Original Article

DOI: 10.14235/bas.galenos.2023.41103
Bezmialem Science 2023;11(4):371-379

In Vitro Investigation of the Effect of Different Mouthwashes Applied to Restorative Dental Materials on Adhesion of *Streptococcus mutans*

Restoratif Dental Materyallere Uygulanan Farklı Ağız Gargaralarının *Streptokokus mutans* Adezyonunda Etkisinin *İn Vitro* Olarak İncelenmesi

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ABSTRACT

Objective: This study aims to evaluate the effect of chlorhexidine gluconate (CHX), Listerine, and boric acid (BA), applied to three different restorative dental materials, on *Streptococcus mutans* (*S. mutans*) adhesion.

Methods: A total of 120 samples were prepared in the study: Composite (Group CR; n=40), glass ionomer cement (Group GIC; n=40) and compomer (Group C; n=40). The upper and lower surface roughness of the samples were measured. After bacterial adhesions, each group was separated into four subgroups (n=10). Three mouthwash and distilled water (DS) were applied for one minute. Subsequently, the remaining *S. mutans* biofilms were examined by colony forming unit count (CFU) and MTT methods. Data were evaluated (p<0.05).

Results: The GIC was the highest, and CR was the lowest in terms of surface roughness. There was a difference among three groups (p<0.001). Log CFU efficiency of bacterial counts of all 3 mouthwashes was higher than distilled water (p<0.001). The effect of all three mouthwash and DW on *S. mutans* log cfu and MTT

ÖZ

Amaç: Bu çalışma, üç farklı restoratif dental materyale uygulanan klorheksidin glukonat (CHX), Listerin ve borik asidin (BA) *Streptococcus mutans* (*S. mutans*) adezyonu üzerindeki etkisini değerlendirmeyi amaçlamaktadır.

Yöntemler: Çalışmada toplam 120 adet numune hazırlandı: Kompozit reçine (Grup CR; n=40), cam iyonomer siman (Grup GIC; n=40) ve kompomer (Grup C; n=40). Numunelerin alt ve üst yüzey pürüzlülükleri ölçüldü. Bakteriyel adezyonlardan sonra her grup dört alt gruba ayrıldı (n=10). Bir dakika boyunca üç gargara ve distile su uygulandı. Ardından, kalan *S. mutans* biyofilmleri koloni oluşturan birim sayısı (CFU) ve MTT yöntemleriyle incelendi. Veriler istatistiksel olarak değerlendirildi (p<0,05).

Bulgular: Yüzey pürüzlülüğü açısından GIC en yüksek, CR en düşüktü. Üç grup arasında fark vardı (p<0,001). Üç gargaranın tümünün bakteri sayımlarının Log CFU etkinliği, distile sudan daha yüksekti (p<0,001). Üç gargara ve distile suyun *S. mutans* log CFU ve MTT değerleri üzerindeki etkisi gruplar arasında farklılık gösterdi (p<0,001). CHX en etkili olanıydı. Restoratif materyal-

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Received: 11.03.2023
Accepted: 16.07.2023

Cite this article as: Türkyılmaz SP, Dülger K, Durukan İ, Kılıç AO, Baltacıoğlu E, Hakkı S, Baran I, Uzun Ü. *In Vitro* Investigation of the Effect of Different Mouthwashes Applied to Restorative Dental Materials on Adhesion of *Streptococcus mutans*. Bezmialem Science 2023;11(4):371-379

ABSTRACT

values differed between the groups (p<0.001). CHX was the most effective. In terms of restorative material-mouthwash interactions, differences were found between the groups (p<0.05). There was a positive moderate statistically significant correlation between log cfu and MTT values (r=0.636; p<0.001).

Conclusion: BA can be an alternative to other mouthwashes due to its natural structure and the minimal side effects.

Keywords: Bacterial adhesion, restorative dental material, restorative dental treatment, colony forming unit

ÖZ

gargara etkileşimleri açısından gruplar arasında fark bulundu (p<0,05). Log CFU ve MTT değerleri arasında pozitif orta derecede istatistiksel olarak anlamlı bir korelasyon vardı (r=0,636; p<0,001).

Sonuç: BA, doğal yapısı ve yan etkilerinin minimal olması nedeniyle diğer gargaralara alternatif olabilir.

Anahtar Sözcükler: Bakteriyel adezyon, restoratif dental materyal, restoratif dental tedavi, koloni oluşturan birim

Introduction

The aim of restorative dentistry is to regain the natural tooth appearance after correct diagnosis and complete treatment. Many different dental materials have been used to restore teeth (1). These materials have different therapeutic effects (2). In addition, their application in the mouth is closely related to oral hygiene and aesthetics (3). Although finishing and polishing processes remove plaque accumulation and smoothen the surface of the tooth, the adhesion of bacteria cannot be prevented. Dental materials may create a suitable environment for oral microorganisms to adhere to (4).

The bacterial colonization of tissues is one of the most important etiological factors for dental caries, gingivitis, and periodontal diseases. *S. mutans* is the primary microorganism responsible for the formation of dental caries. Therefore, reducing the number of these bacteria in the mouth will greatly contribute to preventing dental caries (5,6).

Brushing and flossing are important for oral hygiene. However, these protective measures are not sufficient to completely destroy bacterial plaque (7). In dentistry, the preventive-therapeutic properties of antimicrobial mouthwashes have been used for many years (8). Therefore, dentists recommend antimicrobial mouthwashes to prevent dental caries and periodontal diseases (9). The level of antimicrobial activity of mouthwashes used today is still not clear.

Chlorhexidine gluconate (CHX) is recognized as the gold standart antimicrobial mouthwash however it has various disadvantages such as pigmentation, taste changes, increased supragingival plaque, and mucosal desquamation (10). Thus it is important to determine alternative mouthwashes compared to CHX in terms of *S. mutans* adhesion.

The aim of this study is to evaluate the effectiveness of various mouthwashes applied to composite resin (CR), compomer (C), and glass ionomer cement (GIC) materials, which are frequently used against *S. mutans*, the primary microorganism responsible for dental caries in the bacterial plaque structure. As a result, comparing the effectiveness of different mouthwashes on different dental materials in reducing *S. mutans* adhesion and colonization and inhibiting plaque metabolism will also be useful in determining the best mouthwash that dentists can recommend to those at high risk of dental caries.

Methods

Specimen Preparation

In this study, three different dental restorative materials were used: GIC (Ketac Molar Easy Mix, 3M Espe, Germany), CR (Tokuyama Estelite® Asteria natural enamel composite, Japan), and C (Dyract® eXtra Compomer, Dentsply, Germany). The properties of the restorative materials and mouthwashes are shown in Table 1.

Using a Teflon ring, 40 disk-shaped specimens (10 mm in diameter x2 mm thick) were prepared from each material. Each material was inserted into the Teflon ring and pressed between the Mylar strips and glass slides to extrude excess material and to produce a smooth surface. The manufacturer's instructions were followed in preparing 120 samples of restorative materials. Specimens were prepared by the same operator (SPT) to eliminate operator-dependent variables. CR and C specimens were polymerized from each surface with a light-curing unit (Elipar S10, 1,200 mW/cm^2, 3M ESPE, St. Paul, MN, USA) in accordance with the instructions for use. The prepared samples were kept at 37 ºC and 100% humidity for 24 hours. Each sample was polished for two minutes using polishing discs (Sof-Lex, 3M ESPE, St. Paul, MN, USA). New polishing discs were used for each sample. The polished samples were subjected to ultrasonic cleaning in DW for 15 minutes.

Surface Roughness Measurement

The surface roughness of the specimens was measured using a profilometer (Marsurf PS 10, Mahr Gmbh, Germany) with a tracing length of 5.6 mm and a cut-off value of 0.8 mm. The profilometer device was recalibrated after the measurement of each sample. Three different measurements were made on the surface of each sample. The average surface roughness value was calculated by averaging the obtained data.

Investigation of Surface Morphology with Scanning Electron Microscope (SEM)

One sample from each group was randomly selected for surface examination. The surfaces of the samples were coated with gold

Table 1. Characteristics of the restorative materials and mouthwashes used in the study

Brand	Type	Chemical composition	Manufacturer
Estelite asteria (NE)	Supra nano-spherical composite Lot W614B	Matrix: Bis-GMA, Bis-MPEPP, TEGDMA, UDMAFiller: Uniform supra-nano spherical silica and zirconia fillers (200 nm). 82 wt %, 71 vol %	Tokuyama Dental, Tokyo, Japan
Dyract XP compomer	Polyacid modified glass ionomer (Compomer) Lot 1910000402	UDMA, TCB resin, TEGDMA, trimethacrylate resin (TMPTMA), dimethacrylate resin, ethyl-4 (dimethylalumino) benzate, BHT, strontium-alumino-sodium-fluoro-phosphorus-silicate glass, strontium fluoride, silicon dioxide, camphorquinone, UV stabilizer	DENTSPLY DeTrey GmbH Konstanz, Germany
Ketac molar easy mix	Glass Ionomer Cement Lot 6383514	Powder: glass fluoro-alumino-silicate, strontium, lanthanum, pigments Liquid: polycarboxylic acid , tartaric acid, water	3M/ESPE GmbH, Seefeld, Germany
Listerin total care	Esansial oils based mouthrinse	Water, ethanol, menthol, eucalyptol, thymol, methyl salicylate, benzoic acid, poloxamer 407, fluoride, zinc chloride, and flavor	Johnson & Johnson USA
Klorhex	Chlorhexidine gluconate based mouthrinse	0.2% chlorhexidine gluconate, glycerin, lemon and peppermint extract esansı	DROGSAN Ankara, Turkey
Boric acid	Boron containing mouthrinse	0.75% boric acid	-

using a coating device (SPI Module Sputter Coater, SPI Supplies, USA). Surface images were recorded using a scanning electron microscope (SEM) (EVO LS10, Zeiss, Oberkochen, Germany) (Figure 1).

Preparation of Artificial Saliva

Dental restorative specimens were sterilized in an autoclave at 121 ºC for 15 minutes before applying bacterial adhesion. Synthetic saliva was applied to sterilized restorative specimens prior to bacterial adhesion to form the pellicle layer. For synthetic saliva, 128 mg NaCl, 16.7 mg $CaCl_2$, 12.5 mg $MgCl_2$ ($6H_2O$), 9.5 mg KCl, 150.75 mg CH_3COOK, 38.6 mg $K3PO_4$ ($3H_2O$) were prepared in one liter of DW, and its pH was adjusted to 7 (11).

Application of Saliva to Samples and Ensuring Bacterial Adhesion

For the bacterial adhesion, an *S. mutans* ATCC 25175 strain obtained from Karadeniz Technical University, Faculty of Medicine, Department of Medical Microbiology was grown in Tryptic Soy Agar (TSA) medium at 37 ºC in 5% CO_2 for 48 hours (12). A single colony was taken from the agar medium and inoculated in a test tube containing 10 mL of TSB medium, and a liquid culture was prepared with a 24-hour incubation. The sterilized samples were placed in 24-well plates after their surface roughness was measured, and 1 mL of the prepared synthetic saliva was added to them and kept at room temperature for 1 hour to form a pellicle. After each sample was washed twice with phosphate buffer and freed from artificial saliva, 1 mL of the liquid bacterial suspension with optical density (OD) 600≅0.5 ($1.5x10^8$ CFU/mL) was added, and the cultures were incubated for 24 hours (11).

The pH Measurements of Mouthwashes

The pH values of the mouthwashes used in the study were measured using a pre-calibrated pH meter (Hanna edge®, USA). For BA, 0.75% concentration was selected according to our previous study (13).

***S. mutans* Adhesion Analysis**

At the end of the incubation period, the samples of which bacterial adhesion was complete were removed from the previous plates and placed in new 24-well plates. One milliliter of the mouthwashes used in the study (Table 1) was added to the samples and left for one minute. After removing the mouthwashes, each sample was transferred to Falcon tubes containing 3 mL of TSB and 0.5 mm glass beads (Sigma-Glass Beads, Germany). The solution in the tubes was vortexed at 1,200 revolutions per minute (rpm) for 5 minutes in a vortex device (Isolab, Laborgerate GmbH, Germany), allowing bacteria to pass into the medium. From the suspension containing the bacteria, dilutions with TSB from 10^{-1} to 10^{-5} were prepared. Cultures were incubated at 37 ºC for 48 hours by seeding 0.1 mL of smear on TSA agar plates from the prepared dilutions. The formed colonies were counted, and the number of colonies per milliliter was determined as a CFU.

Bacteria Viability Analysis

The MTT is a method of expressing cell survival and growth. This method is based on the measurement of metabolically active bacteria metabolizing the yellow tetrazolium salt of

3-(4.5-dimethyl-thiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) and forming purple formazan molecules inside the cells (14). Ninety µL of the bacterial suspension released in TSB from the vortexed samples were taken and transferred to 96-well ELISA plates. After adding 10 µL of MTT solution (5 mg/mL MTT in Phosphate Buffered Saline (PBS) and 10 µL of glucose (20%) (15), it was incubated for four hours at 37 ºC in an oven containing 5% CO_2. To dissolve the formazan crystals, 110 µL of dimethyl sulfoxide was added to the bacterial

suspension and incubated for 60 minutes, stirring at 60 rpm at room temperature. Absorbance values were measured at 595 nm with a microplate reader.

Imaging with a Confocal Laser Scanning Microscope (CLSM)

Five hundred µL of overnight liquid culture of *S. mutans* was transferred to microcentrifuge tubes, and the cells were centrifuged at 12,000 rpm for one minute. The precipitated bacteria were suspended in 200 µL of PBS. Cell suspensions prepared in three sets were treated with CHX (Klorhex, Drogsan, Ankara), essential oil-containing mouthwash (Listerine, Johnson & Johnson, USA), and BA for one minute. The mouthwash solutions were centrifuged after the cells had settled, and the treated cells were stained for 15 minutes under light protection using the LIVE/DEAD® BacLight™ Bacterial Viability and Enumeration Kit (Invitrogen, Carlsbad, California, USA). Stained bacteria were observed on a CLSM (Leica DMI8, Leica Microsystems, Germany) with 63x magnification optical lenses using wavelengths of 488 and 532 nm (Figure 2).

Statistical Analysis

Data were analyzed with IBM SPSS V23. The conformity to the normal distribution was evaluated using the Shapiro-Wilk test. Two-way analysis of variance method was used to examine group and solution main effects and interactions on roughness and Log CFU and MTT values. Bonferroni correction was used for multiple comparisons. The Spearman Rho correlation coefficient was used to analyze the relationship between variables. The results were presented as mean and standard deviation for quantitative data. The significance level was taken as $p<0.05$.

Results

Surface Roughness Values

Statistically significant differences were found between the surface roughness values of the materials (Table 2). The average of the surface roughness values of the CR group was the lowest (0.0853 µm), the average surface roughness values of the C group were moderate (0.1405 µm), and the average surface roughness values of the GIC group were the highest (0.4359 µm) ($p<0.001$).

The Examination of Surface Morphology by SEM

SEM images of 2 different magnifications of each of the 3 dental restorative materials are shown in Figure 1.

The pH Values of Mouthwashes

The pH values of the mouthwashes used in the study were measured as 5.8 for CHX, 4.3 for Listerine, and 4.8 for BA, respectively.

S. mutans Adhesion Analysis

In order to determine the adhesion of *S. mutans*, a liquid bacterial culture was added to the surfaces of the test samples. At the end of the 24-hour incubation period, mouthwashes were applied. The number of CFU of the bacteria remaining on the surface of the samples was expressed in CFU/mL. After the mouthwashes were

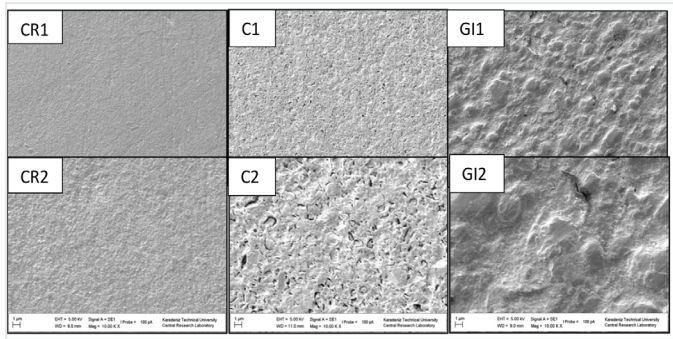

Figure 1. 1 and 2 are 3.00Kx and 10.00Kx magnifications of the SEM images of the groups, respectively

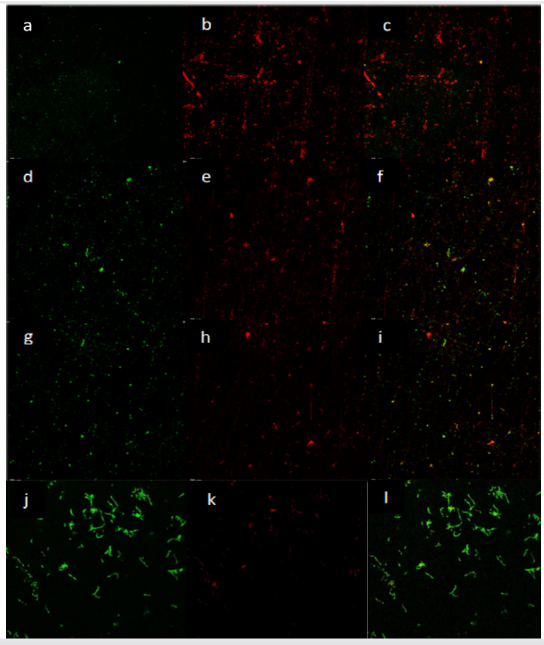

Figure 2. Confocal microscopic analysis of live and dead bacteria after mouthwash applications. (a) CLSM image of viable bacteria after application of CHX. (b) CLSM image of dead bacteria after application of CHX. (c) CLSM image of live and dead bacteria after application of CHX. (d) CLSM image of viable bacteria after application of Listerine. (e) CLSM image of dead bacteria after application of listerine. (f) CLSM image of live and dead bacteria after application of Listerine. (g) CLSM image of viable bacteria after application of BA. (h) CLSM image of dead bacteria after application of BA (i) CLSM image of live and dead bacteria after application of BA. (j) CLSM image of viable bacteria after application of distilled water. (k) CLSM image of dead bacteria after application of distilled water. (l) CLSM image of live and dead bacteria after application of distilled water

CLSM: Confocal laser scanning microscopy, CHX: Chlorhexidine gluconate, BA: Boric acid

Table 2. Comparison of average roughness values in terms of restorative materials

	Composite	Compomer	Glass ionomer cement
	0.0853±0.0203[a]	0.1405±0.0252[b]	0.4359±0.0740[c]

[a-c]There is no difference between groups with the same letter

applied to the restorative materials, the logarithmic calculation of the bacterial counts was made. The logarithmic mean values are shown in Figure 3.

Regardless of the materials, the effect of mouthwash types on CFU was statistically significant (p<0.001). CFU values were statistically significant (p<0.001) according to the restorative material and mouthwash interactions.

Regardless of the restorative material difference, the highest logarithmic value for bacterial adhesion was 6.04 for DW, while the lowest logarithmic value was 4.68 for CHX. The logarithmic values of 4.9 for Listerine and 5.04 for BA showed similar results (Table 3). No significant difference was found between the mean log CFU values obtained from the interactions of GIC-essential oil-containing mouthwash, CIC-CHX, and C-CHX. In particular, the average log CFU value (4.58) in the C-CHX interaction was found to be lower than the others. Other multiple comparison results are presented in Table 3.

No statistically significant correlation was found between the roughness values of the materials and log CFU (r=-0.153; p=0.095) (Figure 4). In the statistical analysis, the lowest surface roughness was on the surface of the CR group (0.0853 µm),

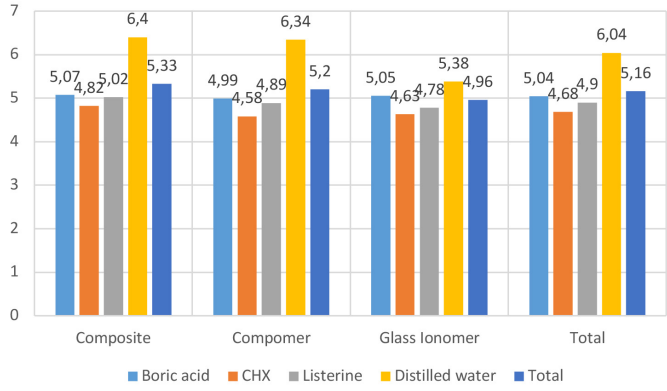

Figure 3. Logarithmic mean values of the number of *S. mutans* attached to the experimental groups

Table 3. CFU values by groups and moutwashes

	Composite	Compomer	Glass ionomer cement	Total
Chlorhexidine	4.82±0.20^{CDEF}	4.58±0.17^{F}	4.63±0.14^{EF}	4.68±0.20^{b}
Listerine	5.02±0.18^{CD}	4.89±0.19C^{DE}	4.78±0.11D^{EF}	4.90±0.19^{c}
Boric acid	5.07±0.21^{C}	4.99±0.22^{CD}	5.05±0.21^{C}	5.04±0.21^{a}
Distilled water	6.40±0.18^{A}	6.34±0.14^{A}	5.38±0.07^{B}	6.04±0.49^{d}
Total	5.33±0.66^{a}	5.20±0.71^{b}	4.96±0.32^{c}	5.16±0.60

$^{a-d}$There is no difference between material/mouthwash types in groups with the same letter, $^{A-F}$There is no difference between interactions between materials with the same letter and mouthwash type, CFU: Colony forming unit count

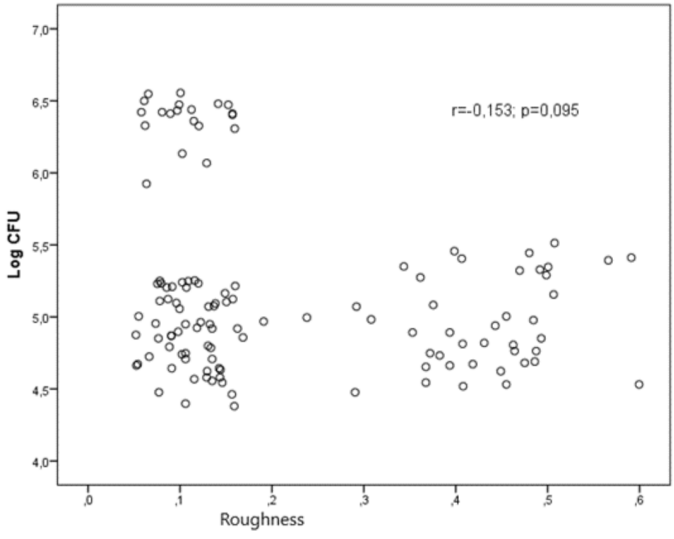

Figure 4. The surface roughness of materials and bacterial adhesions after mouthwashes application to the materials

and the average of *S. mutans* adhesion was determined as the highest. The highest surface roughness was in the GIC group (0.4359 µm), and the average amount of *S. mutans* adhesion was determined as the lowest. No statistically significant correlation was found between surface roughness and *S. mutans* adhesion (p>0.05).

Bacteria Viability Analysis

Regardless of the materials, the effect of the mouthwashes was found to have a statistically significant effect on the MTT values (p<0.001). The mean MTT of DW was 0.296, the mean of the CHX was 0.209, the mean of Listerine was 0.238, and the mean of BA was 0.254 (Table 4). A statistically significant impact was found on the MTT values of the group and mouthwash interaction (p=0.009). The highest average value was obtained in DW (0.353) of the CR group, while the lowest average value was obtained in the CHX (0.185) of the GIC group (Table 4).

Discussion

Adhesion of cariogenic bacteria and biofilm formation are among the most important causes of dental caries (16,17). Although many studies have been conducted on bacterial adhesion on various restorative dental materials (18-23), there is no study with similar experimental design in the literature investigating the effect of mouthwashes applied to these materials on *S. mutans* adhesion.

There is a known significant relationship between the surface roughness of restorative materials and bacterial adhesion (22,24). Bollen et al. (25) stated the critical surface roughness (Ra) value of dental materials for bacterial colonization as 0.2 µm. In another study, the area occupied by the adherent bacteria was found to strongly correlate with the surface roughness of 0.2-0.8 µm Ra (26). Park et al. (27) reported that streptococcal adhesion decreased at a Ra roughness value of approximately 0.15 µm. Considering the relationship between bacterial adhesion and surface roughness in the present study, the roughness values of CR and C were lower than the critical surface roughness value of 0.2 µm, while the roughness value of GIC was higher than this level (p<0.001). SEM images also confirm these findings (Figure 1). In studies dealing with the roughness of restorative dental materials, in parallel with the present study, GIC showed greater surface roughness (28,29). The CR used in this study, on the other hand, had a very low roughness since it had nanofill particles. In the present study, no statistically significant correlation was found between the roughness of the restorative materials and the adhesion of *S. mutans* (Figure 4). This may be due to the detection of *S. mutans* adhesion after applying mouthwashes on restorative dental materials.

The composition of dental restorative materials is another factor that affects the adhesion of *S. mutans* (30). In a study, *S. mutans* adhesion on dental restorative materials was investigated without mouthwash, and it was stated that fluoride release in the materials reduced *S. mutans* adhesion, especially in the early stages of biofilm formation (31). In another study (32), it was reported that conventional GIC inhibited the growth of streptococci with fluoride release. Bis-GMA monomer is often found in the structure of resin-based dental products (33), while TEGDMA monomer is used as a diluent (34). It was reported that the presence of Bis-GMA and TEGDMA products in CR and C materials increased the proliferation of *S. mutans* (35).

In this study, *S. mutans* adhesion (Log CFU =5.38) on the GIC treated with DW according to the colony count method was found to be statistically significantly less than CR (Log CFU =6.40) and C (Log CFU =6.34) materials applied with DW (Table 3). In the MTT method, a statistically different decrease was observed in terms of *S. mutans* adhesion in GIC treated with DW (Table 4). In the light of this information, it is thought that the presence of more fluoride in the structure of the GIC and the presence of Bis-GMA and TEGDMA in the structure of CR and C materials will affect the statistically low *S. mutans* adhesion on the GIC.

Pathogenic bacteria in the oral biofilm are the main factors of periodontal diseases and dental caries. Effective oral hygiene can be achieved by eliminating pathogenic bacteria using mouthwashes (36). In our study, three different mouthwashes were applied to eliminate *S. mutans*, which showed adhesion to dental materials used for restorative purposes. These three mouthwashes showed a statistically significant difference compared to the negative control group, which used DW. Since 0.2% CHX was frequently used in oral antiseptic treatment (37), this concentration of CHX was also preferred in the present study. There is no comparable study reporting the effectiveness of 0.2% concentration of CHX against *S. mutans*, which adheres to CR, C, and GIC in vitro. In clinical studies (38-41), on the other hand, CHX was shown to be the most effective in terms of reducing salivary *S. mutans* and plaque scores. In this study, CHX was the most effective in reducing *S. mutans* adhesion on all restorative materials analyzed by both methods (Table 3 and 4). This may be due to the bactericidal effect of CHX (42).

Table 4. MTT values by groups and mouthwashes

	Composite	Compomer	Glass ionomer cement	Total
Chlorhexidine	0.2382 ± 0.0448^{BC}	0.2045 ± 0.0102^{AC}	0.1854 ± 0.0216^{A}	0.2094 ± 0.0359^{b}
Listerine	0.2921 ± 0.0094^{E}	0.2171 ± 0.0268^{AB}	0.2057 ± 0.0095^{AC}	0.2383 ± 0.0424^{a}
Boric acid	0.3143 ± 0.0384^{E}	0.2276 ± 0.0206^{BC}	0.2206 ± 0.0135^{AB}	0.2542 ± 0.0503^{a}
Distilled water	0.3534 ± 0.0295^{F}	0.2817 ± 0.0267^{DE}	0.2536 ± 0.0319^{BD}	0.2962 ± 0.0513^{c}
Total	0.2995 ± 0.0305^{a}	0.2327 ± 0.0210^{b}	0.2163 ± 0.0191^{c}	0.2495 ± 0.0449

$^{a-c}$No difference between groups with the same letter, $^{A-F}$No statistically significant difference between interactions with the same letter

However, after long-term use of this mouthwash, side effects such as pigmentation, taste changes, increased supragingival plaque, and mucosal desquamation can be seen on teeth and restorations. Therefore, long-term use is not recommended (43). The orientation toward alternative substances with reduced side effects for biofilm control is an important measure.

This study evaluated the effectiveness of essential oil-containing Listerine and BA against *S. mutans* as an alternative to CHX. It has been reported that Listerine is highly effective against the biofilm formed by *S. mutans*. The reason for this is attributed to the antibacterial mint, thyme, and eucalyptus it contains (44). Fine et al. (45) concluded that daily use of essential oil-containing mouthwash would be beneficial in addition to mechanical oral hygiene since it significantly reduced the level of *S. mutans*. Bugno et al. (46) stated that individuals in the group treated with essential oil-containing mouthwashes had lower bacterial counts than those treated with saline solution, and the antimicrobial activity of mouthwash containing essential oil was better than that of 0.12% CHX. According to the data obtained in our study, although the effect of a mouthwash containing an essential oil was statistically significantly higher than DW, it was less than CHX. At the intersection of both methods, Listerine and CHX showed similar activity on GIC when compared. In this case, in individuals with high caries risk, after restoration using GIC, their treatment may be continued with Listerine due to the side effects of CHX. However, some side effects of Listerine are also stated. Zamora-Perez et al. (47) stated that after 30 days of using Listerine containing ethanol, there might be nuclear anomalies associated with DNA damage. It was stated that phenolic compounds in Listerine might cause cell damage to a certain extent. While oral bacteria convert ethanol to acetaldehyde, they also metabolize nicotine to nitrosamine in smokers. The absorption of these products may increase further with phenolic compounds and may have a carcinogenic effect (48).

Boric acid is used in many areas of dentistry. It has been suggested that BA may reduce some clinical measures such as bleeding on probing and alveolar bone loss in treatments performed within the scope of periodontology (49). While it was stated that 6% BA concentration for root canal disinfection was as effective as NaOCl against *Enterococcus faecalis* biofilms (50), it was emphasized that BA at 0.75% concentration could be used in addition to root surface smoothing in the treatment of chronic periodontitis (51). The concentration of BA used in this study was 0.75%. Boron regulates human hormonal metabolism, is an antioxidant, contributes to bone development, strengthens the immune system, accelerates wound healing, reduces the risk of cancer and weight gain, increases mental performance, and cures anemia (52). For these reasons, and due to the side effects of mouthwashes containing CHX and essential oil, BA can be recommended as a natural treatment alternative in cases where it has similar efficacy to these mouthwashes against *S. mutans* adhesion on restorative materials (Table 3 and 4). BA also has anti-inflammatory and antioxidant property (53).

Bacteria were evaluated in experimental samples by plate essay, OD measurement, or a cell cytometer. Plate essay is the most widely used among these methods (54). Although it is inexpensive and widely available, this technique has some disadvantages. These are the large number of Petri plates on which the smear is spread, the need for a large amount of laboratory space for the incubation period, the long incubation time required for colonies to grow, and the manual analysis of each Petri plate one by one (54). MTT, a tetrazolium salt, is a substance that is actively absorbed into cells and is reduced to colored, water-insoluble formazan by a mitochondrial-dependent reaction (14). The MTT-reducing property of cells is taken as a measure of cell viability. Also, the dye density obtained from MTT analysis correlates with the number of viable cells (14). The results found in both colony counting and MTT methods show a positive and moderate significant relationship, as is the case with the correlation graph (Figure 5). However, although the MTT test method is beneficial for the researchers in terms of time, the existing gaps in the knowledge of the application of MTT in bacterial protocols make both methods to be used together in the experiments.

There are some limitations of the present study. Since it was an *in vitro* study, the conditions in the mouth could not be

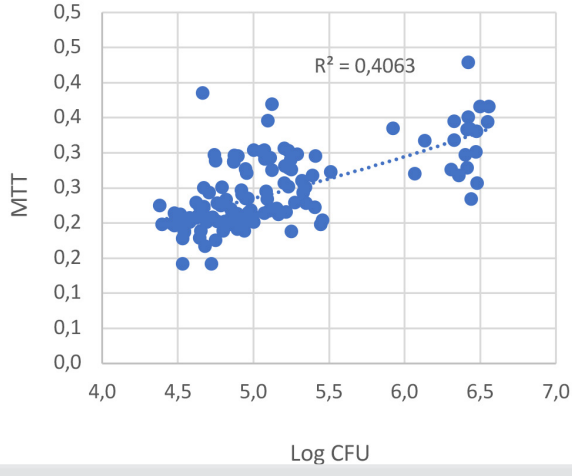

Figure 5. Scatter plot showing the relationship between logCFU and MTT

fully imitated. The effectiveness of mouthwashes on a single bacterium, *S. mutans*, was studied. Individual differences, such as salivary characteristics and nutritional habits, were not considered. In addition, the aging process was not applied to dental materials. The mouthwashes were applied only once. In the future, *in vitro* and *in vivo* studies with different experimental designs examining the effect of BA on other bacteria associated with dental caries are recommended.

Ethics

Ethics Committee Approval: It is an *in vitro* study and an ethics committee certificate is not required.

Informed Consent: *In vitro* study.

Peer-review: Externally peer reviewed.

Authorship Contributions

Consept: S.P.T., K.D., Design: S.P.T., K.D., İ.D., A.O.K., I.B., Data Collection or Processing: S.P.T., İ.D., A.O.K., Analysis or Interpretation: S.P.T., K.D., E.B., S.H., Ü.U., Literature search: S.P.T., Writing: S.P.T., K.D., A.O.K., E.B., S.H.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: This study was supported by Karadeniz Technical University Scientific Research Projects Unit (project number: TDH-2020-8650).

Acknowledgments: This study is based on a thesis. The authors thank to Karadeniz Technical University Scientific Research Projects Unit.

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