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In vitro evaluation of artificial ageing on surface properties and early *Candida albicans* adhesion to prosthetic resins

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Abstract *Objective* The aim of this in vitro study was to examine potential changes and influences of prosthetic resin surface properties on *Candida albicans* adhesion after surface treatment or artificial ageing. *Materials and methods* Standardized specimens of a denture base resin and a veneering composite were prepared, polished, and randomly subjected to different surface roughness treatments or artificial ageing protocols (storage in ethanol or artificial saliva for 7/90 d, thermocycling). Surface roughness (Ra) and surface free energy were determined prior and after each treatment. Specimens were incubated with phosphate buffered saline or whole saliva for 2 h at 37°C, and later with *Candida albicans* suspension (2.5 h, 37°C). Adherent viable fungi were quantified using a bioluminescence assay. *Results* Artifical ageing did not affect substratum surface roughness, yet slight increases in substratum surface free energy and significant increases in *Candida albicans* adhesion were observed. Saliva coating marginally influenced *Candida albicans* adherence to reference and surface treated specimens, yet more pronounced differences in *Candida albicans* adhesion between the various artificially aged specimens were found. *Conclusion* No correlation between substratum surface roughness or surface free energy and *Candida albicans* adhesion could be established.

1 Introduction

It is a well known fact that dental materials that are exposed to the oral milieu are immediately covered by salivary constituents and later by oral microorganisms. Oral biofilms may contribute to the occurrence of oral diseases such as caries, parodontopathia or denture stomatitis [1]. The yeast *Candida albicans* has been identified as a pivotal causative agent for the pathogenesis of denture stomatitis [2–5], and has been found to adhere to numerous dental materials such as hard denture base acrylic resins as well as to silicone-based denture soft liners in vitro and in vivo [6–9].

Four phases during *Candida albicans* adhesion have been identified: transport to the surface, initial adhesion, attachment and colonization [7]. Substratum surface roughness as well as surface free energy have been found to influence these phases decisively. Surface free energy influences the initial adhesion rather than surface roughness, whereas surface roughness is of upmost importance during the adhesion phase [7]. However, some in vitro studies found that surface roughness rather than surface free energy correlates with *Candida albicans* adhesion to dental biomaterials [9, 10].

The film of salivary constituents on the tooth or restorative surface, which is usually referred to as the salivary pellicle [11], influences the adherence of microorganisms. Numerous studies found that saliva coating levels differences in surface free energy between various materials [12]. Some researchers, however, maintain that the original substratum surface properties are transferred even through the pellicle protein layer ("shine-through-effect") [13], and still influence microbial adhesion.

Most of the previous studies investigating *Candida albicans* adhesion to dental materials used specimens immediately after preparation [14]. As prosthetic restorations are exposed to the oral milieu, they are subjected to intense ageing due to permanent humidity and aggressive liquids as well as mastication forces. Ageing might affect

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surface parameters such as surface roughness or surface free energy and therefore microbial adhesion. Some studies found increased adhesion of *Candida albicans* to aged specimens [14, 15]. A decrease of mechanical material parameters such as flexural and tensile strength as well as marginal adaptation after artificial ageing has been reported [16]. Surprisingly little information is available concerning potential changes in surface properties and *Candida albicans* adhesion after artificial ageing. Thus, the aim of this in vitro study was to investigate the influence of artificial ageing on the physicochemical resin surface properties, and to correlate these findings with the initial adhesion of *Candida albicans* to the artificially aged resins in the absence and presence of a preformed salivary pellicle.

2 Materials and methods

2.1 Materials

Round specimens (diameter 10 mm, 2 mm thick) of a cold-curing denture base resin (*Palapress vario*, Heraeus Kulzer, Hanau, G) and a veneering composite (*Sinfony*, 3 M Espe, Seefeld, G) were prepared according to the manufacturers' instructions. The composite resin was polymerized by light using *Visio Alpha* and *Visio Beta* (3 M Espe, Seefeld, G) after covering the resin with a transparent plastic film to prevent the formation of an oxygen inhibited layer. The denture base resin was polymerized in a tempered water bath (40°C, 25 min, 2.2 bar).

All specimens were smoothed prior to further treatment using silicone carbide paper (grain 1000 and 4000, Buehler GmbH, Düsseldorf, G) and a rotating grinding disc apparatus (Motopol 8, Buehler Ltd., Coventry, UK). The discs were subsequently polished to high gloss using universal polishing paste (Ivoclar Vivadent, Schaan, FL) and conventional burnishers. All specimens were stored in distilled water that was exchanged daily for six days prior the experiments for minimizing influences of residual monomers or toxic constituents on cell viability.

All specimens were alloted to various ageing and surface treatment regimes (cf. Table 1). After each treatment, samples were carefully cleaned using ethanol and applicator brush tips (3 M Espe, Seefeld, G), and the surface free energy of the specimens was calculated from contact angle measurements using the sessile drop method and an automated contact angle measurement device (OCA 15plus, DataPhysics Instruments GmbH, Filderstadt, G). For measurements, three liquids differing in hydrophobicity were used: deionized water, diiodomethane (Sigma-Aldrich, St. Louis, IL, USA) and ethylene glycol (Merck KgaA, Darmstadt, G). Nine drops for each liquid (0.2 µl) were examined on three specimens for each material, and left and right contact angle of each drop were averaged. The surface free energy and its polar and disperse components were calculated according to the Owens, Wendt, Rabel and Kaelble (OWRK) method.

Peak-to-valley surface roughness (Ra) was determined after each treatment at three randomly selected spots of each sample (two at the margins, one in central position) using a profilometric contact surface measurement device (Perthen S6P, Feinprüf-Perthen, Göttingen, G). A distance of 1.75 mm was measured in one single line scan perpendicular to the expected grinding grooves using a standard diamond tip (tip radius 2 µm, tip angle 90°) and a cut off level of 0.25.

2.2 Treatment protocols

Specimens were alloted to various surface treatment and ageing regimes ($n = 22$ for each material and protocol, cf. Table 1). Different surface finishing was achieved by treating specimens with silicone carbide papers varying in roughness (grain sizes 500, 1000 and 4000, respectively).

Table 1 Surface roughness treatment and artificial ageing protocols used in this study

Protocol	Procedure
Surface roughness (SR rough)	Treatment with silicone carbide paper (grain 500), 1 min
Surface roughness (SR intermediate)	Treatment with silicone carbide paper (grain 1000), 1 min
Surface roughness (SR smooth)	Treatment with silicone carbide paper (grain 4000), 1 min, high gloss; no further treatment
Thermocycling	Treatment with silicone carbide paper (grain 4000), 1 min, high gloss; Thermocycling, 6000 cycles 5/55°C, 2 min each
Artificial saliva 7 d	Treatment with silicone carbide paper (grain 4000), 1 min, high gloss; Storage in artificial saliva (7 d)
Artificial saliva 90 d	Treatmsent with silicone carbide paper (grain 4000), 1 min, high gloss; Storage in artificial saliva (90 d)
Ethanol 7 d	Treatment with silicone carbide paper (grain 4000), 1 min, high gloss; Storage in ethanol (7 d)
Ethanol 90 d	Treatment with silicone carbide paper (grain 4000), 1 min, high gloss; Storage in ethanol (90 d)

Artificial ageing was carried out by thermal cycling (6000 cycles 5/55°C, 5 min each). Storage (7 or 90 days, 25°C, dark) was performed in ethanol (Ethanol 96%, Carl Roth GmbH + Co KG, Karlsruhe, G) or artificial saliva. The artificial saliva used consisted of 4.1 mM KH_2PO_4, 4.0 mM Na_2HPO_4, 24.8 mM $KHCO_3$, 16.5 mM NaCl, 0.25 mM $MgCl_2$, 4.1 mM citric acid, and 2.5 mM $CaCl_2$ [17]. The pH of the artificial saliva solution was adjusted to 6.7 with 10 N HCl, and the solution was subsequently sterilized using single use 0.22 µm filtration devices (Vacuflo, Schleicher & Schüll Microscience GmbH, Dassel, G) [18]. Ethanol and artifical saliva were exchanged every week during the artificial ageing period.

2.3 Yeast preparation

The strain *Candida albicans* ATCC 10231 was incubated in Universal Medium for Yeast (YM; German Collection of Microorganisms and Cell Cultures, Braunschweig, G) at 37°C. After 18 h, yeast cells were harvested by centrifugation (2200 rpm, 19°C, 5 min), washed twice with 5 ml of phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, IL, USA) and resuspended in PBS. The optical density was standardized and adjusted to 0.3 at 540 nm using a spectrophotometer (Genesys 10-S, Thermo Spectronic, Rochester, USA).

2.4 Saliva preparation

Unstimulated whole saliva was collected in chilled tubes by expectoration from two volunteer adults (1 male, 1 female) aged 22 and 25 years, who refrained from ingestion and oral hygiene for at least two hours and gently rinsed their mouth with water prior saliva collection to minimize microbial contamination. Saliva was pooled and frozen immediately after collection, and was carefully defrosted and sterilized using single-use filtration devices (0.45 and 0.22 µm, successively) directly before the experiments.

2.5 Test assay

After surface or ageing treatment, specimens were equilibrated by rinsing with ethanol and distilled water, transferred into 48 well cell clusters (48 Well Cell Cluster, Corning Inc., Corning, USA) and incubated with 1 ml of PBS ($n = 11$ for each material and protocol) or human whole saliva ($n = 11$) for pellicle formation in a thermo shaking device (OrbitalShaker, ThermoForma, Marietta, USA). After 2 h, specimens were gently rinsed with PBS (1 ml) to remove saliva residues and incubated with 1 ml of *Candida albicans* suspension. After 2.5 h, specimens were gently rinsed twice with 1 ml of PBS to remove unbound cells, and transferred to chilled centrifuge tubes (one specimen per tube) for quantification of adherent *Candida albicans*.

2.6 Quantification of adherent *Candida albicans*

Adherent *Candida albicans* was quantified using a bioluminescence cytotoxicity and cell proliferation assay for viable eucaryotic cell quantification (ViaLight MDA Assay, Lonza, Basel, CH). The kit is based on the measurement of adenosine triphosphate (ATP), which is ubiquitous in viable cells. The enzyme luciferase is employed for catalyzing the formation of light from ATP and luciferin. The emitted luminescence intensity correlates linearly with the amount of ATP, and thus the number of adherent cells.

A total of 500 µl of 0.3 M perchloric acid were added to each tube, and tubes were vortexed for 30 sec for cell lysis. After neutralisation with 500 µl of 0.3 M $NaHCO_3$ the aliquot was transferred to a cup (Reaktionsgefäß 2 ml, Carl Roth GmbH + Co KG, Karlsruhe, G) and centrifuged at 11900 rpm for 15 min at 4°C. A total of 100 µl of the aliquot were transferred to an opaque dark microplate (nunc, Roskilde, DK), and 100 µl of ViaLight MDA Assay Buffer were added to each well. Luminescence intensity was measured four times for each well using an automated multi-detection plate reader (Fluostar Optima, bmg Labtech, Offenburg, G).

2.7 Statistical analysis

Means and standard deviations were calculated for surface free energies and surface roughness (Ra); statistical analysis was performed using one-way analysis of variance (ANOVA) and the Tukey-Kramer multiple comparison test for post-hoc analysis. The level of significance (α) was set to 0.05. Medians and 25/75 percentiles were calculated for relative luminescence intensities; statistical analysis was performed using the Kruskal-Wallis test ($\alpha = 0.05$) and the Bonferroni-adjusted Mann-Whitney U-test ($\alpha = 0.0018$, 8 groups).

Calculations and graphic display were carried out using SPSS 13.0 for Windows (SPSS Corporation, Chicago, IL, USA).

3 Results

3.1 Surface parameters

3.1.1 Surface roughness

One-way ANOVA revealed statistically significant differences in surface roughness between the various treated denture base and composite specimens ($P = 0.000$). For

both materials, surface roughness increased significantly after treatment with intermediate (grain 1000; mean (SD) surface roughness denture base resin/veneering composite 0.31 (±0.14)/0.18 (±0.07) µm) and rough grinding paper (grain 500; 0.68 (±0.19)/0.53 (±0.17) µm; $P = 0.000$). No correlation between the artificial ageing regimes and surface roughness could be observed, as post-hocs found statistically similar values between smooth (treated with fine grinding paper, grain 4000; 0.05 (±0.03)/0.05 (±0.02) µm) and the various aged specimens.

3.1.2 *Surface free energy (cf. Table 2)*

No significant differences were observed in surface free energies of the veneering composite after the various ageing protocols (ANOVA, $P = 0.058$). For the denture base resin, some significant changes in surface free energy were observed after the various ageing protocols (ANOVA, $P = 0.005$). Post-hocs revealed significantly higher surface free energy for specimens that had been subjected to TCML and to ageing in artificial saliva for one week than for untreated specimens ($P = 0.025$ and $P = 0.020$). Significantly lower surface free energy was calculated for specimens that had been stored in ethanol for one week than for those that had been subjected to thermocycling and storage in artificial saliva for one week ($P = 0.033$ and $P = 0.027$).

3.2 *Candida albicans* adhesion

3.2.1 *Uncoated specimens*

For denture base resin specimens (cf. Fig. 1), significantly higher luminescence intensities indicating higher adhesion of *Candida albicans* were measured for specimens treated with intermediate grinding paper (median luminescence intensity 175) than for specimens treated with fine (76; $P = 0.001$) or rough grinding paper (62; $P = 0.001$). Significantly higher luminescence intensities were measured for the artificially aged specimens than for untreated,

Table 2 Surface free energy (SFE, mJ/m^{-2}) of new and artificially aged specimens (Means, SD)

Treatment	Substratum	
	Denture base resin	Veneering composite
Surface roughness (SR smooth)	33.93 (1.21)	31.08 (2.19)
Thermocycling	42.65 (2.39)	39.02 (2.31)
Artificial Saliva 7 d	42.95 (2.25)	38.24 (2.30)
Artificial Saliva 90 d	40.69 (3.44)	36.54 (4.80)
Ethanol 7 d	34.32 (4.47)	34.58 (2.56)
Ethanol 90 d	39.18 (2.01)	34.79 (2.42)

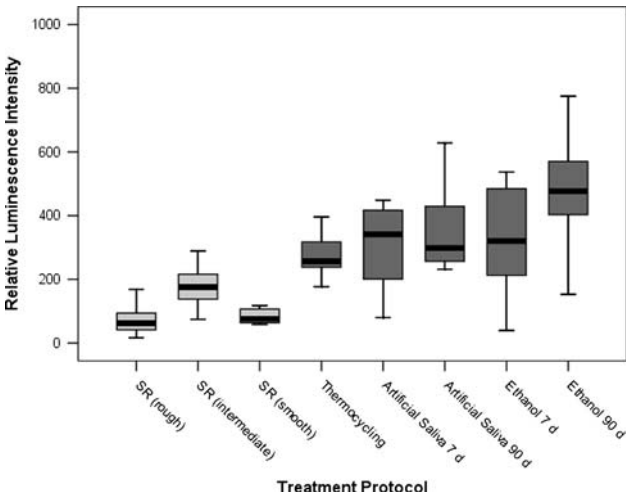

Fig. 1 Relative Luminescence Intensities [no units] to surface-treated and artificially aged *denture base resin* without saliva coating (Median, 25/75%)

smooth specimens ($P = 0.000$). Similar values were observed after thermocycling (257), and storage in artificial saliva for 7 d (342) and 90 d (298) as well as for storage in ethanol for 7 d (320). For specimens that had been stored in ethanol for 90 d significantly higher luminescence intensities (476) were observed than for thermocycled specimens ($P = 0.001$).

For composite resin samples (cf. Fig. 2), similar luminescence values were found for specimens treated with fine (88) and intermediate (93) grinding paper, but significnantly higher values were observed for specimens treated with rough grinding paper (157) than for those treated with intermediate grinding paper ($P = 0.001$). Luminescence intensities measured for smooth, untreated specimens differed significantly from those measured for aged specimens

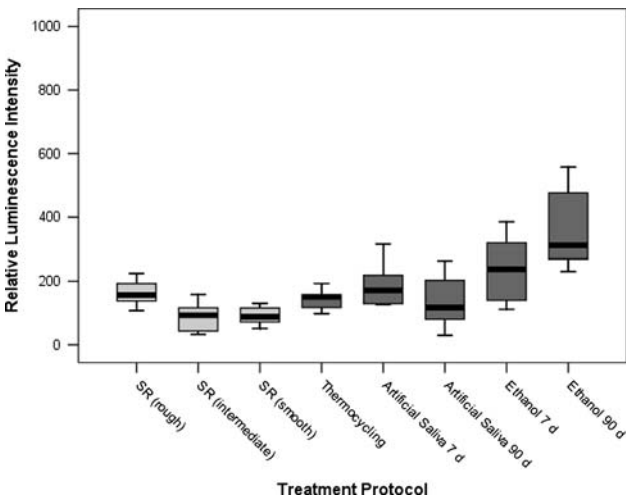

Fig. 2 Relative Luminescence Intensities [no units] to surface-treated and artificially aged *veneering composite* without saliva coating (Median, 25/75%)

($P = 0.000$). Significantly highest values were recorded after ageing in ethanol for 90 d (311), which were significantly higher than for the specimens alloted to all of the other ageing regimes but those stored in artificial saliva ($P = 0.013$) and ethanol ($P = 0.003$) for 7 d. Storage in artificial saliva (171) or ethanol (238) for 7 d increased luminescence intensities compared to smooth, untreated specimens, which yielded similar values as thermocycled specimens (151) and specimens stored in ethanol for 90 d (118).

3.2.2 *Saliva coated specimens*

Similar luminescence intensity was recorded for the various surface treated denture base resin specimens (cf. Fig. 3), with values ranging between 127 and 195. Significantly highest luminescence was observed for thermocycled specimens (919, $P = 0.000$), and significantly lowest values were observed for specimens that had been stored in ethanol for 7 d (71), which were significantly different from all other specimens. Intermediate values were found for specimens that had been stored in artificial saliva for 7 d (290) or 90 d (196), or ethanol for 90 d (301).

For composite specimens (cf. Fig. 4), significantly higher values were measured for intermediate specimens (176) than for smooth specimens (98; $P = 0.001$), but no differences were detected between rough (131) and intermediate ($P = 0.065$) or smooth ($P = 0.101$) specimens. Significant differences in luminescence intensity were found between the surface treated and aged specimens ($P = 0.000$). Significantly highest luminescence was measured for thermocycled specimens (493) and specimens

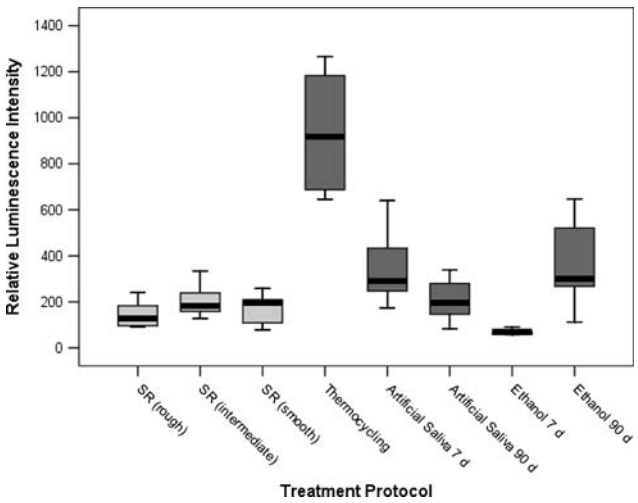

Fig. 3 Relative Luminescence Intensities [no units] to surface-treated and artificially aged *denture base resin* after saliva coating (Median, 25/75%)

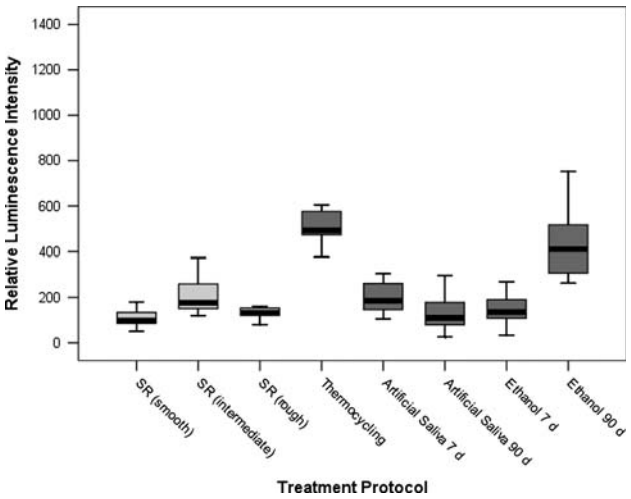

Fig. 4 Relative Luminescence Intensities [no units] to surface-treated and artificially aged *veneering composite* after saliva coating (Median, 25/75%)

stored in ethanol for 90 d (411), which were significantly higher than values measured for all other specimens ($P = 0.000$). Storage in ethanol for 7 d (135) and artificial saliva for 7 d (135) or 90 d (109) yielded similar values compared to the surface treated specimens.

4 Discussion

The purpose of this in vitro study was to investigate the influence of artificial ageing on the surface properties and *Candida albicans* adhesion to a commonly used denture base resin and a veneering composite resin. Furthermore, specimens of both resins were subjected to treatment with grinding paper differing in grain size to estimate the influence of surface roughness on *Candida albicans* adhesion, and to correlate these findings to fungal adhesion to the artificially aged specimens.

Serrano-Granger and co-workers highlighted that the comparability of studies on *Candida albicans* adherence are limited due to distinct methods applied for quantification [10]. In this study, a bioluminescence assay has been used to quantify adherent *Candida albicans*, which features the advantage over conventional microscopy or SEM quantification that only viable cells are subjected to quantification.

Surface roughness influenced *Candida albicans* adhesion to both resins decisively. The findings on the veneering composite correlate with other studies, where a linear relationship between surface roughness and *Candida albicans* adhesion was found [8, 9]. After saliva coating, only minor differences in *Candida albicans* adhesion were detected, which hints that it is possible that the protein film masks differences in surface roughness. These results were

surprising, as surface roughness values higher than 0.2 μm have been found to promote microbial adhesion in vivo and in vitro decisively [19]. For specimens treated with rough grinding paper, surface roughness values about 0.6 μm have been detected; thus, increased adhesion of *Candida albicans* had been expected due to the larger surface area or the protection from rinsing action [7]. With regard to this aspect, it might, however, be thinkable that assays simulating oral shear stress more realistically such as a flow chamber yield results differing from the outcome of this study.

Most of the artificial ageing protocols increased the adhesion of *Candida albicans*, a phenomenon that has been observed in other studies, too. However, previous studies applied only one ageing protocol [14, 15], which cannot simulate the various oral ageing conditions satisfactory; thus, five protocols differing in ageing regime and ageing time have been used in this study for covering a broad range of established artificial ageing protocols. In the oral cavity, dental materials are exposed to permanent humidity, and continuous exposition to saline solutions such as saliva or other aggressive liquids during ingestion may contribute to material degeneration, which justifies storage in artificial saliva or ethanol as in vitro ageing protocols. For evaluating the influence of prolonged ageing, surface properties and *Candida albicans* adhesion have been evaluated after 7 and 90 days of storage in artificial saliva or ethanol. Moreover, repeated temperature changes may take place intraorally during ingestion; these circumstances have been simulated in vitro using thermocycling. Both thermocycling and storage in chemical liquids have widely been used in previous studies investigating the influence of artificial ageing on material mechanical features [17, 20]. For some of the artificially aged specimens, broad variations in *Candida albicans* adherence were observed; this phenomenon has also been observed in previous studies on this topic [10, 21, 22]. Saliva coating only marginally influenced the potential of *Candida albicans* adherence to the reference and surface treated specimens, yet more pronounced differences in *Candida albicans* adhesion between the various ageing treatments were found for the pellicle coated specimens. Moreover, particularly saliva coated thermocycled specimens yielded decisively higher luminescence intensities than uncoated thermocycled specimens. These findings underline the relevance of the salivary pellicle for microbial adhesion, and confirm the hypothesis that substratum surface properties, which might influence microbial adhesion, may be transferred through the protein layer on the substratum surface [13]. However, further extensive analysis is necessary to investigate the impact of artificial resin ageing on possible differences in pellicle composition and conformational changes of salivary macromolecules forming the pellicle layer on the specimens, and to correlate these findings to the *Candida albicans* adhesion.

Surprisingly, the findings of this study suggest no influence of artificial ageing on the surface roughness of the specimens. These findings imply that the increased adherence of *Candida albicans* to the artificially aged specimens cannot be attributed to differences in surface roughness, although higher adhesion was found for some of the artificially aged specimens compared to the surface treated specimens. These results are only in partial accordance with studies by Yip and co-workers, who found slight increases in surface roughness for some resin composites after ageing in artificial saliva for three weeks [23]. Tari and co-workers found increased surface roughness of soft denture liners after artificial ageing using a weathering chamber and exposition to ultraviolet and visible light [24]. It might, however, be argued that ageing protocols different from those applied in this study might influence surface roughness more strongly; thus, further studies are necessary to establish standardized ageing protocols. Furthermore, it must be borne in mind that not all surface defects and irregularities can be detected by profilometry as used in this study. For instance, resolution of very low surface roughness values may be limited due to the form of the stylus; thus, future studies might employ additional methods such as atomic force microscopy for the determination of surface roughness.

Inconsistent data are available concerning the influence of substratum surface free energy on initial *Candida albicans* adhesion. Minagi and co-workers found increased adherence of *Candida albicans* to specimens with high surface free energy than to those with low surface free energy [21], whereas Klotz and co-workers determined greater adhesion of *Candida albicans* to substrata with hydrophic surface properties which yield rather low surface free energy [25]. However, recent studies on this topic found no correlation between substratum surface free energy and *Candida albicans* adhesion at all [9, 10, 26]. In contrast to these studies, the aim of the present study was to assess changes in surface free energy after artificial ageing, and to make an attempt to correlate these findings to the decisively higher adhesion of *Candida albicans* to the aged specimens. Slight increases in surface free energy were observed for most of the specimens after artificial ageing, which supports the hypothesis by Minagi and co-workers finding increased adhesion to high surface free energy surfaces [21]. However, only very few statistically significant changes were found, which indicates that resin ageing and changes in substratum surface free energy do not necessarily correlate with increased *Candida albicans* adhesion. For the denture base resin, more distinct differences in surface free energy were observed than for the veneering composite. This might be attributed to the higher

content of inorganic fillers in the resin matrix of the composite that might account for reduced elution of matrix constituents. It might also be possible that differences are due to the different absorption of water, which has to be clarified by further analysis.

However, it is obvious that the increased adhesion of *Candida albicans* to artificially aged specimens cannot be explained sufficiently with changes in substatum surface roughness or surface free energy. In previous studies, the content of residual monomer has been accounted for higher adhesion of *Candida albicans* to new resin specimens than to aged specimens [15, 27]. However, it is doubtful whether the outcome of this study can be attributed to the content of residual monomer, as all specimens were stored in distilled water that has been exchanged daily. This procedure was performed to minimize the influence of residual monomers and potentially toxic constituents on viable fungi. However, with regard to this aspect, future studies might measure the elution of residual monomers from new and artificially aged specimens, or investigate whether polishing after artificial ageing causes a decline in *Candida albicans* adhesion to the artificially aged but polished specimens.

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