method. In brief, ten-fold serial dilutions of the bacterial suspensions were prepared in 0.9 % sodium chloride and plated on agar plates. After incubating the agar plates for 48 h, the colony-forming units (CFU) were counted according to FDA guidelines (only plates with 25–250 colonies were considered) and the CFU/ml were calculated [18].

2.4. pH

For determining the pH created by the materials in an aqueous environment, disc-shaped specimens (dimensions: 10 mm diameter, 2 mm thickness) were prepared as described above. Six discs of each material were submerged in individual falcon tubes containing 15 ml of deionized water and incubated at 37 °C. pH measurements were performed after 0 h, 6 h,12 h, 24 h, 48 h, 3 d, 5 d, 7 d, 14 d, 4 weeks, and 3 months (827 pH Lab, Deutsche Metrohm, Filderstradt, Germany) with (n = 3) and without (n = 3) changing the water after every point of measurement.

2.5. Statistics

All statistical evaluations were performed in *Python 3.8.8* using the packages *scipy* and *scikit* for inferential statistics and *matplotlib* for the descriptive analysis [19]. Equality of variances was assessed with the Levene's test and data were tested for normality with the Shapiro-Wilk test. Normally distributed data with equal variances were analyzed with an analysis of variances (ANOVA) and Tukey's HSD post hoc analysis. For normally distributed data with unequal variances, Welch's ANOVA and Tamhane's T2 post hoc analysis was performed. Contingency tables were analyzed with Fisher's exact test. The alpha level was set to 0.05.

3. Results

3.1. Marginal tightness of restorations in human extracted teeth

Fig. 1a shows the proportion of margins with (colored) and without (grey) bacterial penetration. The discrete values of all margins (including 0 µm) were visualized with a categorical scatterplot (Fig. 1b). The minimum, maximum, mean and median penetration depths of all margins with a penetration depth greater than zero are shown in Table 2.

In general, bacterial penetration along the restoration margins occurred less frequently in group 1 (VD, AB, CF) than in group 2 (KU, EF, SO). Bacterial microleakage was detected in 14.7 % (VD), 7.1 % (AB), 2.9 % (CF), 47.6 % (KU), 34.0 % (EF) and 55.7 % (SO) of the examined margins. The proportion of cavities with bacterial penetration was significantly higher in group 2 materials compared to group 1 materials (Fisher's exact, $p < 0.05$ for comparisons between each member of group 1 and group 2).

Among all margins where penetration occurred, the mean penetration depths in the adhesive group were higher than in the self-adhesive group and reached into dentin more frequently, whereas bacterial colonization along the margins of cavities restored with KU, EF, SO was predominantly limited to the enamel. Representative images of margins with and without bacterial penetration are shown in Fig. 2 for each material.

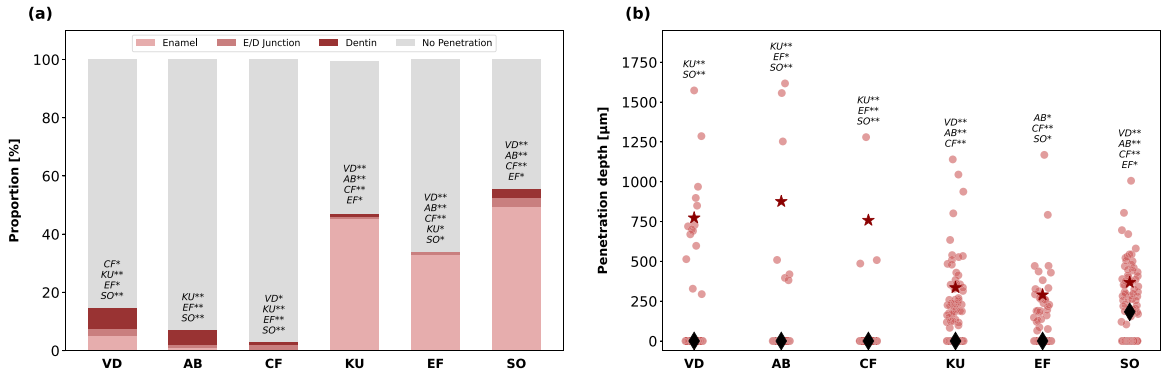

Fig. 1. **(a)** Proportion of margins with and without bacterial leakage stratified according to the affected tooth tissues limiting bacterial colonization. Analysis of contingency tables was performed with Fisher's exact test. **(b)** Discrete values of all measured penetration depths [µm] including 0 µm measurements. Rhombus: median of all values (including 0 µm); star: mean of values >0 µm. P-values obtained with Dunn's test with a Bonferroni correction. *, $p < 0.05$; **, $p < 0.001$.

Table 2
Depth of bacterial colonization along the margins of the restorations. Only margins with penetration >0 were included in the table.

	N with penetration	Min [µm]	Max [µm]	Mean [µm] (Std [µm])	Median [µm]
VD	14	294.22	1573.05	772.82 (341.43)	710.46
AB	7	381.48	1617.16	876.02 (573.46)	508.94
CF	3	486.24	1279.77	757.96 (452.02)	507.88
KU	49	82.68	1140.10	335.10 (238.21)	248.32
EF	33	65.26	1168.51	289.54 (213.86)	258.26
SO	54	103.89	1006.06	367.86 (174.12)	345.89

3.2. Antimicrobial activity

The antimicrobial effects of the materials against planktonic bacteria were assessed in a modified DCT using a multi-species suspension with cariogenic bacteria. The viable bacteria (CFU/ml) after 24 and 48 h of incubation are shown in Fig. 3. After 24 h, the CFU/ml were significantly reduced in the presence of AB, KU, EF and SO compared to the control group ($p < 0.05$). However, SO decreased the number of viable bacteria by almost 3 log-scales, whereas the antimicrobial effects of AB, KU and EF against planktonic bacteria were comparatively weak (0.5–1 log-scales). After 48 h, all materials except CF led to a significant reduction of CFU/ml. Again, the largest difference compared to the control group was in the presence of SO.

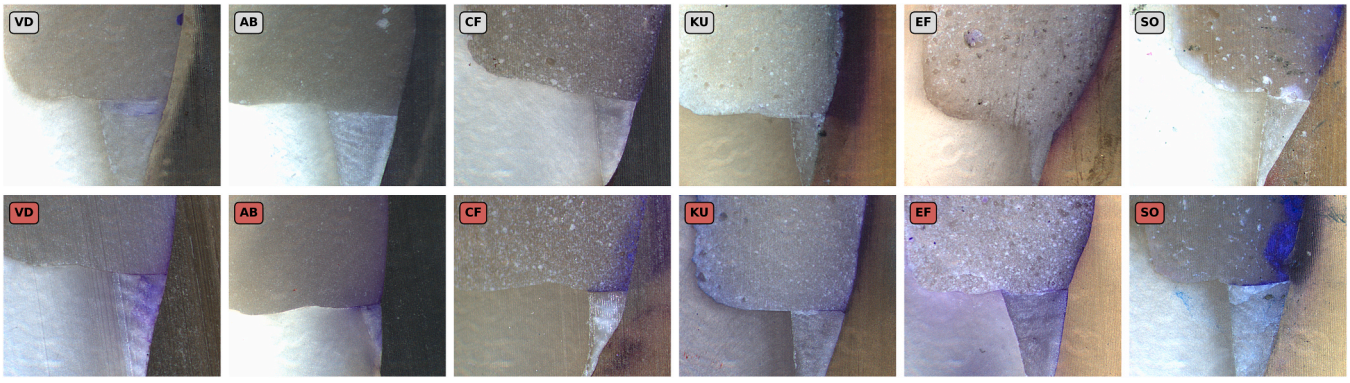

Fig. 2. Representative images displaying cross-sections of the restored teeth. Upper row: restorations showing no leakage; lower row: restoration margins with bacterial colonization visualized with modified gram-staining (purple).

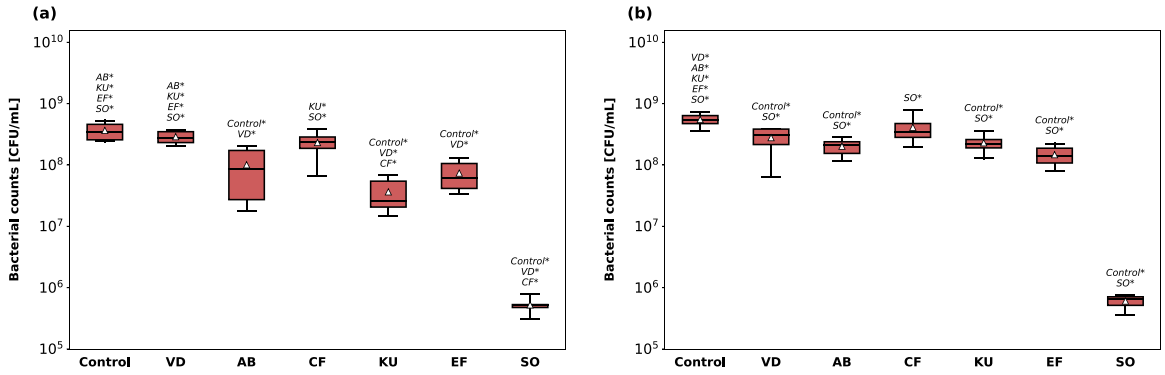

Fig. 3. Number of viable planktonic bacteria (CFU/ml) after **(a)** 24 h and **(b)** 48 h of incubation with restorative materials. P-values obtained with Welch's ANOVA and Tamhane's T2 post hoc test. * , $p < 0.05$.

3.3. pH

Fig. 4 shows the pH of the solutions containing specimens of the restorative materials at different timepoints for a total measurement period of 3 months. At baseline (0 h), VD and CF solutions were slightly alkaline, whereas KU, EF and SO solutions were slightly acidic. The pH of CF solutions increased continuously during the first 24 h, reaching an alkaline pH of 9.6–9.8. In the experimental setup involving replacement of the water after every measurement, the pH of the CF solutions stagnated at 9.8 for the entire observation period. In the setup using the same water for the whole period, the pH of CF solutions began to drop after 7 days and continued decreasing slightly until the end of the measurement. All other solutions levelled off at pH 6–7 after 24 h, irrespective of their initial acidity or basicity.

4. Discussion

As dental treatments are becoming more minimally invasive and biologically oriented, there is a growing scientific and clinical interest in bioactive restorative materials. These materials are essential, as they not only ensure biocompatibility but also actively contribute to the preservation and regeneration of dental tissues. Current advancements in bioactive restorative materials with reactive fillers are driven by the need to address the limitations of resin composites, which are susceptible to marginal gap formation due to polymerization shrinkage [20]. Despite the lack of consensus on the definition of bioactivity, the term is often used to describe the naturally occurring ability of a substance to interact with its environment and elicit a specific response [21]. A recent FDI policy statement suggested certain prerequisites for classifying a material as bioactive: the desired effects exerted by the material should be intentional, local and non-toxic, and not interfere with the material's principal purpose [22]. Moreover, scientific evidence for these features should be obtained at least in vitro or in situ, and ideally confirmed by clinical trials. In a restorative context, the intended response from a bioactive material is considered the formation of a hydroxyapatite-like layer at the tooth/restoration interface so as to enhance the marginal seal and slow down secondary caries formation [23].

Several in vitro methods for examining the marginal integrity of dental materials have been previously described [24]. These techniques frequently focus on the microscopical detection of marginal gaps, usually accompanied by dye penetration for visualization. However, the use of dye tracers is limited by possible alterations to the properties of the dye depending on the surrounding pH or by the presence of ions [25]. Moreover, the penetration depth along the marginal gaps is determined by the molecular size of the dye, which can lead to an over- or underestimation of the marginal seal. It is important to mention that secondary caries does not develop as a natural consequence of the mere presence of marginal gaps, unless these gaps are further infiltrated by oral fluids, salivary enzymes, and bacterial cells. The direct cause of caries at the margins is the presence of cariogenic plaque harboring bacterial byproducts such as lactic acid, that eventually demineralize the tooth structure [26]. Therefore, detecting marginal gaps that allow the passage of bacteria and subsequent biofilm formation seemed most relevant from a clinical point of view. The size of these gaps is expected to be at least in the range of 0.5–1 μm or larger, i.e., the average size of a bacterial cell [24]. However, gaps in these sizes, even when colonized by bacteria, are still not a warrant for tooth decay; restoration defects yielding gap sizes larger than 60 μm have been identified as threshold for facilitated secondary caries development, especially when masticatory forces propelling fluid and biofilm exchange into the tooth-restoration interface are present [27,28]. Nonetheless, the differences in initial marginal colonization which we demonstrated in our study can be viewed as possible predictors of future caries lesions.

Controversy exists over which bacterial model best simulates bacterial demineralization around dental restorations. While bacterial microleakage studies frequently utilize *S. mutans* alone, doubts about the correspondence of such single-species models to clinical reality have been raised. Although *S. mutans* is a key pathogen in caries lesions, this bacterial species is just part of an oral biofilm that nurtures a complex microbial consortium. Therefore, it has been suggested that mixed

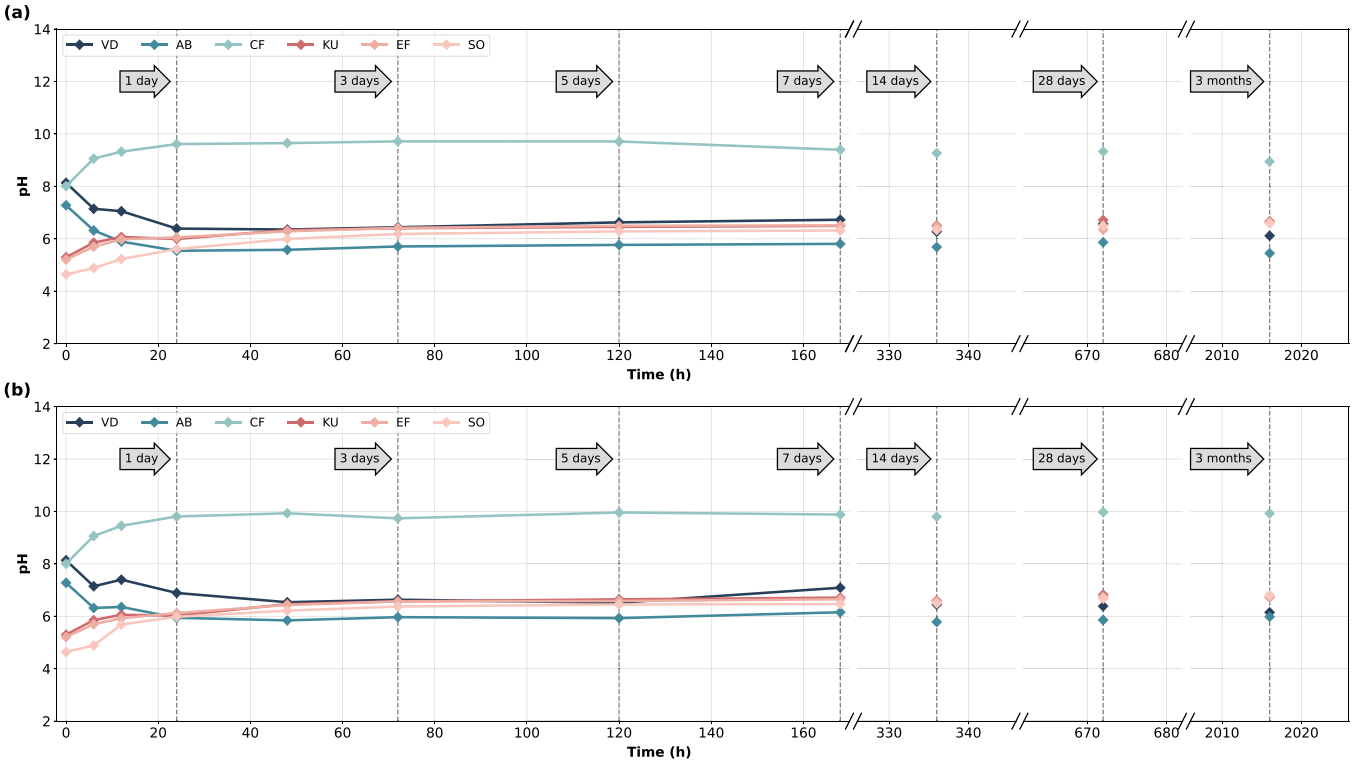

Fig. 4. pH environment created by specimens of the restorative materials immersed in deionized water for 3 months **(a)** without replacing water and **(b)** with replacing water extracts with fresh water after every measurement.

biofilms, comprising at least two different species, better represent biofilm formation in the oral environment [29]. For our bacterial microleakage model we chose five bacterial species that are strongly associated with cariogenic biofilms. The non-mutans streptococci *S. oralis* and *S. sanguinis* are frequent pioneer colonizers during initial biofilm formation and support the attachment of further microorganisms [30]. By interacting with other bacterial species via coaggregation mechanisms, they contribute to a complex biofilm ecology [31]. The acid production resulting from carbohydrate metabolism by the early colonizing streptococci creates an environment conducive to incorporating more acidogenic and aciduric bacteria such as mutans streptococci, *Actinomyces* spp. and *Lactobacillus* spp. into the biofilm [15]. Given the polymicrobial nature of oral biofilms, we employed these bacteria as a representative multi-species suspension for our microleakage model.

The method for visualizing bacterial penetration was based on a modified gram staining protocol using a 1 % Crystal violet staining reagent. Counterstaining with basic fuchsin or safranin was omitted due to the absence of gram-negative bacteria in the multi-species bacterial suspension [17]. Since the bacteria colonizing the restoration margins are embedded in biofilms, no additional fixation with iodine was needed as with traditional gram staining protocols that are employed to visualize planktonic cell suspensions on glass slides. Moreover, an advantage of this staining method is that crystal violet not only stains gram-positive cells, but also the biofilm matrix, thereby visualizing the entire microbial community at the restoration/tooth interface [32].

In order to compare the extent of bacterial penetration among the different restorative materials, the penetration depth was categorized based on the affected tooth tissues. In general, group 1 materials, that were used with a universal adhesive, allowed bacterial colonization into dentin, whereas bacterial microleakage in group 2 restorations was almost entirely limited to the restoration interface with enamel. It should be noted that bacterial leakage was generally a rare event with group 1 materials, but in the few cases where leakage did occur, penetration depths were more severe than in group 2 restorations. Ionomer-like materials are self-adhesive and their adhesion is based on two mechanisms: micromechanical interlocking caused by the self-etching polyacid components as well as ionic forces that operate between the material and the dental hard tissues [13,33]. The chemical bond is formed by carboxylate groups from the polyacid interacting with the calcium ions from the tooth, eventually forming an ion-exchange layer at the interface which provides the foundation for a tight marginal seal [34]. In contrast, the bonding mechanism of composites to dentin is based on the formation of a hybrid layer, where adhesive monomers infiltrate the demineralized dentin collagen network [35]. This hybrid layer creates the link between the adhesive and dentin yet is vulnerable to biodegradation through different mechanisms, such as hydrolysis of the methacrylates in the presence of water, proteolytic activity of the bacteria within the biofilm, and loss of mechanical integrity [36]. As far as composite adhesion to enamel is concerned, etching with phosphoric acid provides a higher level of enamel demineralization and a more pronounced microretentive enamel surface than the acid properties of self-adhesive materials [37,38]. Previous data have demonstrated that the bond strength of ionomer-like materials to the underlying tooth varies depending on the tooth substrate. Particularly for EF and SO there is evidence that the bond strength to dentin is somewhat greater than to enamel [39]. This feature may have contributed to a tighter seal at the restoration interface with dentin, which stopped further bacterial microleakage at the EDJ. Although AB is classified as resin-modified GIC, the application procedure requires cavity conditioning with an adhesive following selective enamel etching. Studies that examined the bond strength of AB without any pretreatment of the cavity found increased rates of retention loss due to adhesive failures [40,41]. In our study, AB provided a relatively tight marginal seal, however the mandatory use of an adhesive with AB makes the self-adhesive features of this material redundant. Moreover, this application procedure calls for the same precautions during placement as with traditional resin composites, especially regarding the necessity to maintain a dry working field. In summary, the prerequisites of AB for sufficient placement are not vastly different from nanohybrid composites, which questions the usefulness of AB for cases that require a quick and resilient application procedure.

To unravel the mechanisms behind resistance to marginal bacterial penetration, we performed a direct contact test to assess potential intrinsic antibacterial effects, using the same multi-species bacterial suspension employed in the bacterial penetration model. The lowest viable count after 24 or 48 h of incubation was observed in the presence of SO, whereas CF did not exhibit any antibacterial effect. After 24 h, AB, KU, and EF led to a significant growth inhibition compared to the control group. However, this initial antibacterial effect only resulted in a 0.5–1 log reduction, which concurs with the increased bacterial counts after 48 h of incubation in these groups. SO seemed to have led to a more persistent growth arrest, given that the number of viable bacteria after 24 and 48 h of incubation with SO were in a similar range as the inoculum. This bacteriostatic effect may be attributed to the specific material composition and the curing mode of SO. According to the manufacturer, SO cures chemically within six minutes when used in self-cure mode, but also has a light curing option that speeds up polymerization within the upper layers of the restoration. SO contains a modified polyacid system which acts as copolymerizing crosslinker between the ionic and covalent network, thereby forming a hydrolytically stable diluent with acidic moieties alongside polymerizable acrylates. To maintain consistency in the application procedure among all group 2 materials, we chose to omit the light curing step and allowed SO to self-cure for a minimum of six minutes. This decision was supported by previous data indicating that the shear bond strength and further mechanical properties of this material are barely affected by the curing mode [39,42]. However, perhaps the crosslinkers within SO were not stable enough without additional light curing and allowed the liberation of acidic components, which concurs with the pH dropping down to 4.6 right after immersion of the specimens into water. Since the experiment was performed in a closed system without any buffering system, this initial acidity may have been toxic to the planktonic bacteria. Indeed, acid killing of bacteria has been reported for planktonic *S. sanguinis* and *A. naeslundii* cultures, which, despite being tolerant up to a certain acidity level, are more sensitive to low pH environments than mutans streptococci and can undergo cell lysis at pH levels somewhat higher than 4 [43]. Moreover, polyacrylic acid containing copolymers have been shown to exert non-specific bactericidal effects. This feature is reportedly due to an ion-exchange effect, where the negatively charged acidic moieties attract membrane balancing divalent cations, resulting in destabilization of the phospholipid bilayer and eventually, in collapse of the bacterial cell membrane [44]. Assuming that this mechanism of action took place in the case of all materials containing polyacrylic acid, it is plausible that we observed a CFU/ml reduction in the presence of the AB, KU, and EF. Higher levels of fluoride release from SO may have also contributed to bacterial growth arrest in vitro, considering that the bacteria were exposed to the materials in a closed system [45]. Nonetheless, the antibacterial effects of the restorative materials did not concur with the frequency and the depth of bacterial penetration along the restoration margins, since SO was found to exhibit the most events with marginal bacterial colonization, whereas CF showed the lowest number of bacterially colonized restoration margins despite having the least antimicrobial effects in vitro. Therefore, the mechanisms of resistance to bacterial penetration were unlikely due to intrinsic antibacterial properties but rather stem from physicochemical interactions.

We assessed the pH environment created by the restorative materials immersed in deionized water. The fact that KU, EF, and SO led to an initial pH drop is not surprising, since the setting reaction of all three materials is an acid-base reaction where the acidic moieties leach out during the first 24 h after mixing. AB and VD maintained a neutral pH, ranging between pH 6 and 8 throughout the entire measurement period.

However, CF specimens led to a continuous increase in pH during the first 24 h, which eventually levelled off at a high pH of nearly 10. Interestingly, the pH remained this high for at least up to three months when the water extracts were regularly replaced with fresh water, whereas in the setup using the same medium for the entire measurement period, the pH started to gradually decrease after 7 days. These results suggest that CF has an ion recharging and re-release capacity which ensures continuous liberation of pH-regulating ions. In fact, this property has been previously examined for Cention N, the predecessor of CF, along with other ion-leaching bioactive restorative materials [46]. The authors demonstrated that alkasites exhibit a high calcium and phosphate release and have excellent recharging and re-release properties for these ions. Since orthophosphate (PO_4^{3-}) ions act as proton acceptors, their release from set alkasite specimens leads to a local pH increase by the withdrawal of H^+-ions from aqueous solutions. Local alkalinity creates an unfavorable environment for bacterial adhesion, thus reducing the chances for bacterial biofilm formation along the restoration margins [43,47]. The alkalizing potential of CF may also influence glucan synthesis by mutans streptococci, which is markedly affected by environmental factors, including the pH and ion concentrations [48]. Moreover, the fact that the ions released from CF mainly target the environment adjacent to the restoration refutes any concerns that the use of adhesives with bioactive restorative materials may impede ion diffusion [12]. Structural analyses from a previous study have shown that CF, used with its complementary adhesive, forms a calcium-fluorosilicate glass that resembles the reactive fillers of bioactive glass, which is a possible explanation for the excellent ion delivery exhibited by CF specimens [46].

Aside from the general in vitro nature of this study, which does not entirely replicate the complex environment of the oral cavity, there are limitations to the bacterial microleakage model that need to be addressed: the penetration depths were measured in cross-sections through the center of the restorations, providing only a two-dimensional view of the colonized margins and not accounting for the width or volume of bacterial penetration. For an approximation of the true bacterial penetration depth, we employed a large sample size in each group with the goal to overcome possible over- or underestimations. A further limitation of our method is the uncertainty regarding the extent to which each species engaged in the biofilm formation process, as the individual species within the mature biofilm were not quantified. Each material may have also affected bacterial growth of each species differently, which our experimental setup does not account for. Moreover, the restored teeth were not exposed to simulated mastication and our findings therefore only reflect the materials' initial susceptibility to bacterial colonization. Shear forces that occur during mastication can affect the marginal integrity of the restorations and accelerate possible secondary caries processes. In this study, chewing simulation was deliberately omitted due to sterility concerns with this particular setup, because placing the restored teeth in a chewing simulator would have required disinfecting them prior to performing the bacterial microleakage experiments. Disinfecting agents effective against bacterial or fungal contamination such as ethanol are absorbed by materials with an ionomer matrix, potentially affecting their cohesion and altering the outcomes of this study. Since it is uncertain whether the gaps allowing bacterial leakage emerged from loss of adhesive or cohesive forces, the progression of these gaps can vary greatly when exposed to masticatory forces. Therefore, further investigations focusing on the marginal integrity under functional loading are necessary to confirm our findings.

5. Conclusions

The bioactive restorative materials evaluated in this study demonstrated varying susceptibility to bacterial microleakage upon initial placement in class II cavities. Restoration materials used with a self-etching universal adhesive showed significantly fewer colonized margins compared to self-adhesive materials. However, the penetration depths differed significantly among the materials, with marginal penetration being limited to enamel in self-adhesive restorations, while reaching the enamel-dentin junction or into dentin in cavities restored with a self-etching adhesive. The alkasite showed the most promising results in terms of a tight marginal seal, likely due to continuous ion release and local pH regulation. There was no direct correlation between inherent antibacterial properties and bacterial microleakage.

In conclusion, optimizing the marginal seal of dental restorations is essential for preventing secondary caries development. Advancements in material chemistry focusing on an enhanced marginal resistance to bacterial colonization contribute greatly to the lifespan of dental restorations. Restorative materials containing improved reactive glass fillers exhibit significant resilience to bacterial microleakage, especially when used alongside complementary adhesives. This makes bioactive materials strong contenders as future alternatives to nanohybrid composites.

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Declaration of Competing Interest

None.

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