CHAPTER 10

Biofilm formation on the Provox ActiValve: Composition and ingrowth analyzed by Illumina paired-end RNA sequencing, fluorescence in situ hybridization and confocal laser scanning microscopy

A.J. Timmermans
H.J.M. Harmsen
C. Bus-Spoor
K.J.D.A. Buijsen
C. van As-Brooks
M.C. de Goffau
R.H. Tonk
M.W.M. van den Brekel
F.J.M. Hilgers
B.F.A.M. van der Laan

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ABSTRACT

Background: The most frequent cause of voice prosthesis failure is microbial biofilm formation on the silicone valve, leading to destruction of the material and transprosthetic leakage. The Provox ActiValve valve is made of fluoroplastic, which should be insusceptible to destruction. The purpose of this study was to determine if fluoroplastic is insusceptible to destruction by Candida-species.


Results: IPES (n=10) showed that Candida albicans and Candida tropicalis are dominant populations on fluoroplastic and silicone. Microbial diversity is significantly lower on fluoroplastic. L. gasseri is the prevalent bacterial strain on most voice prostheses. FISH and CLSM (n=23): in none of the cases was ingrowth of Candida-species present in the fluoroplastic.

Conclusions: Fluoroplastic material of Provox ActiValve seems insusceptible to destruction by Candida-species, which could help improve durability of voice prostheses.
INTRODUCTION

Total laryngectomy (TL) is still an important treatment option for advanced stage larynx cancer and is often the only remaining curative choice for recurrence after (chemo) radiotherapy. After TL, the vocal tract and upper digestive tract are separated and the trachea is attached to the base of the neck, forming a permanent stoma. Because the voice box is removed, an alternative sound source has to be found in order to restore oral communication. Options are an external sound source in the form of an electrolarynx or using the reconstructed pharynx as the new sound source, either enabling esophageal speech with air injected into and then expelled from the esophagus, or tracheoesophageal speech. In the latter case a voice prosthesis, containing a one-way valve mechanism, is implanted into a tracheoesophageal puncture tract to allow pulmonary air to be diverted into the esophagus. Previous research has demonstrated that tracheoesophageal speech, utilising a silicone prosthesis is superior in terms of quality and intelligibility. Op de Coul et al. (2000), for instance, reported a success rate with respect to voice quality (fair to excellent rating) of 88% (1). Because of its high success rate and ease of acquisition, tracheoesophageal prosthetic speech has become the method of choice for voice and speech rehabilitation after TL (1). A variety of voice prostheses, mostly made out of silicone rubber, have been developed in the past few decades, e.g. Blom-Singer, Groningen, Nijdam, and Provox (2, 3). The lifespan of these devices varies from a few weeks to several years. A retrospective study conducted at the Netherlands Cancer Institute reported a mean lifespan for the Provox2 of 163 days and a median of 89 days. In most cases, voice prostheses have to be replaced because of transprosthetic leakage (1).

The main reason for this leakage is microbial biofilm formation on the valve, causing failure of the valve mechanism and sometimes also blockage and/or an increased airflow resistance (4). The biofilm consists of a mixture of bacteria and fungi and starts developing from the moment the voice prosthesis is implanted into the tracheoesophageal puncture. In particular, Candida-species grow into and subsequently build up on the silicone rubber (5). To extend the lifespan of the device, the use of oral and/or topical fungicidal drugs on a regular basis is proposed. To date, however, this has not been substantiated in properly conducted clinical studies, and regular use of antifungals might induce resistance or cause side effects (6). Other options that could extend the lifespan of the device are flushing water or air through the lumen of the prosthesis under light pressure or using a dedicated brush to clean the inside of the prosthesis (7). Some studies reported the reduction of biofilm formation by the use of certain dairy products, such as probiotics, which also extends the clinical device lifespan (8).

To solve this problem in a material-technical way, a special voice prosthesis was developed: the Provox ActiValve (Atos Medical AB, Hörby, Sweden; Figure 1) (9). The valve and valve seat
of this voice prosthesis are solely made out of fluoroplastic, which is deemed insusceptible to ingrowth of Candida-species (Figure 2). Closure of the valve is achieved “actively” with 2 magnets (one in the valve and one in the valve seat), when the tracheoesophageal airflow stops. The Provox ActiValve is available in three versions depending on magnet strengths (Light, Strong and XtraStrong), which are applied according to the “underpressure” in the esophagus. Although the clinical effectiveness of the Provox ActiValve has been substantiated in several retrospective and prospective studies (10-12), the lack of a destructive effect of Candida-species on the fluoroplastic material has so far not been visualized in appropriate studies. Furthermore, the composition and diversity of the biofilm on fluoroplastic valves have not been described before. Buijssen et al. already showed that the biofilm on silicone rubber voice prostheses is composed of lactobacilli as the predominant bacterial genus and Candida as the main fungal component (5). The composition and diversity of the biofilm on the fluoroplastic valve of the Provox ActiValve, however, have not yet been studied, and increasing insight in the behavior of Candida-species and the composition of the biofilm on fluoroplastic material could be helpful to further improve durability of voice prostheses in a material-technical way.

**Figure 1.** Overview of the Provox ActiValve voice prosthesis (S: silicone material; F: Fluoroplastic –blue-material; M: magnets).

**Figure 2.** Macroscopic image of a Provox ActiValve, which was 364 days in situ, illustrating the amount of biofilm on the silicone material and on the fluoroplastic valve.

The first purpose of this study, therefore, was to determine the composition and diversity of the biofilm of both the silicone and the fluoroplastic material of the Provox ActiValve. This was done by analyzing both the bacterial and fungal communities on these samples using Illumina paired-end sequencing (IPES) (13). This is the first time IPES will be used to analyze...
microbial communities by combining amplicons sequencing of the bacterial 16S rRNA gene and the eukaryotic ITS regions on these voice prostheses. The second purpose was to confirm the hypothesis that the fluoroplastic material is not susceptible to destruction by Candida species. For this purpose, fluorescence in situ hybridization (FISH) and a confocal laser scanning microscopy (CLSM) were used (5). FISH is especially suitable for the identification of multiple species in a biofilm. CLSM has the ability to control the depth of the field, to reduce background information and to collect serial optical sections from thick samples.

MATERIALS AND METHODS

The study period lasted from November 2011 to June 2013. During this 19-month period, we collected 33 consecutive dysfunctional Provox ActiValve prostheses of patients visiting the outpatient clinic of the Department of Head and Neck Oncology and Surgery of the Netherlands Cancer Institute (Amsterdam, the Netherlands), or of the Department of Otorhinolaryngology of the University Medical Center Groningen (Groningen, the Netherlands). Of these 33 prostheses, the first 23 prostheses were used for FISH and CLSM after fixation within 24 hours and storage at 4°C. The subsequent 10 prostheses were analyzed using IPES and stored at -20°C, both numbers being sufficient for the envisaged analyses.

Composition and diversity of the biofilm using IPES

Composition and diversity of the biofilm were determined by the IPES method (13). The explanted prosthesis was cut into cross-sections using a surgical blade. Cross-sections of the fluoroplastic part of the valve and of the silicone material of the esophageal flange were stored at -20°C. When all 10 prostheses were collected, cut and stored, DNA was isolated and purified from both the fluoroplastic part of the valve and of the silicone material of the esophageal flange. We added a lysis buffer (500 mM NaCl, 50 mM Tris-HCl (pH 8), 50 mM EDTA, 4% sodium dodecyl sulfate (SDS)) to the samples and heated the samples at 70°C. To disrupt cell walls in order to obtain DNA, zirconium beads (0.1 mm) and glass beads (3 mm) were added and the samples were mechanically disrupted at room temperature at 5.5 ms–1 for 3 times 1 minute. In between, the samples were cooled on ice. Then the samples were heated at 95°C for 15 minutes and shaken by hand every 5 minutes. Samples were centrifuged for 5 minutes at 4°C to collect the supernatant. Fresh lysis buffer was added to the lysate tube and the samples underwent the same steps of mechanical disruption again in order to obtain a higher yield. Afterwards, the corresponding supernatants were pooled. Then, 10 M ammonium acetate was added to each lysate tube, mixed and incubated on ice for 5 minutes. After centrifugation at 4°C for 10 minutes the pellet was discarded. Samples were mixed 1:1 with isopropanol and were incubated on ice for 30 minutes. After centrifugation for 15 minutes, the supernatant was removed by decanting. The pellet was
washed with 500 ml 70% ethanol for 2 minutes and was air-dried after removal of most of the ethanol. The nucleic acid pellet was dissolved AE buffer (200 ml per sample) overnight at 4°C. DNA purity was measured on the NanoDrop 2000, a UV-Vis Spectrophotometer.

The extracted DNA was subsequently amplified with ITS2 primers for eukaryotic (fungal) DNA (ITS3 and ITS4) (14). For bacteria, primers covering the hypervariable V3 and V4 region of bacterial 16S rRNA genes were used (15, 16). The length per read was around 465 bases for bacteria and around 345 bases for fungi.

**Visualization of the biofilm using FISH and CLSM**
The biofilm of the fluoroplastic part of the valve and of the silicone hinge was visualized using FISH and CLSM. The explanted prosthesis was transferred into sterile PBS (phosphate buffered saline, 0.15M, pH 7.3), fixed within 24 hours in 4%-paraformaldehyde solution in PHEM-buffer (0.2M, pH 6.9) and stored at 4°C. After 24 hours the prosthesis was conserved in an ethanol/PBS (1:1) solution until the time of analysis. During this procedure, the prosthesis was stored at -20°C. For analysis, the valve of the prosthesis was cut in four thin slices using a surgical blade and glued onto glasses with a silicone gel. A plastic ring was glued around each slide to enclose the later applied probe and buffer. Subsequently, the glass slides were fixed in 96% ethanol for 10 min. To increase permeability of the bacterial cell membrane Labmix enzyme mixture was used prior to hybridization (17). Subsequently, FISH was performed with two DNA probes, i.e. a rhodamine-labeled EUB338 probe and a fluorescein-isothiocyanate (FITC)-labeled EUK516 probe. The EUB338 probe is specific for bacteria and provides a red signal. The EUK516 probe is specific for eukaryotes and provides a green signal. The slices on the glass slides were hybridized in 50 µl of pre-warmed hybridization buffer (0.9M NaCl, 20 mM Tris, pH 7.2, and 0.01% SDS) containing both probes (5 ng/µl each). Subsequently, the slides were incubated at 50°C in a dark chamber and hybridized overnight. To remove unbound probes, the slides were washed in a washing buffer (50°C; 0.9 M NaCl, 20 mM Tris, pH 7.2) for 15 minutes. Then, the slides were cleaned with Millipore water and dried with compressed air. Vectashield (Vector Laboratories, Burlingame, CA, USA) was applied for fluorescence. To visualize Eukaryotes (Candida) and bacteria after hybridization we used a confocal laser scanning microscope (model LEICA TCS SP2; Leica Microsystems Heidelberg GmbH, Heidelberg, Germany).

**Statistical analysis**
For the description of patients, tumor and prosthesis characteristics, descriptive statistics were performed. Software that was used to analyze the data received from IPES, included PANDaseq (18), QIIME and ARB (19). Principal component analysis (PCA) was performed to find clusters of similar groups of samples or species. PCA is an ordination method based on multivariate statistical analysis that maps the samples into a reduced number of relevant dimensions of variability. The Simpson index was used as a measure of microbial diversity.
Non-parametric tests were used, as microbial abundances are never or rarely normally distributed. Mann-Whitney U, Spearman ρ or Wilcoxon tests were used as indicated. All tests were two-tailed and a p < 0.05 was considered to indicate statistical significance. All statistical analyses were performed using IBM® SPSS® Statistics 20.0.

RESULTS

Thirty-three voice prostheses were analyzed of 22 patients (18 males and 4 females). Some patients had multiple replacements during the study period and were thus included two (n=5) or three times (n=3) in this study. The mean age at time of TL was 56.5 years (± 10.1 years) and at time of the (first) Provox ActiValve prosthesis replacement 68.8 years (± 9.7 years). Patients underwent a TL for several indications: 8 patients underwent a TL as primary treatment of larynx cancer, 17 patients as a salvage procedure after primary treatment with radiotherapy for larynx or hypopharynx cancer or after total thyroidectomy for a papillary thyroid cancer (n=1). In 6 patients a TL was performed because of a second primary tumor and in 1 patient for a dysfunctional larynx after primary treatment with chemoradiotherapy. The following Provox ActiValve prostheses were used: Light (n=17), Strong (n=15) and XtraStrong (n=1) in the sizes 4.5, 6, 8, 10 and 12.5 mm. The median device lifespan was 168 days (range 5 to 738). All patient and prostheses characteristics are shown in Table 1a and 1b. In the majority of patients the prosthesis was removed because of leakage through the prosthesis (see Tables 1a and 1b for all reasons). Median follow-up time from TL until (last) replacement was 161 months (range 3 to 249).
## Table 1a. Patient and prosthesis characteristics (Illumina paired-end sequencing)

<table>
<thead>
<tr>
<th>No.</th>
<th>Gender</th>
<th>Age¹</th>
<th>TN-classification primary tumor</th>
<th>Primary tumor</th>
<th>Indication for TL</th>
<th>RT² pre- or postoperatively?</th>
<th>Magnetic force in PAV⁴</th>
<th>Size of PAV⁵</th>
<th>Device lifetime (days)</th>
<th>Reason of replacement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>69</td>
<td>T4N0</td>
<td>Transglottic</td>
<td>Primary</td>
<td>Yes, post-TL</td>
<td>Light</td>
<td>4.5 mm</td>
<td>260</td>
<td>Leakage through the prosthesis</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>64</td>
<td>T1N0</td>
<td>Hypopharynx</td>
<td>2nd primary</td>
<td>Yes, pre-TL</td>
<td>Strong</td>
<td>12.5 mm</td>
<td>301</td>
<td>Overgrowth of biofilm</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>42</td>
<td>T1bNX</td>
<td>Glottic</td>
<td>Salvage</td>
<td>Yes, pre-TL</td>
<td>Strong</td>
<td>10 mm</td>
<td>332</td>
<td>Demonstration during Provox course</td>
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<td>4</td>
<td>Male</td>
<td>47</td>
<td>T2N0</td>
<td>Glottic</td>
<td>Salvage</td>
<td>Yes, pre-TL</td>
<td>Strong</td>
<td>12.5 mm</td>
<td>251</td>
<td>No leakage*</td>
</tr>
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<td>5</td>
<td>Male</td>
<td>62</td>
<td>T4N0</td>
<td>Transglottic</td>
<td>Primary</td>
<td>Yes, post-TL</td>
<td>Strong</td>
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<td>T3N0</td>
<td>Supraglottic</td>
<td>Primary</td>
<td>Yes, post-TL</td>
<td>Strong</td>
<td>8 mm</td>
<td>132</td>
<td>Overgrowth of biofilm</td>
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<tr>
<td>7</td>
<td>Male</td>
<td>65</td>
<td>T2aN0</td>
<td>Glottic</td>
<td>Salvage</td>
<td>Yes, pre-TL</td>
<td>Strong</td>
<td>6 mm</td>
<td>157</td>
<td>Inadequate sizing</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>43</td>
<td>T4N2a</td>
<td>Supraglottic</td>
<td>2nd primary</td>
<td>Yes, pre-TL</td>
<td>Strong</td>
<td>6 mm</td>
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<td>Leakage around the prosthesis</td>
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<td>9</td>
<td>Male</td>
<td>43</td>
<td>T4N2a</td>
<td>Supraglottic</td>
<td>2nd primary</td>
<td>Yes, pre-TL</td>
<td>Strong</td>
<td>6 mm</td>
<td>&lt; 10**</td>
<td>Leakage through the prosthesis</td>
</tr>
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<td>10</td>
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<td>41</td>
<td>T2N0</td>
<td>Glottic</td>
<td>2nd primary</td>
<td>Yes, pre-TL</td>
<td>Light</td>
<td>8 mm</td>
<td>5</td>
<td>Leakage through the prosthesis</td>
</tr>
</tbody>
</table>

¹ Age was calculated at time of TL
² TL = Total laryngectomy
³ TNM classification according to the American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC) staging manual
⁴ RT = Radiotherapy
⁵ PAV = ProvoxActiValve
* No leakage, this patient went abroad and received a free ProvoxActiValve XtraStrong
** The exact date of insertion of the voice prosthesis remained unknown. We knew however that the device lifetime was within 10 days. For calculating the median device lifetime we used a device lifetime of 9 days for this patient
# Table 1b. Patient and prosthesis characteristics (Fluorescence In Situ Hybridization and Confocal Laser Scanning Microscopy)

<table>
<thead>
<tr>
<th>No.</th>
<th>Gender</th>
<th>Age at TL¹</th>
<th>TN-classification primary tumor²</th>
<th>Primary tumor</th>
<th>Indication for TL</th>
<th>RT¹ pre- or postoperatively?</th>
<th>Magnetic force in PAV³</th>
<th>Size of PAV</th>
<th>Device lifetime (days)</th>
<th>Reason of replacement</th>
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<td>57</td>
<td>T4N0</td>
<td>Glottic</td>
<td>Salvage</td>
<td>Yes, pre-TL</td>
<td>Light</td>
<td>6 mm</td>
<td>168</td>
<td>Leakage through/around the prosthesis</td>
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<td>Male</td>
<td>47</td>
<td>T2N0</td>
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<td>Salvage</td>
<td>Yes, pre-TL</td>
<td>Strong</td>
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<td>Salvage</td>
<td>Yes, pre-TL</td>
<td>Light</td>
<td>6 mm</td>
<td>177</td>
<td>Leakage around the prosthesis</td>
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<td>4</td>
<td>Male</td>
<td>64</td>
<td>T1N0</td>
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<td>Strong</td>
<td>12.5 mm</td>
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<tr>
<td>5</td>
<td>Male</td>
<td>43</td>
<td>T2N2c</td>
<td>Hypopharynx</td>
<td>Salvage</td>
<td>Yes, pre-TL</td>
<td>Light</td>
<td>8 mm</td>
<td>311</td>
<td>Leakage through/around the prosthesis</td>
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<td>Primary</td>
<td>Yes, post-TL</td>
<td>Light</td>
<td>4.5 mm</td>
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<td>Strong</td>
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<td>Yes, post-TL</td>
<td>Light</td>
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<td>Light</td>
<td>6 mm</td>
<td>280</td>
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<td>Hypopharynx</td>
<td>2nd primary</td>
<td>Yes, pre-TL</td>
<td>Strong</td>
<td>10 mm</td>
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<td>Inadequate sizing</td>
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<td>Dysfunctional larynx</td>
<td>Yes, pre-TL*</td>
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<td>23</td>
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<td>Supraglottic</td>
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<td>Light</td>
<td>8 mm</td>
<td>112</td>
<td>Leakage through the prosthesis</td>
</tr>
</tbody>
</table>

¹ Age was calculated at time of TL
² TL = Total laryngectomy
³ TNM classification according to the American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC) staging manual
⁴ RT = Radiotherapy
⁵ PAV = Provox ActiValve
* This patient also received chemotherapy concomitant with radiotherapy
Composition and diversity of the biofilm using IPES

A total of 10 Provox ActiValve voice prostheses were analyzed.

Fungal populations

Figure 3 shows an overview of the proportion per fungus of all fungal species. Six species in total were found: Candida albicans, Candida dubliniensis, Candida glabrata, Candida tropicalis, Candida xylopsoci and Saccharomyces cerevisiae. Both on the fluoroplastic and on the silicone material, Candida albicans and Candida tropicalis were the dominant populations. Figure 4 shows the Simpson index for fluoroplastic and silicone material respectively as a measure of microbial diversity. An increasing Simpson Index indicates increasing microbial diversity. For almost all prostheses, the microbial diversity is higher on the silicone material. Microbial diversity is significantly lower on the fluoroplastic material (p = 0.017, Wilcoxon test); Candida albicans or Candida tropicalis are the main species present on the fluoroplastic material, as is depicted in Figure 3.

Figure 3. Histogram of all the fungal populations on the fluoroplastic and silicone material. F indicates fluoroplastic and S indicates silicone material. The number in front of F or S indicates the number of the voice prosthesis. These numbers correspond with the numbers in Table 1a. Both on the fluoroplastic and on the silicone material, Candida albicans and Candida tropicalis were the dominant populations.
Biofilm formation on the Provox ActiValve

Figure 4. Simpson index of the fungal species on fluoroplastic and silicone material. An increasing Simpson index indicates a higher diversity and more equal distribution of fungal species. For almost all prostheses, the microbial diversity is higher on the silicone material. Each number in the graph represents a voice prosthesis.

Bacterial populations
The most dominant bacterial species are presented in Figure 5. Especially L. gasseri is highly prevalent on most prostheses. L. gasseri and L. johnsonii were grouped together as they cannot be distinguished from one another using 16S rRNA sequencing. The Streptococcus genus colonized the fluoroplastic material to a lesser extent than the silicone material (p = 0.047, Wilcoxon test). On most prostheses L. gasseri tended to be relatively abundant on the fluoroplastic material when compared to the silicone material (p = 0.059, Wilcoxon test). Similarly, bacterial diversity usually tended to be lower on the fluoroplastic material (p = 0.14). PCA analysis clearly shows the inverse relationship between the abundance of L. gasseri and microbial diversity (Figure 6). It should furthermore be noted that PCA analysis demonstrates that one sample pair (sample 6) represented an extreme outlier. Removal of this outlier results in all of the above described patterns reaching statistical significance; p = 0.008 for the increase of L. gasseri, p = 0.011 for the decrease in streptococcal abundance and p = 0.038 for the decrease in diversity.
Figure 5. Histogram of the dominant bacterial populations. F indicates fluoroplastic and S indicates silicone material. The number in front of F or S indicates the number of the voice prosthesis. Note that numbers do not count to 100% because we only show the most dominant bacterial populations. These numbers correspond with the numbers in Table 1a. Especially *L. gasseri* is highly prevalent on most prostheses. The *Streptococcus* genus colonized the Fluoroplastic material to a lesser extent than the silicone material.

Figure 6. Difference in diversity between fluoroplastic and silicone material analysed with the principal component analysis. The biofilm on fluoroplastic material of most prostheses is less diverse, with a subsequent increase in the proportion of *L. gasseri* and *L. Johnsonii* together. PC = principal component.
Visualization of the biofilm using FISH and CLSM

A total of 23 Provox ActiValve voice prostheses were analyzed. In 11 out of 23 voice prostheses the biofilm on the fluoroplastic material was visualized. It was not possible to visualize the biofilm of the other 12 voice prostheses because there was too little biofilm (7 prostheses), the valve in the prosthesis was lost (probably during processing; 3 prostheses) or the prosthesis was frozen (2 prostheses). Because it has already been shown by several authors that Candida-species grow into the silicone material (4,5), we focused on the fluoroplastic material. To allow comparison with earlier reported results, visual analysis of the biofilm formation on silicone material was performed in two of the Provox ActiValve prostheses.

The median lifespan of the 23 voice prostheses was 177 days (range 14 to 738 days). On most prostheses, the biofilm on the valve was visible to the naked eye. Figures 7 and 8 show images of the biofilm on the fluoroplastic valve and the silicone material after different prosthesis lifespans. In both figures the bacteria (red signal) are located ‘on’ the yeasts (green signal). In the fluoroplastic material no ingrowth of the biofilm was found in any of the specimens (Figure 7). In contrast, the silicone material does show ingrowth of Candida-species as in-growing bags of yeast colonies without visual hyphae, as can be seen in Figure 8.

Figure 7. Confocal laser scanning microscopy images of the fluoroplastic material of the Provox ActiValve after fluorescence in situ hybridization with the rhodamine-labeled EUB338 probe (red signal, specific for bacteria) and a fluorescein-isothiocyanate (FITC)-labeled EUK516 probe (green signal, specific for eukaryotes). A-D: no ingrowth of the Candida-species was observed for any of the fluoroplastic material-samples. A: a 311 (prosthesis 5) day old biofilm. B: a 112 (prosthesis 23) day old biofilm without ingrowth of Candida-species. C: a 136 (prosthesis 10) day old biofilm. D: a 543 (prosthesis 1) day old biofilm.
Figure 8. Confocal laser scanning microscopy image of the silicone material of the Provox ActiValve after fluorescence in situ hybridization with the rhodamine-labeled EUB338 probe (red signal, specific for bacteria) and a fluorescein-isothyocyanate (FITC)-labeled EUK516 probe (green signal, specific for eukaryotes). A 311 (prosthesis 5) day old biofilm.

DISCUSSION

The Provox ActiValve is “a problem-solving device” for those patients requiring frequent replacements (every few weeks) because of biofilm overgrowth or inadvertent opening of the valve during swallowing or inhalation (9). As already clinically proven, the Provox ActiValve has a longer device lifespan than the Provox2 (10, 11). In the present study, we could confirm the hypothesis that Candida-species do not destroy the fluoroplastic valve material of the Provox ActiValve. This is most likely due to the nature of the material, which, as has been shown in this study, is not permeable by Candida-species. Thus, patients requiring frequent replacements of their usual voice prosthesis because of leakage through the prosthesis can benefit from the Provox ActiValve. Nevertheless, the silicone material of the body and hinge of the Provox ActiValve prosthesis can still be damaged or destroyed by Candida-species, as has been published before (5), ultimately leading to failure of the valve mechanism and transprosthetic leakage, which in this series also proved the main reason for its replacement.

We further found that, although the overall composition of the biofilm on both material components is about the same, the diversity of bacterial and fungal species is lower on the fluoroplastic material. On both the fluoroplastic and the silicone material the predominant bacterium was L. gasseri and the predominant fungi were Candida albicans and C. tropicalis. With regard to the bacteria, the abundance of L. gasseri had increased on the fluoroplastic material relative to other bacterial species - or, more precisely, the other bacterial species had decreased in abundance. The fungal diversity was also lower on the fluoroplastic material and usually only C. albicans or C. tropicalis can be found. Buijssen et al also found that L.
gasseri was the predominant bacterium on silicone material (5). Lactobacilli are common bacteria in the normal oral cavity and account for about 1% of cultivable oral microbiota (20). Their presence on voice prostheses is thus not surprising. This also holds for Candida-species, which are normal commensals of humans and have already been identified as the most important causative species for failure and/or destruction of the silicone valve (5).

The head and neck region is a non-sterile environment. Bacteria and fungi belonging to the oral microbiota include lactobacilli, streptococci, staphylococci and Candida. Voice prostheses become rapidly colonized by these organisms that subsequently develop into a biofilm. The species in the biofilm are embedded within a self-produced matrix of extracellular material. C. albicans in particular is a dominant fungus in the biofilm. Candida species however do not exist alone in a biofilm and are thought to interact with the dominant bacteria: streptococci, staphylococci and lactobacilli. Candida changes morphologically and forms hyphae. These hyphae form the organisms’ virulence and invasiveness. It has been suggested that lactobacilli in combination with Candida reduce the thickness of the biofilm in vitro, which possibly extends the lifespan of the device (5).

To visualize the biofilm we used FISH and CLSM. These methods have already been used by Buijssen et al (2012) to visualize the biofilm on silicone material (5). FISH is especially suitable for the identification of multiple species in a biofilm. For visualization of the biofilm it was logical to opt for CLSM, an optical microscope with a laser beam; CLSM has the ability to control the depth of the field, to reduce background information and to collect serial optical sections from thick samples. The latter was very useful in the present study, because the fluoroplastic material was difficult to cut into thin slices.

For the identification of bacterial and fungal species IPES was used. This is the first time this technique was used to analyze microbial communities by combining amplicons sequencing of the bacterial 16S rRNA gene and the eukaryotic ITS regions on these voice prostheses. In the present study, the combined analysis in a single Miseq run turned out to be quite successful and is now preferred for analysis of microbial diversity.

This study clearly shows that fluoroplastic material is not susceptible to destruction by Candida-species. This might be useful in the further improvement of the durability of voice prostheses.

In conclusion, the fluoroplastic valve components of the Provox ActiValve appear not to be susceptible for ingrowth and destruction by Candida-species. Furthermore, although the composition of the biofilm on both material components of the Provox ActiValve is not significantly different from the composition of the biofilm on silicone voice prostheses, there is less diversity in the biofilm on the fluoroplastic material. These findings provide evidence of material-technical progress in voice prosthesis development.
REFERENCES


