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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

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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.

Methods. Thirty-three dysfunctional Provox ActiValves (collected 2011–2013). Biofilm analysis was performed with Illumina paired-end sequencing (IPES), assessment of biofilm-material interaction with fluorescence in situ hybridization (FISH), and confocal laser scanning microscopy (CLSM).

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. *Lactobacillus 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.

Conclusion. Fluoroplastic material of Provox ActiValve seems insusceptible to destruction by *Candida* species, which could help improve durability of voice prostheses. © 2015 Wiley Periodicals, Inc. *Head Neck* 38: E432–E440, 2016

KEY WORDS: voice rehabilitation, total laryngectomy, voice prosthesis, biofilm, *Candida*

INTRODUCTION

Total laryngectomy is still an important treatment option for advanced stage laryngeal cancer and is often the only remaining curative choice for recurrence after chemoradiotherapy. After total laryngectomy, 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 1-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 utilizing a silicone prosthesis is superior in terms of quality and intelligibility. Op de Coul et al¹ (2000), for instance, reported a success rate with respect to voice quality (fair to excellent rating) of 88%. 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 total laryngectomy.¹ A variety of voice prostheses, mostly made out of silicone rubber, have been developed in the past few decades (eg, 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

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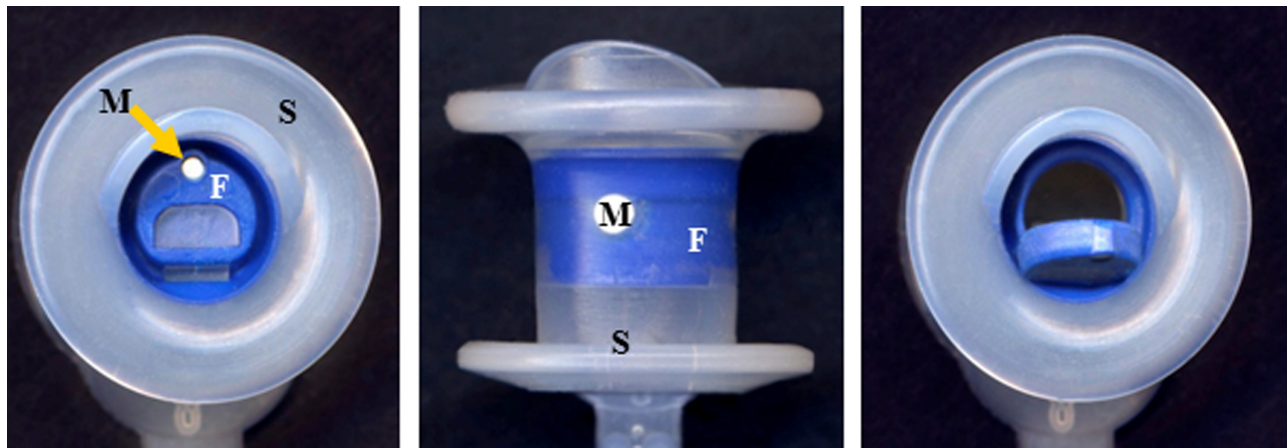


FIGURE 1. Overview of the Provox ActiValve voice prosthesis. S, silicone material; F, fluoroplastic blue material; M, magnets. The arrow points at the magnet (M).

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 (see 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 3 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 al⁵ 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. 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.

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



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.

TABLE 1. Patient and prostheses characteristics (Illumina paired-end sequencing).

No.	Sex	Age, y* at TL	TNM classification primary tumor [†]	Primary tumor	Indication for TL	RT preoperatively or postoperatively	Magnetic force in PAV	Size of PAV	Device lifetime, d	Reason for replacement
1	Male	69	T4N0	Transglottic	Primary	Yes, post-TL	Light	4.5 mm	260	Leakage through the prosthesis
2	Male	64	T1N0	Hypopharynx	2nd primary	Yes, pre-TL	Strong	12.5 mm	301	Overgrowth of biofilm
3	Male	42	T1bNX	Glottic	Salvage	Yes, pre-TL	Strong	10 mm	332	Demonstration during Provox course
4	Male	47	T2N0	Glottic	Salvage	Yes, pre-TL	Strong	12.5 mm	251	No leakage [‡]
5	Male	62	T4N0	Transglottic	Primary	Yes, post-TL	Strong	6 mm	106	Leakage around the prosthesis
6	Female	56	T3N0	Supraglottic	Primary	Yes, post-TL	Strong	8 mm	132	Overgrowth of biofilm
7	Male	65	T2aN0	Glottic	Salvage	Yes, pre-TL	Strong	6 mm	157	Inadequate sizing
8	Male	43	T4N2a	Supraglottic	2nd primary	Yes, pre-TL	Strong	6 mm	20	Leakage around the prosthesis
9	Male	43	T4N2a	Supraglottic	2nd primary	Yes, pre-TL	Strong	6 mm	<10 [§]	Leakage through the prosthesis
10	Male	41	T2N0	Glottic	2nd primary	Yes, pre-TL	Light	8 mm	5	Leakage through the prosthesis

Abbreviations: TL, total laryngectomy; RT, radiotherapy; PAV, Provox ActiValve.

* Age was calculated at time of TL.

[†] TNM classification according to the American Joint Committee on Cancer/Union for International Cancer Control staging manual.

[‡] No leakage, this patient went abroad and received a free Provox ActiValve XtraStrong.

[§] The exact date of insertion of the voice prosthesis remained unknown. We knew, however, that the device lifetime was within 10 days. To calculate the median device lifetime, we used a device lifetime of 9 days for this patient.

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 Illumina paired-end sequencing

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) 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 3 times for 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. Afterward, 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 µl 70% ethanol for 2 minutes and was air-dried after removal of most of the ethanol. The nucleic acid pellet was dissolved in AE buffer (200 µl per sample) overnight at 4°C. DNA purity was measured on the NanoDrop 2000, Thermo Fisher Scientific Inc, Wilmington, DE, USA, 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

TABLE 2. Patient and prostheses characteristics (fluorescence in situ hybridization and confocal laser scanning microscopy).

No.	Sex	Age, y* at TL	TNM classification primary tumor [†]	Primary tumor	Indication for TL	RT preoperatively or postoperatively	Magnetic force in PAV	Size of PAV	Device lifetime, d	Reason for replacement
1	Male	57	T4N0	Glottic	Salvage	Yes, pre-TL	Light	6 mm	168	Leakage through/around the prosthesis
2	Male	47	T2N0	Glottic	Salvage	Yes, pre-TL	Strong	12.5 mm	137	Leakage through/around the prosthesis
3	Male	50	T2N0	Supraglottic	Salvage	Yes, pre-TL	Light	6 mm	177	Leakage around the prosthesis
4	Male	64	T1N0	Hypopharynx	2nd primary	Yes, pre-TL	Strong	12.5 mm	148	Unknown
5	Male	43	T2N2c	Hypopharynx	Salvage	Yes, pre-TL	Light	8 mm	311	Leakage through/around the prosthesis
6	Male	69	T4N0	Transglottic	Primary	Yes, post-TL	Light	4.5 mm	518	Leakage through/around the prosthesis
7	Male	50	T1bNX	Glottic	Salvage	Yes, pre-TL	Strong	10 mm	543	Leakage through the prosthesis
8	Female	59	T4N0	Supraglottic	Primary	Yes, post-TL	Strong	6 mm	731	Leakage through the prosthesis
9	Male	48	T2N0	Glottic	Salvage	Yes, pre-TL	Light	10 mm	26	Leakage through/around the prosthesis
10	Female	56	T3N0	Supraglottic	Primary	Yes, post-TL	Light	8 mm	136	Prosthesis distorted
11	Female	78	T2N0	Glottic	Salvage	Yes, pre-TL	Light	4.5 mm	70	Leakage through/around the prosthesis
12	Male	44	T4N0	Glottic	Salvage	Yes, pre-TL	XtraStrong	4.5 mm	388	Infected fistula
13	Male	47	T2N0	Glottic	Salvage	Yes, pre-TL	Strong	12.5 mm	180	Leakage through the prosthesis
14	Male	64	T2N0	Glottic	Salvage	Yes, pre-TL	Light	8 mm	311	Leakage through the prosthesis
15	Male	55	T4N0	Transglottic	Salvage	Yes, pre-TL	Light	6 mm	280	Unknown
16	Male	64	T1N0	Hypopharynx	2nd primary	Yes, pre-TL	Strong	10 mm	14	Inadequate sizing
17	Female	78	T2N0	Glottic	Salvage	Yes, pre-TL	Light	4.5 mm	136	Leakage through the prosthesis
18	Male	58	T4aN1	Supraglottic	Primary	Yes, post-TL	Light	8 mm	21	Unknown
19	Female	61		Papillary thyroid	Salvage	No	Light	6 mm	43	Leakage through the prosthesis
20	Male	57	T4N0	Glottic	Salvage	Yes, pre-TL	Light	6 mm	189	Unknown
21	Male	55	T3N2	Nasopharynx	Dysfunctional larynx	Yes, pre-TL [‡]	Strong	6 mm	334	Leakage through/around the prosthesis
22	Male	73	T2N0	Glottic	Salvage	Yes, pre-TL	Light	6 mm	738	Leakage through the prosthesis
23	Female	56	T3N0	Supraglottic	Primary	Yes, post-TL	Light	8 mm	112	Leakage through the prosthesis

Abbreviations: TL, total laryngectomy; RT, radiotherapy; PAV, Provox ActiValve.

* Age was calculated at time of TL.

[†] TNM classification according to the American Joint Committee on Cancer/Union for International Cancer Control staging manual.

[‡] This patient also received chemotherapy concomitant with RT.

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 fluorescence in situ hybridization and confocal laser scanning microscopy

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 phosphate buffered saline (0.15 M, 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/phosphate buffered saline (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 into 4 thin slices

using a surgical blade and glued onto glass slides 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 minutes. To increase permeability of the bacterial cell membrane, Labmix enzyme mixture was used before hybridization.¹⁷ Subsequently, FISH was performed with 2 DNA probes (ie, 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 (0.9 M NaCl, 20 mM Tris, pH 7.2, and 0.01% sodium dodecyl sulfate) containing both probes (5 ng/µl each). Subsequently, the slides were incubated at 50°C in a dark chamber and hybridized

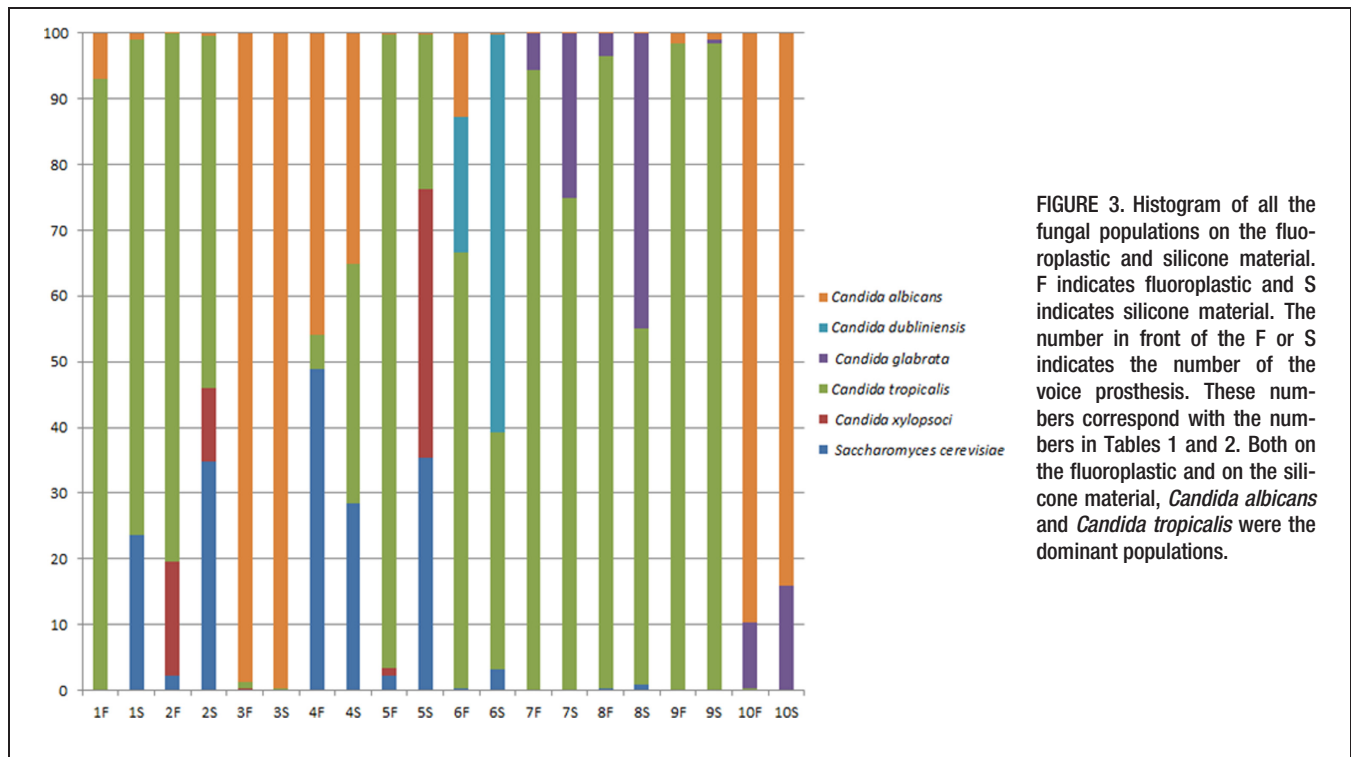


FIGURE 3. Histogram of all the fungal populations on the fluoroplastic and silicone material. F indicates fluoroplastic and S indicates silicone material. The number in front of the F or S indicates the number of the voice prosthesis. These numbers correspond with the numbers in Tables 1 and 2. Both on the fluoroplastic and on the silicone material, *Candida albicans* and *Candida tropicalis* were the dominant populations.

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) 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, tumors, 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 was performed to find clusters of similar groups of samples or species. Principal component analysis 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. Nonparametric 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 2-tailed and a $p < .05$ was considered to indicate statistical significance. All statistical analyses were performed using IBM SPSS Statistics 20.0 (IBM, Armonk, NY).

RESULTS

Thirty-three voice prostheses were analyzed of 22 patients (18 men and 4 women). Some patients had multiple replacements during the study period and were thus included 2 ($n = 5$) or 3 times ($n = 3$) in this study. The mean age at the time of total laryngectomy was 56.5 years (± 10.1 years) and at the time of the (first) Provox ActiValve prosthesis replacement 68.8 years (± 9.7 years). Patients underwent a total laryngectomy for several indications: 8 patients underwent a total laryngectomy as

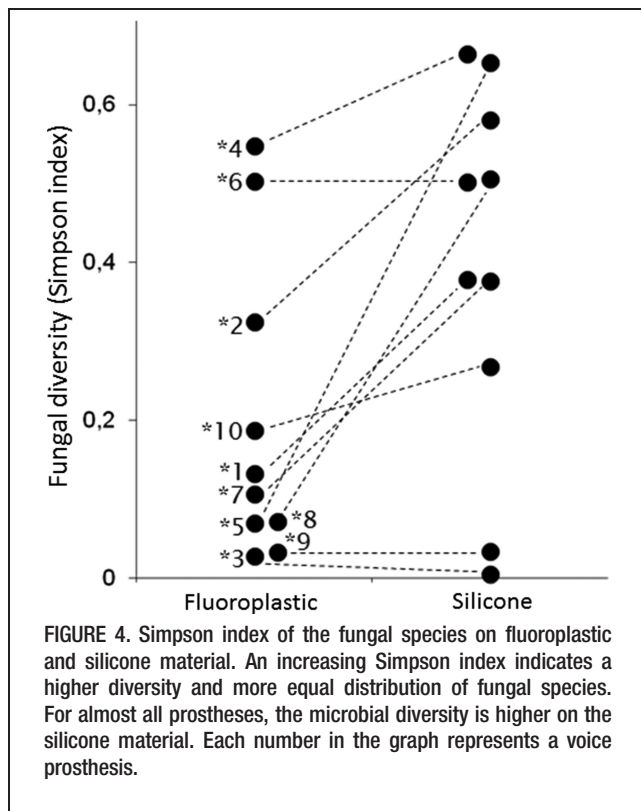
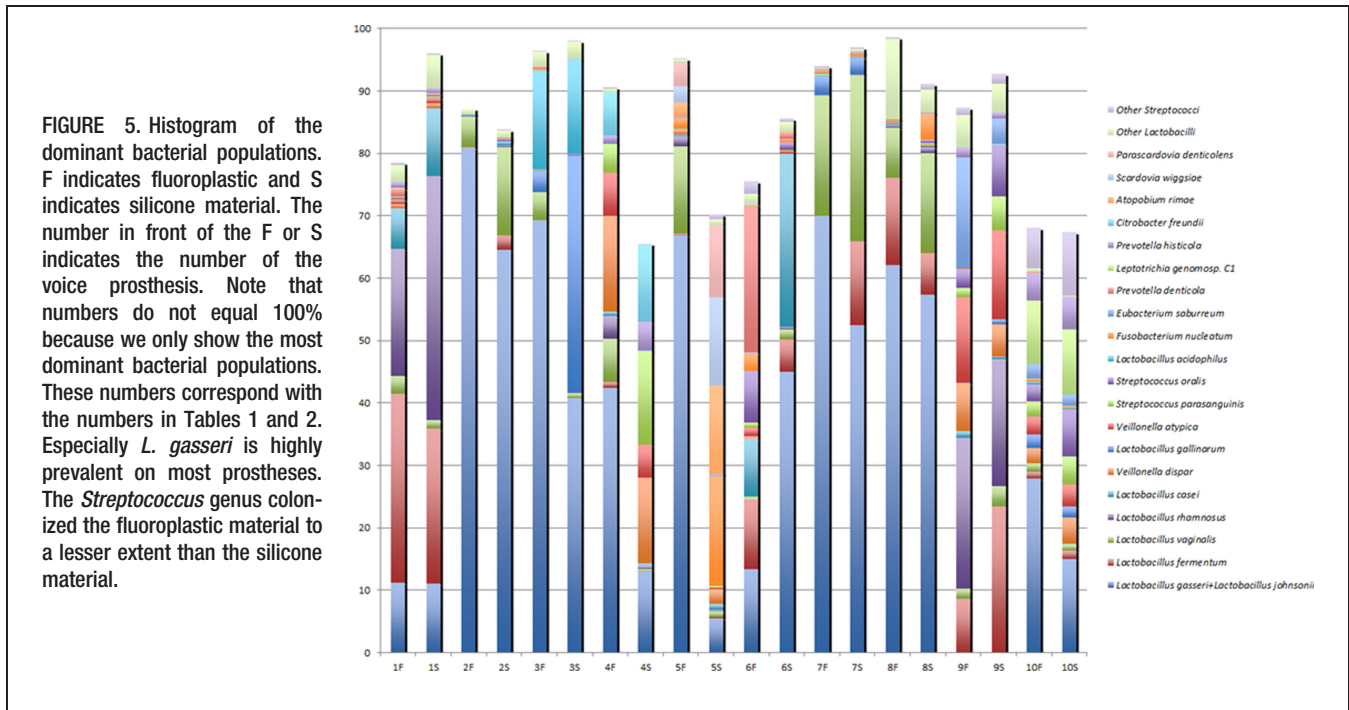
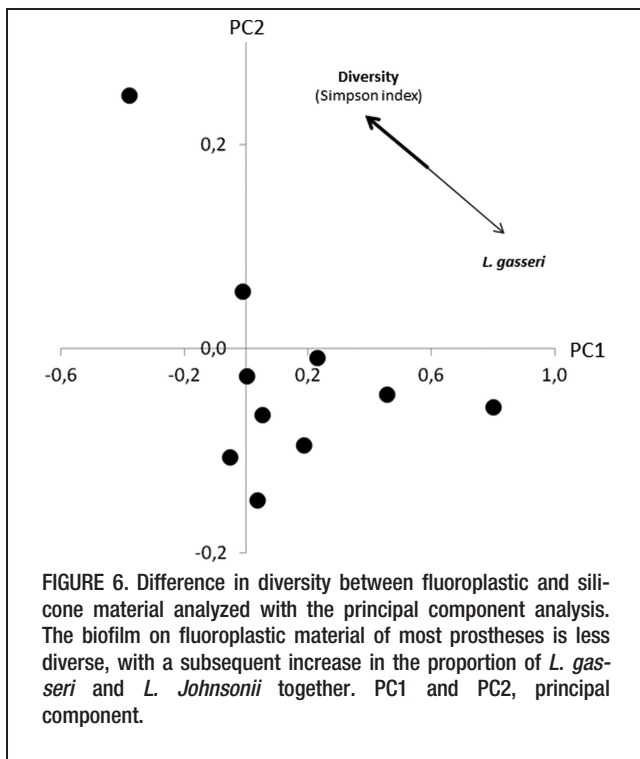


FIGURE 4. Simpson index of the fungal species on fluoroplastic and silicone material. An increasing Simpson index indicates a higher diversity and more equal distribution of fungal species. For almost all prostheses, the microbial diversity is higher on the silicone material. Each number in the graph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



primary treatment of laryngeal cancer; 17 patients as a salvage procedure after primary treatment with radiotherapy for laryngeal or hypopharyngeal cancer or after total thyroidectomy for a papillary thyroid cancer ($n = 1$). In 6 patients, a total laryngectomy 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, 5–738 days). All patient and prostheses characteristics are shown in Tables 1 and 2. In the majority of patients, the prosthesis was removed because of leakage through the prosthesis (see Tables 1 and 2 for all reasons). Median follow-up time from total laryngectomy until (last) replacement was 161 months (range, 3–249 months).



Composition and diversity of the biofilm using Illumina paired-end sequencing

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 ($p = .017$, Wilcoxon test); *Candida albicans* or *Candida tropicalis* are the main species present on the fluoroplastic material, as is depicted in Figure 3.

Bacterial populations

The most dominant bacterial species are presented in Figure 5. Especially *L. gasseri*, which is highly prevalent on most prostheses. *L. gasseri* and *L. johnsonii* were

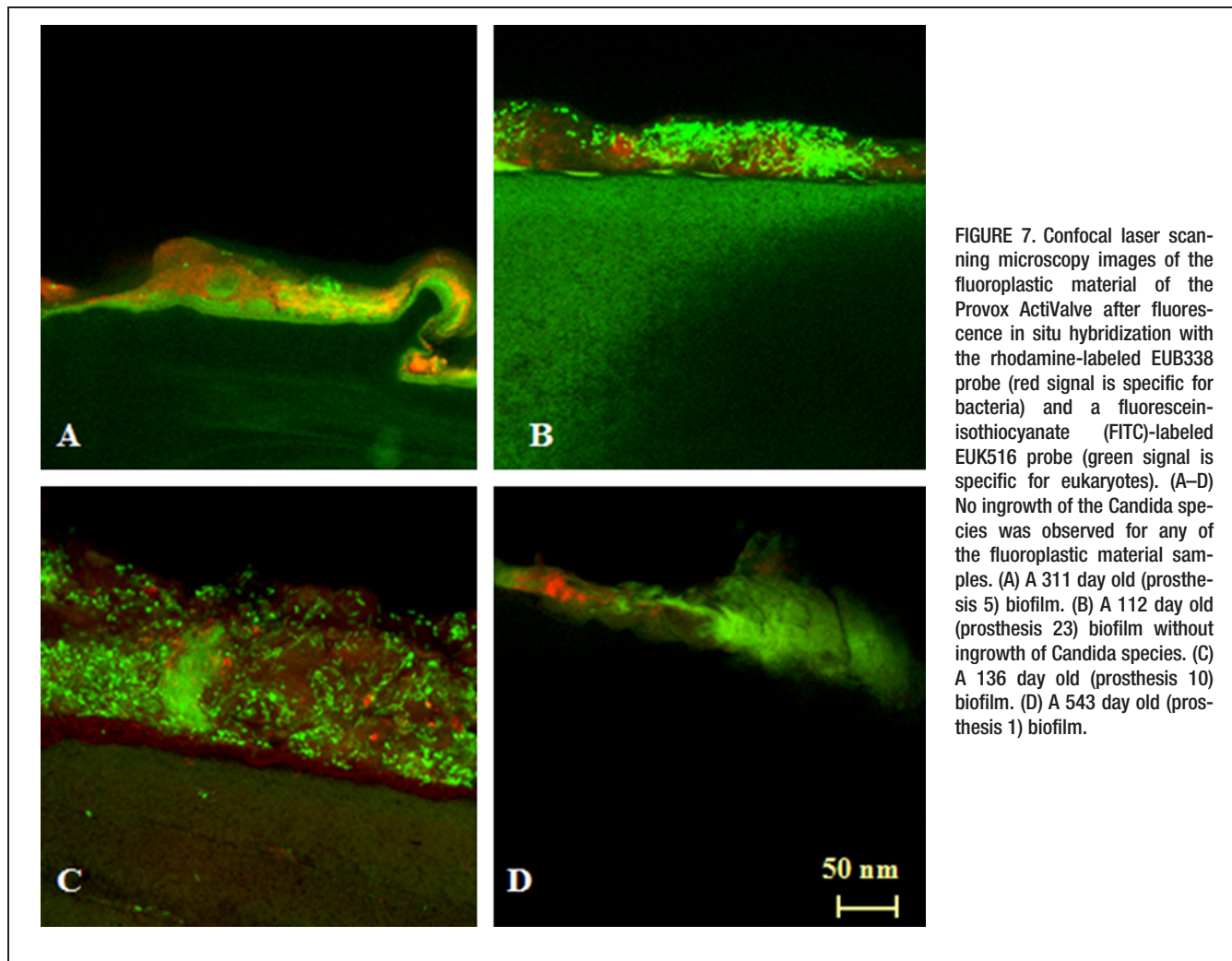


FIGURE 7. Confocal laser scanning microscopy images of the fluoroplastic material of the Provox ActiValve after fluorescence in situ hybridization with the rhodamine-labeled EUB338 probe (red signal is specific for bacteria) and a fluorescein-isothiocyanate (FITC)-labeled EUK516 probe (green signal is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

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 ($p = .047$, Wilcoxon test). On most prostheses, *L. gasseri* tended to be relatively abundant on the fluoroplastic material when compared to the silicone material ($p = .059$, Wilcoxon test). Similarly, bacterial diversity usually tended to be lower on the fluoroplastic material ($p = .14$). Principal component analysis clearly shows the inverse relationship between the abundance of *L. gasseri* and microbial diversity (see Figure 6). It should furthermore be noted that principal component analysis demonstrates that 1 sample pair (sample 6) represented an extreme outlier. Removal of this outlier results in all of the above described patterns reaching statistical significance; $p = .008$ for the increase of *L. gasseri*, $p = .011$ for the decrease in streptococcal abundance, and $p = .038$ for the decrease in diversity.

Visualization of the biofilm using fluorescence in situ hybridization and confocal laser scanning microscopy

A total of 23 Provox ActiValve voice prostheses were analyzed. In 11 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 2 of the Provox ActiValve prostheses.

The median lifespan of the 23 voice prostheses was 177 days (range, 14–738 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 (see Figure 7). In contrast, the silicone material does show ingrowth of *Candida* species as ingrowing bags of yeast colonies without visual hyphae, as can be seen in Figure 8.

DISCUSSION

The Provox ActiValve is “a problem-solving device” for those patients requiring frequent replacements (every

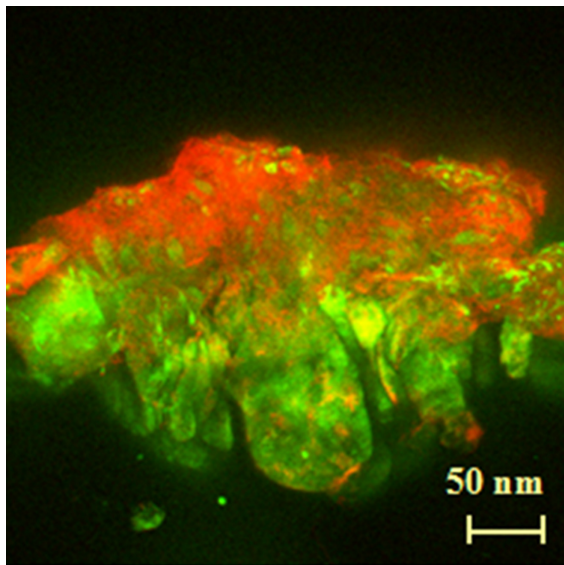


FIGURE 8. Confocal laser scanning microscopy image of the silicone material of the Provox ActiValve after fluorescence in situ hybridization with the rhodamine-labeled EUB338 probe (red signal is specific for bacteria) and a fluorescein-isothiocyanate (FITC)-labeled EUK516 probe (green signal is specific for eukaryotes). A 311 day old (prosthesis 5) biofilm.

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 because of 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 al⁵ also found that *L. gasseri* was the predominant bacterium

on silicone material. 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 nonsterile 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 al⁵ (2012) to visualize the biofilm on silicone material. 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 seem 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.

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