Streptococcus Mutans adhesion to dental restorative materials after polishing with various systems: A Confocal Microscopy study

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ABSTRACT

Bacterial adhesion and biofilm formation on restoration surfaces could lead to secondary caries and even inflammation of pulpal nerve. Finishing and polishing procedures are crucial to form resistance of materials to bacterial adhesion. The aim of the present study is to compare Streptococcus Mutans (*S. mutans*) adhesion on restorative materials polished with one- or multi-step systems. 2x5 mm disc-shaped samples were prepared from a resin-modified glass ionomer (RMGI), a compomer, a conventional flowable composite and two flowable bulk-fill composites. Specimens of each group were divided into two groups according to polishing systems (n = 9): One-step (OG) or Multi-step (SL) systems. Surface roughness values were examined by profilometry and one sample of each group were examined for bacterial on confocal laser scanning microscope (CLSM). *S. mutans* counts were calculated by broth cultivation. Results were analyzed with one-way ANOVA and Bonferroni/Dunn tests. Two flowable bulk-fill composites showed superior roughness values than the conventional flowable and RMGI. Specimens polished with OG system had no significant difference among bacterial counts ($p>0.05$). After polishing with SL system, Tetric Evo Bulk Flow showed significantly the lowest bacterial adhesion followed by the RMGI and the compomer. CLSM images were in consistent with microbiological culture. All tested materials had lower bacterial adhesion when polished with multi-step system. Multi-step systems should be used with flowable bulk-fill composites to have optimum results in terms of lowering bacterial adhesion and improving surface properties. CLSM images supplies accordance with broth culture of *S. mutans* thus, this method could be useful on detecting bacterial adhesion.

Keywords: Broth culture, confocal microscopy, flowable composites, Mutans adhesion, biomaterials.

Adhesión de Streptococcus Mutans a materiales de restauración dental después del pulido con varios sistemas: estudio de Microscopía Confocal

RESUMEN

La adhesión bacteriana y la formación de biopelículas en las superficies de restauración pueden provocar caries secundarias e incluso inflamación del nervio pulpar. Los procedimientos de acabado y pulido son cruciales para formar la resistencia de los materiales a la adhesión bacteriana. El objetivo del presente estudio es comparar la adhesión del Streptococcus Mutans (*S. mutans*) en materiales de restauración pulidos con sistemas de uno o varios pasos. Se prepararon muestras en forma de disco de 2x5 mm a partir de un ionómero de vidrio modificado con resina (RMGI), un compómero, un compuesto fluido convencional y dos compuestos de relleno masivo fluidos. Las muestras de cada grupo se dividieron en dos grupos según los sistemas de pulido (n = 9): sistemas de uno (OG) o de varios pasos (SL). Los valores de rugosidad de la superficie se examinaron mediante perfilometría y se examinó una muestra de cada grupo en busca de bacterias en un microscopio de barrido láser confocal (CLSM). Los recuentos de *S. mutans* se calcularon mediante cultivo en caldo. Los resultados se analizaron con ANOVA de una vía y pruebas de Bonferroni/Dunn. Dos compuestos de relleno masivo fluidos mostraron valores de rugosidad superiores a los fluidos convencionales y RMGI. Las muestras pulidas con el sistema OG no tuvieron diferencias significativas entre los recuentos bacterianos ($p>0.05$). Después de pulir con el sistema SL, Tetric Evo Bulk Flow mostró significativamente la adhesión bacteriana más baja seguida por el RMGI y el compómero. Las imágenes de CLSM estuvieron en consonancia con el cultivo microbiológico. Todos los materiales probados presentaron una menor adhesión bacteriana cuando se pulían con un sistema de varios pasos. Los sistemas de varios pasos deben usarse con compuestos de relleno masivo- fluidos para obtener resultados óptimos en términos de reducción de la adhesión bacteriana y mejora de las propiedades de la superficie. Las imágenes CLSM se suministran de acuerdo con el cultivo en caldo de *S. mutans*, por lo que este método podría ser útil para detectar la adhesión bacteriana.

Palabras claves: Cultivo en caldo, microscopia confocal, compuestos fluidos, adhesión de Mutans, biomateriales.

INTRODUCTION

In the dental market, there is a wide spectrum of restorative materials with different chemical formulas, all of which have various physical, mechanical and esthetic properties. In selecting the most suitable restorative material, it is crucial to understand the properties of the material in question, which can be enhanced by a number of different manufacturing techniques, such as finishing and polishing (f/p). In essence, these adjustments ensure the desired anatomical contour of the restorations and improve the final surface characteristics by smoothing out scratches and eliminating irregularities [1].

There are many different f/p systems for direct restorative materials, such as aluminum oxide discs for resin-based materials in the buccal/palatal or incisal areas [2]. Additively cone-, midi-, and I-shaped silicon carbide burs, brushes, and wheels with aluminum oxide pastes are preferable for clinical practice [3, 4]. These materials have certain advantages and generally exist in multi- or one-step systems.

The application of f/p systems directly affects the bacterial adhesion of the restorative material. Since oral pathogens thrive in protected areas where they can easily feed and reproduce [5], surface deteriorations are optimal areas for bacterial adhesion. Accordingly, polishing the material surface significantly inhibits initial adherence and subsequent colonization [6]. Moreover, polished surfaces result in reduced surface free energy (SFE), which is unfavorable to *Streptococcus mutans* (*S. mutans)*, a pioneering bacterium in the development of dental caries that prefers to adhere to surfaces with high SFE [7]. Bacterial adhesion is also connected to the formation of secondary caries, gingivitis in the area approximal to restorations, and pulpitis [8]. Therefore, to inhibit bacterial adhesion, it is important to improve the surface characteristics of restoratives.

Composite resins are often modified to improve mechanical properties and reduce polymerization shrinkage [9]. For instance, bulk-fill composites have recently been introduced, the main advantage of which is the ability to cure in 4-mm layers without increasing polymerization shrinkage or reducing the degree of conversion [10]. Moreover, flowable bulk-fill composites can be used at the top layer of cervical restorations. In addition, according to *in vitro* studies, the marginal sealing of dentin in restoring Class V cavities is better when bulk-fill flowable composites are used as an intermediate layer or as a restorative material compared with conventional resin composite [11, 12]. Although bulk-fill flowable composites have superior marginal sealing and self-adapting properties, they cannot achieve 100% monomer conversion, which negatively affects the final surface characteristics and prevention of bacterial adhesion [13].

Unfortunately, studies are currently lacking with respect to evaluating the bacterial adhesion of flowable bulk-fill composites. Accordingly, in this paper, the bacterial adhesion of flowable composites polished with one- or multi-step systems is examined using confocal laser scanning microscopy (CLSM). The samples include a resin-modified glass ionomer, a compomer, two bulk-fill composites, and a conventional flowable composite. The null hypothesis is twofold: (1) there will be no significant differences between the surface roughness values of the tested materials, and (2) there will be no significant differences between *S. Mutans* adhesion to the sample surfaces subjected to different f/p methods.

MATERIALS AND METHODS

Two flowable bulk-fill resin composites, one conventional flowable composite resin, one resin-modified glass ionomer, and one compomer were used as substrates. The specimens were polished with multi-step (Sof-Lex XT Finishing & Polishing Discs, 3M Espe, IL, USA) or one-step (One Gloss PS, Shofu Inc., Kypoto, Japan) polishing systems. Manufacturers, lot numbers, and main material compositions are given in Table 1.

Table 1. Compositions of the tested materials. *

Type of Material	Material	Brand	Lot number	Composition
Resin-modified glass ionomer (RMGI)	**Fuji II LC Capsule**	GC	1801191	Liquid: Polyacrylic acid, HEMA, proprietary ingredient, 2,2,4-trimethyl hexamethylene dicarbonate, TEGDMA Powder: fluoroalumino silicate glass
Compomer	**Dyract XP**	Dentsply Sirona	1609000333	TCB resin, UDMA, Strontium-fluoro-silicate glass, strontium fluoride, photoinitiator, stabilizers (0.8 µm, 47% wt, 50% vol. fillers)
Flowable bulk-fill composite	**Tetric Evo Bulk Flow**	Ivoclar Vivadent	W15989	BisGMA, UDMA, BisEMA, silicate glass, ytterbium trifluoride, barium aluminium, additives, initiators, stabilizers and pigments (550 nm (mean), 80% wt., 60% vol. fillers)
Flowable bulk-fill composite (Giomer)	**Beautifil-Bulk Flowable**	Shofu	071721	Bis-GMA, UDMA, Bis-MPEPP, TEGDMA, S-PRG based filler, fluoro-alumino-silicate glass, Reaction initiator, others (72.5% wt., 51% vol. fillers)
Flowable composite	**Ecu Sphere Flow**	DMG	788549	Bis-GMA, Glass filler, pigments, additives, catalysts, (0.02 – 1.5 µm, 77% wt., 57% vol. fillers)
Finishing & Polishing system (f/p)	**Sof-Lex**	3M ESPE	N940009	Al_2O_3 coated flexible discs Coarse: 100µm, Medium: 29 µm, Fine: 14 µm, Super-fine: 5 µm
Finishing & Polishing system (f/p)	**One Gloss**	Shofu	0217220	Matrix: Polyvinylsiloxane Abrasive: Al_2O_3, SiO_2

* TCB resin: A reaction product of butane tetracarboxylic acid and hydroxyethyl metarcrylate. Bis-GMA: bisphenol-A glycidyl dimethacrylate, HEMA: 2-hydroxyethyl methacrylate; UDMA: urethane dimethacrylate, TEGDMA: triethylene glycol dimethacrylate, Bis-MPEPP: Bisphenol A polyethoxy methacrylate, Bis-EMA: ethoxylated bisphenol-A-dimethacrylate, TEGDMA: triethyleneglycol dimethacrylate. wt%: weight percentage, vol%: volume percentage.

Specimens preparation.

A total of 90 specimens were prepared to measure the surface roughness and bacterial adhesion values (n = 9). One specimen from each group was selected randomly for the CLSM imaging of bacterial viability on the surfaces. The disc-shaped specimens were prepared by placing the uncured compomer, bulk-fill composites, and conventional flowable resin into custom-made metallic molds, with a diameter of 5 mm and a thickness of 2 mm. Thereafter, the materials were covered with glass over mylar strips and gently pressured to eliminate excess material. The finalized surfaces were then cured with Elipar DeepCure (3M ESPE, St. Paul MN, USA) by placing the tip of the light guide over the glass for 20 seconds. The standard mode of the light-curing unit had a power density of 1,200 mW/cm, which was verified by a light-emitting diode (LED) radiometer (Demetron LED Radiometer, Kerr Corporation, Middleton, WI, USA). After light polymerization, the specimens were carefully removed from the plate and kept in distilled water on a stove at a temperature of 37°C to complete the polymerization process. After 24 hours, the specimens were removed from the stove and dried with air spray. Then, the specimens were numbered with a waterproof pen on the side that was not subjected to light polymerization and divided into two subgroups according to f/p procedures, which are outlined below.

Multi-step finishing & polishing.

The first procedure was multi-step f/p, hereafter referred to as the SL method. Sof-Lex XT discs were mounted on a low-speed instrument and assorted in order from dark shades (coarse grit) to light shades (fine grit). The coarse-grit discs were used for finishing at 10,000 rpm for 15 seconds with light pressure, after which the samples were rinsed and dried with an air–water syringe for six seconds. The medium-grit discs were used for finishing at 10,000 rpm for 15 to 20 seconds with light pressure, after which the samples were rinsed and dried with an air–water syringe for six seconds. The fine- and superfine-grit discs were used for polishing at 30,000 rpm for 15 to 20 seconds with light pressure, after which the samples were rinsed and dried with an air–water syringe for six seconds.

One-step finishing & polishing.

The second procedure was one-step f/p, hereafter referred to as the OG method. The IC finisher and polisher was selected from the One Gloss PS set and mounted on a low-speed instrument. For the finishing procedures, the IC finisher and polisher was applied with a high pressure for 15 seconds at 10,000 rpm, after which the specimen surfaces were rinsed for 10 seconds. For the polishing procedures, IC finisher and polisher was applied with minimal pressure for 15 seconds at 10,000 rpm, after which the specimen surfaces were rinsed for 10 seconds. The f/p procedures were achieved in slight and intermittent pressure at one direction and by a single operator. The one-step discs were replaced after being used once; the multi-step discs were replaced after being used three times. The specimens were light cured for 20 seconds with the same LED unit used at the beginning of the experiment. The treatment and control groups were stored in distilled water at 37°C ± 1°C for an additional 24 hours to ensure complete polymerization of the sealing resin.

Surface roughness measurement.

Specimens were placed on a metallic plate to obtain the necessary measurements. The surface roughness test was performed with a profilometer (Surtonic 25, Taylor Hobson Ltd., Leicester, England), the cut-off value of which was 0.25 mm; the evaluation length was 1.25 mm. The stylus was moved at a crosshead speed of 0.25 mm/s to record the arithmetic roughness (Ra). Three successive measurements were recorded from a line in the middle of the specimens; the Ra values were then averaged. During the measurements, the tester was periodically calibrated. Before bacterial adhesion, the specimens were vibrated in an ultrasonic cleaner (Sonica, Soltec Srl, Milan, Italy) filled with 10% ethanol for three minutes to disinfect the surfaces.

Assessment of S. mutans adhesion.

Freeze-dried strains of *S. mutans* (ATCC 25175) were inoculated on brain–heart infusion (BHI) broth and incubated for 24 hours at 37°C. At the end of the incubation period, the bacterial suspensions were inoculated on BHI agar and incubated for 24 hours at 37°C. Bacterial samples were collected from the BHI broth, the turbidity of which was adjusted to the McFarland 0.5-turbidity standard. Disc-shaped materials were placed on a flat-bottom 96-well plate, with one specimen for each well; 100 µl of the bacterial suspension was added to each well. After incubation at 37°C for 24 hours, the test materials were washed three times with 200 µl of sterile saline to remove non-adhering cells, after which the adhered cells were collected by swabbing and transferred into Falcon tubes filled with 5 ml of saline. The tubes were vortexed for 60 seconds to detach the bacteria from the swabs. The detached cells from the surface of one specimen from each group were separated for microscopic analyses. Bacterial suspensions were serially diluted in saline (1:100 series) for culture analysis. For bacterial viability imaging and adhesion determination, 100 µl of bacterial suspension was spread over a plate with BHI agar three times and incubated at 37°C for 24 hours. The bacterial count in a 1-mm^2 area on the sample surface was calculated according to the surface area and dilution factor.

Examination with confocal laser scanning microscopy. Two specimens from each subgroup were randomly selected for microscopic evaluation with CLSM (SP8 Lightning Confocal Microscope, Leica Microsystems, Wetzlar, Germany). Detached cells in the 100-µl broth were stained using the Cell Check Viability/Cytotoxicity bacterial kit (ABP Biosciences, Rockville, MD, USA). Following the instructions, 1.5 µl of NucView Green and 1.5 µl of propidium iodide were added to a tube with 7 µl of saline solution, after which 10 µl of bacterial broth was taken by a swab and mixed with 1 µl of dye solution in a

tube. Then, the tube was incubated in the dark at room temperature for 15 minutes. The mixture was spread over a glass plate; the cell images were observed under ×5/×10 magnifications.

Statistical analysis.

The statistical analysis was conducted using the Statistical Package for the Social Sciences version 24.0 with a significance level of 0.05. The means and standard deviations between and within the groups were calculated, and the observed differences for each sample were analyzed by Student's t-test. Further statistical analyses were conducted to compare the five restorative materials by one-way analysis of variance; post-hoc evaluations were conducted by the Bonferroni test.

RESULTS AND DISCUSSION

Surface roughness.

Table 2 summarizes the surface roughness values obtained from all groups. According to results, there was significant interaction between the polishing methods and surface roughness values of restorative materials ($p<0.001$). Except for Tetric Evo Bulk-fill group, all of the restorative materials showed significantly lower roughness values when polished with SL. One-way ANOVA showed that there was statistically significant difference among all tested materials in which Fuji II LC had significantly higher surface roughness values among all groups ($p<0.001$). In SL groups, Bonferroni test showed that Fuji II LC had significantly higher roughness values that all groups and Ecu Sphere showed significantly rougher surfaces than Dyract XP ($p<0.05$). According to OG groups, Fuji II LC and Beautifil-Bulk Flowable groups had significantly higher roughness values than all other groups ($p<0.001$) and there is no significant difference in the surface roughness values between Dyract XP, Tetric Evo Bulk Flow and Ecu Sphere groups.

Table 2. Mean +SD and statistical significances of surface roughness values of restorative materials either polished with Multi-step (SL) or One-step (OG) systems. *

Groups	n	Polishing method	Mean ± SD	p
Fuji II LC	8	Multi-step	0.81 ± 0.04^{A}	< 0.001
	8	One-step	0.91 ± 0.10^{d}	
Dyract XP	8	Multi-step	0.24 ± 0.06^{B}	< 0.001
	8	One-step	0.41 ± 0.12^{e}	
Tetric Evo Bulk Flow	8	Multi-step	$0.30 \pm 0.07^{B,C}$	0.089
	8	One-step	0.38 ± 0.10^{e}	
Beautifil-Bulk Flowable	8	Multi-step	$0.27 \pm 0.04^{B,C}$	< 0.001
	8	One-step	0.65 ± 0.12^{f}	
Ecu Sphere Flow	8	Multi-step	0.35 ± 0.09^{C}	0.035
	8	One-step	0.45 ± 0.09^{e}	
Among groups				< 0.001

*Different uppercase letters show significant differences among SL groups and different lowercase letters show significant differences among OG groups.

Bacterial adhesion.

Table 3 summarizes the surface roughness values obtained from all groups. Results showed that there was statistically significant interaction between polishing method and bacterial adhesion values of tested materials ($p<0.001$).

All of the materials showed significantly lower bacterial adhesion after polishing with SL. Besides, there was significant effect between tested materials and bacterial adhesion as well ($p<0.001$). Ecu Sphere Flow showed significantly higher bacterial adhesion than Tetric Evo Bulk-fill and Fuji II LC materials. Similarly, Tetric Evo Bulk-fill had significantly lower bacterial adhesion when compared to Dyract XP and Beautifil-Bulk Flowable materials ($p<0.05$). In SL groups, Bonferroni test showed that Tetric Evo Bulk-fill had significantly lower bacterial adhesion among all groups ($p<0.001$). Fuji II LC showed significantly lower bacterial adhesion than Beautifil-Bulk Flowable and Ecu Sphere groups and Ecu Sphere had significantly higher bacterial adhesion when compared to Dyract XP and Beautifil-Bulk Flowable materials ($p<0.05$). There was no significant interaction between

OG groups of all materials regarding bacterial adhesion ($p>0.05$).

Table 3. Mean +SD and statistical significances of bacterial adhesion of restorative materials either polished with Multi-step (SL) or One-step (OG) systems. *

Groups	n	Polishing method	Mean	± SD	p
Fuji II LC	8	Multi-step	8762,215^{A}	775,232	<0.001
	8	One-step	15648,884^{e}	778,156	
Dyract XP	8	Multi-step	13253,328^{A}	820,039	<0.001
	8	One-step	15662,218^{e}	883,283	
Tetric Evo Bulk Flow	8	Multi-step	6496,662^{B}	819,618	<0.001
	8	One-step	15804,44^{e}	894,054	
Beautifil-Bulk Flowable	8	Multi-step	14519,996^{C}	1407,664	<0.001
	8	One-step	16871,107^{e}	831,899	
Ecu Sphere Flow	8	Multi-step	15044,44^{D}	794,463	<0.001
	8	One-step	16453,33^{e}	1191,243	
Among groups					<0.001

*Different uppercase letters show significant differences among SL groups. Lowercase letters show that there is no significant difference among OG groups.

CLSM analysis.

One random specimen from each group has analysed by CLSM. Representative images of *S. Mutans* biofilm on the surface of the specimens are shown in figure 1(a–e). The density of the loadings of bacteria (green-coloured bacteria are viable and red-coloured ones are non-viable) were observed for all groups. Specimens polished with one-step system seemed to present greater amounts of viable bacteria. It is also obvious that specimens of Tetric Evo Bulk Flow, Fuji II LC and Dyract XP groups had lower bacteria adhered to surfaces.

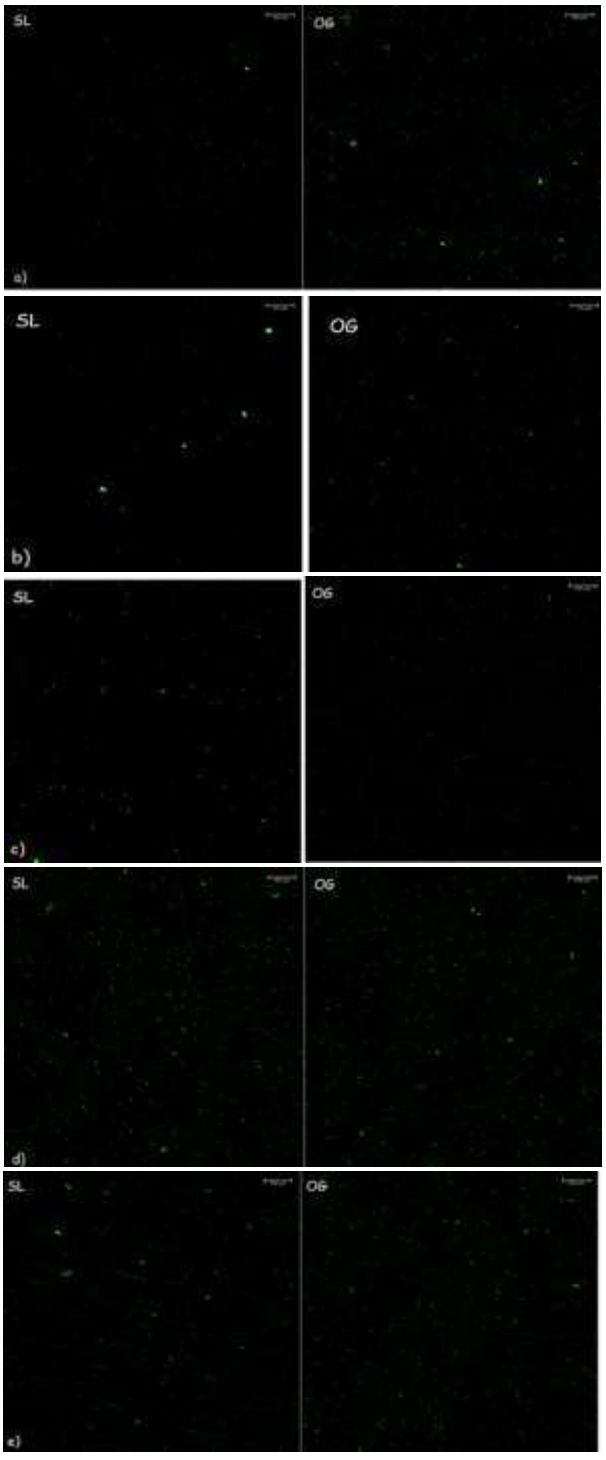

Fig. 1. (a-e). *S. mutans* biofilm on the surfaces of randomly selected specimens of groups.*

*Representative confocal laser scanning microscobe (CLSM) images (10×) of the biofilm formed on the specimens of tested materials: (a) Fuji II LC, (b) Dyract XP, (c) Tetric Evo Bulk Flow, (d) Beautifil, (e) Ecu Sphere Flow. Groups polished with multi-step system termed as (SL) and with one-step system termed as (OG). Green colour refers live bacteria and red colour refers non-viable bacteria

Bulk-fill composites are advantageous due to a number of properties, such as time-saving manipulations, low polymerization shrinkage, and being user friendly [14, 15]. Bulk-fill materials were first introduced as low-viscosity composites; however, inferior mechanical properties limit their application in occlusal areas [16]. With further formula development, bulk-fill materials have been proven to be safe and effective for at Class V cavities due to their low elastic modulus, super handling, and self-adapting properties [17]. Accordingly, the surface characteristics of these materials are crucial with respect to preventing plaque retention and bacterial adhesion, especially for Class V restorations in close proximity to gingival tissue [18]. The present *in vitro* study compared the surface roughness values and bacterial adhesion between bulk and conventional resin-based restorative materials subjected to SL and OG polishing systems. The results suggest that the Fuji II LC groups have significantly higher surface roughness values when polished with both systems. Therefore, the first null hypothesis can be rejected.

Studies regarding bacterial adhesion generally compare materials with a high fluoride content. Accordingly, in the present study, a resin-modified glass ionomer material (Fuji II LC) and a compomer (Dyract XP) were included [7, 19, 20]. The Fuji II LC has the ability to release fluoride, which, over time, may cause surface irregularities [21]. Moreover, it contains HEMA, which is a highly hydrophilic monomer that can increase water sorption characteristics to up to 80% of the material weight. Accordingly, the specimens were kept in distilled water during the experiment; the inferior roughness results of Fuji II LC can be attributed to its fluoride release and matrix properties. Moreover, the intrinsic physicochemical properties, such as filler size, loading, and shape, directly determine the surface roughness values [9]. Thus, the significantly high surface roughness values among all materials can be attributed to the largest filler size (5.9 µm) of Fuji II LC.

In the current study, Dyract XP showed the smoothest surfaces among groups polished with the LS method; following Guler and Unal's [22] study, no significant differences could be found between the Beautifil-Bulk Flowable and the Tetric Evo Bulk Flow group. The smooth surfaces results can be attributed to the similar content of Dyract XP with other bulk-fill composites. It includes a common UDMA monomer along with TCB resin; it also has a similar filler size as the other materials. The Beautifil-Bulk Flowable group had significantly higher roughness values after being polished with the OG method. It has recently developed filler, which is called S-PRG in the resin matrix. However, these materials lack a binding component connecting the resin matrix and S-PRG filler; many studies have reported surface deterioration, water absorption, and high roughness values [23, 24]. Although there were no significant differences between Beautifil-Bulk Flowable, the compomer and other flowable groups subjected to the SL polishing, the Beautifil-Bulk Flowable group polished with OG had significantly higher roughness values. Thus, due to its unstable content, Beautifil-Bulk Flowable had inferior surface roughness values.

Nanoparticles are often incorporated into the resin matrices of dental materials to enhance surface quality and polish retention [25]. Studies suggest that generally, materials with larger fillers show more surface roughness than those with smaller fillers [24, 26]. In the present study, the bulk-fill composites showed superior surface roughness values compared with the micro hybrid composites (Ecu Sphere Flow) when polished with SL. However, in the OG groups, only one bulk-fill composite (Tetric Evo Bulk Flow) had smoother surfaces than the micro hybrid composite which could be attributed to its nano-sized fillers (~550 nm). The roughness results regarding the effect of filler sizes were obtained during the SL method. Indeed, both the polishing time and the physicochemical structures of the polishing systems affect the surface smoothness of the resin composite restorations

[27]. The gradual decrease in grain size for the SL system reveals the difference between the results. The SL system had a prolonged polishing time due to the number of discs used in asorti. The results also suggest that the discs in the SL groups have more flexibility than those in the OG groups [27, 28], which is beneficial since flexible systems result in reduced surface scratching, which can be explain the inferior scores of the OG system.

Bacterial adhesion is affected by the intrinsic physicochemical properties of the restorative materials, such as filler size, shape, loading, and monomer type and structure [8, 9]. The initial colonization was seen in sheltered areas where the bacteria is protected from insistent chewing forces. Indeed, to ensure sufficient time to change from reversible to irreversible plaque formation, irregular surfaces are preferable for bacteria. However, according to Yu [29], rough surfaces attract bacteria only in the early stages of attachment; i.e., two to four hours. In the present study, the biofilm formed on the surface of the specimens over 24 hours; thus, it is possible that the roughness results are not consistent with the bacterial counts. Alternatively, the monomer content of the restorative materials determines their hydrophobicity and hydrophilicity, both of which attract different types of bacteria. For instance, *S. mutans* have a hydrophobic cell membrane that it can thrive on hydrophobic surfaces [30]. OH^- ions or ester bonding in the structure of monomers can affect hydrophobicity properties so that BisGMA and TEGDMA are hydrophilic monomers, whereas UDMA monomer is hydrophobic. The ethoxylated version of BisGMA (BisEMA) and a novel monomer, BisMPEPP, have lower water sorption than BisGMA; accordingly, these monomers have an average value of hydrophobicity [30, 31]. Therefore, the lowest bacterial adhesion of Tetric Evo Bulk Flow fill can be attributed to its hydrophobic monomer structure, including BisGMA, UDMA, and BisEMA. In comparison, Beautifil-Bulk Flowable has a hydrophilic monomer (TEGDMA) in its matrix, so the inferior results can be attributed to its content. Moreover, as obviously monitored in CLSM images (figure 1a-b), Fuji II LC and Dyract XP groups had reduced bacterial adhesion. However, aside from their monomers, the high fluoride content may have affected the results. Similarly, Beautifil-Bulk Flowable has its own S-PRG filler, which releases fluoride along with five other ions (sodium, borate, aluminum, silicate, and strontium). According to the existing literature, restorative materials with S-PRG filler have superior results than those without S-PRG filler [32, 33]. However, some limitations have been identified with respect to the releasing of ions from the filler. Ions can only be released if the pH of the media turns acidic, and they are effective only if *S. mutans* are on the active growth and able to conduct sugar metabolism [34]. The inferior results of the Beautifil-Bulk Flowable group could also be attributed to the fact that the present study did not imitate all the elements for biofilm formation, which means that the S-PRG fillers were not activated.

After biofilm formation, *S. mutans* colonize on non-shedding hard surfaces of teeth and restorative materials. Surface properties, such as surface roughness, SFE, contact angle, and wettability, play an important role in microbial adhesion and biofilm formation [35]. The f/p systems influence the final surface properties and thereby directly affect microbial adhesion and biofilm formation. On the basis of the current study, the second null hypothesis can be rejected since the specimens polished with the OG system have significantly higher bacterial adhesion. Bacterial adhesion is a complex mechanism that includes several parameters, such as SFE. Polished material surfaces have low SFE, which is unfavorable to *S. mutans* [30]. However, the purpose of the present study was not to investigate factors affecting bacterial adhesion, so SFE was not evaluated. As supported by the CLSM images, there were no significant differences among the bacterial adhesion scores for the OG groups. Therefore, the results of could be attributed to the SFE of the lack of polished surfaces of OG groups.

CLSM is a common method for qualitative and quantitative studies; it does not require one to remove bacteria from the substrate nor does it damage the surface [1]. However, to investigate f/p systems, many studies use SEM imaging [36, 37]. Although SEM is an effective way to assess surface configurations, it only provides a qualitative measurement.

In the present study, roughness values were obtained from the profilometer, which is a quantitative assessment and bacterial adhesion is counted on the basis of cultivation results. So it was aimed to compare the materials' properties and f/p systems regarding bacterial adhesion and to evaluate qualitatively with CLSM images however, the current method was not without its own limitations. First, the *in vitro* model investigated the adhesion of *S. mutans* at neutral pH and under conditions without any fundamental oral elements.

Therefore, the study does not fully reflect the oral environment. Second, only *S. mutans* adhesion was tested, which means that mature plaque with many different types of cariogenic bacteria was not included. Third, the environment was not acidic enough for the bioactive S-PRG filler to release ions. Fourth, an insufficient number of parameters related to the adhesion mechanism in terms physicochemical properties were investigated. Therefore, further *in vitro* studies are required to mimic the oral environment for all included compounds with the diversity of cariogenic bacteria.

CONCLUSIONS

Due to the findings of the present study, it is advisable to polish various resin-based materials with multi-step systems to obtain optimum results with respect to reducing bacterial adhesion and improving surface properties. Unfortunately, the relation between the flowable material composition and the parameters affecting bacterial adhesion requires further study. Bacterial counts of broth cultivation were in accordance with CLSM images. Thus, CLSM is the suitable method for imaging bacterial viability on dental materials.

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