

Biofilm assessment on voice prosthesis used for vocal rehabilitation in laryngectomized patients

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ABSTRACT

Background. Voice rehabilitation in patients undergoing total laryngectomy is fundamental for the social reinsertion of the patient. The gold standard to fulfill this objective is the implantation of a voice prosthesis (VP). The main limitation of VP use is the blockage with fluids and deterioration due to biofilm colonization, which leads to frequent replacements.

Objectives. The purpose of the study is to identify the microorganisms that develop within the biofilm colonizing the collected VPs surfaces and the implantation situ and to determine whether that biofilm formation is the main reason for deterioration of VP proper function, leading to frequent replacements of VP.

Material and methods. In our retrospective observational study we assessed the biofilm composition of 41 dysfunctional VP from 10 laryngectomized patients by collecting samples for microbiologic tests. The morphology of the biofilm was also observed using scanning electron microscopy, a precious tool for providing an extensive understanding of the microbial colonizing the behavior of the medical device.

Outcomes. The microbiologic evaluation revealed a complex, mixed bacterial-fungal multispecies composition and the average time of exploitation for each VP was established. Deterioration of the silicone properties contributes to a large extent to the malfunction of VP.

Conclusions. Further research in the medical engineering field and innovative approaches are expected to improve the device lifespan, by proving anti-biofilm properties and thus enhancing the quality of life for laryngectomized patients.

Keywords: laryngeal cancer, voice prosthesis, biofilm

INTRODUCTION

Total laryngectomy is the gold standard in the treatment of far-advanced stages of laryngeal malignancies

and the method of choice in the situation of recurrence after primary organ preservation treatment. Consequently, the patient loses the physiological function of

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speech, associated with social hardship. Thus, vocal rehabilitation is fundamental to improve their quality of life.

The most successful method of voice rehabilitation in laryngectomized patients is the implantation of voice prosthesis (VP) in the situ created by the tracheoesophageal puncture (TEP). The technique was developed by Eric Blom and Mark Singer in the late 1970s and resided in creating a simple tracheoesophageal puncture between the posterior wall of the tracheostomy and the upper esophagus, inserted the one-way silicone valve [1]. The tracheoesophageal speech is based on rerouting the exhaled air towards the pharynx by means of a surgically established, constant communication, the tracheoesophageal fistula. The tracheoesophageal fistula can be done either primarily, simultaneously with the total laryngectomy or secondary, as a postponed procedure, after healing the wound.

Biofilms were identified on ENT accessory medical devices such as endotracheal tubes, tracheostomy tubes and voice prosthesis regardless of the nature of the material, including silicone, polyvinyl chloride, sterling silver or stainless steel. Voice prosthesis, composed of silicone rubber, becomes rapidly colonized with mixed biofilms of bacteria and yeast, mostly *Candida* species, and is one of the most widely studied otolaryngologic devices. Because of the biofilm settlement, the airflow faces increased resistance and the rubber undergoes a rapid process of deterioration, requiring regular replacements due to improper function.

The structure of the majority of biofilms is represented, for more than 90%, of the matrix, whereas the microorganisms represent less than 10% of the dry mass. The biofilm cells are embedded in the extracellular material that is mostly autogenerated by the organisms, while the framework consists of the extracellular polymeric substance (EPS), a cluster of various types of biopolymers [2].

The infrastructure of biofilms is based on colonies of microorganisms that adhere to the surface, organic or inorganic, under the shield provided by the EPS. Disregarding the composition of the surface that is being colonized or even the biofilm structure, every process of biofilm development passes through five identifiable stages. The initiation of the first stage, which is reversible starts with cell migration followed by their adhesion to a surface. On condition that the environmental factors are favorable, the adhering cells trigger the biofilm formation on the surface and generate their shield based on small amounts of exopolymeric material. In the second stage, the extracellular exopolymeric substance is being secreted by the adhering cells that become now irreversibly attached to the surface, stimulating the aggregation of cells and the matrix formation. The biofilm maturation marks the third stage of devel-

opment, the architectural evolution being represented by the multi-layers display, due to the growth of colonies and water channel formation. The biofilm fulfills its highest cell density in the fourth stage, exhibiting a three-dimensional community. The process is accomplished during stage five, when the mature biofilm improves the formation and delivery of microcolonies of cells from the central community, that migrate to adjacent surfaces, expanding the colonization and then the infection to other locations [3].

The particularity of microorganisms developing in biofilms is their adaptation ability to various environmental conditions that rely on the cell-to-cell communication mechanism, namely quorum sensing (QS), which is density-dependent and consists of the contact-conditioned exchange of chemical substances and signaling connected to an electrical impulse [4]. QS functions by observing cell density through chemical signals composed of small quantities of chemical substances (often called autoinducers) that facilitates communication between bacteria to regulate the expression of genes that influence virulence, competition, pathogenicity and resistance [5].

Various microorganisms either facultative anaerobic or mandatory anaerobic, are settled in the upper respiratory tract. Most of these strains, such as species of *Haemophilus*, *Streptococcus*, or *Neisseria* genre, are hardly ever pathogen agents when placed in other environments than normally sterile sites, meaning that they do not cause infections. There are, nevertheless, opportunistic pathogens inhabiting these anatomic areas such as *Streptococcus pyogenes*, *Streptococcus pneumoniae*,

Staphylococcus aureus, *Neisseria meningitidis*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and several bacteria of the *Enterobacteriaceae* family. The neopharynx commensal flora, in addition to tracheostoma created in laryngectomized patients, are factors that promote the adherence of the listed microorganisms to the surface of VP. Moreover, different fungi species find the silicone material providing the proper ambient for their development, leading to the damage of the valve, followed by the leakage of fluids into the tracheal lumen and increased resistance to air flow during the process of voice formation. All of these factors impede the patient's speech [6].

MATERIALS AND METHODS

Our retrospective observational study took place between January 2019 and August 2021. We subscribed 10 patients from the ENT department of SUUMC Bucharest in the study, 9 men and one woman that underwent total laryngectomy. The inclusion criteria were: indication for total laryngectomy surgery in patients

with no contraindications for general anesthesia, followed by primary or secondary vocal rehabilitation. We collected 41 dysfunctional Provox voice prostheses from the 10 aforementioned laryngectomized patients. All patients were informed about the aim and methods of the study prior to the replacement procedure and all patients agreed to participate in the study. The study methods check the principles indicated in the Declaration of Helsinki, whereas the experiments were conducted in agreement with the ethical principles of the assigned institutional board. (identified with the authorization number 5/06.02.2020)

Some patients had replacement procedures prior to the start of the study – there were 13 such cases, therefore these prostheses were not included. In a particular case, the replacement was required by the enlargement of the puncture site of implantation that was resolved using the technique of purse-string suture, without losing the voice prosthesis.

Four other patients had their prosthesis implanted for the first time hence there were no secretion samples collected. All of them opted for the vocal rehabilitation by implantation of the vocal prosthesis after completing the adjuvant radiotherapy cures, the mean delay after the laryngectomy being one year.

The aim of the study was to identify the major reason for VP replacement and to evaluate the biofilm composition of the medical device and of the site implantation. The central element of the research was the microbiological assessment of samples collected from the dysfunctional voice prostheses after their removal and of the trachea-esophageal fistula respectively. The samples were tested in order to identify the colonizing microorganisms, either bacteria or fungi and to provide the antibiogram or antifungigram spectra.

We have sent the used vocal prosthesis covered with biofilm for SEM preparation. The sample was fixed for 1h in 2.5% glutaraldehyde in Phosphate-Buffered Saline (PBS), followed by 3 times gentle washing with PBS. Afterwards, the sample was incubated at room temperature for 30 minutes using the following Ethanol solutions: 70%, 90% and respectively 100%. Then, the sample was incubated for 6 minutes using each of the HMDS (hexamethyldisilazane): Ethanol solutions: 25-75 %, 50-50%, respectively 100% HMDS. At the end, the HMDS was removed, and the sample was left to dry overnight.

After the fixation process, four samples were obtained by performing sections through the silicon valve and were afterward assessed using SEM. The first section plan included the esophageal flange of the prosthesis, the second one was represented by the prosthetic content, the third one was the connecting part (or neck) of the prosthesis and the fourth one was the exterior flange.

RESULTS

The main reason for voice prosthesis replacement was the loss of function due to biofilm colonization of the medical device, followed by silicone deterioration. The accumulation of biofilm is an issue of interest due to the fact that it represents a reservoir of discharge for various microorganisms that could lead to threatening infections of the lower respiratory pathways, especially pulmonary infections.

The number of replacement visits for each patient ranged from two to nine, depending on different objective factors such as the first time of voice prosthesis placement, and subjective factors centered on patient's compliance on proper nurturing.

The mean device lifespan was 8.5 months, equal percentages of patients addressing replacement after a period of 8 to 12 months (30% for each category). In contrast, one patient had replacements every six months due to an accelerated process of voice prosthesis deterioration, which could be related to diabetes as main comorbidity.

TABLE 1. Average time of exploitation of VP for each patient

Nr. crt	Time of exploitation
1	8 months
2	8 months
3	12 months
4	9 months
5	8 months
6	12 months
7	>12 months (per primam, with no visit for revision)
8	10 months
9	12 months
10	6 months

TABLE 2. Number of visits for replacing the VP

Nr. Crt	Number of replacements
1	9
2	4
3	2
4	3
5	8
6	2
7	1 (first time)
8	4
9	3
10	2

There was a large variety of microorganisms identified on the tested vocal prosthesis, respectively, at the implantation site. Thus, 7 different bacterial species and 5 fungal species there were isolated.

The bacteria species that were identified, from both the biofilm at the site of implantation and from the vocal prosthesis, were the following, in decreasing order of frequency: *Pseudomonas Aeruginosa*, *Staphylococcus Aureus*, *Proteus Mirabilis*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Streptococcus constellatus* and *E. coli*.

All of the identified fungal strains within the biofilm samples belong to *Candida* genus-*Candida krusei*, *Candida tropicalis*, other *Candida non-Albicans species (C. Glabrata)*, *Candida albicans*.

From the total number of 23 analyzed plague secretion (tracheo-esophageal fistula), the biofilm composition consisted of a mix of bacterial and fungal species (35%), followed by an equal composition of exclusively bacteria (30% of the analyzed samples), respectively fungal (30%). No growth was identified in one sample. The detailed distribution for each bacterial and fungal species identified within the VP implantation site is summarized in Figure 1 and Figure 2, respectively.

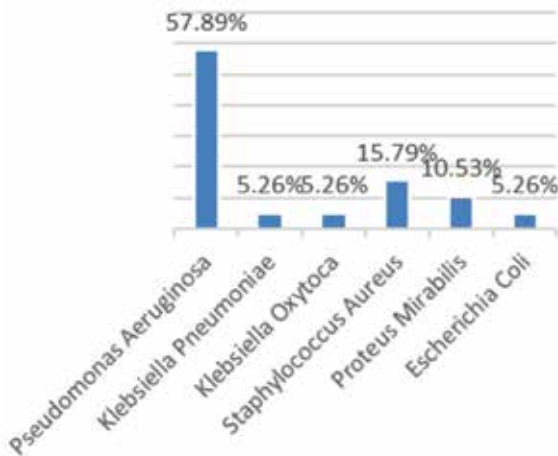


FIGURE 1. Percentage of identified bacteria within the implantation site

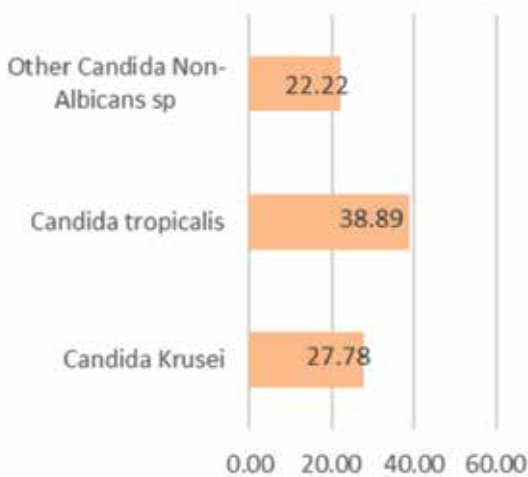


FIGURE 2. Percentage of identified fungi species within the implantation site biofilm

On the investigated voice prosthesis samples, the nature of the majority of the biofilm samples was mixed bacterial and fungal species (39%), followed by exclusively fungal colonization on 31% of samples and exclusively bacterial on other 26%, while on 4% of samples there was no growth detected.

Regarding the biofilm composition colonizing the surface of VP, we observed a similar distribution of bacterial species, compared to plague secretions results, whereas *Candida Krusei* represented the most recurrent strain of fungi. The occurrence of the most frequent species of bacteria and fungi species is illustrated in Figures 3 and 4.

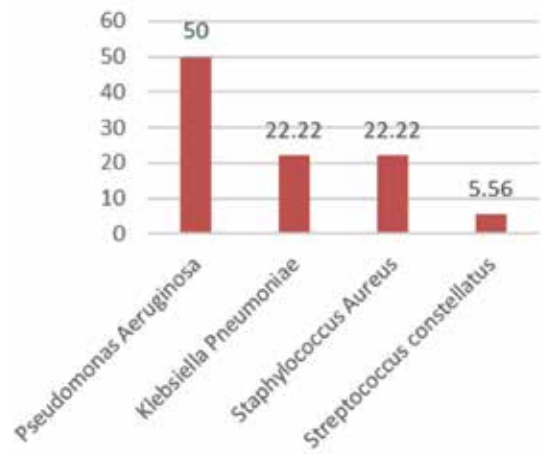


FIGURE 3. Percentage of identified bacteria within voice prosthesis biofilm

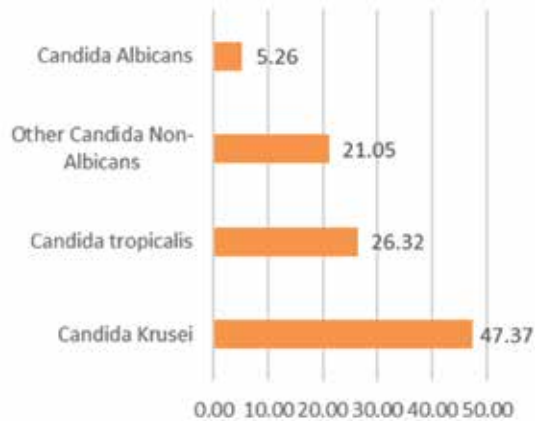
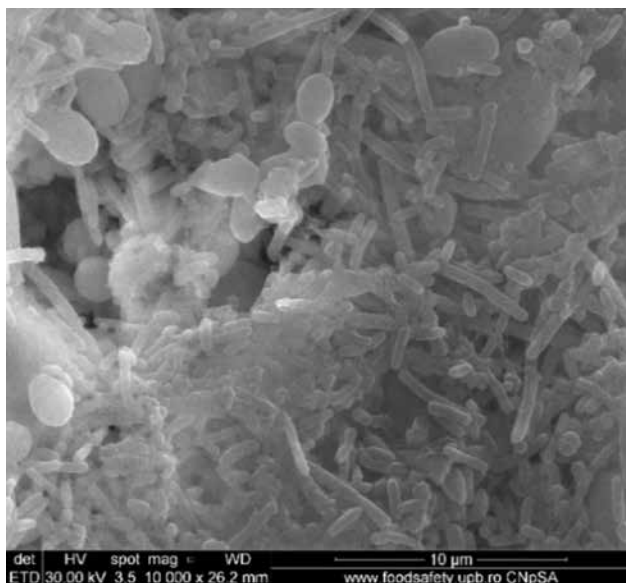
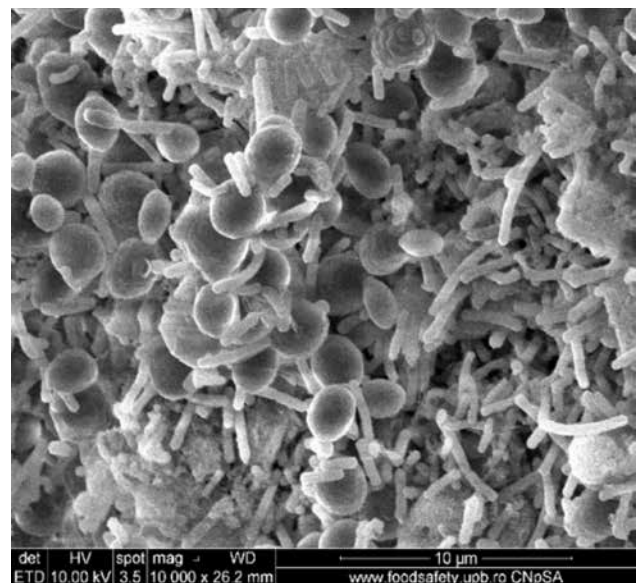


FIGURE 4. Percentage of identified fungi within the voice prosthesis biofilm

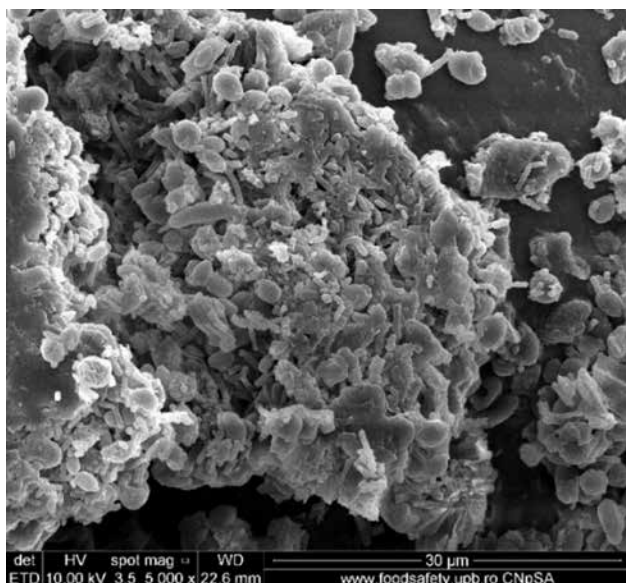
All patients used Provox prosthesis. The causes of device replacement fell within two main categories: device-related issues or fistula-related. The central device-related problem was the blockage of the prosthesis due to biofilm secretions, followed by leakage through the silicon valve, leading to an increased air-flow resistance. Leakage around the voice prosthesis, infection, or even hypertrophy of tissues surrounding



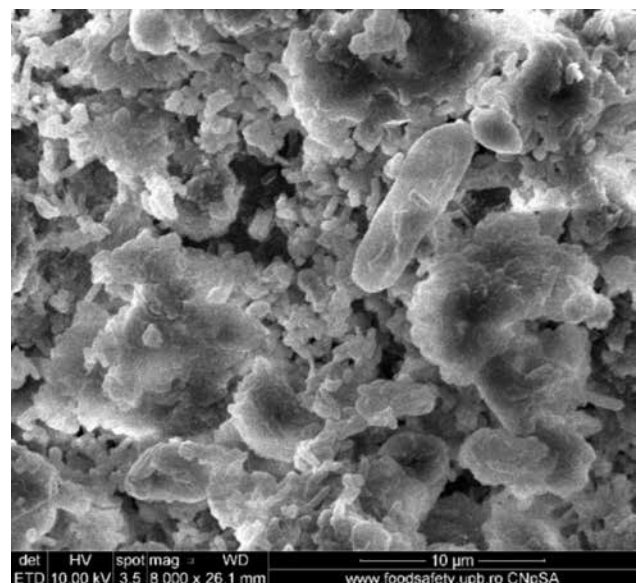
a)



b)



c)



d)

FIGURE 5. SEM morphology of biofilm structure on the surface of voice prosthesis
a) Trachea-esophageal flange, b) the content of the prosthesis c) the connecting piece (or neck) of the prosthesis, d) external (tracheal) flange

the trachea-esophageal fistula were fistula-related problems that occurred less frequently.

In Figure 5 (a-d), we can observe the density and composition of the biofilm covering the described samples of the prosthesis. One can clearly observe a dense organic biomass composed of bacteria and fungi. The rod-like morphology of the cells emphasized in all pictures is characteristic for *Pseudomonas aeruginosa* [7], while the round-shaped cells are most probably the *Staphylococcus Aureus* [8]. The aspect of the biofilm is compact due to the *Candida* fungal presence (filamentous matrix and fusiform beads) [9], however one can clearly distinguish the bacterial population within this mass. The images of the samples from the neck

piece of prosthesis and respectively the external flange emphasize the presence of another fragmented element, which can be represented by degradation residues of the silicone component of the prosthesis.

DISCUSSIONS

Our results identified that the replacement of VP was mainly due to the malfunction of the device, which is in concordance to other publications [10]. This is caused by the blockage of the valve, but also by the deterioration and deformation of the silicon surface as a primary effect of biofilm formation and hostile environmental conditions. The observation that silicon under-

goes a mechanical degradation can be explored using SEM to exhibit areas of erosion underneath the mycelites and microorganisms that adhere and are able to infiltrate into the silicone structure is in line with another conducted study [11].

This study highlighted that upper respiratory tract commensal flora expands to and develops on the polymeric surface of the VP and proliferates by producing the infrastructure of a biofilm, represented by an extracellular matrix of polysaccharides (the glycocalyx) and structural proteins [12]. Furthermore, when exposed to the biofilm, the silicone endures a degradation process that alters its structure uniformity, including its elasticity.

Other device-related complications of VP use that we encountered in our group and that are related to a similar study [13] were represented by mild local inflammatory reaction, infection or bleeding at the implantation site, or even dislodgement of the prosthesis itself caused by widening of the trachea-esophageal fistula. The use of VP was not related to pulmonary nor to systemic infection, every patient being investigated using pulmonary X-ray, without any notable modifications. This findings are in agreement with the study conducted by Spatek et al., who that did not identify any symptoms of systemic infection or aspiration pneumonia [14].

Aiming to expand the VP lifespan, we informed the patients on the requirement of constant cleaning of the prosthesis to prevent microbial colonization by removing the mucus, crust, and even food particles. Although proper nurturing of VP was addressed in other studies, it is agreed to be inadequate to prolong the device's lifetime [15]. This finding outlines the need of implementing adjuvant methods to prevent biofilm formation, as reported by other surveys [16,17].

Regarding the lifespan of the voice prosthesis, multiple studies show that the mean exploitation period ranges from 4 to 6 months [1,14,18]. Our study results, showed a mean exploitation time of 8 months, which agree with a previously conducted study on 38 VP that had an average device duration of 207 days [19]. Various other factors can influence the VP lifespan, including diet, patient's compliance and adherence to device nurture, voice expectations, and other local hostile conditions, namely GERD [20].

SEM is an excellent method to illustrate the morphology of the biofilm, by collecting high-resolution images, being a valuable tool in the evaluation of bacterial interaction EPS organization and which assists in a deeper understanding of the formation and presence of the biofilm [21]. As other studies reported, here we also observed that the esophageal flap is mostly exposed to biofilm colonization [14], due to impeded approach for manual cleaning.

The most frequently identified pathogen was the Gram-negative bacillus *Pseudomonas Aeruginosa*, found either in mono-species colonization or multi-species colonization (*Proteus Mirabilis* or *Klebsiella pneumoniae*) or even in association with different *Candida* species such as *Candida albicans* or *Candida non-albicans* such as *Candida tropicalis* and *Candida Krusei*. Our results are in agreement with other studies [22], and it is worth mentioning an interesting correlation found by Bertl et al. regarding the possible influence of oral cavity anaerobic microorganisms on VP biofilm colonization [23,24]. Although bacterial infections are more common, fungi also play an important role in infections, and become an increasing problem [25], especially in patients having comorbidities, the most important being uncontrolled diabetes, as we observed in our study, analogous to other studies [26].

The main identified limitation is that the *in vivo* biofilm which develops on the surface of medical devices is complex and has a mixed bacterial-fungal composition being an on-going process. Most *in vitro* studies focus on single or dual-species biofilms and frequently assess the incipient biofilm formation phases, up to only few hours. This observation lines up with other similar studies, and it is clear that in order to fulfill the requirement of designing novel anti-biofilm agents, more complex multi-species biofilm models must be tested.

Thus, the expensive manufacturing processes of effective but complex material improvements and testing for biocompatibility often exceed the economic potential of advanced more durable prostheses.

CONCLUSIONS

Vocal rehabilitation is a fundamental objective in laryngectomized patients, and the use of vocal prosthesis is the gold standard for this aim.

The main limiting factor for VP use is represented by the biofilm colonization of the device implanted in laryngectomized patients. The most common fungal species isolated from the VP were *Candida tropicalis*, *Candida krusei*, *Candida non-Albicans*, *Candida albicans*, while the most common bacterial species was *Pseudomonas Aeruginosa*, followed by *Staph Aureus*, *Proteus Mirabilis* and *Klebsiella pneumoniae*.

The morphology of biofilm covering different medical devices, including voice prosthesis, can be properly assessed after prior fixation and preparation using SEM. Thus, further knowledge on multi species biofilm, which is characteristic for in-vivo colonization, can be achieved.

Biofilm formation prophylaxis demands further research in order to develop more efficient methods to prevent its settlement and remains a challenge for various medical fields. Novel polymeric material used for

VPs production should focus on preventing deformation and rupture of surface during use and the design of enhanced anti-biofilm agents such as different coating

nanostructures or targeted delivery antimicrobial agents that would improve the VP exploitation time.

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