

# ORAL MICROBIOTA AND IMPLANT TYPE MEMBRANES

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## KEY WORDS

Oral implant  
Oral membranes  
Microorganisms adhesion  
*Candida* spp  
*Streptococcus* spp  
*Staphylococcus*  
*Porphyromonas* spp  
*Actinomyces* spp  
*Rhodotorula* spp

*Candida albicans* (Ca), *Staphylococcus aureus* (Sa), *Streptococcus sanguis* (Ss), *Actinomyces naeslundii* (An), *Actinomyces odontolyticus* (Ao), *Porphyromonas* spp (*P* spp), *Candida glabrata* (Cg), *Candida krusei* (Ck), and *Rhodotorula* spp (*R* spp) were tested with equal pieces of biodegradable membranes. Membranes pretreated with saliva or chlorhexidine and nontreated control membranes were tested in three different culture media containing 0.1 mL homologous suspension for each strain under study. Incubation was performed at 37°C for 48 hours for aerobiosis and for five days for anaerobiosis. Macroscopy and microscopy were carried out. Membranes were removed, washed, and resuspended. Samples were sonicated, and the supernatant was disseminated on brain heart infusion broth or blood agar. Incubation was repeated, colony-forming unit counts were performed, and statistical analysis was carried out using analysis of variance transforming results to  $\text{Log}_{10}(x + 1)$ , the highest interaction level was used to calculate standard error. Orthogonal contrast was used to compare the different microorganisms under study. Highest adhesion was found with Ca, Cg, Ck, Sa, and Ss. A sufficient quantity of *Actinomyces* could not be recovered from the membranes. Results with *P* spp were poor, confirming lower gram-negative adhesion. Replicate flasks with Ss and Ca were cultivated. Membranes were removed after washing and subjected to scanning electron microscopy, as were untreated control pieces. A cavelike surface was observed. *Streptococcus sanguis* adhering to the membranes showed extracellular projections. *Candida* and gram-positive cocci showed great recovery capacity.

## INTRODUCTION

The oral cavity is an open ecosystem that is comparable to an ongoing culture because of its humidity and temperature and the presence of nutrients. A variety of microbiota and biological elements (*ie*, teeth, mucosa, and fluids) share this environment. These biotic or

biological elements possess different immune mechanisms. Occasionally, different materials or abiotic structures are introduced into this environment. These materials do not normally cause alterations in the host *per se*; nevertheless, microorganisms colonize their surfaces, and they have no defensive response. Previous studies carried out

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at our laboratory have demonstrated the capacity of *Candida albicans* (*Ca*) to form plaque *in vitro* and adhere to acrylics, sealers, and light-cured glass ionomers.

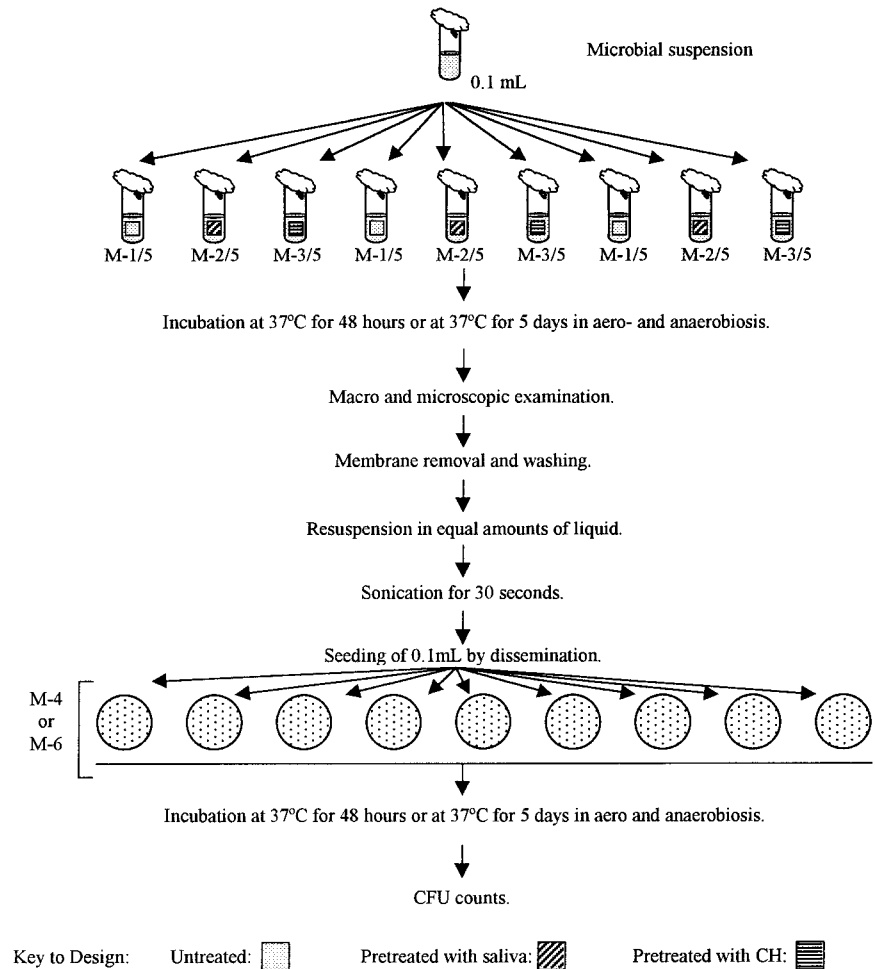
The aim of this study is to analyze the capacity of certain microorganisms that are representative of the oral biota to adhere to membranes typically used in periodontal or other types of oral surgery.

## MATERIALS AND METHODS

We used five strains of *Ca* (*Ca* 19, *Ca* 5, *Ca* 6, *Ca* 18, and *Ca* 26789) (our collection), one strain of *Staphylococcus aureus* (*Sa*) (previously isolated at our laboratory), one strain of *Streptococcus sanguis* (*Ss*) (Lafahi, supplied by CIMIPLADE), one strain of *Actinomyces naeslundii* (*An*) (FF 10) (isolated and typified by MBN), one strain of *Actinomyces odontolyticus* (*Ao*) (ATCC N 746), one strain of *Porphyromona* spp (*P* spp) (supplied by the Malbran Institute), one strain of *Candida glabrata* (*Cg*) (supplied by the Mycology Center of Buenos Aires), one strain of *Candida krusei* (*Ck*) (supplied by the Mycology Center of Buenos Aires), and one strain of *Rhodotorula* spp (*R* spp) (supplied by the Mycology Center of Buenos Aires).

The culture media consisted of basal medium plus sucrose used for *in vitro* plaque formation, brain heart infusion (BHI) broth plus 1% glucose, BHI broth plus 1% horse serum, BHI agar, hemina-menadione and vitamin K added to tioglycolate (this medium was used for *P* spp), and blood agar for anaerobic microorganisms (for *P* spp). Sterile distilled water, tioglycolate broth, saliva pool (sterilized by filtering), and clorhexidine gluconate (CH) (Plac-out, Bernabo Laboratory) were also used. We used biodegradable collagen membranes (BCMs) (Denticol, MR, Tesla Gumter, Switzerland) and pork skin membranes (PLPMs) (Pelcupron, MR, Disp MS and AS 480, Laboratorio Asmpul, Argentina).

A standardized square grid and sterile instruments were used to cut each membrane into 0.5-cm squares. Three



membranes from each group were incubated for 30 minutes in sterile saliva, and another three were incubated for 30 minutes in CH; the remaining membranes were used without any treatment.

## Procedure

Dehydrating solutions for scanning electron microscopy (SEM) were prepared as follows: microbial suspensions were prepared, 0.1 mL of which was inoculated into sets of nine tubes. Using McFarlane's metric turbidity scale, microbial suspensions equivalent to  $1.5 \times 10^7$  germs/mL were prepared (see flow chart).

*Ca*, *Sa*, *Ss*, *Cg*, *Ck*, and *R* spp were incubated at 37°C for 48 hours, whereas *P* spp, *An*, and *Ao* were incubated in anaerobiosis in a Gas-Pak jar at 37°C for 5 days. Macroscopic examination to

assess turbidity and microscopy to examine growth of seeded microorganisms were carried out. Membranes were removed and washed in 5 mL of distilled water, or tioglycolate for anaerobic strains, and were then resuspended in the same amount of liquid. Sonication in an L&R Transistor-Ultrasonic T.9 sonicator was carried out for 30 seconds to dislodge adhered microorganisms. Then, 0.1 mL of supernatant was seeded in petri dishes with BHI agar or blood agar for anaerobic microorganisms. Specimens were incubated at 37°C for 48 hours for aerobiosis and at 37°C for five days for anaerobiosis. Finally, colony-forming unit (CFU) counts were performed. Analysis of variance was performed considering the greatest level to estimate standard error; results were transformed to  $\text{Log}_{10}(X + 1)$ . Orthogonal

TABLE 1  
Analysis of Variance

Factor*	gl	SC	CM	F	p†
A	1	0.12	0.12	0.98	NS
B	2	0.29	0.15	1.21	NS
C	2	0.27	0.13	1.11	NS
D	11	280.80	25.53	212.56	<0.005
AB	2	0.30	0.15	1.26	NS
AC	2	0.07	0.04	0.30	NS
AD	11	0.83	0.08	0.63	NS
BC	4	0.47	0.12	0.98	NS
BD	22	2.81	0.13	1.06	NS
CD	22	2.74	0.12	1.04	NS
ABC	4	0.17	0.04	0.35	NS
ABD	22	2.21	0.10	0.84	NS
ACD	22	2.69	0.12	1.02	NS
BCD	44	6.43	0.15	1.22	NS
ABCD	44	5.28	0.12		
Total	215	305.48	27.09		

\*A, membranes; B, medium; C, factor alone or saliva or chlorhexidine gluconate added; D, microorganism.  
†NS, not significant.

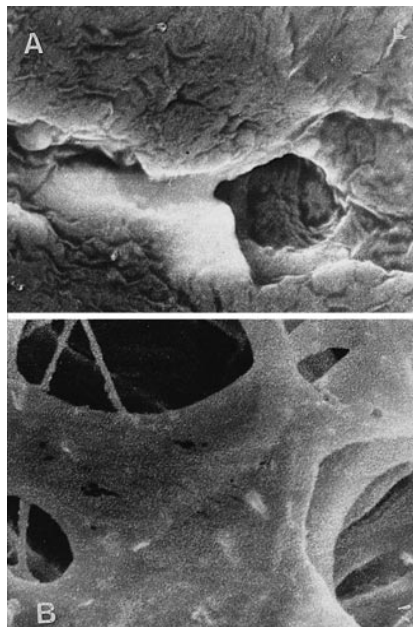


FIGURE 1. (A) Scanning electron micrograph of PCPM at ×3000. (B) Scanning electron micrograph of BCM at ×3000.

comparisons among the different groups of microorganisms were also carried out.

A replicate set of samples prepared with *Ss* and *Ca* 19 in MCB were subjected to preparation of microbial suspensions, inoculation into tubes, incubation, macroscopic and microscopic examination, and washing as described above. The membranes were then re-

moved, fixed, dehydrated, and mounted for observation by SEM, which was also used to study membranes that had not been seeded and noninoculated membranes immersed in the culture medium to determine whether the procedure caused any modification. A JSM25SII (Jeol) microscope was used at 15 kV at ×3000 and ×7000 magnification.

**RESULTS**

Two sets of the 13 strains were processed (one set with each type of mem-

brane) for a total of 234 determinations (26 determinations with nine variables each). The actual growth of the seeded microorganisms was confirmed by the microscopic examination. Macroscopic examination of samples with *Ca*, *Cg*, *Ck*, *Sa*, and *Ss* revealed considerable turbidity, whereas very little deposit was found in *R* spp, *P* spp, *An*, and *Ao* tubes. Scarce deposits of *R* spp and *P* spp evidenced growth, although the scarce quantity of CFU showed that washing dislodged the microorganisms due to their poor adhesion.

The CFU counts were somewhat difficult for certain strains, and the small differences in results could not be attributed either to the different culture media or the membrane treatment, as shown in Tables 1 and 2.

The results obtained with both types of membranes were practically the same. However, differences were found among the microorganisms regarding their ability to grow and their likely adhesion capacity.

Observation of untreated membranes by SEM revealed an uneven, cavelike surface (Fig 1). Immersion in culture medium did not alter their appearance. *Ss* shown in Fig 2 exhibit a lack of strands and bridges, and some extracellular projections (arrow) are

TABLE 2  
Contrast Orthogonal Comparison

	SC	F	p
Different <i>Candida</i> spp and <i>Streptococcus sanguis</i> versus the other	243.82	2030.18	<0.005
<i>Candida</i> spp versus <i>Streptococcus</i> spp	0.00	0.00	NS*
<i>Candida glabrata</i> versus other <i>Candida</i> spp	2.55	21.24	<0.005
<i>Staphylococcus aureus</i> versus <i>Streptococcus sanguis</i>	0.48	4.04	NS
<i>Actinomyces naeshlundii</i> and <i>Actinomyces odontolyticus</i> versus <i>Porphyromona</i> spp and <i>Rhodotorula</i> spp	33.75	281.00	<0.005
<i>Actinomyces naeshlundii</i> versus <i>Actinomyces odontolyticus</i>	0.09	0.79	NS
<i>Porphyromona</i> spp versus <i>Rhodotorula</i> spp	0.01	0.11	NS

\*NS, not significant.

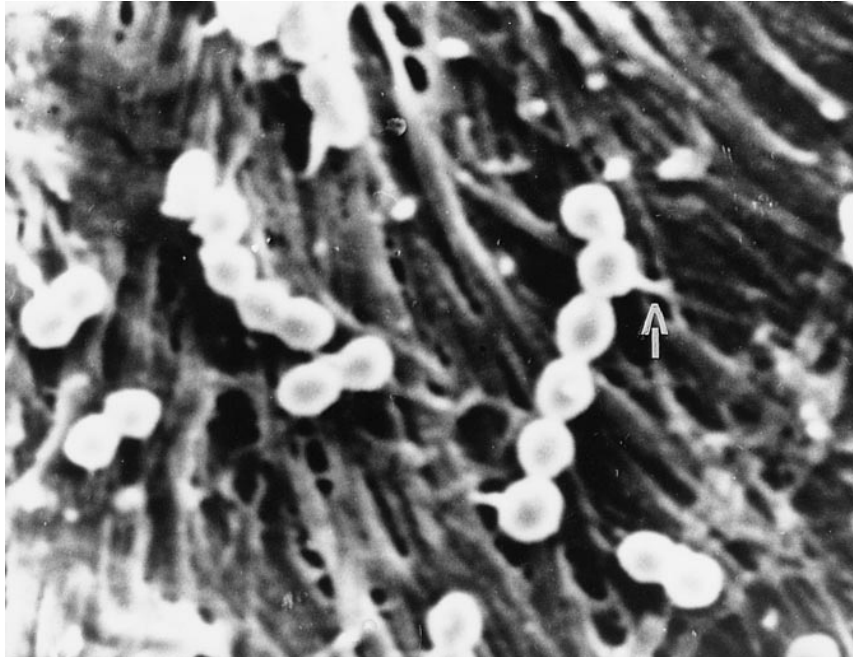


FIGURE 2. Scanning electron micrograph of *Ss* seeded in basal medium plus sucrose with untreated membrane. The arrow indicates extracellular projection at  $\times 7000$ .

present that might facilitate adherence. *Ca* fail to show these structures.

#### COMMENTS

The methodology used in this study is an original design. A considerable number of trials were needed to establish the inoculum dosage that would allow recovery of countable amounts of the different microorganisms under study.

The incubation period was calculated as microorganism generation time, and it took into account that membranes are usually placed in the mouth for more than a few hours.

An attempt was made to reproduce certain *in vivo* conditions as closely as possible. For this purpose, glucose was added to some media, since it is a usual component of most people's diets and also facilitates adhesion. Serum was added to other media to reproduce the high serum level in postsurgery conditions. To demonstrate the effect of saliva at the onset of microorganism adhesion to teeth, some of the membranes were preincubated in this fluid. Because mouth rinses are routinely prescribed for surgery patients,

particularly those undergoing periodontal surgery, some membranes were pretreated with this antiseptic, which is known for its substantivity. Sucrose added to basal medium for *in vitro* plaque formation has been used by us and by other authors to obtain microbial clumps under different culture conditions. Because of its different salt content, this medium is considered synthetic.<sup>1-3</sup>

The aim of this study was to compare results obtained using different culture media; no significant variations were found (Table 1). Previous works have demonstrated growth of *Actinomyces* and *R* spp in BHI broth, BHI agar, and basal medium, but at a slower rate.<sup>4</sup> Thus, a different culture medium was not required for their cultivation. Modification of culture media and washing liquid was required for *P* spp, since it is a strictly anaerobic microorganism. Nevertheless, very little growth was encountered.

Samples were washed before sonication to dislodge microbes that were not completely adhered. However, sonication may have failed to dislodge all the attached cells. Further subcultures

are deemed necessary to study this possibility.

Not only *Ca*, but also *Cg*, *Ck*, and *R* spp were used. Over the last few years, clinical manifestations originated by non-*Ca* have increased and have been proven to be azole resistant, thus rendering treatment difficult. It is important to point out that both *Cg* and *Ck* showed high CFU values. Very little growth of *R* spp was encountered, even though previous studies have successfully isolated it in the mouth and it is known to form abundant *in vitro* plaque.

Different species of *Candida* colonize catheters placed in patients subjected to intravenous nutrition, forming a layer commonly known as slime, which is a source of infection. A large number of works on *Candida* adhesion have shown that surface receptors similar to integrins that participate in protein binding are involved in the formation of this fungus. Other studies have shown that it can adhere to type IV collagen, laminins, and fibronectines.<sup>5-7</sup> Similar results have been obtained with strains of *Sa*, which have the same capacity and, in these cases, behaves like a virulent pathogen.<sup>8</sup>

The results obtained in this study prove yet again that gram-positive cocci, as well as *Candida*, adhere easily to membranes (Tables 1, 2; Figs 3, 4). It is likely that the characteristics of the membrane surface facilitate this mechanism, as revealed by SEM (Fig 1). These materials are not strictly abiotic but could nevertheless interfere both through their surface, which would act as a physical trap, and through their composition, which is capable of generating nutrients for microorganisms and provides sites for chemical binding.

As shown in the first list of microorganisms presented in Materials and Methods, both membranes were seeded with *Cg*. High CFU counts were obtained with PCP membranes whereas results were negative with collagen membranes. After repeating the procedure several times, we noticed that

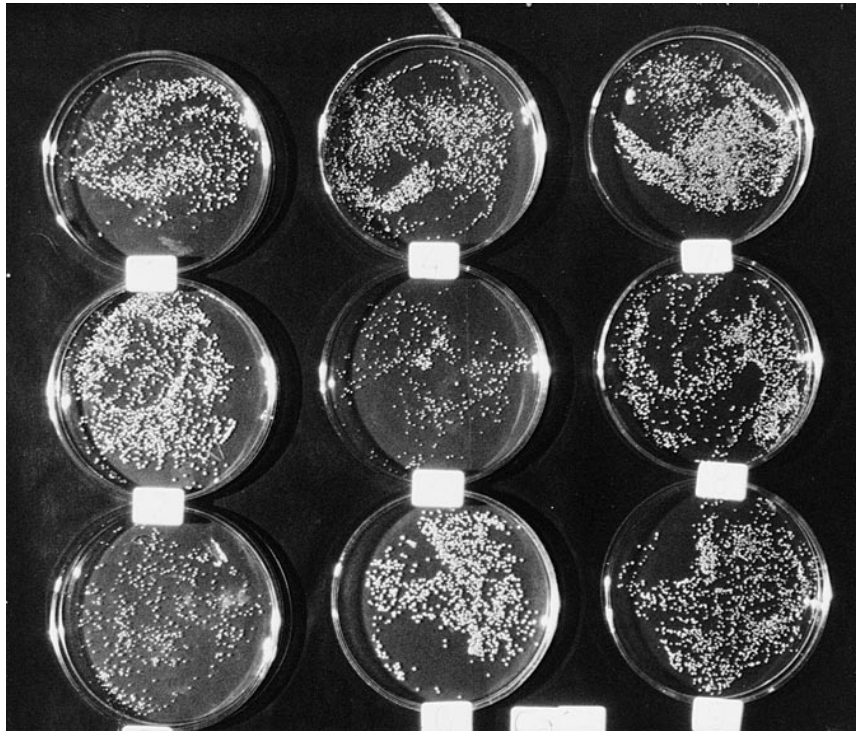


FIGURE 3. *Ca* (strain 19) seeded with BCM. 1: basal medium plus sucrose c/m alone; 2: basal medium plus sucrose c/m with saliva; 3: basal medium plus sucrose c/m with CH; 4: BHI broth plus 1% glucose c/m alone; 5: BHI broth plus 1% glucose c/m with saliva; 6: BHI broth plus 1% glucose c/m with CH; 7: BHI broth plus 1% horse serum c/m alone; 8: BHI broth plus 1% horse serum c/m with saliva; 9: BHI broth plus 1% horse serum c/m with CH.

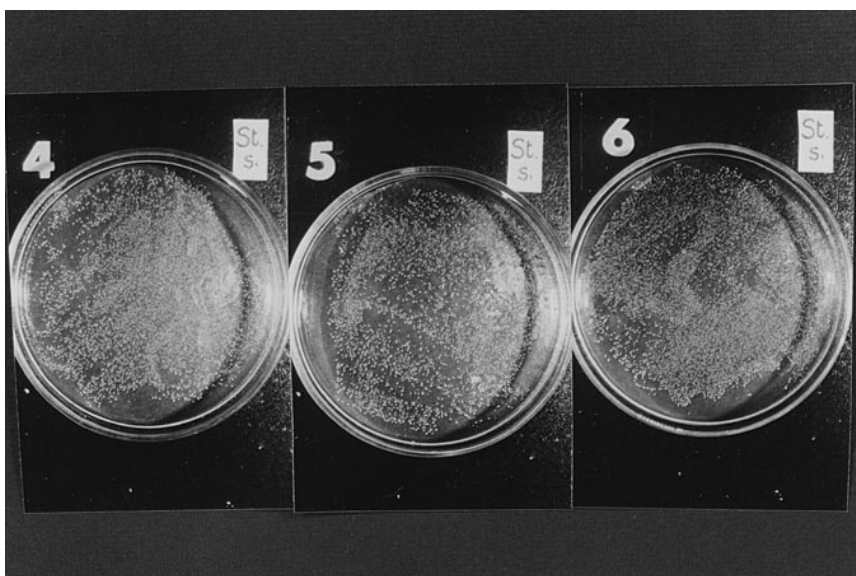


FIGURE 4. *Ss* seeded with PCPM. BHI broth plus 1% glucose c/m alone; 5: BHI broth plus 1% glucose c/m with saliva; 6: BHI broth plus 1% glucose c/m with CH.

the membranes belonged to a different batch. *In vitro* studies lead us to the conclusion that an effective antimicrobial (ATM) had been added to the membranes. Attempts to obtain information from the manufacturers were unsuccessful. This strain was not included in the statistical analysis. We believe that in our country at least, clinicians should have preference for this type of membrane with ATM agents.

The gram-negative, strictly anaerobic strain tested in this experiment demonstrated the known fact that anaerobic microorganisms are less likely to colonize these surfaces primarily. Thus, these microorganisms act as secondary colonizers inside the oral cavity. For this reason, no other types of gram-negative microorganisms were tested in this study. As previously stated, the methodology used in this study is original. In our opinion, this is the only method that will allow extrapolation of an experimental design to *in vivo* conditions.

Although it holds true that biodegradable membranes are reabsorbable, resorption does not occur within the time period used in this study. Clinical experience has shown that membranes must be removed after 16 weeks for resorption to occur. We will carry out further studies along these same lines, using other methodological variables and other types of membranes.

The results obtained in this study under these experimental conditions with these particular infectious agents would lead to the conclusion that colonization of these implants by the tested microorganisms is likely. Previous studies have shown *in vivo* colonization of polytetrafluorethylene membranes using SEM and TEM. One of these studies indicates the presence of *Treponemes* and spherical bodies, and another report found *Streptococcus*, *Actinomyces*, *Haemophilus*, and *Candida*. Patients included in both studies had received oral hygiene training and had been instructed to use CH mouth rinses.<sup>9,10</sup> Infection with *Candida* might be facilitated by antimicrobial drug ther-

apy prescribed to patients undergoing oral surgery. Consequently, these membranes could act as a source of infection to neighboring tissues. Few studies on the role played by microorganisms in implant techniques have been published. Our laboratory previously presented two cases of periimplantitis involving *Candida* sp that remitted with treatment. *Actinomyces* sp was detected in another patient with diabetes.<sup>11</sup>

Thus, we can conclude that placement of these elements requires the utmost care. A thorough preparation of the mouth is mandatory, and microbiological monitoring prior to treatment to determine possible hosting of certain species is recommended.

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