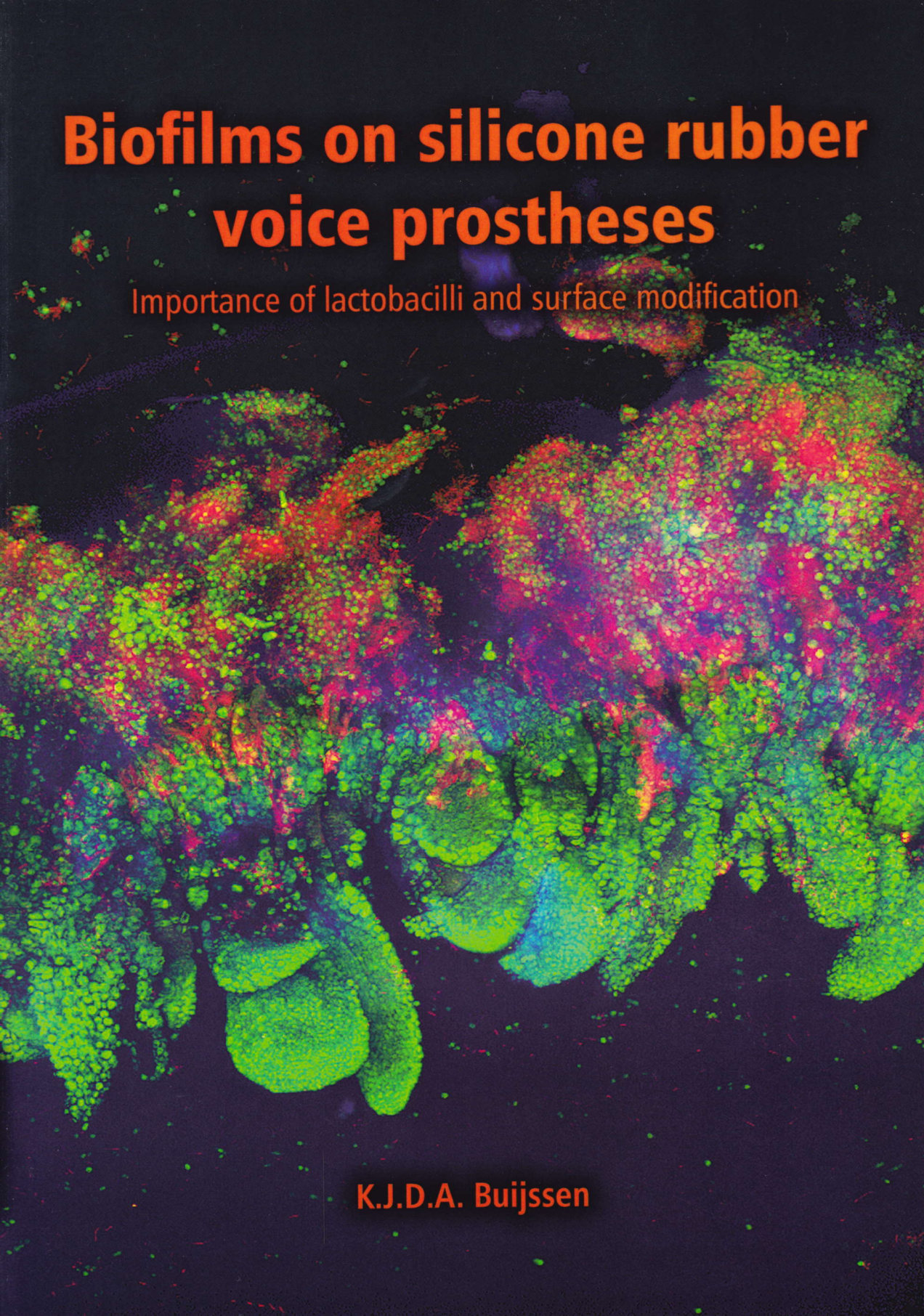
Biofilms on silicone rubber voice prostheses

Importance of lactobacilli and surface modification



K.J.D.A. Buijssen

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Cover: Biofilm on a silicone rubber voice prosthesis made with a confocal laser scanning microscope after Fluorescence in situ Hybridization with rhodamine labeled EUB338 probe (red), FITC-labeled EUK516 probe (green), and Calcofluor white stain (blue).

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Biofilms on silicone rubber voice prostheses

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Aan mijn ouders

CONTENTS

1 General Introduction

TOTAL LARYNGECTOMY AND VOICE PRODUCTION

Laryngeal cancer has a major impact on the function of speech and swallowing. This is partly due to the destructive process of the disease itself, and also to the effects of surgery, radiotherapy, and/or chemotherapy. In the cases of more extended tumours (T4 carcinomas) or in case of recurrent disease after radiotherapy, surgery is the best treatment option. The first successful laryngectomy for laryngeal cancer was carried out by Billroth in 1873. The anatomy is changed by a laryngectomy (Figure 1). The entire larynx including the vocal folds are removed and the lower respiratory tract is separated from the vocal tract and from the upper digestive tract. After a total laryngectomy, patients breath through a tracheostoma.

There are several options in voice rehabilitation available for patients after a total laryngectomy, e.g. oesophageal speech, artificial larynx speech, and tracheoesophageal speech. The last one has become the preferred method. In this method a silicone rubber voice prosthesis is positioned in a tracheoesophageal puncture made during laryngectomy.

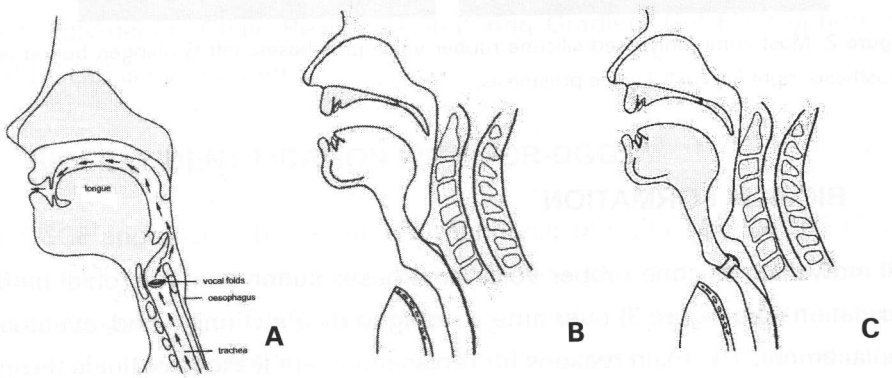

Figure 1. Anatomy of the larynx. (A) Normal situation, before laryngectomy; (B) Situation after laryngectomy, with a tracheostoma as a newly created airway; (C) After laryngectomy, with a voice prosthesis as a tracheoesophageal shunt

There are a variety of different voice prostheses on the market, such as the Blom-Singer[1] and Panje[2], and indwelling devices such as the Groningen button[3], a prosthesis of Nijdam[4], Provox®[5], and Staffieri and Staffieri[6]. All voice

prostheses vary according to length, diameter, retention flange, and resistance to airflow. The Provox® voice prostheses together with the Groningen voice prostheses are the most commonly used devices in Europe at present (Figure 2), but the production of the Groningen voice prostheses recently stopped.

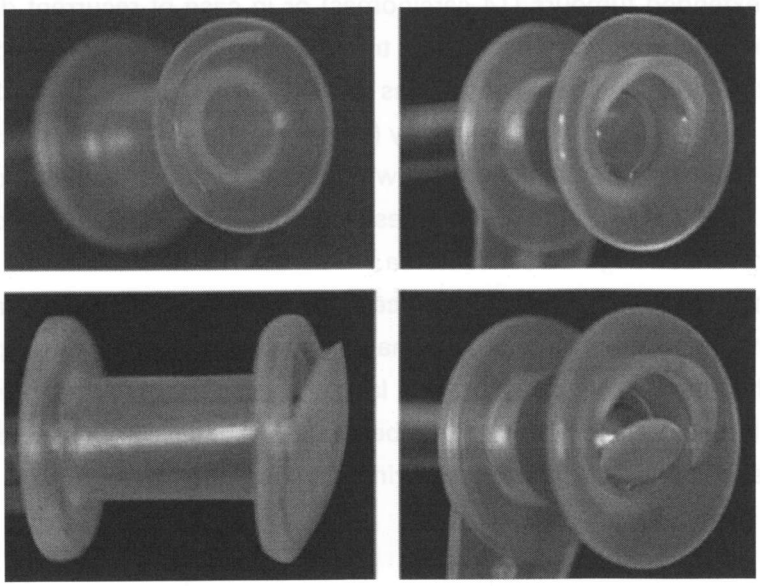

Figure 2. Most commonly used silicone rubber voice prostheses: left Groningen button voice prosthesis, right Provox® 2 voice prosthesis.

BIOFILM FORMATION

All indwelling silicone rubber voice prostheses suffer from microbial biofilm formation (see Figure 3) over time, leading to dysfunctioning and, eventually, replacement. The main reasons for replacement are leakage of fluids through the prosthesis or an increased airflow resistance during phonation.

Several studies have investigated the microbial composition of the biofilm on explanted voice prostheses. The microorganisms found are often members of the normal oral microflora and commensals of the skin, but also bacterial strains originating from food and dairy products were identified[7]. The composition of the microorganisms in the biofilm will differ individually influenced by several factors like irradiation, volume of irradiated salivary gland

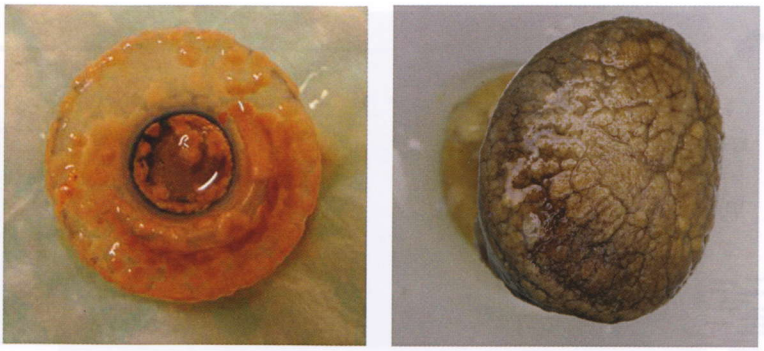

Figure 3. Silicone rubber voice prostheses covered by a biofilm. Provox® 2 (left) and Groningen Ultra Low Resistance (right) voice prosthesis.

tissue, residual salivary flow rate, time passed after irradiation or insertion of a prosthesis and surgical therapy[7,8,9]. The biofilm is a mixture of bacteria and fungi, in which Candida are held responsible for the dysfunctioning of the voice prostheses because of their ingrowth into the silicone rubber. For the integrity of the biofilm however, bacteria are thought to be essential[7,10-14]. Still not all microorganisms in voice prosthetic biofilms have been identified[7]. Several advanced techniques can now be used for identification of microorganism of which Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) is one of them.

BIOFILM IDENTIFICATION WITH PCR-DGGE

The DGGE approach is based on electrophoresis of PCR-amplified 16S rDNA (or another gene) fragments in polyacrylamide gels containing a linearly increasing gradient of DNA denaturants (a mixture of urea and formamide): a molecular fingerprinting technique that separates PCR-generated DNA products. The principle of DGGE relies on the electrophoretic separation of PCR amplicons of equal length in a sequence-specific manner using a polyacrylamide gel containing a denaturing gradient of urea and formamide. Essentially, PCR-DGGE comprises three steps (Figure 4): (1) extraction of total community DNA from the sample; (2) PCR-controlled amplification using specific oligonucleotide primers; and (3) separation of the amplicons using DGGE. For this purpose, a reproducible and efficient method for total DNA

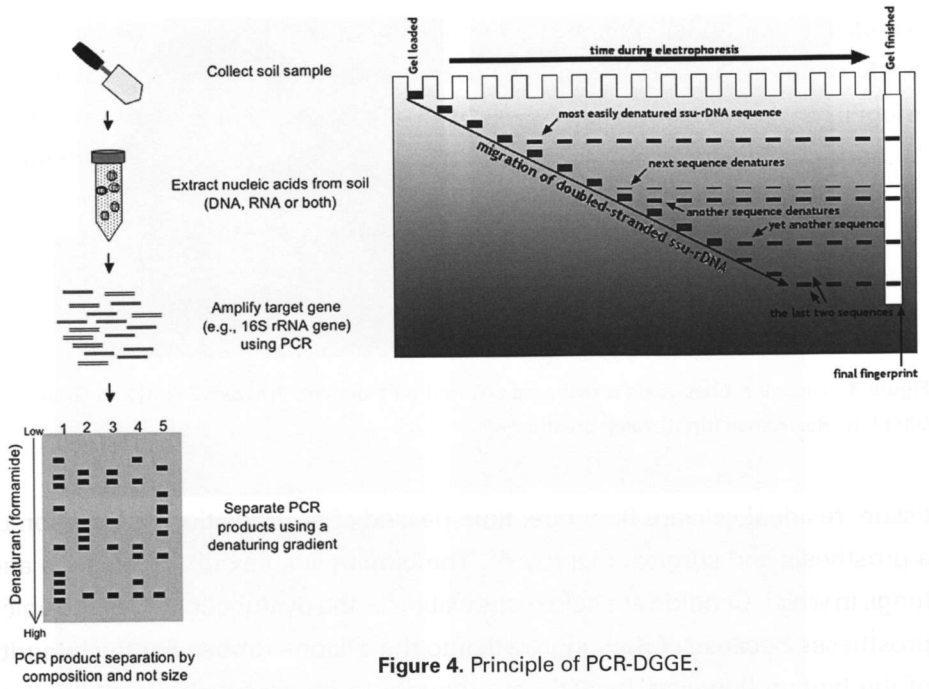

Figure 4. Principle of PCR-DGGE.

extraction is indispensable and needs to be evaluated and optimized depending on the nature of the sample. In the subsequent PCR step, multiple PCR primer sets with different resolution can be used. The use of universal primers allows any microbial community to be analyzed, although in ecosystems with a high diversity only the dominant microbiota will be visualized. In order to focus on specific subpopulations, group-specific PCR primers can be used.

VISUALIZATION OF BIOFILMS

So far, biofilms on silicone rubber voice prostheses have been visualized by scanning electron microscopy (SEM)[12] as shown in Figure 5. Visualizing specific members of biofilms on biomaterials using stains is challenging because staining both yeasts and bacteria and also the extracellular matrix is difficult to penetrate by many stains.

A major drawback of SEM is that the specimen must be dehydrated, which reduces the total volume of the extracellular matrix and alters its architecture.

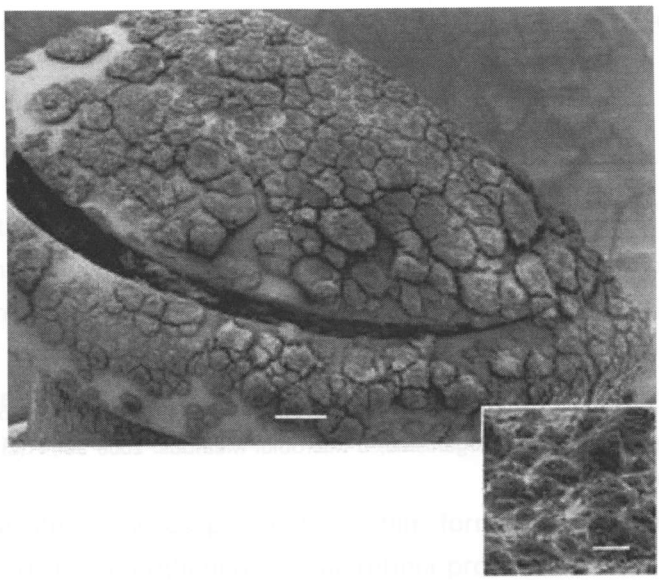

Figure 5. Scanning electron microscopy of Groningen ULR voice prosthesis covered by a biofilm (from Neu, Van der Mei, Busscher, Biomaterials 1993).

Fluorescence in situ Hybridization (FISH) is a useful method allowing the detection of bacteria and fungi within biofilms without disrupting its natural structure. Over the last decades, sensitivity and speed have made FISH a powerful tool for phylogenetic, ecologic, diagnostic, and environmental studies in microbiology[15]. FISH detects nucleic acid sequences by a fluorescently labelled probe that hybridizes specifically to its complementary target sequence within the intact cell. The procedure includes the steps shown in Figure 6: fixation of the specimen, preparation of the sample, hybridization with probes, washing steps to remove unbound probes and lastly mounting, visualization and documentation of results.

Confocal laser scanning microscopy (CLSM) offers several advantages over conventional optical microscopy, including the ability to control the depth of field, elimination or reduction of background information away from the focal plane (that leads to image degradation), and the capability to collect serial optical sections from thick specimens.

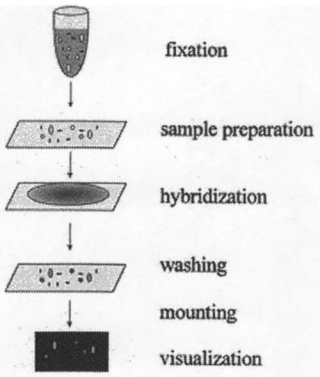

Figure 6. Flow chart of a typical FISH procedure (Reprinted from "Moter A., Göbel U.B.: FISH for direct visualization of microorganisms; J Microbiol Methods. 2000 Jul;41(2):85-112." With permission from Elsevier).

PREVENTION OF BIOFILMS

Different approaches have been taken to reduce or prevent biofilm formation on voice prostheses, with the aim of elongating the clinical life-time of voice prostheses.

External influences, for example the use of antibiotics or antimycotics can decrease biofilm formation, but it is preferable not to use them due to unpredictable patient compliance and the risk of inducing resistant strains[16].

Another strategy is to modify the silicone rubber surface to inhibit biofilm formation and consequently to prolong the lifetime of voice prostheses. Several methods used for silicone rubber surface modification have been described: metal coatings, plasma surface treatment, perfluoro-alkylsiloxane surface treatment, covalently coupled quaternary ammonium silane coatings, the use of surface active molecules (biosurfactants)[17-21]. A possible other strategy to decrease biofilm formation on voice prostheses could be to smoothen the material itself. In dentistry and in ophthalmology (especially on contact lenses), it has been shown that rough surfaces will promote biofilm formation[22,23].

There are also alternative prophylactic treatments to prolong voice prostheses lifetime, and for example the use of dairy products, probiotics,

caffeinated soft drinks has been demonstrated before to yield some sort of efficacy[24,25].

AIMS OF THIS STUDY

The aim of this thesis is

1) to identify the microbial composition and architecture of voice prosthetic biofilms using PCR-DGGE and FISH in combination with CLSM

2) to determine the role of specific bacterial strains in voice prosthetic biofilms with respect to the clinical life-time of prostheses

3) to evaluate the voice prosthetic biofilm formation on antimicrobially-modified and smoothened silicone rubber prostheses.

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Identification of biofilms on used voice prostheses by Denaturing Gradient Gel Electrophoresis (DGGE)

2

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Buijssen K.J.D.A., van der Laan B.F.A.M., van der Mei H.C., Atema-Smit J, van den Huijssen P, Busscher H.J., Harmsen H.J.M. (2011 Sept), Head and Neck [Epub ahead of print]. Composition and Architecture of Biofilms on voice prostheses.

INTRODUCTION

Laryngeal cancer is usually treated with radiation therapy or surgery. Total laryngectomy, surgical removal of the total larynx, is performed in more advanced stages of laryngeal cancer or when treatments with radiation or minor surgery (CO_2-laser) fail to conserve the larynx. After total laryngectomy patients no longer have the ability to speak normally because the whole voice box has been removed and they now breathe through an opening called a tracheostoma. Voice functions can be replaced (I) by the use of an electrolarynx, (II) oesophageal speech, or (III) a silicone rubber prosthetic device placed in a surgically created tracheooesophageal puncture[2]. The provision of a voice prosthesis permits a laryngectomee to attain a more natural manner of speech. However, a great disadvantage is the deterioration by a biofilm on the valve of the voice prosthesis that yields leakage through the prosthesis into the trachea or increased airflow resistance impeding speech[1,15]. Due to this biofilm formation the prostheses have to be replaced every three months on average[1].

Studies using culture-dependent procedures have revealed that the microorganisms associated with tracheoesophageal shunt prosthetic failure are diverse[7,11]. Candida, especially *Candida albicans* are found to be responsible for prostheses dysfunctioning. Elving et al.[7] mentioned that the bacterial strain *Rothia dentocariosa* was especially found on voice prostheses with an implantation period of less than 9 months but they also noticed that 51 out of 92 bacterial isolates from voice prostheses could not be classified with the MicroPlate, Biolog system (Hayward, California, USA) they used. These unidentified microorganisms can also play a role in the development of biofilms and therefore can be responsible for prosthesis failure.

Culturing methods are insufficient to study the full diversity of complex microbial biofilms. In 1993 Muyzer[14] described for the first time a molecular approach to analyze the genetic diversity of complex microbial population, based on the separation of PCR-amplified fragments of the 16S rRNA-gene by denaturing gradient gel electrophoresis (DGGE). The combination of PCR and DGGE allows the identification of a high number of bacterial and fungal species in the complex ecosystems of the human body and on biomaterials.

This study was conducted to gain insight into the total bacterial and fungal population on silicone rubber voice prostheses and to search for the most important bacteria and fungi in voice prosthetic biofilm formation. The composition of biofilms on silicone rubber voice prostheses was assessed by PCR-DGGE and sequence analysis.

MATERIALS AND METHODS

A total of 66 biofilms isolated from silicone rubber voice prostheses which were removed from patients for medical reasons were analyzed in this study. These prostheses were all removed because of leakage through the prosthesis, from patients visiting the outpatient clinic of the Department of Otorhinolaryngology of the University Medical Center Groningen. Directly after removal, explanted prostheses were conserved in sterile PBS (phosphate buffered saline, 10 mM potassium phosphate and 150 mM NaCl, pH 7.0).

DNA isolation and purification. DNA was extracted from the samples using a modification of the phenol/chloroform extraction method described by Zijnge et al.[22] Biofilms were removed from the voice prostheses (within 24h after removal) and diluted in 500 μl TE-buffer (10 mM Tris-HCl, 1 mM EDTA pH 7.4). 400 μl phenol was added to the sample after which the samples were mechanically disrupted in a Mini-BeadBeater-8TM (BioSpec Products Inc., Bartlesville, OK) with zirconium beads (0.1-0.5 mm) twice for 30 s. After disruption 400 μl chloroform/isoamylalcohol (24:1) was added and samples were centrifuged for 10 min at 14,000 *g*. After collecting the supernatant two more phenol chloroform extractions were performed, with 200 μl chloroform/ isoamylalcohol (24:1) and 200 μl phenol and centrifuged for 5 min at 14,000 *g*. After collecting the supernatant after the last extraction the DNA was precipitated by adding an equal amount of isopropanol and incubating at -80°C for 1 h. After centrifugation for 15 min at 14,000 *g*, the supernatant was discarded and the pellet was washed twice in 70% ethanol and again centrifuged for 15 min at 14,000 *g*. The supernatant was removed and the pellet was dissolved in 50 μl TE-buffer and stored at -20°C. DNA was purified using the QIAamp® DNA mini kit (QIAGEN Benelux B.V., Venlo, the Netherlands)

with a modification of the tissue protocol of the manufacturer, 75 µl sample was diluted to 200 µl with ATL tissue lysis buffer with Rnase (1.5 ml ATL + 4 µl Rnase) and incubated for 2 min at room temperature. Subsequently, 200 µl AL lysis buffer was added, mixed by vortexing and 200 µl 96% ethanol was added, mixed again and centrifuged to remove drops from inside the lid. The entire solution was applied to the QIAamp spin column and centrifuged for 1 min at 6000 *g*. Next the column was washed with 500 µl AW1 and 500 µl AW2 washing buffers respectively and the DNA was eluted from the column with 50 µl AE low-salt elution buffer after incubation for 5 min at room temperature. Samples were stored at -20°C.

Polymerase chain reaction (PCR) amplification. Extracted DNA was subsequently used as a template to amplify conserved regions of the16S rRNA-genes of bacteria or the 26S rRNA genes of fungi. The PCR reaction-mixture for both assays contained 5 µl reaction buffer (100 mM Tris-HCl, pH8.3, 500 mM KCl, and 15 mM $MgCl_2$), 1.25 units of Taq polymerase (both TaKaRa SHUZO Ltd, Otsu, Japan) 400 nM of each primer, 200 µM of dNTP's, and around 20 ng of template DNA in a total volume of 50 µl. For the amplification of bacterial DNA the universal primers UI341fGC (5' CCTACGGGIGGCIGCA-3') and UI533r (5' TIACCGIIICTICTGGCAC) were used, described by Watanabe et al.[21] To make the PCR-product suitable for DGGE a previously described GC-clamp was added to the forward primer[16]. The temperature profile included 5 min at 95°C, followed by 35 cycles at 94°C for 45 s, 49°C for 30 s and at 72°C for 1 min, followed by 5 min at 72°C. For amplification of fungal DNA the universal primer GC-NL1-f; -(5'-GCATATCAATAAGCGGAGGAAAAG-3') with the following GC-clamp attached to the 5'end (5'-CGCCCGCCGCGCCCCGCGCC-CGTCCCGCCGCCCCCGCCCG-3') and primer LS2-r (5'-ATTCCCAAACAACTCGACTC-3') as described by Rantsiou et al.[17]. The amplification temperature profile included 5 min at 95°C, followed by 30 cycles at 94°C for 1 min, 52°C for 45 s and at 72°C for 1 min, followed by 5 min at 72°C. After agarose gel-electrophoresis, the ethidiumbromide-stained PCR-products were analyzed by UV light.

DGGE. DGGE analysis of PCR products was performed as described by Muyzer[13]. PCR products were loaded on a 8% (w/v) polyacrylamide gel in 0.5 TAE (0.02 M Tris base, 0.01 M acetic acid, 0.5 mM EDTA, pH 7.5). The denaturing

gradient consisted of 30 to 70% denaturant (100% denaturant equals 7 M Urea and 40% formamide). Gels were poured using a gradient mixer. A 10 ml stacking gel without denaturant was added on top. Electrophoresis was performed for 16h to 18h at 120 V and 60°C using a INGENYphorU-2x2 system (INGENY International, Goes, The Netherlands). Gels were stained with silver nitrate[14,16].

Clone library. Three voice prostheses biofilm samples were selected for cloning based on their high diversity in DGGE analysis. Extracted DNA of these samples were amplified with universal bacterial primers Bact 11f 5'-GAGTTTGAT(C/T)(A/C)TGGCTCAG-3' and Bact 1492 5'-GGTTACCTTGTTACGACTT-3'. PCR products were purified with the QIAquick gel extraction kit (Qiagen, Germany) and subsequently ligated into pGEM-T Easy Vector and ligation products were transformed by heat shock into JM 109 High Efficiency Competent Cells (Promega, Madison, USA) as specified by the manufacturer. The recombinant cells containing a plasmid with an insert were selected and plasmid extraction by minipreparations and subsequent sequencing was carried out by an external commercial service (Baseclear, Leiden, The Netherlands). Dideoxy-DNA sequence reactions were carried out with primer 341 forward primer 5'-CCTACGGGAGGCAGCA-3' and analysed with a single long run. The resulting sequences were analyzed and a phylogenetic tree based on the neighbour-joining method was constructed with the ARB software package[9,10] using a RDP9 database of the ribosomal database project[5]. The neighbour-joining tree is based on a distance matrix including 800 nucleotide positions invariable in 50% of all bacterial sequences between *Escherichiae coli* position 431 and 1222. Bootstrap percentages at nodes, based on 1000 replications, display the significance of these nodes; only values above 95% are given.

Analysis of the DGGE profiles. The fungal gels bands of interest were excised after silver staining, suspended in 100 μl DNA free water and grinded with a pipette tip to extract the amplicons from the gel. This was used as a template for the fungal PCR amplification. The products were purified and the sequence was analysed using the forward PCR-primer. Fungal markers were created with the use of bacteria and fungi cultured on agar plates from voice prostheses in earlier research. One colony of every strain from the agar plate was transferred into 100 μl of DNA free water to induce lyses. This served as

a template for fungal PCR amplification of the rRNA-gene after which DGGE-analysis was performed.

Bacterial markers were created by using the DNA of selected clones identified by sequencing for PCR/DGGE with the universal bacterial primers described above. The bands from the bacteria and fungi from the voice prosthesis were compared with the markers in order to identify the strains. The bacteria and fungi were scored (presence/absence) for all samples and were related to the in situ lifetime of the voice prostheses.

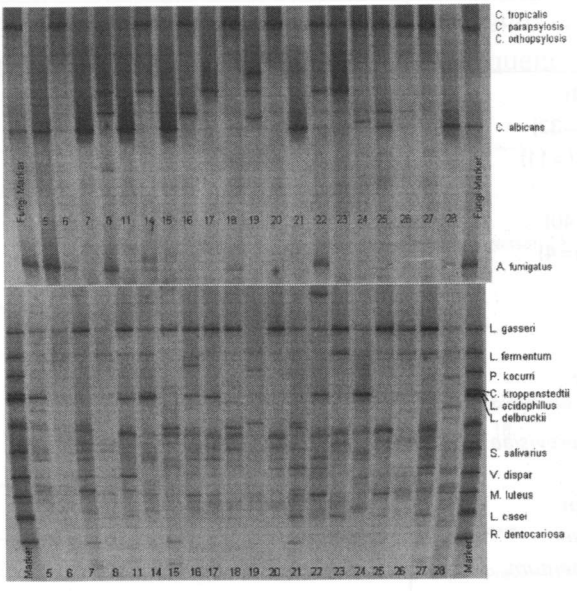

Figure 1. Example of DGGE-gels showing lanes from voice prosthetic biofilms and marker lanes. The markers contain bands from a mixture of single species fungal (upper gel) and bacterial (lower gel) strains isolated from voice prostheses that have been identified by 16S rRNA-gene sequence analysis. The corresponding species are indicated at the right.

RESULTS

A total of 66 biofilms from different silicone rubber voice prostheses were analyzed for the bacterial and fungal community population: 49 Provox®2 voice prostheses, 16 Groningen Ultra Low Resistance (ULR) voice prostheses and one Provox® ActiValve voice prosthesis. The lifetime of the investigated

voice prostheses differs widely, from only 5 days till 594 days. In 100% of the cases the indication for removing the prosthesis was leakage of fluids through the prosthesis. Forty-four biofilms (33 Provox2 and 11 Groningen ULR voice prostheses) out of 66, yielded DNA and subsequent DGGE-fingerprints, from 22 biofilms no PCR product could be obtained for DGGE analysis. The used voice prostheses have a median device in situ lifetime of 75 days and a mean device in situ lifetime of 99 days (see Table 1).

	% of total group	*Mean lifetime (days)*	*Median lifetime (days)*
Total group (n=44)	100	99	75
Provox® group (n=33)	75	100	73
Groningen group (=11)	25	96	92
Gender (male, n=40)	91	100	74
Gender (female, n=4)	9	93	108
Candida (group)	98	99	75
Candida albicans	77	96	75
Candida tropicalis	52	106	92
Candida glabrata	46	116	95
Saccharomyces cerevisiae	23	86	82
Lactobacilli (group)	100	99	75
Lactobacillus gasseri	96	101	75
Lactobacillus fermentum	73	106	74
Lactobacillus acidophilus	52	113	88
Lactobacillus delbrueckii	36	74	76
Lactobacillus casei	30	120	92
Planococcus kocurii	21	58	74
Corynebacterium kroppenstedtii	9	217	107
Streptococcus (group)	61	82	74
Veillonella dispar	40	100	92
Micrococcus luteus	50	121	94
Rothia dentocariosa	16	59	61

Table 1. Characteristics of all prostheses (Provox®2 and Groningen Ultra Low Resistance). Frequency of microorganisms is defined as percentage of voice prostheses on which given bacterium or yeast was found relative to total number of voice prostheses in the group.

For the Provox®2 prosthesis and the Groningen ULR prosthesis the median device in situ lifetime was 73 and 92 days respectively and the mean was 100 and 96 days respectively. DGGE analysis showed different band profiles from the biofilms of different individuals. An example of DGGE patterns is shown in Figure 1. In all lanes the presence of lactobacilli is observed. Yeast strains identified with PCR-DGGE were mainly *C. albicans*, *C. tropicalis*, *C. glabrata* and *Saccharomyces cerevisiae*.

To analyze the bacterial diversity in more detail three biofilm samples with a high number of DGGE bands were selected for cloning of the 16S rRNA-genes and subsequent sequence analysis. In Figure 2, a phylogenetic tree is showing the relationships of the biofilm clone sequences. These clones

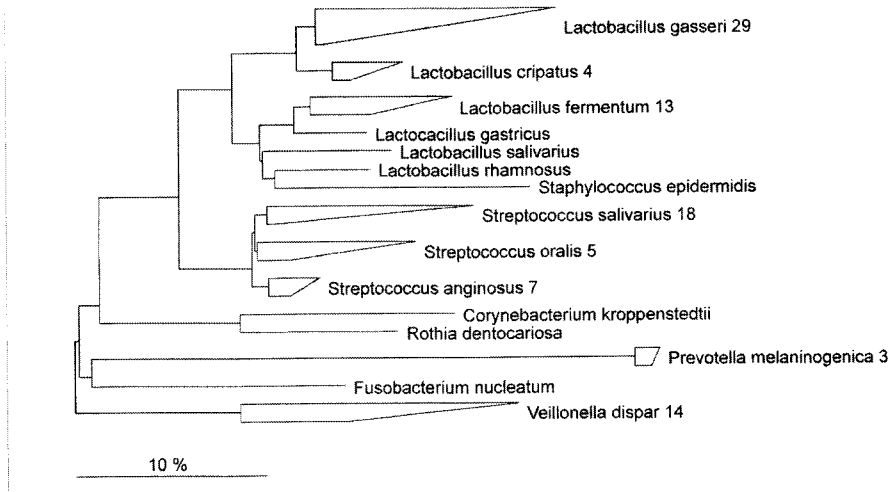

Figure 2. Phylogenetic tree showing the relationships between sequences of 100 clones derived from 3 biofilm samples. The sequences are grouped based on similarity and represented in boxes, the species with which the group has the highest similarity is indicated behind the box. The sizes of the boxes reflect the number of clones that is indicated behind the species name, and their shape reflects the phylogenetic depth of the corresponding group. Single sequences are indicated as single lines. The tree was based on an alignment of the 16S rRNA sequences of the clones to reference sequences of the indicated species. The tree is a neighbor-joining tree of a distance matrix including 800 nucleotide positions invariable in 50% of all bacterial sequences between *E. coli* position 431 and 1222. Bar shows 10% sequence divergence.

are identified based on most closely related reference sequences of described bacteria and other Gram-positive and Gram-negative bacteria from the RDP-database.

Numbers of clones are also mentioned behind the species. *Lactobacillus gasseri*, *L. fermentum*, *Streptococcus salivarius* and *Veillonella dispar* were most frequently present in the clone libraries. Table 1 shows the presence and the in situ lifetime characteristics of the 44 biofilms that were further analyzed. Microorganims that are most present (>50%) are *C. albicans* (77%), *C. tropicalis* (52%), *L. gasseri* (96%), *L. fermentum* (73%), *L. acidophilus* (52%) and *Streptococci* (61%). The median in situ lifetime of voice prosthesis from which these microorganisms are isolated differs from 74 till 92 days. In all biofilms lactobacilli are present and in 98% of the biofilms fungi. The presence of *Corynebacterium kroppenstedtii* (9%), is detected on the voice prosthesis with the longest lifetime and *Rothia dentocariosa* (16%) with the

	< 75 days (n=22)	*>75 days (n=22)*
Candida (group)	95	100
Candida albicans	77	77
Candida tropicalis	41	64
Candida glabrata	36	55
Saccharomyces cerevisiae	23	23
Lactobacilli (group)	100	100
Lactobacillus gasseri	95	95
Lactobacillus fermentum	73	68
Lactobacillus acidophilus	50	68
Lactobacillus delbrueckii	36	36
Lactobacillus casei	27	32
Planococcus kocurii	23	18
Corynebacterium kroppenstedtii	5	14
Streptococcus (group)	68	55
Veillonella dispar	32	25
Micrococcus luteus	45	55
Rothia dentocariosa	27	5

Table 2. Fungi and bacteria identified from voice prostheses subdivided according to "in situ lifetime", Two groups: in situ lifetime < 75 days, > 75 days.

shortest lifetime. Table 2 shows the frequencies of identified microorganisms subdivided in two groups according to the in situ lifetime. Among the *Candida* strains, more *C. tropicalis* and *C. glabrata* are found in the long in situ lifetime-group (> 75 days) compared to the short in situ life-time group. As mentioned before, in all voice prosthetic biofilms lactobacilli were identified, especially *L. gasseri*, independently of the in situ lifetime. *L. acidophilus* is detected more often in biofilms over 75 days whereas *R. dentocariosa* is detected more often in the short lifetime group.

DISCUSSION

In the present study the bacterial and fungal diversity on silicone rubber shunt prostheses was examined with the use of PCR-DGGE.

PCR-DGGE indicated for the first time that lactobacilli were present on all explanted tracheoesophageal shunt prostheses. Rod-shaped organisms have often been observed in voice prosthetic biofilms[7], but have never been identified as lactobacilli. Sequence analysis of the 16S rRNA-gene showed that these strains were especially *L. gasseri, L. fermentum* and *L. acidophilus.* It is known that lactobacilli belong to the normal oral microflora. Köll-Klais et al.[8] mentioned that the most prevalent strains in healthy persons are *L. gasseri* and *L. fermentum* also found in this study. Therefore it is not surprisingly that lactobacilli belong to the microflora in voice prosthetic biofilms.

The most prevalent fungus in voice prosthetic biofilms is *C. albicans*. It is described that Candida species and especially *C. albicans* are ubiquitously accepted as the predominant causative organisms for prosthesis failure[11], although Candida alone is incapable of forming a biofilm[5].

It is unclear whether the presence of lactobacilli in voice prosthetic biofilms should be associated with failure of the prostheses or not. The Candida-lactobacilli interaction likely plays an important role in the mixed biofilm on tracheoesophageal voice prostheses. *L. acidophilus* at varying concentrations suppresses candidal biofilm formation in vitro and therefore it is considered that there is a possible mechanism by which lactobacilli have a beneficial effect in mucosal candidal infections[20]. Furthermore, lactobacilli are amongst the most widely used probiotic organisms. Lactobacilli as

probiotics exert their benificial effects through competitive displacement and exclusion of pathogens, affinity for tissues and materials to be protected, co-aggregation with pathogens to be eliminated, H_2O_2, lactic acid, bacteriocin and biosurfactant production. Lactobacillus therapy has been applied especially to improve the intestinal microbial balance and is used for the prevention of symptoms of lactose intolerance, treatment of acute diarrhea, attenuation of antibiotic-associated gastrointestinal side effects and the prevention and treatment of allergy manifestations[3,6]. Also, lactobacillus therapy is known to restore a healthy urogenital microflora, preventing urinary tract infections[18]. Recently the consumption of a fermented dairy product containing a particular strain of probiotic lactobacilli, *Lactobacillus casei* Shirota, has been associated with a prolonged life-time of voice prostheses in patients[19].

Mixed species biofilms is a complex process because hyphal formation of the fungi is required for a robust biofilm which is adhering strongly to the surface, but still little is known about the influence of the bacterial presence on the expression of hyphae in fungi. In vitro on silicone rubber, adhesive interactions between yeasts and bacteria have been reported and in the presence of saliva, *R. dentocariosa* and *Staphylococcus aureus* enhanced adhesion of yeasts, especially *C. albicans*[12], which could be a reason why *R. dentocariosa* has been identified more in biofilms with a shorter in situ lifetime than a longer.

In summary, this is the first time that lactobacilli have been identified to be general colonizers of tracheoesophageal voice prostheses in vivo. It is the predominant bacterium in voice prosthetic biofilms. *C. albicans* is the most important fungus but also *C. tropicalis* en *C. glabrata* have been identified, especially in biofilms with an extended in situ lifetime.

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Visualization by Fluorescence in situ Hybridization and Confocal Laser Scanning Microscopy of Biofilm Architecture on used Voice Prostheses

3

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Buijssen K.J.D.A., van der Laan B.F.A.M., van der Mei H.C., Atema-Smit J, van den Huijssen P, Busscher H.J., Harmsen H.J.M. (2011 Sept), Head and Neck [Epub ahead of print]. Composition and Architecture of Biofilms on voice prostheses.

INTRODUCTION

Laryngeal cancer is a cancer of the throat that affects the vocal cords and voice box, also called the larynx. Cancer of the larynx is first treated with radiotherapy or surgery (CO_2-laser), but requires a total laryngectomy when these treatments are insufficient or in the more advanced stages of the disease. In a total laryngectomy, the entire voice box is removed (see Fig. 1A, B). Although the life-expectancy of patients after laryngectomy is generally good, the loss of voice is a devastating consequence of the treatment. There are three voice rehabilitation techniques after total laryngectomy: electromechanical speech, esophageal speech and tracheoesophageal (shunt) speech. Since its introduction by Blom and Singer in 1980[3], the insertion of a silicone rubber voice prosthesis in a surgically created tracheoesophageal shunt is still the best and most frequently used method for voice rehabilitation after total laryngectomy (see Fig. 1C). The prosthesis essentially is a one-way valve that prevents the leakage of food and fluids into the trachea, and opens when the patient wants to speak. Breathing normally occurs through the surgically created stoma (see Fig. 1B,C), but upon closing the stoma with a finger, the valve can be opened and air flow through the esophagus into the oral cavity stimulates the vibration of remaining muscular structures to produce a voice. The functional lifetime of voice prostheses varies enormously from patient to

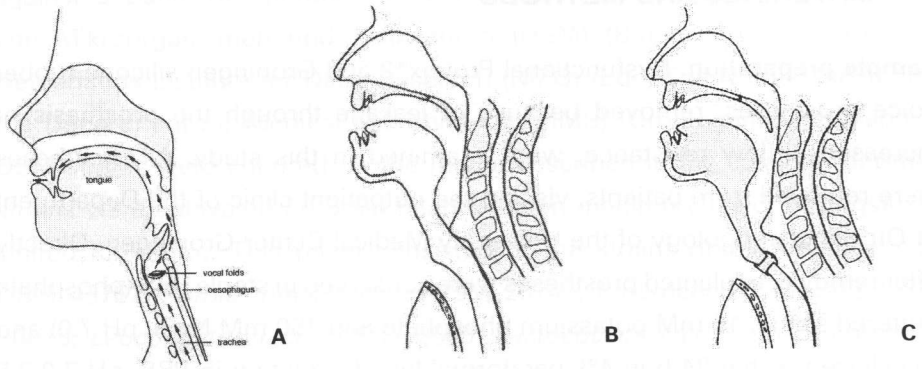

Figure 1. Anatomy of the larynx. (A) Normal situation, before laryngectomy; (B) Situation after laryngectomy, with a tracheostoma as a newly created airway; (C) After laryngectomy, with a voice prosthesis as a tracheoesophageal shunt

patient and ranges from several days to more than four years. Nevertheless, the functional lifetime is short, with an average of around three months.

Biofilms containing a mixture of different bacterial and yeast strains on the valve side of the prostheses are considered responsible for dysfunctioning of the valve, either causing increased airflow resistance or leakage through the prosthesis. Although several studies have identified strains and species involved in voice prosthetic biofilms[5,10] and electron microscopy has indicated that microorganisms even grow into the silicone rubber[14], only a part of the microbes colonizing voice prostheses have been identified and a good visualization of the biofilm architecture is lacking. Knowledge of the biofilm architecture would contribute to a better understanding of the symbiosis between yeasts and bacteria in voice prosthetic biofilms.

Fluorescence in situ Hybridization (FISH) using rRNA-targeted oligonucleotide probes and Confocal Laser Scanning Microscopy (CLSM) can be applied to investigate microbial biofilms[12,19,22] and in the past FISH has also been used as a diagnostic tool[7,8,9,18] or to investigate the microbial diversity in infections[13].

The aim of the present study is to visualize the biofilm architecture on silicone rubber voice prostheses used for different lengths of time and to identify microorganisms in the biofilm with the use of FISH and CLSM.

MATERIALS AND METHODS

Sample preparation. Dysfunctional Provox®2 and Groningen silicone rubber voice prostheses, removed because of leakage through the prosthesis or increased airflow resistance, were examined in this study. All prostheses were removed from patients, visiting the outpatient clinic of the Department of Otorhinolaryngology of the University Medical Center Groningen. Directly after removal, explanted prostheses were conserved in sterile PBS (phosphate buffered saline, 10 mM potassium phosphate and 150 mM NaCl, pH 7.0) and transferred within 24 h in 4%-paraformaldehyde solution in PBS, pH 7.2-7.5 (Paraformaldehyde, MP Biomedicals, LLC, Eschwege, Germany). After 24 h fixation at 4°C, prostheses were conserved in an ethanol/PBS (1:1) solution

to store the samples (-20°C) until preparation for visualization. Subsequently, the valves of the silicone rubber prostheses were cut into small cross-sections and glued onto glass slides with a silicone gel. Parts of six voice prostheses were also used for identifying microorganisms in the biofilms. Deposits were scraped from the parts and dissolved in an ethanol/PBS (1:1) solution and 15 µl of this suspension was spread out on a gelatin coated glass slide, air dried, and then dehydrated in 96% ethanol for 10 min.

Oligonucleotide probes. FISH with DNA probes was mostly performed with a rhodamine-labeled EUB338 probe, specific for the domain bacteria providing a red signal and a fluorescein-isothyocyanate (FITC)-labeled EUK516 probe, complementary to a 18S rRNA region conserved for eukarya providing a green signal. The sequences of these probes and probes used for further identification of bacteria are listed in Table 1. Besides the listed probes several experimental probes for some bacteria were also used. All oligonucleotide probes were commercially obtained (Eurogentec, Seraing, Belgium).

Besides those oligonucleotide rRNA-targeted probes, we used Calcofluor-white stain (Sigma), 0.048 g/ml diluted 250x for the visualization of Extracellular Polymeric Substances (EPS) in the biofilms.

Supplementary, a specific probe was designed to detect bacteria of the Rothia group since *Rothia dentocariosa* has been associated with early failure of voice prostheses[5]. The probe was designed as described before[7] and 5' end-labeled with FITC. The specificity of the probe was confirmed by testing against a panel of reference strains obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM) (Braunschweig, Germany), Netherlands Institute for Dairy Research (NIZO) (Ede, The Netherlands), and the Department for Medical Microbiology (MMB) (Groningen, Netherlands). DSM strains were cultured on the media described in the catalog. All other strains were cultivated on brain heart infusion medium (Oxoid, Basingstoke, United Kingdom). This panel consisted of the strains *Rothia dentocariosa* DSM 43762, *Rothia mucilaginosa* DSM 20746, *Enterococcus faecalis* DSM 20478, *Leuconostoc lactis* NIZO B630, *Lactococcus lactis* subsp. *cremoris* DSM 20069, *Streptococcus mitis and Salmonella typh*i (both clinical isolates MMB), and *Escherichia coli* DSM 25922. For this experiment, the EUB338 probe was used as a positive control and the anti-sense probe non-EUB338 as

TABLE 1. Oligonucleotide probes used in this study.

Probe	Sequence (5'-3')	Target group	Used permeabilization	Hybridization	Reference
EUK516	ACCAGACTTGCCCTCC	Eukarya	-	Over night, 50°C	3
EUB338	GCTGCCTCCCGTAGGAGT	Bacteria	-	Over night, 50°C	3
Ca.al	GCCAAGGCTTATACTCGCT	*Candida albicans*	-	Over night, 50°C	12
Str493	CCAGAAAGGGACGGCUAAC	Streptococci, Lactococci	20 min Lysozyme	Over night, 50°C	13
Ato291	GGTCGGTCTCTCAACCC	*Atopobium*	-	Over night, 50°C	11
Lab158	GGTATTAGCA(C/T)CTGTTTCCA	Lactobacilli, Enterococci	50 min Labmix	Over night, 50°C	10
Pseudaer	GGTAACCGTCCCCCTTGC	*Pseudomonas aeruginosa*	-	Over night, 50°C	32
EC1531	CACCGTAGTGCCTCGTCATCA	*Enterobacteriaceae*	-	2h, 50°C	26
Roth192	GGUGUUGGUGGAAAGCGU	*Rothiae*	20 min Lysozyme	Over night, 50°C	This study
Bac303	CCAATGTGGGGGACCTT	*Bacteroides, Prevotella*	-	2h, 50°C	18
Haeinf	CCGCACTTTCATCTTCCG	*Haemophilus influenzae*	-	Over night, 50°C	12
PNA FISH Staph aureus[a]	-	*Staphylococcus aureus*	-	1½h, 55 °C	-
Veil223	AGACGCAATCCCCTCCTT	*Veillonella*	-	Over night, 50°C	11

[a] Obtained commercially (AdvanDx Inc., Woburn, MA, USA)

a negative control. For the evaluation of the new designed probe (Roth192), an Olympus BH2 epifluorescence microscope was used.

Fluorescence in situ Hybridization (FISH). FISH was basically performed as described previously[2,12]. The cross sections of used silicone rubber voice prostheses were hybridized with minor modifications. For three probe hybridizations, 50 µl enzyme mixture was used prior to hybridization to allow better permeabilization of the bacterial cell membrane (Table 1). The samples were hybridized in a volume of 50 µl pre-warmed (50°C) hybridization buffer (0.9 M NaCl, 20 mM Tris, pH 7.2, and 0.01 % SDS) containing specific oligonucleotide probes. To be sure that the hybridization buffer with the probes would submerge the sample, plastic rings were used around the sample inhibiting the solution from drifting away. The slides were kept in a closed moist chamber for 17-19 h (if hybridized overnight) in a dark chamber at 50°C. The slides were washed in a pre-warmed (50°C) washing buffer (0.9 M NaCl , 20 mM Tris, pH 7.2) for 15 min to remove unbounded probes. Before microscopic analysis, samples were rinsed with Millipore water, carefully dried with tissues, mounted in Vectashield® medium for fluorescence (Vector Laboratories, Inc. Burlingame CA 94010, USA) and covered with a coverslip.

Confocal Laser Scanning Microscopy. A confocal laser scanning microscope model LEICA TCS SP2 (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany) equipped with a UV laser, a He-Ne laser and an Ar laser and supplied with the latest version of Leica Confocal Software was used to visualize the hybridizations of the biofilms on used voice prostheses and to make records of the sections. Signals were recorded in the blue channel (excitation 400 nm, emission 490 nm) for detection of Calcofluor white, green channel (excitation 488 nm, emission 514 nm) for detection of FITC-labeled probes and red channel (excitation 543 nm, emission 580 nm) for detection of rhodamine-labeled probes. The confocal images were obtained using 20x and 63x oil immersion objectives. The surface of the valve of the silicone rubber voice prosthesis was scanned thoroughly and series of images were made of the biofilm at randomly chosen locations on the surface and stacked into overlay projections.

RESULTS

For this study, 22 different voice prostheses have been investigated: 10 Groningen Ultra Low Resistance voice prostheses and 12 Provox®2 voice prostheses. Table 2 shows an overview, together with a macroscopic score of the amount of visual biofilm. On most prostheses, deposits could be seen with the naked eye and on the prostheses with a more extended lifetime also ingrowth into the silicone rubber was observed. The lifetime of the investigated voice prostheses differs widely, from only 7 days till 1532 days. The biofilm thickness generally increased in time.

Figure 2 demonstrates the variety in biofilm on the silicone rubber voice prostheses in time. It shows that even in a short time (Fig. 2C, 7 days)

Groningen/Provox	Amount of biofilm	Lifetime (days)
Provox	Minor	6
Provox	Minor	7
Provox	Minor	7
Provox	Minor	11
Provox	Minor	39
Groningen	Minor	47
Provox	Minor	50
Groningen	Minor	54
Provox	Minor	60
Provox	Extensive	67
Groningen	Moderate	76
Provox	Moderate	87
Groningen	Moderate	89
Groningen	Minor	94
Provox	Moderate	149
Provox	Extensive	233
Groningen	Extensive	370
Groningen	Extensive	318
Groningen	Extensive	331
Provox	Extensive	About 3 years
Groningen	Extensive	525
Groningen	Extensive	1532

Table 2. Overview of the voice prostheses removed from patients because of dysfunctioning, as used in this study, together with a macroscopic assessment of the amount of biofilm and the prosthesis clinical lifetime.

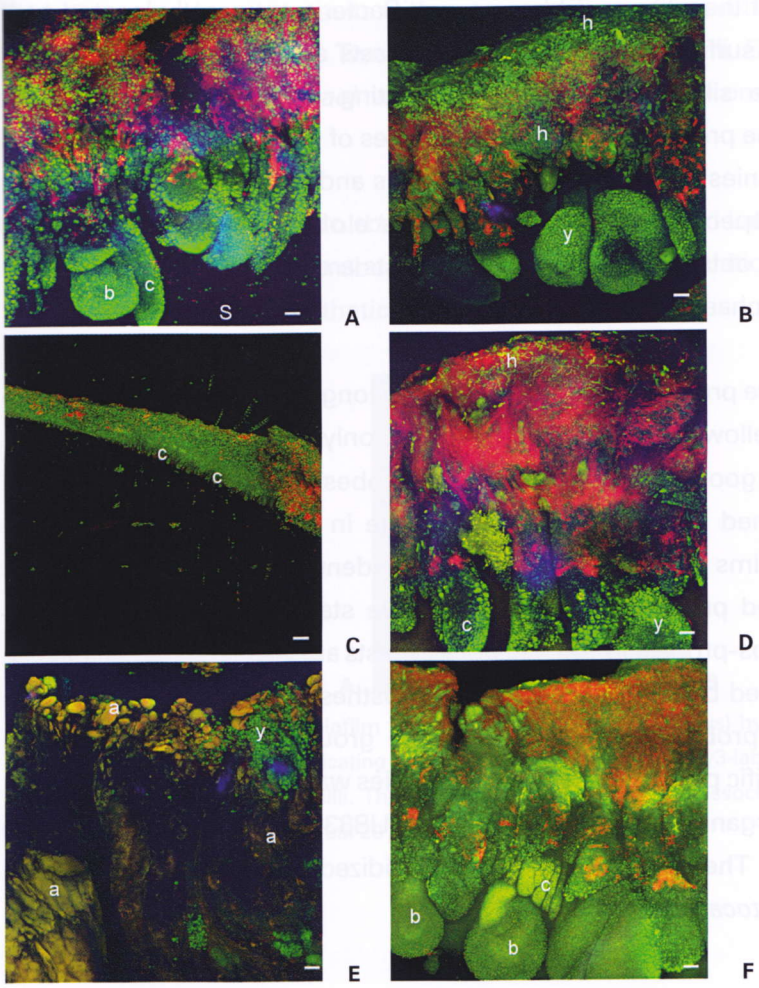

Figure 2. CLSM images (A-E) of voice prosthetic biofilms after FISH with rhodamine labeled EUB338 probe (red), FITC-labeled EUK516 probe (green), and Calcofluor white stain (blue). Note, no Calcofluor white stain was applied on the 7 days old biofilm. Bars equal 20 μm. S = silicone rubber, b = bag, c = comb, y = yeasts, h = hyphae, a = autofluorescence (yellow-brown).

A. 67 days old biofilm

B. A 370 days old biofilm illustrating the morphological differences of *Candida*: hyphae (h) in the outermost surface of the biofilm and close to the silione rubber. Colonies of yeasts (y) without hyphae can be seen in the bags (b) in the deteriorated silicone rubber.

C. Biofilm of 7 days old

D. Biofilm of 318 days old

E. Biofilm of 1532 days old

F. Image of the 318 days old biofilm using the FITC-labeled *Candida albicans* probe (green) and rhodamine-labeled EUB338 probe (red) demonstrating the presence of *C. albicans*.

deterioration of the silicone rubber occurs. Bacteria are mostly located near the outermost surface of the biofilm and yeasts are predominantly located near or in the silicone rubber, demonstrating ingrowing bags of yeast colonies and the presence of crevices. Analyses of the cross sections showed ingrowing colonies of bacteria above the bags and combs of yeasts. Hyphae of yeasts are especially seen around the surface of the silicone rubber and in the outerlayers of the biofilm. Ingrowing yeasts are grouped together and do not express hyphae.

On several voice prostheses with an extremely long lifetime, autofluorescence was seen as yellow brown. In these biofilms, only some parts on top of the biofilm show a good staining with the used probes (i.e. Fig. 2E).

EPS (stained with Calcofluor white, blue in Fig. 2) was usually more present in biofilms with a longer lifetime. The identification of specific yeasts on several used prostheses shows a positive staining with a FITC-labeled *Candida albicans*-probe (Fig. 2F), but not all yeasts are visualized as *C. albicans*.

The isolated biofilms from six voice prostheses were investigated with more specific probes for different bacterial groups. For this purpose, an additional specific probe to detect *Rothia* species was designed and evaluated. All reference organisms hybridized with the EUB338 probe and none with the nonEUB probe. The Roth192 probe only hybridized with *Rothia mucilaginosa* and *Rothia dentocariosa.*

Table 3. Results of the identification using specific bacterial probes on isolated voice prosthetic biofilms (only probes with a positive result are listed in this table).

	EUB338	Str493	Lab158	EC1531	Roth192	Veil223	Bac303	Haeinf	Saur
7 days	+++	+	+	+	+	-	-	+	+
67 days	+++	-	+	+	-	-	-	-	-
94 days	+++	-	+	+	+	+	-	-	+
233 days	+++	-	+	+	+	-	+	-	+
318 days	+++	-	++	+	-	-	-	+	-
1532 days	+++	-	++	++	+	+	+	-	-

- no fluorescence, + fluorescence of a few bacteria, ++ fluorescence of several bacteria, +++ fluorescence of (almost) all bacteria. Saur = Staphylococcus aureus PNA FISH.

Table 3 summarizes the results with more specific probes on the isolates from the used voice prostheses. Two probes, Lab158 (Lactobacilli, *Enterococci)* and EC1531 (*Enterobacteriaceae)* were positive for biofilms from all prostheses investigated.

Figure 3 shows a 318 days old biofilm with the Cy3-labeled Lab158 probe and the FITC-labeled EUK516 probe. It demonstrates the association of lactobacilli and yeasts in a voice prosthetic biofilm.

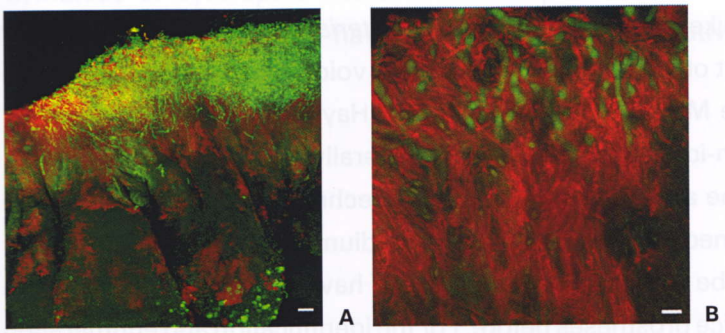

Figure 3. Overlay-images of a biofilm from a used prosthesis (318 days) hybridized with the a FITC-labeled EUK516 probe indicating all yeasts (with hyphae) and Cy3-labeled Lab158 probe illustrating presence of lactobacilli. The magnification (B) shows the association of lactobacilli (red) and yeasts (green). Bars equal 20 μm (A) and 5 μm (B).

DISCUSSION

In this study biofilm architecture on silicone rubber voice prostheses has been further explored using FISH and CLSM, preserving the intact structure of biofilms. This is the first time, that the complex biofilms on clinically used silicone rubber voice prostheses are visualized using FISH. We have illustrated that FISH in combination with CLSM is a good method that allows detailed analysis of the biofilm architecture. The large variety in biofilm architecture from patient to patient and the concurrent differences in prosthesis lifetimes have been suggested to be due to differences in irradiation dose, volume of irradiated salivary gland tissue, residual salivary flow rate, time passed after irradiation or insertion of the prosthesis, surgical or drug therapy, or

prosthetic tooth replacement[5]. All silicone rubber voice prostheses in this study were colonized with a multi species biofilm. This is not surprising, as the esophagus is an ideal incubator. It has a high humidity, a temperature close to 37°C, and regular provision of nutrients. Moreover, the organisms colonizing the prosthesis are inaccessible by host defense mechanisms and antibiotics.

In the past it has been suggested that especially *C. albicans* deteriorates the silicone rubber, as confirmed in this study, but also other yeast species and groups of bacteria were observed near the ingrowing colonies of yeasts. Interestingly, we were able to detect bacterial strains that have not been identified before, like lactobacilli and *Enterobacteriaceae*. Elving et al.[5] already noticed that 51 out of 92 bacterial isolates from voice prostheses could not be classified with the MicroPlate, Biolog system (Hayward, California) and they described the non-identified bacteria as "generally rod-shaped organisms". It is known that the accuracy of the culturing technique is dependent on the selectivity of the medium. The fact that the medium used was not specific for lactobacilli could be a reason why lactobacilli have not been cultured from silicone rubber voice prostheses before. For the identification and enumeration of lactobacilli, FISH using specific probes is a more direct approach than elaborate culture methods[6]. Increased salivary levels of lactobacilli, but also of *C. albicans* in patients after radiation therapy are quite common[1,21], combined with a reduced salivary pH. It is unclear whether the presence of lactobacilli in voice prosthetic biofilms should be associated with failure of the prostheses or not, but recently the consumption of a fermented dairy product containing a particular strain of probiotic lactobacilli, *Lactobacillus casei* Shirota, has been associated with a prolonged life-time of voice prostheses in patients[17]. A possible general beneficial influence of the presence of lactobacilli might also be inferred from the observation that lactobacilli are predominantly found on prostheses after prolonged use (see Table 3).

EPS is an important characteristic of any biofilm[4] and usually difficult to preserve for scanning electron microscopy, unless special drying procedures are applied. After appropriate staining with Calcofluor white, and in combination with FISH we here use the CLSM to visualize the EPS in voice prosthetic biofilms. EPS is especially present in biofilms with an extended lifetime and previously it was suggested that biofilm integrity is ensured by

EPS[15], therewith contributing to the lifetime of Provox prostheses, which have an intrinsically weaker closing force than Groningen button voice prostheses.

In conclusion, FISH is a method for better visualization of biofims on indwelling medical device and to identify microorganisms difficult to culture. Knowledge of the biofilm architecture can contribute to a better understanding of the symbiosis between yeasts and bacteria in biofilms. Lactobacilli have been identified for the first time in voice prosthetic biofilms, and seem to have an important interaction with yeasts. It would be intriguing to find out what symbiotic or syntrophic interactions take place between these organisms, especially since they can have both positive and negative effects on voice prosthetic biofilms.

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Co-adhesion between Candida and lactobacilli and other commensal bacterial strains on silicone rubber.

4

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INTRODUCTION

Verbal communication in patients after laryngectomy can be restored by placing a silicone rubber voice prosthesis into a surgically created puncture between the trachea and oesophagus. Due to the nonsterile environment, voice prostheses become rapidly colonized by microorganisms, leading to increased airflow resistance or leakage of food and liquid[1], which results in frequent replacements of voice prostheses. *Candida albicans* is regarded as one of the main microbial strains in voice prosthetic biofilms, and seldom exists alone in a biofilm. Next to *C. albicans* species embedded in a biofilm matrix, several other bacterial strains and species have been detected in voice prosthetic biofilms, mainly streptococci, staphylococci and lactobacilli, bacteria that are members of the commensal oral flora[2,3]. Amongst Candida species, *C. albicans* is the most commonly isolated species from the oral cavity and is responsible for most superficial and systemic fungal infections[4].

The important aspect of Candida pathogenicity is the morphogenetic conversions between yeast and filamentous forms of hyphae[5]. The hyphal form is associated with the invasive characteristic of the fungus and hyphae have been demonstrated to growth into silicone rubber when exposed to cycles of feast and famine[6]. In order to colonize a surface, *C. albicans* must first adhere to a biomaterial surface. The initial attachment of *C. albicans* and other microbial strains to biomaterials surfaces is closely followed by cell division, proliferation and biofilm development. A mature biofilm on a prosthesis surface consists of densely packed fungi and bacteria, embedded within an exopolysaccharide matrix[7]. The morphogenetic conversion of *C. albicans* from yeast to hyphae plays a pivotal role in biofilm development. The presence of hyphae in the biofilm is essentially for the biofilm structural integrity and perhaps for material deterioration on voice prostheses[3,8].

It is known that the complex architecture of most biofilms is intrinsically stratified into spatially organized populations of mixed species communities with a high degree of interspecies interaction[9] and there seems to be a phenomenon of coaggregation and coadhesion between Candida and different bacteria on a spatio-temporal basis. The presence of bacteria is especially essential for the integrity of voice prosthetic biofilms[10], and like in the oral

cavity, metabolic cooperation among bacteria may be of importance to the establishment of stable biofilm communities[11]. Interestingly, combination of different bacterial strains, like *Rothia dentocariosa* with *C. albicans* have been associated with a decreased clinical life-time of voice prostheses, while combinations with lactobacilli have been suggested to prevail in prostheses with an elongated life-time[2,3]. It is currently unknown why certain combinations of bacteria with *C. albicans* are more or less harmful than others.

The aim of this study was to investigate the interaction between Candida (*C. albicans* and *C. tropicalis*) and commensal bacteria, especially lactobacilli in biofilm formation on silicone rubber. All strains and species used in this study were originally isolated from biofilms on explanted voice prostheses.

MATERIALS AND METHODS

Silicone rubber

In this study, commercially available silicone rubber tubes were used (Rubber B.V., Hilversum, The Netherlands). Silicone rubber surfaces were characterized by water contact angles measurements, taken at 25°C using the sessile drop (3μl) technique and a homemade contour monitor. This monitor registers the contour of a liquid droplet based on grey value thresholding after which contact angles are calculated from the height and base width of a droplet.

Biofilms

A modified Robbins device was used to grow biofilms in silicone rubber tubes[12]. During an experiment, the tubes were maintained at a temperature between 36°C and 37°C. To grow biofilms, the silicone rubber tubes were inoculated for 5 h with a combination of a bacterial strain and *C. albicans* or *C. tropicalis*. The combination comprised *C. tropicalis* GB 9/9 or *C. albicans* GBJ 13/4A, with *Staphylococcus aureus* GB 2/1, *Staphylococcus epidermidis* GB 9/6, *Streptococcus salivarius* GB 24/9, *Rothia dentocariosa* GBJ 52/2B or a Lactobacillus strain (*Lactobacillus casei* ATCC 393, *Lactobacillus fermentum* ATCC 9338, *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus crispatus* ATCC 33820 or *Lactobacillus johnsonii* ATCC 11506) and was cultured in a mixture of 30% brain heart infusion broth (OXOID, Basingstoke, UK) and 70% defined

yeast medium (per litre: 7.5 g glucose, 3.5 g $(NH_4)_2SO_4$, 1.5 g L-asparagine, 10 mg L-histidine, 20 mg DL-methionine, 20 mg DL-tryptophane, 1 g KH_2PO_4, 500 mg $MgSO_4.7H_2O$, 500 mg NaCl, 500 mg $CaCl_2.2H_2O$, 100 mg yeast extract, 500 μg H_3BO_3, 400 μg $ZnSO_4.7H_2O$, 120 μg Fe(III)Cl_3, 200 μg $Na_2MoO_4.2H_2O$, 100 μg KI, 40 μg $CuSO_4.5H_2O$).

After inoculation, a biofilm was allowed to grow in the silicone rubber tubes during three days, by filling the tubes with growth medium. From day four till day seven, the tubes were perfused three times a day with 250 ml phosphate buffered saline (10 mM potassium phosphate and 150 mM sodium chloride, pH 6.8). Subsequently, the prostheses were left in the moist environment of the artificial throats. At the end of each day, the devices were filled with growth medium during 30 min and left overnight in the moist environment of the drained artificial throats.

On day eight of the experiment, the silicone rubber tubes were removed to assess the number of colony forming yeast and bacteria. To this end, biofilms were removed by scraping and sonication in Reduced Transport Fluid (NaCl 0.9 g/l, (NH4)2SO4 0.9 g/l, KH2PO4 0.45 g/l, Mg2SO4 0.19g/l, K2HPO4 0.45 g/l, EDTA 0.37 g/l, L-Cysteine HCl 0.2 g/l, pH 6.8), after which the resulting suspension was serially diluted and plated on MRS (de Man, Rogosa and Sharpe) agar plates for yeasts and blood agar plates for bacteria. Plates were incubated at 37°C in an aerobic incubator for 3 days prior to enumeration. In case of combinations with lactobacilli, the resulting suspension was plated on MRS plates with incubation in 5% carbon dioxide incubator, while Candida strains were plated on Sabouraud agar plates (incubation at 25/27°C).

All experiments were done in quadruplicate and data were compared using a Student t-test, accepting $p < 0.1$ as statistically significant.

RESULTS

Silicone rubber

Water contact angles of the silicone rubber tubing were slightly larger (110 ± 1 degrees) than on Groningen Ultra Low Resistance voice prostheses (104 ± 1 degrees), included for comparison, but this difference was not considered relevant in terms of the study.

Biofilms

Table 1 summarizes the total numbers of CFU/cm^2 on the silicone rubber tubes.

In general, bacteria in combination with *C. tropicalis* form a more extensive biofilm than combinations with *C. albicans*. Only *C. tropicalis* in combination with *L. johnsonii* formed a biofilm comparable with *C. albicans* with *L. johnsonii* (4.4. x 10^5 and 3.5 x 10^5 CFU/cm^2, respectively).

Yeast strain combined with	*CFU/cm^2*	*C. albicans (%)*	*C. tropicalis (%)*
Rothia dentocariosa	$4.4x10^5 \pm 0.4 \times 10^5$	1.1	
Streptococcus salivarius	$3.5x10^5 \pm 0.3 \times 10^5$	15.7	
Staphylococcus epidermidis	$1.7x10^6 \pm 1.3 \times 10^6$	3.2	
Staphylococcus aureus	$2.4x10^4 \pm 1.2 \times 10^4$	3.4	
Lactobacillus casei	$5.0x10^5 \pm 0.5 \times 10^5$	45.4	
Lactobacillus fermentum	$7.2x10^5 \pm 1.5 \times 10^5$	18.4	
Lactobacillus acidophilus	$1.3x10^5 \pm 0.3 \times 10^5$	74.7	
Lactobacillus crispatus	$9.2x10^4 \pm 1.0 \times 10^4$	36.5	
Lactobacillus johnsonii	$3.5x10^5 \pm 0.5 \times 10^5$	84.4	
Rothia dentocariosa	$5.0x10^6 \pm 0.5 \times 10^6$		0.3
Streptococcus salivarius	$9.0x10^5 \pm 0.6 \times 10^5$		0.6
Staphylococcus epidermidis	$4.7x10^6 \pm 0.3 \times 10^6$		5.4
Staphylococcus aureus	$2.8x10^7 \pm 0.7 \times 10^7$		0.3
Lactobacillus casei	$2.0x10^7 \pm 0.3 \times 10^7$		27.2
Lactobacillus fermentum	$4.9x10^6 \pm 1.0 \times 10^6$		9.5
Lactobacillus acidophilus	$6.2x10^6 \pm 0.1 \times 10^6$		8.9
Lactobacillus crispatus	$1.5x10^6 \pm 0.3 \times 10^6$		34.9
Lactobacillus johnsonii	$4.4x10^5 \pm 0.4 \times 10^5$		65.5

Table 1. Number of CFU/cm^2 (bacteria and yeast) grown on silicone rubber in an eight days time period after inoculation with a combination of a bacterial strain and a *C. albicans* or *C. tropicalis* strain, together with the percentage prevalence of the yeast in the final biofilm.All results are from quadruplicate experiments with separate microbial cultures and are presented ± SD.

C. albicans in combination with lactobacilli resulted in less biofilm formation, especially the combinations with *L. acidophilus* and *L. crispatus*. The most extensive biofilm was formed in the presence of *C. albicans* in combination with *S. epidermidis* (1.7×10^6 CFU cm^2). In the presence of *C. tropicalis* the largest amount of biofilm is formed in combination with *S. aureus* (2.7×10^7 CFU/ cm^2). Results were not significant in combinations with *C. albicans* as well as combinations with *C. tropicalis* ($p > 0.1$). Biofilm growth between the two groups of Candida showed no significance either.

Table 1 also summarizes the percentage prevalence of both Candida strains in biofilms. In general, combinations with lactobacilli result in a higher prevalence of Candida strains in the biofilms than combinations with other bacterial strains. In presence of *L. casei, L. crispatus and L. johnsonii*, both *C. albicans* and *C. tropicalis* comprise even more than 25% of all biofilm organisms. The highest percentage of Candida is found for *C. albicans* in combination with *L. johnsonii* (84%).The presence of *C. albicans* and *C. tropicalis* is low in combination with *R. dentocariosa*, repectively 1.1 and 0.3%.

DISCUSSION

In the present study, growth of dual species biofilms on silicone rubber was studied for combinations of Candida with bacterial strains that are commensals of the oral cavity and the skin, including lactobacillus strains. Recently, it has been described that lactobacilli are always part of the bacterial colonisation on silicone rubber voice prostheses[3], especially in prostheses after extremely long clinical life-times and Lactobacillus interacting with Candida have been suggested to prolong the clinical life-time of voice prostheses[13]. Interestingly, the current study shows that combinations of lactobacillus strains with Candida yield less biofilm, but at the same time an increased prevalence of Candida as compared with combinations involving other bacterial strains. This constitutes a controversy, since Candida have been described as the main causative organism for the deterioration of silicone rubber[1,14,15].

Lactobacilli are innocuous commensals living in close association with the human organism and they make up approximately 1% of the cultivable oral flora[16]. It has also been indicated that lactobacilli may play a crucial role in the maintenance of the micro-ecologic balance in the oral cavity[17]. Dental caries has been described to result from a shift toward increased proportions of acid-producing and acid-tolerating species, such as mutans streptococci and Lactobacilli[18]. In the acid environment caused by lactobacilli, Candida might increase its prevalence. More importantly, the interaction between the bacterium and the fungus has been described to regulate *C. albicans* morphogenesis and therewith its virulence and invasiveness when in the hyphal form. *C. albicans* germination is regulated by multiple signals and signaling pathways, amongst which short chain fatty acids produced by lactobacilli inhibited hyphal formation. In addition, culture supernatants of lactobacilli as well as live lactobacilli also inhibited *C. albicans* morphogenesis. Co-growth of *C. albicans* with lactobacilli had inhibited hyphal formation and invasion by 23-44%[19].

The above observations may explain why the higher prevalence of *C. albicans* in biofilms together with lactobacilli is still beneficial to the *in situ* lifetime of voice prostheses. Likely, it is not the presence of *C. albicans* that is detrimental to the silicone rubber, but its transition to the hyphal form and it

is particularly this transition that appears inhibited by lactobacilli, while it may be stimulated by other bacterial species. Also it has been demonstrated that a fermented milk drink containing *L. casei* Shirota reduced biofilm formation on silicone rubber voice prostheses *in vitro* and *in vivo*. Daily consumption of the fermented milk drink (with *L. casei* Shirota) increased the lifetimes of Provox®2 prostheses in laryngectomized patients and reduced the number of prosthesis replacements[13]. We suggest that the deterioration of silicone rubber proceeds according to the scheme presented in Figure 1, while lactobacilli will delay the morphogenetic conversion of *C. albicans* to the hyphal growth from making the biofilm as a whole less invasive.

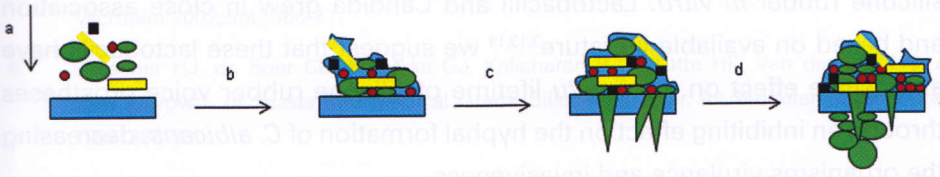

Figure 1. Schematic model of the sequence of events that lead to invasive growth of multi-species biofilms into silicone rubber.
(a) planktonic Candida and bacteria in suspension,
(b) microbial adhesion to a surface e.g. silicone rubber,
(c) proliferation of Candida in a multi species biofilm embedded in a self-produced exopolysaccharide matrix, with morphological changes resulting in hyphal formation and deterioration of silicone rubber, depending on the bacterial strain present
(d) morphogenic transition of hyphae back to the yeast form after invasion.
(adapted from Ten Cate et al., 2009)

It is reasonable to believe that in a later stadium, when the biofilm is more mature, Candida will make a transformation back from hyphae into ingrowing bags and combs of yeasts, to be more harmful to the silicone rubber resulting in dysfunctioning of the voice prostheses. The influence of other bacterial strains on the growth of Candida seems to be less distinct in this study and little or nothing is known on how these strains affect Candida morphogenesis, although coagreggation of *C. albicans* with oral streptococci has been extensively analyzed in the human oral cavity. In the presence of salivary components *C. albicans* attaches to oral streptococci and colonizes the oral cavity[17] and there are bacterial strains known to modulate *C. albicans*

biofilm formation in the mixed environments like the oral cavity[20]. Elving *et al.*[2] observed that the bacterial strain *R. dentocariosa* was especially found on voice prostheses with an implantation period of less than 9 months. In the extended lifetime group (>9 months) *R. dentocariosa* was found with a fourfold lower isolation frequency and *C albicans* was found with a twofold lower isolation frequency. They concluded that this bacterium plays a significant role in the early failure of Groningen silicone rubber voice prostheses. This suggests that *R. dentocariosa* may have a stimulating influence on the hyphal formation by *C. albicans*, but this remains to be confirmed by experiments

In summary, it has been indicated that different commensal bacterial strains and especially lactobacillus strains influence the biofilm formation on silicone rubber *in vitro*. Lactobacilli and Candida grew in close association and based on available literature[3,13,19] we suggest that these lactobacilli have an positive effect on the *in situ* lifetime of silicone rubber voice prostheses through an inhibiting effect on the hyphal formation of *C. albicans*, decreasing the organisms virulence and invasiveness.

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Effects of Quaternary Ammonium Silane Coatings on Mixed Fungal and Bacterial, Tracheoesophageal Shunt Prosthetic Biofilms

5

Oosterhof J.J.H., Buijssen K.J.D.A., Busscher H.J., van der Laan B.F.A.M., van der Mei H.C. (2006), Appl environ microbial. 72:3673-3677.

INTRODUCTION

Biofilm formation is the leading cause for the failure of biomedical prostheses[9,14], including tracheoesophageal shunt prostheses, used for speech rehabilitation in patients after total laryngectomy because of a malignant laryngeal tumor. Tracheoesophageal shunt prostheses are made of a silicone rubber tube capped on one end with a one-way valve and are placed between the esophagus and the trachea. The valve of the prosthesis constitutes its esophageal side and the one-way mechanism allows air to pass from the tracheal side, but fluids passing the esophagus are blocked from entering the trachea. Microorganisms readily form a biofilm on the esophageal side of a prosthesis, which leads to dysfunctioning of the valve and induces leakage of fluids in the trachea or increased air-flow resistance during speech[2,16]. Consequently, the useful lifetime of a voice prosthesis varies between 3-6 months[10,11,16].

Tracheoesophageal shunt prosthetic biofilms contain a mixture of yeast and bacteria, including *Candida* species, *Staphylococcus* and *Streptococcus* species, as well as *Rothia dentocariosa*. Especially *C. albicans*, *C. tropicalis* and *R. dentocariosa* are known to reduce the lifetime of tracheoesophageal shunt prostheses in vivo[5]. Both antibiotics and or antimycotics have been administered to patients in order to eradicate these biofilms[19], but the biofilm offers effective protection against antimicrobials, to which planktonic organisms are usually susceptible[3,7]. Therefore, preventive measures seem a better way to deal with these biofilms. Gottenbos et al.[8] described the antibacterial properties of a positively charged, 3-(trimethoxysilyl)-propyldimethyloctadecylammonium chloride (QAS), coating on silicone rubber against a variety of different bacterial strains in vitro, and moreover demonstrated effectiveness of QAS-coatings against a *S. aureus* biofilm in vivo. Similarly Biocidal ZF, a commercially available disinfectant, containing quaternary ammonium compounds as active ingredients, is used for coating incubators and sterile cabinets to protect cell cultures from microbial contamination. Neither the QAS-coating nor Biocidal ZF has ever been investigated for their efficacy against mixed fungal and bacterial biofilms.

The aim of this study was to evaluate the inhibitory effect of QAS- and Biocidal ZF-coatings against the development of a mixed fungal and bacterial

biofilm on silicone rubber tracheoesophageal shunt prostheses in vitro, in order to develop new strategies for the prevention of microbial colonization of silicone rubber voice prostheses.

MATERIALS AND METHODS

Tracheoesophageal shunt prostheses. "Ultra Low Resistance" silicone rubber Groningen button tracheoesophageal shunt prostheses were supplied by Médin Instruments and Supplies (Groningen, The Netherlands). The "Ultra Low Resistance" Groningen button tracheoesophageal shunt prosthesis consists of a shaft with two flanges with a semicircular slit of 210° in the hat of the esophageal flange, functioning as a one-way valve. The prosthesis is made of implant grade silicone rubber.

Silanization and surface characterization. The tracheoesophageal shunt prostheses were cleaned in a 2% RBS 35 detergent solution (Omniclean, Breda, The Netherlands) under simultaneous sonication and thoroughly rinsed in demineralized water, sterilized in 70% ethanol, washed with sterile Millipore-Q water and dried overnight at 80°C under sterile conditions. For coating with Biocidal ZF, tracheoesophageal shunt prostheses were sprayed twice with Biocidal ZF (WAK-Chemie Medical GmbH, Germany), fully covering the valve with the Biocidal, followed by exposure to ambient air and drying for 20 h, under sterile conditions. For QAS-coating[8], prostheses were oxidized in a glow-discharge reactor (a DC modified Edwards sputter coater S150B) through an argon plasma treatment, done under 5 mbar argon pressure, at a power of 7 W for 5 min, followed by exposure to ambient air. Subsequently, each oxidized voice prosthesis was immediately immersed in 0.5 % QAS (Dow Corning Corporation, USA) in Millipore water. Coated tracheoesophageal shunt prostheses were allowed to react and dry at 80°C for 20 h[8], under sterile conditions. Sheets of silicone rubber (SR) (25 x 76 mm) were similarly treated for surface characterization.

For surface characterization, QAS- and Biocidal-coated SR was washed for 30 min in PBS followed by rinsing with demineralized water. The chemical composition of SR, QAS-and Biocidal-coated SR surfaces were determined by X-ray photoelectron spectroscopy (XPS) using an S-Probe spectrometer

(Surface Science Instruments, Mountain View CA, USA) at a spot size of 250 ´ 1000 mm and X-rays were produced using an aluminum anode. A scan of the overall spectrum in the binding energy range of 1-1200 eV at low resolution (pass energy 150 V) was recorded, followed by scans over a 20 eV binding energy range at high resolution (pass energy 50 eV) for C_{1s}, O_{1s}, N_{1s}, Si_{2p} and Cl_{2p}. The area under the peak, after linear background subtraction, was used to calculate the peak intensities after correction with sensitivity factors provided by the manufacturer. The elemental surface compositions were expressed in atomic % setting %C + %O + %N + %Si + %Cl to 100%.

Zeta potentials of the surfaces were derived from the pressure dependence of the streaming potentials employing a parallel plate flow chamber of which the top and bottom plate were constituted by SR, QAS- or Biocidal-coated SR sheets fixed on Perspex plates (25 x 76 mm), separated by an 0.2 mm Teflon gasket. Two rectangular platinum electrodes (5.0 x 25.0 mm) were located at both ends of a parallel plate flow chamber[18]. Streaming potentials were measured during 1 h in PBS (10 mM potassium phosphate and 150 mM NaCl, pH 7.0), at ten different pressures ranging from 37.5 to 150 Torr and each pressure was applied for 10 s in both directions.

Advancing type water contact angles were measured at room temperature with a home-made contour monitor using the sessile drop technique.

Determination of in vitro cytotoxicity (ISO 10993-5). In order to ensure that potential future applications of these coatings would later on not be impeded because of cytotoxicity, we performed an in vitro cytotoxicity test, in which the biological reactivity of a mammalian monolayer, L929 mouse fibroblast cell culture, in response to a QAS coated silicone rubber was determined. Silicone rubber sheets, made from exactly the same silicone rubber as used for the voice prosthesis, were used in stead of voice prostheses to create a larger surface area. Extracts were prepared at 37°C for 24 h by using 12.1 ml minimum essential medium supplemented with serum (MEM complete) for 72.5 cm^2 QAS coated silicone rubber. Positive (natural rubber) and negative (bare silicone rubber) controls were prepared to verify the proper functioning of the test system. The maintenance medium on the cell cultures was replaced by the filter sterilized extracts from the QAS coated silicone rubber or controls in triplicate and the

cultures were subsequently incubated for 48 h at 37°C in a humidified atmosphere containing 5% carbon dioxide. Biological reactivity was rated on the following scale: grade 0 (no reactivity), grade 1 (slight reactivity), grade 2 (mild reactivity), grade 3 (moderate reactivity and grade 4 (severe reactivity). QAS coated silicone rubber may be considered non-cytotoxic if none of the cultures exposed show greater than mild reactivity. The toxicity measurements were performed at a notified test institute (TOXICON Europe NV, Leuven, Belgium).

Biofilm formation. A modified Robbins device made of stainless steel was used as an artificial throat (see Fig.1) to grow biofilms[15]. Each artificial throat was equipped with three Groningen Ultra Low Resistance tracheoesophageal shunt prostheses an uncoated, a QAS-coated and a Biocidal-coated prosthesis. During the experiment, the artificial throat was maintained at a temperature between 36°C and 37°C, as in a laryngectomized patient.

To grow tracheoesophageal shunt prosthetic biofilms as found in laryngectomized patients, artificial throats were inoculated for 5 h with a combination of bacteria and yeasts, previously isolated from explanted Groningen tracheoesophageal shunt prostheses. This combination comprised *Candida tropicalis* GB 9/9, *Candida albicans* GBJ 13/4A, *Staphylococcus aureus* GB 2/1, *Staphylococcus epidermidis* GB 9/6, *Streptococcus salivarius*

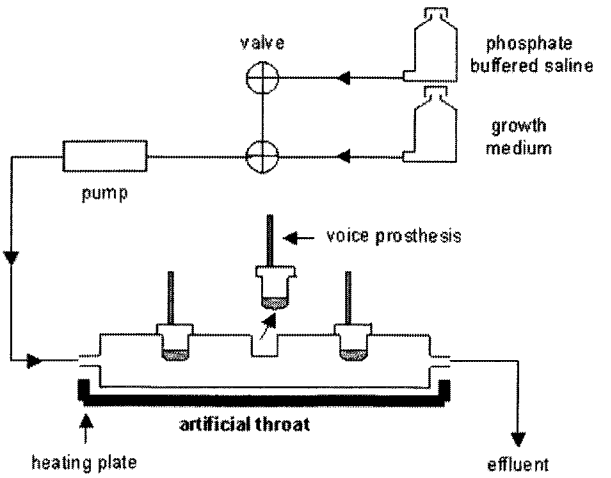

Figure 1. Schematic presentation of the modified Robbins device, used as an artificial throat, and equipped with three Groningen voice prostheses.

GB 24/9 and *Rothia dentocariosa* GBJ 52/2B and was cultured in a mixture of 30% brain heart infusion broth (OXOID, Basingstoke, Great Britain) and 70% defined yeast medium (per liter: 7.5 g glucose, 3.5 g $(NH_4)_2SO_4$, 1.5 g L-asparagine, 10 mg L-histidine, 20 mg DL-methionine, 20 mg DL-tryptophane, 1 g KH_2PO_4, 500 mg $MgSO_4.7H_2O$, 500 mg NaCl, 500 mg $CaCl_2.2H_2O$, 100 mg yeast extract, 500 μg H_3BO_3, 400 μg $ZnSO_4.7H_2O$, 120 μg Fe(III)Cl_3, 200 μg $Na_2MoO_4.2H_2O$, 100 μg KI, 40 μg $CuSO_4.5H_2O$). After inoculation, a biofilm was allowed to grow on the tracheoesophageal shunt prostheses during three days, by filling the devices with growth medium. From day four till day seven, the artificial throats were perfused three times a day with 250 ml PBS. After each perfusion the prostheses were blown through, with compressed air at three different pressures (10, 15 and 20 cm H_2O), to mimic shunt esophageal speech and to mobilize the valve system.

Subsequently, the prostheses were left in the moist environment of the artificial throats. At the end of each day, the devices were filled with growth medium during 30 min and left overnight in the moist environment of the drained artificial throats. The tracheal sides of the prostheses were left in ambient air, similar to the situation with a stoma. Previously, this cycle of feast and famine and exposure to ambient air has been demonstrated essential to grow biofilms with features that cannot be distinguished from in vivo biodeterioration seen on explanted prostheses[15].

Evaluation of biofilms. On day eight of an experiment, tracheoesophageal shunt prostheses were removed from the artificial throats. Biofilm formation on the valve side of the prosthesis was assessed by determining the number of colony forming yeast and bacteria (CFUs). To this end, biofilms were removed by scraping and sonication and subsequently serially diluted. After plating the serial dilution on MRS (de Man, Rogosa and Sharpe) agar plates for yeasts and blood agar plates for bacteria, plates were incubated at 37°C in an aerobic incubator for 3 days prior to enumeration. The number of bacterial and yeast colony forming units on the esophageal surface of each prosthesis was determined separately and expressed as a percentage with respect to the control. The consistency of the biofilm formation in each run was secured by comparison with the control throat.

Two artificial throats were used for imaging biofilm formation on the valve side of the prostheses with Confocal Laser Scanning Microscopy (CLSM). Voice prostheses of one artificial throat were visualized after Fluorescence in Situ Hybridization (FISH) with rRNA-targeted oligonucleotide probes. FISH was performed using a modification of previously described protocols[1,6,13]. After removal from the artificial throat the tracheoesophageal shunt prostheses underwent the following preparation steps: conservation for 24 h in sterile PBS, fixation for 24 h in a 4%-paraformaldehyde solution at 4°C and conservation for at least 24 h in an ethanol/PBS (1:1) solution. After those preparation steps the valves of the prostheses were cut into small cross-sections and attached on glass slides. The fixed samples were hybridized (in a closed moist chamber by 50°C) in a volume of 200 μl pre-warmed (50°C) hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl, pH 7.2, and 0.01% SDS) mixed with 10 μl, 100 ng/μl rhodamine labeled EUB-338 probe (5'-GCTGCCTCCCGTAGGAGT-3') for detecting bacteria and 10 μl, 100 ng/μl fluorescein-isothyocyanate (FITC) labeled EUK-516 probe ('5-ACCAGACTTGCCCTCC-3') for visualization of the yeasts[1]. After 17-19 h the slides were washed in a pre-warmed (50°C) washing buffer for 15 min to remove unbound probes, rinsed with sterile Millipore water, carefully dried with tissues, mounted in Vectashield® medium for fluorescence (Vector Laboratories, Inc. Burlingame CA 94010) and covered with a coverslip. The confocal images were obtained using a 20x oil immersion objective of a CLSM model LEICA TCS SP2 (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany).

Samples from the second artificial throat intended for imaging were subjected to live/dead *bac*light® staining kits for yeasts and bacteria. Directly after removing the prostheses from the artificial throat, prostheses were stained for 15 min in the dark at room temperature with both the live/dead viability stain containing SYTO 9 dye (3.34 mM) and propidium-iodide (20 mM) and the live/dead Yeast viability kit containing FUN-1 cell stain (10 μM) and Calcofluor White M2R staining (25 μM), but in our experience the two-color fluorescent probe, FUN-1, sufficed for determining yeast viability. Series of about 20 images were made of each biofilm on the prostheses using a 20x water immersion objective and stacked into overlay projections.

Statistical analysis. All experiments in the artificial throats were done quadruple and the quantitative data were statistically compared with respect to the control. A Wilcoxon signed rank test was used for the statistical analysis, accepting $P < 0.05$ as statistically significant.

RESULTS

The surface characteristics of the silicone rubber prior to and after QAS- and Biocidal-coating are summarized in Table 1. The presence of a QAS-coating increases %N and %Cl relative to the uncoated SR, whereas the presence of the Biocidal-coating is not evident from the XPS data, likely because its layer thickness is too thin for detection by XPS. Water contact angles are similar on QAS-coated (100 degrees) and uncoated SR (108 degrees), but the Biocidal-coating creates a more hydrophilic SR surface (40 degrees). Most importantly, the zeta potential of SR, authentically negative, becomes positive after QAS- and Biocidal-coating (16 and 29 mV, respectively). The Biocidal-coating however, becomes negatively charged within one hour whereas the QAS coating stays positively charged.

Surface property	Untreated	QAS-coated SR	Biocidal-coated SR
%C	49	63	49
%O	26	19	25
%Si	25	14	26
%N	0	2.6	0
%Cl	0	2.3	0
Equilibrium water contact angle	108	100	40
Zeta potential	- 15 mV	+ 16 mV	+29 mV

Table 1. Chemical surface composition, equilibrium water contact angles (degrees) and zeta potentials (mV) in PBS of untreated silicone rubber, Quaternary Ammonium Silanized silicone rubber (QAS-coated SR) and Biocidal-coated SR.

The cytotoxicity of the QAS coated silicone rubber was tested and mild biological reactivity (Grade 2) was observed in the L929 mammalian cells at 48 h, post exposure. The observed cellular response obtained from the positive control extract (Grade 3) and the negative control extract (Grade 0) confirmed the suitability of the test system. The QAS coated silicone rubber can therefore be considered non-cytotoxic.

The percentages of viable yeast and bacteria harvested from QAS- and Biocidal- coated SR are shown in Table 2. Significantly ($p < 0.05$) less viable bacteria and yeast are harvested from the QAS coating than from authentic silicone rubber prostheses, while the Biocidal-coating shows a reduction as well, that is however not significant. These numbers are confirmed qualitatively in Fig. 2 showing CLSM images of the prostheses surfaces after live/dead staining. Note some hyphae on the Biocidal-coating are observed, which are absent on the QAS coated prosthesis.

Table 2. Decrease in percentage of viable bacteria and yeast isolated from the tracheoesophageal shunt prostheses coated with QAS or Biocidal with respect to untreated prostheses, as obtained in four independent experiments (± SD). Both for bacteria and yeasts, the number of organisms of the untreated prostheses was set at 100%.

Coatings	Percentage of bacteria[a]	Percentage of yeast[a]	Total microorganisms (CFU/cm^2)
Untreated	100[a]	100[a]	$2.5\ 10^6 \pm 1.5\ 10^6$
QAS	$36^* \pm 16$	$12^* \pm 9$	$0.8\ 10^{6*} \pm 0.3\ 10^6$
Biocidal	27 ± 32	16 ± 15	$0.6\ 10^{6*} \pm 0.7\ 10^6$

[a] = the number of viable bacterial and yeast colony forming units on untreated silicone rubber prostheses amounted respectively 2.1×10^6 and 3.8×10^5 per cm^2 on the esophageal side of the Low Resistance Groningen Button tracheoesophageal shunt prostheses; * = significantly different from untreated prostheses (Wilcoxon signed rank test, $p < 0.05$) from the control.

Figure 3 shows CLSM images of cross-sections of biofilms on the valve of the silicone rubber tracheoesophageal shunt prostheses prior to and after coating. As can be seen, the biofilm on the untreated prosthesis is thicker than on the coated ones. The high magnifications for the control and Biocidal coating show ingrowth of hyphae into the silicone rubber. No hyphae of yeasts in the biofilm on the QAS-coated surface have been observed.

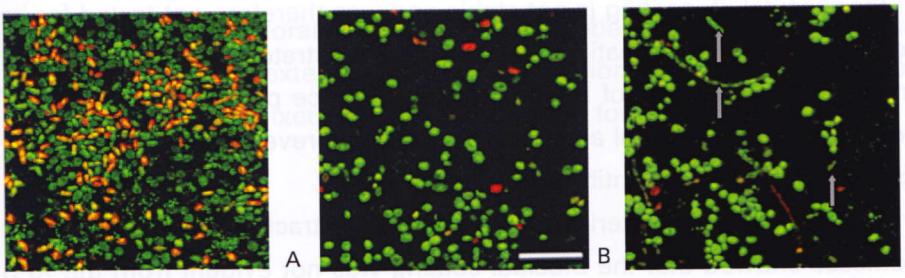

Figure 2. Confocal Laser Scanning Microscopy images (CLSM) of surfaces of Groningen button tracheoesophageal shunt prostheses, after live/dead staining for yeasts and bacteria. The arrows are pointing to hyphae. Bar denotes 40 μm. A) Untreated prosthesis; B) Quatenary Ammonium Silanized Silicone Rubber (QAS) coating; C) Biocidal coating.

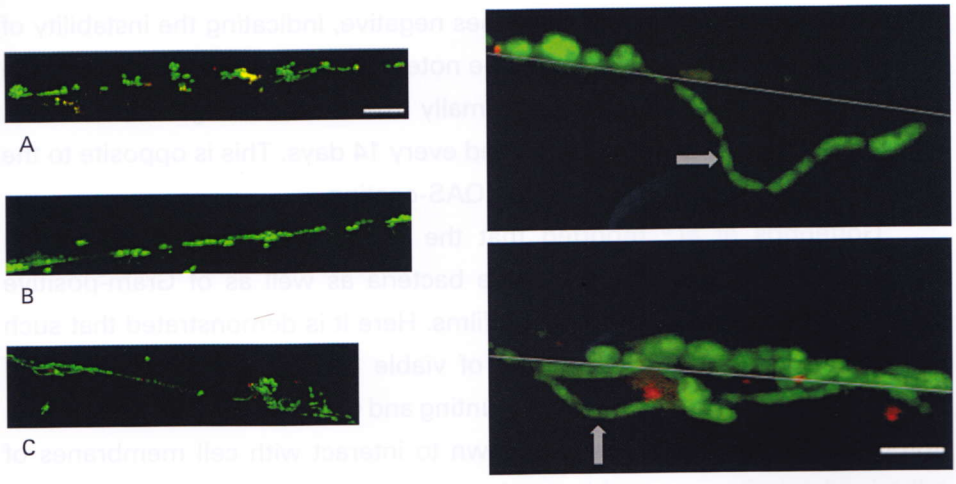

Figure 3. Confocal Laser Scanning Microscopy images (CLSM) of cross-sections of tracheoesophageal shunt prosthetic bioflims after in situ hybridization with fluorescence-labelled oligonucleotide probes, making bacterial cells appear red and yeast appear green. The arrows in the magnifications are pointing to hyphae. Bar denotes 40 μm for the overview and 10 μm for the magnification. A) Untreated prosthesis; B) Quatenary Ammonium Silanized Silicone Rubber (QAS) coating; C) Biocidal coating.

DISCUSSION

In this study silicone rubber tracheoesophageal shunt prostheses were coated with QAS- and Biocidal ZF-coatings to evaluate their inhibitory effect against the development of a mixed fungal and bacterial biofilm on these prostheses. QAS-coatings turned out to be stable coatings that were not cytotoxic in a first evaluation due to the stable bound state of the QAS-molecules (note that the Biocidal ZF-coating is not stable and was therefore not tested for its cytotoxicty). Thus QAS-coatings constitute a new strategy for the prevention of microbial colonization of silicone rubber of voice prostheses but also of medical devices in general and can be helpful in prevention of resistance of microorganisms against antibiotics or antimycotics.

The surface characteristics of the coated tracheoesophageal shunt prostheses showed that the Biocidal-coating was not evident from the XPS data, probably because the Biocidal-coating is thinner than the depth of information of XPS (3-5 nm). In contrast, water contact angles and the zeta potential, both measuring on the outer surface layer with an information depth of several, clearly demonstrated the presence of the coating. The zeta potential of the Biocidal-coating quickly becomes negative, indicating the instability of the coating. In this respect it should be noted that the commercially available antimicrobial fluid, Biocidal ZF, is normally used as a coating for incubators, which have to be cleaned and recoated every 14 days. This is opposite to the chemical bonding established for the QAS-coating

Gottenbos et al.[8] reported that the positively charged QAS-coating affects the viability of Gram-negative bacteria as well as of Gram-positive bacteria in single strain, bacterial biofilms. Here it is demonstrated that such a coating reduces also the number of viable bacteria and yeast in mixed biofilms, as demonstrated by plate counting and CLSM after live/dead staining. Immobilized QAS molecules are known to interact with cell membranes of adhering bacteria, presumably causing membrane leakage and cell death[8,12]. The mechanisms of action of QAS causing death in yeast is not known, but it seems to impede the formation of hyphae (see Fig. 3). Alternatively, little is also known about the influence of the bacterial presence on the expression of hyphae in yeast. Consequently, the absence of hyphae could either be a direct

effect of the coating or an indirect effect caused by the absence of bacteria on QAS-coated surfaces.

This study demonstrates for the first time that the viability of both yeast and bacteria in mixed biofilms is affected by positively charged QAS-coatings on silicone rubber. Since a QAS-coating is non-toxic, clinical application could increase the useful lifetime of tracheoesophageal shunt prostheses by decreasing biofilm formation in vivo, as ingrowth of yeasts is mainly held responsible for deterioration of the silicone rubber in vivo[4]. The relevance of the current findings extends, however, to all biomedical and environmental applications where mixed biofilms develop and form a problem.

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In vitro biofilm formation on smooth indwelling voice prostheses and their clinical lifetime in laryngectomized patients.

6

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Submitted.

INTRODUCTION

In 1955, the first silicone rubber product for medical application, a hydrocephalus shunt, was made[1]. A wide range of medical devices followed through time, among which catheters, breast implants, contact lenses, and cochlear implants[2-5]. The widespread use of silicone rubber is due to its inactivity to the immune system, good sterilization opportunities, anti-adhesive and stable properties during long term residence in human body and minimum negative tissue response[6]. Another positive characteristic is its ease of processing into different shapes by moulding. This became even easier when liquid silicone rubber was introduced in 1979. Liquid silicone rubber has the low viscous appearance of the raw material and can be moulded in any shape before curing. Curing takes several days at room temperature (20°C), but at temperatures above 140°C the reaction is completed within a few seconds[7]. The process of turning liquid silicone rubber into an end product involves filling, packing/holding and cooling of the mould. During the first stage, the hot polymer melt rapidly fills a cold mould, representing a cavity of the desired product shape. During the packing/holding stage, the pressure is raised and extra material is forced into the mould to compensate for shrinkage due to temperature decreases and the development of crystallinity during solidification. The cooling stage starts the solidification of the final product and when the solid layer on the mould surface reaches a thickness sufficient to assure the required rigidity, the product is ejected from the mould[8].

Silicone rubber has a hydrophobicity in the so-called bio-abhesive range, which constitutes a reason for its widespread use as a biomaterial, because infection due to biofilms is common in the clinical application of biomaterial implants, and almost inevitably leads to removal of the implant[9-11].

Over the past decades, silicone rubber has turned out to be the biomaterial of choice for the preparation of voice prostheses in laryngectomized patients. In essence, a voice prosthesis is a one-way valve connecting the trachea and the oesophagus. The clinical lifetime of voice prostheses is limited by biofilm formation, despite the fact that they are made out of bio-abhesive silicone rubber. Especially the functioning of the valve, positioned in the unsterile environment of the oesophagus, can be severely hampered by rapid biofilm

formation. Malfunctioning of the valve causes voice prostheses to fail within an average of 3-6 months[12-14]. The biofilm on voice prostheses consists of a mixed biofilm of bacteria and fungi. The variety in oropharyngeal microflora between different patients results in different mixtures of bacteria and fungi in voice prosthetic biofilms, and probably relates to the large differences in in vivo lifetimes between prostheses of different patients. Elving et al. identified a group of microorganisms which were significantly more present in biofilms of prostheses that failed within 4 months compared to prostheses failing after 9 months from the time of insertion[15]. This group of microorganisms (*Candida tropicalis*, *Candida albicans*, *Rothia dentocariosa*) supplemented with *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus salivarius* are comprised in our *in vitro* model to grow biofilm on the voice prostheses[16].

The roughness of silicone rubber is a second important property next to hydrophobicity influencing biofilm formation and is determined largely by the mould and the viscosity of the silicone rubber. Studies in the human oral cavity have demonstrated that roughness is more important for biofilm formation under conditions of fluctuating shear, like in the supra-gingival area than in region of low and more constant shear, like sub-gingivally on implant surfaces[17]. In addition, the roughness of acrylic resin and silicone rubber has been shown to affect biofilm formation by oral bacterial and fungal strains[18]. Consequently, it can be expected that the roughness of silicone rubber will also have a major influence on voice prosthetic biofilm formation.

The primary aim of this study is to produce a voice prosthesis, based on the regular Groningen prosthesis, with decreased surface roughness using liquid silicone rubber and smoother moulding and to compare in vitro biofilm formation on thus prepared smooth and regular voice prostheses. Secondly, the clinical lifetime of smooth prostheses is determined in a selected group of laryngectomized patients, requiring frequent replacement of their voice prosthesis.

MATERIALS AND METHODS

Voice prostheses

Commercially available "Ultra Low Resistance" (ULR) silicone rubber Groningen voice prostheses were supplied by Médin Instruments and Supplies (Groningen, The Netherlands) and later by Atos Medical BV (Zoetermeer, The Netherlands). The ULR Groningen voice prosthesis consists of a shaft with two flanges with a semicircular slit of 210 degrees in the hat of the oesophageal flange, functioning as a one-way valve. The prosthesis is made of implant grade silicone rubber MED-4750 NuSil Technology (Carpinteria, USA). It is a two-part, high tear strength silicone elastomer, that consists of dimethyl and methylvenyl siloxane copolymers and reinforcing silica. To process prostheses using MED-4750, Part B is softened first on a cooled two-roll mill, and then Part A is softened. An equal portion by weight of softened Part B is added to Part A, and next these components are cross-blended until thoroughly mixed. The temperature of the blended material is kept as low as possible to give maximum table life. The curing process is done at 116°C for 10 min[19].

In addition to the commercially available prostheses, a smoother ULR variant was made by a simple injection moulding technique utilizing a single cavity hardened steel mould. To produce a smooth silicone rubber surface, an extreme level of polishing was applied to the relevant mould surfaces. The highly smooth finish was achieved by polishing using pure diamond particles of varying sizes from 45 μm to 1 μm to create a final surface roughness R_a between 0.05-0.2 μm. The silicone rubber MED-4850 (NuSil Technology, Carpinteria, USA) applied here is a two part translucent silicone system, with a viscous liquid physical state[20]. To produce the prostheses, the materials were mixed in a 1:1 ratio in a vacuum mixer before being loaded into a 10 ml syringe. The mould surfaces were sprayed with MACSIL silicone release agent (Polymed limited, Cardiff, UK) and the three parts of the mould were tightly bolted together. The MED-4850 was then injected through the injection port and the mould was placed in a dry oven at 100°C for 2 h to ensure full curing. After curing, the prostheses were left in dry air for cooling.

Surface characterization

Elemental surface compositions of the silicone rubber surfaces of the commercially available and newly made, smooth voice prostheses were determined with X-ray photoelectron spectroscopy (XPS). The XPS used was an S-Probe spectrometer (Surface Science Instruments, Mountain View CA, USA) with a spot size of 250 × 1000 μm and monochromatic X-rays were produced using an aluminium anode. A scan of the overall spectrum in the binding energy range of 1-1100 eV at low resolution (pass energy 150 V) was recorded for the measurement of elemental surface concentrations.

The roughness of the silicone rubber surfaces of the commercially available and the newly made prostheses were measured using Atomic Force Microscopy (AFM). The AFM was a Nanoscope III Dimension^{tm} 3100 Digital Instruments, (Santa Barbara, CA, USA) operated in the contact mode using a Si_3N_4 cantilever tip with a spring constant of 0.06 Nm^{-1}. The valves of the voice prostheses with their concave sides up were put below the cantilever of the AFM to obtain height images in three dimensions at six places per sample, from which its mean roughness (R_a) was calculated. R_a indicates the average distance of the roughness profile to the centre plane of the profile.

Water contact angles were measured at room temperature using the sessile drop technique. Droplets were placed with a syringe and (1-1.5 μl) and resulting contact angles were calculated from droplet profiles determined with a home-made contour monitor. Five droplets were placed over the surface of three different voice prostheses.

Biofilm formation

A modified Robbins device made of stainless steel was used as an artificial throat (Figure 1) to grow biofilms on silicone rubber voice prostheses[16]. Each artificial throat was equipped with original Groningen ULR voice prostheses and the newly produced, smooth voice prostheses in order to evaluate both in the same artificial throat. During an experiment, the artificial throat was maintained at a temperature between 36°C and 37°C, as in a laryngectomized patient.

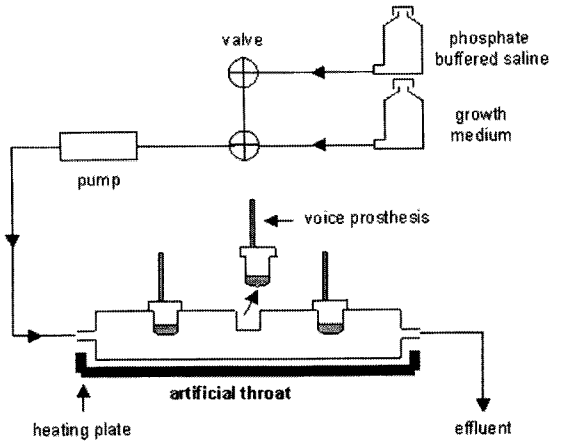

Figure 1. Schematic presentation of the modified Robbins device, used as an artificial throat, and equipped with three Groningen voice prostheses.

To grow voice prosthetic biofilms as found in laryngectomized patients artificial throats were inoculated for 5 h with a combination of bacteria and yeasts, previously isolated from explanted Groningen voice prostheses. This combination comprised *C. tropicalis* GB 9/9, *C. albicans* GBJ 13/4A, *S. aureus* GB 2/1, *S. epidermidis* GB 9/6, *S. salivarius* GB 24/9 and *R. dentocariosa* GBJ 52/2B and was cultured in a mixture of 30% brain heart infusion broth (OXOID, Basingstoke, UK) and 70% defined yeast medium (per litre: 7.5 g glucose, 3.5 g $(NH_4)_2SO_4$, 1.5 g L-asparagine, 10 mg L-histidine, 20 mg DL-methionine, 20 mg DL-tryptophane, 1 g KH_2PO_4, 500 mg $MgSO_4.7H_2O$, 500 mg NaCl, 500 mg $CaCl_2.2H_2O$, 100 mg yeast extract, 500 μg H_3BO_3, 400 μg $ZnSO_4.7H_2O$, 120 μg Fe(III)Cl_3, 200 μg $Na_2MoO_4.2H_2O$, 100 μg KI, 40 μg $CuSO_4.5H_2O$). After inoculation, a biofilm was allowed to grow on the voice prostheses during three days, by filling the devices with growth medium. From day four till day seven, the artificial throats were perfused three times a day with 250 ml phosphate buffered saline (10 mM potassium phosphate and 150 mM sodium chloride, pH 6.8). Subsequently, the prostheses were left in the moist environment of the artificial throats. At the end of each day, the devices were filled with growth medium during 30 min and left overnight in the moist environment of the drained artificial throats. The tracheal sides of the prostheses were left in ambient air, similar to the situation with a stoma.

On day eight of the experiment, voice prostheses were removed from the artificial throats to assess the number of colony forming yeast and bacteria on the valve side of the prostheses. To this end, biofilms were removed by scraping and sonication and subsequently serially diluted. After plating, the serial dilutions on MRS (de Man, Rogosa and Sharpe) agar plates for yeasts and blood agar plates for bacteria, plates were incubated at 37°C in an aerobic incubator for 3 days prior to enumeration. In each experimental run, original and newly made silicone rubber prostheses were inserted. The number of bacterial and yeast colony forming units on the oesophageal surfaces of the newly made prostheses was determined and expressed as a percentage of the mean number of bacterial and yeast colony forming units of the original prostheses.

The experiments in the artificial throats were done in quadruplicate and data for both types of prostheses were compared using a Wilcoxon signed rank test and accepting $p < 0.1$ as statistically significant.

Clinical lifetime

Patients with a Groningen ULR voice prosthesis or Provox®2 voice prosthesis having received at least 3 replacements in the past 6 months were selected. During the course of the study however, two patients had to be excluded. One patient requested a replacement with no clinical signs of leakage or increased airflow resistance. Another patient developed an infection of the tracheoesophageal fistula with the necessity to remove the voice prosthesis. Finally, eleven male patients with a mean age of 66 years (range, 44-87 years) completed the clinical evaluation.

The mean in situ lifetime of the voice prostheses used in the 6 months before entering this study was calculated for each patient and served as an individual, historic control. After entering the study patients, received a newly prepared, smooth voice prosthesis. Also when a replacement was necessary during the experimental period of 6 months, this was done with a smooth prosthesis. Replacement was carried out because of clinically observable leakage through the prosthesis or because of an increased airflow resistance impeding speech. The study ended after 6 months and was performed according to the rules approved and set out by the hospital's ethical review

board (approval reference METc 2004.029, University Medical Center Groningen).

For each patient, the smooth prosthesis lifetime was expressed as a normalized lifetime. Normalized lifetimes were defined as the ratio between the smooth prosthesis lifetime and the average lifetime of the prostheses used by a patient during 6 months preceding the study, i.e. the historic control.

RESULTS

The physico-chemical surface characteristics of the original and smooth voice prostheses are summarized in Table 1. The elemental surface compositions and water contact angles are nearly identical, while the main difference between both prostheses is in the surface roughness. The mean surface roughness of the newly made prosthesis amounts 8 nm, which is significantly ($p < 0.05$) smoother than of the original prosthesis, possessing a roughness of 46 nm.

Surface property	%C	%O	%Si	Water contact angle (degrees)	Surface roughness (nm)
Original	48	26	26	108	46
Smooth	48	27	25	112	8

Table 1. Elemental surface composition, water contact angles and roughness of original and newly made, smooth silicone rubber voice prostheses.

The percentages of viable yeast and bacteria harvested from original and smooth voice prostheses with respect to the mean numbers found on the original prostheses, set at 100%, are summarized in Table 2. Both bacterial and yeast prevalence are reduced by about 40% on smooth prostheses with respect to the original one.

Table 2. Percentage of viable bacteria and yeast isolated from original and newly made, smooth voice prostheses. Results were obtained in four independent experiments (± SD); percentage was expressed with respect to the mean number of bacteria and yeast found on original prostheses, set at 100%.

Voice prostheses	Percentage of total bacteria	Percentage of total yeast
Original	100[a]	100[a]
Smooth, mean ± SD	60 ± 31*	60 ± 29**

[a] The number of viable bacterial and yeast colony forming units on original silicone rubber prostheses amounted 2.0 x 10^7 and 8.8 x 10^5 per cm^2, respectively.

* Significantly different from original prostheses (Wilcoxon signed rank test, $p < 0.1$) from the control.

** Significantly different from original prostheses (Wilcoxon signed rank test, $p < 0.15$) from the control.

Table 3 shows the clinical lifetimes and number of replacements during the historic control and use of the smooth prostheses. The median and mean clinical lifetime of the original prosthesis before entering the study were 52 and 61 days respectively (range: 33-101 days). The median and mean clinical lifetime of the smooth prosthesis were 103 and 130 days respectively (range 58-334). This implies that the clinical lifetime of the smooth prosthesis had more than doubled with respect to the original one (normalized clinical lifetime 2.09, $p < 0.005$). This increase in lifetime was concurrent with a statistically significant ($p < 0.005$) decrease in the number of prosthesis replacements from 44 in the 6 months preceding the study to 26 replacements in the 6 months period during which patients used a smooth voice prosthesis. Sixteen of the 26 prostheses with increased lifetimes could be used longer than two times the SD with respect to the historic control. The reasons for replacement of a smooth prosthesis were leakage through the prosthesis in 92% of cases ($n = 24$) and increased airflow resistance in 8% ($n=2$).

Table 3.

Differences in clinical lifetime and number of voice prostheses replaced during 6 months before entering the study (historic control) and during 6 months using a smooth prosthesis.

Patient number	Historic control		Use of a smooth prosthesis			Lifetime extended by	
	Lifetime ± SD (days)	Number of replacements	Lifetime ± SD (days)	Normalized life time* ± SD	Number of replacements	> 1 SD	> 2 SD
1	52 ± 23	4	151 ± 22	2.90 ± 0.15	2	0	2
2	101 ± 28	3	334 ± 0	3.31 ± 0#	1	0	1
3	42 ± 11	5	103 ± 15	2.45 ± 0.14	2	0	2
4	82 ± 56	3	97 ± 9	1.18 ± 0.09	2	0	0
5	51 ± 4	4	120 ± 34	2.35 ± 0.28	2	0	2
6	37 ± 10	5	64 ± 33	1.73 ± 0.52	3	0	2
7	45 ± 15	5	58 ± 44	1.29 ± 0.76	5	0	1
8	33 ± 21	4	73 ± 21	2.21 ± 0.29	3	0	2
9	54 ± 48	5	85 ± 64	1.57 ± 0.75	3	0	1
10	87 ± 24	3	194 ± 0	2.16 ± 0#	1	0	1
11	84 ± 18	3	151 ± 24	1.80 ± 0.16	2	0	2
Mean	61 ± 23	44	130§ ± 24	2.09§ ± 0.28	26§	0	16
Median	52		103				

**For each patient, the life time of the smooth prosthesis was normalized with respect to the historic control.*

#These patients required no new smooth prosthesis during the study.

§Significant differences (paired Student's t test, $P < 0.005$) from the control.

DISCUSSION

In this study, the original Groningen ULR silicone rubber voice prosthesis was modified through the use of a different mould and liquid silicone rubber filling, resulting in a decreased surface roughness. Though during the course of this study, the Groningen button voice prosthesis has been taken off the market, we believe that the conclusions reached in this study will have great relevance for other types of voice prostheses, like e.g. Provox®, Bivona® and Blom-Singer, all containing silicone rubber valves. The design of the Groningen button however, is quite simple and allows easier and more reliable quantification of biofilm formation than other prostheses, often containing multiple-parts to be sampled whereas the Groningen button merely consists of a flat valve to be sampled. Moreover, smoother moulding of silicone rubber voice prostheses to prevent biofilm formation as demonstrated here, will likely also provide benefits for other silicone rubber implants and devices that clinically malfunction due to biofilm formation, hydrocephalus shunts, catheters, breast implants, contact lenses, and cochlear implants.

Roughness has been shown in the literature to be an important aspect in biofilm formation and a roughness above 200 nm is said to facilitate oral biofilm formation on dental restorative materials[21]. Bruinsma et al. described an increase in deposition of *Pseudomonas aeruginosa* when rigid gas permeable contact lenses had a surface roughness exceeding 14 nm[22]. The effect of a rougher surface on biofilm formation has been attributed to the increased surface area of rough surfaces, allowing stronger microbial adhesion forces and therewith providing protection against shear off forces in the environment[22,23]. Especially streptococcal adhesion has been described to increase with increasing surface roughness, which are primary colonizers in the oral cavity and have been found in voice prosthetic biofilms before. Moreover, also the shape and size of micro-organisms may play an important role in relation to the shape and dimension of the surface roughness in order to establish strong attachment. *S. aureus* (a 1 μm coccal bacterium) was more easily removed from titanium oxide surfaces with a mean surface roughness of 8.7 nm compared to a surface with 500 nm roughness, while *P. aeruginosa* (a 1 μm x 3 μm rod shaped bacterium) showed an opposite result[24]. Surface

roughness of voice prostheses however, has never been subject of research before.

In this study, a large decrease in roughness from 46 nm to 8 nm was established on newly made, smooth voice prostheses compared to the original prostheses. This decrease was created by using a different silicone rubber MED-4850 instead of MED-4750 in combination with a mould with a smoother surface. These two silicone rubbers differ significantly in their physical state: rubber-crepe for MED-4750, which is a high consistency elastomer, and viscous liquid for MED-4850, which is a liquid silicone rubber. Because of this difference in physical state, MED-4850 is easier to mix and more fully and intimately filling the mould. Moreover, an extreme level of polishing was applied to the surface of the mould, using pure diamond particles of varying sizes from 45 μm to 1 μm to create the smooth finish of the mould with R_a values in the range of 0.05-0.2 μm. Standard polish levels for injection mould tools are in a R_a range of 0.4-1 μm. Our smooth voice prostheses have shown a significant reduction in biofilm formation in vitro albeit at $p < 0.1$, both with respect to bacterial (40% reduction), as well as with respect to fungal reduction. Concurrently, the clinical lifetime was increased by a factor of 2.1. Since the literature indicates that adhering bacteria are more easily removed from surfaces with a roughness in the range of 8-10 nm[24], this may indicate that the smooth silicone rubber surface has become self-cleaning under the dynamic conditions of the oropharynx. Interestingly, the self-cleaning character of smooth silicone rubber does not become so much clear in vitro, where less bacterial and fungal biofilm formation was observed but with a relatively low statistical significance due the absence of dynamic detachment forces.

Although biofilm formation is generally accepted to be the main cause of failure for voice prostheses, it remained unclear at this stage as to whether the reductions in biofilm formation observed in vitro contributed significantly to an elongated clinical life time of the prostheses. Clinically, smooth voice prostheses had a doubled clinical lifetime as compared with other prostheses. This emphasizes that a simple measure like a change in moulding can significantly impact the clinical performance of silicone rubber implants

without increasing the production costs, and the current finding may extend to the further development of other prostheses.

Life time assessments of indwelling silicone rubber voice prostheses, always yield highly variable results. Compared with other clinical lifetime studies in which newly designed indwelling voice prostheses were evaluated, this is one of a few studies that shows an significant extension in the device lifetime[25,26]. Recently a prospective clinical study has been published in which a newly designed prosthesis with advanced design components to reduce airflow resistance during speech and to reduce biofilm formation (the Provox® Vega) was compared with the original Provox®2 voice prosthesis[27]. The clinical lifetimes of both designs turned out to be comparable with each other however, which may be due to the relatively complicated designs of the Provox® prostheses with multiple parts and niches.

Consequently the elongation of the clinical lifetime of our newly made, smooth voice prostheses can be considered as quite unique, especially taken the simplicity of the adjustments and their low costs into account. There is only one study in which a similar elongation has been described through the daily intake of Yakult light fermented milk drink[28]. However the daily use of the product results in extra costs and may not be attractive to each and every patient.

In conclusion, adjustment of the moulding process of silicone rubber implants, most notably voice prostheses in the current study, may provide a highly cost-effective way to elongate their clinical lifetime. Although the Groningen ULR voice prosthesis , as used in the current study, has been removed from the market, this study importantly demonstrates that the choice of the material and its surface finish in particular, may be determinant factors with respect to the clinical lifetime of silicone rubber implants and devices failing due to biofilm formation.

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General Discussion 7

GENERAL DISCUSSION

Biofilms on voice prostheses

Biomaterials are frequently used in otorhinolaryngology, e.g. tympanostomy tubes, endotracheal tubes, cochlear implants and silicone rubber voice prostheses. Different types of voice prostheses have been developed since the introduction of the first reliable voice prosthesis by Blom and Singer[1]. The most frequently used prosthesis in The Netherlands nowadays is the Provox® silicone rubber voice prosthesis. Although the development of these biomaterial implants can be considered successful, a drawback of biomaterial implants in the human body is that microorganisms have the tendency to adhere to the surfaces: a biofilm will be formed.

A biofilm is defined as a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a self-produced matrix of extracellular polymeric substances (EPS) and exhibit an altered phenotype with respect to growth rate and gene transcription[2]. The formation of these biofilms on medical devices is undesirable. The formation of biofilms involves several main processes, including adhesion, growth and subsequent dispersal. Attachment is known to be complex and is regulated by the available growth medium, substratum, and cell surface. Once the biofilm has been established it comprises microbial cells and EPS matrix in a defined architecture, which provides an optimal environment for growth and exchange of genetic material between cells. Biofilm formation on silicone rubber voice prostheses starts

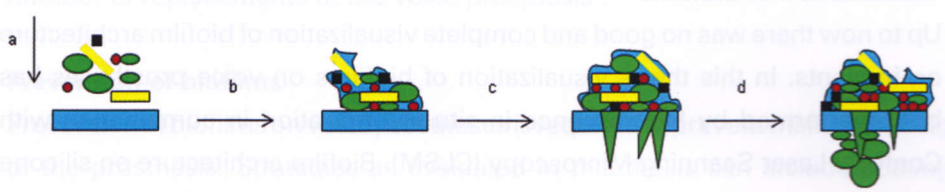

Figure 1. Schematic model of multi-species biofilm formation. (a) Candida and bacteria as planktonic microorganisms, (b) attachment of microorganisms to a surface e.g. silicone rubber, (c) proliferation of Candida in a multi-species biofilm embedded in a self-produced exopolysaccharide matrix, with morphological changes resulting in hyphal formation and deterioration of silicone rubber, (d) dispersal of yeasts after invasion (adapted from Ten Cate et al. 2009)

developing from the first moment the device is placed. Figure 1 shows the steps in biofilm formation as occurs on silicone rubber voice prostheses.

Identification of microorganisms

Although several studies have identified the strains and species involved in voice prosthetic biofilms, still not all microorganisms have been identified. Culturing methods are insufficient to study the full diversity of complex microbial biofilms. In 1993 Muyzer[4] described for the first time a molecular approach to analyze the genetic diversity of complex microbial populations, based on the separation of PCR-amplified fragments of the 16S rRNA-gene by denaturing gradient gel electrophoresis (DGGE). Combination of PCR and DGGE allows identification of a high number of bacterial and fungal species in complex ecosystems in the human body and on biomaterials.

In this thesis, the PCR-DGGE method has been used for identification of microorganisms involved in voice prosthetic biofilm formation. By this method we were able to determine for the first time that lactobacilli were present on all explanted tracheoesophageal shunt prostheses. It is known that lactobacilli belong to the normal oral microflora and therefore it is not surprisingly that lactobacilli also belong to the microflora in voice prosthetic biofilms. For us it was unclear whether the presence of lactobacilli in voice prosthetic biofilms should be associated with failure of the prostheses or not, but it was for sure that the Candida-lactobacilli interaction likely plays an important role in the mixed biofilm on tracheoesophageal voice prostheses and was interesting for further research.

Visualization of biofilms

Up to now there was no good and complete visualization of biofilm architecture on implants. In this thesis visualization of biofilms on voice prostheses has been performed by Fluorescence in situ Hybridization in combination with Confocal Laser Scanning Microscopy (CLSM). Biofilm architecture on silicone rubber voice prostheses has been further explored using FISH and CLSM, a technique preserving the complete structure of biofilms. For the first time, the complex biofilm was visualized on clinically used silicone rubber voice prostheses using FISH. We have illustrated that FISH in combination with

CLSM is a good method that allows detailed analysis of biofilm architecture. Interestingly, we were able to visualize lactobacilli with a specific probe, which showed the close interaction between lactobacilli and Candida in biofilms on clinically used voice prostheses, especially those that had been *in situ* for prolonged periods of time.

Co-adhesion between lactobacilli and Candida

This thesis shows that a combination of lactobacillus strains with Candida yields an increased prevalence of Candida. This constitutes a controversy, since Candida has been described as causative to the deterioration of silicone rubber. On the other hand in dental caries it has already been shown that there is a shift toward increased proportions of acid-producing and acid-tolerating species, such as mutans streptococci and lactobacilli[5]. In the acid environment influenced by lactobacilli, Candida could be increased in prevalence. The interaction between the bacterium and fungus regulates the *Candida albicans* morphogenesis. It has been described that lactobacilli have an inhibiting effect (23-44%) on germ tube formation and hyphal invasion by *C. albicans*[6] through the production of large quantities of biologically active short chain fatty acids (SCFA). As illustrated in figure 1, hyphal formation will be inhibited (step c). This explains why the presence of lactobacilli could be beneficial to the *in situ* lifetime as already has been demonstrated for fermented milk drink, containing *Lactobacillus casei* Shirota, which reduced biofilm formation on silicone rubber voice prostheses *in vitro* and *in vivo*. Daily consumption of the fermented milk drink (with *L. casei* Shirota) increased the lifetimes of Provox®2 prostheses in patients with voice prostheses and it reduced the number of replacements of the voice prosthesis[7].

Prevention of biofilms

Prevention of biofilms on voice prostheses is required to prevent malfunctioning of the prosthesis. Strategies as evaluated in this thesis can include coating of prostheses, development of biomaterials that are less prone to biofilm formation, and as mentioned in literature and explained in this thesis, the use of probiotics.

In literature different coatings on different biomaterials are described. Metal coatings for example have been reported, like gold or titanium coatings, but also silver impregnation. Balasz et al.[8] reported that silver impregnation of vinyl chloride completely inhibited *Pseudomonas aeruginosa* adhesion and efficiently prevented colonisation over a longer period. In a pilot study we also tested the use of a solution of colloidal silver motivated by a letter of a patient (Appendix 1). Unfortunately we did not observe a decrease of biofilm formation on silicone rubber voice prostheses *in vitro*. Furthermore, silver is toxic when ingested in higher concentrations, which is an obvious drawback.

Gottenbos et al.[9] determined the antimicrobial activity of 3-(trimethoxysilyl)-propyldimethyloctadecylammonium chloride (QAS) coating on silicone rubber. Antimicrobial activity of QAS-coated silicone rubber was demonstrated *in vitro* and *in vivo*. They reported that the positively charged QAS-coating affected the viability of Gram-negative bacteria as well as of Gram-positive bacteria in single strain, bacterial biofilms. In this thesis it is demonstrated that such a coating reduces also the number of viable bacteria and yeast in mixed biofilms. The mechanisms of action of QAS causing death in fungi is not known, but it seems to impede the formation of hyphae, as we demonstrated with CLSM. Since a QAS-coating is non-toxic, clinical application could be possible.

Smooth prostheses

In the last chapter of this thesis a modulated smooth voice prosthesis based on the Groningen Ultra Low Resistance voice prosthesis was produced and tested *in vitro* and *in vivo*. This was achieved by using a smoother mold and a more liquid silicone rubber. This method did not bring in extra costs for production which is important in society nowadays. This prosthesis showed reduced biofilm formation *in vitro* compared to regular prostheses of the same type and the clinical lifetime was increased by a factor of 2.1. In a study of Harms et al.[10], it is mentioned that that there are no significant differences in lifetime between Groningen ULR and Provox®2 voice prostheses found, mean lifetimes were 106.2 and 102.7 days, and median lifetimes were 76 and 65 days for Groningen ULR and Provox® respectively. In this thesis (chapter 2) we also calculated the lifetimes: mean lifetimes were 95.9 and 100.2 days,

and median lifetimes were 92 and 73 days for Groningen ULR and Provox®2 respectively. No significant differences in lifetime were found and that is why Groningen ULR as well as Provox®2 voice prostheses were both used.

In conclusion we believe that the use of the proposals in this thesis will be of direct benefit for laryngectomized patients by lengthening the *in situ* lifetime of silicone rubber voice prostheses. Because of the importance of lactobacilli and especially the interaction with Candida, as demonstrated in this thesis, further research in their mechanisms in biofilm formation will be interesting. Maybe this can bring us another step forward in the prevention of biofilm formation on silicone rubber voice prostheses in the near future.

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APPENDIX

Letter and explanation of colloidal silver from a patient who uses a solution of colloidal silver (in Dutch).

Hierbij stuur ik je een kopie van het artikel over colloidaal zilver.
Dit heeft mij geweldig geholpen. Twee jaar geleden was mijn button ong. om de 2 à 3 weken lek. Dan sukkelde ik door tot 2 maanden. (Drinken met een rietje eten).
Mijn vriendin en ik zijn de bieb in gedoken, om meer te weten te komen over de Indiaanse kultuur i.v.m. zilver. Dit medicijn is al heel oud. Werd ook in de reguliere geneeskunst gebruikt tegen allerlei infekties en ontstekingen vóór de penicilline.
Ik ben begonnen met de kleine flesjes. maar het is veel voordeliger het zelf te maken met de CS-generator.
Toen ging de button lekkage naar gem. 5 maanden. Geweldig!
Misschien hebben meer patiënten hier baat bij.

Hartelijke groet.

COLLOÏDAAL ZILVER:

WAT IS COLLOÏDAAL ZILVER (hierna genoemd CZ).

Een Colloïdaal deeltje is zo klein dat de volgende halte op weg naar kleiner, het atoom zelf is. Colloïden zijn van nature de kleinste deeltjes waarin een stof gesplitst kan worden met behoud van de individuele, unieke eigenschappen

ZILVER was al bekend en gebruikt door de Romeinen om water te zuiveren. In de middeleeuwen werd dit water door de adel gebruikt als preventie i.v.m. o.a. de pest. NASA heeft ook zilver in de door hun gebruikte waterfilters.

Door zuiver (+99,9%) zilvermetaal om te zetten in een wolk van microscopische kleine deeltjes wordt het oppervlakte enorm vergroot (en dus de helende werking) terwijl het ook dieper in het lichaam doordringt.
Omdat de zilverdeeltjes geladen zijn, willen ze zich hechten aan andere elementen. Sporenelementen treffen we zelfs in gedistilleerd water aan, en wanneer de geladen zilverdeeltjes zich verenigen met een specifiek sporenelement, dan kan de oplossing diverse kleuren krijgen. Over het algemeen zal het zich als een mist/wolkachtige verschijning voordoen.
Het element dat het zilver kiest is niet belangrijk; eenmaal in het lichaam ontdoet het zilver zich van zijn binding en gaat op zoek naar een sterkere aanhechting om zijn lading te stabiliseren.
Als zilver colloïde in het lichaam komt, passeren de zilverdeeltjes (ongeveer ter grootte van 15 atomen) snel de maagwand en komen dan in het bloed, waar ze ongeveer een week blijven circuleren voordat ze worden uitgescheiden.

WAT DOET CZ?
Zuivert en heelt. Het doodt bijna elk soort lichaamsvreemd micro-organisme en kiem, steunt het immuunsysteem, beteugelt infecties, laat tandplaque verdwijnen, ondersteunt verjonging.
CZ is het meest veilige en effectieve geneesmiddel op aarde. (stelling)

Een breed –spectrum geneesmiddel.
De helende eigenschappen van CZ zijn zeer uiteenlopend; ze doodt niet alleen ziekte verwekkende organismen maar bevorderd ook de heling van beschadigd weefsel met meer dan 50%.
"Veel stammen van pathogene microben –virussen, schimmels, bacteriën of enig ander eencellig pathogeen organisme- resistent tegen andere antibiotica worden gedood in contact met CZ en zijn niet in staat te muteren. Maar het schaadt niet de cel-enzymen of vriendelijke (eigen) bacteriën".
Meer dan 650 micro-organismen, uitgetest in laboratoria, zijn niet opgewassen tegen CZ en leggen het loodje.

Ondersteuning van het immuunsysteem en verjongende werking van cz.
CZ bevordert de groei van een nieuw soort cellen die eruit zien als de cellen bij kinderen. Deze cellen groeien snel en vormen een uitgebreid assortiment van primitieve celvormen, welke in staat zijn zich in snel tempo te vermeerderen en vervolgens te differentiëren in de specifieke cellen van een orgaan of weefsel dat beschadigd is, zelfs bij patiënten boven de 50 jaar. (Volgens een onderzoek.) Het geeft heling en herstel bij beschadigingen van de huid.

WAT DOET CZ NIET?
Het werkt niet in op andere medicijnen (behalve wanneer daarin metaal zit, zie Wanneer CZ niet) en heeft geen enkel neven- of bij effect op mens en dier.

WANNEER NIET CZ?
Bij medicatie waarin metaal is verwerkt (zink, chroom e.d.) CZ ziet die stoffen als lichaamsvreemd en bestrijd dat.
Als iemand allergisch is voor Zilver.

Summary

8

The most widely used and most successful technique of voice restoration following a total laryngectomy, because of laryngeal cancer, is the use of a silicone rubber voice prosthesis. Unfortunately, biofilm formation on the oesophageal side of the prostheses limits their lifetime to 2-3 months on average. **Chapter 1** gives an introduction in which biofilm formation on silicone rubber voice prostheses was described in general and the main techniques used in this thesis are described. Because still not all fungi and bacteria playing a role in biofilm formation have been identified, the aim of this thesis was to identify the microbial composition and architecture of voice prosthetic biofilms using molecular techniques and to determine the role of specific bacterial strains in voice prosthetic biofilms. Besides that this thesis concerns the evaluation of voice prosthetic biofilm formation on antimicrobially-modified and smoothened silicone rubber voice prostheses.

In **Chapter 2**, Denaturing Gradient Gel Electrophoresis (DGGE) was used for the analysis of PCR amplified ribosomal genes of the bacterial and fungal population obtained from voice prosthetic biofilms. Most commonly identified fungi in the biofilms were *Candida albicans*, *Candida tropicalis* and *Candida glabrata*. In all isolated voice prosthetic biofilms lactobacilli (*Lactobacillus gasseri*, *L. fermentum*, *L. casei*, *L. delbrueckii* or *L. acidophilus*) were identified as the dominant bacterial group. Biofilms isolated from voice prostheses with a longer lifetime than 75 days showed more *C. tropicalis* and *C. glabrata* than in the shorter life time group. This chapter demonstrated that with DGGE more microorganisms can be identified in voice prosthetic biofilms than with traditional culturing techniques. It also showed that lactobacilli were always present in voice prosthetic biofilms and nearly always in combination with Candida.

Although several studies have identified strains and species involved in voice prosthetic biofilms and electron microscopy has indicated that microorganisms even grow into the silicone rubber, still a good visualization of the biofilm architecture is lacking. The aim of **Chapter 3** was to visualize the biofilm architecture on voice prostheses in time and identifying microorganisms by Fluorescence in situ Hybridization (FISH) and Confocal Laser Scanning Microscopy (CLSM). Dysfunctional Provox®2 and Groningen silicone rubber

voice prostheses after varying durations of clinical use were considered for this study. FISH was performed on cross-sections of the valves using rRNA-targeted oligonucleotide probes and biofilms were examined using CLSM. The results demonstrate that there is a large variety in biofilm formation. *Candida* species are present as hyphae in the biofilm and as yeasts deteriorating the silicone rubber. Bacteria are mostly seen near the surface of the biofilm but bacterial colonies are also seen near and between ingrowing yeasts. Generally in time there is an increase in biofilm thickness, and an increase in the amount of extrapolymeric substances. The isolation of biofilms from several voice prostheses and the use of more specific oligonucleotide probes resulted in the identification of mainly lactobacilli and *Enterobacteriaceae*. CLSM analysis showed for the first time that mostly lactobacilli are the predominant bacteria in the biofilms and grow intertwined with yeasts.

This interaction between lactobacilli and Candida on silicone rubber was studied in more detail in **Chapter 4.** In a modified Robbins device biofilms were grown in commercially available silicone rubber tubes. *C. albicans* in combination with lactobacilli resulted in less biofilm formation, especially the combinations of *C. albicans* with *L. acidophilus* and *L. crispatus.* Interestingly, the current study showed that combinations of lactobacillus strains with Candida yield an increased prevalence of Candida as compared with combinations involving other bacterial strains. It is plausible that the interaction between the bacterium and fungus regulates the *C. albicans* morphogenesis because it has been described that lactobacilli have an inhibiting effect (23-44%) on germ tube formation and hyphal invasion by *C. albicans*. This is why lactobacilli could be beneficial to the *in situ* lifetime of silicone rubber voice prostheses.

In **Chapter 5** two quaternary ammonium silanes were used to coat silicone rubber tracheoesophageal shunt prostheses, yielding a positively charged surface. One QAS-coating ((trimethoxysilyl)-propyldimethyloctadecylammonium chloride) was applied through chemical bonding, while the other Biocidal ZF coating was sprayed on the silicone rubber surface. The sprayed coating lost its stability within an hour, while the chemically bonded one appeared stable. Upon incubation in an artificial throat model, allowing

simultaneous adhesion and growth of yeast and bacteria, all coated prostheses showed a significant reduction in the numbers of viable yeast (to 12%-16%) and bacteria (to 27%-36%), as compared with silicone rubber controls, and as confirmed using CLSM after live/dead staining of the biofilms. *In situ* hybridization with fluorescence-labelled oligonucleotide probes showed that yeast expressed hyphae on the untreated and on Biocidal ZF coated prostheses, but not on the QAS-coated ones. Whether this is a result of the positive QAS-coating or due to the reduced number of bacteria is currently unknown. This is the first report on the inhibitory effects of positively charged coatings on the viability of yeast and bacteria in mixed biofilms. Although initially aimed at reducing voice prosthetic biofilms, its relevance extends to all biomedical and environmental surfaces where mixed biofilms develop and form a problem.

Moulding technique can have a major influence on the roughness of the prosthesis surface, but the importance of moulding is mostly underestimated. In **Chapter 6** we aimed to produce a voice prosthesis, based on the regular Groningen ultra low resistance (ULR) prosthesis, with a decreased surface roughness and to compare *in vitro* biofilm formation on smooth and regular Groningen ULR voice prostheses. In addition, the clinical lifetimes of smooth and regular prostheses are evaluated in laryngectomized patients. The use of a smoother mould and less viscous silicone rubber yielded a decrease in surface roughness of the prostheses from 46 nm to 8 nm and was accompanied by a 40% reduction in the prevalence of bacteria and yeast in *in vitro* formed biofilms. Clinically, the lifetime of smooth prostheses was significantly ($p < 0.005$) increased by a factor of 2.1 with respect to prostheses with a regularly rough surface. This suggests that smoother moulding techniques may favourably impact the infection rates of biomedical implants and devices made of silicone rubber.

In the general discussion, **Chapter 7,** the results of the previous chapters are discussed and suggestions are made whether to use the proposals in this thesis. Summarizing, PCR-DGGE and FISH in combination with CLSM are molecular techniques that give us a more complete view of the biofilm on

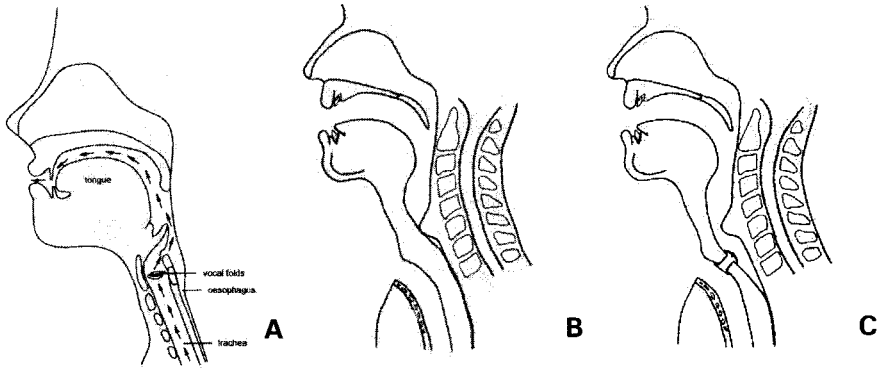
silicone rubber voice prostheses. These techniques show that lactobacilli are important in biofilm formation interacting with Candida and probably inhibit hyphal formation, resulting in a prolongation of the *in situ* lifetime of the prostheses. This is interesting for extensive further research.

The use of QAS-coatings or smoother surfaces prevent biofilm formation on voice prostheses and it is recommended to use these findings on voice prostheses available nowadays.

Samenvatting

Bij patiënten met een maligniteit van het strottenhoofd zijn, afhankelijk van de grootte van de afwijking, verschillende behandelingen mogelijk. Bij patiënten die niet meer in aanmerking komen voor bestraling, of waarbij bestraling of eerdere plaatselijke chirurgische behandeling niet voldoende effectief is gebleken en de aandoening is teruggekeerd, zal een laryngectomie (chirurgische verwijdering van het strottenhoofd) uitgevoerd moeten worden. Bij deze operatie wordt het gehele strottenhoofd, inclusief de stembanden verwijderd en wordt de verbinding tussen de mond en de luchtpijp (trachea) opgeheven (zie figuur 1). De ademhaling vindt plaats via een opening in de hals (tracheostoma) die tijdens de operatie wordt gemaakt door het inhechten van het restant van de luchtpijp in de huid net boven het borstbeen. De verbinding tussen de mond en de slokdarm is wel intact gehouden, waardoor eten via de normale weg gaat.

Een laryngectomie heeft wel consequenties voor de voedselpassage, ademhaling en het stem- en spraakvormend vermogen. Het slikken kan lastiger gaan door toch een veranderde vorm en vaak circulair vernauwde ingang van de slokdarm (neopharynx). De bijdrage van de neus als filter en warmte- en vochtwisselaar tijdens de ademhaling, is als het gevolg van het permanente tracheostoma komen te vervallen. Door het verlies van het strottenhoofd inclusief de bijbehorende stembanden is de fysiologische bron van het basisgeluid niet meer aanwezig. Het niet meer normaal kunnen

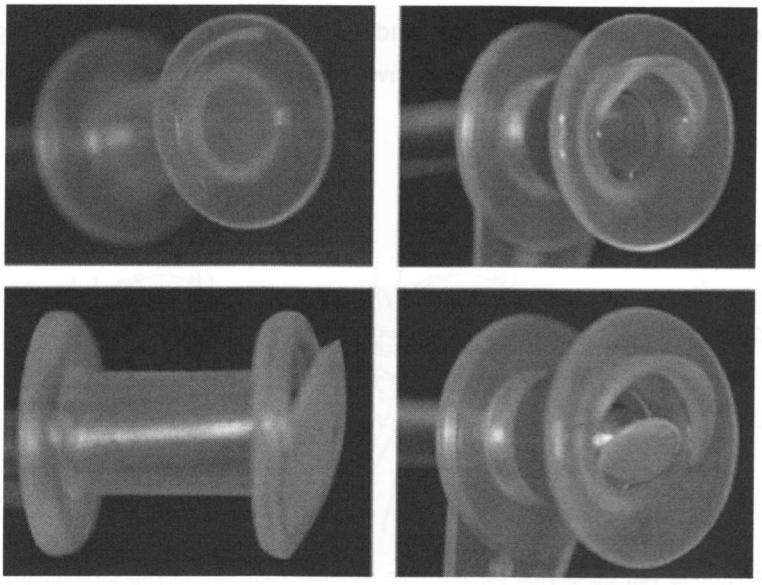


Figuur 1. Anatomie van de larynx. (A) Normale situatie, vóór een laryngectomie; (B) Situatie na een laryngectomie, met een tracheostoma als nieuwe toegang tot de luchtweg; (C) Na een laryngectomie, met een spraakprothese tussen de trachea en oesophagus.

spreken is voor patiënten sterk invaliderend en psychisch belastend. Een snelle en adequate stem- en spraakrevalidatie is dan ook erg belangrijk.

In het verleden zijn er verschillende methoden geïntroduceerd en gebruikt voor vervangende stemgeving. Hiervan is de meest succesvolle methode met behulp van een siliconen rubberen spraakprothese, die in een chirurgisch gemaakte opening tussen de slokdarm en luchtpijp is geplaatst (figuur 1C). Door het tracheostoma met een duim of vinger af te sluiten, gaat lucht vanuit de longen via de prothese, door opening van een klepmechanisme, naar de slokdarm en door de mond naar buiten. Deze luchtstroom zal het weefsel ter hoogte van de slokdarmingang in trilling brengen en op deze manier kan geluid geproduceerd worden. Het klepmechanisme van de spraakprothese voorkomt ook dat er geen speeksel, drank of voedsel vanuit de slokdarm in de luchtpijp lekt.

Door de jaren heen zijn sinds 1980 veel verschillende spraakprothesen ontworpen. In Nederland werden tot kort geleden de Groningen spraakprothese en de Provox®2 spraakprothese het meest gebruikt (figuur 2).



Figuur 2. Groningen Low Resistance spraakprothese (links) en Provox®2 spraakprothese (rechts) met gesloten en geopende klep.

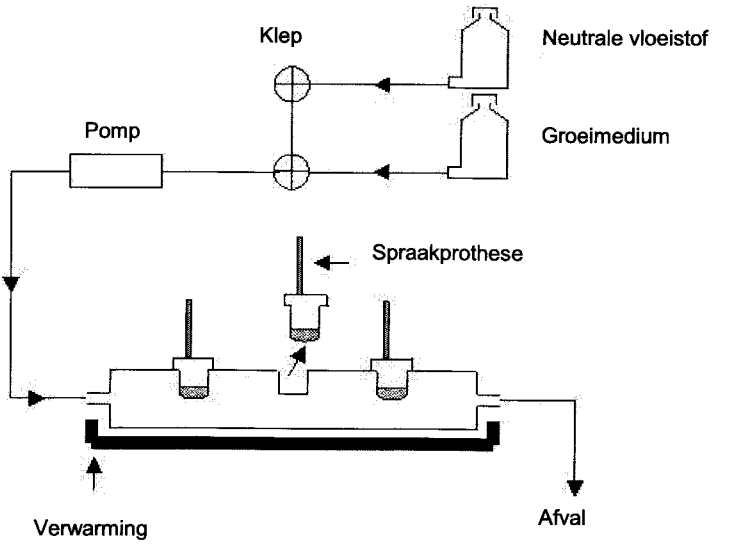
Sinds twee jaar is de Groningen spraakprothese niet meer in productie en worden in Nederland voornamelijk verschillende soorten Provox® spraakprothesen (Provox®2, Activalve, Vega) gebruikt.

De levensduur van siliconen rubberen spraakprothesen is beperkt. Dit komt doordat er bacteriën en gisten in een slijmlaag (extracellulaire polymere substanties) gaan hechten op het oppervlak van de prothese aan de slokdarmzijde. Deze hechting van bacteriën en gisten wordt biofilmvorming genoemd. Doordat er zich een biofilm vormt, werkt het klepmechanisme van de prothese op den duur niet meer goed. Er zal dan lekkage optreden van speeksel, voedsel of drank vanuit de slokdarm in de luchtpijp of de luchtstroomweerstand zal toenemen waardoor het spreken moeizamer gaat. De prothese zal in deze gevallen poliklinisch door een keel-, neus-, en oorarts vervangen moeten worden. De gemiddelde levensduur van spraakprothesen is ongeveer drie maanden, echter de levensduur kan sterk verschillen tussen patiënten. Soms moet een prothese al na enkele dagen vervangen worden, wat erg belastend voor de patiënt is. Het vinden van een methode die de levensduur van spraakprothesen kan verlengen, zal een belangrijke positieve invloed hebben op de kwaliteit van leven van deze patiëntengroep.

In het verleden is vaak gekeken naar de microbiële samenstelling en opbouw van de biofilm, maar nog steeds zijn niet alle microorganismen geïdentificeerd. Het eerste doel van dit proefschrift is dan ook om de samenstelling en architectuur van de biofilm verder te onderzoeken en de rol van bepaalde bacteriën.

Daarnaast is gekeken naar oplossingen om de biofilmvorming op spraakprothesen te verminderen om de levensduur van de prothesen te verlengen en zo dus de kwaliteit van leven van patiënten te verbeteren.

In een aantal hoofdstukken is de biofilmvorming in het laboratorium nagebootst. Dit werd gedaan met een zogenaamde kunstkeel (figuur 3). Dit is een roestvrijstalen buis waarin spraakprothesen geplaatst kunnen worden. De buis wordt op lichaamstemperatuur gebracht en door de buis kunnen vloeistoffen met bacteriën en gisten gespoeld worden. Hierdoor vormt er zich een biofilm op de spraakprothesen, net zoals dat in een patiënt zou gebeuren.



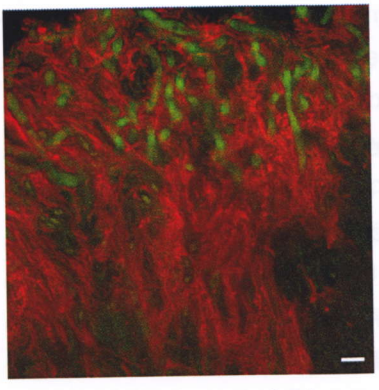
Figuur 3. Schematische tekening van een kunstkeel.

Voor de identificatie en visualisatie van micoorganismen in de biofilm zijn twee moderne microbiële technieken gebruikt, namelijk Denaturing Gradient Gel Electrophoresis (DGGE) voor de identificatie van microorganismen en Fluorescentie in situ Hybridisatie (FISH) voor de visualisatie van de biofilm.

Ieder organisme heeft erfelijke informatie: DNA. In **hoofdstuk 2** is DGGE gebruikt om biofims van spraakprothesen die in patiënten hebben gezeten, te analyseren. Met de moleculaire techniek DGGE wordt er als het ware een vingerafdruk van het DNA gemaakt. Op deze manier zijn de bacteriën en gisten in de biofilms geïdentificeerd. De meest voorkomende gisten waren *Candida albicans*, *Candida tropicalis* en *Candida glabrata*. *Candida albicans* kwam het meeste voor. In alle biofilms die onderzocht waren, kwamen lactobacillen het meeste als bacteriën voor (*Lactobacillus gasseri*, L *fermentum*, *L.casei*, *L. delbrueckii* of *L. acidophilus*). Hiermee zijn lactobacillen voor het eerst in biofilms op spraakprothesen geïdentificeerd, maar ook meteen als de meest belangrijke omdat ze op alle spraakprothesen die onderzocht waren voorkomen.

In **hoofdstuk 3** zijn biofilms op spraakprothesen van verschillende levensduur gevisualiseerd. Bij FISH worden fluorescerende labels (probes)

gebruikt die hechten aan bepaalde cellen in de biofilm (DNA). De probe is zo samengesteld (een bepaalde volgorde aan basen) dat het complementair is aan het stuk DNA in de biofilm dat we willen onderzoeken en zal zich binden (hybridiseren) aan dat stuk DNA. "In situ hybridisatie" kan je daarom ook lezen als "zich ter plaatse hechten". Er zijn verschillende fluorescente merkers die aan de probes gekoppeld kunnen worden, die elk met een eigen kleur oplichten onder de microscoop (confocale laser scanning microscoop). Daardoor is het mogelijk verschillende stukken DNA en dus microorganismen tegelijk te onderzoeken en de biofilm goed te visualiseren. Om de biofilms te onderzoeken werden doorsnedes van het klepmechanisme onder de microscoop na FISH bekeken. Dit toonde ten eerste aan dat er een zeer grote variatie in biofilmvorming is, met over het algemeen een toename van biofilmdikte in de tijd en ook een toename van slijm in de biofilm (EPS). Candida werden in verschillende vormen gezien: als gistbollen en als uitlopers (hyphen). De hyphen dringen het oppervlak van het siliconen rubber binnen, omringd door bacteriën en transformeren dan als ingroeiende "kegels" dieper in het siliconen rubber. Door specifieke probes te gebruiken, konden wederom veel lactobacillen aangetoond worden in de biofilm. Deze konden goed in beeld gebracht worden in de biofilm en leken een grote relatie te hebben met Candida (figuur 4).



Figuur 4: Interactie tussen hyphen van Candida (groen) en lactobacillen (rood).

De interactie tussen lactobacillen en Candida op siliconen rubber werd verder onderzocht in **hoofdstuk 4**. In een soort kunstkeel werden biofilms in siliconen

rubberen slangetjes gegroeid. Er werd gezien dat *C. albicans* in combinatie met lactobacillen minder totale biofilm vormde, vooral de combinaties van *C. albicans* met *L. acidophilus* en *L. crispatus*. In dit hoofdstuk kwam ook naar voren dat er procentueel meer Candida groeien in combinatie met lactobacillen in vergelijking met andere bacteriën. Doordat de interactie tussen lactobacillen en Candida zo duidelijk is, is het aannemelijk dat lactobacillen invloed hebben op de morfogenese van C. albicans: lactobacillen remmen de ontwikkeling van hyphen en daarmee de ingroei in het siliconen rubber. Daarom zouden lactobacillen een gunstig effect kunnen hebben op de levensduur van de spraakprothese.

Om biofilmvorming op de spraakprothese te verminderen werd gekeken naar de invloed van verschillende oppervlaktebehandelingen.

In **hoofdstuk 5** zijn twee quaternaire ammonium silaan coatingen (QAS) onderzocht. Eén coating [(trimethoxysilyl)- propyldimethyloctadecyl-ammonium chloride] werd door chemische binding aan de spraakprothese gekoppeld. De andere coating, Biocidal ZF, werd op de klep van de spraakprothese gesprayed. Beide coatingen werden in de kunstkeel getest en lieten significante verminderingen zien van het aantal levende gisten en bacteriën ten opzichte van de controle prothese zonder coating. Op de biofilm werden kleuringen toegepast waarmee levende en dode bacteriën en gisten aangetoond kunnen worden. Hierbij kon ook gezien worden dat er minder biofilm op de spraakprothesen met coating aanwezig was. De gesprayde coating verloor echter zijn stabiliteit binnen een uur, terwijl de chemisch gebonden coating stabiel leek te blijven. Na de toepassing van FISH (zoals eerder beschreven in hoofdstuk 3) werden er ook hyphen gezien. Deze werden alleen op de onbehandelde en de Biocidal gecoate prothesen gezien en niet bij de QAS-gecoate prothesen. Dit is de eerste keer dat remmende effecten van positief geladen coatingen op de levensvatbaarheid van gisten en bacteriën in gemixte biofilms is aangetoond.

In **hoofdstuk 6** is beschreven wat het effect is van een minder ruw oppervlak van de spraakprothese. De originele Groningen "Ultra Low Resistance" siliconen rubberen prothese werd aangepast door het gebruik van een andere mal en een vloeibare siliconen rubberen vulling waardoor het

oppervlak van de prothese minder ruw werd. Bij experimenten in de kunstkeel werd een vermindering gezien van biofilmvorming op de gladde prothesen in vergelijking met de originele Groningen spraakprothese. Deze nieuwe gladde prothese werd ook in een patiëntengroep getest en liet een significante verdubbeling van de levensduur zien. Deze methode is een simpele methode om toe te passen en brengt weinig extra kosten met zich mee.

CONCLUSIES

Goede stemrevalidatie na een totale laryngectomie is erg belangrijk. De meest gebruikte methode om dit te bereiken is met behulp van een siliconen rubberen spraakprothese in een chirurgisch gemaakte verbinding tussen de oesophagus en trachea.

Biofilms die op de prothese groeien beperken echter de levensduur. Het gebruik van moderne microbiële technieken, zoals DGGE en FISH, hebben in dit proefschrift aangetoond een completer beeld te geven van de samenstelling en architectuur van de biofilm. In het verleden is altijd genoemd dat Candida verantwoordelijk zijn voor het niet goed functioneren van de spraakprothese, echter de invloed van bacteriën en dan vooral lactobacillen lijkt hierin erg belangrijk te zijn. Lactobacillen komen namelijk altijd voor in de biofilm op spraakprothesen in interactie met Candida. Ze hebben invloed op de hyphevorming en daarmee ook op de ingroei van het siliconen rubber. Deze interactie is interessant en zal dan ook verder onderzocht worden.

Om aanhechting van biofilm te verminderen werden coatings op prothesen en gladdere prothesen onderzocht. Met name de QAS-coating heeft een gunstige invloed op zowel de vorming als ingroei van de biofilm, echter een mogelijk nadeel kan het gebruik van deze coating in patienten zijn, omdat de invloed op lange termijn niet bekend is. Daarnaast brengt deze coating ook extra kosten met zich mee. Het gebruik van een gladdere prothese is daarom veel interessanter, omdat het makkelijk toepasbaar is, ook een duidelijk gunstig effect op de levensduur van de prothese heeft en weinig extra kosten met zich meebrengt.

Dankwoord

Dit proefschrift zou niet compleet zijn zonder een woord van dank aan de mensen die op één of andere manier (zowel wetenschappelijk als privé) hebben bijgedragen aan het tot stand komen van dit boekje.

Meerdere patiënten hebben al eens aangegeven niet bedankt te willen worden, omdat ze juist door dit soort onderzoeken geholpen zijn. Toch wil ik dit dankwoord beginnen om die patiënten te bedanken die bereid zijn geweest deel te nemen aan de patiëntenstudie.

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CURRICULUM VITAE

Kevin Jorge Dave Andrew Buijssen was born in 1977 in Geldrop, the Netherlands. After graduating from High School (St. Willibrord Gymnasium in Deurne) in 1996, he studied Medicine in Gent, Belgium for one year and in 1997 he enrolled to study Medicine at the University of Groningen, until 2004. In November 2004 he started research for this thesis at the University of Groningen (Departments of Otorhinolaryngology, BioMedical Engineering and Medical Microbiology) and from May 2007 he started his residency in Otorhinolaryngology at the University Medical Center Groningen that he has completed in April 2012. Since May 2012 he works as an ENT-surgeon and staff member in the University Medical Center Groningen and in September 2012 he will start to work as an ENT-surgeon in the Albert Schweitzer Hospital (Dordrecht, Sliedrecht, Zwijndrecht; The Netherlands).

The author is married to Mariëlle Elise Francino and has one son (Rowan) and one daughter (Noémy).

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