Quantitative Assessment of Primary Colonizer Adhesion on Different Resin-Based Restorative Materials Using SYBR-Green qPCR

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Article

Quantitative Assessment of Primary Colonizer Adhesion on Different Resin-Based Restorative Materials Using SYBR-Green qPCR

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Abstract

The aim of this study was to investigate the adhesion of primary colonizers of the oral biofilm on five adhesive restorative materials. For each material (Admira Fusion, Clearfil AP-X, Durafill VS, Filtek Supreme XTE, Venus Diamond) sixteen test specimens were prepared according to a standardized protocol. For pellicle formation, the specimens were incubated for two hours at 37 °C with sterile-filtered inactivated human saliva. The bacteria (*S. oralis, S. gordonii, S. sanguinis, S. mitis, A. spp.*) were cultivated and suspended. A bacteria mix was prepared from the suspensions. The specimens with pellicles were wetted with the bacterial mix and incubated at 37 °C for 8 hours. The total genomic DNA of the adhered bacteria was isolated and subsequently quantified using SYBR Green qPCR. For *S. gordonii*, *S. oralis* and *A. spp.*, no significant differences in the amount of adhered bacterial DNA were found between the different materials. DNA concentration of *S. mitis* was significantly higher on Filtek Supreme XTE compared to the other materials. Significantly higher DNA concentrations of *S.sanguinis* could also be detected on Filtek Supreme XTE compared to Clearfil AP-AX and Durafill VS.

Keywords: primary colonizers; adhesion; restorative materials

1. Introduction

The occurrence of secondary caries is one of the most common causes of composite filling failure, requiring replacement or repair [1–4]. Microbial biofilms that adhere to tooth structures or filling materials play a major role in this process and are therefore the main cause of carious lesions and gingival inflammation [5,6]. It is also known that the risk of developing secondary caries and periodontal inflammation increases with the amount of adhered plaque [7]. The extent to which biofilms adhere to restorations is therefore of great significance and importance for the longevity of restorations in the oral cavity [5,8].

In biofilm formation, the adhesion of initial colonizers is an important step that also influences the later composition of mature plaque [9]. The formation of a pellicle is of essential importance for the development of a pathogenic biofilm as it enables subsequent microbial adhesion [10–12].

In terms of the longevity of restorations, it is important to reduce or even completely prevent bacterial adhesion [13–16]. There is a growing demand for materials that minimize plaque formation [17]. In the field of dental resins in particular, materials are used that have different surface properties due to varying compositions of ingredients and particle sizes which has an impact on bacterial

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adhesion [18–20]. For example, the growth of cariogenic bacterial species could be influenced by the monomer matrix of composites [21,22].

Previous studies on filling materials have primarily focused on the adhesion of *Streptococcus mutans* as it is considered the principal bacterium associated with carious plaque [23–31]. However, there are already studies that examine the adhesion of primary colonizers [10,18,19,27,32–40]. In these studies, the bacteria are examined in mixtures [10,18,19,35,36,38–40] or as individual species [24–30,32–34,37,41], on surfaces coated with [10,18,19,24,25,27,29,32,33,35,36,38,39] and without [26,28,30,34,36,37,41] pellicle. The detection methods used in these studies are usually microscopic [24,25,27,32–37,41]. Quantitative investigations using polymerase chain reaction (PCR) [10,18] are rare, although it was pointed out that molecular biological investigations such as PCR, real-time PCR, and DNA sequencing should be used in the investigation of the dental microbiome [42].

To date, there are no studies investigating the adhesion of initial colonizers in a mix of five species, including streptococci and actinomycetes, to adhesive filling materials coated with pellicle. In addition, possible metabolic interactions between bacterial species during biofilm formation should be investigated [39], which underscores the need to analyze different species of initial biofilm formation as a mix. To the best of our knowledge there are currently no studies that detect bacterial DNA using SYBR green qPCR.

Thus, the aim of this in vitro study is to investigate the adhesion capacity of primary colonizers of oral biofilm on composites of different material groups to re-evaluate the respective materials in terms of their suitability for dental restorations by comparing the initial plaque affinity.

2. Materials and Methods

2.1. Test Specimen Preparation

Based on a preliminary test with all five species on ten test specimens of Filtek Supreme XTE material and case number planning, it was decided to produce n = 16 test specimens per material.

Five different adhesive filling materials were selected for the preparation of the test specimens, including four composites of different material classes and one ormocer (Table 1). The test specimens were prepared and polished according to a defined protocol, which is in the following.

Table 1. Materials used in this investigation with material classes and batch numbers.

Product	Material class	Batch	Company
Admira Fusion	Ormocer	2044350	Voco, Cuxhaven, Germany
Clearfil AP-X	Hybrid Composite	9E0731	Kuraray, Chiyoda, Japan
Durafill VS	Microfiller Composite	K010230	Kulzer, Hanau, Germany
Filtek Supreme XTE	Nanocomposite	NC10955	3M Espe, Landsberg am Lech, Germany
Venus Diamond	Ultra-fine particle hybrid composite	K010201	Kulzer, Hanau, Germany

The adhesive materials were first heated to 50 to 60 °C in a heating cabinet (P30, Memmert GmbH, Schwalbach, Germany). Meanwhile, all surfaces and instruments that would get in touch with the materials during the preparation of the test specimens were disinfected and the room was darkened for working with light-curing materials.

To form 16 test discs per material with a diameter of 10+/-0.1 mm and a thickness of 1+/-0.1 mm, the materials were placed in a PTFE template with a length of 140 mm, a width of 330 mm, and a height of 10 mm, which contained the punches in the corresponding size. A new PTFE template was used for each material. Modeling instruments such as Heidemann spatulas and ball pluggers were used for insertion. The respective material was inserted into the mold using a compule gun and then

 doi:10.20944/preprints202602.2025.v1

shaped with ball pluggers. During insertion, it was important to ensure that the material was flush with the metal splint so that there were no over- or under-contours. To achieve this, excess material at the edge was carefully scraped off.

The top of the metal splint was then placed, and the material was pressed for one minute at 2000 to 3000 bar in a hydraulic press. After removal from the press, the metal construction was opened again, and the material was polymerized with a Bluephase polymerization lamp at full intensity of >1000 watts/cm² for 40 seconds per side. The performance of the polymerization lamp was checked regularly in a ten-test specimen cycle. Finally, the test specimens were removed from the template and excess material was removed with a sharp scalpel.

Al_2O_3-coated Super-Snap polishing discs (Shofu Dental GmbH, Ratlingen, Germany) with grain sizes of 20 µm (green) and 7 µm (pink) were used for the two-step polishing of the test specimen surface. The test specimens were moistened with sterile water and polished with a contact pressure of 0.3 to 0.7 N at 10,000 rpm for one minute each, first with the green and then with the pink polishing disc. A new polishing disc was used for each test specimen. After polishing, the test specimens were first disinfected with ethanol and then immersed in 70% ethanol for one minute. After five minutes of air drying, the test specimens were stored in well plates in distilled water at 37 °C in a heating cabinet (P30, Memmert GmbH, Schwalbach, Germany) for 7 days until the start of the test.

The test specimens were produced and stored in accordance with ISO 399075.

2.2. Preparation of Bacterial Mix

For cultivation, the bacteria stored at -80 °C in cryotubes (Table 2) were removed from the freezer and thawed for approx. 2 minutes at 37 °C in a water bath (MPC E, Mechatronik GmbH, Darmstadt, Germany) with swirling movements.

Table 2. Species used in this investigation with strain specifications.

Species	Strain
A.naeslundii	DSM 43013
A.spp.:	+ 1:1
A. oris	DSM 23056
S. mitis	DSM 12643
S. oralis	DSM 20627
S. sanguinis	DSM 20068
S. gordonii	DSM 6777

40 to 50 µl of the bacterial suspension were applied to a blood agar plate that had been brought to room temperature, spread with a sterile inoculating loop, and left to dry for 2 minutes. For incubation, the inoculated agar plates were stacked upside down in an anaerobic pot, which had previously been provided with an Anaerocult C packet (Merck KGaA, Darmstadt, Germany) moistened with 6 ml of water. The agar plates were incubated at 37 °C for 48 hours in a heating cabinet (P30, Memmert GmbH, Schwalbach, Germany). The cultivation and storage of the bacterial species complied with ISO 399075.

After 48 hours, 1 to 2 colonies per species were transferred from the agar plates to a Falcon tube containing 8 ml of reduced anaerobic growth medium and cultivated for a further 24 hours in a heating cabinet (P30, Memmert GmbH, Schwalbach, Germany).

Of the six cultivated species, 1:6 dilutions were prepared from 2 ml of bacterial suspension and 10 ml of anaerobic growth medium in 15 ml falcons. These were incubated with loose lids for 2 hours in a large anaerobic chamber with two packets of Anaerocult-C (Merck KGaA, Darmstadt, Germany) at 37 °C in a heating cabinet (P30, Memmert GmbH, Schwalbach, Germany) The 1:6 dilution corresponded to an optical density of 0.2 to 0.3 at a wavelength of 600 nm, which was verified using a spectrophotometer (BioMate3S, Thermo Fisher Scientific, Waltham, MA, USA). To prepare the bacterial mixture, 7 ml of each species was added to a Falcon tube.

 doi:10.20944/preprints202602.2025.v1

2.3. Pellicle Formation on Test Specimens

The saliva required for pellicle formation was collected from five healthy subjects after consultation with the Ethics Committee of the Medical Faculty of Goethe University Frankfurt, which determined that no vote was required. For this purpose, the subjects were asked to repeatedly provide saliva samples within one hour; no stimulation of salivation, for example with paraffin blocks, was performed.

After collection, the saliva was centrifuged for 30 minutes at 4500 rpm and 4 °C using a Universal 16R centrifuge (Hettich GmbH, Tuttlingen, Germany), then pooled and sterile-filtered (Millex-GV, 0.45 µm, PVDF, 3 mm, Millipore Inc., Billerica, MA, USA). The pooled saliva was inactivated at 56 °C in a heating cabinet (P30, Memmert GmbH, Schwalbach, Germany) for 30 minutes and then diluted with DPBS in a ratio of 1:2.

The test specimens, which had been stored in water up to this point, were air-dried and transferred to new 24-well plates (one plate per material). 250 µl of the inactivated saliva was pipetted onto each test specimen. The test specimens were incubated at 37 °C in a heating cabinet (P30, Memmert GmbH, Schwalbach, Germany) for 2 hours to allow pellicle formation.

2.4. Incubation of Test Specimens with Bacterial Mix

The liquid components of the saliva were washed off the test specimens by rinsing twice with DPBS and pipetting, leaving only the pellicle. 350 µl of the pooled bacterial mix was applied to each test specimen. The well plates with the test specimens were incubated in an anaerobic pot with 2 packets of Anaerocult-C (Merck KGaA, Darmstadt, Germany) for 8 hours at 37 °C in a heating cabinet (P30, Memmert GmbH, Schwalbach, Germany).

2.5. DNA Isolation

After incubation, the supernatant was first removed, and each test specimen was carefully washed with 300 µl PBS at 37 °C to remove all non-adherent bacteria. The prepared test specimens were then transferred with the adhering bacteria to new 24-well plates for DNA preparation.

DNA isolation was performed using the DNA Preparation Kit from Jena Bioscience (#PP-214-L, Jena Bioscience, Jena, Germany).

To this end, 300 µl of resuspension buffer was first added to each test specimen, followed by 2 µl of lysozyme, and everything was thoroughly mixed by pipetting up and down. This was followed by further incubation at 37 °C for one hour. The mixture was then centrifuged for one minute at 10,000 g (G570E, Scientific Industries, Bohemia, NY, USA). The supernatant was removed and discarded. 300 µl of lysis buffer and 2 µl of RNase were added to each test specimen, and everything was mixed thoroughly again. Now 8 µl of protein kinase K was added, and everything was mixed again. The test specimens were incubated again for 10 minutes at 60 °C. The bacterial suspension was rinsed off the test specimens and transferred to a 1.5 ml reaction vessel using a pipette. 300 µl of binding buffer was added to each sample and mixed thoroughly. The samples were then cooled at 4 °C for 5 minutes and subsequently centrifuged at 10,000 g for another 5 minutes. A column was then placed on a 2 ml collection tube, mixed with 100 µl activation buffer, centrifuged at 10,000 g for 30 seconds, and the flow was discarded. The sample, i.e., the supernatant after centrifugation, was then loaded onto the column, centrifuged again at 10,000 g for one minute, and the flow was discarded. 500 µl of washing buffer was added to each column, which was then centrifuged again for 30 seconds at 10,000 g, and the flow was discarded. This step was performed twice in total. After the second run, the flow was removed, and the columns were centrifuged again for one minute at 10,000 g to remove any remaining washing buffer and to dry the columns. The columns were then transferred to new 1.5 ml reaction vessels and 40 to 50 µl of elution buffer was pipetted directly onto the membrane per column. After incubating for one minute at room temperature and then centrifuging for 2 minutes at 10,000 g, the samples were stored at -20 °C until SYBR Green qPCR was performed.

 doi:10.20944/preprints202602.2025.v1

2.6. SYBR Green qPCR

9 µl master mix, consisting of 5 µl Mastermix Plus for SYBR Green I-NO ROX (RT-2N2X-03+NR, Bio & Sell GmbH, Feucht, Germany), 2 µl primer mix (1 µl forward, 1 µl reverse) and 2 µl distilled water, was added per well. The final primer design (Table 3) (MWG-Biotech GmbH, Ebersberg, Germany; metabion GmbH, Planegg-Martinsried, Germany; Eurogen- tec, Seraing, Belgium) was calculated with the Primer Express Software (Applied Biosystems Inc., Foster City, CA, USA) for *S. mitis* and taken from the literature for the other bacteria [18,43]. One µl of the now thawed DNA was pipetted into the master mix. The plates were sealed with MicoSeal B seal foil (Bio-Rad Laboratories Inc., Hercules, California, USA) and then centrifuged for 30 seconds at 2250 rpm (Plate Fuge, Benchmark Scientific, Sayreville, NY, USA). Subsequently, duplex SYBR Green qPCR was performed for quantitative DNA detection using the Pro Rad CFX96 Optic Module C1000 Touch Thermo Cycler from Bio-Rad Laboratories Inc. (Hercules, California, USA).

Table 3. Design of the specific primers used in this investigation.

Species	Primer	Reference
A. spp.	F GGT CTC TGG GCC GTT ACT GA	Henrich et al., 2016 [18]
	R TGG CCC CCA CAC CTA GTG	
A. naeslundii	F GGG CCT GGG AAA GAT TG	Henrich et al., 2016 [18]
	R TGA CCG TGC ACC CTC TCA	
A. oris	F TGC CTG CTG CAT GGT GG	This study
	R AAA GGG ACA GGC CTG CTT C	
S. mitis	GAGCTTGCTTCTCCGGATGA	Henrich et al., 2016 [18]
	AATTGCACCTTTTAAGCAAATGTCA	
S. oralis	F TCC CGG TCA GCA AAC TCC AGC C	Hoshino et al., 2004 [43]
	R GCA ACC TTT GGA TTT GCA AC	
S. sanguinis	F GGA TAG TGG CTC AGG GCA GCC AGT T	Hoshino et al., 2004 [43]
	R GAA CAG TTG CTG GAC TTG CTT GTC	
S. gordonii	F CTA TGC GGA TGA TGC TAA TCA AGT G	Hoshino et al., 2004 [43]
	R GGA GTC GCT ATA ATC TTG TCA GAA A	

2.7. Statistical Analysis

The statistical analysis was performed using the software "BiAs. For Windows" (version 11.12 ©1989-2023 Epsilon-Verlag) in collaboration with the Institute for Biostatistics and Mathematical Modeling at Goethe University Frankfurt am Main. For the statistical analysis, a Kolmogorov-Smirnov test was first performed to check for normal distribution. Since there was no normal distribution, the analysis was then performed using the Friedmann test with Bonferroni-Holm correction for pairwise post-hoc comparisons. A significance level of $p \leq 0.05$ was used. Graphical illustrations used R (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

The materials were evaluated regarding the percentage of adhered bacteria species on the different materials and the median DNA concentration of the tested bacteria adhered to the materials. Using the median instead of the mean is of advantage as it is more robust against outliers.

3.1. S. gordonii

 doi:10.20944/preprints202602.2025.v1

Diffferent materials showed different percentages of adhered *S. gordonii* DNA on the test specimens. DNA adhered to Venus Diamond by 68,75%, to Clearfil APX by 50%, to Filtek supreme XTE by 37,5%, to Admira Fusion by 31,25% and to Durafil by 25%. The median adhesion concentration detected on Admira Fusion, Durafil and Filtek Supreme XTE did not differ (0.0000 ng/30µl DNA). Compared to those materials, there was a higher median DNA concentration adhered to Clearfil APX (0.0069 ng/30µl DNA) and Venus Diamond (0.0010 ng/30µl DNA). However, there were no statistically significant differences between the materials (Figure1).

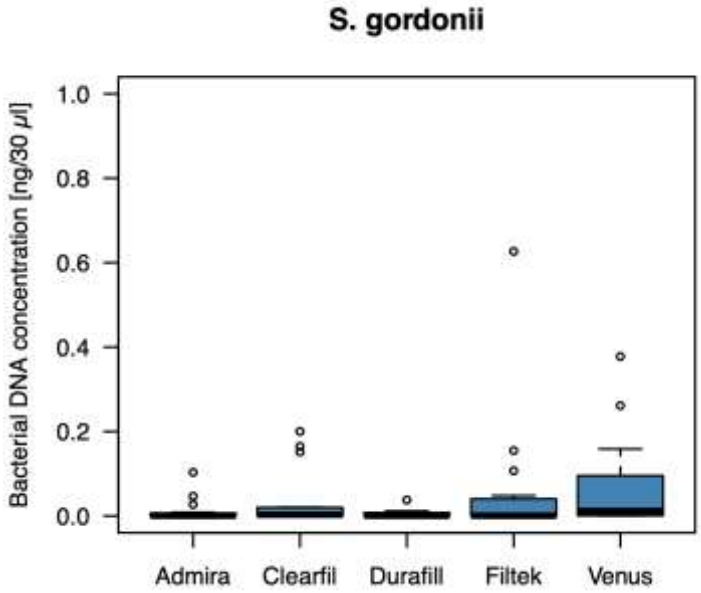

Figure 1. Results *S. gordonii*. Adhesion of bacterial DNA when compared between different materials. No significant differences were observed.

3.2. S.oralis

Most DNA of *S. oralis* adhered to Venus Diamond (81.25%) followed by Filtek supreme XTE (68.75%), Clearfil APX and Admira Fusion (62.5%) and Durafil (37.5%). The median adhesion concentration detected on Filtek Supreme XTE was 1.8138 ng/30µl DNA, on Durafil 0.0000 ng/30µl DNA, on Clearfil APX 0.1222 ng/30µl DNA. There was also no statistically significant difference compared to Admira Fusion (0.2388 ng/30µl DNA) or Venus Diamond (0.4208 ng/30µl DNA). Comparison between the materials revealed no statistically significant differences (Figure 2).

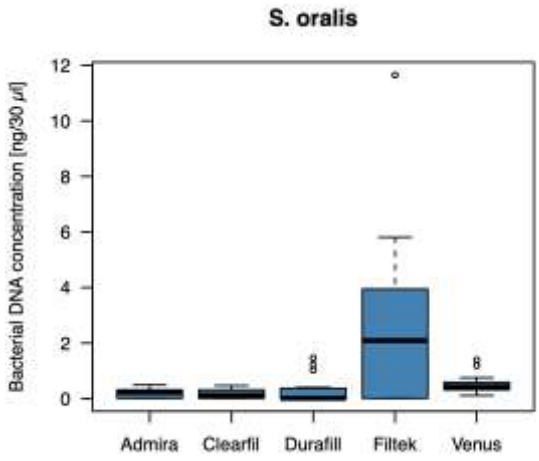

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Figure 2. Results *S. oralis*. Adhesion of bacterial DNA when compared between different materials. No significant differences were observed.

3.3. A. spp.

A. spp. DNA adhered by 100% to the test specimens of all materials. The median adhesion concentration detected differed for Admira Fusion (0.9953 ng/30µl DNA), Clearfil APX (0.6105 ng/30µl DNA), Durafil (0.5119 ng/30µl DNA), Filtek Supreme XTE (1.0124 ng/30µl DNA) and Venus Diamond (0.8839 ng/30µl DNA) but the different results were not statistically significant when compared to each other (Figure 3)

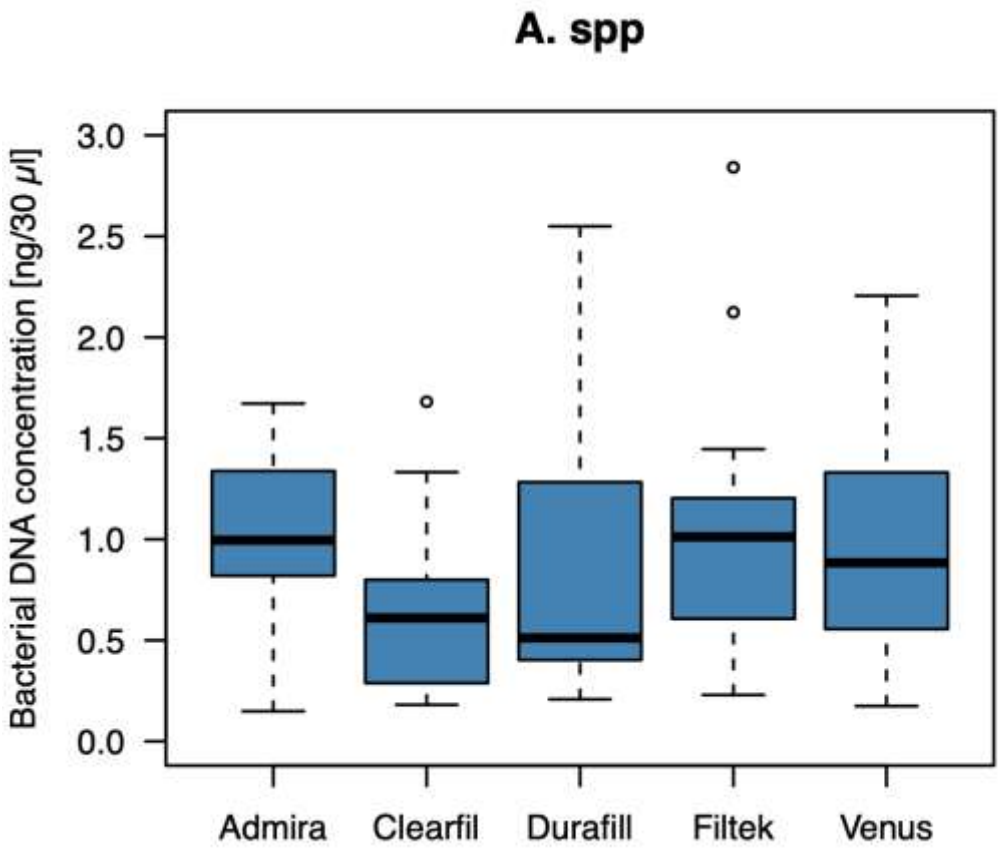

Figure 3. Results *A. spp*. Adhesion of bacterial DNA when compared between different materials. No significant differences were observed.

3.4. S.mitis

DNA of *S. mitis* adhered on Admira Fusion, Clearfil APX and Filtek supreme XTE by 93,75% and on Durafil and Venus Diamond by 87,5%. Global comparison results in significant differences (p=0.001). Pairwise post-hoc tests showed that detected DNA concentration was significantly higher on Filtek supreme XTE (0.8119 ng/30µl DNA) compared to Admira Fusion (0.0681 ng/30µl DNA; p=0.001), Clearfil APX (0.1186 ng/30µl DNA; p=0.003) and Venus Diamond (0.1061 ng/30µl DNA; p=0.008). There were no statistically significant differences between Filtek supreme XTE and Durafill (0.2317 ng/30µl DNA) or the other materials compared to each other (Figure 4).

 doi:10.20944/preprints202602.2025.v1

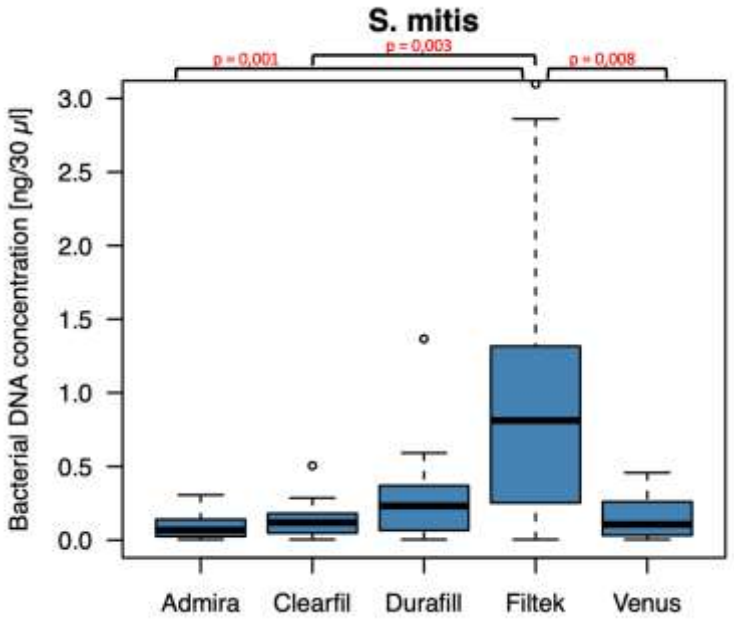

Figure 4. Results *S. mitis*. Adhesion of bacterial DNA when compared between different materials. Significant pairwise comparisons are marked.

3.5. S. sanguinis

DNA of *S. sanguinis* showed high adhesion to the materials. 100% adhered to Filtek supreme XTE and Venus Diamond and 93,75% to Admira Fusion, Clearfil APX and Durafill. Pairwise post-hoc tests showed that detected DNA concentration was significantly higher on Filtek Supreme XTE (0.4005 ng/30µl DNA) compared to Durafill (0.0848 ng/30µl DNA; p=0.001, p=0.001) and Clearfil APX (0.0861 ng/30µl DNA; p<0.001). There was no statistically significant difference compared to Admira Fusion (0.1557 ng/30µl DNA) or Venus Diamond (0.1702 ng/30µl DNA). Comparison between other materials revealed no statistically significant differences either (Figure 5).

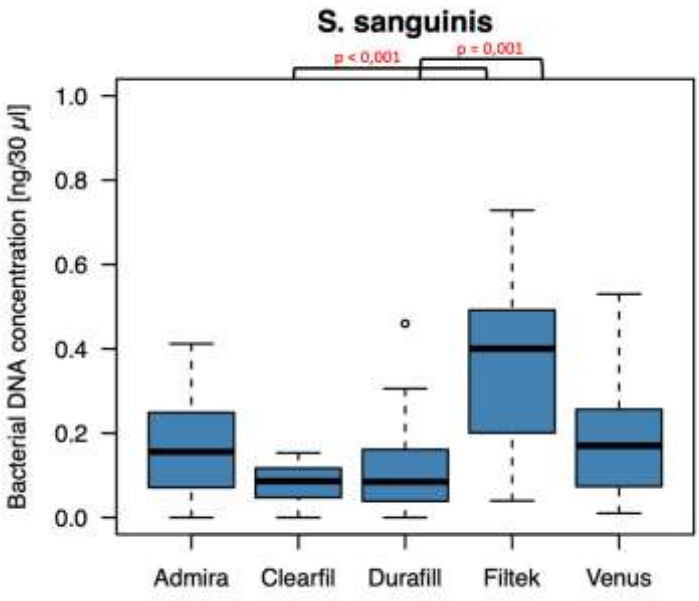

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Figure 5. Results *S. sanguinis*. Adhesion of bacterial DNA when compared between different materials. Significant pairwise comparisons are marked.

Overall, no significant differences in bacterial adhesion between the different materials are shown for *S. gordonii*, *S. oralis* and *A. spp*. In contrast, such significant differences are observed for *S. mitis* and *S. sanguinis*. At a significance level of $p < 0.05$, there were no significant difference in adhesion of DNA to the various adhesive filling materials for three of the five primary colonizers, namely for *S. gordonii*, *S. oralis* and *A. spp*. However, the results showed a significant difference regarding *S. mitis* and *S. sanguinis* DNA-adherence to different materials.

4. Discussion

As minimal invasive procedures in restorative dentistry are on the rise with modern resin-based composites (RBCs) extending their field of indication and their steady improvement in aesthetic and mechanical features, the question of durability of RBCs is of increasing importance. One major aspect compromising the long-term success of RBCs is the formation of secondary caries which may lead to the failure of restorations [2].

The selection of different adhesive material classes used in this study aims at representing a wide range of conventional adhesive filling materials, including ormocers, hybrid composites, microfiller composites and nanocomposites. Due to their good mechanical and aesthetic properties, these materials are the most commonly used filling materials and are therefore highly clinically relevant [44].

For this study, five primary colonizers were examined in a mixture regarding the extent of adhesion to five different filling materials. Bacteria are never isolated within the natural oral flora, and within a mixture adhesion of individual bacterial species can be promoted or prevented [45]. Thus, we decided to use a bacterial mix for the present study in order to mimic the natural conditions of the oral cavity and thereby provide more transferable and clinically relevant results [46]. As mentioned above, another important reason for using a mix is that primary colonizers interact with each other [47] and coaggregate, enabling and promoting adhesion to materials and the formation of biofilm [48]. The bacterial species selected for this study are classic primary colonizers, meaning they are among the first bacteria to colonize pellicle-coated surfaces in the oral cavity [49]. Investigating streptococci and actinomycetes in the mix is useful because these species contribute to plaque formation in different ways: streptococci stabilize the biofilm matrix, while actinomycetes contribute to its structure [50]. The selection of species also represents different adhesion mechanisms, as streptococci often adhere via protein-receptor interactions, while actinomycetes can adhere to surfaces by forming a sticky polysaccharide matrix [50]. As reflected in our results where *A. spp*. adhered by 100% to the materials, previous research has shown that Actinomyces species have strong adhesion properties because of type 1 fimbriae which allow attachment to proline-rich proteins and type 2 fimbriae which further allow attachment to streptococcal structures [51]. The other streptococci species differ in their characteristic, e.g., *S. gordonii* seems to bind to amylase, an enzyme present in saliva which may vary in amount from individual to individual and might coat differently on different surfaces [52]. Other studies have found different adhesion capacity of different subspecies of *S. oralis* indicating a general high variability of adhesion capacity of bacteria which is in accordance with our results [53]. Furthermore, the selected bacteria enable other potentially pathogenic species to colonize the biofilm [15] and can behave pathogenically themselves [54–56]. It has long been known that *S. mitis* in particular occurs in carious dentin in teeth affected by root caries [54]. On the other hand, *S. sanguinis* is associated with oral health [57] and inversely with caries [58] and could have an antagonistic effect on cariogenic bacterial species such as S. mutans [57–59]. At the same time, however, *S. sanguinis* is also considered to be a cause of infectious endocarditis [55,56].

In the present study, the incubation period of the bacteria on the test specimens was selected to be eight hours. During the first six to eight hours of biofilm formation, primary colonization takes place which lay the foundation for the further growth of the biofilm [48]. After this time period,

 doi:10.20944/preprints202602.2025.v1

maximum adhesion of primary colonizers is reached [60]. Thus, a longer incubation period of eight hours is appropriate in order to achieve and represent maximum possible adhesion. Thus, a longer incubation period is not necessary for the investigation of primary colonizers as secondary colonization begins at this point [61] which exceeds the aim of our study. A shorter incubation time, on the other hand, might not fully capture the complete extent of adhesion [60].

The results revealed no statistically significant differences in bacterial adhesion between the materials for three (*S. gordonii*, *S. oralis*, *A. spp.*) of the five bacterial species examined. This is consistent with the results of other studies that have also investigated primary colonization and found no significant results [19,26,27,38]. Significant differences in bacterial adhesion to different adhesive materials were demonstrated in the present study for *S. mitis* and *S. sanguinis*. Regarding *S. mitis*, this contradicts the findings of Bilgili et al., who were unable to find any significant differences in the adhesion of *S. mitis* to the bulk-fill materials they examined [32]. Possible reasons for this could be methodological, as this study differs significantly from the present study in terms of incubation time and *S. mitis* was examined in monoculture rather than in a mixture [32]. In other studies that included *S. mitis* [10,18,36,39] the focus of the researchers differed from that our study, e.g. by focusing on the influence of pellicle [36] or surface roughness [34] on the interactions of different species on adhesion [39] or on the investigation of experimental composites [10,18]. Therefore, the results cannot be compared with those of the present study. However, in a study where adhesion on rough and smooth titanium surfaces were examined, *S. mitis* adhered significantly less to the rough surface compared to the smooth one [62]. However, Actinomyces species showed no significance difference in adhesion between the different surfaces [62]. Those results may be comparable to the findings in our study where *S. mitis* appeared to be more adherent to the less rough nano-textured surface compared to the other materials while no significant difference was found for A. spp.. Another study has also found *S. mitis* to be adhere more to smoothened resin surfaces of dentures [63].

Studies on bacterial adhesion can only be compared to a limited extent due to a wide variability in methodology and a large variance in the definition of composite material groups both in the literature and in manufacturer specifications. In addition, the results of the present study should be interpreted and considered within the limitations of an *in vitro* study. This study was conducted as an *in vitro* experiment in the laboratory under static conditions which can only represent the conditions prevailing in the oral cavity to a limited extent. Therefore, the design of this study lacks dynamic factors such as salivary flow, pH and temperature fluctuations, chewing pressure, and abrasion or attrition processes which could influence bacterial adhesion [64,65]. In addition, this study focuses on a selection of five primary colonizers, although there is a much greater diversity of bacterial species in the intraoral environment [66]. *Fusobacterium nucleatum* should be mentioned here as an important bacterium that connects the primary colonizers and thus acts as a bridge builder [67]. It is therefore an important component of initial biofilm formation, as it is able to aggregate with many other species [67].

An additional limitation of the present study is that no additional physicochemical surface characterization (e.g., surface roughness, contact angle measurement, or surface free energy) was performed, although these parameters are known to influence bacterial adhesion to restorative materials [15]. Although all specimens were polished using a standardized two-step polishing protocol under controlled pressure and rotational speed, subtle differences in filler size, filler distribution, and matrix composition may still have resulted in differences in surface topography and hydrophilicity, as variations in composite formulation have been shown to affect surface characteristics after polishing [68]. These parameters are known to influence protein adsorption and subsequent bacterial adhesion [69]. Therefore, the observed differences in adhesion, particularly for *S. mitis* and *S. oralis*, may partially reflect material-specific surface characteristics rather than solely compositional differences [69].

Additionally, there are noteworthy limitations regarding the qPCR. Contamination of substrates is a major limitation for qPCR. It has been shown that even contamination within reagent kits, buffer

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or water may be common [70]. Other potential sources of contamination include cross-contamination between the samples or through aerosolized amplification products from PCR that may contaminate the laboratory environment including reagents and equipment [71]. Further, internal contamination is possible as potential untargeted primers are amplified during PCR and serve as substrates for following cycles causing large amounts of the amplified product, this phenomenon is described as carryover [72]. Therefore, validation of primers and the use of negative controls should be highly encouraged in order to prevent falsification of data obtained from PCR [73]. In contrast to qPCR, real-time PCR (RT-PCR) eliminates the risk of carryover as signals are directly measured and scrutinized [74]. However, as our aim was to evaluate the mere DNA concentration of bacteria and not specific gene expression, qPCR was appropiate for this study.

Furthermore, quantitative determination of bacterial DNA using SYBR green qPCR does not provide any information about the vitality of the adhering bacteria; this could be determined by further investigations such as live-dead staining.

5. Conclusions

A final statement on the recommendation of specific filling materials cannot be given. Due to the significant importance of secondary caries as a common cause for the need for replacement of composite fillings [2] and the fact that bacterial adhesion significantly increases the risk of developing secondary caries [7], further research on this topic is of great interest. In addition, with the EU-wide ban on amalgam from 2025, materials such as self-adhesive composites, self-adhesive composite hybrids, glass carbomers, alkasites and giomers may gain importance, as they could be used as amalgam alternatives in standard care. Thus, investigating the adhesion of initial colonizers to these materials could contribute to their establishment. The investigation of other filling materials such as glass ionomer cements, other composites or typical materials used in pediatric dentistry, such as compomers, is also useful for gaining further insights and improving the comparability of studies.

Moreover, the composition of the bacterial mix could be adjusted to the same concentration ratio as they occur *in vivo*. Studies with other species, such as *Fusobacterium nucleatum*, could also be of interest.

Further research to investigate the causes of the results of this study, e.g. using experimental composite formulations, is needed for improving material. In the long term, to the aim should be advancement and optimization resulting in materials that are less susceptible to plaque.

The transfer of the *in vitro* study protocol to *in vivo* studies may also be of interest as a future research approach. Study protocols with a split-mouth design and *in situ* carrier splints are conceivable here.

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