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Research Article

Reexamination *In Vitro* and *In Situ* of an Antibacterially Modified Experimental Dental Resin Composite with Molecular Methods: A Pilot Study

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Purpose. To introduce additional methods to detect and to quantify single pathogens in the complex biofilm formation on an antibacterial dental material. *Materials and Methods*. A conventional (ST) and an antibacterial dental composite (B) were manufactured. *In vitro*: specimens were incubated with a mixture of early colonizers. Bacterial adhesion was analyzed by TaqMan PCR after 8/24 h. *In situ*: TaqMan PCR and 16S rRNA Next Generation Sequencing (NGS) were performed. *Results*. *In vitro*: after 8 h incubation, B was covered by 58.6% of the bacterial amount that was attached to ST. After 24 h, the amount of attached bacteria to ST remained constant on ST only slightly lower on B. *In situ*: after 8 h the amount of adhering *A. viscosus* and *S. mitis* was prominent on ST and reduced on B. NGS revealed that *S. sanguinis*, *S. parasanguinis,* and *Gemella sanguinis* were the mainly attached species with *S. sanguinis* dominant on ST and *S. parasanguinis* and *G. sanguinis* dominant on B. *Conclusions*. Initial biofilm formation was altered by B. A shift between actinomycetes and streptococci was observed *in situ*. TaqMan PCR and 16S rRNA NGS revealed comparable results *in situ* and demonstrated the usefulness of NGS to characterize complex bacterial communities.

1. Introduction

Pellicle and bacterial adherence is considered to play an important role in early plaque formation [1–3]. It is of interest to avoid or at least to diminish bacterial adherence not only on hard tooth tissues but also on dental restorative materials [4–7]. Hence many attempts have been made to modify dental resin composites to that effect [1, 8–13]. To test the expected antibacterial effects mainly confocal laser scanning microscopy [2, 13–15] or fluorescence microscopy [1–3, 8, 16] and predominantly bacteria as *S. gordonii* [17], *S. mutans* [13, 18–21], *S. mitis* [1, 8, 18, 22, 23], *S. oralis* [1, 8, 17, 18, 20], *S. sanguinis* [1, 8, 17, 24], *A. naeslundii* [1, 2, 8, 15], *A. odontolyticus* [21, 25], and *A. viscosus* [8, 26–28] were used.

This work focuses on the reexamination of the bacterial attachment to an experimental dental resin composite, called material B [1]. Material B is modified with 4% (m/m) of the antibacterial monomer (5-chloro-2-2,4-dichlorophenoxy)phenyl methacrylate (called Methacryl-Irga in the following); its antibacterial properties were examined in

Table 1: Raw material.

Code	Product/properties	Batch	Company
Photoini	α,α-dimethoxy-α-phenylacetophenone	0066162S	Ciba Specialities Chemical Inc., Basel, Switzerland
Stab	Pentaerythritol tetrakis[3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate]	26099IC3	
TTEGDMA	Tetraethyleneglycole dimethacrylate, standard monomer, functionality = 2, MW = 330 [g mol^{-1}], good chemical and physical properties, very low viscosity (14 Pa s, 25°C), diluting	J1620	Cray Valley, Paris, France
UV-Stab	2-Hydroxy-4-methoxybenzophenone	411351/143302	Fluka, Buchs, Switzerland
UDMA	7.7.9-Trimethyl-4,13-dioxo-3,14-dioxa-5,12-diaza-hexadecan-1,16-diol-dimethacrylate, standard monomer, functionality = 2, MW = 471 [g mol^{-1}], flexible, tough, very good chemical resistance, medium viscosity (10000 mPa s, 25°C)	330503057	Rahn AG, Zürich, Switzerland
Bis-GMA	Bis-GMA, standard monomer, functionality = 2, MW = 513 [g mol^{-1}], rigid, very good chemical resistance, very high viscosity (4500 mPa s, 60°C)	2008218303	
CQ	D,L-camphorquinone	0148990002	
Amine	Ethyl-4-(dimethylamino)-benzoate	310170	
Glass	Strontium borosilicate glass (Glass G0 18-093, 0.7 μm). Silaned (3-methacryloyloxypropyltrimethoxy silane), D = 2.6 [g cm^{-3}]	Lab14701	Schott Electronic Packaging GmbH, Landshut, Germany
Methacryl-Irga	5-Chloro-2-(2,4-dichlorophenoxy)phenyl methacrylate		University laboratory

Information is based on the manufacturers' technical data sheets.

monoclonal *in vitro* settings in comparison with a standard, called ST, with fluorescence microscopy [1]. (5-chloro-2-2,4-dichlorophenoxy)phenol is known under the trademarks Irgasan or Triclosan, respectively, and is a well-proven broad spectrum antimicrobial agent. It inhibits the enoyl-acyl-carrier protein reductase component of type II fatty acid synthase in bacteria, the mammalian fatty acid synthase, and provides anticariogenic activity [29, 30]. Irgasan/Triclosan was used to synthesize Methycryl-Irga [1]. The aforesaid investigation reports good antibacterial effectiveness for material B for the streptococci *S. oralis* and *S. sanguinis* and *S. mitis* and for the actinomycetes *A. naeslundii* and *A. viscosus* [1].

The goal of the present investigation was to analyze bacterial mixtures instead of a single isolated species of the aforesaid bacteria in their *in vitro* and *in situ* attachment to ST and the antibacterially modified material B by real-time PCR (TaqMan-PCR) and Next Generation Sequencing (NGS). Since the published TaqMan-PCRs to detect *A. naeslundii* [31, 32], *A. viscosus,* or *S. mitis* [31] were not found to be sufficient specific and sensitive when reevaluated they were newly designed. Although the present work must be considered as a pilot study it is thought to be valuable because additional methods are introduced to detect and to quantify simultaneously single pathogens in the complex situation of biofilms. It was hypothesized that there is no difference in total bacteria load between the materials ST and B.

2. Material and Methods

For detailed information about manufacturing of the test materials, refer to the literature [1]; the respective raw materials are listed in Table 1. The standard ST represents a conventional dental resin composite. Material B differs from ST by replacing 4% (m/m) of the resin matrix with (5-chloro-2-2,4-dichlorophenoxy)phenyl methacrylate (see Table 2). N = 28 discs (thickness: 1 ± 0.1 mm; diameter: 10 ± 0.1 mm) were made and cured 40 s each side and then stored for 14 days in water at 37°C, disinfected, and wet-polished (sterile water) on one side with fine and superfine polishing-discs according to [1]. Twenty-four discs were used for the *in vitro* and four for the *in situ* adhesion assay.

2.1. Bacteria

2.1.1. Bacterial Plate Cultures. To test the analytical specificities and the sensitivities of primers and probes bacteria (Table 3) were grown on COS blood agar plates (bioMerieux GmbH, Nürtingen, Germany) and incubated for 48 h at 37°C: actinomycetes under anaerobic conditions and streptococci under 5% (v/v) carbon dioxide. The same culture conditions were utilised for counting the CFUs of each strain.

2.1.2. Bacteria Liquid Cultures for In Vitro Adhesion Assay. Bacteria were grown overnight at 37°C in 2.98% (w/v) thioglycollate (THIO-) broth (Merck GmbH, Darmstadt, Germany), streptococci by shaking under atmospheric air, and actinomycetes under anaerobic conditions. Dilutions of 1 : 6 to 1 : 10 were cultivated for 2–4 h up to a turbidity of 1 McFarland, which corresponds to an optical density of 0.2–0.3 OD at 600 nm. Cells were harvested by centrifugation (5 min at 4,000 ×g) and suspended in the same volume of PBS, and a mixture of bacterial suspensions in equal parts was made. Total genomic DNA of 350 μL bacterial mixture

Table 2: Formulations of experimental resin-based restorative materials (ST = standard).

Experimental resin-based filling materials formulations [weight %]		
ST	B	Raw material
73.00	73.00	Glass
—	4.00	Methacryl-Irga
27.00	23.00	Matrix

Matrix: UDMA = 44.10, Bis-GMA = 30.00, TTEGDMA = 25.00, photoinitiator = 0.30, CQ = 0.20, amine = 0.10, and stabilizer = 0.10.

was prepared for subsequent quantification of each species by TaqMan-PCR.

2.2. In Vitro Adhesion Assays. Human saliva was collected from ten healthy volunteers, prepared, and sterilized according to Rüttermann et al. [8]. Approval from the ethics committee was obtained (Ethics Committee of the Medical Faculty of Heinrich-Heine-University, Düsseldorf, Germany, internal study number: 2912). The participants gave their consent verbally, as they all were part of the academic faculty staff of the department. Documentation of the oral consent was done by making a list of prospective saliva donors. All donors who subsequently gave their oral consent were checked-off on this list. The oral consent was explicitly judged as adequate, and the need for written informed consent from the participants was waived by the ethics committee. For each test material (ST, B) and incubation period (8 h/24 h), six discs (24 discs in total) were placed with the polished side up in a 24-well plate and preincubated with 250 μL sterile saliva (1 : 2 in PBS) for 2 h at 37°C. Next, the disks were gently rinsed two times with PBS (37°C) and 350 μL of the bacterial mixture was added to each pellicle coated disk and incubated at 37°C under 5% CO_2 (v/v) for 8 h or 24 h. PBS was used as negative control. After 8 h or 24 h of incubation the supernatant was removed and the disks were gently washed with 300 μL PBS (37°C) and transferred to a clean well of a 24-well plate before preparation of total genomic DNA.

2.3. In Situ Adhesion Assays. The Ethics Committee of the Medical Faculty of Heinrich-Heine University Düsseldorf, Germany, stated that there is no need for further vote as the two participants of the *in situ* experiment were two of the authors themselves (UP and SR), who gave their written consent. Their saliva samples and mucosal swabs were taken for analysis. One disk of each material was separated into four parts to obtain suitable pieces to be inserted in individual oral splints on the upper jaw at each molar on the buccal side [33, 34]. The splints were steadily worn for 8 hours overnight, removed, and gently washed with 300 μL PBS (37°C) and all quarters of a disk were transferred to one clean well on a 24-well plate prior to preparation of total genomic DNA.

2.4. Preparation of Total Genomic DNA. Total genomic DNA was isolated with the Gram-positive protocol of the DNA Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany). (a) Cells of the bacterial suspensions were collected by centrifugation (10 min at 13,000 ×g), resuspended in 300 μL enzymatic lysis buffer, and incubated for 30 min at 37°C. (b) Bacterial-coated disks were incubated with 300 μL lysis buffer and the 8 h specimens after overnight storage at −20°C were incubated for at least 30 min at 37°C.

30 μL Proteinase K (>600 mU/mL) and 300 μL Buffer AL (Qiagen GmbH) were added to each sample and incubated at 56°C for 1 h. After addition of 300 μL ethanol mixtures were transferred to DNeasy Mini spin columns (Qiagen GmbH). Total genomic DNA was eluted in 100 μL and stored at −20°C until use.

Table 3: Bacterial strains used for *in vitro* experiments.

Species	Strain
Achromobacter xylosoxidans	Isolate SP 6486-2012
Actinomyces spp.	
A. europeus	INSTAND-strain 2004
A. neuii anitratus	INSTAND-strain 2003
A. naeslundii	ATCC 12104 = DSM 43013
A. odontolyticus	ATCC 17929 = DSM 43760
A. odontolyticus	Isolate SP6320-2012
A. spp (formerly viscosus)	DSM 43329
A. urogenitalis	INSTAND-strain 2002
A. viscosus	ATCC 15987 = DSM 43327
Escherichia coli	K12 derivate DH5α
Haemophilus influenzae	Isolate SP 299-2013
Streptococcus spp.	
S. gordonii	DSM 6777
S. intermedius	Isolate SP14964-2012
S. mitis	ATCC 49456 = DSM 12643
S. oralis	ATCC 35037 = DSM 20627
S. sanguinis	DSM 20068
Veillonella parvula	ATCC 17745

2.5. Design of Primers and Probes. Multiple sequence alignments were calculated with the MegAlign algorithm of the DNASTAR sequence analysis software (DNASTAR Inc., WI, Madison, USA). Gene sequences were retrieved from the nucleotide database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/nucleotide/). The final design of the specific primers and probes (Table 4) (MWG-Biotech GmbH, Ebersberg, Germany; metabion GmbH, Planegg-Martinsried, Germany; Eurogentec, Seraing, Belgium) was either calculated with the Primer Express Software (Applied Biosystems Inc., Foster City, CA, USA) or taken from the literature [35].

2.6. TaqMan-PCR. In house TaqMan-PCRs were carried out in a total volume of 25 μL consisting of 12.5 μL Eurogentec PCR MasterMix NO ROX (Eurogentec GmbH Reference: RT-QP2X-03NR, containing buffer, dNTPs with dUTP, HotGoldStar DNA polymerase, 5 mM $MgCl_2$, Uracil-N-glycosylase, and stabilizers), 300 nM of each forward and reverse primer,

Table 4: Design of the specific primers and probes.

Species/ acc. number	Gene	Primers/ probes	Sequences (5′-3′)	Amplicon length/bp	References
Eubacteria	16S	Euba-F	TGGAGCATGTGGTTTAATTCGA	143	This study
X58308	rDNA	Euba-R	TGCGGGACTTAACCCAACA		
		Euba-S	TexRed- CACGAGCTGACGACARCCATGCA- BHQ2		
Actinomyces spp.	16S	Act-F	GGT CTC TGG GCC GTT ACT GA	112	Bizhang et al., 2011 [35]
AJ635359	rDNA	Act-R1	TGG CCC CCA CAC CTA GTG		
		Act-R2	GRC CCC CCA CAC CTA GTG		
		Act-S	CTG GTA GTC CAC GCC GTA AAC GTT GG		
A. odontolyticus	16S	Act-F	GGT CTC TGG GCC GTT ACT GA	123	This study
AB818950	rDNA	Aodo-R	CGCAGAAACCACGGGTGG		
		Act-S	CTG GTA GTC CAC GCC GTA AAC GTT GG		
A. naeslundii	16S	Anae-F	GGGCCTGGGAAAGATTG	131	This study
M33911	rDNA (3 pro genome)	Anae-R	TGACCGTGCACCCTCTCA		
		Anae-S	FAM-TTTTTGGTGTTTTTGGTGGGGGATC-BHQ-1		
A. viscosus	nanH	Avis H-F	GTCGACAACCAGACGGGC	76	This study
EU805603	(1 pro genome)	Avis H-R	CGGCCTGTGAGTGGCC		
		Avis H-S	FAM-TTCCACGTCAAGTCCTTCGACCAGG-BHQ-1		
S. gordonii	gtfG	Sgor-F	AGCTAGTGCGGCAACCAATC	87	This study
U12643	(1 pro genome)	Sgor-R	TGTCTGGAGTCAGTTCTTGAAGTTTC		
		Sgor-S	FAM-AGCTAACGAACAGACGACGCAACAGGAT-BHQ1		
S. mitis	16S	Smit-F	GAGCTTGCTTCTCCGGATGA	141	This study
AB682354	(4 pro genome)	Smt-R	AATTGCACCTTTTAAGCAAATGTCA		
		Smit-S	Hex-TTGCGAACGGGTGAGTAACGCGT-BHQ1		
S. oralis	gtfR	Sora-F	GGCCCTTATTGTAGCACCAAAG	101	This study
AB025228	(1 pro genome)	Sora-R	TCTGAGCTGTAGCAGGTTCTTGA		
		Sora-S	FAM-TCCATGCGGATGATGTAAAGCAGGTTGTAG-BHQ1		
S. sanguinis	gtfP	Ssan-F	GCAAAAAAGACTGTTACAGACAARATTG	96	This study
AB252650	(1 pro genome)	Ssan-R	AGCTATCGCTCCCTGTCTTTGA		
		Ssan-S	HEX-AGGTTGCAAAGAAAGATCGCTTGCCA-BHQ1		

200 nM labelled probe, and 2.5 μL of template DNA. Quantification standards were amplicon carrying plasmids in three different concentrations (10^5, 10^3, and 10^2 copies/μL) (Table 4). Each sample was analyzed twice. Thermal cycling, fluorescent data collection, and analysis were done with the iCycler (BioRad Laboratories, Munich, Germany). The cycling program was as follows: 1 cycle at 50°C for 10 min and 1 cycle at 95°C for 10 min followed by 45 cycles at 95°C for 15 s and 60°C for 1 min.

2.7. PCR Amplicon Library Preparation. PCR amplification of the 16S rDNA hypervariable region V6 was done with a pool of five degenerated forward and four degenerated reverse primers expected to target the known groups of bacteria according to Huber et al. [36]. The V6 region was bidirectional sequenced with two different primer sets: (a) 5′-ends of the forward primers fused with the A-adaptor and the reverse primers 5′-ends fused with truncated P1 (trP1)-adaptors (Life Technologies GmbH, Darmstadt, Germany, User Guide #4468326C, Ion Amplicon Library Preparation, Fusion Method) and (b) trP1 fused to the 5′-ends of the forward primers and A to the 5′-ends of the reverse primers.

Per 25 μL PCR reaction the amplicon library preparation consisted of 1 μL DNA (1% (v/v) total DNA-preparation/sample, volunteer 1 only), 1 U Platinum Taq DNA Polymerase High Fidelity, 2 mM $MgSO_4$, 0.2 mM dNTPs, and 0.4 μM of each forward and reverse primer. The cycling was as follows: 94°C/3 min, 36 cycles: 94°C/15 s, 58°C/30 s, 68°C/1 min, and finally 68°C/30 min and hold at 4°C. The PCR-quality was checked with gel electrophoresis on 2% (w/v) agarose gel in 1x Tris Borat EDTA buffer. Amplicon library was purified (QIAquick PCR Purification Kit, Qiagen GmbH) and quantified (Qubit Quant-iT HS DNA Assay, Invitrogen GmbH, Darmstadt, Germany) and finally quality and size

of amplicons were determined with the Agilent 2100 Bioanalyzer DNA Lab Chip 1000 (Agilent Technologies GmbH, Böblingen, Germany). The concentration of the amplicon library for clonal amplification was adjusted to 6 pmol/L.

2.8. Emulsion PCR and Sequencing. The emulsion PCR for enrichment of template positive IonSphere particles was done with the Ion OneTouch 200 Template Kit (Life Technologies GmbH) and the quality of the IonSphere particles was checked with the Qubit 2.0 Fluorometer (Invitrogen GmbH) and the IonSphere Quality Control Kit (Life Technologies GmbH). The amplicon libraries were sequenced twice to increase sequencing depth with the Ion Torrent Personal Genome Machine (PGM), the Ion Torrent 314 Chips, and the Ion PGM 200 Sequencing Kit (Life Technologies GmbH) according to the respective protocol.

2.9. NGS (Next Generation Sequencing) Analysis

2.9.1. Sequencing Read Preprocessing. Raw sequencing reads were filtered for low quality and PCR primer sequences applying three consecutive quality filter steps to the two individual sequence data sets per *in situ* sample: (a) reads <10% of bases quality score ≤25 were discarded (FASTQ quality filter/FASTX-Toolkit, http://cancan.cshl.edu/labmembers/gordon/fastx_toolkit), (b) PCR primer sequences were removed by semiglobal alignment with Cutadapt (http://journal.embnet.org/index.php/embnetjournal/article/view/200), and (c) in Cutadapt the minimum length of the trimmed reads was set to 50 bases.

2.9.2. Diversity and Taxonomic Analyses. Preprocessed high quality sequence reads were by BLAST (Basic Local Alignment Search Tool) applying the Human Oral Microbiome Database (HOMD), version 13.2, 16S rRNA RefSeq, according to Chen et al. [37]. BLAST analyses were calculated with the CLC Genomics Workbench Software Version 6.0.5 (Qiagen GmbH) applying CLC standard BLAST parameters. Query sequences matching more than one target sequence with equal BLAST scores were randomly assigned.

Matching reads per taxon were normalized for the total number of query sequences for each sample to compare the relative bacterial composition of the two *in situ* samples. The cut-off for comparative analysis of relative abundances was set to 0.1% after normalization to focus on a more robust subset of the identified taxa.

2.10. Statistics

2.10.1. In Vitro Adhesion Assay. Each material, ST and B, was analyzed sixfold at 8 h and 24 h, respectively. In TaqMan-PCR, DNA samples were analyzed in duplicate. Descriptive statistics were accomplished; that is, means and standard deviations were calculated.

2.10.2. NGS Analysis. Technical reproducibility of the Ion Torrent sequencing reaction was controlled using linear regression coefficient analysis.

3. Results

3.1. TaqMan-PCRs. Species-specific regions for the bacteria tested and the number of copies per genome are shown in Table 4. Due to three (*A. naeslundii*) and four (*S. mitis*) 16S rDNA copies per genome, these TaqMan-PCRs had higher sensitivities than the other ones which targeted single copy genes. For *A. viscosus*, *S. oralis*, *S. sanguinis*, and *S. gordonii*, the glucosyltransferase (GTF) gene (existent in most streptococci [38]) was used for targeting *S. gordonii* (*gtf*G), *S. oralis* (*gtf*R), and *S. sanguinis* (*gtf*P) and the sialidase gene (*nan*H) for targeting *A. viscosus*. The efficiency of the TaqMan-PCRs ranged from 94.5% (*S. sanguinis*) to 99.9% (*A. odontolyticus*) and the coefficient of determination ranged from 0.990 to 0.999 (ideal value = 1.000). Cross reactions with cognate species amounted to less than 0.001%.

3.2. In Vitro Adhesion Assay. The quantification of the different species in the inoculum with TaqMan-PCRs proved the bacterial concentration of a McFarland 1 suspension as species-specific but not always of the same value (Figure 1). Streptococci were overrepresented in the inoculum in contrast to the actinomycetes. After 8 h of incubation the antibacterial material B was covered with 58.6% of the bacterial amount of the standard material ST. After 24 h the amount of attached bacteria remained constant on material ST (99.8%) and increased on material B to 92.6% of the bacterial amount on ST. The bacterial composition on both materials was quite similar. More than 90% of the attached bacteria accounted for *S. sanguinis*, *S. oralis*, and *S. gordonii* with 36% *S. oralis* after 8 h and 50% after 24 h of incubation. *A. viscosus* (3%), *A. naeslundii* (<1%), and *S. mitis* (1%) were underrepresented on both materials ST and B after 8 h which further declined after 24 h (Figure 1 and Table 5).

3.3. In Situ Adhesion Assay. The bacterial loads in saliva of volunteers 1 and 2 were similarly concentrated ($1.18E+7$ and $2.2E+7$, resp.), dominated by *A. odontolyticus* in volunteer 1 (Figure 2(a1)) and *A. odontolyticus* and *S. mitis* in volunteer 2 (Figure 2(a2)). The diversity of bacteria and their quantities varied in the mucosal swabs volunteer-specifically (Figures 2(b1) and 2(b2)). Volunteer 1 showed less bacterial load than volunteer 2. *A. viscosus* was missing and the streptococci dominated. Volunteer 2 showed 1,000-fold increased actinomycetes and streptococci with a dominance of streptococci.

After 8 h of *in situ* carriage, *A. viscosus* and *S. mitis* dominated on ST with respect to both volunteers (Figures 2(c1) and 2(c2)). The load of these species was heavily reduced on material B of volunteer 1, but on material B of volunteer 2 the reduction was only due to the disappearance of *A. viscosus* (Figure 2(c2)). Volunteer 1 showed 10-fold increased bacterial load on the test materials compared to volunteer 2.

3.4. Next Generation Sequencing (NGS). Reproducibility of the 16S amplicon sequencing reactions was confirmed by comparing two duplicated data sets from each of the materials ST and B which showed very good correlations of 99.97%

Table 5: Results of the *in vitro* adhesion assay after 8 h and 24 h. Amounts (mean (StDev)) of *A. viscosus* (Avis), *A. naeslundii* (Anae), *S. mitis* (Smit), *S. oralis* (Sora), *S. gordonii* (Sgor), and *S. sanguinis* (Ssan) on materials ST and B.

		Ssan	Sgor	Sora	Smit	Anae	Avis
Input	CFU*	1,23*E* + 06	1,72*E* + 06	3,56*E* + 05	2,94*E* + 05	3,11*E* + 05	8,57*E* + 05
		(6,04*E* + 05)	(1,83*E* + 06)	(2,15*E* + 05)	(3,75*E* + 05)	(1,27*E* + 05)	(6,79*E* + 05)
	GE	7,30*E* + 07	4,57*E* + 07	5,98*E* + 07	1,31*E* + 07	6,02*E* + 06	1,66*E* + 07
		(3,66*E* + 07)	(2,18*E* + 07)	(6,61*E* + 07)	(6,14*E* + 06)	(5,22*E* + 06)	(1,30*E* + 07)
8 h	ST	1,19*E* + 07	1,24*E* + 07	1,44*E* + 07	4,05*E* + 05	1,04*E* + 05	1,12*E* + 06
		(2,3*E* + 06)	(1,63*E* + 07)	(7,66*E* + 06)	(2,59*E* + 05)	(3,74*E* + 04)	(6,58*E* + 05)
	B	8,85*E* + 06	5,27*E* + 06	8,50*E* + 06	2,30*E* + 05	4,96*E* + 04	7,53*E* + 05
		(2,07*E* + 06)	(7,87*E* + 06)	(4,36*E* + 06)	(1,46*E* + 05)	(2,63*E* + 04)	(4,39*E* + 05)
24 h	ST	1,13*E* + 07	7,89*E* + 06	2,01*E* + 07	2,90*E* + 05	4,72*E* + 04	6,45*E* + 05
		(5,99*E* + 06)	(8,27*E* + 06)	(1,86*E* + 07)	(2,03*E* + 05)	(2,14*E* + 04)	(4,20*E* + 05)
	B	1,18*E* + 07	5,64*E* + 06	1,92*E* + 07	2,41*E* + 05	3,15*E* + 04	4,57*E* + 05
		(8,03*E* + 06)	(6,12*E* + 06)	(1,46*E* + 07)	(1,38*E* + 05)	(1,62*E* + 04)	(3,72*E* + 05)

*CFU: colony forming units; GE: genome equivalents.

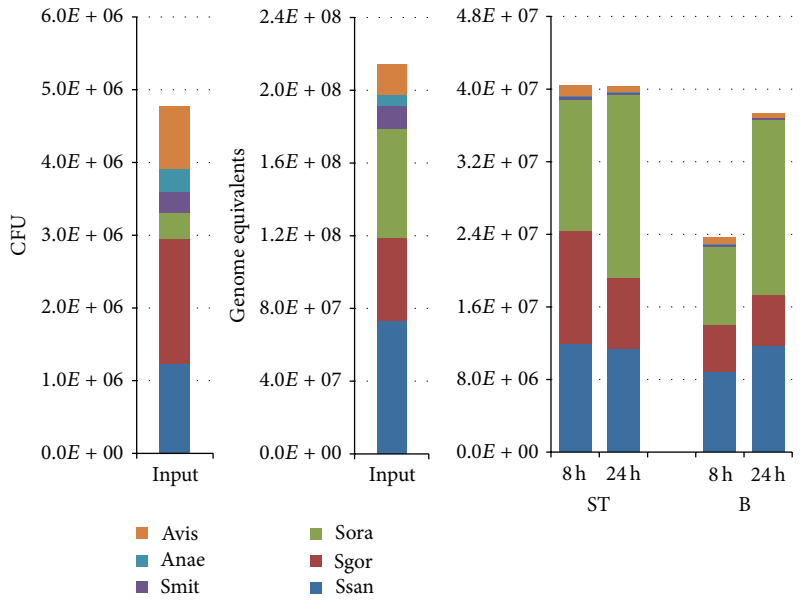

Figure 1: Results of the *in vitro* adhesion assay after 8 h and 24 h. Colony forming units and TaqMan-PCR genome equivalents of *A. viscosus* (Avis), *A. naeslundii* (Anae), *S. mitis* (Smit), *S. oralis* (Sora), *S. gordonii* (Sgor), and *S. sanguinis* (Ssan) in the inoculum (input) and attached to materials ST and B after 8 h or 24 h incubation.

and 99.51%, respectively. Therefore, after preprocessing the two filtered sequence data sets per *in situ* sample were combined for subsequent analyses to increase sequencing depth. Consequently, after 8 h *in situ* the specimens of volunteer 1 showed 504,710 for ST and 415,258 for material B high quality sequence reads, probably indicating a lower bacterial load of 82% on material B.

BLAST analyses of the test materials' sequences with HOMD 16S rRNA database yielded five taxa on the phylum and 11 taxa on the genus level for both of the materials (Figure 3(a)). Firmicutes were the dominant phylum, *Streptococcus* and *Gemella* were the dominant genera, and bacteroidetes were almost absent. Material B had higher amounts of actinobacteria especially of *Rothia* and *Actinomyces* than ST. Both materials showed 247 taxa species-wise with two sequence reads for the respective rRNA target sequence, and the main species were *S. sanguinis*, *S. parasanguinis*, and *G. sanguinis*. *S. sanguinis* (approx. 37% of the microbiota) and *S. parasanguinis* (approx. 25% of the microbiota) dominated on material ST, whereas *G. sanguinis* (approx. 25% of the microbiota) dominated on material B (Figure 3(b)). *R. mucilaginosa* and *A. odontolyticus* showed

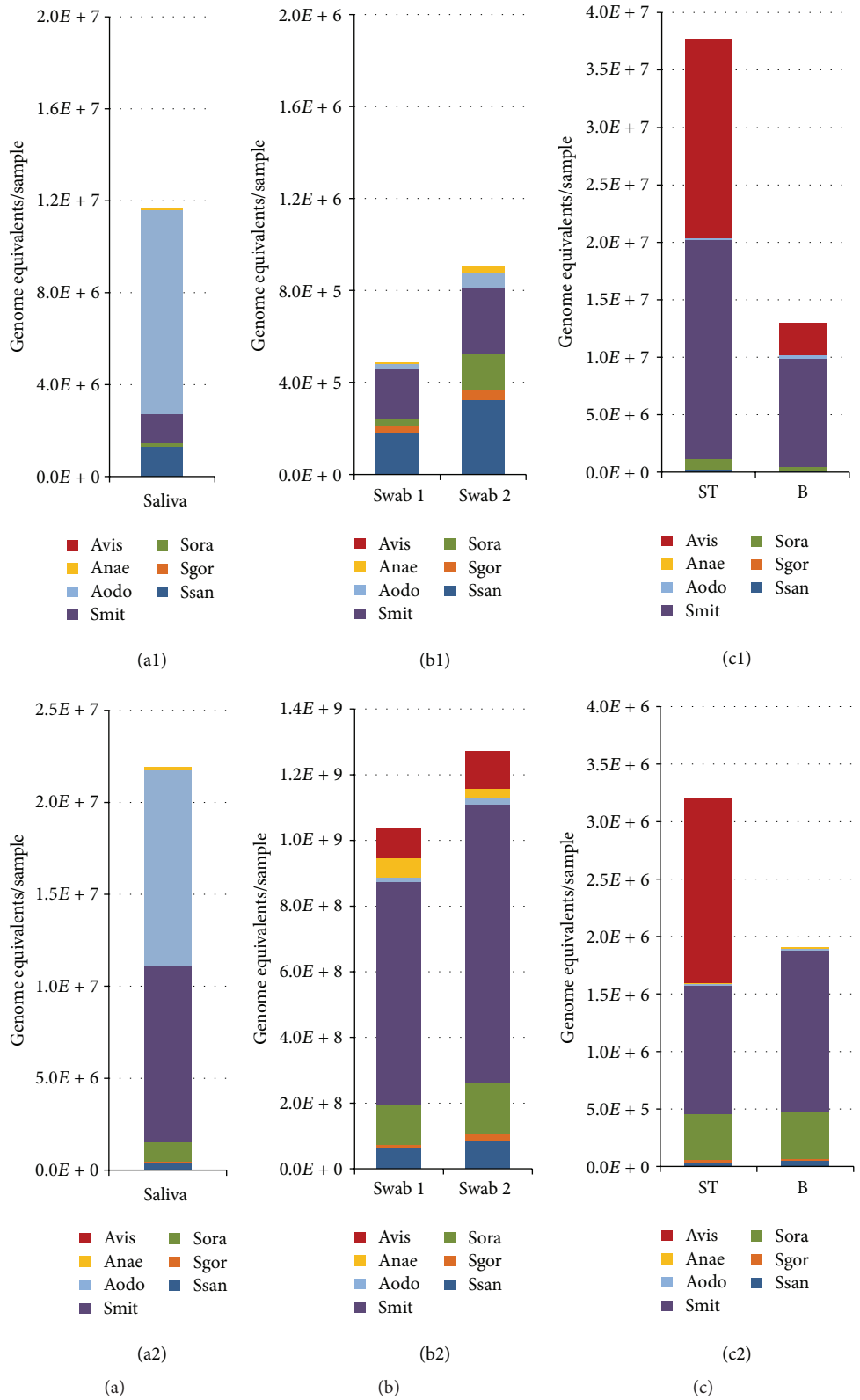

Figure 2: Results of the *in situ* adhesion assay after 8 h. TaqMan-PCR results of salvia (a1/a2), mucosal swabs (b1/b2), and the amount of *A. viscosus* (Avis), *A. naeslundii* (Anae), *S. mitis* (Smit), *S. oralis* (Sora), *S. gordonii* (Sgor), and *S. sanguinis* (Ssan) (c1/c2) after 8 h on ST and material B for volunteers 1 (a1, b1, and c1) and 2 (a2, b2, and c2).

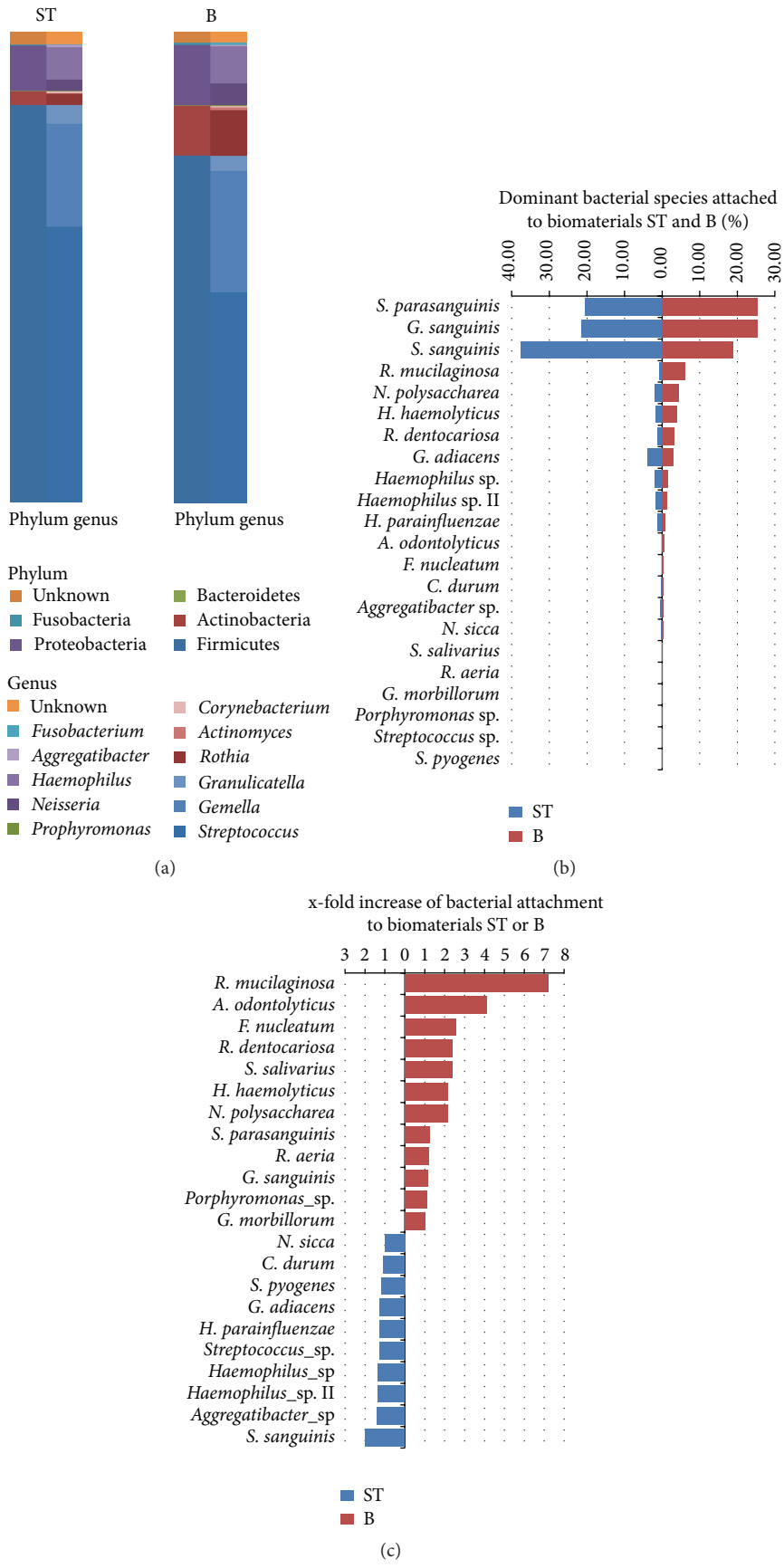

Figure 3: NGS results for materials ST and B for volunteer 1 after 8 h. (a) Phyla and genera, (b) species > 0.1% of microbiota, and (c) species x-fold increased.

seven- and fourfold higher amounts on material B, whereas *S. sanguinis* load was twofold higher on ST (Figure 3(c)).

4. Discussion

This pilot study reevaluates the antibacterial effect of the experimental dental resin composite material B in comparison with a standard dental resin composite ST [1] with more sophisticated methods. The test materials (raw materials, manufacturing/synthesis, formulation, properties, antibacterial effects, and preparation of specimens and saliva) and the rationale for their choice as well as for the selection of the bacteria (*A. viscosus, A. naeslundii, S. mitis, S. oralis,* and *S. sanguinis*) have already been thoroughly described by Rüttermann et al. [1, 8]. The results of the aforesaid literature are strongly limited because the attachment of only single isolated bacteria was investigated on the test materials with fluorescence microscopy [1]. Biofilm formation is very complex and does not include only the few bacteria individually tested. Much more bacteria live in the oral cavity and coexist with saliva and protein components. Therefore, it is certainly of scientific value to investigate the test materials' antibacterial effects on complex bacteria mixture *in vitro* and preferably *in situ*. This justifies the present pilot study which introduced TaqMan-PCRs and NGS to observe simultaneously distinct pathogens in complex bacterial mixtures. Though more bacteria were identified by TaqMan-PCRs and especially by NGS, only the formerly investigated species *A. viscosus, A. naeslundii, S. mitis, S. oralis,* and *S. sanguinis* [1] are discussed here.

Although TaqMan-PCRs for *A. naeslundii* [31, 32], *A. viscosus,* and *S. mitis* had been published, these PCRs were reevaluated and found to be less specific and sensitive. Therefore, the TaqMan-PCRs were newly designed for all investigated species.

The results of the *in vitro* adhesion assay, which were quantified by TaqMan-PCR (Figure 1), showed slightly fewer bacteria after 24 h on material B than on ST. The greatest difference was observed after 8 h of incubation, as the amount of attached bacteria on material B counted only 58.6% of the bacterial load on standard material ST. This contradicted our formerly published observation, found by fluorescence microscopy, that there was no difference of the total bacterial load between the incubation periods [1]. This might be due to the different incubation methods (PBS instead of medium) or to an altered adherence mechanism of the individual bacterial species in a polymicrobial mixture. Considering the differences between the test materials the present investigation and the former analysis of Rüttermann et al. [1] coincide that there are definitely fewer bacteria, considered overall as well as species-wise, on material B than on ST, especially in the first hours of biofilm formation (8 h). In contrast to the other streptococci, the adherence capacity of *S. mitis* was reduced on both materials tested (Figure 1). This finding is also in good accordance with formerly published results of our research group [1]. Although attachment of *A. viscosus* and *A. naeslundii* was reduced to less than 5% of the total bacterial load on both test materials, still fewer actinomycetes loads were detected on material B than on ST. Concluding, the distinct actinomycetes and streptococci species of the polybacterial mixture showed a similar attachment-behaviour like the monobacterial cultures [1]. In using Taqman PCR as a molecular approach, quantification of single species in complex bacterial mixtures is now possible, which was impractical by fluorescence microscopy. However, in contrast to the fluorescence staining method, in which the ratio between vital and nonvital cells can be estimated, amplification of bacterial DNA by TaqMan-PCR does not distinguish between DNA of a vital or nonvital cell. By implementation of propidium monoazide technique as a discriminator [38] in future, it should be possible to differentiate between vital and nonvital cells even in Taqman-PCR.

One should keep in mind that we used a static biofilm model to simulate the oral situation. We decided to do so, because the advantages of a dynamic model are not crucial for the short observation periods for the analysis of the initial biofilm (8/24 h).

The *in situ* TaqMan-PCRs (Figure 2) did not coincide in all aspects with its *in vitro* version after 8 h of incorporation. The mucosal swabs showed substantial differences in the bacterial composition and load between the volunteers (Figures 2(b1) and 2(b2)), which was also the case with the test materials (Figures 2(c1) and 2(c2)). The fact that some of the six species tested were not found in the saliva or rarely detected in the mucosal swabs but showed dominant adherence to the test materials illustrated that the interactions among the magnitude of oral species and between the species and the formulation of the test material play an important role in the resulting bacterial adhesion. Future studies should try to clarify these phenomena. Attempts that were made in biofilm research to simulate the oral cavity [39–41] revealed very low discriminatory power of single species, indicating that the oral cavity is certainly the best biofilm reactor at present.

Thus, Next Generation Sequencing has the opportunity to characterise the complexity of the oral microbiome in saliva and mucosal swab and attached to the test material and, depending on the discriminatory power of the target region, to enable a relative quantification of the bacterial families, genera, or species. The target region of the NGS analysis used here (Figure 3) was already described in the literature [42] to elucidate shifts in bacterial communities in subgingival plaque. The comparison of NGS and TaqMan-PCRs showed higher PCR-derived genome equivalents than NGS sequencing reads, but both confirmed lower loads of *A. viscosus* and *A. naeslundii* and higher loads of *A. odontolyticus* on material B than on ST (Figure 4). It was difficult to identify and to compare the quantities of the streptococci due to the low discriminatory power of the short V6 region in streptococci (e.g., *S. oralis*, *S. mitis*, *S. sanguinis*, *S. pyogenes*, and *S. gordonii*). TaqMan-PCRs proved lower loads of these streptococci to material B (Figure 4(b)) than NGS analysis. Identical NGS reads complicated the differentiation between *S. oralis*, *S. mitis,* or *S. sanguinis* (Figure 4(a): Ssan/Sora) and between *S. gordonii* and *S. pyogenes* (Figure 4(a): Sgor/Spyo). These results indicated that longer stretches of the hypervariable 16S rDNA regions are required for microbiota analyses down to species-level.

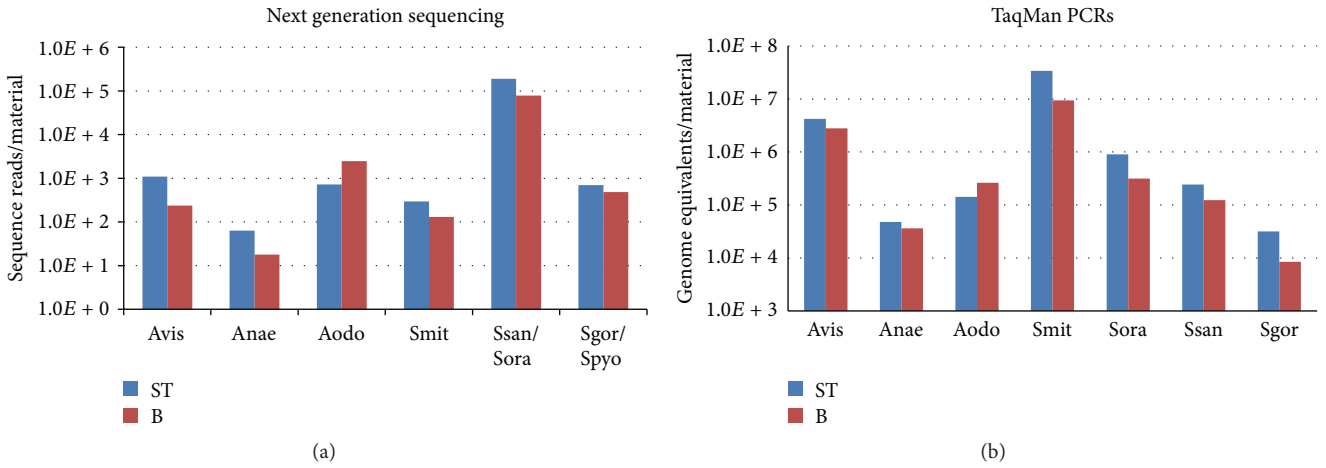

Figure 4: Comparison of NGS and TaqMan-PCR. (a) NGS and (b) TaqMan-PCR for materials ST and B. *A. viscosus* (Avis), *A. naeslundii* (Anae), *A. odontolyticus* (Aodo), *S. mitis* (Smit), *S. oralis*/*S. sanguinis* (Ssan/Sora), and *S. pyogenes*/*S. gordonii* (Sgor/Spyo).

5. Conclusion

The following can be concluded:

(i) *In vitro* and *in situ* TaqMan-PCRs and NGS analyses coincided with the literature [1] that fewer bacteria adhered on material B than on ST.

(ii) The literature's [1] species-wise testing of antibacterial effects gave only tentative results.

(iii) The literature [1] and the *in vitro* TaqMan-PCRs did not coincide with the *in situ* TaqMan-PCRs and the NGS in the differentiation of the adhered bacteria.

(iv) Sound results can only be obtained by *in situ*/*in vivo* testing.

(v) The following limitations need to be considered:

(a) TaqMan-PCR should be upgraded to differentiate between vital and nonvital cells.

(b) Target region of NGS analysis needs to be optimized to better differentiate between the (early oral colonizing) bacteria.

Although the statistical power of this study is too low to give detailed results, the data reveal that TaqMan-PCR and NGS analysis are highly valuable molecular methods to investigate biofilms on restorative materials in the oral cavity. Sound results can only be achieved by clinical studies and a statistically sufficient number of volunteers which will follow this pilot study.

Competing Interests

The authors declare that they have no competing interests.

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