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Original Article

Enhanced susceptibility of *Candida albicans* to chlorhexidine under anoxia

Andressa Marafon Semprebom[1], Ana Cláudia Azevedo Isidoro[1], Maria Ângela Naval Machado[1], Patrícia Maria Stuelp Campelo[1], José Francisco Höfling[2], Lakshman Perera Samaranayake[3], Edvaldo Antonio Ribeiro Rosa[1]

[1] Laboratory of Stomatology, Dental School, Pontifícia Universidade Católica do Paraná (PUCPR), Curitiba (PR), Brazil
[2] Laboratory of Microbiology and Immunology, Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas (Unicamp), Piracicaba (SP), Brazil
[3] Oral Biosciences Unity, Dental School, University of Hong Kong, Hong Kong, Hong Kong SAR.

Abstract

Aim: Periodontal pockets can be colonized not only by bacteria, but also by *Candida albicans*. However, its role in periodontitis is unknown. This study evaluated the inhibitory performance of chlorhexidine digluconate under normoxic and anoxic conditions against 16 strains of *C. albicans* from periodontal pockets and other 20 from the oral mucosa. **Methods**: Strains were grown in normoxia and anoxia to adapt themselves to the different atmospheric conditions. Microdilution-based assays were carried out to determine the minimum concentrations of chlorhexidine that may restrain the conditioned candidal strains, in normoxia (normoxic MIC) and anoxia (anoxic MIC). The Mann-Whitney U test was used to evaluate the antimicrobial effect of chlorhexidine on *C. albicans* under normoxic and anoxic conditions ($\alpha = 0.05$). **Results**: The normoxic MIC of chlorhexidine varied broadly from 150 to 1200 µg/mL, whereas its anoxic MIC varied narrower from 2.34 to 37.5 µg/mL. Regarding the origins of strains, no statistically significant differences ($p > 0.05$) were found. **Conclusions**: These results indicate that anoxic environmental conditions, compatible with periodontal pockets, tend to enhance *C. albicans* susceptibility to chlorhexidine.

Keywords: *Candida albicans*, chlorhexidine, anoxia.

Introduction

Periodontitis is a multifatorial inflammatory disease process that leads to the destruction of the periodontal tissues supporting the teeth[1]. The etiologic factor of periodontitis is the dental biofilm associated or not with calculus[2]. The progression of the disease is related to gingival crevice colonization by microorganisms such as *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*, *Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia*, and *Treponema denticola*[1,3,4].

Although bacteria has a major role in the pathogenesis of periodontal disease, the yeast *Candida albicans* has also been isolated from periodontal pockets[2], with prevalence ranging from 14 to 19%[5]. In a previous study, yeasts were found in 19.7% of individuals with periodontal pockets > 7 mm and in 15.6% of subjects with pockets ≤ 7 mm[6]. This organism has important virulence factors such as proteolytic activity and capacity to adhere and invade the epithelium[7,8]. Although the presence of *C. albicans* in the periodontal pocket *per se* may not be

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Correspondence to:
Edvaldo Antonio Ribeiro Rosa
Faculdade de Odontologia, Pontifícia Universidade Católica do Paraná.
Rua Imaculada Conceição, 1155
CEP 80215901 – Curitiba (PR), Brazil
E-mail: edvaldo.rosa@pucpr.br

directly associated with periodontitis, this yeast may take part in the pathogenic microbiota of some forms of periodontitis[6].

The chemotherapeutical eradication of periodontal yeasts does not follow the protocols indicated for bacteria once they are not affected by drugs commonly used in periodontics. As no antifungal therapy is routinely used, antiseptics may play an important adjuvant role. Chlorhexidine {1,1'-hexamethylene-bis[5-(p-chlorophenyl) biguanide]}, a widely used antimicrobial agent, adversely affects the microbial eukaryotic plasma membrane by nonspecific electrostatic binding[9] to negative protein and phospholipid moieties, causing alteration in the cellular membrane structure and in the cellular osmotic balance[10,11]. Normal fungal cells have a negative internal charge[12,13] that explains their susceptibility to chlorhexidine.

Based on the premise that periodontal sites are anoxic and no prior studies investigated the inhibitory effects of chlorhexidine on *C. albicans* in such environmental condition, the present study evaluated the performance of this biguanide on periodontal *C. albicans* strains under normoxic and anoxic conditions.

Material and methods

Sampling

Sixteen periodontium-related (so called "PP" strains) strains from periodontal pockets ≥ 4 mm were used. These strains were obtained from the culture collection of Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas (Unicamp), Brazil. Twenty mucosa-related *C. albicans* isolates (so-called "OM" strains) were obtained from the culture collection of the Dental School of the Pontifícia Universidade Católica do Paraná (PUCPR), Brazil. The research project was reviewed and approved by the Ethics Committee of the second institution.

Culture media

The culture broth used throughout the study[14] contains (*per* 900 mL of distilled water) 4 g of KH_2PO_4, 3.2 g of NaH_2PO_4, 1.2 g of L-proline, and 0.7 g of $MgSO_4.7H_2O$. L-proline was replaced by 0.5 g of L-lysine and 1 g of yeast extract was added. After autoclaving, the broth received 40 mL of 20% glucose, 0.5 mL of vitamin mixture, and 0.25 mL of mineral mixture. The vitamin mixture contains (*per* 100 mL of 20% ethanol) 2 g of biotin, 20 mg of thiamine-HCl, and 20 mg of pyridoxine-HCl. The mineral mix contains (*per* 100 mL of 100 mM HCl) 0.5 g of $CuSO_4.5H_2O$, 0.5 g of $ZnSO_4.7H_2O$, 0.8 g of $MnCl_2.4H_2O$, and 0.5 g of $FeSO_4$. The vitamin and mineral mixtures were filter-sterilized with 0.22 μm pore-sized cellulose nitrate membranes (Whatman, Maidstone, UK), and stored at 4 °C. For anaerobic growth of *C. albicans*, the broth was supplemented with 200 μL of 1 mM oleic acid in 100% methanol, 200 μL of 4 mM nicotinic acid, and 1 mL of 500 mM NH_4Cl. Sterile L-cysteine was added up to 0.01%. The pH of the complete broth was 5.0. This modified broth was distributed in sterile disposable 96 wells polystyrene plates (Difco Laboratories, Detroit, MI, USA) at 100 μL *per* well and stored at -20°C.

Aerobic inoculum preparation

Both sets of strains (PP and OM strains) were inoculated in 3 mL of broth and incubated under normoxia at 37 °C for 24 h. After growth, the cells were harvested, washed three times in sterile deionized water, and suspended at 2×10^7 cells/mL. The suspensions were stored at 4°C for no more than four hours.

Anaerobic inoculum preparation

One hundred microliters of aerobic inocula were inoculated in 3 mL of modified broth and incubated in hermetically sealed jars supplied with two disposable Anaerobac® anoxia generator cartridges (Probac Co., São Paulo, SP, Brazil) at 37 °C for 48 h. One hundred milliliters of culture were transferred to 3 mL of modified broth and anaerobically incubated at 37 °C for 48 h. This procedure aimed turning the cells totally adapted to the anoxic condition. After growth, the cells were harvested, washed three times in sterile deionized water, suspended until obtaining a concentration of 2×10^7 cells/mL, and stored at 4 °C in vials whose headspaces were filled with sterile CO_2.

Susceptibility tests

The technique of broth microdilution was used for determining the minimum inhibitory concentration (MIC)[15] for chlorhexidine digluconate (Pharma Nostra Co., São Paulo, Brazil). Chlorhexidine was diluted in modified broth and transferred to microdilution plates to obtain a range of 12 wells with doubling increased concentrations ranging from 0.122 to 500 μg/mL. Each well received 10 μL of suspension of normoxic or anoxic *C. albicans*, obtaining final densities of 1×10^6 cells/mL. The microdilution plates were statically incubated at 37 °C in normoxic or anoxic atmospheres. The anoxic conditions were obtained using hermetically sealed jars supplied with two disposable Anaerobac® anoxia generator cartridges (Probac Co.) at 37°C, as stated before. The normoxic growth was monitored up to 48 h and the anoxic growth was monitored up to 72 h. The cellular growth was visually compared to the growth in wells containing only culture broth. The MIC determination assays were done in triplicate on three independent occasions.

The Mann-Whitney U test was used to evaluate the antimicrobial effect on *C. albicans* under normoxic and anoxic conditions. A p value of 0.05 was assumed as threshold for differences.

Results

During inoculum preparation, we observed that the normoxic cultures achieved an OD_{520nm} of 0.450 ± 0.030 after 24 h of incubation at 37 °C, whereas anoxic-adapted cells achieved an OD_{520nm} of 0.123 ±

0.018 after 24 h and an OD_{520nm} of 0.420 ± 0.023 after 48 h. The predominant cell shape in normoxic cultures was budding yeast-like with some cells forming pseudo-hyphae; on the other hand, most part of cells grown in anoxia was true hyphae. By multiple Gram staining, it was estimated that less than 5% of the fungal load grew as budding yeast-like cells in anoxia.

Figures 1 and 2 indicate the variation in the susceptibility performances for consensual values after nine repetitions of PP and OM strains, respectively. For the PP strains, the results showed that chlorhexidine promoted growth restrain of all *C. albicans* strains with MIC values varying from 15.62 to 125 μg/mL (mean = 70.31 ± 50.06 μg/mL) in normoxic conditions *versus* a decreased range from 0.97 to 15.62 μg/mL (mean = 8.45 ± 6.84 μg/mL) in anoxia. For the OM strains, chlorhexidine promoted growth restrain of all *C. albicans* strains with MIC values varying from 15.62 to 125 μg/mL (mean = 70.31 ± 50.06 μg/mL) in normoxic conditions *versus* a decreased range from 0.97 to 7.81 μg/mL (mean = 5.09 ± 3.18 μg/mL) in anoxia.

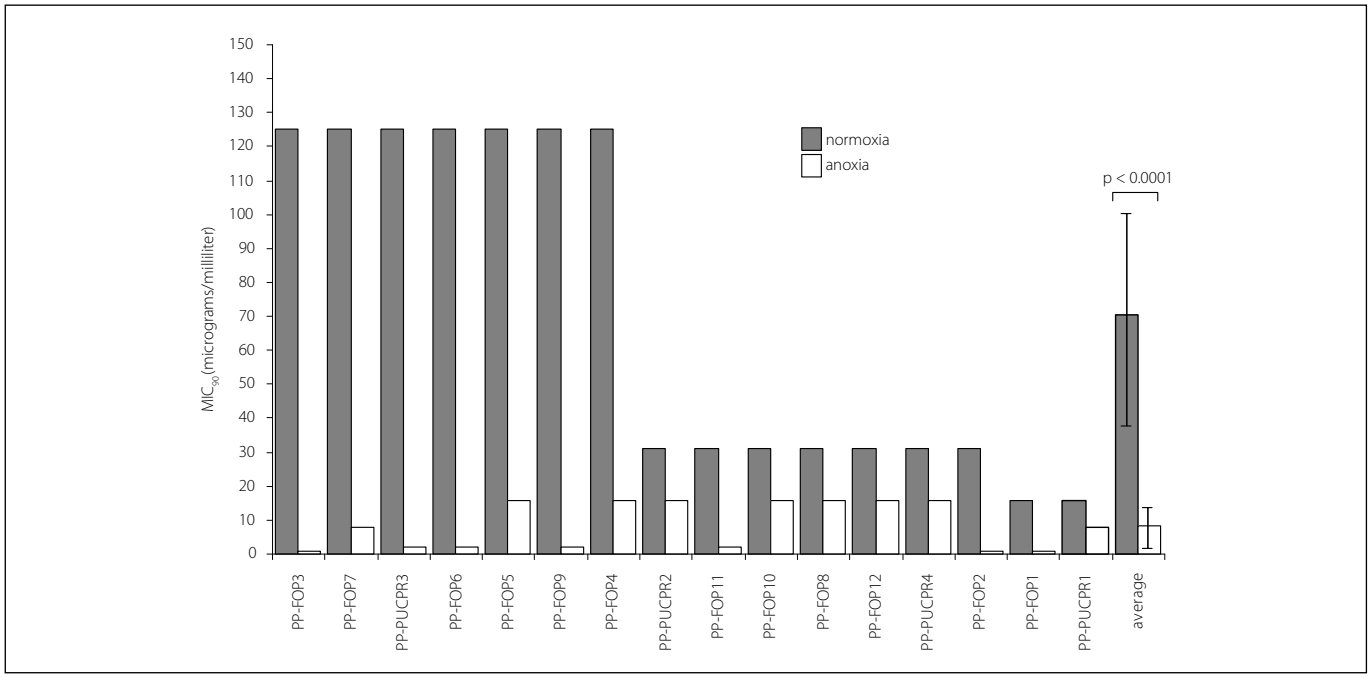

Figure 1. Minimum inhibitory concentration (MIC) of chlorhexidine digluconate for periodontium-related (PP) *Candida albicans* strains in normoxia and anoxia.

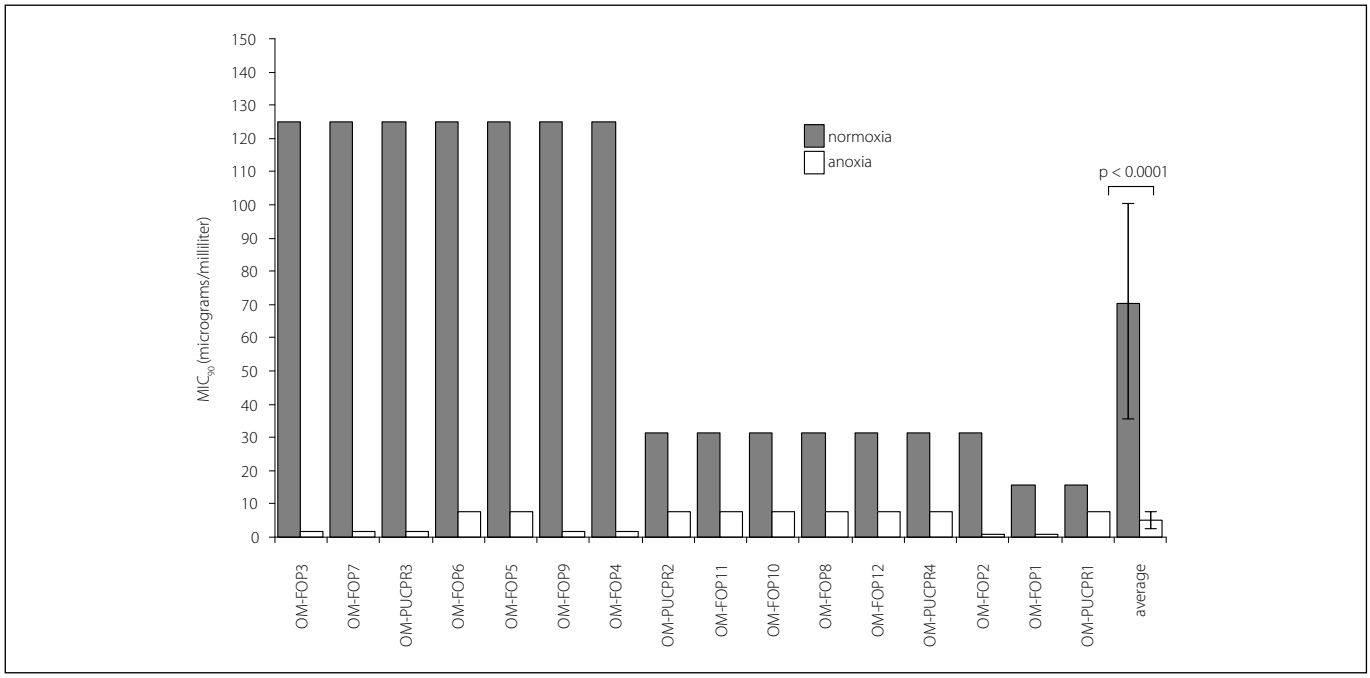

Figure 2. Minimum inhibitory concentration (MIC) of chlorhexidine digluconate for mucosa-related (OM) *Candida albicans* strains in normoxia and anoxia.

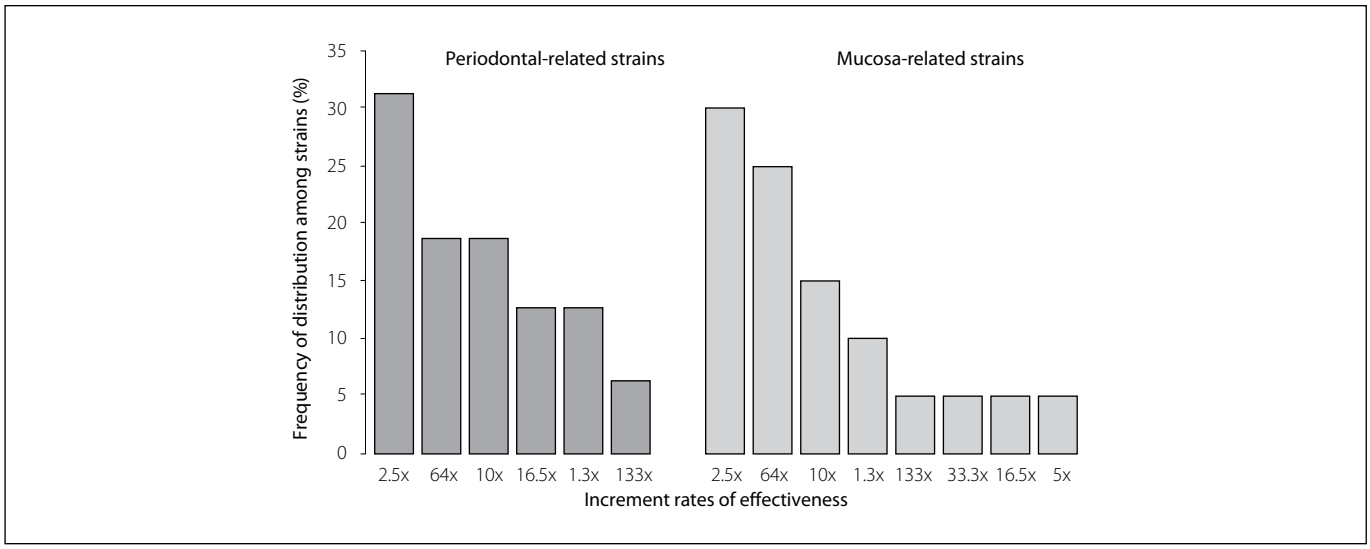

Figure 3. Increment rate of effectiveness (IRE) for minimal inhibitory concentrations (MIC) of chlorhexidine digluconate for periodontium-related (PP) and mucosa-related (OM) *Candida albicans* strains in normoxia and anoxia.

In order to determine de minimum candidacidal concentration (MCC), after the MIC determination, contents of all wells were independently recovered and transferred to tubes with 3 mL of broth without chlorhexidine. The growth in those tubes followed the same MIC assay results. Thus, it could be concluded that the MIC and MCC were the same for these strains under the experimental conditions.

In all cases, the nine values obtained (three repetitions in three independent situations) were exactly the same and no standard deviations could be noticed. The Mann-Whitney U test demonstrated a significant reduction in the resistance to chlorhexidine under anoxia. This test also showed no significant differences for MIC in relation to the anatomic origin of strains ($p = 0.9584$).

In order to evaluate the influence exerted by atmospheric oxygen on MIC performance, the increment rate of effectiveness (IRE) for each isolate was calculated through the equation: $IRE = MIC_{normoxia}/MIC_{anoxia}$. **Figure 3** shows that the IRE values for the PP strains varied from 2.0-fold to 128.8-fold, with higher frequencies of 2.0-fold (37.5%), 16-fold (18.75%), and 66.8-fold (18.75%). OM strains had their susceptibility ranging from 2.0-fold to 66.8-fold, with higher frequencies of 4.0-fold (37.5%), 66.8-fold (32.25%), and 16-fold (18.75%).

Discussion

Studies addressing the action of chlorhexidine on yeasts living in anoxia are scarce[16]. Until the present moment, no data referring to the susceptibility of PP *C. albicans* strains to antimicrobials under anoxia are available.

It has been shown that *C. albicans* cells living in anoxic environments are protected against the action of most common antimycotics[14]. This anaerobic-related resistance to antifungal probably derives from a suppression of ergosterol biosynthesis at fungal cell membrane. As the ergosterol biosynthetic pathway is the main target of azoles and such via is not used when candidal cells are under anoxia, these antimycotics completely lose their efficacy. On the other hand, as ergosterol appears not to be synthesized under anoxic conditions, polyenes do not manifest their membrane interactive behavior either. Furthermore, it has been shown that histatin-5, a potent salivary antifungal peptide, might have such effect abolished, once the mitochondrial energy level is very low when in anoxia[17,18].

In this study, the results showed that chlorhexidine had its candidacidal capacity increased in such environmental conditions. Two plausible hypotheses to explain such phenomenon are proposed.

Firstly, this increased susceptibility to chlorhexidine in an oxygen-free environment may be better discussed taking into account that oxygen is also a positively charged element[19] and has a cationic behavior. Based on this premise, we herein postulate that, in normoxia, the existing atmospheric oxygen competes with chlorhexidine for binding sites, whereas it does not occur in an anoxic environment. Plaut et al.[20] calculated the sorption enthalpy for chlorhexidine and stated that it may be mediate by electrostatic-like bonding interactions. Such interactions are weak and depend on the molecular size. Akaho and Fukumori[21] stated that an area near to 548 $Å^2$ is required for the complete absorption of chlorhexidine molecule to an amphyphylic surface in order to accommodate its ionic and hydrophobic moieties responsible for the adsorption to solid surfaces. This value is much higher than the 1.2 Å of molecular oxygen, commonly absorbed for the aerobic respiration. Additionally, the fact that the deenergization caused by the dropdown in aerobic respiration rates affects significantly the plasmatic membrane of eukaryotes leading to a reduction in the negative potentials[22-25]. Anoxic conditions cause a release of previously accumulated lipophilic cation tetraphenylphosphonium (TPP^+) into *Saccharomyces cerevisiae*[26], which is compatible with the assumption that anoxia reduces de negative feature of fungal membranes. Interestingly, the data hereby presented show that the inhibitory efficiency of chlorhexidine increases in such unfavorable environmental conditions. Our assumption that the better action of chlorhexidine in the absence of oxygen must be derived from

a disruption in the competition rates is reinforced by the fact that some cations may reduce its adsorption and antimicrobial effectiveness by competitive ways[20,27,28].

Secondly, it has been previously reported that chlorhexidine substantially increases the cell permeability on apical and sub-apical segments of early-stage filamentous forms higher than for yeast forms[29]. This is compatible with the proposed mechanism of disruption of the membrane followed by rapid permeabilization. Therefore, it is possible that fungal cells had their susceptibility have increased by the anaerobically-induced filamentation.

Interestingly, the origin of strains did not exert any influence on the susceptibility ($p = 0.9584$). However, as strains from culture collections were used in the present study, the long-term storage or the multiple re-inoculations might have decreased their susceptibility. Although the sampling have been done six months before the study (data not shown), it was not possible to ensure whether or not it actually occurred. Further studies enrolling freshly isolated strains may possibly clarify this issue.

The extensive variation observed in the increase of effectiveness rates indicates a great heterogeneity for this characteristic. Some strains presented only an increment of mere 1.3-fold susceptibility, whereas others had increased their effectiveness rates in 133 times. It may be suggested that it is variable according to the strains and no generalizations may be done.

The results of the present study are applicable for growing planktonic cells. For *C. albicans* grown in biofilms, Lamfon et al.[30] previously reported that the resistance to chlorhexidine increases up to 8-fold the MIC in relation to planktonic counterparts grown in normoxia. Such result contrasts with those of the present study since it is widely accepted that the accessibility of oxygen to internal layers of biofilms is limited. There are two points that may clarify the differences between the results from Lamfon's et al.[30] study and those expected after assuming that anoxia increases chlorhexidine effectiveness. Firstly, those authors grew their biofilms under normoxic conditions; secondly, the extracellular matrix present in the biofilm may act as a barrier against the antiseptic diffusion.

According to the results obtained in the present study, it may be concluded that chlorhexidine is effective to restrain *C. albicans* grown in the anoxic periodontal pocket with minor concentrations than those needed to kill cells living on surfaces under normoxic conditions. The origin of strains seems not to influence the growth of *C. albicans* under neither normoxia nor anoxia. However, despite these encouraging results, our opinion is that clinicians should not indicate lower chlorhexidine doses to their patients. The main advantage of lower MIC is the maintenance of inhibition effects throughout the lixiviation of chlorhexidine.

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