CHAPTER 3

MICROBIAL BIOFILMS ON FACIAL PROSTHESES


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Abstract

The composition of microbial biofilms on silicone rubber facial prostheses was investigated and compared with the microbial flora on healthy and prosthesis-covered skin. Scanning electron microscopy showed presence of mixed bacterial and yeast biofilms on and deterioration of the surface of the prostheses. Microbial culturing confirmed presence of yeasts and bacteria. Microbial colonization was significantly increased on prosthesis-covered skin compared to healthy skin. *Candida* spp. were exclusively isolated from prosthesis-covered skin and from prostheses. Biofilms from prostheses showed least diverse band-profile in denaturing gradient gel electrophoresis (DGGE) whereas prosthesis-covered skin showed the most diverse band-profile. Bacterial diversity exceeded yeast diversity in all samples. It is concluded that occlusion of the skin by prostheses creates a favorable niche for opportunistic pathogens as *Candida* spp. and *Staphylococcus aureus*. Biofilms on healthy skin, skin underneath the prosthesis and on the prosthesis have a comparable composition, but appearance in number differs per microorganism.
Introduction

Facial prostheses are fabricated to mask defects in disfigured patients because of acquired or congenital defects in the facial area when reconstructive surgery is not feasible [1-6]. These prostheses are usually made from silicone elastomers, a material that is intrinsically and extrinsically colored to match the color of the skin [7, 8]. For retention, usually adhesives or percutaneous implants in combination with bar/clip or magnets are used [1, 6, 9].

It is well acknowledged that the use of silicone elastomers for facial prostheses is accompanied by clinical problems such as gradual discoloration, and degradation of physical and mechanical properties [10-12]. This limits the mean longevity of the prostheses to 13 – 28 months, depending on the location of the prosthesis (auricular, orbital or nasal) [6, 13]. This limited longevity requires patients to make frequent hospital visits to replace the prosthesis [6], which is inconvenient to the patient and brings high costs to society.

Microbiological research within the facial prosthetic field is almost exclusively directed at the percutaneous implant, which connects the prosthesis to tissue underneath it and is prone to peri-implantitis [14-17]. Skin irritation in prosthesis-skin contact area, not adjacent to implants, is also a problem in patients, but to our knowledge the microbiology related to this has received no attention. It is postulated that this irritation is caused by surface microbial colonization (biofilms) of the prostheses in direct contact with the skin [6].

On the skin there is a subtle balance of symbiosis between skin microbial flora and the host. This balance may become disturbed upon application of a silicone elastomer prosthesis to the skin. It is well established that medical limb prostheses made from plastics may lead to dermatitis due to pressure, occlusion, heat and friction [18]. Dermatitis could result from wearing a facial prosthesis for similar reasons. The face is the second most affected body site from physical irritant contact dermatitis [18]. However, the occurrence of microbial biofilms on facial prostheses, their effect on the composition of the microflora of the skin and their potential for deterioration of prosthesis material have never been investigated.
The aim of this study was to investigate the composition of microbial biofilms on facial prostheses and to compare their microbial composition relative to that of the skin underneath the prosthesis and to mirroring unaffected, healthy, skin. Microbial colonization of worn facial prostheses was investigated using scanning electron microscopy (SEM). Microbial composition of biofilms was compared to samples from unaffected skin, and skin underneath the prosthesis using microbiological culturing and PCR/denaturing gradient gel electrophoresis (DGGE).

Materials and methods

Patient selection and sampling sides

Between 2005 and 2009, a total of 43 malfunctioning prostheses for facial defects on orbital, nasal or auricular region was collected at the Department of Oral and Maxillofacial Surgery and Maxillofacial Prosthetics, University Medical Center Groningen, The Netherlands. Due to the destructive nature of the analyses only malfunctioning prostheses were collected.

The facial prostheses were all made from pigmented silicone elastomer VST-50HD (Factor 2, Lakeside, AZ, USA) colored with intrinsic pigment paste (Factor 2, Lakeside, AZ, USA) and worn by the patient for 0.6 to 3 years (mean 1.6 years). Prosthesis were immediately after collection placed in a closed container, stored in a refrigerator and transported to the laboratory the same day. The surface of the prosthesis in direct contact with the skin was analyzed with SEM. Moreover, for microbial analyses the surface of the skin in direct contact with the prosthesis and of the healthy skin on the mirroring side, was sampled by a swab. The collected microorganisms were cultured on blood agar (OXOID, Basingstoke, UK) and CHROMagar (BBL-Becton Dickinson, Breda, The Netherlands) plates; for community composition DGGE was used.
Scanning electron microscopy analysis

As all prostheses showed signs of degradation of the silicone elastomer, 6 out of these 43 prostheses were selected randomly and subjected to SEM analysis. These prostheses had been worn for 1-2 years. SEM analysis of biofilms on silicone elastomer facial prostheses was performed as described previously [19]. Briefly, samples were fixed in 2% w/v glutardialdehyde (Sigma-Aldrich, Vienna, Austria) and 0.1 M cacodylate (Sigma-Aldrich) buffer (pH 7.4) for at least 48 h. Post-fixation was performed in 1% OsO4 for 2 h. The samples were washed with water and dehydrated using ethanol series followed with tetramethylsilane treatment. In order to investigate material degradation in the presence of microbial biofilms, samples were freeze fractured to obtain prosthesis top and side-views. Samples were sputter-coated with 3 nm Pd/Au and images taken using low-voltage SEM (SM-6301F, JEOL, Japan) at 2 kV. When indicated, prostheses were brushed for 30 s using a sterile cotton tip, wetted with sterile PBS prior to sample preparation to mimic cleaning of the prosthesis. The prosthesis side in contact with the skin was analyzed with SEM, because this side was most heavily deteriorated (see Figure 1a).

Microbiological culturing skin

A skin area of approximately 3 cm² that has been in direct contact with the prosthesis sample was swabbed for 30 s using a sterile flocked swab with breaking point (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) moistened with filter sterile reduced transport fluid (RTF, 0.9 g/l NaCl, 0.9 g/l (NH₄)₂SO₄, 0.45 g/l KH₂PO₄, 0.45 g/l K₂HPO₄, 0.19 g/l MgSO₄, 0.37 g/l Na₂EDTA, 0.2 g/l L-Cysteine HCl, pH 6.8). A healthy part (3 cm²) of the skin was swabbed in the same manner. This healthy part mirrored the sampling site of skin underneath the prosthesis. The tip of the swab was broken and placed into a sterile 2 ml Eppendorf tube containing 1 ml of RTF. The tube was capped and vortexed for 30 s to suspend the microorganisms.
Prosthesis

From 25 prostheses, we were able to culture microorganisms and determine the most dominant organisms present. A 2 x 5 mm section of the prosthesis area (Figure 1a) that was in contact with the skin was cut and placed in tubes containing 4.5 ml sterile phosphate buffered saline (PBS; 0.15 M NaCl and 10 mM potassium phosphate, pH 7.0). The tubes were vortexed for 10 s, sonicated for 3 min in an ultrasonic waterbath and vortexed again for 10 s.

The samples from the prosthesis and the skin were serially diluted and 100 µl of each dilution was plated on blood agar plates and incubated at 37°C under aerobic and anaerobic (10% H₂, 85% N₂ and 5% CO₂) conditions for 24 and 48 h, respectively. To distinguish fungal isolates, the same samples were also plated on CHROMagar Candida which were incubated at 37°C for 48 h.

The most prominent bacterial and yeast species from skin and prostheses samples, as observed visually on each blood agar and CHROMagar plate, were selected based on macroscopic and microscopic characteristics. Pure cultures were prepared on blood agar plates and sent for identification to the Department of Medical Microbiology, University Medical Center Groningen, The Netherlands. Staphylococci were determined by catalase 3% and API Staph-plus (bioMérieux, Marcy l’Etoile, France) and streptococci by catalase 3% and API Strep (bioMérieux). Corynebacteria were determined by Gram-staining, catalase 3% and API Coryne (bioMérieux), while Propionibacterium avidum was determined using Gram-staining, catalase 15%, Indol tube/nitrate disk (both homemade) and Esculin Diatabs (Rosco, Taastrup, Denmark). As for bacilli, Gram-staining and spore forming were essential. Pseudomonas was determined using oxidase, growth at 42°C and King A/B (Mediaproducts BV, Groningen, The Netherlands). Gram-negative bacteria (Proteus mirabilis, Enterobacter cloacae, Serratia marcescens) typing was done using VITEK2 (bioMérieux). Yeast was typed using CHROMagar Candida.
FIGURE 1 (a) Example of facial prosthesis with sampling side cut made at the margin that is in contact with the skin. Prosthesis showing degradation/tear on the periphery (black arrow) and brownish deposition (white arrow) at the area that is in contact with the skin; (b) SEM image of the biofilm on the cut area of the prosthesis (bar = 100 µm); (c) SEM image at high magnification (bar = 10 µm) of a microbe-filled pocket in the prosthesis. The left inset is a detailed image showing the presence of bacteria (diameter ~ 1 µm) within the pocket while the right inset shows the presence of yeast (diameter ~ 3 µm) around the same pocket.

**DGGE analysis**

Only from 12 patients enough DNA was isolated for further examination using PCR/DGGE from the prostheses, skin samples from underneath the prosthesis and from a mirroring healthy, contra lateral side. DNA was extracted using a modification of the microwave technique [20]. Briefly, suspended biofilm in the Eppendorf tube was centrifuged for 3 min at 18,000 × g. The supernatant was
aspirated and the cells were heated in the microwave for 2 min at 700 W. Fifty µl water was added and the tube was centrifuged at 18,000 × g for 1 min. The nucleic acid concentration was estimated using a spectrophotometer (NanoDrop ND 1000 V3.5, Isogen, Maarsen, The Netherlands) and stored in -80°C freezer for subsequent use. Prior to PCR, the extracted DNA was dissolved in buffer (0.1% Dextran 10000 (Serva Feinbiochemica, Heidelberg, Germany), 0.3 M sodium acetate (Merck, Darmstadt, Germany), 1% Triton-X 100 (Boom, Meppel, The Netherlands), and 0.1% bovine serum albumin (Sigma, Louis, MO, USA) and isopropanol (Merck) precipitated to concentrate the DNA. The PCR primers used in this study, targeting the V2-V3 region of the 16S rRNA gene in bacteria or the D1 region of the 26S rRNA gene of fungi, are listed in Table 1. Each PCR reaction consisted of 12.5 µl PCR Master Mix (Fermentas, St. Leon-Rot, Germany), 1 µl of each forward and reverse primer, 0.01 % Tween 20 (Boom) and 100 ng template DNA in 25 µl total reaction volume. Initial denaturation was performed at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 45 s, 58°C (bacteria) or 54°C (yeast) annealing for 45 s, 72°C for 60 s primer extension and final extension at 72°C for 5 min. Presence of the expected PCR product was confirmed by electrophoresis of 5 µl on a 2% (w/v) agarose gel (Sigma-Aldrich, Zwijndrecht, The Netherlands) in 45 mM Tris-Borate and 1 mM EDTA buffer stained with ethidium bromide.

DGGE was performed using an Ingeny Phor-U machine (Ingeny International BV, Goes, The Netherlands) according to the manufacturer’s instruction. Eight percent (w/v) polyacrylamide gel was used with a 30 to 70% denaturant gradient across the gel to analyze PCR products. A 5 ml stacking gel with 0% denaturant was decanted on top. Gels were run at 60°C for 16 h at 120 V in 0.5 × TAE buffer (0.04 M Tris base, 0.02 M acetic acid, 1.0 mM EDTA pH 7.5) then the gels were stained using silver nitrate [21].


**TABLE 1** PCR primers F357GC-R518 for bacteria and NL1GC-LS2 for yeast

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position</th>
<th>Sequence (5'→3')</th>
<th>Product size</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>F357GC</td>
<td>357 – 372</td>
<td>CGC CCG CCG CGC CCC GCG CCC GGC CCG CCC TAC GGG AGG CAG CAG</td>
<td>161</td>
<td>[22]</td>
</tr>
<tr>
<td>R518</td>
<td>518 – 534</td>
<td>ATT ACC GCG GCT GCT GG</td>
<td></td>
<td>[22]</td>
</tr>
<tr>
<td>NL1GC</td>
<td>63 – 88</td>
<td>GCC CGC CGC GCC CGC CGC CGC TCC CGC CGC CCC CGC CCG GCC ATA TCA AGC GGA GGA AAA</td>
<td>203</td>
<td>[23]</td>
</tr>
<tr>
<td>LS2</td>
<td>266 – 285</td>
<td>ATT CCC AAA CAA CTC GAC AAA</td>
<td></td>
<td>[23]</td>
</tr>
</tbody>
</table>

*GC clamp is present on forward primer

Corresponding to numbering in *Escherichia coli* sequence for bacteria and *Saccharomyces cerevisiae* for eukarya

**Statistical analysis**

The distribution of CFU counts was tested for normal distribution using Kolmogorov-Smirnov test. Differences in CFU counts obtained from the skin underneath the prosthesis and from the healthy side were compared using Mann-Whitney U test with P < 0.05 indicating significant differences.

Gel images were analyzed with GelCompar II software (Applied Maths, Gent, Belgium). The bands present in each lane were automatically detected and then checked manually. A similarity matrix based on Dice coefficient was made [24]. Cluster analysis was based on the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and dendrograms were constructed based on UPGMA.
Results

**SEM analysis of prostheses**

Silicone elastomer facial prostheses were examined by SEM, the presence of microbial biofilms, observed as brownish deposition (Figure 1a), was evident in the area of the prosthesis that was in contact with the skin. Using 250-fold magnification, areas with obvious signs of degradation of the silicone material were covered by a microbial biofilm (Figure 1b). Higher magnifications (1000-fold) showed in-growth of the microorganism and deterioration of the prosthesis material (Figure 1c). When the surfaces of the prostheses were cleaned as instructed to the patient, the biofilm covering the prosthesis was removed, but microorganisms could still be observed buried in the material (Figure 2). It is important to note that biofilm formation on, and degradation of the prosthesis was limited to areas in contact with the skin.

**FIGURE 2** SEM images of silicone surface of prostheses (bar = 10 µm). (a) Unused prosthesis; (b) biofilm on top of prosthesis and deterioration of prosthesis before cleaning with microorganism corresponding to size of bacteria (1µm); (c) despite cleaning, there were microorganisms and remnants of microorganism embedded in the defects of the prosthesis where the biofilm was removed.
**Microbiological culturing of prosthesis covered and healthy skin**

Skin swabs with approximately equal surface area were obtained from the healthy skin side and from skin in direct contact with the prosthesis and the culturable microbial load was analyzed using serial dilution plating, the total CFU was not normally distributed (P < 0.05, Kolmogorov-Smirnov test).

The median aerobic bacterial CFU counts (CFU/cm²) on the prosthesis side was approximately 10-fold higher than on the contra lateral, healthy side (Figure 3). Similar results were obtained for anaerobic bacteria and yeast. Typing of the most prominent isolates from the skin showed that both mirroring healthy and prosthesis sides harbor coagulase-negative staphylococci.

In addition, bacteria that are normally not found on the skin, such as *Pseudomonas*

![FIGURE 3](image-url)  
**FIGURE 3** CFU counts (CFU/cm²) of aerobic, anaerobic bacteria and yeast comparing the skin underneath prosthesis (prosthesis side) and healthy skin (healthy side). The green lines and squares represent the orbital prostheses, the blue lines and triangles the nasal prostheses and the red lines and circles the auricular prostheses.
aeruginosa, were also isolated from the healthy and skin underneath the prosthesis. Interestingly, Candida spp. were exclusively cultivated from the skin underneath the prosthesis (Table 2) and were not detected on the healthy skin. There was not any difference observed in microbial population for the orbital, nasal or auricular region (see also Figure 3) neither could an ageing effect of the biofilm be observed due to a great variation between individuals.

**TABLE 2** Typing results for the most prominent bacterial and yeast colonies from culturing of healthy skin (healthy side) and skin underneath prosthesis (prosthesis side).

<table>
<thead>
<tr>
<th>Healthy side</th>
<th>Prosthesis side</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulase negative Staphylococcus</td>
<td>Enterobacter cloacae</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td><em>Bacillus spp</em></td>
<td>Serratia marcescens</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td></td>
<td><em>Proteus mirabilis</em></td>
</tr>
<tr>
<td></td>
<td>Coagulase negative Staphylococcus</td>
</tr>
<tr>
<td></td>
<td><em>Propionibacterium avidum</em></td>
</tr>
<tr>
<td></td>
<td><em>Corynebacterium amycolatum</em></td>
</tr>
<tr>
<td></td>
<td><em>Candida spp</em></td>
</tr>
<tr>
<td></td>
<td><em>Candida parapsilosis</em></td>
</tr>
</tbody>
</table>

**Microbiological culturing of prostheses**

Both bacteria and yeast were cultured by sampling the prostheses. *Staphylococcus epidermidis*, *Staphylococcus schleiferi*, *Staphylococcus xylosus* and
Staphylococcus capitis were the most frequently detected bacterial species while Candida albicans, Candida parapsilosis and Candida famata were the most frequently detected yeast species. SEM analysis revealed that it was impossible to completely remove all microbial cells from the surface by the care system applied by the patients (Figure 2). Therefore, the CFU/cm² could not be determined reliably for prostheses. Hence, prosthesis samples were not included in Figure 3.

**DGGE similarity profile of prostheses, prostheses covered and healthy skin**

DGGE profiles of the prosthesis, healthy skin and skin underneath the prosthesis were compared. An example of DGGE profiles for the prosthesis, healthy skin and skin underneath the prosthesis using both primer sets is shown in Figure 4. The profiles obtained for the bacterial primers showed many bands (high diversity) with a high level of similarity between samples (Figure 4a). A prominent band was observed at the same level for the S. aureus and S. epidermidis marker for all samples. In contrast to the high diversity observed for the bacterial primers, the fungal primer set resulted in less diversity but still with a considerable similarity between the samples (Figure 4b).
Dice coefficients were used to calculate similarity patterns between samples. Patterns of bacterial and yeast profiles were calculated separately. Similarity values of bacterial DGGE band profiles combined for all samples ranged from 20 to 85.7%. Yeast DGGE band profiles combined for all samples ranged from 9.9 to 92.3%. Dendrograms show that for the bacteria (Figure 5a) and yeast (Figure 5c) isolated from the healthy skin and skin underneath the prosthesis of the same patient group together for 11 out of 12 patients. The similarities per patient can be very different as for example the similarity for patient #96 is very high (86%), whereas for patient #94 it is only 50% for the bacterial profile on the healthy skin and skin underneath the prosthesis (see Figure 5a). Note that the patient where
the bacterial profile is not grouping together is not the same patient as in the yeast group. However, when the prosthesis was compared with the skin underneath the prosthesis (Figures 5c, 5d) only 8 out of 12 for bacteria and 7 out of 12 yeast samples of the patients clustered together.

**Discussion**

All silicone elastomer facial prostheses that were evaluated with SEM revealed presence of a mixed bacterial and yeast biofilm on the area in direct contact with the skin. Microorganisms of the biofilms penetrated into the silicone elastomer facial prosthesis similar as has been observed for voice prostheses [19]. The bag-like defect caused by the microbial biofilms on the facial prostheses (see Figure 1) could be responsible for the clinically observed deterioration of the prosthesis material. SEM analysis of “cleaned” prostheses (Figure 2) revealed that standard cleaning regimes described in the routine care program suggested to patients do not remove all the microbial cells from the prosthesis surface [25]. This recalcitrance towards mechanical cleaning illustrates the need for improved cleaning guidelines for these prostheses or alternatively for new materials that are more resistant to microbial biofilm formation.
Microbial typing from skin samples underneath the prosthesis showed presence of commensal microorganisms such as coagulase negative staphylococci, however, also opportunistic pathogens such as *S. aureus*, *P. aeruginosa* and *Candida spp.* were isolated. Interestingly, *Candida spp.* could only be detected by culturing on the skin underneath the prosthesis and the prosthesis itself, in contrast with DGGE yeasts are also detected on the skin. Although *Candida spp.* are commensals of the skin, their abundance is generally too low for culturing, but not for non-culturing techniques as DGGE. The association of *Candida spp.* with silicone elastomer is observed more often, for instance in silicone denture liners [26, 27] and voice prostheses [28, 29]. Failure of silicone elastomer voice prostheses was strongly related to the microorganisms present in the biofilm. According to these studies, *Candida spp.* were predominantly isolated from short lifetime voice prostheses compared to extended lifetime prostheses. In the extended lifetime group *C. tropicalis* was never isolated, whereas *C. albicans* and *Rothia dentocariosa* were isolated two and four fold lower than in the short lifetime group [28]. In this study it is shown that the specific niche related to prosthesis occlusion of the skin favors the presence of opportunistic bacterial and fungal pathogens.

The DGGE profiles illustrate that in contrast to the prosthesis microflora, the species composition of microflora on the skin is not significantly altered by the presence of the prosthesis. This seems to be at odds with the culturing result of the skin (Table 2). It should be noted that DGGE analysis is a culture independent and not a true quantitative method. Culturing results reflected the predominant
cultivable microorganisms, but not the whole profile of the samples. Importantly, a significant difference was observed in total number of cultivable microorganisms; skin occluded by a prosthesis showed a 10-fold increase in total culturable microflora compared to mirroring healthy skin. Prosthesis-covered skin provides a unique niche for bacteria and yeast, which is caused by occlusion of the skin by the prosthesis resulting in increased humidity and temperature [18]. In addition, microflora on the prosthesis is different by enrichment of some microorganisms whereas others are diminished, which could be related to material specific properties such as surface roughness or hydrophobicity. In general, rougher and more hydrophobic surfaces enhance biofilm formation [30]. Silicone elastomer is a hydrophobic material and its roughness is directly correlated with the roughness of the handmade stone mold used to fabricate the prostheses. Coating of stone molds with various materials to facilitate separation of the prosthesis from the mold, did not result in less roughness of silicone material [31].

Clinical experience indicates that most skin irritation in patients will decrease upon local treatment with water, soap combined with antibiotics. However, the skin will soon become irritated again once the antibiotic treatment has been stopped [6]. Successful treatment with local antibiotics illustrated that most skin irritations could be a consequence of the specific niche created by the prosthesis-covered skin, as shown in our study. Importantly, it should be noted that yeasts are abundantly present on prosthesis (see also Figure 2) and antibiotic treatment would further favor fungal overgrowth due to removal of competing bacterial flora. The observed rapid recurrence of irritation could be related to reinfection with microorganisms derived from the prosthesis, especially because mechanical cleaning does not remove all microorganisms, as illustrated in the present study. This hypothesis is supported by the clinical observation that for patients with recurrent irritation, replacing the old prosthesis with a new one usually resolves the problem.

In conclusion, results from this study indicate that facial prostheses are generally colonized by complex microbial biofilms. The presence of microbial biofilms has several implications, importantly; their occurrence could be related to material
degradation and skin irritation, ultimately leading to dysfunction of the facial prostheses. The skin microflora underneath the prosthesis is very similar to healthy skin microflora in species composition, but significantly higher in total microbial load. In contrast, the microflora on the prosthesis is probably derived from skin, but due to specific material properties of the prosthesis, selection for yeasts occurs.

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