Research Article

Relationship between In Vitro Physical Properties and In Situ Biofilm Formation of Fissure Sealants

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The aim of this 2-part study was to investigate whether surface properties of two resin sealants (K1, K2), a glass-ionomer (Ci) sealant, and a biomimetic hydroxyapatite (BHAP) had any effects on *in situ* biofilm formation.

Standardized specimens manufactured from 4 materials and human enamel (E) were subjected to *in vitro* microhardness, surface roughness, and contact angle measurements. Then, 3 fissure sealant samples and BHAP blocks were placed on the upper removable appliances of 20 children. In the first week, biofilm was allowed to form *in situ*, then a hydroxyapatite (HAP) paste was used with renewed materials for the second week. The biofilm developed on the surfaces was analyzed using SEM and image analyzing programs.

There was a statistically significant difference between the roughness of dental materials ($p < 0.05$). There was no difference between the microhardness of K1 and K2, whereas a statistical difference between Ci and other materials ($p<0.05$) was found. Ci had also statistically higher contact angle measurements than other materials ($p<0.05$). The *in situ* biofilm formation was highest in Ci and lowest in BHAP materials, but not statistically different, and the biofilm formation was significantly decreased in all groups ($p<0.05$) with the use of the HAP paste.

Within the limitations of this study, the roughness of materials correlated with the biofilm formation on BHAP, glass-ionomer, and resin sealants. The use of the HAP paste contributed to reduced biofilm formation.

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1. Introduction

The pits and fissures on the occlusal surfaces of primary and permanent teeth are deep enough to hold microorganisms and food residues. These areas are subject to tooth decay mostly in school-age children, and the risk of dental caries is remarkably high at this age group. The most used dental materials for protective purposes in pediatric dentistry are resin- or glass ionomer-based fissure sealants. They are aimed to effectively reduce these caries-prone regions for the growth of oral bacteria by blocking the occlusal surfaces of the teeth. Commercially available fissure sealants are resin-based and/or glass ionomer fillers which are light-activated and contain fluoride [1][2].

The physical, mechanical, and/or biological properties of an ideal protective material are of great importance. Depending on whether it is resin-based or not, the physical properties of a fissure sealant include its hardness, the amount and the particle sizes of the fillers, and the release level of its ions. It is generally reported that low physical properties are seen with high fluoride release [3][4]. The differences in the structures, as well as the aesthetic properties of restorative materials, including fissure sealants, affect the physical properties and the usage of these materials and are important in their clinical success [5][6].

Although many studies have been conducted on the formation of oral biofilms on dental tissues, the adhesion mechanisms of the oral microflora to the surface of dental materials have not yet been fully elaborated. Studies have shown that the factors involved in this process and the correlation between bacterial adhesion and oral biofilm formation can include saliva proteins, hydrophobicity or hydrophilicity of the substrate, organic and inorganic contents of the dental materials and their solubility, microhardness, surface free energy, surface tension, and surface roughness. [7][8][9]. *In vitro* studies have shown that adding materials such as fluoride, CPP-ACP, xylitol, chlorhexidine, and HAP has the potential to inhibit the growth of cariogenic bacteria in oral biofilms [10][11][12]. The results regarding the anti-carcinogenic effects of these dental materials obtained from *in vivo* and *in situ* studies are open to debate, and a consensus has not been reached yet. Dental literature is mainly interested in the wear, retention, microleakage, and caries prevention effects of fissure sealants [13] [14][15]. Understanding oral biofilm and its interaction with the physicochemical characteristics of dental material surfaces holds potential for enabling improvements in oral health [16]. We hypothesized that the physical properties of the sealant materials can also influence *in vivo*

accumulation and adhesion of oral bacteria onto these products in caries-prone children, leading to caries risk for those children.

In this 2-part experimental study, firstly, the physical properties of 3 different fissure sealant materials, a new biomimetic hydroxyapatite (BHAP), and human enamel (E) samples were compared under in vitro conditions; and then secondly, *in situ*-formed biofilms on these materials, either with or without a HAP-containing paste, were evaluated using SEM. Finally, both experiments were combined to correlate the physical properties of the materials to biofilm formation.

2. Materials and Methods

Three dental sealant materials (2 resins: Helioseal F (K1), Ultraseal XT Hydro (K2), and 1 glass ionomer: Fuji Triage Capsule (Ci)), human molar enamel samples (E), and hydroxyapatite discs (pressing biomimetic hydroxyapatite BHAP) were used in this *in vitro* and *in situ* combined study.

Forty samples of each resin dental material were set in previously prepared 2 mm diameter, 10 mm height standard Teflon moulds, placed on a transparent tape and glass, and polymerized from the bottom and top using a polymerization LED device (LK-G13-1 DY400-4, Denjoy Dental, China) for 40 seconds according to the manufacturer's instructions. K1 is a hydrophilic, highly filled (53%), thixotropic white resin sealant, and K2 is a hydrophilic, highly filled (40%), fluoride-releasing, white resin sealant. The glass ionomer capsules were mixed for 10 seconds, and 40 samples were prepared in 2 mm diameter, 10 mm height standard Teflon moulds, placed on a transparent tape and glass according to the manufacturer's instructions. Ten samples of each material were left unpolished; the remaining surfaces were sanded for 30 seconds with 500- and 800-mesh carbon disc sandpaper and polished accordingly to obtain a smooth surface.

Biomimetic hydroxyapatite powder (BHAP) was synthesized using calcium nitrate tetrahydrate [Ca(NO3)2.4H2O] and diammonium hydrogen phosphate with a 1.64 ratio of Ca/P molar ratio and 2.10 g/cm^3 density. Details of the synthesis method were given in the previous study [16]. Hydroxyapatite discs (n = 70) were prepared by pressing BHAP under 350 MPA pressure in 10x2 mm special moulds for 1 minute at 1100°C. They were obtained by sintering with a heating speed for 4 hours. BHAP discs were cut into 5x5x2 mm dimensions under water cooling using a precision cutting device (Struers Manitom), and a total of 40 pieces were prepared.

Twenty surgically removed third molars were used in the *in vitro* study. The crowns were first separated from the roots using a diamond bur at the enamel-cement junction and then divided into buccal and lingual parts; thus, 40 tooth specimens were obtained. A total of 40 pieces of 10x10x2 mm enamel samples were embedded in acrylic under water-cooling.

All 200 samples were placed separately in tubes containing 2 ml of distilled water and stored in an incubator at 37°C for 24 hours.

For the *in situ* experiment, a total of 160 samples were redesigned from K1, K2, Ci, and BHAP, i.e., 40 of each material. All specimens were prepared in 5x5x2 mm dimensions in the same manner and stored as the *in vitro* study.

2.1. In Vitro Experiments

Ten discs of K1, K2, Ci, BHAP, and E were prepared as described above, and a 10-gr load was applied to each sample at five different indentation points, measured (HMV Microhardness Tester, Shimadzu), and averaged according to the Vickers surface microhardness test.

Surface roughness of the previously prepared discs of 10 BHAP, 10 E, as well as 10 polished and 10 unpolished samples of the 3 different fissure sealant materials was measured using a profilometer device (Dektak 6M Profilometer Veeco). Measurements were made with a 4-μm diamond stylus, 90° reading angle, and 0.80 mm cut-off length at five different locations of each sample surface. Average surface roughness was expressed by the mean roughness value (Ra) and root mean square roughness (Rq) value for each sample and recorded in μm.

Wettability of 5 different materials was measured using the CAM 200 (KSV NIMA) contact angle measurement instrument. Uniform drops of 5 μl distilled water were carefully released from a height of 10 mm on the surfaces of 5 different materials using Teflon syringes, and both right and left contact angles were spontaneously calculated with the software.

2.2. In Situ Experiments

The ethical approval of the *in situ* study and the extracted teeth of the *in vitro* study was obtained from the Clinical Research Ethics Committee of Yeditepe University (No: 2012/270) in accordance with the Declaration of Helsinki. Inclusion criteria consisted of 20 children who were aged between 7 and 12, had been referred to the Pediatric Dentistry Clinics of Marmara University for dental treatments, and had not used antibiotics in the last three months, had dft + DMFT more than 2, all decayed teeth

restored, and an indication for an upper removable space maintainer. In the intraoral examination, necessary information, including oral hygiene instructions, was given to all children and their parents/legal guardians, and then written consents were obtained.

2.2.1. Preparation of Removable Space Maintainers

The participating children were 8 girls and 12 boys, and the mean dft+DMFT index was 4.95 ± 1.76. Impressions from the volunteers' upper jaws were taken with alginate (Alginmax, Major, Italy), and plaster models were prepared. For each appliance, four wax moulds (Dentsply Pinnacle, Germany) of 7x7x3 mm diameter were prepared in the palatal areas of the appliances. After melting the wax boxes, K1, K2, CI, and BHAP samples were attached randomly 1 mm below the appliances' surfaces by using a light-cured temporary filling material (Clip, Voco, US) and polymerized for 20 seconds (Figure 1).

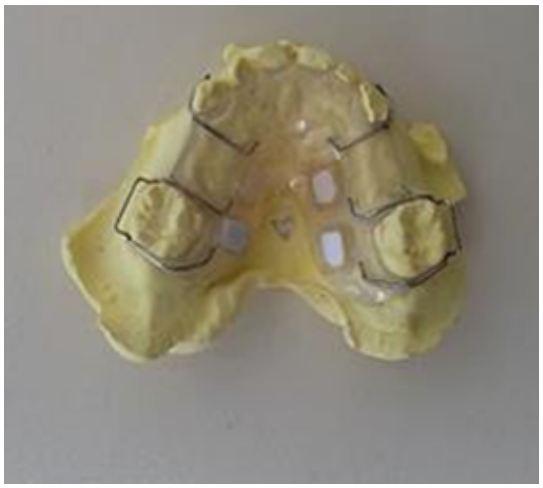

Figure 1. Removable space maintainer with 4 different specimens

2.2.2. Forming Biofilm in the Oral Environment

In the first week, the children used their space maintainers for one week to create biofilms on the specimens. Instructions were given to remove the appliances only while eating and to keep them in storage boxes filled with tap water to prevent biofilm dehydration during mealtime. The volunteers had their teeth brushed with toothpaste containing 1450 ppm fluoride (Sensodyne Pronamel for Children, GlaxoSmithKline, UK) twice a day and continued their regular diet throughout the experimental period. The space maintainers were collected from the volunteers at the end of the 7th

day. The specimens were removed from the appliances by pushing them from the clip edges with a hand piece.

In the second week, new materials were inserted onto the space maintainers, and beside the regular diet and dental hygiene, the children were instructed to wipe their teeth with a disposable product containing HAP crystals, xylitol, and 1450 ppm fluoride (Remin Pro, Voco, Germany) at night after brushing with the same 1450 ppm toothpaste for one week. There was good compliance among the volunteers, and all participants fulfilled the requirements and completed the 2-week experiments without any complications and/or withdrawal.

2.2.3. Preparation of Oral Biofilm for Scanning Electron Microscopy

In the end of both weeks, care was taken not to damage the biofilm layers, and immediately the specimens were removed and placed in code-numbered plastic tubes containing a freshly prepared 2.5% (0.1 M phosphate buffer (PBS), pH 7.4) glutaraldehyde solution. After fixing for 4 hours, they were kept in the refrigerator at 4 ºC for 12 hours. The samples were washed with buffer for 12 hours, then removed from the pad and kept in osmium for 1 hour. For the dehydration process, the samples underwent incremental alcohol solutions and were completed in a hexamethyldisilane solution for 5 minutes. The dehydrated samples were then dried and gold-plated (Sputter Coater 108, Creesington, UK) with 15 nm thick gold for 1 minute at a pressure of 5x10 millibars and an electrical voltage of 10 milliamps. *In situ* biofilm accumulations on four different test materials detached from removable space maintainers were processed and examined under Scanning Electron Microscopy (SEM), and photographs were taken at X50, X150, X500, and X2000 magnifications.

2.2.4. Evaluation of Oral Biofilm Photographs with Image Analysis Program

In our study, 2 different image analysis programs (Clemex Vision Lite Scientific Image Analysis for Microscopy, Canada, and Image J, USA) were used with the similar principle [17]. The processes and filters required for image analysis were defined in grey, and then the binarization process (black and white) was applied to the image after the filtering processes. X50 magnifications were used in this process, and biofilm accumulation area percentages were measured from photographs for each sample.

2.3. Statistical Analysis

The statistical analyses were performed using SPSS (Statistical Package for Social Sciences) for Windows 15.0 program. The Kruskal-Wallis test was used to compare the quantitative data that did not fit the normal distribution of the parameters. The relationship between parameters in the study was assessed with the Pearson correlation test. Results were evaluated at a 95% confidence interval and significance level of $p < 0.05$.

3. Results

3.1. In vitro assessments

Table 1 showed the results of overall *in vitro* assessments of 5 different materials: K1, K2, Ci, BHAP, and E.

3.1.1. Microhardness

The mean microhardness measurement was highest in enamel (272.6±9.90), followed by the BHAP material (225.75±3.02). The glass ionomer had lower (65.60±2.00) microhardness, and two resin sealants were the lowest, but they had similar microhardness (K1: 13.05±1.67; K2: 11.36±0.73). A statistically significant difference was observed between all materials. The null hypothesis of the distribution of microhardness, which was the same across categories of materials, was rejected ($p = 0.000$). There was no difference between the microhardness of K1 and K2, whereas a statistical difference between Ci and other materials ($p<0.05$) was found.

3.1.2. Roughness

In this study, we measured Absolute Surface Roughness (Ra) and Corrected Surface Roughness (Rq) of enamel and BHAP materials, with the polished and unpolished surfaces of the 3 sealant materials, using Profilometric Analysis, and compared them with artificial BHAP and natural enamel specimens. Table 1 showed the results obtained with statistical significance.

		K1	K2	Ci	BHAP	E	p
Microhardness		13.05±5.43	11.36±0.78	65.41±2.00	225.76±3.02	272.61±9.90	**0.000**
Contact Angle		64.86±5.43	67.79±6.06	94.20±11.55	64.17±9.47	62.05±9.40	**0.000**
Ra (μm)	**Unpolished**	0.72±0.16	0.61±0.10	0.90±0.22	0.48±0.06	0.35±0.09	**0.000**
	Polished	0.49±0.07	0.48±0.05	0.48±0.06			**0.000**
Rq (μm)	**Unpolished**	0.88±0.24	0.71±0.10	1.11±0.31	0.54±0.06	0.44±0.09	**0.000**
	Polished	0.58±0.04	0.60±0.05	0.54±0.06			**0.000**

Table 1. *In vitro* measurements of surface properties of materials.
(K1: Helioseal F; K2: Ultraseal XT Hydro; Ci: Fuji Triage Capsule, BHAP: pressed Biomimetic Hydroxyapatite; E: human enamel) (Ra: mean roughness; Rq: root mean square roughness)

Ra and Rq values of unpolished Ci material showed the highest measurements (0.90±0.22 and 1.11±0.31, respectively), followed by K1 (0.72±0.16 and 0.88±0.24), K2 (0.61±0.10 and 0.71±0.10), respectively. BHAP had lower roughness (0.48±0.06 and 0.54±0.06), and E had the lowest (0.35±0.09 and 0.44±0.09), respectively. A statistically significant difference was observed between all materials (p=0.000). Polishing the fissure sealant materials reduced the mean Ra and Rq values significantly, by 31.9% and 34.1% for K1; 21.3% and 15.5% for K2; 7.8% and 9.0% for Ci materials, respectively. In the study, a statistically significant difference was observed in Ra and Rq measurements of the unpolished and polished materials. The null hypothesis of the distribution of unpolished and polished materials that was the same across categories of materials was rejected (p=0.000).

3.1.3. Wettability

In contact angle measurements, the Ci (94.20±11.55) value was found to be statistically significantly higher than that of M (62.05±9.40), BHAP (64.17±9.47), K1 (64.86±5.43), and K2 (67.79±6.06). The null hypothesis of the distribution of wettability measurements that was the same across categories of materials was rejected (p=0.000). Ci had statistically higher contact angle measurements than the other materials (p<0.05).

3.1.4. Correlations between physical properties of the materials

Table 2 showed the correlations and significance observed between 5 different materials. Microhardness measurements revealed a negative correlation with all other parameters. The highest negative correlations were obtained with unpolished samples, whereas a decrease was seen after polishing the materials. Contact angle measurements were positively correlated with roughness measurements, and an increase was seen after polishing the materials.

		Ra unpolished	**Rq unpolished**	**Ra polished**	**Rq polished**	**Contact Angle**
Microhardness	**Pearson Correlation**	-,639**	-,606**	-,371**	-,400**	-,298*
	Sig. (1-tailed)	,000	,000	,004	,002	,018
Ra unpolished	**Pearson Correlation**		,949**	,808**	,806**	,579**
	Sig. (1-tailed)		,000	,000	,000	,000
Rq unpolished	**Pearson Correlation**			,730**	,839**	,596**
	Sig. (1-tailed)			,000	,000	,000
Ra polished	**Pearson Correlation**				,901**	,672**
	Sig. (1-tailed)				,000	,000
Rq polished	**Pearson Correlation**					,709**
	Sig. (1-tailed)					,000

Table 2. Correlations between the measurements of surface properties (Ra: mean roughness; Rq: root mean square roughness)

**. Correlation is significant at the 0.01 level (1-tailed).

*. Correlation is significant at the 0.05 level (1-tailed).

3.2. SEM Evaluation

3.2.1. Bare surfaces with biofilm

SEM examinations showed that 2 resin fissure sealant materials and BHAP had uniform surfaces, no cracks, and were similar in appearance. K1 and K2 materials were sparsely covered with microorganism groups (Figure 2a, b), and CI material had small groups of residues spread over a larger surface area (Figure 2c). BHAP (Figure 2d) showed the smoothest surface and the most negligible biofilm formation. In addition, Streptococci-like groups can be clearly observed on the materials under X500 and X2000 magnification (Figure 3a, b, c).

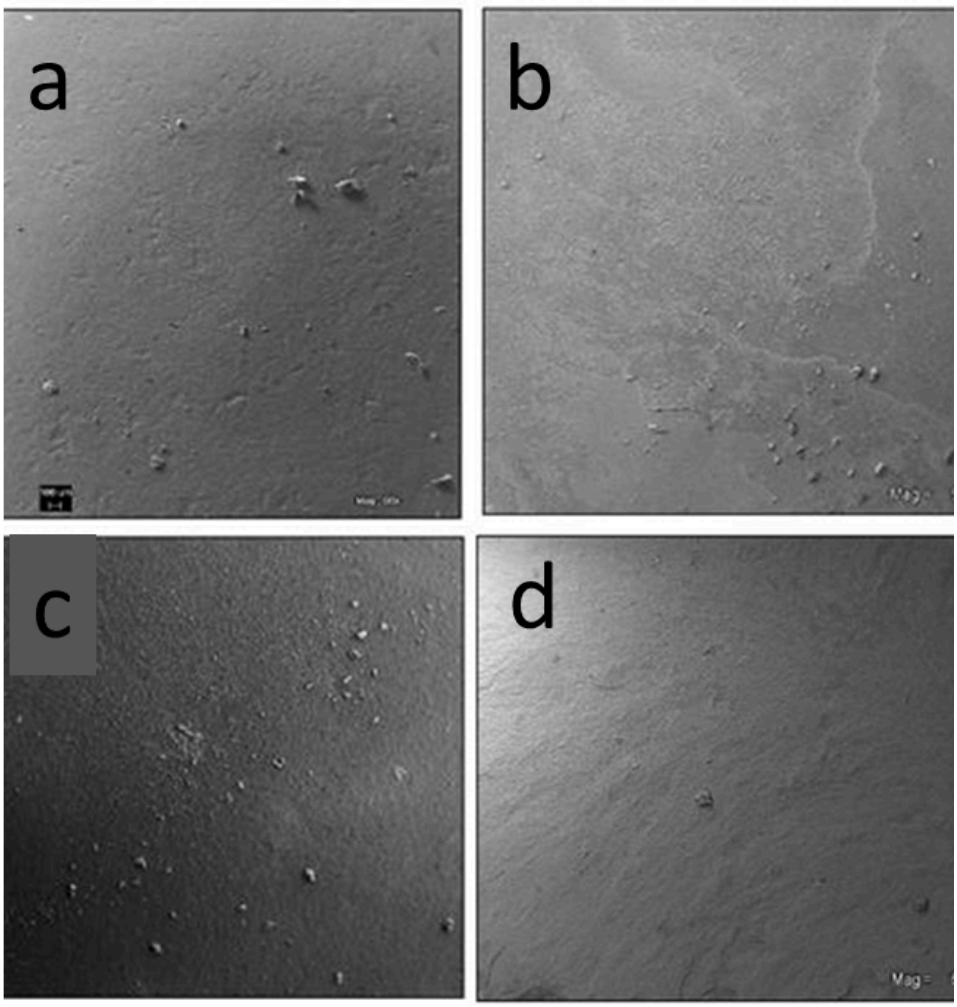

Figure 2. Materials with biofilm at X50 magnification a-Helioseal F, b-Ultraseal XT Hydro, c-Fuji Triage Capsule, d-pressed Biomimetic Hydroxyapatite

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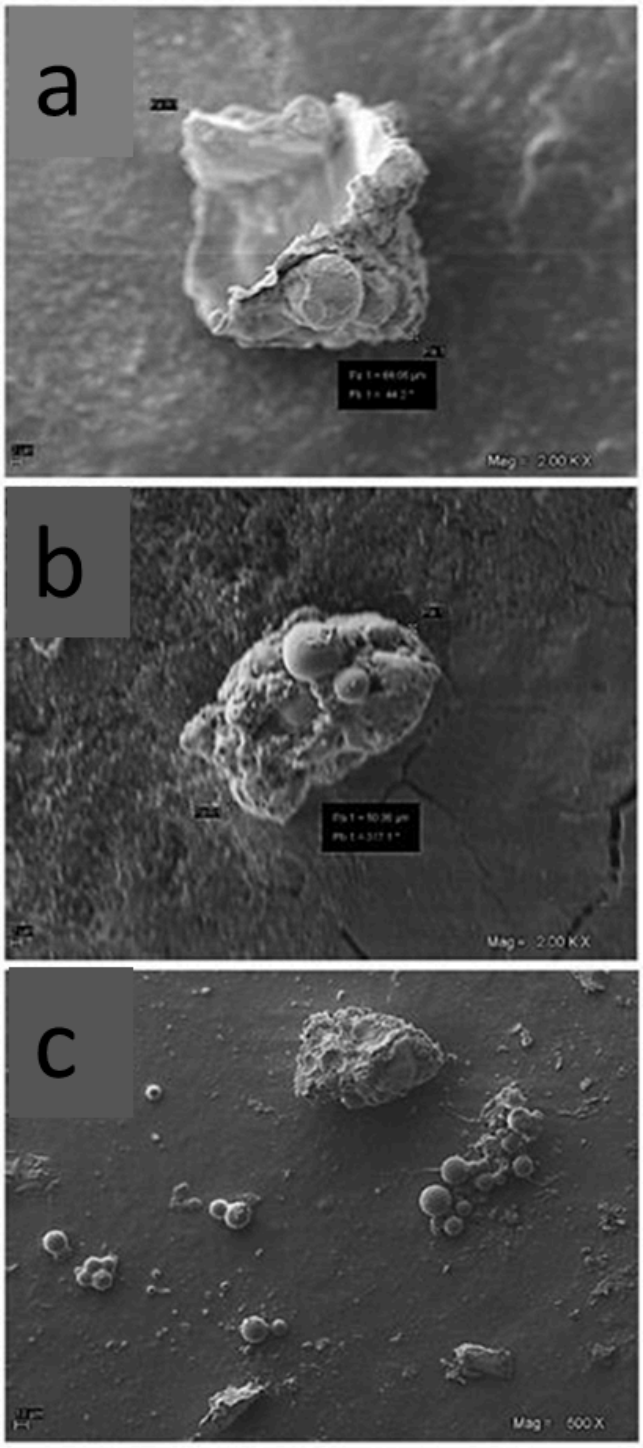

Figure 3. a-Helioseal F b-Fuji Triage Capsule X2000 Magnification; c- Ultraseal XT Hydro at X500 Magnification

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3.2.2. HAP Treated Surfaces with Biofilm

When SEM photographs of the biofilm under the biofilm-containing HAP were examined at 50X magnification, the irregularity of all material surfaces and the biofilm area on the surfaces were similar. A decrease in the biofilm-covered surface area due to the use of HAP was noticed in all samples, and microorganisms forming separable colonies rather than clustered groups were observed throughout the investigated surfaces (Figure 4).

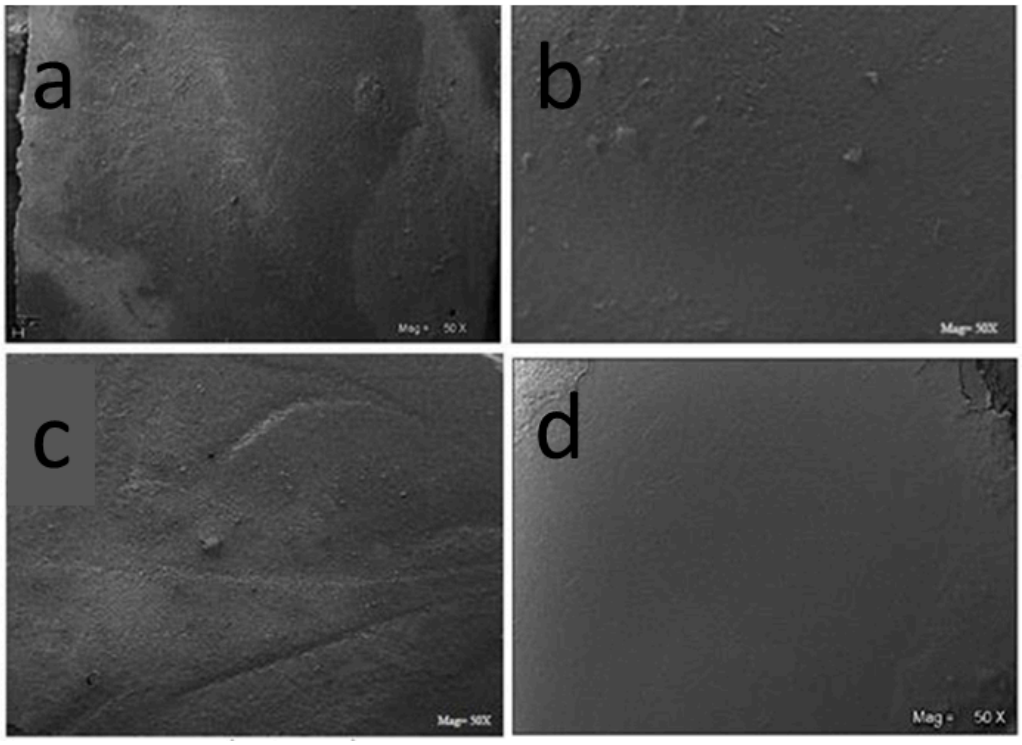

Figure 4. Materials with Remin Pro-treated surfaces with biofilm at X50 magnification a-Helioseal F, b-Ultraseal XT Hydro, c-Fuji Triage Capsule, d-pressed Biomimetic Hydroxyapatite

3.3. Examination of Image Analysis Programs

Two image analysis programs working with the same principle were used in our study. Table 3 showed that there was a positive correlation between the 2 analysis programs for biofilm calculation in the 1st week and in the 2nd week with HAP paste usage.

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biofilm		Clemex-HAP	ImageJ-HAP	Imagej
Clemex	Pearson Correlation	,770**	,907**	,932**
	Sig. (1-tailed)	,000	,000	,000
Clemex-HAP	Pearson Correlation		,738**	,769**
	Sig. (1-tailed)		,000	,000
ImageJ-HAP	Pearson Correlation			,942**
	Sig. (1-tailed)			,000

Table 3. Correlations between 2 different image analysis programs

**. Correlation is significant at the 0.01 level (1-tailed).

At the end of the first week, the highest *in situ* biofilm formation was seen on Ci, followed by equal accumulations on K1 and K2; then, the lowest was on BHAP. When HAP paste was applied in the second week, Clemex showed that the percentage reductions of biofilms were 65.3%, 62.1%, 54.8%, and 28.7% on K1, K2, BHAP, and Ci, respectively. ImageJ showed that the percentage reductions of biofilms were 65.3%, 62.1%, 54.8%, and 28.7% on K1, K2, BHAP, and Ci, respectively. (Figure 5) However, no statistically significant differences were observed in the percent biofilm accumulations of all materials either with or without HAP paste in both programs (Table 4) ($p > 0.05$).

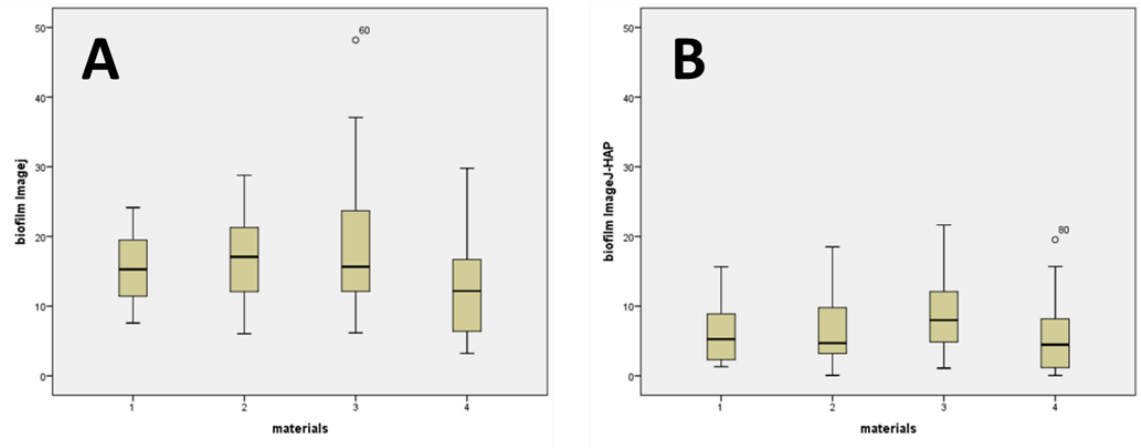

Figure 5. Box plot of percent biofilm formation calculated by the ImageJ program in *in situ* study. A: Bare surfaces with biofilm; B: Remin Pro-treated surfaces with biofilm 1: Helioseal F; 2: Ultraseal XT Hydro; 3: Fuji Triage Capsule, 4: Pressed Biomimetic Hydroxyapatite

		K1	K2	Ci	BHAP	p
Clemex	**Biofilm**	16.75±6.65	17.11±4.53	18.53±10.63	12.98±5.86	0.157
	HAP±Biofilm	5.82±4.88	7.01±5.53	9.25±8.18	5.86±3.56	0.139
Image J	**Biofilm**	15.73±5.12	16.91±6.34	18.34±10.46	12.63±7.27	0.552
	HAP±Biofilm	5.75±4.07	6.70±5.32	8.98±6.05	5.59±5.34	0.188

Table 4. *In situ* percentage biofilm formation on the surface of materials:
(K1: Helioseal F; K2: Ultraseal XT Hydro; Ci: Fuji Triage Capsule, BHAP: pressed Biomimetic Hydroxyapatite)

3.3.1. Comparisons of in vitro and in situ results

Enamel was not used in the *in situ* study with a concern to contaminate the participating children, and artificially prepared BHAP was considered as a substitute material for enamel. So, Table 5 showed the correlation between physical properties of 3 fissure sealants and BHAP materials calculated *in vitro* and biofilm formation on these materials *in situ*.

Even though both image processing programs did not differ in their results, when the children did not use HAP paste, biofilm accumulation measured by the Clemex Program was associated only with the microhardness values, which showed a weak negative correlation (r=-0.431; p=0.003). On the other hand, in the Image J program, biofilm formation correlated to the surface roughness of the unpolished materials positively (r=0.362; p=0.003) and to microhardness negatively (r=- -0.550; p=0.000). When the children used the HP paste, no correlation was seen in the Clemex program, but a positive correlation was observed between unpolished and polished samples, as well as contact angle measurements of the materials. The microhardness did not reach the significance level (Table 5).

Biofilm		**contact Angle**	**Micro hardness**	**Ra unpolished**	**Ra polished**	**Rq unpolished**	**Rq polished**
Clemex	**Pearson Correlation**	-,185	**-,431****	,103	-,092	,033	-,129
	Sig. (1-tailed)	,126	**,003**	,263	,287	,420	,214
Clemex ± HAP	**Pearson Correlation**	-,051	-,086	-,037	,021	-,121	-,010
	Sig. (1-tailed)	,376	,299	,411	,449	,228	,477
Image j	**Pearson Correlation**	,093	**-,550****	**,362***	,225	**,291***	,166
	Sig. (1-tailed)	,283	**,000**	**,011**	,081	**,034**	,153
Image J ± HAP	**Pearson Correlation**	**,326***	-,258	**,424****	**,479****	**,364***	**,396****
	Sig. (1-tailed)	**,020**	,054	**,003**	**,001**	**,010**	**,006**

Table 5. Correlations between *in vitro* vs *in situ* results.

**Correlation is significant at the 0.01 level (1-tailed)

*Correlation is significant at the 0.05 level (1-tailed)

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4. Discussion

4.1. In Vitro Experiment

Since this study involved an *in situ* experiment in children, our concerns about the contamination risks of natural enamel pieces in children's mouths and embedding them in the palatal appliances led to the search for alternative materials. In this study, we used BHAP as a replacement for enamel. So first, we compared the *in vitro* properties of human enamel specimens with BHAP disks manufactured in our laboratory.

Tooth enamel is approximately 96% inorganic matter, and 4% organic structure and water. The Ca/P ratio of enamel is 1.64. Calcium phosphates in the hard tissues of bones and teeth are found in the form of calcium-poor or carbonated hydroxyapatite, which has a chemical formula and composition whose physical and mechanical properties differ from synthetic hydroxyapatites [18]. For medical applications, HAP powder synthesized by biomimetic techniques with 30-40 nm particle size and a 1.64 Ca/P ratio is commonly preferred for biomedical applications [19][20]. In this present study, disc-shaped HAP powder was produced using the biomimetic method. As seen in Table 1, BHAP had comparable values in microhardness, roughness, and wettability measurements to natural human enamel. It has been shown that the crystal properties of blocks obtained from HAP powder produced by the biomimetic method have the same properties in terms of chemical composition and morphology as the enamel HAP crystals [21].

The physical properties of dental products, which include surface topography, roughness, hardness, as well as surface free energy, wettability, and ion charge, may have a significant impact on the biofilm development over the dental material. This can be realized through physical, chemical, mechanical, and biological connections. [7][22] Here, we examined the microhardness, wettability as expressed by the contact angle, and the surface roughness of the 3 fissure sealants. Ci was the hardest of all sealant materials, and its surface showed more irregularities and less hydrophilicity than the resin sealants. So clearly, its physical properties differed from those of the resin sealants, as mentioned in other studies [3][23][24][25] The difference in this glass ionomer material seems to be due to the size of the particles in its structure as well as extra content to ameliorate sealant retention and fluoride release. Adding fluoride appeared to have no influence on the microhardness of the resin materials, as seen in other studies [9][26].

To provide an aesthetic appearance, a bright, smooth, and stable surface is essential for dental materials. This also averts the formation of pigmentation from food and microorganism retentions, which diminish the clinical success of the dental material. Surface properties also affect the fracture resistance of brittle materials such as resin composites [27][28]. In a study comparing the surface roughness values of eight different resin-based and non-resin-based restorative materials, it was concluded that the resin-based restorative materials had a statistically significant smoother surface compared to non-resin-based materials [29]. Our microhardness and roughness results for fissure sealants and enamel were compatible with previous reports. [25][30][31][32] and the surface roughness of resin composites is found to be reduced by polishing but not to the extent of enamel [33][34][35][36] Our results showed that polishing the glass ionomer sealant reduced Ra values less than the resin composites, also in accordance with the dental literature [11][32]. Bürger et al. examined the roughness of the fissure sealants before and after thermocycling and found no change for glass ionomer but higher values in resin sealants after the aging procedures, especially after thermocycling [30]. On the other hand, we have to keep in mind that polishing the surfaces of the fissure sealants is not a routine procedure.

Hydrophobicity has been described as one with a contact angle of > 90° with water or other liquids, while other cut-off points (e.g., 65°) have also been suggested [37]. In our study, the average contact angles were determined to be equally hydrophilic for K1, K2, BHAP, and E at 64.86°, 67.79°, 64.17°, and 62.05°, respectively [3][38][39][40]. Ci, on the other hand, had a higher value of 94.2°, which can be considered hydrophobic [32][41]. The surfaces of the materials can have different surface tensions, and this surface tension can be affected by surface roughness. Depending on the surface tension, the materials have different wettability characteristics. Sure enough, we calculated a positive correlation between the roughness and the contact angle values, and the correlation improved as the polishing procedure was applied to dental materials [42][43].

4.2. In Situ Experiment

In vitro studies reported that the crystal structures of the synthetic HAP crystals were similar to the enamel HAP crystals and that in the presence of bacteria, surface properties of HAP crystals were an alternative to tooth enamel and could be easily operated. [19][44]. Elliot et al [18] focused on the biofilm development on the HAP-coated glass surface and the plain glass surface and reported that there was

no difference between the two surfaces. Xiao et al [45] preferred HAP discs covered with saliva. They investigated the 3-dimensional structure of the oral biofilm and the virulence of the strains in the biofilm. To calculate the biofilm-covered surface of BHAP, we used 2 different freely available pieces of software, and both programs indicated an area of only 13% on the surface, which was the least value on SEM images.

In vitro studies offer standardized bacterial colonies and/or conditions, but they hardly mimic natural environments. Their standardization may be better solved by isolating the respective strains from patients' saliva. In *in situ* situations, volunteers wear intra-oral splints, removable appliances to expose the specimens of test materials to the oral environment for short periods of time [23][24][46]. We believe that *in vivo* biofilms differ significantly from those formed *in vitro* [8] Auschill et al [47] found *in situ* from 3 volunteers that more dead microflora got from a glass ionomer than from a resin composite, and a study demonstrated *in vitro* models to have negligible clinical relevance in predicting the *in vivo* effect [48].

Nevertheless, dental literature showed that *in vitro* studies are preferred in most cases to elaborate on the interaction between biofilms and dental products. They focused on single aspects of materials, microorganisms, or other factors influencing biofilm formation and/or on the biological activity of these biofilms. [22][23][25][31][32][33][34][35][40][41][43] While surface properties like wettability or surface free energy influence biofilm formation to a certain extent, the most relevant surface properties are material roughness followed by surface chemistry [38]. In this *in situ* study, when we compared *in vitro* results, we could not find any correlation in bare surfaces of the materials (i.e., no HAP paste application). Studies showed that the roughness above an Ra threshold of 0.2 μm facilitated microbial adhesion [34][49] and Ra can be considered as an appropriate roughness parameter to predict initial bacterial accumulation on dental biomaterial surfaces *in vitro* and *in situ* [49][50]. In this study, the least biofilm formation was detected on BHAP with the least Ra and Rq values *in vitro*. The sealant materials revealed *in vitro* Ra values above the 0.2 μm, and indeed, we found an average positive correlation between the Ra values and the percentage area of biofilm formation in the *in situ* study, especially with the Image J program (Table 5).

It is obvious that the softer the surface becomes, the more biofilm formation will occur. A negative correlation was seen between the microhardness and biofilm formation on the bare specimens (i.e., no HAP paste application) in the present study. Barbosa et al [24] investigated the microhardness of

dental materials using *in situ* methodology and showed the softening of the Knoop microhardness of aesthetic restorative materials from the oral environment and biofilm formation. A similar phenomenon, followed by wear of the surface, could be seen in fissure sealant materials and needs to be explored *in situ* further. In an integrated review, Faria et al [51] revealed a negative effect of acid challenge on the biofilm on the wear of the fissure sealants. While the content, size, and type of fillers in the product influenced their resistance, the corrosive impact of an acidic environment was higher on glass ionomer sealants when compared to resin composite sealants.

4.3. Hydroxyapatite (HAP) Paste Treatment

The structural resemblance of the HAP nanocrystals to ground enamel crystallites may allow HAP particles to reduce bacterial adherence onto the surfaces via interaction with bacterial adhesins and reduction in biofilm formation [52]. Since we observed up to 65% reduction of biofilm formation on the BHAP, 62 to 54% on the resins, and 32% on the glass ionomer fissure sealant specimens, it seemed to be the case in our *in situ* study. The reduction pattern also seemed to confirm this phenomenon. Nel et al [53] introduced a list of the main bio-physicochemical influences on the interface between nanomaterials, including the size, shape, surface charge, roughness, porosity, and hydrophobicity of the materials. Here, even though there were no differences in the percentage of biofilm distribution between the surfaces of the 4 materials, we observed that biofilm establishment occurred according to divergent properties of the different materials. In the *in situ* part of our study, the volunteers used the protective HAP paste not directly on the dental materials; that is why we expected to get an indirect effect on biofilm formation. Luo et al [54] did not find a significant effect of nano-HAP suspension on the composition of multi-species biofilms in vitro but detected an inhibition of the metabolism and acid production of oral bacteria. Wear, microleakage, and partial loss of fissure sealants were considered as important disadvantages for microbial adhesion and caries formation [14][51], giving us the impression that the main outcome of this study was that using an HAP paste after sealant application may reduce the adhesion of biofilm to its surface. A recent review of the dental applications of systems based on HAP nanoparticles concluded that they can be used as a reinforcing material to increase the quality of available dental materials [55].

Limitations of this *in vitro* study obviously included excluding numerous other surface parameters like ion contents, surface topography, surface free energy, etc.; we also did not conduct thermocycling, wearing, degrading, or aging of the materials, which will influence the surface characteristics. Each

parameter would add more time and energy to the study. The adhesion of biofilm to the dental materials is a complex procedure and is dependent not only on materials but also on the oral environment. *In situ* trials cannot control the metabolism, thickness, and/or the composition of the biofilm and oral fluids like saliva. Our volunteers performed well, and we urged them to wear the space maintainers as much as possible, but we cannot guarantee that they did a good job. Having a biofilm on the surfaces of fissure sealants does not necessarily mean a cariogenic and/or erosive potential. Finally, recent studies use sophisticated methods of fluorescence microscopic visualization and quantification of biofilms, and deep sequencing of biofilm microbiomes with uncertain clinical significance.

5. Conclusion

In this 2-part study, first, microhardness measurements showed negative correlations with the roughness and contact angle parameters of fissure sealants, BHAP, and enamel specimens. In the *in situ* part, while the bare surfaces of 3 fissure sealant and BHAP samples deposited similar biofilm areas, when HAP paste was applied to the oral environment, the reductions of biofilm formation were highest on resin sealants, followed by BHAP, and the least on glass ionomer. When considering both parts together, this study provided some insights into biofilm adhesion, which is a complex process controlled by the interplay between the physical, chemical, mechanical, and topographical surface properties of the dental sealants.

Declaration of interest

The authors declare no potential competing financial or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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