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Biofilm composition and composite degradation during intra-oral wear

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ABSTRACT

Objectives. The oral environment limits the longevity of composite-restorations due to degradation caused by chewing, salivary and biofilm-produced enzymes and acids. This study investigates degradation of two resin-composites in relation with biofilm composition *in vitro* and *in vivo*.

Methods. Surface-chemical-composition of two Bis-GMA/TEGDMA composites was compared using X-ray-Photoelectron-Spectroscopy from which the number ester-linkages was derived. Composite-degradation was assessed through water contact angles, yielding surface-exposure of filler-particles. Degradation *in vitro* was achieved by composite immersion in a lipase solution. In order to evaluate *in vivo* degradation, composite samples were worn in palatal devices by 15 volunteers for 30-days periods in absence and presence of manually-brushing with water. PCR-DGGE analysis was applied to determine biofilm composition on the samples, while in addition to water contact angles, degradation of worn composites was assessed through surface-roughness and micro-hardness measurements. **Results.** *In vitro* degradation by lipase exposure was highest for the high ester-linkage composite and virtually absent for the low ester-linkage composite. Filler-particle surface-exposure, surface-roughness and micro-hardness of both resin-composites increased during intra-oral wear, but filler-particle surface-exposure was affected most. However, based on increased filler-particle surface-exposure, the high ester-linkage composite degraded most in volunteers harvesting composite biofilms comprising *Streptococcus mutans*, a known esterase and lactic acid producer. This occurred especially in absence of brushing. **Significance.** Degradation during intra-oral wear of a low ester-linkage composite was smaller than of a high ester-linkage composite, amongst possible other differences between both composites. *S. mutans* herewith is not only a cariogenic, but also a composite-degradative member of the oral microbiome.

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1. Introduction

The primary cause of dental caries is adhesion of bacteria forming biofilms on oral hard tissues [1,2]. Use of resin composites is the primary option for the repair of dental hard tissues affected by caries [3–5]. Though involving small volumes of biomaterial per restoration, this makes resin composites the most frequently used biomaterial worldwide in an environment that is highly demanding to the properties of the biomaterial. Oral bacteria not only adhere to and form biofilms on oral hard and soft tissues, but also on composite restorations and along the composite-tooth interface [6–8]. Biofilms can cause degradation of resin composites through bacterial production of degradative enzymes and acids [9–12] and thereby limit the functional and esthetic longevity of a restoration [13–15]. All materials used for the restoration of oral function, including metals, ceramics and composites, are prone to oral biofilm formation [16]. Although hydrophobic biomaterials attract less oral biofilm than hydrophilic ones [17,18] and rough surfaces more than smooth surfaces [19], the clinical relevance of “less or more”, i.e. “thin and thick” biofilms is hard to demonstrate.

Dental resin composites have four major components: (1) an organic polymer matrix, usually methacrylate based and containing ester-linkages, (2) inorganic, usually silica filler particles, (3) coupling agents such as silanes and (4) an initiator-accelerator polymerization system. Monomers can be added by manufacturers to reduce viscosity and increase polymerization. Inorganic filler-particles are intended to reinforce the resin matrix [20]. Conventionally, dental composites are classified by filler-size and type: macro-, micro-, nano-filled and hybrid ones, containing filler-particles with different sizes [21].

Degradation of composites is frequently studied *in vitro*, using models that only mimic one aspect of the oral environment, such as exposure to water [22], effects of organic and inorganic salivary components [23–25], thermocycling [26], growth of bacterial single- or multispecies laboratory-biofilms [27–29] or combinations of artificial saliva and acids [11]. In many cases, a relation between *in vitro* evaluations with the clinical performance of a composite during intra-oral wear is not clear [30] and dependent on the method applied to evaluate actual composite degradation.

The (near-)surface region plays a crucial role in the degradation of dental resin composites through adsorption and absorption of oral fluid components [31] and mechanical wear [32]. Whereas the onset of degradation is a surface phenomenon, absorption of enzymes and acids from saliva, food or beverage components into the composite and along the composite-tooth interface will eventually lead to softening of the bulk matrix [25]. Thus, degradation will first become obvious from altered surface properties of a composite, followed by changes in bulk properties. Accordingly, selection of surface-sensitive methods to detect early degradation of composites either after *in vitro* experiments or intra-oral wear is important, as use of more surface-sensitive methods will allow shorter experimentation times to reach statistically significant conclusions. Conventional methods to study composite degradation comprise (micro-)hardness measurements

[11,33], evaluation of material loss [10,12,34], surface morphology or roughness measurements [27,28]. Contact angle measurements exhibit an extreme surface-sensitivity at the level of a molecular monolayer, which make them highly suitable for the analysis of early composite degradation [11]. Differences in wettability of the relatively hydrophobic composite matrix versus the relatively hydrophilic filler-particles are reflected in equilibrium, advancing and receding water contact angles [11], and allow to calculate a percentage surface-exposure of filler-particles using the Cassie and Baxter equation (see Section 2 for more details) [35]. Surface modeling based on water contact angles measured on composite surfaces after artificial ageing in distilled water (180 days) or citric acid (14 days) [36] and after 14 days exposure to biofilms *in vitro* [37] has quantitatively indicated early composite degradation by increased surface-exposure of filler-particles, not readily detectable by more conventional micro-hardness and surface roughness measurements.

Biofilm formation on composite restorations will never be entirely preventable, while different biofilm inhabitants may not produce the same amount and types of enzymes and acids that may degrade composites during intra-oral wear, possibly pointing to the existence of a composite-degradative microbiome. Therefore, the aim of this study was firstly to compare the degradation of two different Bis-GMA/TEGDMA composites during *in vitro* exposure to a lipase, dissolved in an acidic and neutral aqueous solution and secondly to relate *in vivo* composite degradation during intra-oral wear with the microbial composition of the oral biofilm formed on the composites. Such a relation would support the existence of a hypothetical “composite-degradative oral microbiome”. Composition of the oral microbiome is assessed from PCR-DGGE of oral biofilm formed on the composites during intra-oral wear. In addition, the sensitivity of conventional micro-hardness and surface roughness measurements to reflect composite degradation will be compared with the sensitivity offered by filler-particle exposure calculated from measured water contact angles.

2. Materials and methods

2.1. Resin composites

Beautiful II and Lava Ultimate CAD/CAM resin composites were selected for this study. Both composites are different in several aspects: Beautiful II (Shofu Inc., Kyoto, Japan) is a nano-hybrid Bis-GMA/TEGDMA composite with glass ionomer particles (83 wt%), while Lava Ultimate CAD/CAM Restorative (3M ESPE Dental, St. Paul, MN, USA) is a CAD/CAM milled, nano-ceramic Bis-GMA, UDMA, TEGDMA, PEGDMA, bis-EMA resin (80 wt% filled).

Samples (2 mm thickness and 5 mm diameter) were made of the two different composites. Beautiful II samples were covered with a 100 μm thick, translucent Mylar strip and pressed to create a smooth surface (average surface roughness, R_a equal to $0.4 \pm 0.1 \mu\text{m}$) and prevent formation of an oxygen inhibition layer. Light-curing (Woodpecker, 1000–1200 mW/cm^2) was subsequently done in direct contact with the Mylar strip for 20 s. Lava Ultimate CAD/CAM is available as blocks (14 \times 10 \times 10 mm), that were cut to the desired sample size

using the 'IsoMet' sectioning device, yielding a comparable surface roughness R_a ($0.3 \pm 0.1 \mu\text{m}$), as obtained for Beautifil II samples.

In order to determine the surface chemical composition of the resin composites, X-ray Photoelectron Spectroscopy (XPS) was carried out immediately after preparation with an S-Probe Spectrometer (Surface Science Instruments, Mountain View, CA, USA), equipped with an aluminum anode (10 kV, 22 mA) and a quartz monochromator. The direction of the photoelectron collection angle to the specimens was 55° and the electron flood gun was set at 10 eV. Binding energies were determined by setting the binding energy of the C_{1s} component due to C–C bonds at 284.8 eV. A survey scan was made with a $1000 \times 250 \mu\text{m}$ spot at a resolution of 150 eV in the range of 1–1200 eV, after which narrow scans were made of the C_{1s} (280–300 eV) and O_{1s} (520–540 eV) electron binding peaks. The C_{1s} and O_{1s} peaks were decomposed in 4 and 2 components with a full width at half maximum set at 1.4 eV and 1.9 eV, respectively.

2.2. Water contact angles and surface-exposure of filler-particles of the composites prior to and after *in vitro* lipase exposure or intra-oral wear

Water contact angles represent an extremely sensitive measure for changes in surface composition of a material and can be applied to quantitate the fractional exposure of filler-particles at a composite surface [37]. Because of their demonstrated extreme surface-sensitivity, water contact angles were chosen to compare filler-exposure prior to and after *in vitro* lipase exposure or intra-oral wear. Samples were analyzed immediately after preparation, while samples after *in vitro* lipase exposure or intra-oral wear and biofilm removal were further cleaned by sonication five times for 1 min in demineralized water and dried in air. Next, water droplets of 1.5–2 μL ultra-pure water were placed on the composite surface with a microsyringe. While keeping the syringe in the droplet, the droplet volume was increased to yield advancing contact angles while subsequently reducing the volume to create receding angles. Contact angles were measured from the droplet contours after black–white thresholding using a customized instrument connected with a camera. After release of the syringe from the droplet and the formation of a sessile droplet, equilibrium contact angles were measured from the droplet contour.

The different types of water contact angles allow calculation of the fractional surface-exposure of filler-particles at the composite surface. The equilibrium contact angle, determined in part by the matrix and the filler-particles exposed, can be expressed according to [37]

$$\cos \theta_E = (1 - f_{\text{filler}}) \times \cos \theta_A + f_{\text{filler}} \times \cos \theta_R \quad (1)$$

where " θ_E " is the equilibrium water contact angle, " θ_A " the advancing angle reflecting the more hydrophobic matrix, " θ_R " the receding angle reflecting hydrophilic silica particles and " f_{filler} " the fractional surface-exposure of filler-particles at the composite surface.

2.3. Surface roughness of the composites prior to and after *in vitro* lipase exposure and intra-oral wear

Surface roughness of the composite surfaces prior to and after *in vitro* lipase exposure or intra-oral wear was measured by means of optical profilometry (Proscan 2000, Scantron, Taunton, England). Specimens were scanned with 300 Hz speed in two different directions ($5 \times 5 \text{ mm}$), and the mean R_a surface roughness was calculated. R_a is arithmetical mean deviation of the assessed surface profile which we used as a roughness parameter in this study.

2.4. Hardness of the composites prior to and after *in vitro* lipase exposure and intra-oral wear

Micro-hardness of the composites prior to and after *in vitro* lipase exposure or intra-oral wear was evaluated on a Shimatzu instrument (Tokyo, Japan), using a Vickers diamond tip, a pyramidal diamond indenter with a facing angle of 136° . Three indentations were made on different positions on each sample disc under a 981 mN load, applying a dwell time of 10 s, leaving an indentation of the diamond tip on the sample. The indentation lengths were measured with $40\times$ optical microscope.

2.5. *In vitro* lipase exposure of resin composites

After preparation of composite samples, samples were exposed to lipase (2% (w/v); >100,000 units/g SIGMA-ALDRICH, Saint Louis, MO, USA), dissolved in an acidic and neutral fluid. The lipase was diluted 10 times in saliva buffer (50 mM potassium chloride, 2 mM potassium phosphate, 1 mM calcium chloride) with pH adjusted to 5 or 7, mimicking the pH range occurring in oral biofilm and saliva. Samples were exposed to the lipase solution for 7 days at 37°C , after which they were cleaned by sonication five times for 1 min in demineralized water and dried in air prior to measurements.

2.6. Intra-oral wear: study setup and volunteers

The study was setup as an *ex vivo* within-volunteer comparison, with permission from the Medical and Health Research Ethics Committee. Sixteen healthy volunteers (5 males; 11 females) between 18–30 years of age, with full maxillary dentition, controlled oral hygiene and able to comply with the experimental protocol were included. The 16th female volunteer however, dropped out immediately after enrolling due to unwillingness to comply with the experimental protocol. Accordingly, only data from 15 volunteers were available for analyses. Volunteers undergoing active orthodontic treatment were excluded. During the study, the volunteers were not consuming any drugs, particularly not those that have anticholinergic action (antidepressants, anxiolytics, antipsychotics, antihistaminic, antihypertensive) or antimicrobial effects. Additional oral hygiene measures other than the prescribed oral hygiene regime were not allowed. There were no restrictions on consumption of food and beverages. Verbal and written information was provided and written informed consent was obtained prior to enrolment.

2.7. Oral hygiene regime and randomization of volunteers during intra-oral wear

Volunteers were equipped with a palatal appliance containing cavities in which the sample discs could be fitted. A palatal appliance was chosen in order to be able to study only the influence of the biofilm and the oral environment and to exclude, as much as possible, mechanical wear due to chewing. Two samples of each composite were randomly embedded at anterior or posterior positions in an appliance. Each appliance was equipped with a thermosensitive chip (TheraMon[®], Orthosmart, Heerhugowaard, The Netherlands) to monitor wearing time. Appliances were provided with metal clasps in the molar or pre-molar region to create stable retention in the oral cavity.

Volunteers were asked to perform oral hygiene according to one of the following instructions in each phase of the study: manual-brushing of the dentition including the palatal appliance with water twice a day for 2 min using a soft-toothbrush (Pepsodent double care sensitive toothbrush, Unilever, Indonesia) or refraining from brushing of the appliance. In the latter case, volunteers were instructed to remove the appliance and rinse it under flowing water after which volunteers were allowed to brush their teeth with toothpaste (Colgate Total[®], Colgate-Palmolive company, Guanzhou, China) according to their daily oral hygiene routine. Before each phase of intra-oral wear, biofilm levels in each volunteer were returned to baseline through treatment by a professional oral hygienist, applying a washout period of 60 days between phases.

Thirty-two palatal appliances were prepared for use during the two phases of the study, i.e. intra-oral wear in absence and presence of manual-brushing with water. Temperature of the palatal appliance was recorded every 15 min through the thermosensitive chip throughout the experimental period. Wearing and non-wearing times were determined at a cut-off temperature of 30 °C and temperatures exceeding 30 °C were categorized as 'wearing time'. Wearing time data were considered as outliers when the 'wearing time' was more than 1.5 IQR (interquartile range), but less than 3 IQR from the end of the boxplot according to a normal distribution test. Volunteers presenting such data were considered 'non-compliant' and their data discarded.

2.8. Biofilm removal from composite surfaces after intra-oral wear and DNA extraction

After 30 days of biofilm formation in the oral cavity on the composites and different brushing regimes, biofilm was gently scraped off with a pocket probe from the composite surfaces and put in 10 μ L Tris-EDTA buffer (10 mM tris(hydroxymethyl)aminomethane, 1 mM ethylenediamine tetraacetic acid pH 7.4) and inserted in small Eppendorf cups, 5 μ L 7.5 M NH₄Ac (ammonium acetate) and 20 μ L isopropanol were added. Composite sample surfaces were microscopically examined to confirm complete biofilm removal. The biofilm in suspension was mixed carefully, and stored for at least 3 h at –80 °C. The suspended biofilm was taken out of the freezer, thawed and centrifuged in an Eppendorf centrifuge for 15 min at 16,000 g. The supernatant was carefully removed

and 10 μ L 70% ethanol was added, vortexed for 1 min and centrifuged again for 15 min. The ethanol was removed and the DNA pellet was air-dried. The DNA samples were stored till all samples were collected. The quality and quantity of the DNA samples were measured using a NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, DE, USA) at 230 nm.

2.9. PCR-DGGE analysis of biofilms

Per sample, 100 ng of DNA was employed for PCR amplifications with a T-gradient thermocycler. PCR products were analyzed by electrophoresis on a 2.0% agarose gel containing 0.5 mg/mL ethidium bromide. Denaturing gradient gel electrophoresis (DGGE) of PCR products generated with the F357-GC/R-518 primer set, was performed [38]. PCR products were applied on 0.08 g/mL polyacrylamide gel in 0.5 TAE buffer (20 mM Tris acetate, 10 mM sodium acetate, 0.5 mM EDTA, pH 8.3). The denaturing gradient consisted of 30%–80% denaturant (100% denaturant equals 7 M urea and 37% formamide). A 10 mL stacking gel without denaturant was added on top. Electrophoresis was performed overnight at 120 V and 60 °C. Gels were stained with silver nitrate [39]. Each DGGE gel was normalized according to a marker consisting of 10 reference species comprising common bacterial species associated with oral health and disease [40]. Reference strains included *Streptococcus oralis* ATCC35037, *Streptococcus mitis* ATCC9811, *Streptococcus sanguinis* ATCC10556, *Streptococcus salivarius* HB, *Actinomyces naeslundii* ATCC51655, *Lactobacillus* sp., *Streptococcus sobrinus* ATCC33478, *Streptococcus mutans* ATCC10449, *Porphyromonas gingivalis* ATCC33277 and *Prevotella intermedia* ATCC49046 [41].

2.10. Statistical analysis

DGGE gel images were converted and transferred into a microbial database with BioNumerics version 7.6.1 (Applied Maths N.V, Sint-Martens-Latem, Belgium). Similarities in bacterial composition of the biofilms were analyzed using a band-based similarity coefficient with a tolerance of 0.4% and a non-weighted pair group method with arithmetic averages to generate dendrograms indicating similarities in composition [42].

Matrix compositions, water contact angles, filler-exposures, surface roughnesses and micro-hardnesses (indentation lengths) of the two composites selected prior to and after intra-oral wear were compared using a parametric Student's t test. Filler-exposure, surface roughness and hardness were analyzed after intra-oral wear using a non-parametric Chi-square Test of Independence to determine whether a significant relationship between degradation in absence or presence of *S. mutans* existed. All statistical analyses were performed in SPSS, Version 22 (IBM Corp). $p < 0.05$ was considered to be statistically significant.

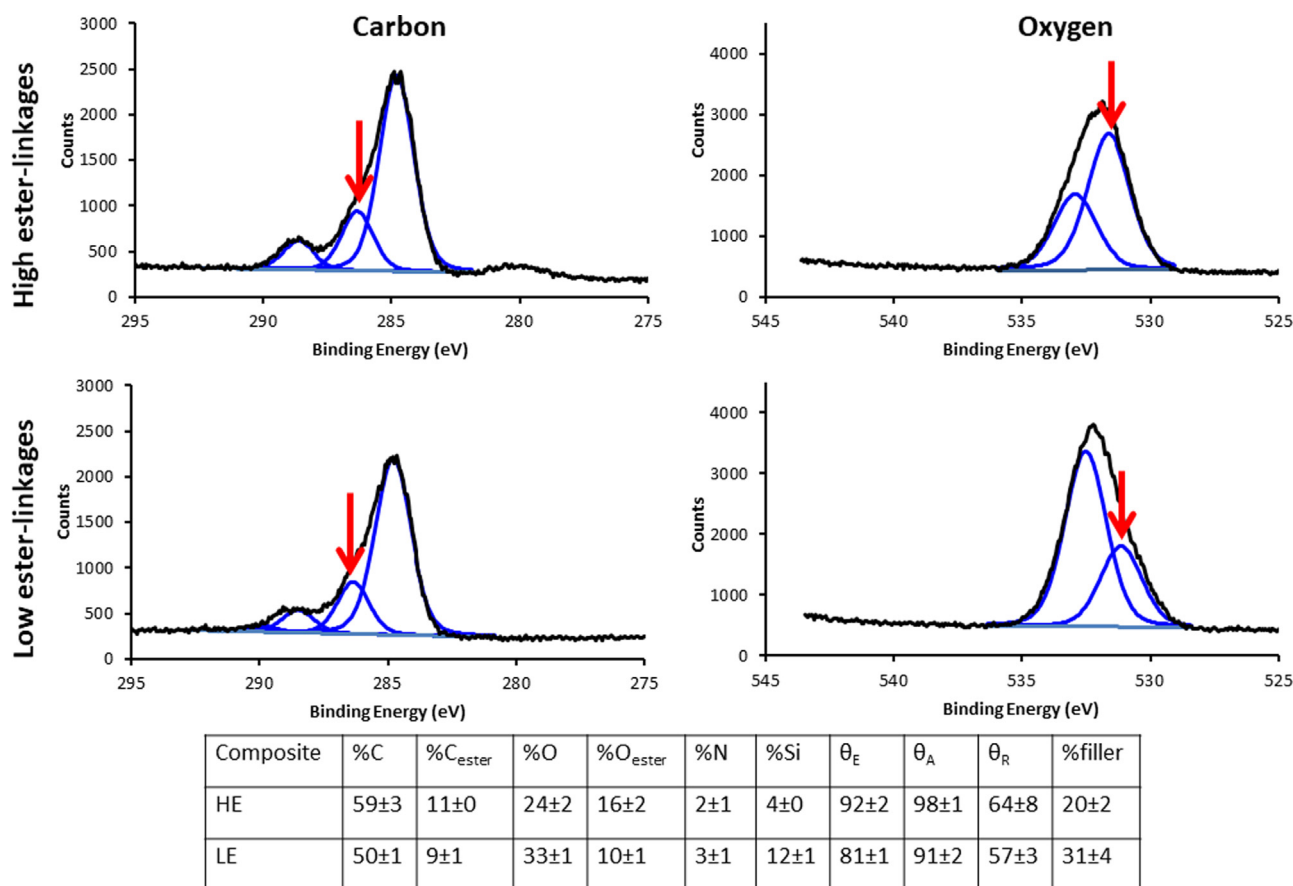


Fig. 1 – Matrix composition of the high (HE) and low (LE) ester-linkage composites used in this study, as determined by XPS and including the percentages of the carbon ($C_{286.3}$ eV) and oxygen ($O_{531.4}$ eV) involved in ester-linkages (red arrows). Surface-exposures of filler-particles at the composite surfaces were calculated from the equilibrium (θ_E), advancing (θ_A) and receding (θ_R) water contact angles ($^\circ$), as also presented in the table part to this figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3. Results

3.1. Characterization of the composite surface prior to degradation

Matrix surface chemical compositions of both composites prior to exposure to lipase or intra-oral wear as derived from XPS analyses are shown in Fig. 1. The major difference between both composites appeared in their O_{1s} photoelectron binding energy peaks, showing lower oxygen exposure but with a roughly twofold higher component at 531.4 eV for the Beautiful II than for the Lava Ultimate composite. Attributing the percentage carbon and oxygen involved in ester-linkages (C–O/C–N ($C_{286.3}$ eV) and O–C ($O_{531.4}$ eV)) to be indicative of ester-linkages, it could be calculated that the Beautiful II composite had almost twice as much of its oxygen involved in ester-linkages (16%) than the Lava Ultimate composite (10%). A higher number of ester-linkages in the Beautiful II composite could also be calculated from the carbon peak and its decomposition, but on a percentage basis, carbon involved in ester-linkages in the Beautiful II composite (11%) was only slightly higher than in the Lava Ultimate

one (9%). In addition, the Beautiful II composite was slightly more hydrophobic, regardless whether judged from advancing, receding or equilibrium water contact angles (Fig. 1), which translated according to Eq. 1 into a lower percentage surface-exposure of filler-particles for the Beautiful II (20%) than for Lava Ultimate (31%). According to the XPS data, the Beautiful II and Lava Ultimate composites will from here on be indicated as high (HE) and low ester-linkage (LE) composites, respectively, while their initial surface roughness was the same (0.3 – 0.4 μm), according to measurements done during sample preparation (see Section 2).

3.2. In vitro lipase degradation

After exposure to lipase, water contact angles on both composites decreased, regardless whether advancing, receding or equilibrium angles (Table 1). The percentage surface-exposure of filler-particles derived from these contact angles increased significantly with 16% (pH 7) and with 24% (pH 5) for the HE composite and increased with only 6% (not statistically significant) for the LE one, regardless of pH. There were no significant increases in surface roughness after *in vitro* exposure to lipase in either HE and LE composites (0.4 ± 0.1 and

Table 1 – Advancing (θ_A), equilibrium (θ_E) and receding (θ_R) water contact angles ($^\circ$) on the high (HE) and low (LE) ester-linkage composites involved in this study prior to and after *in vitro* exposure to a lipase solution (0.2% (w/v) at different pH, together with the resulting percentage filler-particle exposed at the composite surface calculated from the measured contact angles (see Eq. 1). Data are presented as averages over 3 samples \pm standard deviations.

Composite		Prior to lipase exposure	Lipase exposure pH 7	Lipase exposure pH5
HE	θ_A	98 \pm 1	61 \pm 4 ^a	44 \pm 9 ^{a,b}
	θ_E	92 \pm 2	52 \pm 2 ^a	50 \pm 3 ^a
	θ_R	64 \pm 8	32 \pm 4 ^a	25 \pm 3 ^{a,b}
	%filler	20 \pm 2	36 \pm 7 ^a	44 \pm 9 ^a
LE	θ_A	91 \pm 2	72 \pm 6 ^a	72 \pm 4 ^a
	θ_E	81 \pm 1	62 \pm 7 ^a	60 \pm 3 ^a
	θ_R	57 \pm 3	38 \pm 2 ^a	35 \pm 3 ^a
	%filler	31 \pm 4	37 \pm 8	37 \pm 14

^a Indicates a significant ($p < 0.05$) difference between the composite prior to and after lipase exposure at pH 7.

^b Indicates a significant ($p < 0.05$) difference between the composite prior to and after lipase exposure at pH 5.

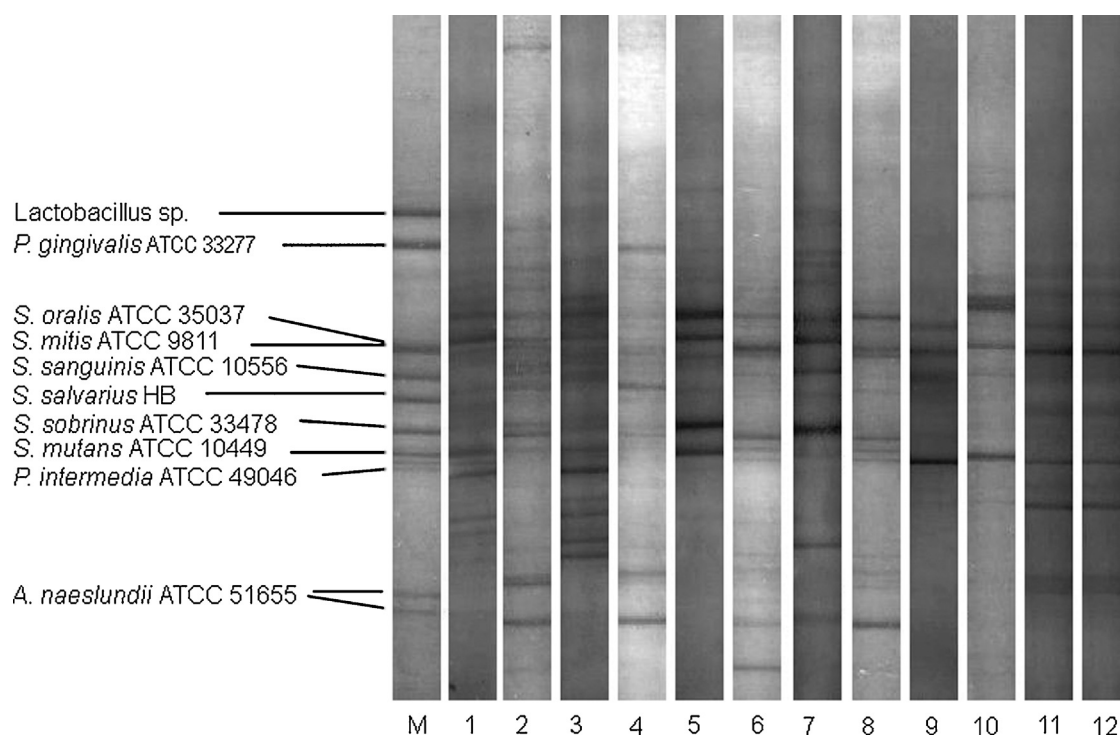


Fig. 2 – Example of a DGGE gel of PCR-amplified bacterial 16S rRNA gene segments from biofilms formed on high (HE) and low (LE) ester-linkage composite samples after 30 days of intra-oral wear under different oral health care regimes for three volunteers. Lanes 1, 5, 9: HE composite biofilms, no brushing Lanes 2, 6, 10: HE composite biofilms, water brushing Lanes 3, 7, 11: LE composite biofilms, no brushing Lanes 4, 8, 12: LE composite biofilms, water brushing M is the marker lane with the reference strains.

$0.3 \pm 0.0 \mu\text{m}$ for HE and LE composites, respectively), nor in indentation lengths (29.6 ± 0.1 and $24.4 \pm 0.0 \mu\text{m}$ for HE and LE composites, respectively). This collectively attests to the extreme surface sensitivity of water contact angles in detecting early composite degradation.

3.3. Biofilm composition and composite degradation after intra-oral wear

Fig. 2 presents an example of a PCR-DGGE gel of oral biofilm formed during intra-oral wear for each composite under both brushing regimes for 3 different volunteers, including ref-

erence strains. The microbial composition of the biofilms formed on the composites under both brushing regimes did not show any sex-related differences and were compared in cluster-trees. Fig. 3a shows the cluster trees marking the two brushing regimes, while in Fig. 3b the cluster tree is colored marking the LE and HE composites. However, full grouping of the data either according to oral hygiene regime or composite type did not occur, attesting to the multi-factorial nature of oral biofilm formation. Microbial composition on composites was further analyzed from the prevalence of the marker strains either according to oral hygiene regime or composite type (Table 2). In absence of brushing, prevalence of most

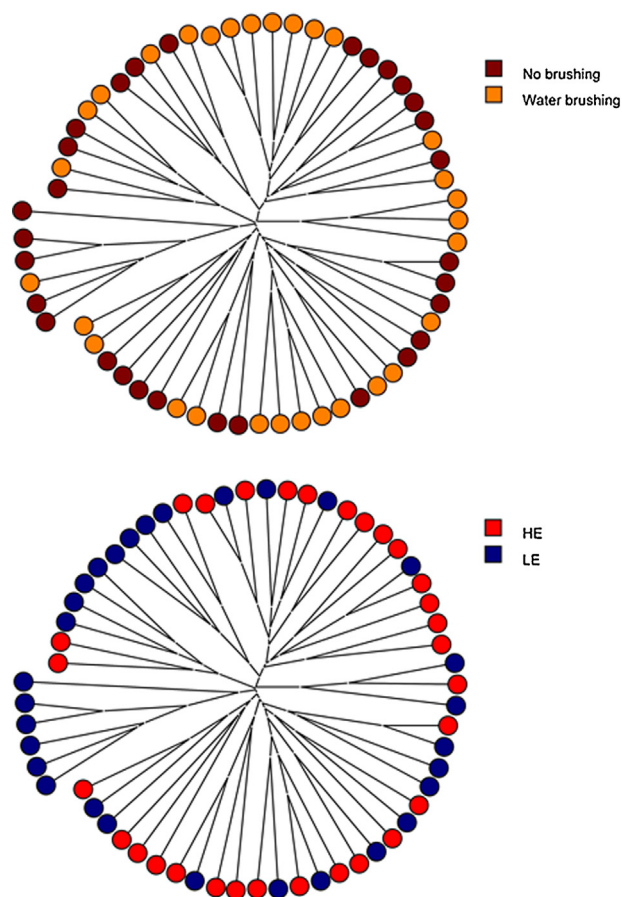


Fig. 3 – Cluster-tree of DGGE analyses of biofilms isolated from the two composites after 30 days of wear in 15 volunteers, marking whether or not samples were brushed (top figure) or distinguishing between the high (HE) and low (LE) ester-linkage composite (bottom figure). The closer the proximity of similarly colored dots to one another, the more the biofilm composition is alike.

strains was higher than in presence of brushing. Strikingly when combining brushing regimes, prevalence of *S. mutans* was almost two-fold higher on HE than on LE composites.

After intra-oral wear, the HE composite had lost more of its hydrophobicity, judged by decreases in water contact angle, than the LE composite, regardless of the type of water contact angle considered (Table 3). The LE composite only suffered a significant loss in hydrophobicity in absence of brushing. Calculation of the percentage filler-particle surface-exposure

Table 2 – Prevalence of marker strains in biofilm samples on the high (HE) and low (LE) ester-linkage composites for the two different oral hygiene regimes applied, taken over 15 volunteers.

Bacteria	Prevalence according to oral hygiene regime, combining HE and LE composites	
	No brushing	Brushing with water
<i>S. oralis/mitis</i>	93	73
<i>S. sanguinis</i>	47	40
<i>S. salivarius</i>	27	20
<i>A. naeslundii</i>	30	24
Lactobacilli	7	13
<i>S. sobrinus</i>	33	37
<i>S. mutans</i>	30	27
<i>P. gingivalis</i>	17	17
<i>P. intermedia</i>	30	10

Bacteria	Prevalence according to composite type, combining oral hygiene regimes	
	HE composite	LE composite
<i>S. oralis/mitis</i>	83	83
<i>S. sanguinis</i>	43	43
<i>S. salivarius</i>	20	27
<i>A. naeslundii</i>	20	33
Lactobacilli	7	13
<i>S. sobrinus</i>	37	33
<i>S. mutans</i>	37	20
<i>P. gingivalis</i>	13	20
<i>P. intermedia</i>	20	20

100% indicates that biofilm samples from all volunteers in the brushing regime or on the composite contain the indicated marker strain.

of the composites after wear from the contact angle data in Table 4, indicated a significant increase in filler-particle exposure for the HE composite, indicative of its degradation. Also surface roughness and micro-hardness indicated degradation of the HE composite, but this was not significant under all conditions. The LE composite showed no significant degradation after intra-oral wear.

Splitting degradation according to whether or not the corresponding biofilms harvested *S. mutans*, known to produce esterases [9,10], revealed that surface-exposure by filler-particles, surface roughness as well as micro-hardness of the HE composite increased more after intra-oral wear in volunteers harvesting *S. mutans* in composite biofilms, especially in absence of brushing. However, this was only statistically significant for surface-exposure of filler-particles calculated from highly surface-sensitive water contact angles. Degradation

Table 3 – Advancing (θ_A), equilibrium (θ_E) and receding (θ_R) water contact angles ($^\circ$) on the high (HE) and low (LE) ester-linkage composites involved in this study prior to and after intra-oral wear in absence or presence of brushing with water. Data are presented as averages over 15 volunteers \pm standard deviations.

Composite	Prior to intra-oral wear			After intra-oral wear, no brushing			After intra-oral wear, water brushing		
	θ_A	θ_E	θ_R	θ_A	θ_E	θ_R	θ_A	θ_E	θ_R
HE	98 \pm 1	92 \pm 2	64 \pm 8	80 \pm 12 ^a	62 \pm 12 ^a	42 \pm 11 ^a	82 \pm 10 ^a	69 \pm 13 ^a	44 \pm 10 ^a
LE	91 \pm 2	81 \pm 1	57 \pm 3	78 \pm 10 ^a	66 \pm 12 ^a	39 \pm 8 ^a	84 \pm 10	72 \pm 12	42 \pm 8 ^a

^a Indicates a significant ($p < 0.05$) difference between the composite prior to and after intra-oral wear.

Table 4 – Absence or presence of *S. mutans* in biofilms, mean surface-exposure of filler-particles, surface roughness and micro-hardness (indentation length) for the high (HE) and low (LE) ester-linkage composites prior to and after intra-oral wear in absence and presence of brushing with water. Data represent averages \pm SD over the numbers of volunteers in the different groups. For *in vitro* data prior to wear, 3 samples were used.

Composite	Prior to intra-oral wear	After intra-oral wear			
		No brushing		Water brushing	
Number of volunteers		<i>S. mutans</i> (+)	<i>S. mutans</i> (–)	<i>S. mutans</i> (+)	<i>S. mutans</i> (–)
HE	Not applicable	11	4	9	6
LE	Not applicable	8	7	10	5
%Surface-exposure of filler-particles					
HE	20 \pm 2	79 \pm 10 ^{a,b}	46 \pm 8 ^{a,b}	59 \pm 4 ^{a,b}	31 \pm 3 ^{a,b}
LE	31 \pm 4	41 \pm 10	36 \pm 10	44 \pm 14	31 \pm 8
Surface roughness (μ m)					
HE	0.4 \pm 0.1	1.6 \pm 1.2	2.2 \pm 1.3	1.2 \pm 0.8 ^a	0.8 \pm 0.8
LE	0.3 \pm 0.0	0.9 \pm 0.7 ^a	1.2 \pm 0.7 ^a	2.7 \pm 1.2 ^{a,b}	1.0 \pm 0.8 ^b
Hardness-indentation length (μ m)					
HE	29.6 \pm 0.1	31.8 \pm 0.7	28.9 \pm 1.5	35.3 \pm 3.2 ^a	32.5 \pm 0.6
LE	24.4 \pm 0.7	26.0 \pm 0.5	25.5 \pm 0.0	26.1 \pm 0.5	27.5 \pm 1.3 ^a

^a Indicates a significant ($p < 0.05$) difference between the composite prior to and after intra-oral wear.
^b Indicates a significant ($p < 0.05$) difference between composites of the same type harvesting and not harvesting *S. mutans*.

tion during intra-oral wear of the LE composite was generally minor and mostly not significant with respect to its properties prior to intra-oral wear.

4. Discussion

Biofilm formation on resin composites in the oral cavity is a highly multi-factorial process. Here we show that a high (HE) ester-linkage composite harvested oral biofilm that more frequently possesses *S. mutans* than oral biofilm formed on a low (LE) ester-linkage composite. Concurrently, HE resin composite was subject to stronger intra-oral degradation than LE composites, regardless of oral hygiene regime applied. *In vitro* exposure of the composites to lipase support that these differences in degradation observed, may be attributed to different numbers of ester-linkages in both composites. Importantly, surface roughness and water contact angles differed little between both composites prior to degradation.

Whereas the onset of degradation is a surface phenomenon, adsorption and absorption of enzymes from saliva, food or beverage components into the composite and along the composite-tooth interface will eventually lead to softening of the bulk matrix [25]. Thus degradation will first become obvious from altered surface properties of a composite, followed later by changes in bulk properties. In order to compare the surface-sensitivity of the three evaluation methods applied, their output parameters were compared based on the dynamic range of each method, calculated as the difference between the largest and smallest value obtained divided by the median (36%, 0.8 μ m and 26.1 μ m for filler-particle exposure, roughness and indentation length, respectively). Accordingly, the respective dynamic ranges amount 2.19, 5.71 and 0.55, attesting to a lack of surface-sensitivity of micro-hardness measurements. This is confirmed by the absence of a relation between indentation lengths and filler-exposures observed, while surface roughness increases with increasing filler-particle exposure (Fig. 4). The lack of surface-sensitivity

of micro-hardness measurements [43] occurs because the indentation lengths measured will not only reflect degradation of the composite surface but also of the bulk matrix. Therefore, it can be concluded that hardness is not suitable to demonstrate early degradation as a surface phenomenon. The weak relation between surface-exposure of filler-particles with surface roughness, as another conventional parameter to study composite degradation [28,29] on the one hand confirms the surface-sensitivity of roughness measurements, but on the other hand reflects that smooth regions of a composite surface exposing only matrix may exist as well. Such an interference in the evaluation of surface degradation does not exist for water contact angle based calculation of surface-exposure of fillers, as it relies on differences in wettability of the relatively hydrophobic matrix versus the relatively hydrophilic filler-particles. Thus, early degradation of composites after intra-oral wear can best be studied by analysis of equilibrium, advancing and receding water contact angles.

Ester-linkages, as in BisGMA and TEGDMA, are prone to hydrolysis, catalyzed by enzymes. Such catalytic enzymes are not only present in glandular saliva, but also originate from polymorphonuclear leukocytes, macrophages and bacteria [12,13]. *In vitro* studies have shown that seven out of seven *S. mutans* isolates had esterase activity [9] and recently, isolation and characterization of the specific esterase activity in an *S. mutans* strain has been reported [10]. Moreover, *in vitro* growth of *S. mutans* mono-species biofilms on resin composites yielded demonstrable amounts of BisGMA degradation products in the culture medium. Poly(ethylene)glycol-poly(β -amino esters) (PEG-PAE) micelles with Triclosan conjugated to the PAE through an ester-linkage, preferentially killed *S. mutans* in *ex vivo* biofilms from orthodontic patients, which was attributed to the high esterase production of *S. mutans* causing release of the conjugated Triclosan [44]. A possible role of *S. sobrinus* in the degradation of resin composite is less clearly reported, although TEGDMA stimulated growth of *S. sobrinus in vitro* [45].

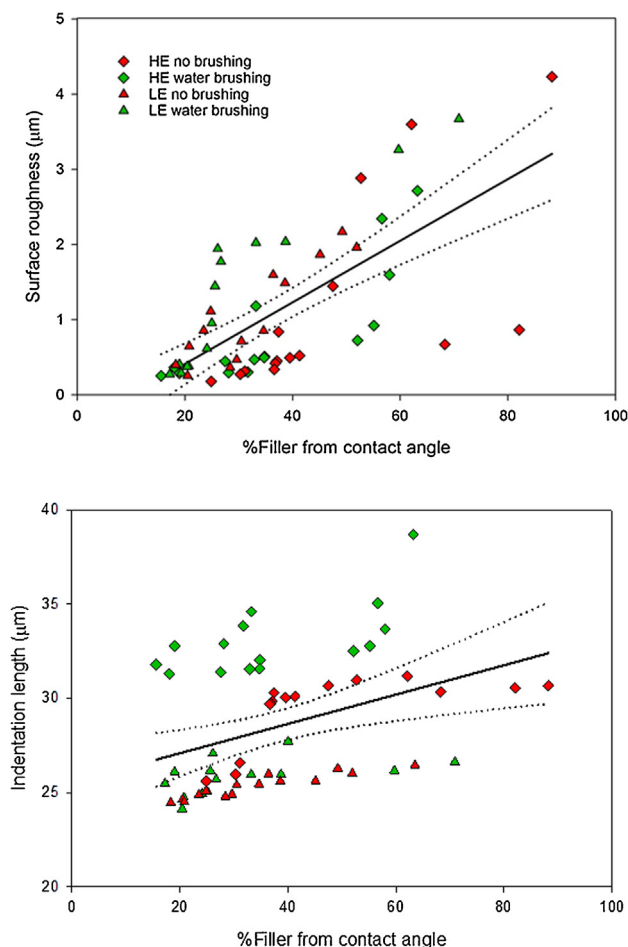


Fig. 4 – Surface roughness (top graph) and indentation length (bottom graph) as a function of the %filler-particles exposed at the surface, calculated from water contact angle measurements using Eq. 1. Each data point represents one of the two composite types under one particular regime of oral health care in each of the 15 volunteers, while the dotted lines indicate the 95% confidence interval.

In vivo it is difficult to demonstrate the role of any specific bacterial strain in composite degradation, as the contribution of salivary enzymes is hard to separate from the influence of specific bacterial strains. Our study is the first study to link higher prevalence of *S. mutans* in clinical biofilms with HE composite degradation, coinciding with the higher killing efficacy of *S. mutans* by ester-linked Triclosan in micellar carriers [44]. Highly sensitive detection of early degradation within 30 days of intra-oral wear was made possible through the use of highly surface-sensitive analysis of water contact angles of advancing, receding and equilibrium type, allowing us to detect large differences in filler-particle exposure at the composite surface as an indication of the onset of composite degradation most notably already within 7 days *in vitro* exposure to lipase. Clearly, degradation of bulk composite will require longer intra-oral wear than the onset of surface degradation [27,28].

Differences in bacterial composition of the planktonic microbiome in saliva and the adhering oral microbiome on teeth have been related to the forces by which different bac-

terial species are attracted to the tooth surface. Species that were found predominantly in the adhering microbiome had significantly higher adhesion forces to saliva-coated enamel (0.60–0.05 nN) than did species mostly present in the planktonic microbiome (0.40–0.55 nN) [46]. By the same token it can be argued that the adhesion forces exerted by different composite types on specific bacterial strains present in saliva regulate the development of a composite-degradative microbiome. The HE composite showed the largest degradation, i.e. increased exposure of filler-particles at its surface, and concurrently the largest prevalence of *S. mutans* after 30 days of intra-oral wear. Interestingly, atomic force microscopy has indicated that *S. mutans* adheres more strongly to a composite with high filler-particle content [47]. Therefore, it can be speculated that as soon as salivary enzymes and acids have stimulated initial degradation of a composite and filler-particles become increasingly exposed, this will yield a stronger selection of *S. mutans* from the salivary microbiome, marking the onset of the development of a degradative microbiome that speeds up degradation.

5. Conclusions

In conclusion, a composite-degradative oral microbiome can be defined that is rich in *S. mutans*. The oral degradative oral microbiome is, together with salivary enzymes and acids, responsible for intra-oral degradation of ester-linkages in Bis-GMA/TEGDMA composites. A degradative microbiome more readily develops on HE composites than on LE ones. Since this is the first time that esterases produced by *S. mutans* are demonstrated to be responsible for intra-oral degradation of composites containing a high number of ester-linkages, this points to a clear pathway for the development of composites less amenable for degradation during intra-oral wear.

6. Registration number: KE/FK/1125/EC

Name of trial registry: Effect of the oral environment on the aging process of dental resin composites and their impact on its physical properties.

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