



Antibacterial Activity of a New Ready-To-Use Calcium Silicate-Based Sealer

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The aim of this study was to evaluate the antibacterial potential of a calcium silicate-based sealer (Bio-C Sealer, Angelus) against common bacteria in primary and secondary endodontic infections. *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus mutans* were exposed to fresh Bio-C Sealer for 24 h by the agar diffusion method (n=5). Additionally, the antibacterial activity was investigated against *E. faecalis* and *S. mutans* biofilms (48 h old) grown in discs with 4 mm in diameter and 2 mm in height. (n=3) of set discs of Bio-C Sealer (Angelus), EndoFill (Dentsply-Mallefer), Sealer 26 (Dentsply), AH Plus (Dentsply), Sealapex (Sybron-Endo) and EndoSequence BC Sealer (Brasseler). The antibacterial activity was evaluated by colony forming unity (CFU) counting using ImageJ software. Data were compared by one-way ANOVA followed by Holm-Sidak test ($\alpha=5\%$). Fresh Bio-C Sealer exhibited antimicrobial activity against all bacteria evaluated by agar diffusion method, except for *S. mutans*. Set discs of all endodontic sealers tested showed similar CFU values for *E. faecalis* ($p>0.05$). *S. mutans* in biofilms showed higher susceptibility to EndoFill compared with the other sealers ($p<0.05$). In conclusion, the results indicate that fresh Bio-C Sealer does not inhibit *S. mutans* growth, but exhibits antibacterial activity against *E. faecalis*, *S. aureus*, *P. aeruginosa* and *E. coli*. After setting, the Bio-C Sealer exhibits an antimicrobial potential comparable to that of the other sealers evaluated in *E. faecalis* biofilm, but lower than that of EndoFill for *S. mutans* biofilm.

Key Words: endodontics, calcium silicate-based sealer, antibacterial activity, biofilm.

Introduction

Studies comparing the composition and properties of endodontic sealers are relevant both to produce new materials with advantageous properties, as well as for the evaluation and improvement of those already produced today (1-3). Several types of sealers with different compositions are currently available, including those based on zinc oxide and eugenol, calcium hydroxide, epoxy resin, methacrylate resin sealers and calcium silicate-based materials (1,3).

Calcium silicate-based sealers exhibit excellent biocompatibility due to their compositions, which resemble biological hydroxyapatite (2). Among the currently available calcium silicate-based sealer, Bio-C Sealer is a new ready-to-use non-resinous sealer, which has been shown to favor the expression of osteoblastic markers and biomineralization when in contact with connective tissues *in vivo* (3,4).

For successful treatment, it is known that the microbial population present in both dental and dental support tissues must be eliminated so that subsequent lesions in the periapical region do not occur. However, the complete elimination of these microorganisms is not always possible, even with a significant bacterial reduction promoted by NaOCl or CHX in association with mechanical

instrumentation, bacteria may still be detected in root canals of teeth with apical periodontitis. The remaining bacteria may utilize necrotic tissue remnants in untouched root canal areas, and a additional nutrient source can be develop from tissue fluids and inflammatory exudates from the periradicular tissues as a consequence of an inappropriate apical seal (5). Thus, it is important that the filling materials used for root canal sealing have antimicrobial activity to prevent infection recurrence, also adding better healing to the affected structures (6).

Complex bacterial communities can be observed at primary and secondary apical periodontics, such as *Enterococcus*, *Staphylococcus* and *Streptococcus*. In fact, the endodontic treatment failures are closely related to Gram-positive and Gram-negative facultative anaerobic and anaerobic bacteria persistence inside the root canal system and periapical tissues (7). Most of the endodontic sealers usually exhibit some discrete antibacterial effects, but only before setting (5). Thus, this study aimed at evaluating the antibacterial potential of the calcium silicate-based sealers (Bio-C Sealer and EndoSequence BC Sealer) compared with sealers based on zinc oxide and eugenol (EndoFill), epoxy resin (AH Plus), epoxy resin with calcium hydroxide (Sealer 26), and methacrylate resin with

calcium hydroxide sealer (Sealapex) on common bacteria in endodontic infections.

Material and Methods

Sealers

In this study, six endodontic sealers were used: Bio-C Sealer (Angelus, Paraná, Brazil), EndoFill (Dentsply-Mallefer, Rio de Janeiro, RJ, Brazil), Sealer 26 (Dentsply Ind. and Com. Ltda, Rio de Janeiro, Brazil), AH Plus (Dentsply, DeTrey GmbH, Konstanz, Germany), Sealapex (Sybron-Endo, Orange, CA, USA) and EndoSequence BC Sealer (Brasseler, GA, USA). Sealer compositions are shown in Table 1. The sealers were mixed and manipulated depending on the manufacturer's instructions under sterile conditions. For biofilm evaluation, discs with 4 mm in diameter and 2 mm height [adapted from Delben et al. (8)] of all sealers were forged in a silicon mold and let to set at 37 °C in a humidified atmosphere. The discs were detached before exposition to the microorganisms.

Bacterial Strains

For the present study, five different reference bacterial strains were used: *E. faecalis* ATCC 4083, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *E. faecalis* ATCC 25923 and *S. mutans* ATCC 25175.

Agar Well Diffusion Test

Bacterial isolates of all strains were removed from stock (-80 °C), thawed and cultured on solid Müller Hinton (MH) agar (Oxoid Basingstoke, Hampshire, England) at 37 °C for 48 h. Then, three to four colonies were picked up and resuspended in 5 mL Luria-Bertani broth (LBb) (Oxoid) and the inoculum was adjusted to match the turbidity equivalent to 0.5 McFarland Standard (approximately 1.5×10^8 CFU/mL) previously to antibacterial evaluation. Muller-Hinton agar plates were prepared, sterilized and inoculated (three

plates for each strain) with the 0.5 mL McFarland scale of microbial suspensions to agar well diffusion test (9). Wells of 3 mm depth and 5 mm diameter were aseptically punched from each plate with a sterile 200 µL tip base. Bio-C Sealer was placed into the wells (n=5). Subsequently, the plates were aerobically incubated at 37 °C for 24 h, except for *S. mutans*, which was incubated in microaerophilia (5% CO₂). Thereafter, the diameter of the inhibition zones around each well was measured with an electronic digital caliper (Digimess, São Paulo, Brazil). The mean diameter of measured zone was analyzed to assess antimicrobial activity of fresh Bio-C Sealer.

Biofilm Formation

For biofilm formation analysis, only *E. faecalis* and *S. mutans* reference bacterial strains were used. The biofilm formation experiments in the sealers were carried out based on Delben et al. (8) with adaptations. After bacterial reactivation in test tubes containing 10 mL of Brain Heart Infusion (BHI) medium and incubation at 37 °C for 18 h under aerobiosis (*E. faecalis*) or microaerophilia (*S. mutans*), the strains were subcultured in solid Mueller-Hinton Agar (Oxoid), under the same conditions described above. The discs of each sealer (n=3) were placed individually in 2 mL polystyrene cryotubes (Corning, São Paulo, SP, Brazil) containing 500 µL of inoculum equivalent to McFarland scale 1 (3×10^8 bacterial cells per mL) and 500 µL BHI, resulting in the final inoculum equivalent to the 0.5 McFarland scale (1.5×10^8 bacterial cells per mL). The discs were incubated under culture conditions and the bacterial inoculum was replaced after 24 h to guarantee the viability of the bacterial cells in the formation of the biofilm. Before renewing the inoculum, the discs were washed with 1 mL of Phosphate Buffered Saline (PBS) to remove planktonic bacterial cells not adhered to the sealers. After 48 h of

Table 1. Sealers compositions according to the manufacturer

Sealer	Composition	Lot number
EndoFill	Powder: zinc oxide, staybelite resin, bismuth subcarbonate, barium sulphate, sodium and borate anhydrate Liquid: eugenol, almond oil and BHT.	349188K
Sealer 26	Powder: calcium hydroxyde, bismuth oxide, methenamine and titanium dioxide. Resin: epoxy.	338639J
AH Plus	Paste A: bisphenol-a epoxy resin; bisphenol-f epoxy resin; calcium tungstate; zirconium oxide; silica and iron oxide. Paste B: adamantine amine; N, N'-dibenzyl-5-oxanonane diamine-1,9; TCD - diamine; calcium tungstate; zirconium oxide; silica and desilicone oil.	350598K
Sealapex	Catalyst: isobutyl salicylate resin, pyrogenic silicic acid (silicon dioxide), bismuth trioxide, titanium dioxide pigment. Base: n-Ethyl toluene sulfonamide resin, pyrogenic silicic acid (silicon dioxide), zinc oxide, calcium oxide.	6738798
Bio-C Sealer	Tricalcium silicate, dicalcium silicate, tricalcium aluminate, calcium oxide, zirconia oxide, silicon oxide, polyethylene glycol iron oxide.	101526
EndoSequence BC Sealer	Zirconium oxide, calcium silicates, calcium phosphate monobasic, calcium hydroxide, filler and thickening agents.	10/19003SP

incubation, the bacterial inoculum was removed and the discs were washed with 1 mL of PBS.

Then, the discs were transferred to new 2 mL tubes containing 1 mL of PBS, shaken in a vortex mixer (Gehaka, São Paulo, SP, Brazil) for 30 s at maximum speed and, subsequently, for 8 min in an ultrasonic vibrator (Kondortech, São Paulo, Brazil) to detach the biofilms. The quantification of biofilms was done by the CFU quantification. For this, 100 μ L of the collected biofilm suspension were transferred to a 2 mL tube containing 900 μ L of PBS, followed by six decimal dilutions (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6}). Aliquots of 100 μ L were inoculated according to the spread plate technique (9), with the aid of a Drigalski loop on Mueller-Hinton Agar (Oxoid), for later total CFU counting. The plates containing the inoculum were incubated for 24 h at 37 °C under the conditions described above. The CFU experiments were carried out in triplicate (n=3) to ensure the reliability of the results obtained and allow statistical analysis. The counting of the CFU's was performed by a single calibrated and trained operator using the ImageJ software (Fiji, Bethesda, MA, USA), counting the total CFU's referring to plate number four of the serial dilution sequence (11). Data were expressed as Log₁₀ (CFU/mL).

Statistical Analysis

The experiments were done in triplicate and the quantitative data was submitted to normality and variance homogeneity tests. Data were compared using one-way ANOVA, followed by Holm-Sidak post hoc test, when appropriate. The level of significance adopted was 5%.

Results

The results of the agar diffusion test revealed that fresh Bio-C Sealer promoted inhibition zone in cultures

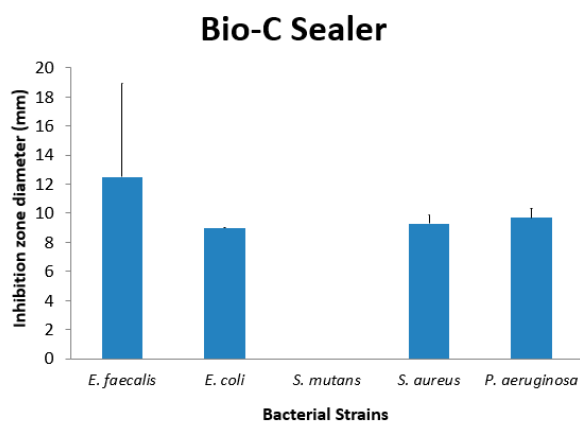


Figure 1. Mean values \pm standard deviation of diameter of inhibition zone formed in cultures of different bacterial strains exposed to fresh Bio-C Sealer.

of *E. faecalis*, *E. coli*, *P. aeruginosa* and *S. aureus*, but not in *S. mutans*. Mean values and standard deviations of the inhibition zone diameters promoted by this sealer in the different bacterial cultures are shown in Figure 1.

The set discs of all sealers tested exhibited the presence of biofilm adhered onto the surface, for both tested bacterial strains. After exposure to *E. faecalis* biofilms, all sealers showed similar values of CFU ($p > 0.05$), while *S. mutans* biofilms showed higher susceptibility to EndoFill compared with the other sealers, with lower CFU values ($p < 0.05$). Log₁₀ (CFU/mL) values are shown in Figure 2, and the outcome of the One-Way ANOVA with Holm-Sidak post hoc test ($\alpha = 5\%$) are displayed in Table 2.

Table 2. Mean values \pm standard deviation of Log₁₀ (CFU/mL) of *Enterococcus faecalis* and *Streptococcus* biofilms grown in different endodontic sealers

Sealer	<i>E. faecalis</i>	<i>S. mutans</i>
EndoFill	8.61 \pm 0.48 a	5.92 \pm 0.20 a
Sealer 26	8.95 \pm 0.63 a	7.36 \pm 0.73 b
AH Plus	9.16 \pm 0.72 a	7.24 \pm 0.73 b
Sealapex	8.75 \pm 0.45 a	6.82 \pm 0.76 b
Bio-C Sealer	8.65 \pm 0.61 a	7.55 \pm 0.58 b
EndoSequence BC Sealer	8.82 \pm 0.56 a	7.40 \pm 0.74 b

Distinct letters in the same column indicate statistically significant difference among the groups (ANOVA, $p < 0.05$).

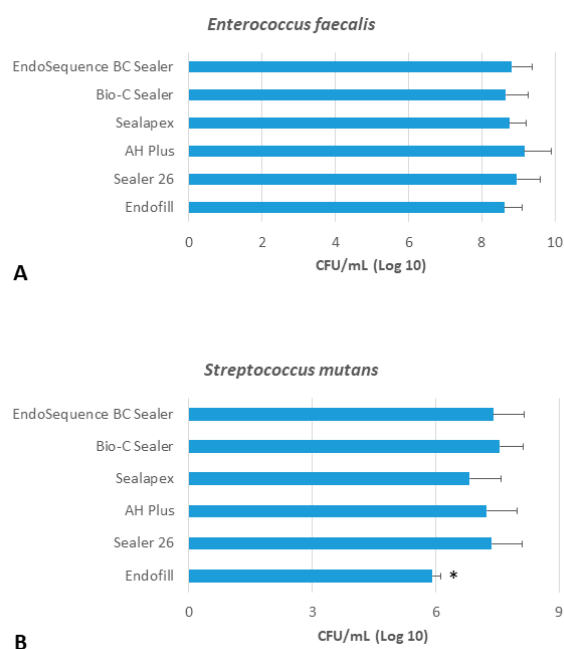


Figure 2. The *in vitro* antibacterial activity of six endodontic sealers against 48h-old *E. faecalis* (A) and *S. mutans* (B) biofilms. Data expressed as mean and standard deviations Log₁₀ (CFU/mL). Asterisk indicates statistically significant difference (ANOVA, $p < 0.05$).

Discussion

Successful endodontic treatment primarily requires effective elimination of the persistent microbial population in the apical and periapical region of a dental element (7). This occurs through mechanical debridement associated with irrigation compounds (12). In addition, the antimicrobial effect of endodontic sealers contributes to achieve this result (2,6). In the present study, the antimicrobial activity of the Bio-C Sealer on common bacteria in endodontic infections was evaluated and compared with some commercially available sealers. The results showed that contact with fresh Bio-C Sealer inhibits the growth of pathogens related to the development of persistent endodontic infections, including *E. faecalis*, *E. coli*, *P. aeruginosa* and *S. aureus*, although it has no effect on *S. mutans* cultures. Comparing the antimicrobial potential against 48h-old biofilms, post-setting discs of the Bio-C Sealer exhibited behavior similar to that of the main endodontic sealers commercially available in *E. faecalis* biofilms, and less antimicrobial potential than EndoFill in *S. mutans* biofilms.

Antimicrobial susceptibility testing is a key factor for the prediction of therapeutic outcome. A variety of laboratory methods can be used to evaluate or screen the *in vitro* antimicrobial activity of an extract or a pure compound (9). In the present study the agar diffusion method, recognized as the most basic method, was used to give an initial screening of Bio-C Sealer antimicrobial capacity. However, there are some limitations to this test, since the bacterial growth inhibition does not mean the bacterial death, this method cannot distinguish bactericidal and bacteriostatic effects, and the results should be interpreted with caution (9). For this evaluation, bacterial strains commonly isolated from primary and secondary endodontic infections were selected. *E. faecalis* is among the main causes of endodontic failure. It is an opportunistic bacterium with great potential for resistance to chemical compounds used for root canal disinfection, in addition to their ability to produce biofilm, invading periapical tissues, and inhibit the defense action of lymphocytes, promoting a higher degree of pathogenicity (13). In addition to *E. faecalis* demonstrating resistance to drugs used during intracanal treatment, it is also known to resist the antibacterial alkaline effect of calcium hydroxide used in intracanal dressings (14). *E. coli*, is a facultative anaerobic Gram-negative bacillus also isolated from periapical infections of endodontic treatments (15). *E. faecalis* is one of the most resistant and frequent bacteria in primary infections and recurrence of endodontic treatments (16). *S. mutans*, a microorganism with strong etiological potential of caries disease and also found in infected root canals associated with apical periodontitis (17). *P. aeruginosa* is

a facultative Gram-negative bacterium frequently found in periodontal infections and has been recovered from primary and persistent endodontic infections (18).

In the present work, fresh Bio-C exhibited antimicrobial activity against all strains except for *S. mutans*. The literature shows that in addition to ability to induce biomineralization after implantation into connective tissue (4), Bio-C Sealer has capability to alkalization reaching pH of 10, up to 21 days (3). It is known that pH higher than 9 can inactivate cell membrane enzymes of microorganisms, causing loss of biological activity or integrity of the plasma membrane. Therefore, it is necessary to maintain high pH levels, as several species remain stable at pH 9 or higher (19). However, the antibacterial potential considering the high pH values, should be evaluated with caution. The pH values from Bio-C samples were observed with *in vitro* studies after immersion in deionized water (3), and, clinically, the samples would be in contact with body fluids, which could alter the pH in contact with the bacterial environment. Nevertheless, considering this sealer setting time (≤ 240 min) and high solubility, the antibacterial effects of fresh Bio-C Sealer could also be related to the significant calcium hydroxide and cement ions release (3), capable of inactivating bacterial endotoxin (LPS) potentially limiting its destructive effects on periodontal tissues. The absence of antibacterial activity against *S. mutans* is probably related to their capacity to quickly recover from pH shock and resume growth (20).

According to a systematic review (21), the majority of the studies on antimicrobial effect of endodontic sealers were done on planktonic bacteria, i.e. single cell isolates floating in water. This type of evaluation does not simulate an *in vivo* or clinical situation because the oral cavity bacteria are presented in a biofilm form, which guarantee a protected mode of growth in a hostile environment (21). Therefore, the present study also evaluates the biofilm formed in set sealers discs to mimic the clinical conditions and gives more reliable antimicrobial evidence. For this evaluation, two bacteria species were selected: *E. faecalis* and *S. mutans*, considering their relationship with persistent periapical infections (14,17).

The antimicrobial potential of Bio-C Sealer on 48h-old biofilms was compared with that of some of the most widely used endodontic sealers. EndoFill is composed of zinc oxide and eugenol (22), a traditional root sealing material with favorable physicochemical properties and low cost. Sealer 26, originated and modified from AH 26 sealer, is based on epoxy resin with calcium hydroxide and bismuth, and has high adhesion power and antibacterial effect (23). Sealapex is a calcium hydroxide-based material, so its formula depends on the release of hydroxyl ions through ionization, which consequently increases the pH value of the medium (24). AH Plus Sealer is an epoxy

resin sealer currently considered the gold standard due to its excellent physical chemical properties and sealing ability (25). EndoSequence BC Sealer is a premixed calcium silicate-based endodontic sealer with an alkaline pH, high calcium ions release and antibacterial activity (2).

In the present study, there were differences in antibacterial effects against *E. faecalis* evaluated by agar diffusion method and biofilm formation. Unlike the agar diffusion method, no antimicrobial activity was observed in the bacterial biofilms formed in the tested sealers. This could be explained either by the differences in the sealers setting or by the biofilm features. The agar diffusion method used fresh sealer and the biofilm used set discs. It has been previously reported that the antimicrobial activity can be lost as the material set, with no inhibitory effect for 2- to 7-day aged sealer discs, probably due to the reduction of antimicrobial components released from the sealer matrix (21,25). Also, in the biofilm, the bacteria aggregate in hydrate polymeric matrix and sessile colonies are resistant to antimicrobial agents (21). However, it has been previously observed that the presence of a membrane between sealer and biofilm did not significantly affect the antibacterial properties of epoxy- and calcium silicate-based sealers, suggesting that the antibacterial activity is primarily mediated by released substances during setting of the material (25).

Interestingly, CFU values of *S. mutans* were significantly reduced in EndoFill discs, indicating lower biofilm formation onto its surface in comparison with all other sealers. Corroborating with this result, the antibacterial activity of Endofill has been previously demonstrated and related to the zinc oxide particles and eugenol diffusion through the medium (26). However, the eugenol also is responsible for the Endofill cytotoxic effects, which has potential for long-term tissue irritation (22).

In conclusion, the results of the present study demonstrated that fresh Bio-C Sealer exhibit antibacterial effects against *E. faecalis*, *E. coli*, *P. aeruginosa* and *E. faecalis* strains, but not against *S. mutans*. After setting, the antimicrobial potential of Bio-C Sealer is comparable to that of the other sealers evaluated in 48h-old *E. faecalis* biofilm, but lower than that of EndoFill for *S. mutans* biofilm. These results suggest that this material could be used for endodontic treatment of teeth with and without apical infection and particularly represents a good option to the retreatment of endodontic failures.

Resumo

O objetivo deste estudo foi avaliar o potencial antibacteriano do novo cimento biocerâmico (Bio-C Sealer, Angelus) contra bactérias comuns em infecções endodônticas primárias e secundárias. Culturas de *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* e *Streptococcus mutans* foram expostas a amostras frescas do Bio-C

sealer durante 24 h pelo método de difusão em agar (n=5). A atividade antibacteriana de amostras dos cimentos Bio-C Sealer (Angelus), EndoFill (Dentsply-Mallefer), Sealer 26 (Dentsply), AH Plus (Dentsply), Sealapex (Sybron-Endo) e EndoSequence BC Sealer (Brasseler) após a presa também foi investigada em biofilmes de 48 h das bactérias *E. faecalis* e *S. mutans*, crescidos em discos com 4 mm de diâmetro e 2 mm de altura. A atividade antibacteriana foi avaliada por contagem das unidades formadoras de colônias (UFC) utilizando o software ImageJ. Os dados foram comparados por ANOVA a um critério seguido pelo pós-teste Holm-Sidak ($\alpha=5\%$). Amostras frescas do Bio-C Sealer exibiram atividade antimicrobiana contra todas as bactérias avaliadas pelo método de difusão em ágar, exceto para *S. mutans*. A análise da formação de biofilme mostrou que todos os cimentos endodônticos testados apresentaram valores similares de UFC para *E. faecalis* ($p > 0,05$), enquanto biofilmes de *S. mutans* foram mais suscetíveis ao EndoFill em comparação com os demais cimentos ($p < 0,05$). Conclui-se que o cimento Bio-C Sealer fresco exibe atividade antibacteriana para *E. faecalis*, *S. aureus*, *P. aeruginosa* e *E. coli*, mas não inibe o crescimento de *S. mutans*. Após a presa, o cimento Bio-C Sealer exibe potencial antimicrobiano similar ao dos demais cimentos avaliados em biofilme de *E. faecalis*, mas inferior ao do EndoFill para *S. mutans*.

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