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Ariani, Nina

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Nina Ariani

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University Medical Center Groningen, University of Groningen
Groningen, The Netherlands

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Nina Ariani
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Promotores

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To my parents

Paranimfen

Gusnaniar

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Table of Contents

CHAPTER 1

GENERAL INTRODUCTION

Maxillofacial prostheses are used to replace parts or complete stomatognathic or craniofacial structures. According to the Glossary of Prosthodontic terms, a facial, also named extraoral, prosthesis is a maxillofacial prosthesis that replaces a portion of the face that is lost or deformed due to, e.g., trauma, congenital abnormalities or ablative surgery [1]. Already the ancient Egyptians were involved in maxillofacial prosthetics as part of their attempt to preserve normal body appearance of the deceased. There is some evidence that around 3000 B.C. the face of deceased subjects was reconstructed with filling the mouth and filling the empty eye socket with artificial eyes made of limestone, calcite, bone or linen with pupils delineated using black paint [2]. In the 16th century A.D. a more lifelike facial prosthesis was made, a nose, fabricated from metal, colored with oil to match the skin and attached with an adhesive to the face (Figure 1a). Later, different materials are used for fabricating facial prostheses (Figure 1b) [2]. Nowadays, these facial prostheses are usually fabricated from silicone rubber and retained either by using adhesive, undercuts, eyeglasses or implants (Figure 1c) [3].

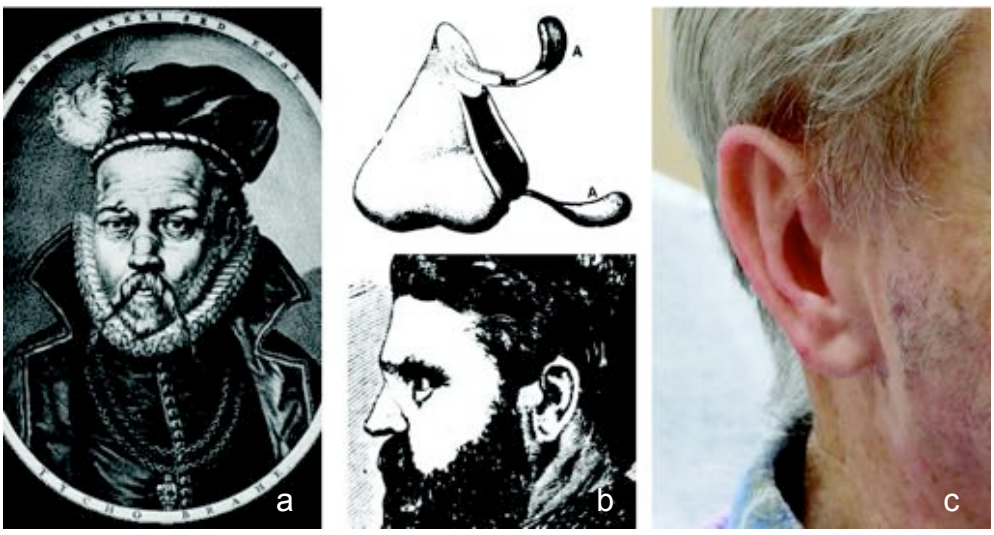

FIGURE 1 (a) Early nasal prosthesis made of metal (16th century) [4]; (b) collodion nasal prosthesis with vulcanite pads for retention (1870) [2]; (c) an implant retained silicone auricular prosthesis.

The inner surface of facial prostheses is in contact with soft tissues and body fluids. Therewith microorganisms can colonize and form biofilms on the facial prostheses. From silicone soft lining materials used for dentures it is known that microorganisms can degrade these softliners [5]. The composition of biofilms on surfaces of prostheses, in particular on surfaces of facial prostheses, and how microorganisms can affect the silicone rubber and other components facial prostheses made from in particular are not yet thoroughly investigated. So far, most studies have focused on biofilms around percutaneous implants used to retain facial prostheses [6-9], i.e. the peri-implant flora, instead of focusing on flora that has colonized the silicone rubber of the prostheses. The latter is of interest as infections of, e.g., the skin underlying the prostheses is a common clinical phenomenon that causes distress to the patient.

Biofilms are of particular interest regarding the maintenance of facial prostheses due to a long held notion that microorganisms within the biofilm have properties to degrade the material facial prostheses made from and to change the color of the prosthesis. In 31% of the cases discoloration was the main reason to fabricate a new prosthesis [10]. Pollution, exposure to UV, natural aging and nicotine all have been presumed to underlie the observed discoloration [11-13]. In addition, the use of intrinsic colors, either alone or as a result of interaction with microorganisms, is thought to be a contributing factor to degradation and discoloration of facial prostheses, but this presumption is in need of further study [10].

Since facial prostheses are in contact with skin for extended time, the surface of facial prostheses that covers the skin creates pressure, heat, higher humidity, occlusion and friction [14]. Biofilms formation are suggested to occur on skin as well [15]. The prevalence and incidence of silicone rubber related adverse skin reactions in facial prostheses patients is unknown, but occlusion and humidity are amongst the factors that promote adverse skin reactions [16, 17]. The occurrence of biofilms on both silicone facial prostheses and skin, in conjunction with aforementioned factors, is thought to contribute to the problem. This is especially true for adhesively retained prostheses, but implant retained prostheses also face this problem. Implant retained prostheses are still accompanied by skin occlusion and humidity because the

margins of the prosthesis need to be properly adapted to the skin as well and there still might be constituents of the prosthesis material that are harmful for the patient. Regarding orbital prostheses, 5% of these prostheses fail due to in-growth of microorganisms [10]. For other type of facial prostheses, i.e. auricular and nasal prostheses, it is unknown what percentage of failure is related to microorganisms, but adhesion of microorganisms to and formation of biofilms on surfaces of prostheses are well known causes for infections of medical devices.

The incidence of medical devices failures due to bacterial contamination ranges from 1-4% for hip prostheses to 100% for urinary catheters [18]. Chronic infections by biofilms are of interest because of resistance of microorganisms present in the biofilm to antibiotics. Furthermore, the architecture of a biofilm, i.e., the layer on the surface of a prosthesis in which the cells are embedded in extracellular polysaccharide matrix, renders poor penetration of antibiotics through that layer [19]. Therefore it is possible that a biofilm on a medical device is 500-1000 times more resistant to antibiotics than planktonic bacteria [20, 21].

New strategies to prevent biomaterial related infections are underway. Among these strategies are modifications of the surface of biomaterials, e.g., through incorporation of antimicrobial agents into the biomaterials itself and use of surface coatings. With these modifications, infections related to medical devices can be inhibited or prevented [22]. While new strategies are being developed, the routine method used currently to prevent biofilm formation on silicone facial prostheses is to instruct patients to clean their prostheses meticulously. As a result, most studies on facial prostheses focus on biofilms and cleansing of the percutaneous implants that are increasingly used to retain facial prostheses currently [23-26] instead of assessing the efficacy of cleansing the prosthesis itself. This approach might be driven to the presumption that rigorous cleaning or use of inappropriate cleaning agents can lead to damage of the silicone rubber [26]. It is also still questionable whether the most inner surface of the prosthesis is sufficiently accessible to optimal hygiene.

Therefore, it is required to focus on efficacy of routine cleaning methods and materials to help establish suitable cleaning methods and or agents, not only for

peri-implant tissues but also for the material the prosthesis made from. When adverse skin reactions persist despite correct hygiene procedures, antibiotics and anti-inflammatory drugs are prescribed with recurring events of adverse skin reactions once the medication is discontinued. Therefore, life-long follow up and studies to improve facial prostheses longevity with low burden to the patients' own tissues are indispensable as well as studies to develop easily applicable, nontoxic and non-prosthesis material damaging procedures to effectively clean a facial prosthesis.

Aim of this thesis

The general aim of this thesis was to make an inventory of biofilms on facial prostheses and to analyze the composition of these mixed species biofilms. In addition, routine methods to clean a facial prosthesis were studied to assess how efficient they are in killing biofilms.

In **chapter 2** the current state of facial prosthetic rehabilitation was reviewed. The main conclusion of this chapter was that facial prostheses are a reliable treatment option to restore maxillofacial defects and to improve quality of life. Significant progress has been made in the utilization of implants and digital technology. Improvements to enhance prostheses longevity include a better understanding of the biofilm on the surface of the prosthesis and to judge which methods are most effective in removing this biofilm from the prosthesis.

In **chapter 3** we studied the composition of biofilms on facial prostheses and the influence of the prosthesis on the microbial composition of the skin underneath prostheses. The main result of this chapter was that occlusion of the skin by the prostheses created a favorable niche for opportunistic pathogens such as *Candida spp.* and *Staphylococcus aureus*. Biofilms on healthy skin, skin underneath prosthesis and the prosthesis had a comparable composition.

In the study described in **chapter 4** the efficacy of cleansing agents to affect the biofilm on facial prostheses was studied. Chlorhexidine mouthrinse showed the

highest efficacy in eradicating bacteria and yeasts in the biofilms, especially after repeated treatment compared to the other cleansing agents.

The case study in **chapter 5** assessed efficacy of cleansing agents in killing biofilms of *ex vivo* silicone facial prostheses. Essential oils and chlorhexidine were effective in reducing microorganisms of *ex vivo* silicone facial prostheses biofilms.

The general discussion described in **chapter 6** places the results of the studies performed in a broader perspective. It is discussed which other factors might affect the longevity of facial prostheses including the *in vitro* effect of pigments on silicone rubber and the effect of adding pigments to silicone rubber on the growth of microorganisms on silicone rubber.

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CHAPTER 2

CURRENT STATE OF CRANIOFACIAL PROSTHETIC REHABILITATION

Nina Ariani, Anita Visser, Robert P. van Oort, Lindawati Kusdhany, Tri Budi W. Rahardjo, Bastiaan P. Krom, Henny C. van der Mei, Arjan Vissink.

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Abstract

Aims To provide an update of the current status of treatment options and materials utilized in the rehabilitation of maxillofacial defects (ear, nose and orbital defects).

Methods A search of MEDLINE and EMBASE databases was conducted for articles pertinent to maxillofacial prostheses. The main clinical stages were the subject of analysis. The references spanned the period from January 1990 to July 2011.

Results A multidisciplinary approach is preferred when rehabilitating maxillofacial defects aiming for optimal patient care and improving patient's quality of life. Surgical reconstruction can be used for smaller defects but larger defects require prosthesis to achieve aesthetic reconstruction. In terms of prosthesis retention, implant-retained prostheses are preferred over adhesives prostheses. Silicone elastomer is currently the best material for maxillofacial prosthesis. However, material longevity and discoloration are still big issues and greatly influenced by UV-radiation, microorganisms and environment. Widespread availability and cost-effective approach of digital systems could improve the workflow and outcome of facial prostheses in the near future both from a clinician's and patient's perspective. Overall patients state high satisfaction with their prosthesis although some areas need improvement.

Conclusion Maxillofacial prostheses are a reliable treatment option to restore maxillofacial defects improving patient's quality of life. During the last decade, most progress in maxillofacial rehabilitation care has been made in the application of implants for retention and digital technology for designing the surgical guides, suprastructures and craniofacial prostheses. Improvements are necessary for longevity of the prosthesis, i.e. quality of materials, color stability and microbial influence on prostheses.

Introduction

Worldwide, patients suffer from maxillofacial defects due to cancer, trauma or congenital diseases demanding high-quality prosthetic treatment [1] because, amongst others, these defects cause aesthetic and psychological problems (Figure 1a) [2].

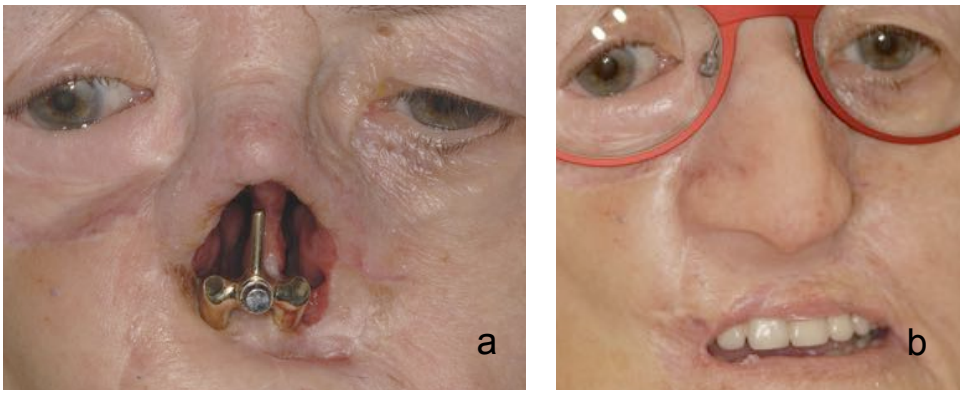

FIGURE 1 Patient treated for a basal cell carcinoma of the nose. (a) A bar suprastructure was placed on two implants in the floor of the nose; (b) the nasal prosthesis was positioned on the bar suprastructure.

In many cases, it is challenging to reconstruct maxillofacial defects and a satisfactory aesthetic outcome is not always easy to achieve. Maxillofacial defects can be treated by surgical reconstruction and prosthetic rehabilitation (Figure 1b) [3-5]. In particular, surgical reconstruction of maxillofacial defects is often very difficult to perform from a technical point of view. Furthermore, there is a high risk of complications and it seldom leads to patients' satisfaction [4]. The aesthetic results can be disappointing, especially for oncologic surgical ear reconstructions. With regard to reconstruction of nose defects resulting from tumor surgery, it has been reported that reconstruction with an expanded forehead flap may be a very good alternative to maxillofacial prostheses [6].

Maxillofacial prosthodontists have a number of options available to rehabilitate patients using prosthetic restorations to improve function and aesthetic [5]. An aesthetic and comfortable maxillofacial prosthesis alleviates many concerns of the patient and improves their quality of life [7, 8] without the risks associated with surgery.

Maxillofacial prostheses can provide a natural-looking cosmetic situation. In many cases, the aesthetic outcomes of maxillofacial prostheses are superior over surgical reconstruction [3, 9]. In the past, maxillofacial prostheses were retained by mechanical tools (e.g. glasses), skin adhesives or undercuts [10], but since 1979 there is a shift towards implant-retained maxillofacial prostheses [11, 12]. Such prostheses are preferred by many patients over conventional maxillofacial prostheses [13, 14].

This narrative review addresses the current status of treatment options and the materials involved in the rehabilitation of maxillofacial defects (ear, nose and orbital defects) and their possible treatment outcomes, as well as the impact of the various treatments on coping of the patient with the rehabilitation of their maxillofacial defect and the patient's quality of life. To the best of our knowledge, such a review is lacking in the current literature despite continuing progress in maxillofacial prosthodontics and the current literature does not allow for a systematic approach.

Literature search

A search of MEDLINE and EMBASE databases was conducted using (a combination of) search terms: facial defect, maxillofacial prosthesis, silicone facial prosthesis, facial prosthodontics, adhesive facial prosthesis, extra-oral implants, nasal defect, orbital defect, sculpturing, digital planning, stereolithography and color matching. Additional references were taken from the bibliography of the references identified through MEDLINE and EMBASE searches. Title and abstracts identified through electronic searches were reviewed by 2 authors independently.

The references spanned the period from January 1990 to July 2011. Only papers written in English, German or Dutch relevant to maxillofacial prosthodontics were incorporated in this review. Case reports were avoided as much as possible.

The multidisciplinary approach

Treatment of maxillofacial defects has evolved to a multidisciplinary treatment modality and consists of a combination of invasive and non-invasive approaches. The reconstruction plan is the result of discussions between various members of the head & neck team, including ablative surgeons, reconstructive surgeons, maxillofacial prosthodontists and maxillofacial technicians. The following factors have to be taken into account with regard to the prosthodontic rehabilitation of the patient: 1) amount of supporting tissue remaining, 2) number, position and condition of remaining dentition, 3) age and medical condition of the patient, 4) pathologic findings, 5) patient's demands to opt for surgical or prosthetic reconstruction, 6) skills of the reconstructive surgeon and prosthodontist, 7) the mental status and manual skills of the patient to deal with a maxillofacial prosthesis, and 8) the availability of adequate supportive care in case the patient is not able to take care of his prosthesis. The resulting treatment plan is discussed with the patient and concerned family. In other words, maxillofacial rehabilitation is an integral part of patient management and is, at least in the high and middle income countries, currently composed of a combination of implantology, technology, advanced surgical and prosthetic procedures, and proper instruction and education of the patient, concerned family and/or care assisting network [15-17]. The latter counts especially for the elderly as elderly may face difficulties in handling the prosthesis and cleaning the suprastructures [18].

The multidisciplinary setting allows the patient the privilege of having treatment provided by a dedicated head & neck team. This team encompasses different ablative, reconstructive and prosthodontic fields including otolaryngology, maxillofacial surgery, plastic/reconstructive surgery, maxillofacial prosthetics, radiology, medical oncology, pathology, psychology, social work, speech and

physiotherapy, and dietetics [19, 20]. All disciplines must cooperate to provide the patient with an optimal, individualized treatment plan by incorporating diagnosis, staging, treatment, rehabilitation, follow-up and supportive care. This way the patients are not just provided with medical care, but also with the best guarantees that their therapy aims for an optimum quality of life and they can cope with their defects [5, 15, 21].

Surgical reconstruction

As this review focuses on craniofacial prosthetic rehabilitation, surgical reconstruction will only be discussed briefly. Advances in imaging modalities (e.g. high resolution CT scanners, MRI), alloplastic materials, surgical techniques and instrumentations have led to highly improved approaches for surgical reconstruction of the maxillofacial area either by the use of autologous and/or alloplastic materials [22, 23]. In extensive ablative procedures, a combination of free tissue transfer, local flap and implant-retained prosthesis rehabilitation is performed. The successful outcome of these approaches is also apparent when psychosocial outcomes are taken into account [24]. While smaller defects can often be reconstructed successfully with surgery in local hospitals [25, 26], larger and more complex defects call for medical centers with greater expertise to reconstruct function and aesthetics of the patient [27, 28]. The surgical procedures of complex cases might require several operations over a prolonged period of time. As an example, surgical reconstruction of a nose with a reasonable aesthetic outcome requires 3 to 15 operations in a time-span of 4 to 49 months [29]. Even then, often a suboptimal aesthetic outcome is obtained, as well as that many patients are not into such an extensive and time-consuming surgical treatment.

Facial prosthetics

Conventionally and adhesively retained prostheses: still a reasonable approach?

Retentive methods for maxillofacial prostheses involve adhesives, undercuts, spectacles and implants for anchorage [30-35]. Prostheses which are conventionally retained using adhesives are often rated as unsatisfactory by the patients because of the difficulty that patients experience for placing the prosthesis properly, and because of prosthesis-movement or dislodgement during daily activities related to surrounding soft tissue movements [36, 37]. Furthermore, adhesives can cause irritation of the skin [17, 31, 38, 39]. Retentive problems that may occur due to loss of adhesive strength of the glue [40] can be solved in part by using a combination of adhesives. Such multi-adhesive layering of two adhesives was shown to have the highest adhesion properties [31]. Unfortunately, there is no superior combination of prosthetic material/adhesives developed during the last decades [38, 41-43].

Implant-retained prosthesis: the current standard?

Implant-retained facial prostheses have evolved to an excellent treatment option in prosthetic rehabilitation and are usually preferred by patients over adhesive prostheses. Implant-retained facial prostheses are easier to put in place, more comfortable to wear and easier to clean compared to adhesive prostheses [11, 16, 17, 30, 32, 34, 49-54]. The surgical technique for osseointegrated implants is relatively simple and associated with a low rate of perioperative and long-term complications [14, 44]. Several retention systems for implant suprastructures are currently available such as bar-clip retention, ball attachment, magnetic retention, locator abutment attachment and the slant lock system [36, 45-48].

For facial application, bar-clip and magnet systems are mostly used [55]. Recent *in vitro* studies shown that the bar-clip system has the highest retention value and is the method of choice for retaining auricular and nasal prostheses [56]. The

disadvantage of this system is, however, that one needs sufficient space inside the prostheses to accommodate the acrylic clip carrier and bar. Magnetic systems have lower shear strength [57], but are very suitable for use in cases where there is not enough space for a bar-clip system and horizontal forces can be avoided. Magnets can also be very useful in case of non-parallel implants. Therefore, they are particularly suitable for orbital prostheses or patients with low manual strength or dexterity.

Some disadvantages of implant-retained prostheses are reported. Percutaneous implants by definition impair the function of the first line of defense, the skin, and as a result are prone to microbial infections [58]. With this respect, it has to be noted that in irradiated skin less peri-implant skin reactions are observed [14] Furthermore, when placed in irradiated bone, the risk of implant failure is three to twelve times higher than in non-irradiated bone [13, 14, 59, 60]. Implants placed in the mastoid area show higher overall success rates than implants placed in the nasal and orbital area [14, 58].

Despite these disadvantages, implant-retained prostheses are by far preferred by patients' above conventional prostheses meanwhile improving patients' daily activities and quality of life [13, 61-66].

Prosthetic materials: suitability, problems and new developments

During the last five decades, silicone elastomers have been clinically the material of choice for fabricating a facial prosthesis [41]. Particularly, the introduction of room temperature vulcanizing polymers (e.g. MDX-4-4210, Dow Corning, Chicago, USA; VST-50, Factor 2, Arizona, USA) has been an improvement compared to poly(methylmethacrylate), poly(vinylchloride) and polyurethane in offering optimal overall properties for facial prosthesis material [19, 73-77]. In a recent trial, a newer material, chlorinated polyethylene, was tested [41]. It was shown that wearers of silicone-based facial prostheses prefer silicone elastomers above chlorinated polyethylene elastomers, while new users had no preference for either material. In

other words, the non-inferiority of chlorinated polyethylene elastomers to silicone elastomers for fabricating facial prostheses cannot be shown in that trial [41].

In the 1990s, Andres et al. [78] and Beumer et al. [19] reported the ideal properties facial prosthetic material should possess. These lists contain a total of 68 criteria, divided into three sections (physical and mechanical properties, processing characteristics, biological properties). The criteria included color stability, margin integrity, edge strength, durability, ease of use, adjustments without remake, costs of production, nontoxicity and short fabrication time. Despite the advances in material technology, a 2010 survey in North America, Europe, Asia and Australia revealed that the same criteria still apply and disadvantages of materials still exist [42]. The most often reported disadvantages are limited longevity of the elastomers, discoloration, non-reparability and degradation (Figure 2) [14, 42, 79, 80].

Longevity

Longevity is an important property for the clinical application of facial prosthetics [81]. Degradation and discoloration of the material requires a remake of the prosthesis. Discolored prostheses can cause esthetic problems and have a negative impact on patient's quality of life. Factors associated with longevity of silicone elastomer prosthesis are the use of skin adhesives, UV radiation, discoloration, loosening of the acrylic clip-carrier to the silicone, aging by environmental influences such as pollution and degradation by microorganisms [5, 14, 82]. On average, facial prostheses have to be (re)made every 1.5 to 2 years which can be considered a considerable burden to the patient and an area that need attention in current and future research [14, 83, 84].

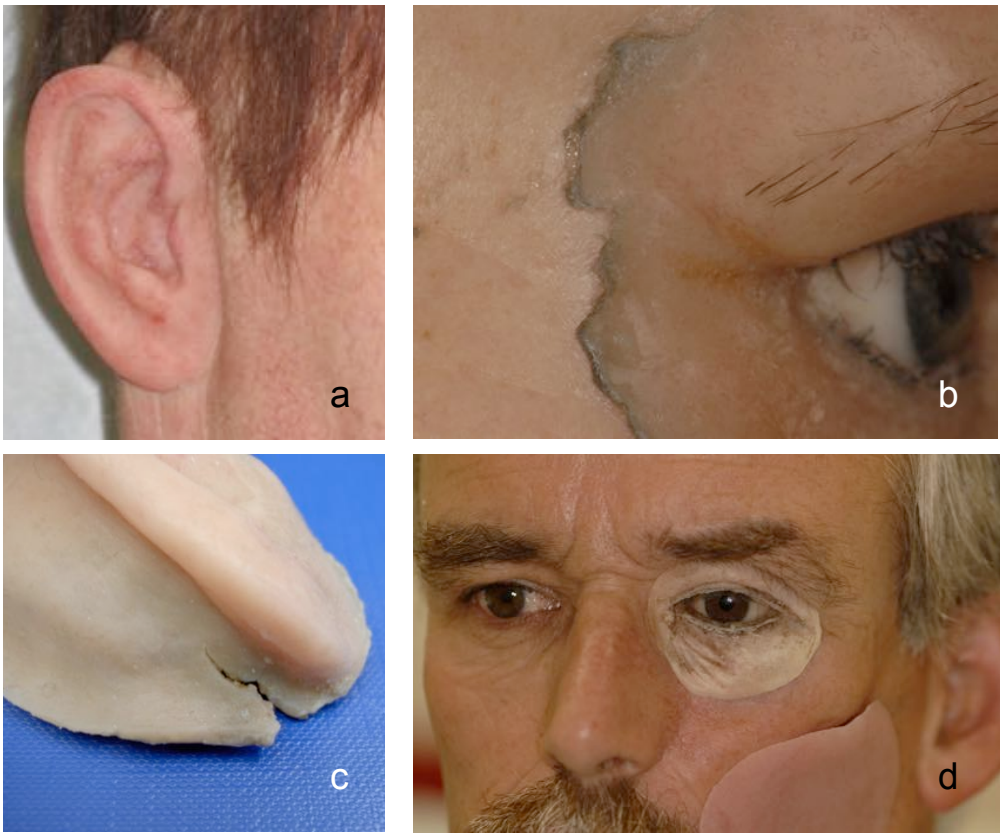

FIGURE 2 Main disadvantages of the materials used in facial prosthodontics. (a) Implant-retained ear prosthesis with proper shape, color and margins directly following placement; (b) discoloration at the edges of an adhesively retained orbital prosthesis after 1 year; (c) rupture of the silicone material of an ear prosthesis due to repeated placement and removal; (d) discolored orbital prosthesis after 18 months.

Color matching: how to mimic nature

Achieving a proper skin color match of a facial prosthesis is known historically to be a procedure based on experience. A skin color match can be achieved by adding suitable pigments to translucent silicone elastomers until an acceptable color match under (preferably) daylight is attained. In addition to pigments, rayon fibers can be incorporated into the polymer network before cure. This method is called intrinsic coloration. For this method to be successful, the pigments must be

dispersible in the polymer and must not have a significant adverse effect on the physical properties of the base material [85]. An already acceptable color match can be further improved by applying pigments dispersed into a solvent on the surface of the prosthesis (extrinsic coloration) [42]. It has to be noted, however, that the pigments used with silicone elastomers do exhibit a color change in due course [79, 86].

Several studies have indicated that the human eye is less sensitive to color differences in darker shades than in light shades [87, 88]. The result of this difference in sensitivity is that the patient's perception is more affected by lighter shades than by darker shades and that there might be a discrepancy between the perception of the patient and the clinician, particularly under different lighting conditions (color metamerism). Therefore, Cheng et al. [89] suggested making three prostheses with slightly different colors to match the skin under natural light. The best match from these three processed prostheses is chosen after custom external coloration. This method provides patients with a range of options related to e.g. the season, and might reduce the need to make another prosthesis due to clinically unacceptable color match as perceived by observers. However, this method is a very costly and uncommon approach.

The use of a spectrophotometer and computerized color formulations may assist the clinician in obtaining a certain degree of objectivity in color matching of silicone facial prostheses [90]. Several color measurement systems are available: spectrophotometer, fiber-optic device and imaging color analyzer module. Of these various systems, the imaging color analyzer module has been shown to provide the best clinical results [91]. Major disadvantages of the other two systems include large minimum size of the measurement area, contact measurement, poor accuracy, poor functionality, poor repeatability and unsuitable acquisition protocol [91]. Comparison of the obtained result between studies is difficult due to non-standardized use of spectral instrumentation and illuminants within the studies [90].

The color matching process with help of an instrument in order to obtain quantitative color measurement for a matching shade of facial structures is still far

from perfect [92-94]. Important questions that remain to be answered include whether a particular instrument indeed records the color correctly (e.g. is black indeed 'read' as black by the instrument thereby also assessing the degree of translucency) and whether the measurements results in a color formula that matches the recorded shade. A new measurement tool in objective color matching system that might overcome these shortcomings is the Color and Translucency Meter. It is a highly sensitive tool that can detect small differences in the scattering properties of translucent materials and takes into consideration the translucent characteristics of the skin on three different distances from light source with a single measurement [95].

Microbiologic challenges

An evaluation of the surface characteristics of facial prosthetic elastomers identified the role of surface texture of materials in harboring organisms [96]. Moreover, a possible link between incorrect elastomer formulation and susceptibility of a facial silicone elastomer to deterioration by ingrowth of fungi has been reported [97]. A recent study showed that *Candida albicans* adherence differs between materials and was least in 12 h room-temperature polymerized silicone elastomers [98].

A cross sectional study on microflora associated with extra oral endosseous maxillofacial implants showed that no single organism emerged as a predominant cause of peri-abutment skin infection [99]. On percutaneous implants, *Staphylococcus aureus*, Gram-negative bacilli and yeasts were all present as potential pathogens in a biofilm mode of growth. Hygiene was one important factor in maintaining peri-implant tissue healthy. Culture and sensitivity results should therefore guide treatment of peri-implant infections [99, 100]. In one of our studies, we observed a mixture of microorganisms including yeast and bacteria, a so-called multispecies biofilm, on silicone facial prostheses. These microorganisms were also present on the margin area that is not directly adjacent to implants. Opportunistic *Candida spp*, however, were only isolated from silicone prosthesis and prosthesis covered skin, but not from healthy skin [101].

Discoloration of facial prostheses has been ascribed as fungal driven [102]. This was the reason that an *in vitro* study was performed to assess whether fungal growth was indeed associated with discoloration, whether antifungal agents incorporated into the silicone inhibit fungal growth *in vitro*, and to determine longevity of antifungal action [102]. From this study, it was concluded that fungi from the genus *Penicillium* were associated with discolored areas of a nasal prosthesis. Addition of clotrimazole to *in vitro* silicone samples was shown to be effective in inhibiting fungal growth, while nystatin was shown to be ineffective [102]. The inhibition of fungal growth indicated a degree of stability and some longevity when samples were stored dry or in water at room temperature.

It has been postulated that biofilm on implant surfaces might complicate the management of peri-implant skin infections and the relative effects of antimicrobial agents, which can play a role in endosseous maxillofacial implants and prosthetic failure [100, 103]. Recombinant human Beta Defensin 3 exhibited antibacterial activity against some oral pathogenic strains on elastomers, but unfortunately no information was provided regarding its activity towards strains isolated from the skin [104].

As is evident from the studies discussed above, endosseous maxillofacial implants and prosthetics face multifactorial infection problems due to the unnatural situation created by the prosthesis. The chronic interruption of the skin surface integrity by the suprastructure fixed on the implants causes poor air circulation, accumulation of moisture and compromised skin hygiene [58, 103]. Therefore, patients, their concerned family and/or care assisting network have to be adequately educated to go for optimal cleansing of the prosthesis, implants, and superstructure [48, 61-63, 105, 106]. In case of improper hygiene by the patient, there may be a need to use local antibiotics, antimycotics and steroids to solve the problem in addition to convincing the patient to perform a meticulous hygiene [32]. Occasionally, surgical thinning and debridement of the skin is needed to return to healthy skin again [34, 103].

Computer-guided implant placement and prosthesis fabrication

With aid of digital technology it is possible to digitally plan and place extra-oral implants in the extra-oral areas and design and fabricate facial prostheses. A major advantage of digital planning is that one can preoperatively visualize and plan the desired implant locations and positions on the computer screen after which a digitally designed surgical guide is designed and fabricated by rapid prototyping (RP) technology (Figure 3).

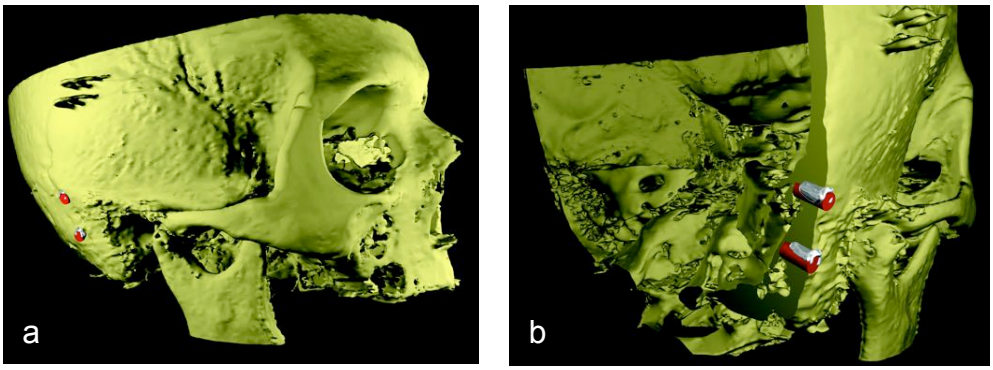

FIGURE 3 Accuracy of digitally planned implants in the mastoid region. (a) By superimposing the preoperative and postoperative cone beam CT data, an impression of the preoperative implant plan *(red)* compared to the actual implant placement can be obtained. The implants *(gray)* were placed in close proximity to the planned locations; (b) sectional plane of the mastoid area with the actual implant positions. The implants were fully surrounded by bone and in close proximity to the planned locations.

The surgical guide for placement of extra-oral implants is designed in such a way that it guides the surgeon during implant placement thereby avoiding damage to vital anatomic structures (e.g. nerves, roots of the teeth), safeguarding a sufficient volume of bone at the implant site as preoperatively planned [107, 108], and limiting the burden of the surgical procedure to the patient. This technique is only scarcely described in literature for extra-oral areas. Van der Meer et al. [109]

recently described a method showing that extra-oral implants indeed can be placed in the preoperatively planned and prosthetically preferred position when applying digital technology, albeit that the implants were not exactly placed at the planned positions (Figure 3) [109]. In fact, the implants were placed in close proximity to their preoperatively planned positions and their position was more than satisfactory from a surgical and prosthodontic point of view to allow for optimum implant-retained prosthodontics.

Before CAD/CAM technology became available, the method to reconstruct a facial form using facial prostheses was by skillful hand carving a wax model. In 2003, Wolfaardt et al. [16] suggested that RP technology, stereolithography and fused deposition modeling gave promise for application in head and neck reconstruction. Recent advances in computer technology allow facial prostheses to be designed digitally [110-112]. Various CAD/CAM applications in facial prosthetics are published and evaluated since that time. A common sequence in applying CAD/CAM technology for making facial prostheses is capturing patients' soft and hard tissue information using imaging techniques such as CT, cone beam CT, MRI, surface scanning and charge-coupled device cameras. Next, by using software (e.g. Mimics, Materialise Leuven, Belgium), this information is converted to an RP model. RP models can be either directly printed in wax or in case it is printed in acrylic it can be transferred into a wax model with duplication techniques. The wax model can be fitted to the patient and final small details are hand carved as RP models are not mimicking the skin curvature exactly. Subsequently, the silicone elastomer prostheses are made according to the conventional molding method after fitting on the model [113-119]. CAD/CAM system can also be used to make immediate facial prosthesis with less time compared to the conventional technique with a form selected from a digital library when the original, for example nose, is deformed [120]. The potential of technology to transform a treatment process from an artistically driven process to a reconstructive biotechnology process cannot be overlooked [121].

A comparison of conventional impression procedure and RP technology in terms of quality, accuracy, required time and ease of production of each technique for making and duplicating prostheses showed that RP has many advantages, but the RP equipment should become more cost effective, user friendly and compact [122, 123]. Compared with the conventional procedure, cost for CAD/CAM prosthesis fabrication seems high at first investment, but on daily basis, the costs are probably lower than manual fabrication by technicians [113]. However, there is no information in the literature regarding availability of CAD/CAM technology in low and middle income countries. The availability of specific centers in the world for CAD/CAM, transmission of files digitally and sending stereolithography models by postal service might further reduce the costs in the future.

How satisfied is the patient?

The ideal prosthesis mimics the missing facial contours as precisely as possible (Figure 1). A successful rehabilitation must allow patients to appear in public without fear of attracting unwanted attention [124-127]. This approach not only applies to the final prosthesis, but also to interim prostheses, because patients might greatly benefit from such a prosthesis when (immediate) surgical repair is not available [128]. A comprehensive and high quality interim rehabilitation can increase the patients' daily activities and quality of life [129]. However, it is advised that patients also get social counseling when provided with a facial prosthesis to further improve their quality of life and to learn to cope with their prosthesis [130].

Patients' attitude and opinions regarding facial prostheses have been assessed in surveys. Responses revealed that although patients express a high degree of satisfaction with their prostheses [13], they wish that their prostheses could last longer and would be more color-stable [14, 80]. In addition, patients were concerned towards the fit of the prostheses [81]. Social acceptance in family and society was also found to be better when a facial defect was adequately covered by a prosthesis and patients' satisfaction was shown to be directly related to prosthodontists' psychological attitude towards gaining patient's confidence [64].

Some patients mentioned their desire to eliminate the use of adhesives, which they found to be awkward and irritating [81]. As such, implant-retained facial prostheses are better accepted by patients compared to adhesive prostheses and offer improvement in the patients' daily activities and quality of life [11, 13, 16, 17, 61-66].

Discussion and conclusion: current limitations and hopes for the future

Currently, the available literature does not allow for robust recommendations based on good quality evidence. Prosthodontic rehabilitation of craniofacial defects is still the skilled manual work of anaplastologists and maxillofacial prosthodontists who try to do their best for the individual patient. In fact they are a kind of artists that use their skills and expertise to rehabilitate the craniofacial defects to the satisfaction of the patient. The current literature on prosthodontic craniofacial rehabilitation predominantly consist of cases and cases series in which the clinicians share their expertise rather than sound clinical trials comparing different treatments with each other aiming for good quality evidence to provide a basis for robust recommendations as how to treat a patient with a craniofacial defect. With the introduction of digital techniques, which may makes craniofacial prosthodontics less demanding on the skills of the artist, a new era is about to start allowing for a more standardized work up and thus for designing sound clinical trials.

However, to the best of our knowledge, there are yet no published papers describing a 100% fully digital workflow by means of scanning, designing and printing facial prostheses that can be placed directly onto the patient without the help of plaster models, wax etc. In the meantime the technology is improving rapidly, we presume a 100% digital workflow will become available within the next decade. Advancements in the digital workflow also aim for implant placement with minimal invasive surgery thus reducing the morbidity of the implant procedures to the patient.

Even when new technology would allow fully digitally manufactured prostheses, some basic issues related to longevity and color stability need to be addressed at the same time. Attempts to overcome material degradation related to microbial biofilm formation and correct repeatable color formulations are pursued at the moment. To achieve these hopes, industrial designers need to cooperate closely with clinicians. Developing new techniques and materials is costly and the group of patients who are in need of this technology is rather small. For that reason the industry is often not interested in cooperating. It is our goal and task as maxillofacial prosthodontists to convince technicians and manufacturers that working closely together will immensely improve the quality of life of the patients.

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CHAPTER 3

MICROBIAL BIOFILMS ON FACIAL PROSTHESES

Nina Ariani, Arjan Vissink, Robert P. van Oort, Lindawati Kusdhany, Ariadna Djais,
Tri Budi W. Rahardjo, Henny C. van der Mei, Bastiaan P. Krom.
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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 cm^2 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 $(NH_4)_2SO_4$, 0.45 g/l KH_2PO_4, 0.45 g/l K_2HPO_4, 0.19 g/l $MgSO_4$, 0.37 g/l Na_2EDTA, 0.2 g/l L-Cysteine HCl, pH 6.8). A healthy part (3 cm^2) 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% H_2, 85% N_2 and 5% CO_2) 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.

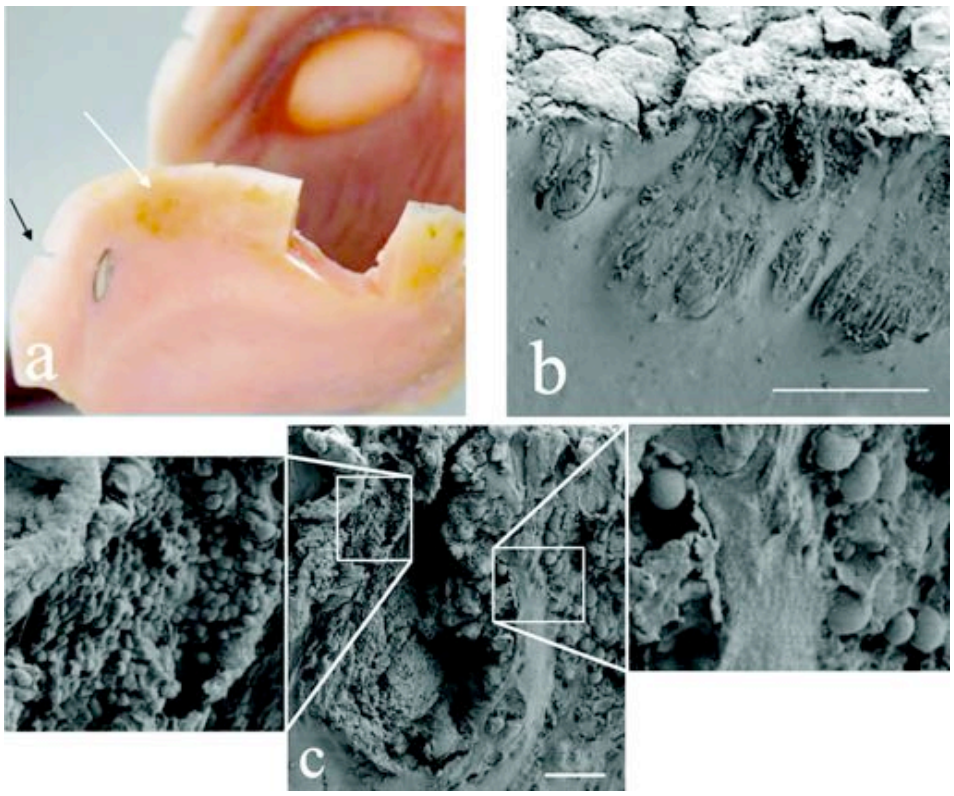

FIGURE 1 (a) Example of facial prosthesis with sampling side cut made at the margin that is in contact with the skin. Prosthesis showing degradation/tear on the periphery (black arrow) and brownish deposition (white arrow) at the area that is in contact with the skin; (b) SEM image of the biofilm on 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

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

[a]GC clamp is present on forward primer
[b]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.

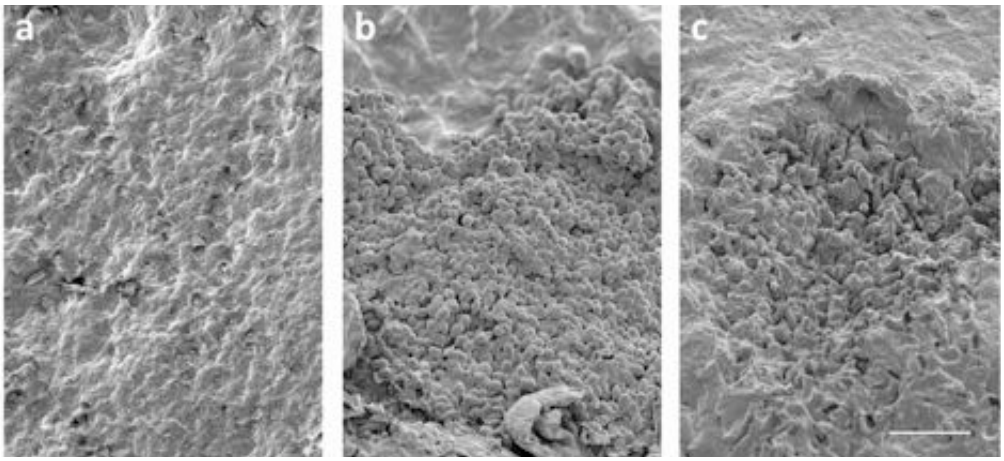

FIGURE 2 SEM images of silicone surface of prostheses (bar = 10 µm). (a) Unused prosthesis; (b) biofilm on top of prosthesis and deterioration of prosthesis before cleaning with microorganism corresponding to size of bacteria (1µm); (c) despite cleaning, there were microorganisms and remnants of microorganism 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/cm^2) 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*

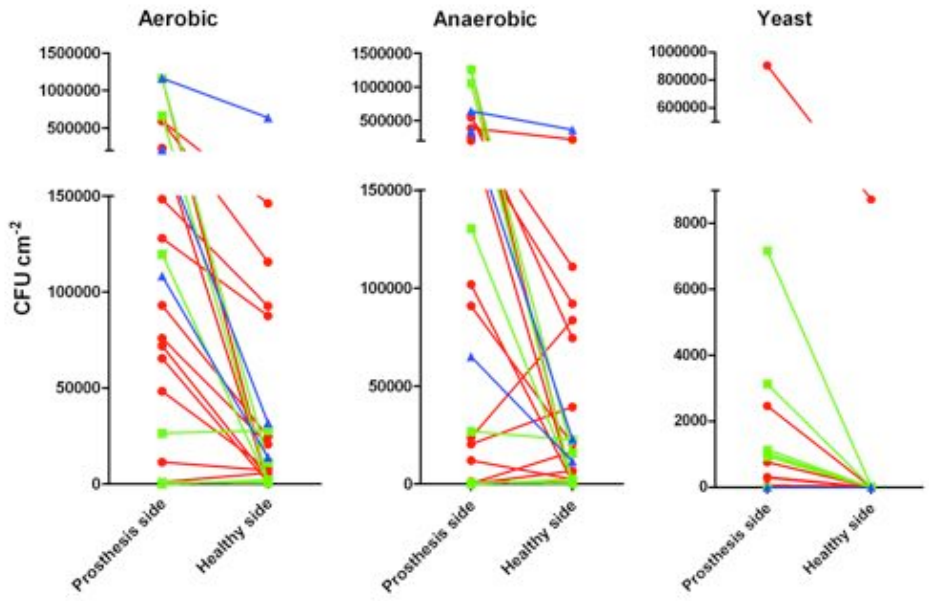

FIGURE 3 CFU counts (CFU/cm^2) of aerobic, anaerobic bacteria and yeast comparing the skin underneath prosthesis (prosthesis side) and healthy skin (healthy side). The green lines and squares represent the orbital prostheses, the blue lines and triangles the nasal prostheses and the red lines and circles 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).

Healthy side	**Prosthesis side**
Coagulase negative *Staphylococcus*	*Enterobacter cloacae*
Pseudomonas aeruginosa	*Staphylococcus aureus*
Bacillus spp	*Serratia marcescens*
	Pseudomonas aeruginosa
	Proteus mirabilis
	Coagulase negative *Staphylococcus*
	Propionibacterium avidum
	Corynebacterium amycolatum
	Candida spp
	Candida parapsilosis

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/cm^2 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).

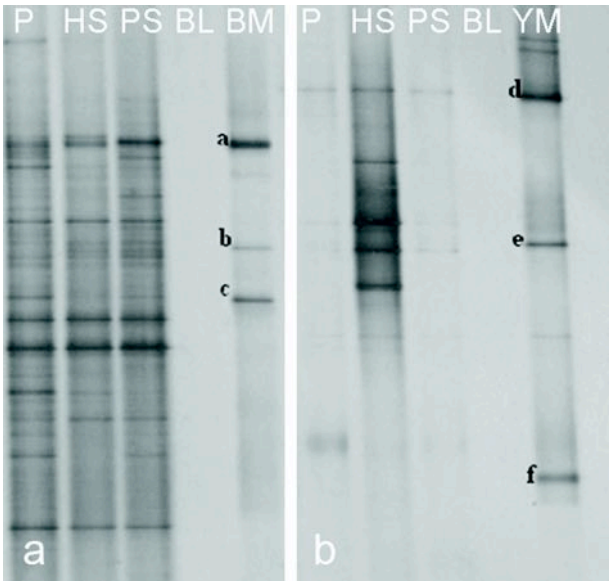

FIGURE 4 (a) Bacterial and yeast DGGE profile of prosthesis and corresponding skin swab sample from one patient. Bacterial profile of prosthesis, healthy skin, and skin underneath prosthesis; (b) yeast profile of prosthesis, healthy skin, and skin underneath prosthesis. P = prosthesis, HS = healthy skin, PS = skin underneath prosthesis, BL = negative control, BM = Bacterial marker: a. *S. aureus*, *S. epidermidis*, b. *S. schleiferi*, c. *Proteus mirabilis*; YM = yeast marker: d. *C. tropicalis, C. parapsilosis, C. famata*, e. *C. albicans*, f. *C. krusei*

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.

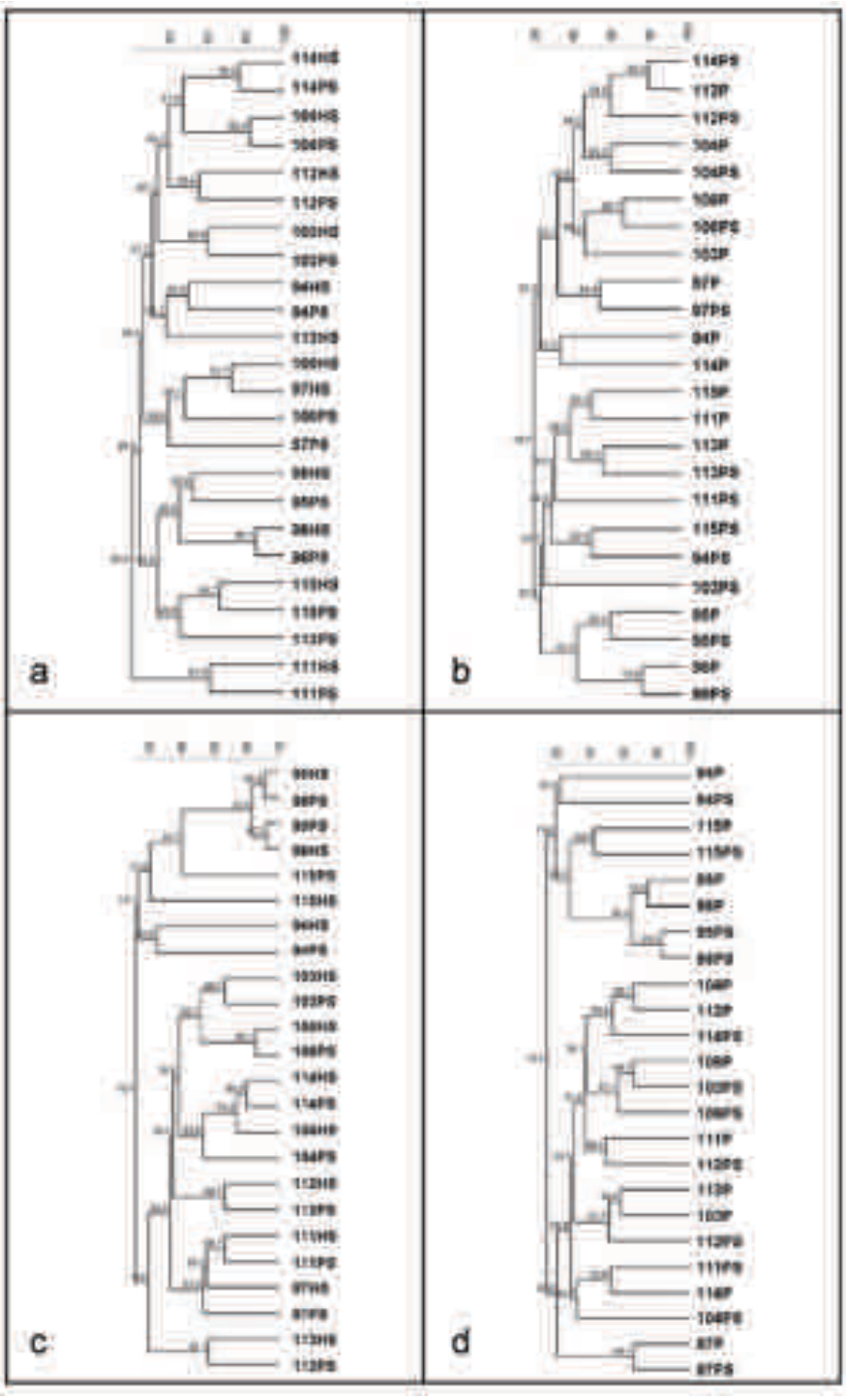
a
b
c
d

FIGURE 5 Dendrograms of DGGE profiles of bacterial and yeast communities from healthy skin, skin underneath prosthesis and prostheses. Numbers on the right side represents samples, numbers on the tree represent percentage of similarity between the profiles. (a) Bacterial similarity profile of healthy skin (HS) and skin underneath prosthesis (PS); (b) bacterial similarity profile of prosthesis (P) and skin underneath prosthesis (PS); (c) yeast similarity profile of healthy skin (HS) and skin underneath prosthesis (PS); (d) yeast similarity profile of prosthesis (P) and skin underneath prosthesis (PS).

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

cultivatable microorganisms, but not the whole profile of the samples. Importantly, a significant difference was observed in total number of cultivatable 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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CHAPTER 4

EFFICACY OF CLEANSING AGENTS IN KILLING MICROORGANISMS IN MIXED SPECIES BIOFILMS PRESENT ON SILICONE FACIAL PROSTHESES

- AN *IN VITRO* STUDY -

Nina Ariani, Anita Visser, Margot R.I.M. Teulings, Melissa Dijk, Tri Budi W. Rahardjo, Arjan Vissink, Henny C. van der Mei.

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Abstract

Objectives The purpose of this study was to assess the efficacy of different cleansing agents in killing mixed species biofilms on silicone facial prostheses.

Materials and methods Two bacterial and three yeast strains, isolated from silicone facial prostheses, were selected for the mixed species biofilms. A variety of agents used to cleans facial prostheses were employed, viz. antibacterial soap, essential oils containing mouthrinse, ethanol 27%, chlorhexidine mouthrinse (CHX) and buttermilk. Colony forming units (CFU) and live/dead staining was analyzed to assess the efficacy of these cleansing agents against 24 h, 2 weeks biofilms and regrown biofilms on silicone samples.

Results CHX was the most effective cleansing agent. CHX killed 8 log units CFUs (> 99.99% killing) in a 24 h biofilm and 5 log units CFUs (> 99.99% killing) in 2 weeks biofilms. Also after regrowth and repeated treatment of the biofilm CHX was the most effective cleansing agent showing no detectable CFUs. The essential oils containing mouthrinse (containing 26.9% ethanol) showed a similar efficacy as ethanol (27%) alone. Antibacterial soap and buttermilk were the least effective agents tested.

Conclusions CHX showed the highest reduction in CFUs in 24 h, 2 weeks and regrown mixed species biofilm of microorganisms isolated from silicone facial prostheses.

Clinical relevance CHX mouthrinse (easily obtainable and relatively inexpensive) is very effective in killing bacteria and yeast present in biofilms on silicone facial prostheses. When applied on a regular basis, cleansing a facial prosthesis with CHX will presumably increase its lifetime and reduce skin irritations.

Introduction

Patients suffering from facial defects caused by trauma, tumor removal or congenital defects are often provided with facial prostheses made from silicone rubber in order to camouflage the defect. These facial prostheses have a limited lifetime of 1.5-2 years on average [1]. This relatively short lifetime of facial prostheses is mainly caused by discoloration, deterioration of the prosthesis material by microbial ingrowth, material rupture and aging [1-3]. These effects are due to the use of skin glue and exposure of the prostheses to environmental factors such as personal hygiene, environmental pollution (e.g., a dusty environment in a workshop), UV, temperature and humidity [3-5]. Furthermore, skin secretions like perspiration and sebum [6,7] and different cleansing treatments, such as using a microwave and commercially available disinfectants, contribute to changes in the silicone rubber of which the prostheses are made from [3,8-10].

Silicone facial prostheses can be retained using a variety of tools of which adhesives (skin glue) and dental implants are currently the most common ones [3]. Maintaining hygiene of the prosthesis is important for the health of the soft tissue underneath the prosthesis and for preserving the prosthesis itself in a good condition. Cleansing a facial prosthesis (with or without glue) or the skin (with or without an implant suprastructure) can be a difficult task, especially for patients with limited manual dexterity or visual problems, which is common in elderly who present the largest group amongst the facial prostheses wearers [1]. This is also reflected by the high prevalence of soft tissue infections around implants. Such skin reactions have been reported to occur in about half of the patients [1,11]. Etiological factors like poor ventilation of the skin, accumulation of moisture and compromised skin hygiene are presumed to be the most important factors causing skin irritations and infections [12,13].

Acrylic resin and silicone facial prostheses may retain microorganisms, depending on the adhesion force with which these microorganisms adhere to the surface [7,13,14] and the cleansing skills of the patient [1]. The surface of the silicone prostheses can act as a reservoir for microorganism and yeast. Surface

irregularities increase the possibility of harboring microorganisms, making the surface more difficult to cleanse [15,16]. Mechanical methods, like brushing, have been shown to be insufficient to eliminate microorganisms colonizing acrylic resin dental prostheses [17]. Soft silicone materials used to reline dental prostheses are more difficult to cleanse than resins and these soft materials are permeable and therefore susceptible to microbial colonization [15]. Fungal ingrowth was observed in nasal silicone prostheses and was associated with the black discoloration of these prostheses [18]. Black discoloration can also be caused by smoking [19].

Water and neutral soap, together with gentle brushing using a soft, nylon bristles toothbrush, are recommended for cleansing facial prostheses [14,20] as well as the implants underneath the prosthesis. The use of chlorhexidine (CHX) has been shown as an excellent auxiliary method to cleanse facial prostheses, along with the use of hydrogen peroxide and isopropyl alcohol [14,20]. In an 18 months clinical longitudinal pilot study assessing the efficacy of a hygiene protocol for cleansing implant-retained facial prostheses, two thirds of the implant-retained facial prostheses had to be replaced due to silicone damage [21]. That pilot study as well as several other studies revealed that silicone rubber damage was caused by rigorous cleansing or use of inappropriate cleansing agents [8-10,21,22]. In line with this observation, a negative advice was given for mechanical cleansing of facial prostheses, e.g., by brushing. Repeated brushing also could contribute to discoloration of silicone rubber prostheses by dissolution and removal of surface pigments [8].

As far as we know, researchers did not yet investigate the efficacy of chemical cleansing with regard to killing of microorganisms present on silicone facial prostheses. A variety of cleansing agents has been used to cleanse silicone facial prostheses, the most common ones include soap, CHX and isopropyl alcohol [14,20], but the efficacy of these agents on killing of microorganisms present in mixed species biofilms on silicone facial prostheses was not yet studied.

Therefore, the aim of the present study was to assess *in vitro* the efficacy of different cleansing agents in killing bacteria and yeast in a mixed species biofilm on

silicone facial prostheses. The bacteria and yeast tested originated from mixed species biofilms that are present on used facial prostheses [13].

Materials and methods

Preparation of silicone samples

Silicone rubber (M511 Maxillofacial Silicone System, Technovent Ltd., South Wales, UK) commonly used to fabricate facial prostheses was used to make 60 x 60 x 1.5 mm sheets of silicone rubber. The silicone sheets were processed using plaster molds similar to molds used for processing facial prostheses. The silicone in the molds was polymerized with 5 Bar pressure, at 45°C for 90 min. To prevent adhesion of the silicone to the plaster molds, the surface of the molds was sprayed with releasing agent (MediMould, Polymed Limited, Cardiff, UK). After polymerization, the silicone was taken out from the mold. The technician wore new medical latex gloves to prevent adhesion of the skin flora onto the silicone sheets. To mimic the clinical situation one side of the sheet was sealed with a silicone sealant (Multisil Sealant, Bredent, Senden, Germany) as used in clinical practice. Application of the sealant was according to the instructions of the manufacturer in order to reduce microbial colonization and microbial penetration, preventing dirt adhesion, easier to cleanse and improving adhesion to skin adhesives. After that, the silicone sheet was cut into samples of 15x15 mm with a sterile scalpel blade on a glass plate that was disinfected with 70% ethanol. All silicone samples were sterilized by 70% ethanol and air-dried under sterile conditions.

Microbial strains, culture conditions and biofilm formation

Two bacterial strains, *Staphylococcus epidermidis* MFP5-5 and *Staphylococcus xylosus* MFP28-3*,* and three yeast strains, *Candida albicans* MFP8*, Candida parapsilosis* MFP16-2 and *Candida famata* MFP29-1*,* were selected for the multi-species biofilms. All strains were retrieved from patients' facial silicone prosthesis [13]. Each strain was grown on Brain Heart Infusion (BHI, OXOID, Basingstoke,

UK) agar over night at 37°C. One colony of a microbial strain was inoculated in 5 ml of 30% BHI and 70% Yeast Nitrogen Base (YNB, BD Difco™, MD, United States; BHI/YNB) and incubated at 37°C for 24 h. Subsequently, all strains were mixed in one to one volume ratio giving a multi-species suspension. The concentration of the bacterial culture was $2x10^9$/ml and yeast culture was $3x10^7$/ml.

One silicone sample was placed in each well of a 12-well plate (Costar, Corning, NY, USA). Six silicone samples were placed with the sealed side up and six with the sealed side down. The wells were inoculated with 2 ml of a multi-species suspension in BHI/YNB media and incubated for 3 h at 37°C under aerobic condition for microbial adhesion. After 3 h incubation, the samples were washed with sterile phosphate buffer saline (PBS; 0.15 M NaCl and 10 mM potassium phosphate, pH 7.4), moved to a new sterile well plate filled with 2 ml fresh BHI/YNB. The biofilm was allowed to grow for 24 h at 37°C under aerobic conditions. All experiments were performed in triplicate.

Treatment of biofilms with various cleansing agents

24 h old biofilms

The following products were chosen: CHX and the essential oils products because they have good antimicrobial efficacies, ethanol 27% in order to exclude that the ethanol in the essential oils product caused the killing of the microorganisms, buttermilk since it was very effective in reducing mixed species biofilm formation on silicone rubber voice prostheses, and antibacterial soap since soap is often advised for cleansing of silicone facial prosthesis.

After 24 h, the silicone rubber samples with biofilm were treated with one of the antimicrobial agents or cleansing solutions as mentioned in Table 1. This was done in order to measure the efficacy of the cleansing agents on killing microorganisms present in the biofilms.

TABLE 1 Antimicrobial agents and cleansing solutions used for treatment of the biofilm on silicone rubber samples.

Antimicrobial agents/cleansing solution	**Manufacturer**	**Active Ingredients**
Control		
Demineralized water		
Agents/cleansing solutions		
Demineralized water and Unicura Balance soap (1:1)	Colgate-Palmolive, Weesp,The Netherlands	1-5% cocamidopropylbetaine (surfactant/antiseptic), PPG-2-hydroxyethyl cocamide (surfactant), C12-16 pareth-7 (emulsifying, surfactant), cacamide MEA (foaming agent, surfactant), triclocarban (antibacterial, antifungal), laureth-4 (surfactant, emulsifier)
Listerine Original	Johnson & Johnson Consumer, Maidenhead, UK	0.092% eucalyptol, 0.06% methyl salicylate, 0.064% thymol, 0.042% menthol
Ethanol 27%*		
Corsodyl mouthwash	GlaxoSmithKline Consumer Healthcare BV, Zeist, The Netherlands	0.2% chlorhexidinedigluconate
Buttermilk	Friesland Campina, Amersfoort, The Netherlands	

*Ethanol percentage in Listerine Original is 26.9%

The samples in the 12-well plates were dipped once in water in order to remove the non-adhering microorganisms and subsequently immersed in 2 ml of the cleansing solution for 1 h at room temperature. Afterwards the samples were dipped once in

water. The attached biofilm on the silicone samples was collected by swabbing with a sterile cotton swab stick and then suspended by vortexing in 1 ml sterile PBS. The suspended biofilms were serially diluted and 100 µl of each dilution was plated on BHI agar plates and incubated at 37°C under aerobic conditions for 24 h before CFUs were counted. The suspended biofilms were also stained for 15 min with live/dead stain (1:1) (*Bac*Light™, Invitrogen, Breda, The Netherlands) and the percentage dead bacteria and yeast was determined. Three images along the sample were taken using a Leica DM4000B Fluorescence Microscope (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany). Note that live/dead staining is not a measure of microbial killing but of membrane damage [23-25]. The membrane of live microorganisms is permeable to SYTO9, staining both live and dead microorganisms and yielding green fluorescence. Propidium-iodide can only enter through damaged membranes, where it replaces SYTO9, yielding red fluorescence of dead or damaged cells.

Two weeks old biofilms

To check the efficacy of cleansing agents on more mature biofilms, biofilms were grown on silicone samples for 2 weeks. The biofilms were grown as above except that the biofilm was allowed to grow for 2 weeks. The growth medium was refreshed every second day. At day 14, the biofilms were treated and the biofilm samples were collected by swabbing and were suspended by vortexing in 1 ml sterile PBS for plating and fluorescence microscopy as described above. A 2 weeks biofilm was tested in order to determine whether a patient can start every moment with the cleansing procedure or that it is only effective for killing microorganisms in young biofilms.

Regrowth of treated biofilm

Immediately after removing the 24 h treated biofilm on the silicone samples, the samples were placed in a 12-well plate with the biofilm side up. The wells were filled with 2 ml BHI/YNB growth medium and incubated for another 24 h at 37°C. This procedure mimics the daily use of the prostheses. After incubation for 24 h, the biofilms were treated with the same cleansing solutions as before and biofilms were analyzed with the same methods as mentioned above to study repeated exposures to cleansing agents. This regimen was used to mimic the efficacy of repeatable cleansing of a facial prosthesis by the patient.

Determination of the MIC and MBC

For the most promising cleansing agents the minimal inhibitory concentration (MIC) and minimal inhibitory bactericidal concentration (MBC) per microbial strain were determined. A microbial suspension in growth media (5.10^4 microorganisms per ml) was incubated together with serially diluted cleansing agents in a 96 wells plate for 24 h at 37°C. The microbial suspension with the cleansing agent which did not show any growth was determined as the MIC. The clear suspensions were plated on agar and when there was no growth this was determined as the MBC.

Statistical analysis

Two-tailed t-test on the log units of CFUs was used to detect differences between the different biofilm and treatment groups. A significance level of $p<0.05$ was used. Note that the sealed and unsealed sides of the silicone samples were taken together resulting in n=6 for statistical analyses.

Results

No statistically significant differences in CFU counts of the mixed species biofilms were observed between data derived from sealed and unsealed sides of the

silicone samples treated with the same cleansing agents. Therefore, data from sealed and unsealed sides of the silicone were combined for each cleansing agent.

When comparing the various cleansing agents with the control (water), all cleansing agents were significantly more effective than the control (Figure 1). CHX was the most and buttermilk the least effective cleansing agent for all time points studied (Figure 1).

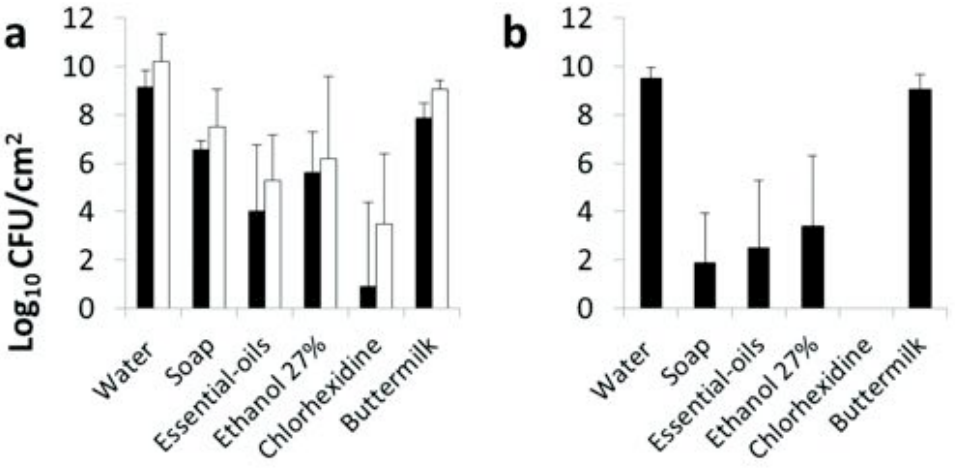

FIGURE 1 CFU of mixed species biofilms on silicone rubber after treatment with different cleansing agents. (a) Log CFU per cm^2 of mixed species 24 h (black boxes) and 2 weeks (white boxes) biofilms on silicone rubber samples after treatment with different cleansing agents and water as a control; (b) log CFU per cm^2 of mixed species biofilm after treatment with various cleansing agents of a regrown 24 h mixed species biofilm.
For statistical significances see Table 2.

The essential oils product (containing 26.9 % ethanol) showed a similar efficacy than when only the ethanol 27 % was tested (Table 2). All cleansing agents were less effective for 2 weeks old biofilm compared to a 24 h biofilm. CHX killed 8 log units CFUs in a 24 h biofilm, whereas in a more mature biofilm (2 weeks) 6 log

units CFUs reduction was observed (Figure 1). After treatment, the regrowth of the treated 24 h biofilm showed a high efficacy for the antibacterial soap, essential oils product, ethanol 27 % and CHX (Figure 1). CHX killed all microorganisms and no growth was detectable. Note that the regrown biofilm has been treated twice, directly after the 24 h growth period and again after the regrowth of the treated biofilm.

TABLE 2 Between groups significance level of CFU counts after exposure of 24h, 2 weeks and regrowth of biofilms to different cleansing agents (n=6).

Time	Agents	Soap	Essential oils	Ethanol 27%	CHX	Buttermilk
24 h	Water	0.000*	0.005*	0.007*	0.000*	0.008*
	Soap		0.067	0.269	0.001*	0.002*
	Essential oils			0.257	0.060	0.016*
	Ethanol 27%				0.003*	0.036*
	CHX					0.000*
2 weeks	Water	0.007*	0.001*	0.031*	0.001*	0.054
	Soap		0.051	0.414	0.019*	0.055
	Essential oils			0.582	0.239	0.004*
	Ethanol 27%				0.169	0.091
	CHX					0.005*
Regrowth of biofilms	Water	0.000*	0.001*	0.019*	0.000*	0.211
	Soap		0.686	0.449	0.083	0.000*
	Essential oils			0.666	0.080	0.002*
	Ethanol 27%				0.105	0.024*
	CHX					0.000*

In Figure 2, the %dead bacteria and yeast are presented. Soap, ethanol, essential oils product and CHX were significantly more effective than the control in killing bacteria and yeast for 24 h and 2 weeks old biofilms. Only the buttermilk was not significantly different from control (water). Tables 3 and 4 depict the efficacy of the various cleansing agents in killing bacteria and yeast.

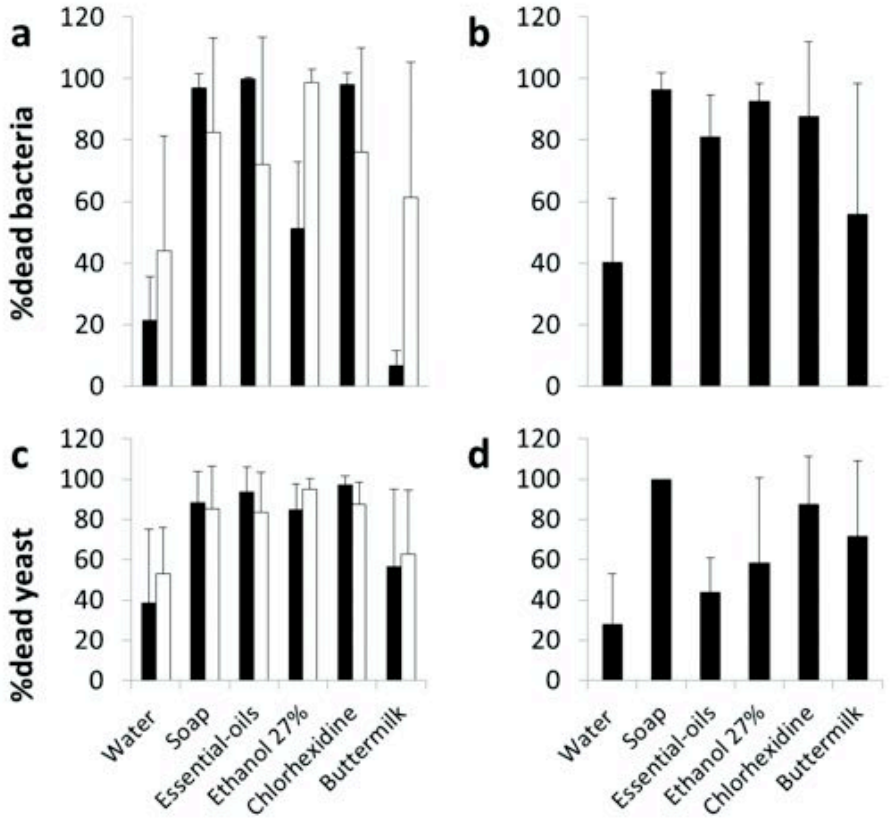

FIGURE 2 Mean and standard deviations of %dead microorganisms in mixed species biofilms on silicone samples that are treated with different cleansing agents compared to water (control). %dead bacteria of 24 h (black boxes) and 2 weeks (white boxes) biofilms. (a) %dead bacteria of 24 h (black boxes) and 2 weeks (white boxes) biofilms; (b) %dead bacteria of regrowth of biofilms after treatment; (c) %dead yeast of 24 h (black boxes) and 2 weeks (white boxes) biofilms (d) %dead yeast of regrowth of biofilms following exposure to different cleansing agents. For statistical significances see Table 3 and 4.

TABLE 3 Between groups significance level of %dead bacteria after exposure of 24h, 2 weeks and regrowth of biofilms to different cleansing agents (n=6).

Time	Agents	Soap	Essential oils	Ethanol 27%	CHX	Buttermilk
24 h	Water	0.000*	0.000*	0.000*	0.000*	0.003*
	Soap		0.038*	0.000*	0.460	0.000*
	Essential oils			0.000*	0.016*	0.000*
	Ethanol 27%				0.000*	0.000*
	CHX					0.000*
2 weeks	Water	0.000*	0.030*	0.000*	0.015*	0.217
	Soap		0.063	0.053	0.143	0.018*
	Essential oils			0.007*	0.752	0.447
	Ethanol 27%				0.022*	0.003*
	CHX					0.298
Regrowth of biofilms	Water	0.001*	0.004*	0.001*	0.001*	0.255
	Soap		0.063	0.204	0.252	0.001*
	Essential oils			0.140	0.493	0.049*
	Ethanol 27%				0.529	0.003*
	CHX					0.017*

The MIC and MBC were determined for CHX, essential oils product and 27% ethanol. For all strains tested in this study the MIC and MBC of CHX was 0.06% chlorhexidine digluconate (30 times dilution of the CHX product). The essential oils product could be diluted up to 4 times (corresponding to 6.7% ethanol) for the MIC for all strains, except for *C. albicans* where the MIC was a 2 times (corresponding with 13.5% ethanol) dilution of the product. The MBC was a 2 times dilution of the essential oils product for all strains. The MIC and MBC of ethanol was 27% for all strains. Note that the MIC and MBC were tested on single strain which can explain the difference in behavior between the essential oils product and 27% ethanol.

TABLE 4 Between groups significance level of %dead yeast after exposure of 24 h, 2 weeks and regrowth of biofilms to different cleansing agents (n=6).

Time	Agents	Soap	Essential oils	Ethanol 27%	CHX	Buttermilk
24 h	Water	0.002*	0.001*	0.003*	0.001*	0.265
	Soap		0.271	0.494	0.037*	0.017*
	Essential oils			0.040*	0.162	0.007*
	Ethanol 27%				0.001*	0.029*
	CHX					0.004*
2 weeks	Water	0.000*	0.000*	0.000*	0.000*	0.263
	Soap		0.322	0.067	0.725	0.002*
	Essential oils			0.016*	0.456	0.017*
	Ethanol 27%				0.017*	0.000*
	CHX					0.004*
Regrowth of biofilms	Water	0.000*	0.226	0.125	0.000*	0.004*
	Soap		0.007*	0.040*	0.095	0.018*
	Essential oils			0.440	0.005*	0.064
	Ethanol 27%				0.130	0.508
	CHX					0.213

Discussion

In this study, the efficacy of cleansing agents on silicone used for silicone facial prostheses was tested on their ability to kill mixed species biofilms. All tested cleansing agents proved to be more effective than control (water) in killing bacteria and yeast that were present in 24 h and 2 weeks old mixed species biofilms as well as in double treated mixed species biofilms. CHX seems to be very effective, while buttermilk was shown to be the least effective agent in killing microorganisms. The latter might be due to the bacteria present in the buttermilk used in this study, which bacteria also grew on agar plates.

No cultivable biofilm was present on the regrowth and double treated biofilm on silicone samples after treatment with CHX. CHX is a widely used antiseptic agent

for prevention of biofilm formation, but also promotes removal of biofilms of, e.g., *S. epidermidis, C. albicans* and *C. parapsilosis* [26-28]. CHX was less effective in killing microorganisms in a 2 weeks old mixed species biofilm, but was still the most effective of the tested cleansing agents in this study. The fact that cleansing agents become less effective for matured biofilms is not surprising as aged biofilms have been shown more resistance to antimicrobials than young biofilms [29-31]. Matured biofilm is embedded in a polysaccharides matrix which reduces penetration of antimicrobials through the biofilm [32]. Other means of how biofilms develop resistance to antimicrobials are changes in the chemical environment within the biofilm that produce zones of slow or no growth, adaptive stress responses, and presence of persister cells [32-34]. Some facial prosthodontists, on basis of their experience, already advise their patients to clean their facial prosthesis with CHX, although there was yet no evidence that this product would be effective for killing the biofilm. This study showed that CHX is indeed effective in killing microorganisms present in the mixed species biofilm on silicone facial prostheses.

Essential oils containing mouthrinse, although not as effective as CHX, are frequently used to reduce the presence of (potentially) pathogenic microorganisms present in oral biofilms [35,36]. The essential oils showed a similar efficacy in killing microorganisms than ethanol 27 %, showing no additional effect of the essential-oils. This was observed earlier in a gingivitis study where the essential-oils product was compared with ethanol [37]. The only difference we observed, between ethanol and essential oils was that ethanol alone had a higher MIC and MBC than essential oils product. MIC and MBC were tested on single strain bacteria which can explain the fact that there was a difference observed. However, essential oils has been shown to be effective as an adjunct to mechanical biofilm removal as well [38]. Netuschil et al. [39] showed that essential oils works best against young and sparse oral biofilms as was also observed in this study for 24 h biofilms [39].

Buttermilk was involved as one of the cleansing agents which might be effective for cleansing silicone facial prostheses as buttermilk has been shown to be very

effective in reducing mixed species biofilm formation on silicone rubber voice prostheses [40,41]. However, buttermilk was not very effective in killing microorganisms present in the biofilm on facial prostheses. In the present study, we only did a single treatment with buttermilk on the biofilm, while in the voice prosthesis study the biofilm in an artificial throat device was perfused with buttermilk three times a day for 9 days [42]. Our study was designed to study the effect of a single exposure of a biofilm to a cleansing agent, so we cannot exclude that buttermilk is effective in killing the studied biofilms when repeatedly exposed to buttermilk. Note, that there are two types of buttermilk available in the Netherlands one contains *Lactococcus lactis* and *Lactococcus cremoris* and the other one is pasteurized buttermilk which contains no viable bacteria. Only buttermilk containing viable *L. lactis* and *L. cremoris* was shown to reduce yeast colonization [42]. Our study used unpasteurized buttermilk, thus buttermilk containing viable bacteria.

The sealant that was applied on one side of the silicone samples was shown to be not effective in preventing microbial colonization of the samples. Although the sealant lowers the surface roughness of the silicone materials, other factors important for biofilms growth, such as nutrients and temperature, were still providing an environment for the microorganisms overpowering the effect of surface modification by the sealant. Further study is needed to confirm the presumed other properties of sealant such as preventing dirt adhesion and improving adhesion to skin adhesives as the sealant did not prevent microbial colonization of the samples.

CFU results showed that the number of microorganisms was lower after regrowth compared to 24 h and 2 weeks biofilm, except for water and buttermilk. The CFU counts for the 2 weeks biofilms were higher than for the 24 h biofilm, because in 2 weeks, biofilms have developed resistance [29]. Thus, the patient has to repeat the cleansing of their facial prosthesis to potentially become effective as possibly some biofilm might reside in niches on the prosthesis. Like for tooth brushing, repeated cleansing is the most effective means of proper cleansing all spots while cleansing

with too long intervals will render in a less effective cleansing action as the biofilms have become more resistant [43,44].

This study adds to the knowledge of how to maintain the facial prosthesis clinically in a good condition and avoid possible skin irritations, which advices are currently mainly based on the experience of maxillofacial prosthodontists who fabricate such prostheses. Clinicians working with patients needing facial prosthodontics have suggested the patients to soak their prosthesis in the mouthrinse with essential oils, believing it helps to reduce skin irritation underneath the prosthesis (personal communication). Our results confirmed their suggestion. In addition, our results also showed efficacy of other cleansing agents that might help patients maintaining health of the skin covered by the facial prosthesis as well as preserving the prosthesis itself. For good antimicrobial efficacy a minimum of 3 log reduction in CFUs is advised, showing that the essential oils product and CHX are both good choices for patients to use for cleaning of their facial prostheses.

Taking both published recommendations on silicone facial prostheses maintenance [14,19] and the results of our study into account, we propose the following maintenance regimen to be advised to patients to prolong the life time of silicone facial prosthesis and to be an asset in reducing the skin problems that occur beneath facial prostheses: cleanse a silicone facial prosthesis by soaking the prosthesis in one of the cleansing agents that were shown to be effective in this study, preferably CHX. This procedure has to be repeated on a daily basis to achieve best prosthesis hygiene and to reduce skin irritation caused by microorganisms.

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CHAPTER 5

EFFECT OF CLEANSING AGENTS ON BIOFILMS ON SILICONE FACIAL PROSTHESES. AN *EX VIVO* CASE STUDY

Abstract

Objectives To assess the efficacy of a variety of cleansing agents in killing mixed species biofilms present on *ex vivo* silicone facial prostheses.

Materials and methods Facial prostheses from patients at the Department of Oral and Maxillofacial Surgery, University Medical Center Groningen, Groningen, The Netherlands, were collected. The effect of 0.2% w/v chlorhexidine mouthrinse (CHX) and its dilutions (up to 30x), an essential oils mouthrinse and a 50% v/v soap solution on the biofilms present on facial prostheses was tested and compared to control (no treatment). Silicone samples derived from prostheses were treated for 1 h with 1 ml of each agent at room temperature every 24 h for 3 consecutive days.

Results Essential oils and CHX were effective in reducing the bacterial load on facial prostheses. At the third treatment, essential oils achieved 82% log reduction and CHX 61%, while 50% soap reached 19% log reduction.

Conclusions Essential oils and CHX both showed antimicrobial efficacies against biofilms of *ex vivo* silicone facial prostheses. These results warrant further research.

Introduction

Several problems related to biofilms on facial prostheses have been identified in the last years:

- Deterioration of maxillofacial silicone rubber by ingrowth of biofilms is linked to incorrect elastomer formulation [1];
- Discoloration of silicone facial prostheses is linked to exposure to fungal biofilms, cleaning methods and agents, ultraviolet rays and human skin oils [2-4];
- The unnatural situation created by introducing silicone facial prostheses disturbs the balance of the skin microbial flora leading to skin or peri-implant problems as well as deterioration of the facial prosthesis [5-7].

In other words, adequate hygiene instruction for cleaning the prosthesis and implant attachments such as bars and magnets is crucial for eradicating the biofilms build-up on silicone facial prostheses [8-10]. Such an approach, when shown effective, might result in less deterioration and discoloration of facial prostheses as well as in more favorable microbial conditions on the skin, prosthesis material and around the implants. However, effective cleansing of prostheses, as commonly experienced by patients and observed by maxillofacial prosthodontists, is very difficult. For example, as observed in denture wearers, *Candida* is associated with denture stomatitis after using dentures for sometime. Insertion of new dentures decreases *Candida* levels, but after 6 months *Candida* levels have returned to baseline [11]. In line with this observation, we have observed that providing a patient with a new facial prosthesis is followed by a temporary reduction of skin irritation underneath the prosthesis.

When looking into the cleansing regimen for dentures, despite the lack of evidence about the specific efficacy of the various denture cleansing methods applied in clinical practice [12], a combination of chemical and mechanical cleansing is usually recommended [13]. With regard to silicone facial prostheses, such a cleansing regimen has to be applied with great care as inappropriate use of mechanical

cleansing is accompanied by damage to the silicone material the prostheses are made from.

In chapter 4 it is shown that chemical treatment of mixed species biofilms with chlorhexidine (CHX) was the most effective in killing bacteria and yeast on silicone samples *in vitro*. As dipping a facial prosthesis in CHX seems to be a promising, easy to apply, non-silicone material destructive procedure for cleansing facial prostheses, it has to be assessed *ex vivo* whether CHX works on existing biofilms of silicone facial prostheses that has been in function for a considerable time too. Therefore, the aim of this case study was to assess *ex vivo* the potential antimicrobial possibilities of CHX and some other agents used for facial prostheses care in daily practice (essential oils mouthrinse, soap) on biofilms present on silicone facial prostheses worn by patients.

Materials and methods

Ex vivo facial prostheses

Three facial prostheses worn by patients treated at the Department of Oral and Maxillofacial Surgery, University Medical Center Groningen, Groningen, The Netherlands, were collected. These prostheses had to be renewed because of discoloration of the silicone material the prostheses were made from (M511 silicone, Technovent, Bridgend South Wales, UK). The prostheses had been in function for 14 months (orbital prosthesis), 13 and 21 months (both auricular prostheses). The prostheses were stored immediately after collection in a refrigerator at 5°C until the start of the experiment (within five days after collecting the prosthesis).

As mixed species biofilms are most prominently present on the skin side of the prosthesis [7], samples (3x3 mm) for the various experiments were all cut from the margins of the prosthesis that were in contact with patient's skin. Under sterile conditions, the cuts were made using a sterile blade.

Treatment of ex vivo silicone facial prostheses with cleansing agents

Silicone samples cut from the prostheses were transferred to 1.5 ml Eppendorf tubes using a sterile pair of tweezers. The samples were treated with Corsodyl mouthrinse (0.2% w/v chlorhexidine digluconate, 7% ethanol; GlaxoSmithKline Consumer Healthcare BV, Zeist, The Netherlands; denoted as CHX), Listerine Original mouthrinse (0.092% eucalyptol, 0.06% methyl salicylate, 0.064% thymol, 0.042% menthol, 26.9% ethanol; Johnson & Johnson Consumer, Maidenhead, UK; denoted as essential oils), 50% v/v Unicura Balance soap (Colgate-Palmolive, Weesp, The Netherlands; mixed 1:1 with water, denoted as soap), or no treatment (control). The samples were treated for 1 h with 1 ml of the product at room temperature. Next, the samples were taken out of the cleansing agent and care was taken that no droplets of the cleansing product tested were left on the silicone samples by blotting them carefully on a sterile tissue. The samples were transferred to a sterile Eppendorf tube with 1 ml phosphate buffered saline (PBS; 10 mM potassium phosphate and 150 mM NaCl, pH 7.0). The pieces were sonicated 3 times for 10 s on ice, vortexed and from the microbial suspension 100 µl of 10 and 100 times dilutions were plated on brain heart infusion agar and incubated at 37°C for 24 h. After 24 h, colony forming units (CFUs) were counted. CFUs of the mixed species biofilm present on the prostheses were analyzed before and after treatment with cleansing agents. After treatment, the silicone samples were stored under sterile conditions at room temperature, and were treated once every 24 h for 1 h in order to mimic the cleansing cycle of a prosthesis worn by a patient. This cleansing cycle was repeated three times.

According to the results described in chapter 4, the minimum inhibitory concentration and minimum bactericidal concentration of CHX *in vitro* was 0.06% (30 times dilution of Corsodyl mouthrinse) for all strains tested. Therefore, in addition to the undiluted CHX, 10, 20 and 30 times dilutions of CHX were tested on samples from the prostheses for three consecutive days, comparable to the design of testing the various cleansing agents.

Results and discussion

The treatment efficacy of the various testing agents applied to cleanse facial prosthesis *ex vivo* is shown in Figure 1. Both CHX and essential oils were effective in reducing CFUs from biofilms from *ex vivo* silicone facial prostheses. The load of the microorganisms had decreased enormously after the third treatment.

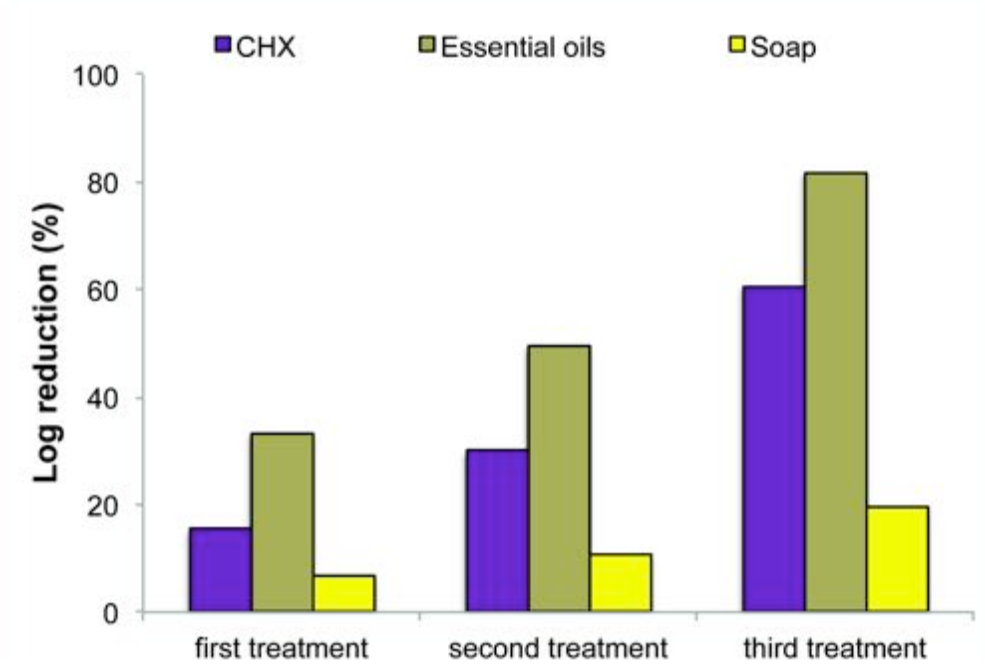

FIGURE 1 Percentage log reduction in CFUs after treatment of *ex vivo* silicone facial prostheses (n=2) with different cleansing agents. The samples were treated once every 24 h for 1 h on three consecutive days. Bars represent log reduction (%) per cm^2 compared to control. The control had 6 log units CFUs per cm^2.

CHX achieved 61±56% log reduction compared to the control, while essential oils reached 82±26% log reduction. Soap had the lowest log reduction (19±3%). The treatment efficacy of dilutions of CHX is depicted in Figure 2.

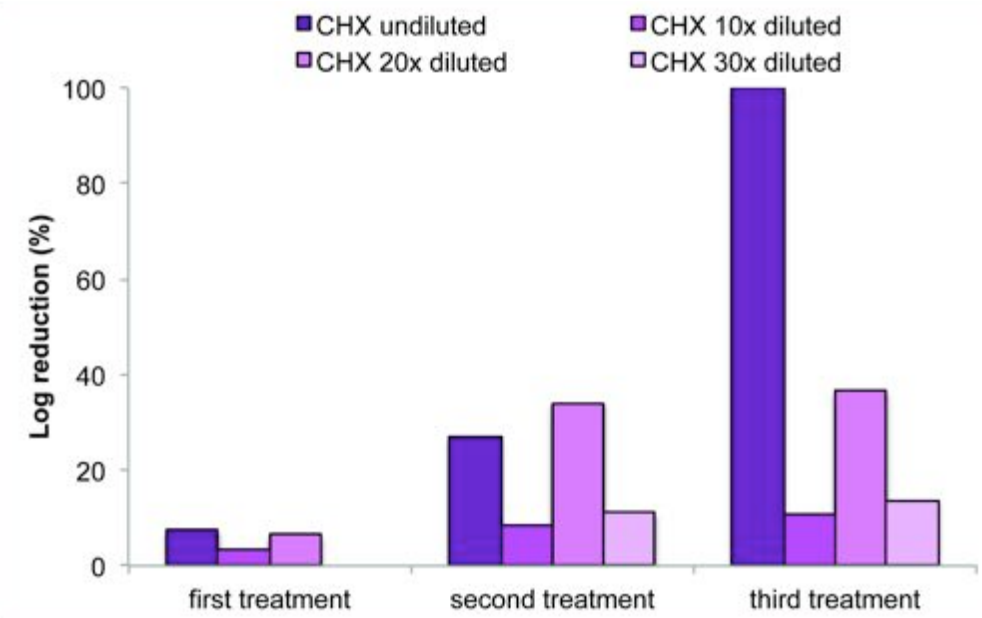

FIGURE 2 Percentage log reduction in CFUs after treatment of *ex vivo* silicone facial prostheses with 0.2% CHX and its dilutions for three consecutive days presented as log reduction (%) per cm^2 compared to control (5 log CFUs per cm^2).

In chapter 4, there were still biofilms present in essential oils group after double treatment and regrowth of the *in vitro* biofilms, while our *ex vivo* result showed that essential oils achieved 82% log reduction on multiple treatments. The differences between the results reported in chapter 4 and this chapter regarding essential oils and CHX can be explained by the experimental design. In chapter 4 we selected certain microorganisms as biofilms constituents, while in this *ex vivo* experiment we worked with biofilms from worn prostheses with unknown microorganisms composition and quantity. The latter may also explain why undiluted CHX resulted in 61% log reduction in the experiments shown in Figure 1 and 100% log reduction in the experiments shown in Figure 2. Furthermore, samples from different patients can contain different microbial species, which may have a different sensitivity to a particular product and number of treatments with that product. Nevertheless, the huge reduction in microbial load of the surfaces of silicone facial prostheses by repeated exposure to CHX or

essential oils underlines the potential efficacy of these regimens in reducing the bacterial load of facial prostheses.

We did not type the microorganisms on the prostheses. Future studies incorporating typing of microorganisms from the prostheses may provide detail on the efficacy of the cleansing agents related to specific microorganisms present in the biofilm on the prostheses as the efficacy of the cleansing agent applied might depend on the composition of the biofilm on an individual prosthesis. Clinically, patients can use either essential oils or CHX to cleanse their prostheses regularly as repeated treatments provide higher microorganisms reductions.

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CHAPTER 6

GENERAL DISCUSSION

Introduction

The last five decades, silicone elastomers have been the material of choice for fabricating facial prostheses [1, 2]. This material is still in use despite its inherent clinical problems including degradation, discoloration, non repairability and limited longevity [1, 3, 4]. These clinical problems are in line with the perception of patients who report high satisfaction with their prostheses, but wish that their prostheses should last longer, should be more color stable and should have a better fit to the skin [5].

Like what is known from voice prostheses, biofilms covering the surface are thought to be a major factor influencing longevity of facial prostheses [6-8]. Interactions between microorganisms and silicone are influenced by surface texture, physico-chemical affinity and lack of antimicrobial properties of silicone [9]. Once microorganisms, especially *Candida* species, harbor silicone, they penetrate into the material, which contributes to deterioration of the material [10-12].

Biofilms on facial prostheses

In chapter 3, the biofilm present on healthy skin, on skin underneath the prosthesis and on the inner surface of silicone facial prosthesis was studied. To date, this study has been the first attempt to identify the microorganisms present in biofilm on silicone facial prostheses. *In vivo* research to gain further understanding of biofilm formation on a facial prosthesis is difficult to perform due to the influence of environmental factors, which was our reason to develop an *in vitro* well-plates model system. The advantage of such a model system is that biofilms can be assessed under laboratory conditions in a controlled environment that mimics the *in vivo* ecosystem as close as possible [13].

In light of biofilm growth on silicone rubber voice prostheses, an ‘artificial throat’ model has been used before in our laboratory to investigate biofilm formation on these devices [14]. In this model, one side of the voice prosthesis is exposed to a culture of microorganisms, media, and buffer solutions and the other side to the

open air, thereby mimicking the condition of a voice prosthesis in the patient [14]. Likewise, a model was developed mimicking the mixed species biofilms on the facial prostheses using a mixture of bacteria and yeasts. The biofilms were grown on the surface of a silicone sample placed in a wells plate. The media was replaced with fresh growth media on a regular basis. The advantage of this model is that it is very user friendly and has a high throughput. Only small volumes of reagents are needed, and composition of growth media, incubation time, temperature and humidity can be adjusted easily and analyses on various stages of biofilm growth can easily be performed [13]. By using the developed *in vitro* model, the efficacy of different cleansing agents in killing bacteria and yeasts strains was assessed (chapter 4). A disadvantage of our model is that it lacks the skin factor, which has to be investigated in the clinic itself.

Cleansing procedures of facial prostheses

Regular cleansing of facial prostheses is advocated to patients [15, 16], but improper mechanical and chemical cleansing can lead to silicone damage [17-19]. On basis of the available literature and the results described in chapter 4, we worked out a protocol for effectively cleansing silicone facial prostheses. That protocol was tested *ex vivo* in chapter 5. The results obtained we suggest that silicone facial prostheses should be cleansed gently and soaked in chlorhexidine or essential oils on a daily basis to avoid biofilm growth on the prosthesis. The addition of cleansing agents, which effect we consider to be worthwhile to add to the common methods to clean a facial prosthesis in daily routine, is not yet commonly advised to patients, however. The most common cleansing methods in daily practice rely heavily on mechanical procedures, although we showed that mechanical cleansing procedures are usually not sufficient to eliminate biofilm on facial prostheses (chapter 3). Soaking the prostheses in a cleansing agent for 1 h, as we suggested, can therefore be considered a potentially great asset in effective biofilms eradication. Further study is needed to work out the optimum soaking time and minimum inhibitory concentrations of the effective cleansing agents when applied under *in vivo* circumstances. With

regard to the agents tested it has to be mentioned that chlorhexidine, the best cleansing agent in our *in vitro* study, has the potential disadvantage that it may affect the color stability of silicone facial prostheses [19]. However, a 20 days exposure to chlorhexidine did not result in discoloration of the silicone material (see section on color stability below).

Other factors affecting longevity of facial prostheses

Besides biofilms, other aspects may also influence the longevity of silicone facial prostheses amongst which are pigments, environmental exposure, and aging of silicone. Ideally, the facial prosthesis material should retain its color and maintain its mechanical properties [20]. With regard to the current prosthesis materials, weathering is presumed to result in color changes and deterioration of silicone facial prosthesis materials [21, 22]. In addition, microorganisms within the biofilms on silicone facial prostheses deteriorate the prosthesis material. Certain microorganisms, especially *Candida* species, have been shown not only to contributes to deterioration of the material [7], but probably also to discoloration of pigmented silicone rubber. Support for this presumption comes from the presence of *Candida* species on discolored facial prostheses [23].

Pigments

There are different types of pigments available on the market to stain facial prostheses which have different effects on material properties [22]. Dry colorants tend to decrease tensile strength and increase hardness. Liquid colorants decrease hardness and tensile strength while increasing tear strength and percent elongation [22]. Oil paint can exert an effect on the polymerization of the silicone, leading to lower hardness in the initial period because oil inhibit catalysis of silicone material [24]. The overall mechanical properties of silicone can be improved by adding nanosized oxides opacifiers (Ti, Zn, or Ce) to the silicone. Adding of these opacifiers resulted in better longevity of the material [25]. In order to prepare the best color match of a facial prosthesis with the color of the patients, skin rayon flocking is used.

Rayon flocking has the disadvantage that it can increase the hardness beyond the range preferred for facial prostheses [20, 22].

To the best of our knowledge no study has yet been performed estimating which color combination result in earlier deterioration of silicone facial prosthesis material. Also it is not clear whether color pigments influence the biofilm formation and mechanical properties of the silicone used for facial prostheses. Therefore, we assessed the influence of color pigments on biofilm formation and the effect of cleansing agents on color stability in two pilot studies.

Pilot study 1: pigments and biofilms

To explore the effect of pigments, we used non-pigmented, red, blue, yellow or white pigmented room temperature vulcanized silicone rubbers (VST-50HD, Factor 2, Lakeside, AZ, USA) and studied biofilm formation on these silicone rubbers. We used a mixed species biofilm consisting of *Staphylococcus epidermidis* MFP5-5*, Staphylococcus xylosus* MFP28-3, *Candida albicans* MFP8, *Candida parapsilosis* MFP16-2 and *Candida famata* MFP29-1. Colony forming units (CFU) were used to determine the effect of adding color pigments to the silicone rubber on bacterial and yeast growth and expressed as log reduction (%).

Based on the results (Figure 1), we can conclude from this pilot study that pigments are not influencing colonization of silicone rubbers with the mixed species biofilm used.

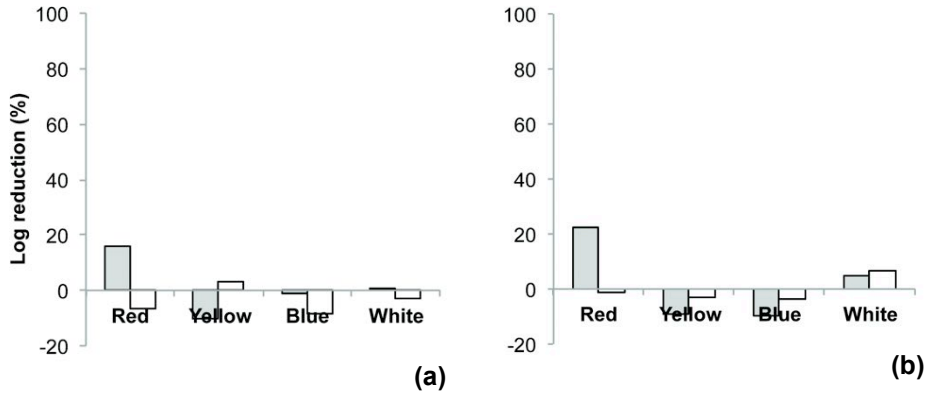

FIGURE 1 Bars represent log reduction (%) per cm^2 on day 3 (grey bars) and day 21 (white bars) for bacteria (a) and yeast (b) grown on a variety of color pigmented silicone rubber samples. Number of samples ranges from 1-3. The log reduction (%) per cm^2 was compared to non-pigmented control. The control had 7 log units CFUs per cm^2 (bacteria) and 5 log units CFUs per cm^2 (yeast).

Pilot study 2: pigments and cleansing agents

To study discoloration of silicone rubber samples due to repeated exposure to cleansing agents, red pigmented silicone rubber sheets (thickness 1.5 mm) of the same silicone material facial prostheses are made of were prepared. Intrinsic staining with red pigments was chosen because clinical experience at the Department of Oral and Maxillofacial Surgery showed that red color changed the most during wearing facial prostheses (personal communication with Robert van Oort). Prostheses collected from patients could not be used for this purpose as they were already discolored, the reason why these patients need new prostheses [3, 26].

The color of the silicone rubber was measured using a calibrated homemade spectrophotometer before and after treatment with cleansing agents. This spectrophotometer was a non-contact spectrophotometer with 90° light source and 20 mm diameter of the measuring area. Calibration was done with a black sample to determine the zero and noise level and a white sample to determine the complete spectrum. The spectrum (390-750 nm) was divided in 7 colors: violet (390-422.5

nm), indigo (422.5-460 nm), blue (460-492.5 nm), green (492.5-542.5 nm), yellow (542.5-582.5 nm), orange (582.5-620 nm) and red (620-750 nm). The counts per color were calculated and expressed in percentages of the total counts. The percentages of the colors of the non-treated silicone samples were compared with the treated ones. The color of the samples was measured after the total treatment period and compared with the untreated piece with the spectrophotometer. The silicone samples were treated with a variety of cleansing agents (chlorhexidine, essential oils, soap; for details see chapters 4 and 5) for 1 h at room temperature, dried, and stored under dry conditions in the dark at room temperature. The control was not treated and was stored in the dark at room temperature in order to exclude influences other than the cleansing agents. The cleansing was repeated every 24 h for 20 consecutive days, mimicking 20 days of daily cleansing in clinical environment.

The various treatments applied had a negligible effect on the color of the silicone samples tested (Figure 2).

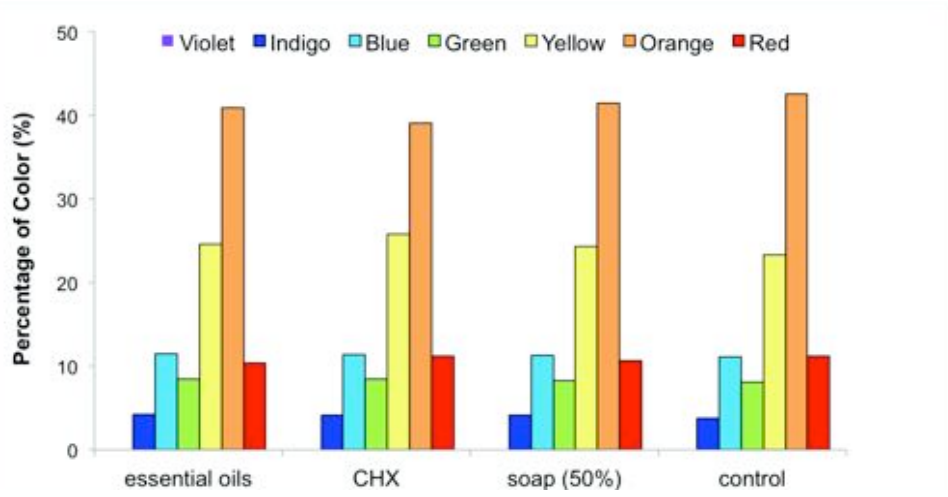

FIGURE 2 Percentage color after treatment with different cleansing agents. The control and treated samples were stored in between treatments at room temperature under dry conditions in the dark (n=1). The color of the control before and after storage for 20 days was identical.

These results suggest that, at least in the short run, cleansing agents do not affect the color of facial prostheses. It is presumed that environmental factors such as weathering and exposure to sunlight probably are more relevant factors to control longevity of facial prostheses. This has to be tested in future studies.

Future perspectives

In vivo research to gain further understanding of biofilm formation on a facial prosthesis is time consuming and difficult to perform due to great variety in environmental and host factors that are difficult to control. Most silicone facial prosthesis material degradation studies used aging chambers that mimic facial prostheses after 1 year of clinical service, but so far the biofilm was not taken into account. Therefore, specific biofilm models that are able to control environmental factors need to be developed. Such a biofilm model is not yet available, but our model is a good start. Preferably this model needs to mimic prosthesis after 6 months or 1 year of clinical service while maintaining a biofilm throughout the course of the study.

There are a lot of factors to consider in understanding the longevity and discoloration problem of silicone facial prostheses material. To overcome these problems, *in vitro* studies to attain which factors are important for longevity and discoloration of the material have to be proposed and incorporated into *in vivo* studies.

Finally, clinical studies comparing prosthesis longevity between different climates should be performed as, for example, a cleansing strategy might be effective in one climate zone, but loses some of its activity in another climate.

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SUMMARY

Summary

Facial prostheses are used to rehabilitate stomatognathic or craniofacial defects. Although facial prostheses function well, there are issues that affect the lifespan of these prostheses.

As mentioned in **chapter 1**, the inner surface of facial prostheses is in contact with soft tissues and body fluids. Therewith microorganisms can colonize and form biofilms on the surface of facial prostheses. These biofilms are of particular interest regarding the maintenance of facial prostheses due to a long held notion that microorganisms within the biofilm have properties to degrade the material facial prostheses are made of and to change the color of the prosthesis. Therefore, the general aim of this thesis was to make an inventory of biofilms on facial prostheses and to analyze the composition of these mixed species biofilms. In addition, routine methods to clean a facial prosthesis were studied to assess how efficient they are in killing biofilms. In **chapter 2,** the current state of the techniques and materials used to rehabilitate maxillofacial defects was reviewed. For this purpose, the MEDLINE and EMBASE databases were searched for articles pertinent to maxillofacial prostheses published from January 1990 to July 2011. This review revealed that a multidisciplinary approach is preferred when rehabilitating maxillofacial defects. Surgical reconstruction can be used for smaller defects, but larger defects require a prosthesis to achieve an esthetic rehabilitation. Moreover, implant-retained prostheses are preferred over adhesive prostheses. Silicone elastomer is currently the best material available for maxillofacial prostheses; however, longevity and discoloration, which are greatly influenced by ultraviolet radiation, microorganisms, and environmental factors, remain significant problems. Furthermore, it is envisioned that in the near future, the widespread availability and cost effectiveness of digital systems may improve the workflow and outcomes of facial prostheses. Finally, it is mentioned that patients report high satisfaction with their prostheses despite some areas that still need improvement. Thus, on basis of this review of the literature it is concluded that facial prostheses are a reliable treatment option to restore maxillofacial defects and improve quality of life.

Significant progress has been made in the application of implants for retention and digital technology for designing surgical guides, suprastructures, and craniofacial prostheses. Further improvements are necessary to enhance longevity of prostheses with a focus on the effect of microorganism in deteriorating the silicone material prostheses are made of and adequate prosthesis maintenance.

Therefore, in **chapter 3,** 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 the presence of mixed bacterial and yeast biofilms on and deterioration of the surface of the prostheses. Microbial culturing confirmed the presence of yeasts and bacteria. Microbial colonization was significantly increased on prosthesis-covered skin compared to healthy skin. *Candida* species were exclusively isolated from prosthesis-covered skin and from prostheses. Biofilms from prostheses showed the least diverse band-profile in denaturing gradient gel electrophoresis 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 such as *Candida* species and *Staphylococcus aureus*. Biofilms on healthy skin, skin underneath the prosthesis and on the prosthesis had a comparable composition, but the numbers present differed according to the microorganism. Furthermore, on basis of the analysis of the microorganisms present in the biofilm on facial prostheses, the composition of the mixed species biofilm was determined and be used in research aiming for effective cleansing of a facial prosthesis.

In the study described in **chapter 4**, the efficacy of different cleansing agents in killing mixed species biofilms on silicone facial prostheses was assessed. Two bacterial and three yeast strains, isolated from silicone facial prostheses, were selected for the mixed species biofilms (see chapter 3 for details). A variety of agents used to cleanse facial prostheses were employed, viz. antibacterial soap, essential oils containing mouthrinse, ethanol 27%, chlorhexidine mouthrinse and buttermilk. Colony forming units (CFU) and live/dead staining was applied to

assess the efficacy of these cleansing agents against 24 h, 2 weeks biofilms and regrown biofilms on silicone samples. The results showed that chlorhexidine was the most effective cleansing agent. Chlorhexidine killed 8 log units CFUs (>99.99% killing) in a 24 h biofilm and 5 log units CFUs (>99.99% killing) in 2 weeks biofilms. Also after regrowth and repeated treatment of the biofilm chlorhexidine was the most effective cleansing agent showing no detectable CFUs (100% killing). The essential oils containing mouthrinse (containing 26.9% ethanol) showed a similar efficacy as ethanol (27%) alone. Antibacterial soap and buttermilk were the least effective agents tested. From this chapter it was concluded that chlorhexidine showed the highest reduction in CFUs in 24 h, 2 weeks and regrown mixed species biofilm of microorganisms isolated from silicone facial prostheses. This premise has clinical relevance as chlorhexidine mouthrinse (easy obtainable and relatively cheap) is very effective in killing bacteria and yeast present in biofilms on silicone facial prostheses. When applied on a regular basis, cleansing a facial prosthesis with chlorhexidine will presumably increase its lifetime and reduce skin irritations.

The efficacy of cleansing agents in killing biofilms on *ex vivo* silicone facial prostheses was investigated in the case study described in **chapter 5**. The effect of 0.2% w/v chlorhexidine mouthrinse and its dilutions (up to 30x), an essential oils mouthrinse and a 50% v/v soap solution on the biofilms was tested and compared to control (no treatment). Silicone samples derived from facial prostheses were treated for 1 h with 1 ml of each agent at room temperature every 24 h for 3 consecutive days. Essential oils and chlorhexidine were effective in reducing the bacterial load on facial prostheses. At the third treatment, essential oils achieved 82% log reduction and chlorhexidine 61%, while the soap solution reached 19% log reduction. In conclusion, essential oils and chlorhexidine showed antimicrobial efficacies against biofilms of *ex vivo* silicone facial prostheses. These results warrant further research.

The general discussion described in **chapter 6** places the results of the studies performed in a broader perspective. It is discussed which other factors might affect the longevity of facial prostheses including the *in vitro* effect of pigments on silicone

facial prostheses material. In two pilot studies, the effect of adding pigments to silicone rubber on the growth of microorganisms on silicone rubber and the effect of cleansing agents on pigmented silicone facial prostheses were studied. These pilot studies showed that pigments were not influencing colonization of silicone rubbers with the mixed species biofilm used and the cleansing agents applied had a negligible effect on the color of the silicone samples. Future *in vitro* studies need to further focus on factors that are important for longevity and discoloration of the material. When these factors are determined, the resulting knowledge has to be translated to well designed, well conducted *in vivo* studies. In addition, clinical studies comparing prosthesis longevity between different climates should be performed.

SAMENVATTING

Samenvatting

Gelaatsdefecten kunnen adequaat worden hersteld met een prothese, maar de levensduur van deze gelaatsprotheses is beperkt. Doordat de binnenzijde van de gelaatsprothese in contact staat met de huid waarop veel micro-organismen zitten vormt zich gemakkelijk een biofilm op het oppervlak van de prothese. Deze biofilm wordt als een belangrijke, levensduur beperkende factor van de prothese beschouwd. Er wordt namelijk verondersteld dat de in de biolfilm aanwezige micro-organismen in staat zijn om degradatie en verkleuring te veroorzaken van het siliconenmateriaal waarvan gelaatsprotheses zijn gemaakt. Degradatie en verkleuring zijn de twee belangrijkste redenen waarom gelaatsprotheses worden vervangen. Daarom was het doel van dit promotieonderzoek om (1) de microbiële samenstelling van de op gelaatsprotheses aanwezig biofilm te inventariseren en (2) te onderzoeken hoe effectief reinigingsproducten zijn in het doden van de in de biofilm aanwezige micro-organismen op gelaatsprotheses (**hoofdstuk 1**).

In **hoofdstuk 2** wordt de huidige stand van zaken m.b.t. de technieken en materialen die gebruikt worden voor gelaatsprotheses beschreven. Er werd een literatuuronderzoek gedaan m.b.v. MEDLINE en EMBASE databases (periode januari 1990 tot juli 2011). Uit het literatuuronderzoek komt naar voren dat herstel van gelaatsdefecten een multidisciplinaire aanpak behoeft. Chirurgische technieken zijn vooral geschikt voor herstel van kleine defecten, terwijl grote defecten, wanneer een fraai esthetisch resultaat wordt nagestreefd, beter kunnen worden hersteld met gelaatsprotheses. Gelaatsprotheses die op implantaten zijn verankerd genieten daarbij de voorkeur boven protheses die zijn vastgeplakt met huidlijm. Met betrekking tot de materialen waarvan gelaatsprotheses kunnen worden vervaardigd, gaat de voorkeur nog steeds uit naar siliconen. Nadelen van de toepassing van siliconen zijn de beperkte levensduur (degradatie, verkleuring), vooral als gevolg van blootstelling aan ultraviolette straling, ingroei van micro-organismen en omgevingsfactoren. Door de wereldwijd steeds betere beschikbaarheid van digitale technieken wordt het steeds eenvoudiger en/of

goedkoper om een gelaatsprothese te vervaardigen en te vervangen. Tenslotte moet worden genoemd dat, hoewel patiënten erg tevreden zijn over hun gelaatsprothese, op veel van de genoemde gebieden nog winst is te boeken. Leidend hierbij zijn het verkrijgen van inzicht in het effect van micro-organismen op de degradatie van het prothesemateriaal en het ontwikkelen van een effectief regiem om een gelaatsprothese te reinigen. Indien hierin een beter inzicht in wordt verkregen en een effectief regiem kan worden ontwikkeld, is het de verwachting dat de levensduur van protheses kan worden verlengd.

Als eerste stap werd de microbiële samenstelling van de biofilm op siliconen gelaatsprotheses geïnventariseerd, waarbij zowel is gekeken naar de samenstelling van de microflora op de gezonde gelaatshuid, de huid onder de prothese en het prothese-oppervlak zelf (**hoofdstuk 3**). Scanning elektronenmicroscopisch onderzoek van gedurende langere tijd gedragen gelaatsprotheses liet zien dat op het prothese-oppervlak zowel bacteriën als gisten aanwezig zijn. Met behulp van kweken werd de aanwezigheid van levende bacteriën en gisten op het prothese-oppervlak bevestigd. Van belang is om hierbij te melden dat significant meer bacteriën en gisten op het prothese-oppervlak aanwezig waren dan op de met de prothese bedekte huid. Bovendien werden *Candida* soorten alleen aangetroffen op het prothese-oppervlak en de huid onder de prothese, en niet elders op de huid in het gelaat. Met behulp van denaturerende gradiënt gelelektroforese kon vervolgens worden aangetoond dat de microbiële samenstelling van de biofilm op de prothese minder divers was dan die van de biofilm op de huid onder de prothese. De bacteriële samenstelling van de biofilm toonde daarbij een grotere variatie dan de diversiteit aan gisten. Uit deze studie werd geconcludeerd dat het bedekken van de huid met een gelaatsprothese een niche creëert die gunstig is voor opportunistische micro-organismen zoals *Candida* soorten en *Staphylococcus aureus*.

Op geleide van de resultaten van de in **hoofdstuk 3** beschreven studie, kon een microbiële samenstelling worden bepaald die representatief is voor de microbiële samenstelling op gelaatsprotheses. Deze gemengde biofilm, bestaande uit bacteriën en gisten, werd gebruikt bij het onderzoek naar de effectiviteit van reinigingsproducten voor gelaatsprotheses met betrekking tot het doden van de in de biofilm aanwezige micro-organismen (**hoofdstuk 4**). Twee bacteriële en drie giststammen, alle geïsoleerd van stammen aanwezig op gelaatsprotheses, werden geselecteerd voor de gemengde biofilm (zie **hoofdstuk 3** voor details). Als reinigingsmiddelen werden een aantal producten gekozen die veelvuldig in de praktijk worden toegepast voor het reinigen van gelaatsprotheses, namelijk antibacteriële zeep, een mondspoelmiddel op basis van essentiële oliën, 27% ethanol (controle; het mondspoelmiddel op basis van essentiële oliën bevat namelijk 26,9% ethanol), een 0,2% chloorhexidine mondspoelmiddel en karnemelk. Om de effectiviteit van deze producten te bepalen werd het aantal kolonievormende eenheden ("colony forming units", CFU) bepaald en een leven/dood kleuring ("live/dead staining") uitgevoerd. Het effect van deze regiems werd bepaald door stukjes siliconenmateriaal, waarop een 24 uur of twee weken oude biofilm aanwezig was, bloot te stellen aan elk van deze producten. Ook werd getest of deze producten effectief waren tegen een biofilm die was hergroeid na een initiële behandeling van de biofilm met één van deze producten. Van de onderzochte producten bleek het chloorhexidine mondspoelmiddel het meest effectief te zijn om de in de biofilm aanwezige micro-organismen te doden. Chloorhexidine doodde 8 log units CFUs (>99.99% afdoding) in een 24 uur oude biofilm en 5 log units CFUs (>99.99% afdoding) in een 2 weken oude biofilm. Ook na hergroei van de biofilm bleek chlorhexidine het meest effectieve onderzochte reinigingsmiddel; er waren na herbehandeling geen CFUs aantoonbaar (100% afdoding). Het effect van het mondspoelmiddel op basis van essentiële oliën was vergelijkbaar met het effect van alleen ethanol. Antibacteriële zeep en karnemelk waren niet of nauwelijks effectief. Uit het in dit hoofdstuk beschreven *in vitro* onderzoek werd geconcludeerd dat een mondspoelmiddel op basis van chloorhexidine het meest effectief is in het doden van micro-organismen in een

gemengde biofilm met bacteriën en gisten. Deze bevinding is veelbelovend voor toepassing in de praktijk aangezien een chloorhexidine mondspoelmiddel vrij en alom verkrijgbaar is, en zeer effectief is in het doden van bacteriën en gisten die aanwezig zijn in de biofilm op gelaatsprotheses. Naar verwachting zal het toepassen van een chloorhexidine mondspoelmiddel bij de dagelijkse reiniging van gelaatsprotheses leiden tot een langere levensduur van de gelaatsprothese en minder huidirritatie.

Als vervolgstap werd de effectiviteit van de in **hoofdstuk 4** onderzochte reinigingsmiddelen op doden van in de biofilm aanwezige micro-organismen onderzocht in de in **hoofdstuk 5** beschreven *ex vivo* pilot studie. Het effect van een 0,2% chloorhexidine mondspoelmiddel (met verdunningen tot 30x), een mondspoelmiddel op basis van essentiële oliën en een 50% zeepoplossing werd vergeleken met een controle (geen behandeling). De onderzochte stukjes siliconenmateriaal waren afkomstig van gedragen gelaatsprotheses die aan vervanging toe waren. De stukjes werden gedurende 1 uur in 1 ml van een bepaald reinigingsmiddel gelegd. De behandeling werd gedurende drie dagen dagelijks herhaald. De monsters werden opgeslagen bij kamertemperatuur De mondspoelmiddelen op basis van essentiële oliën en chloorhexidine bleken beide effectief om de bacteriële belasting op gelaatsprotheses te verminderen. Na de derde behandeling, resulteerde de behandeling met essentiële oliën in een 82% log reductie en chloorhexidine in 61% log reductie. De log reductie van de 50% zeepoplossing bleef daarbij sterk achter (19% log reductie). Op basis van deze resultaten werd geconcludeerd dat mondspoelmiddelen op basis van essentiële oliën of chloorhexidine antimicrobieel effectief zijn om de bacteriële load op *ex vivo* gelaatsprotheses te verminderen. Deze resultaten zijn veel belovend voor toepassing in de algemene praktijk van deze middelen voor reiniging van gelaatsprotheses. Vervolgonderzoek is gewenst.

In de algemene discussie worden de resultaten van de verschillende studies in breder perspectief geplaatst (**hoofdstuk 6**). Factoren die de levensduur van een gelaatsprothese bepalen, worden bediscussieerd, inclusief het effect van de toevoeging van pigment aan de siliconen op de microbiële kolonisatie van het prothesemateriaal en het effect van herhaalde blootstelling aan een reinigingsproduct op de kleur van het prothesemateriaal. Beide effecten werden in twee pilotstudies onderzocht (**hoofdstuk 6**). Uit deze pilotstudies kwam naar voren dat de aanwezigheid van pigment in het siliconenmateriaal de mate van microbiële kolonisatie van het siliconenmateriaal niet beïnvloedt en dat herhaalde reiniging met de onderzochte producten een verwaarloosbaar effect had op de kleur van de prostheses (in ieder geval op de korte termijn). Nadere *in vitro* studies zijn nodig met het focus op welke factoren de levensduur en de degradatie van het siliconenmateriaal waarvan gelaatsprothese zijn gemaakt te bepalen. Zodra deze factoren bekend zijn, kan een *in vivo* studie worden uitgevoerd om de in het laboratorium opgedane kennis te testen in de algemene praktijk. Hierbij moet de factor klimaat niet uit het oog worden verloren, per klimaat zone moet namelijk worden bekeken welke van de vermoede factoren leidend is.

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