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Assessment of antimicrobial effect of Biosilicate® against anaerobic, microaerophilic and facultative anaerobic microorganisms

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Abstract This study assessed the antimicrobial activity of a new bioactive glass–ceramic (Biosilicate®) against anaerobic, microaerophilic, and facultative anaerobic microorganisms. Evaluation of the antimicrobial activity was carried out by three methods, namely agar diffusion, direct contact, and minimal inhibitory concentration (MIC). For the agar diffusion technique, bio glass–ceramic activity was observed against various microorganisms, with inhibition haloes ranging from 9.0 ± 1.0 to 22.3 ± 2.1 mm. For the direct contact technique, Biosilicate® displayed activity against all the microorganisms, except for *S. aureus*. In the first 10 min of contact between the microorganisms and Biosilicate®, there was a drastic reduction in the number of viable cells. Confirming the latter results, MIC showed that the Biosilicate® inhibited the growth of microorganisms, with variations between ≤ 2.5 and 20 mg/ml. The lowest MIC values (7.5 to ≤ 2.5 mg/ml) were obtained for oral microorganisms. In conclusion, Biosilicate® exhibits a wide spectrum of antimicrobial properties, including anaerobic bacteria.

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1 Introduction

For decades, several classes of materials, including metals, polymers, ceramics, and composites, have been used to repair or rebuild parts of the injured muscle-skeletal system. These materials are denominated biomaterials [1]. According to Hench and Wilson [2], biomaterials are classified according to the type of interaction they establish with live tissues. The bioactive material concept was introduced by L. L. Hench, who provided the following definition: "A bioactive material elicits a specific biological response on the interface resulting from formation of a bond between the tissue and the material." [3]. More specifically, bioactive materials are materials which, upon contact with live tissue in the presence of body fluids, produce a hydroxycarbonate apatite (HCA) layer on the surface, thereby promoting an extremely strong chemical bond between the tissue and the implant [2, 3].

This interfacial layer mimics the type of interface that is formed during natural processes of tissue reconstruction and is chemically and structurally equivalent to the bone mineral phase [3]. In the early 1970 s, bioceramic implants were introduced with the use of almost biologically inert ceramics, such as alumina [4]. Other types of bioactive materials have emerged over the years Hydroxyapatite ceramics and glass–ceramics such as Cerabone, Ceravital and Bioverit with different levels of bioactivity [5], are only able to bind to soft tissues.

In general, vitreous materials exhibit the highest bioactivity levels. However, these materials have weak mechanical properties, which prevent their widespread use as implants, particularly in situations where the mechanical demands are greater [4]. Some ceramics and glass–ceramics (also known as vitroceramics and pyrocerams) with higher bioactivity have been specially developed, but the

introduction of crystallinity, necessary for the development of materials with enhanced mechanical properties, reduces their bioactivity levels. Thus, either bioactive glasses with high bioactivity and low mechanical properties, or ceramics and vitroceramics with better mechanical properties and low bioactivity levels [5] are usually obtained.

One strategy to circumvent this limitation has been the development of new glass–ceramics, using methods for the total or partial crystallization of bioactive glasses [6]. Within this context, a fully crystallized bioactive glass–ceramic denominated Biosilicate® was developed by controlled heat treatment for use in the medical and dental areas [7]. An international patent "Process and compositions for preparing particulate, bioactive or resorbable biosilicates for use in the treatment of oral ailments" was filled (PCT/BR 2004/000015). The first studies indicated that totally crystallized Biosilicate® reaches bioactivity levels similar to that of the golden standard bioglass 45S5 developed by Hench, and higher than those achieved with any other glass–ceramics [8, 9].

Bioactive materials, such as bioglass and this new glass–ceramic may have antimicrobial potential. According to Stoor et al. [10], the dissolution of bioglass particles releases calcium and sodium ions into the solution, causing an increase in the osmotic pressure and raising the pH. These two effects, combined with the high Ca^{2+} concentration, eliminate pathogenic microorganisms in the oral environment, for instance after 60 min of contact between the bioglass and bacterial inoculum.

The antimicrobial activities of bioactive glasses have been fully reported [4, 10–18]. According to these authors, who used different evaluation methods (DCT, MIC, AD) this property arises from the intrinsic characteristics of the material or from changes in the culture media caused by material dissolution, such as osmotic pressure, pH, and composition.

Agar diffusion, direct contact, and minimum inhibitory concentration tests are the most commonly employed methods for evaluation of the antibacterial potential of a material. Several modifications have been made to screening methods, in order to obtain more reliable data. This is because such factors as culture medium composition, tested microorganism, pH, and sample solubility can alter the results [19, 20].

In this study we evaluate the antimicrobial activity of the bioactive glass–ceramic (Biosilicate®) against anaerobic, microaerophilic, and facultative anaerobic microorganisms.

2 Experimental

2.1 Sample preparation

High-purity silica, calcium carbonate, sodium carbonate, and sodium phosphate were used to obtain the bioactive glass–ceramic (Biosilicate®, Vitrovita, São Carlos, SP, Brazil) (Table 1). The materials were weighed and mixed for 30 min in a polyethylene bottle. The fusion (1250–1380°C, 3 h) occurred in an electric furnace (Rapid Temp 1710 BL, CM Furnaces Inc., Bloomfield, NJ, USA) in a Pt crucible.

The samples were poured into a stainless steel bar and pressed with another bar, to obtain glass plates. The latter were annealed at 460°C for 5 h. Then, the glass plates were submitted to several cycles of heat treatment, to promote their crystallization. The composition and thermal treatment protocol for Biosilicate® preparation are described in the patent WO 2004/074199 [7–9]. This P_2O_5-Na_2O–CaO silicate glass–ceramic (containing some other minor elements) is >99.5% crystalline.

The material was milled in order to obtain the particulate Biosilicate® in high-energy planetary mill. The resulting particle size was between 20 and 0.1 μm, with surface area of about 2.7 m^2/g.

2.2 Antimicrobial activity evaluation

Before the start of the tests, Biosilicate® powder was sterilized in dry heat at 180°C for 2 h. The clinical bacterial isolates and standard strain (acquired from the American Type Culture Collection–ATCC) employed in this study were maintained at −20°C in a freezer in the Laboratory of Research in Applied Microbiology (LaPeMA) of the University of Franca. The following microorganisms were evaluated: *Enterococcus faecalis* (ATCC 4082), *Streptococcus mutans* (ATCC 25175), *Streptococcus mutans* (clinical isolate), *Streptococcus mitis* (ATCC 49456), *Streptococcus mitis* (clinical isolate), *Streptococcus*

Table 1 Composition of Biosilicate®

Components	Range of composition (% in mass)
SiO_2	40–60
Na_2O	0–30
K_2O	0–30
Li_2O	0–15
P_2O_5	0–18
TiO_2	0–4
B_2O_3	0–10
Nb_2O_5	0–15
SnO_2	0–5
Al_2O_3	0–3
Ag_2O	0–2
SrO	0–8
ZrO_2	0–3
MgO	0–10
CaO	0–25

salivarius (ATCC 25975), *Streptococcus salivarius* (clinical isolate), *Streptococcus sanguinis* (ATCC 10556), *Streptococcus sanguinis* (clinical isolate), *Lactobacillus casei* (ATCC 11578), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 15442), *Kocuria rizophila* (ATCC 9341), *Salmonella* Choleraesuis (ATCC 10708), *Aggregatibacter actinomycetemcomitans* (ATCC 29523), *Candida albicans* (ATCC 28366), *Candida albicans* (clinical isolate), *Porphyromonas gingivalis* (ATCC 33277), *Actinomyces naeslundii* (ATCC 19039), and *Prevotella nigrescens* (ATCC 33563). The assays of antimicrobial evaluation for the growth of anaerobes, the plates were incubated in atmosphere of 10% H_2, 10% CO_2 and 80% N_2 in an anaerobic chamber (MiniMac, Don Whitley Scientific, Bradford, UK). For the growth of facultative aerobes and microaerophilics, in aerobic atmosphere, and 10% CO_2 atmosphere respectively.

2.2.1 Agar diffusion technique (AD)

Porphyromonas gingivalis (ATCC 33277), *A. naeslundii* (ATCC 19039), and *P. nigrescens* (ATCC 33563) were tested on Schaedler agar (SCHa, Difco, Detroit, MI, USA), while the other bacteria were assessed in Brain Heart Infusion agar (BHIa, Difco). Inocula from a 24 h growth of the test aerobics and microaerophilics bacteria and 72 h for the anaerobe bacteria in sterile saline were added to the agar, incubated at 37°C, and allowed to grow. The *C. albicans* inoculum was adjusted to 1.0 McFarland scale, whereas the inocula of other bacteria were adjusted at 0.5 McFarland scale. The antimicrobial activity of the samples was determined by AD, using the well technique and employing the double layer agar system. The strains were suspended in 2.5 ml sterile Brain–Heart Infusion (BHI) medium. A 12.5 ml portion of the BHI agar (50°C) and 2.5 ml of each test suspension were gently mixed and poured onto a previously set layer of 25 ml BHI agar or Schaedler agar (90 × 15 mm Petri dish). After solidification, the seed layer was perforated with a sterilized stainless-steel cylinder (inside diameter = 4 mm), to form the wells. The latter were located 25 mm away from the plate border and 40 mm halfway from each other. Biosilicate®, inert silica, chlorhexidine digluconate solution at 0.12% (Periogard, Colgate-Palmolive, São Paulo, SP, Brazil), and distilled deionized water were applied inside the wells. The plates were kept for 2 h at room temperature, to allow diffusion of the agents through the agar [20]. Afterwards, the plates were incubated at 37°C under appropriate gaseous and temperature condition, for 24 at 72 h. Zones of microbial growth inhibition around the wells containing the test samples were measured (mm) and recorded after the incubation time. The inhibitory zone was considered the shortest distance (mm) from the outside margin of the well to the initial point of microbial growth. Three replicates were accomplished for each microorganism.

2.2.2 Direct contact technique (DCT) [10]

The microorganisms were incubated in BHI broth (Difco) or Schaedler broth (SCHb, Difco) supplemented with 5 μg/ml hemin (Sigma) and 1 μg/ml menadione (Sigma), for 24 h (facultative/microaerophilics) and 72 h (anaerobics). After that, the cultures were centrifuged (4000 rpm/5 min) and washed (once) in sodium chloride solution at 0.9%. The supernatant was discarded, and a new NaCl 0.9% solution was added to the precipitate, until a cell concentration of 1.0 in the McFarland scale (3.0×10^8 CFU/ml) was achieved. Serial dilutions were performed in NaCl 0.9% (from 10^8 to 10^5), and the tube with a 10^6 dilution was used for the technique. The suspension (10 μl) was seeded in the surface of a solid culture medium in order to obtain a viable cell counting (CFU/ml) after incubation.

Fifty milligrams of Biosilicate® were placed in each of two Eppendorf microtubes (Tube 1 and Tube 2) containing 30 μl microbial suspension (10^6 UFC/ml), and the contents were homogenized (10 min). NaCl 0.9% solution (470 μl) was added to Tube 1. Aliquots (20 μl) were removed from this tube and seeded on solid culture media, and serial dilutions in 0.9% NaCl (10^{-1}–10^{-7}) were also performed. Each dilution (50 μl) was seeded in each culture media (BHIa or SCHa), to allow for the CFU/ml counting. Tube 2 was reincubated (36°C/50 min) without stirring, and the same dilution and counting procedures were performed, as previously described for Tube 1. The contact time between Biosilicate® and the microorganism was 60 min.

For the positive control of the technique, the microorganisms were inoculated in culture media without Biosilicate®. For the negative control 100% pure inert quartz powder (Laboratory of Vitreous Materials of the Federal University of São Carlos, SP, Brazil) was used. The bacteria were counted (logarithm), and the average and standard-deviation of each triplicate were calculated.

2.2.3 Minimum inhibitory concentration

The dilution-agar technique [21], was employed. A double dilution of Biosilicate® was made at concentrations of 40.0; 30.0; 20.0; 15.0; 10.0; 7.5; 5.0; and 2.5 mg/ml (tube 8). The tubes were homogenized and poured into coded Petri dishes (15 × 90 mm). A tube containing broth only was used as negative control. Different microorganisms were grown in supplemented BHIa (Difco) or SCHa (Difco) and incubated (37°C/24 h for facultative anaerobes and microaerophilic microorganisms; 37°C/72 h for anaerobic bacteria). After incubation, the bacterial suspensions were prepared according to the 0.5 McFarland scale (1.5×10^8 CFU/ml)

for facultative/microaerophilic microorganisms, and to the 1.0 McFarland scale for anaerobic bacteria (3.0×10^8 CFU/ml). The seeding was accomplished by means of a multi-applicator of "Steers", from the highest (tube 8) to the lowest dilution, and then a stamping/sowing of the plates and their control was carried out in triplicate. Chlorhexidine (Sigma, Aldrich, St. Louis, MO, USA) was used as the positive control of the technique, so the MIC values of this antimicrobial agent against the microorganisms was also calculated. The plates were prepared in concentrations ranging from 40 to 1.25 mg/ml. After drying of the inoculum, the plates were incubated (37°C/24 h for aerobic and 37°C/72 h for anaerobic bacteria). MIC was considered the lowest concentration that prevented visible growth, in triplicates.

3 Results

3.1 Agar diffusion technique

The majority of the microorganisms (14 out of 20) were inhibited by Biosilicate®. The inhibition halos ranged from 9.0 ± 1.0 mm to 22.3 ± 2.1 mm (Table 2). Concerning aerobic and facultative anaerobic microorganisms, Biosilicate® was most active against the standard strain *C. albicans* (17.3 ± 0.6 mm). As for microaerophilic microorganisms, the best Biosilicate® activity was obtained against *A. actinomycetemcomitans* (22.3 ± 2.1 mm) and *S. mutans* (19.0 ± 2.0 mm). Among the anaerobic bacteria, *P. nigrescens* (19.3 ± 3.2 mm) was the most sensitive to the tested agent. This method revealed no growth inhibition by Biosilicate® in the cases of *E. faecalis* (4082), *S. aureus* (6538), *S.* Choleraesuis (10708), *P. aeruginosa* (15442), and *S. mitis* (49456 and clinical isolate). Inert silica, used as negative control, displayed no antimicrobial activity against the studied microorganisms.

3.2 Direct contact technique

Biosilicate® exhibited antimicrobial activity against all the tested microorganisms, except for *S. aureus* (6538). After 60 min of contact, there still were viable cells of the latter microorganism ($2.6 \pm 0.4 = 3.6 \times 10^2$ CFU/ml), but in lower quantities compared with the initial inoculum (Table 3). Twelve bacteria died within the first 10 min, whereas the other microorganisms subsided after 60 min of contact with Biosilicate® (Table 3). The clinical isolates *S. sanguinis*, *S. mutans,* and *C. albicans* were more resistant to Biosilicate® in the first 10 min of direct contact, compared with their respective standard strains. The microorganisms were viable in the positive and negative control.

Table 2 Mean and standard deviation (mm) of the halos of inhibition of microbial growth promoted by Biosilicate®

Microorganisms (ATCC/clinical isolate)	Mean ± SD (mm)	
	Biosilicate®	Periogard®
E. faecalis (4082)	0.0 ± 0.0	14.7 ± 0.6
K. rizophila (9341)	10.7 ± 3.1	23.7 ± 0.6
S. aureus (6538)	0.0 ± 0.0	18.3 ± 0.6
S. choleraesuis (10708)	0.0 ± 0.0	14.3 ± 1.2
P. aeruginosa (15442)	0.0 ± 0.0	10.7 ± 0.6
S. salivarius (25975)	10.3 ± 0.6	15.7 ± 0.6
S. salivarius (clinical isolate)	11.3 ± 0.6	16.5 ± 0.6
S. sanguinis (10556)	9.0 ± 1.0	18.7 ± 1.5
S. sanguinis (clinical isolate)	10.0 ± 0.6	22.0 ± 1.0
S. mutans (25175)	16.3 ± 0.6	23.3 ± 1.5
S. mutans (clinical isolate)	19.0 ± 2.0	24.0 ± 1.7
S. mitis (49456)	0.0 ± 0.0	18.7 ± 0.6
S. mitis (clinical isolate)	0.0 ± 0.0	19.5 ± 0.6
L. casei (11578)	14.0 ± 2.0	29.7 ± 0.6
A. actinomycetencomitans (29523)	22.3 ± 2.1	28.7 ± 1.2
A. naeslundii (19039)	15.7 ± 2.0	21.0 ± 2.0
P. nigrescens (33563)	19.3 ± 3.2	24.0 ± 1.0
P. gingivalis (33277)	15.0 ± 0.6	20.7 ± 0.6
C. albicans (28366)	16.3 ± 1.2	20.0 ± 1.0
C. albicans (clinical isolate)	17.3 ± 0.6	21.3 ± 1.5

AD technique

SD Standard deviation

3.3 Minimum inhibitory concentration

The development of microorganisms was inhibited by Biosilicate®, with MIC values ranging from ≤2.5 to 20 mg/ml (Table 4). The best results were achieved for *S. salivarius* (ATCC and clinical isolate), *S. sanguinis*, *S. mutans* (clinical isolate), and *S. mitis* (ATCC and clinical isolate), with values of MIC ≤2.5 mg/ml. The MIC was determined as the lowest concentration of the sample capable of inhibiting microorganism growth.

4 Discussion

In the case of the AD technique, Biosilicate® inhibited the development of 14 microorganisms. Among other factors, the lack of action against *E. faecalis* (4082), *S. aureus* (6538), *S. Choleraesuis* (10708), *P. aeruginosa* (15442), *S. mitis* (49456), and *S. mitis* (clinical isolate) may have been due to the prevention of the pH elevation in relation to the production of acid by the bacterial metabolism, thus hindering the diffusibility of the material in the agar. Furthermore, the intrinsic resistance of these bacteria to high osmotic pressure may have influenced the results [10].

Table 3 Mean and standard deviations (Log) of microbial development (CFU/ml) in DCT

Microorganisms (ATCC/clinical isolate)	Initial count	Biosilicate®		Positive control (without Biosilicate®)		Negative control (Silica 100% inert)	
Time (min.)	0	10	60	10	60	10	60
E. faecalis (4082)	5.3	0.0 ± 0.0	0.0 ± 0.0	5.3 ± 0.7	5.4 ± 1.7	5.2 ± 1.2	5.5 ± 2.0
K. rizophila (9341)	4.7	2.9 ± 0.1	0.0 ± 0.0	4.8 ± 2.2	4.8 ± 1.7	4.8 ± 2.2	5.2 ± 2.1
S. aureus (6538)	5.3	3.0 ± 1.1	2.6 ± 0.4	5.3 ± 2.9	5.5 ± 2.7	5.3 ± 2.7	6.4 ± 0.0
S. Choleraesuis (10708)	5.8	2.4 ± 0.7	0.0 ± 0.0	5.8 ± 2.4	5.8 ± 2.2	5.8 ± 1.5	5.8 ± 1.1
P. aeruginosa (15442)	6.0	0.0 ± 0.0	0.0 ± 0.0	6.2 ± 1.3	6.4 ± 1.3	6.3 ± 1.1	6.4 ± 1.1
S. salivarius (25975)	5.4	0.0 ± 0.0	0.0 ± 0.0	5.0 ± 1.8	5.5 ± 1.6	5.6 ± 1.9	6.1 ± 0.6
S. salivarius (clinical isolate)	5.6	0.0 ± 0.0	0.0 ± 0.0	5.5 ± 1.1	5.6 ± 1.3	5.6 ± 1.5	5.6 ± 1.2
S. sanguinis (10556)	6.4	0.0 ± 0.0	0.0 ± 0.0	6.4 ± 2.4	6.4 ± 2.5	6.4 ± 3.0	6.5 ± 2.6
S. sanguinis (clinical isolate)	6.3	2.4 ± 0.6	0.0 ± 0.0	6.4 ± 1.6	6.5 ± 1.6	6.7 ± 1.9	6.7 ± 1.6
L. casei (11578)	6.2	0.0 ± 0.0	0.0 ± 0.0	6.0 ± 2.2	6.5 ± 1.0	5.0 ± 1.6	6.6 ± 1.1
S. mutans (25175)	6.2	0.0 ± 0.0	0.0 ± 0.0	6.0 ± 1.1	6.3 ± 1.1	5.6 ± 1.6	4.5 ± 1.3
S. mutans (clinical isolate)	5.7	2.2 ± 0.4	0.0 ± 0.0	5.6 ± 2.2	5.7 ± 2.3	5.0 ± 2.0	6.0 ± 1.1
S. mitis (49456)	5.9	4.2 ± 1.2	0.0 ± 0.0	5.8 ± 2.2	5.9 ± 2.5	5.7 ± 2.4	5.2 ± 0.9
S. mitis (clinical isolate)	5.8	0.0 ± 0.0	0.0 ± 0.0	5.9 ± 1.5	5.9 ± 1.7	5.9 ± 1.4	5.9 ± 1.4
A. actinomycetemcomitans (29523)	6.5	0.0 ± 0.0	0.0 ± 0.0	6.0 ± 1.9	6.5 ± 1.8	5.6 ± 1.5	6.1 ± 0.9
A. naeslundii (19039)	5.8	3.3 ± 1.6	0.0 ± 0.0	5.7 ± 1.7	5.8 ± 2.5	5.5 ± 1.9	6.6 ± 1.3
P. nigrescens (33563)	6.3	0.0 ± 0.0	0.0 ± 0.0	6.5 ± 0.5	6.5 ± 0.3	5.7 ± 1.8	6.6 ± 0.6
C. albicans (28366)	4.3	0.0 ± 0.0	0.0 ± 0.0	4.3 ± 1.4	4.7 ± 0.7	4.3 ± 1.7	5.1 ± 1.5
C. albicans (clinical isolate)	4.5	3.0 ± 0.3	0.0 ± 0.0	4.3 ± 1.3	4.1 ± 1.4	4.2 ± 1.0	4.2 ± 1.0
P. gingivalis (33277)	6.1	0.0 ± 0.00	0.0 ± 0.0	6.0 ± 1.8	6.2 ± 0.5	5.8 ± 1.6	6.7 ± 0.4

The AD technique is often used to evaluate the antimicrobial activity of dental products [17, 22]. However, it is affected by factors such as weight and molecular structure, diffusibility of the antimicrobial agent, electrical charge and concentration of the assessed product, agar viscosity, and the contact between the material and culture medium [23]. Also, the control and standardization of the inoculum density, the choice of microorganisms to be tested and culture medium to be employed, the volume and thickness of the medium, and the temperature of incubation are also limiting factors of AD technique variability [24]. To minimize these limitations, we tried to standardize the experiment and to keep the plates at 25°C (180 min) before incubation, to facilitate the dissemination of Biosilicate® in the agar, as recommended by Möller [20].

The consistency of the results obtained in the experiments carried out in triplicate suggests that there was contact between the material and agar. However, it can be assumed from the variation in the action of Biosilicate® against microorganisms that its diffusion was a limiting factor, although other factors may have been responsible for the material′s antimicrobial effects against the various microorganisms. Furthermore, the results show that, despite its limitations, the AD method was capable of evaluating the activity of the glass–ceramic against oral microorganisms.

The DCT demonstrated the antimicrobial activity of Biosilicate®, which was able to destroy the evaluated microorganisms after 60 min (bactericidal effect), except for *S. aureus*, whose cells were also isolated after the total contact time (2.6 ± 0.4–bacteriostatic effect). Among the 20 studied microorganisms, 8 survived to the osmotic effect in the first 10 min, being 6 of them Gram-positives (*K. rizophila*, *S. aureus*, *S. sanguinis* clinical isolate, *S. mutans* clinical isolate, *S. mitis*, and *A. naeslundii*), 1 Gram-negative (*S. choleraesuis*), and 1 yeast (*C. albicans* clinical isolate). The outer membrane of the Gram-negative bacterium and the yeast cell wall are more efficient permeable barriers compared with the cell wall of Gram-positive bacteria [25]. However, the larger viability of Gram-positive bacteria suggests the involvement of other mechanisms.

Using the DCT, Stoor et al. [10] showed that the standard strains *S. sanguinis* and *S. mutans* as well as the Gram-negative bacterium *A. actinomycetemcomitans* also remained viable after 10 min of contact with the S53P4 bioglass. Moreover, Waltimo et al. [16] observed the reduction of viable cells by more than 99% after 100 min of contact with the bioactive glass 45S5. Conversely, Yli-Urpo et al. [17] demonstrated that the S53P4 bioglass was unable to destroy the standard strains *S. mutans* and

Table 4 MIC results of Biosilicate® and Chlorhexidine® (technique positive control)

Microorganisms (ATCC/clinical isolate)	MIC (mg/ml)	
	Biosilicate®	Chlorhexidine®
E. faecalis (4082)	15	≤1.25
K. rizophila (9341)	15	≤1.25
S. aureus (6538)	20	≤1.25
S. Choleraesuis (10708)	20	≤1.25
P. aeruginosa (15442)	20	≤1.25
S. salivarius (25975)	≤2.5	≤1.25
S. salivarius (clinical isolate)	≤2.5	≤1.25
S. sanguinis (10556)	5	≤1.25
S. sanguinis (clinical isolate)	≤2.5	≤1.25
S. mutans (25175)	5	≤1.25
S. mutans (isolado clínico)	≤2.5	≤1.25
S. mitis (49456)	≤2.5	≤1.25
S. mitis (clinical isolate)	≤2.5	≤1.25
L. casei (11578)	5	≤1.25
A. actinomycetencomitans (29523)	5	≤1.25
A. naeslundii (19039)	2.5	≤1.25
P. nigrescens (33563)	2.5	≤1.25
P. gingivalis (33277)	5	≤1.25
C. albicans (28366))	7.5	≤1.25
C. albicans (clinical isolate)	7.5	≤1.25

C. albicans after 60 min of contact, but there was a considerable decrease in the initial inoculum, confirming the antimicrobial effect of this material. Biosilicate® was effective against anaerobic bacteria, whereas for *P. nigrescens* and *P. gingivalis* the inhibitory action occurred within the first 10 min of contact (Table 3). Similarly, Lepparanta et al. [14] evaluated the antibacterial effect of S53P4 against 17 anaerobic bacteria, confirming the growth inhibition capacity of this material.

Still concerning the DCT and considering the susceptibility of standard strains and clinical isolates, it was observed that in the cases of *S. sanguinis*, *S. mutans*, and *C. albicans* the clinical isolates remained viable after 10 min of contact with Biosilicate®, unlike the corresponding standard strains. This suggests that clinical isolates can support more pH changes, once they are more susceptible to the changes that occur continuously in the oral cavity. These results differ from those obtained by Waltimo et al. [16], who found similar behavior upon comparison between the clinical isolates and standard strains of *E. faecalis* in the presence of the 45S5 bioglass, using the DCT. Among the advantages of the DCT is the fact that it allows the close contact of bacteria with the evaluated material regardless of their solubility, not to mention that it is a reproducible and quantitative technique [26].

MIC results demonstrated the action of Biosilicate®, and the best results were achieved against *K. rizophila*, *E. faecalis* (15 mg/ml), and *C. albicans* (7.5 mg/ml), being the last two microorganisms considered as important pathogenic microorganisms related to oral infections. However, *S. aureus*, *S. Choleraesuis*, and *P. aeruginosa,* commonly employed in the investigation of the antimicrobial activity of intermediate-level disinfectants [27], were more resistant, with MIC values of 20 mg/ml.

Assessing the activity of 8 bioactive materials against 29 bacteria, including *E. faecalis*, *S. mutans*, *S. sanguinis*, *P. aeruginosa*, and *S. aureus* (also used in this work), Munukka et al. [15] observed that there was inhibition of bacterial growth in concentrations of up to 100 mg/ml. Comparing these results with those obtained for Biosilicate®, the latter was more effective because the highest concentration that inhibited bacterial growth was 20 mg/ml. There was action against yeast, and Gram-positive and Gram-negative bacteria, although the lowest concentration of the product necessary for inhibition of bacterial growth was obtained for oral pathogens. These results indicate the antimicrobial potential of Biosilicate® and its future application in dentistry, incorporated into other materials or pure, corroborating its bioactivity reported in the studies of Moura et al. [8], Roriz et al. [9] and Oliveira Jabur et al. [28].

Several dental applications have been suggested for bio glasses and glass–ceramics. For instance, Stoor et al. [10] observed the antimicrobial action of a bioactive glass (S53P4) paste against *A. actinomycetemcomitans* (ATCC 29523), *P. gingivalis* (ATCC 33277), *A. naeslundii* (clinical isolate), *S. mutans* (NCTC 10449), and *S. sanguinis* (NCTC 10904), and these authors demonstrated the action of this material against the microorganisms of supra and subgingival biofilm. The authors affirmed that it could be used in oral care products indicated for the prevention and treatment of caries and periodontal disease. In the present work, Biosilicate® was also proven to be active against *A. actinomycetemcomitans* (ATCC 29523) and *P. gingivalis* (ATCC 33277).

Incorporation of the S53P4 bioactive glass into glass-ionomer cements was tested by Yli-Urpo et al. [17], who observed antibacterial effect against *S. mutans* ATCC 25175. Biosilicate® and S53P4 belong to the quaternary system $CaO–P_2O_5–Na_2O–SiO_2$ and display antimicrobial activity against the same microorganisms.

The antimicrobial effect of Biosilicate® may be due to a series of factors. Considering its composition, the highly crystalline bioactive phase, high surface area, and distribution of ultrafine particles (between 0.1 and 20 μm) of this material makes its surface highly reactive in the presence of water. Biosilicate® quaternary composition is able to increase the pH of aqueous suspensions [10, 11].

The basic pH of the solution renders the bioglass more soluble due to the presence of OH^-, which has the ability to cleave the silica chains [8, 9, 28].

When a bioactive silica-based material is in contact with an aqueous solution, there is an increase in pH due to the leaching of Na^+ and Ca^{2+} ions from the surface of the material into the solution. The particulate Biosilicate® has been demonstrated to raise the pH of aqueous solution from 5.0 to 8.7 in 35 min [8]. This suggests that pH elevation and the consequent change in the osmotic medium leads to cellular injury. The alkalinization of the medium can also cause inactivation of the enzymes produced by bacteria. Another possible explanation is that a proton motivating force (energized state of the membrane) is not established, thus preventing ionic transport, flagellar rotation, and decreased synthesis of adenosine triphosphate–ATP, thereby altering the transport of nutrients and organic components into the cell [21, 25].

About the microorganisms evaluated in this study, they are most commonly associated with oral diseases. *E. faecalis* is a resistant facultative anaerobe microorganism of the mouth, used in several studies of antibacterial activity [26, 29] and associated with failures in endodontic treatment [30]. *S. mutans* [31], *S. salivarius*, *S. sanguinis*, *S. sobrinus*, *S. mitis,* and *L. casei*, among others, may be part of the oral biofilm [32]. *A. naeslundii* [33], *P. nigrescens* and *P. gingivalis* [34], *A. actinomycetemcomitans* [35], and *C. albicans* [36] are related to endodontic treatment failure, periodontal disease, and denture stomatitis. *S. aureus*, *P. aeruginosa*, *K. rizophila*, and *S. Choleraesuis* are indicators of the antimicrobial activity of intermediate-level disinfectants [27].

Since antimicrobial activity is a desirable requirement of a dental material, the antimicrobial action of Biosilicate® can add several advantages [8, 9, 28, 37] and become a potentially applicable product. The importance of seeking new products with antimicrobial action for oral use is based on the fact that biofilms of oral bacteria become tolerant to currently employed antimicrobial agents [38].

5 Conclusion

Based on the experimental conditions used and considering the overall results of this work and we conclude that:

i. Regarding the AD technique, the largest inhibition of halos occurred for *A. actinomycetencomitans* (29523), *P. nigrescens* (33563), and *S. mutans* (clinical isolate), while the lowest were obtained for *S. sanguinis* (ATCC 10556 and clinical isolate) and *S. salivarius* (ATCC 25975 and clinical isolate). There was no action against *E. faecalis* (ATCC 4082), *S. aureus* (ATCC 6538), *S. choleraesuis* (ATCC 10708), *P. aeruginosa* (ATCC 15442), or *S. mitis* (ATCC 49456 and clinical isolate).
ii. As for the DCT, only *S. aureus* (ATCC 6538) was not destroyed after 60 min of contact with Biosilicate®. Among the inactivated microorganisms, 11 were eliminated within the first 10 min.
iii. The best MIC results were obtained for *C. albicans* (7.5 mg/ml), *K. rizophila*, and *E. faecalis* (15 mg/ml). *S. aureus*, *S. Choleraesui*, and *P. aeruginosa* were more resistant to Biosilicate®, with MIC values of 20 mg/ml.

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