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ORIGINAL ARTICLE

Antibiofilm potential over time of a tricalcium silicate material and its association with sodium diclofenac

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Abstract

Objectives The objectives of this study are to investigate, over time, the antimicrobial activity against polymicrobial biofilms and ability to inhibit biofilm formation, of Biodentine (BD) alone and with 5% and 10% sodium diclofenac (DC).

Material and methods The antimicrobial activity of BD alone and modified with 5% and 10% DC against polymicrobial biofilm growth in dentin was determined by a modified direct contact test. The study groups were (1) BD; (2) BD + 5% DC; and (3) BD + 10% DC. The viability of microorganisms after 1 and 4 weeks was quantified by means of an ATP assay and flow cytometry. The antibiofilm efficacy of the materials, preventing polymicrobial biofilm formation over time, was assessed by confocal laser scanning microscopy (CLSM).

Results The results obtained with both the ATP test and flow cytometry showed that BD alone and with 5% and 10% DC exerted antibiofilm activity with respect to the control, in the two evaluated times ($p < 0.001$). Comparison between groups showed a tendency of increased antimicrobial effect, both over time and depending on the DC concentration. These results coincide with those obtained in CLSM analysis, where efficacy increased with time and DC concentration.

Conclusions and clinical relevance Biodentine, over time, showed antimicrobial and antibiofilm efficacy on polymicrobial biofilms. The addition of 5% and 10% DC to BD enhanced this effect, in a concentration- and time-dependent manner.

Keywords Antimicrobial activity · Antibiofilm · Biodentine · Sodium diclofenac · Tricalcium silicate

Introduction

The development of bioactive or bioinductive dental materials has contributed greatly to the current boom in minimally invasive strategies for vital pulp therapy (VPT) and regenerative endodontic procedures (REP) [1–3]. Among them, hydraulic calcium silicate-based materials (CS) such as mineral trioxide aggregate (MTA) are considered of choice due to their ability to induce tissue repair and stimulate mineralization, their biocompatibility, and mechanical properties [1, 4, 5]. Ideally these materials should also possess antimicrobial activity, since they are used in clinical situations where microorganisms exist, mainly growing in the form of biofilms [6].

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Biodentine™(BD) (Septodont, Saint-Maur-des-Fossès, France), a CS-based material, is currently used as an alternative to MTA in pulp capping and pulpotomies, as an apical barrier in apexification, a coronal barrier in REP, and other endodontic applications [1, 4, 5, 7, 8]. It consists of a powder containing tricalcium silicate, calcium carbonate and zirconium oxide and a solution of calcium chloride [9]. It has improved physical properties compared to MTA, such as better handling, a shorter setting time, greater color stability, and the possibility of being used as a temporary restorative material [7]. BD has shown biocompatibility along with bioactivity similar to MTA and can provide for a critical balance between pulp inflammation and regenerative potentials [1, 4, 5, 7].

This material forms hydrated calcium silicate and calcium hydroxide (CH) in contact with water [9, 10]. The release of calcium and hydroxyl ions promotes pulp tissue healing and mineralized tissue formation, while its alkalinity contributes to microbial elimination [1, 5, 10]. Published data on its antimicrobial ability are conflicting, however. Laboratory studies have demonstrated different degrees of antimicrobial

activity. Although the time and methods of evaluation varied, most investigations indicate that BD has efficacy against planktonic bacteria in vitro [10–23], but there is controversy regarding its activity over time [15, 19]. Just two studies report on the in vitro antimicrobial capacity of BD against monospecies [24] or multispecies [25] biofilms, concluding that BD had a limited effect. Meanwhile, its ability to inhibit biofilm formation has only been evaluated in one short-term study [18]. More studies with multispecies biofilms and the quantification of live/dead bacteria are needed to clarify these findings [23].

The incorporation of antimicrobial agents into dental materials has been aimed to provide antibacterial activity. Previous studies have incorporated chlorhexidine, cetrimide, or titanium tetrafluoride to BD, obtaining improved antibacterial properties [12, 19, 20]. Sodium diclofenac (DC), a potent non-steroidal anti-inflammatory, has demonstrated in vitro and in vivo antimicrobial activity on gram-positive and gram-negative bacteria [26–28]. A recently published study shows that 5% and 2.5% DC solutions have a greater antimicrobial effect than triantibiotic and diantibiotic solutions [29]. Also, the addition of 5% DC to CH paste increases the antimicrobial activity against *E. faecalis* biofilms [30].

The objectives of the present study were therefore to investigate, over time, the antimicrobial activity against polymicrobial biofilms as well as the ability to inhibit the formation of new biofilms, of BD alone and with the addition of 5% and 10% DC.

Materials and methods

This protocol was approved by the Ethics Committee of the University of Granada, Spain (no. 1076 CEIH/2020).

In this study, the antimicrobial activity of the BD alone and modified with 5% and 10% DC over time was determined by means of a modified direct contact test (DCT) against polymicrobial biofilms growth in dentin. The DCT reflects a material's ability to kill microorganism growth in biofilm. The viability of microorganisms was quantified by the adenosine triphosphate (ATP) assay and flow cytometry. The antibiofilm efficacy of the materials, preventing the formation of biofilm over time, was assessed by confocal laser scanning microscopy (CLSM).

Materials

Discs of BD (Biodentine™, Septodont) alone and modified with 5% and 10% of DC powder (Spanish Pharmacopeia grade and master formulation) were prepared under aseptic conditions using sterile silicone molds, 5 mm in diameter, and 1.5 mm high. BD was mixed according to the manufacturer's recommendations. To prepare the modified materials, 5% and 10% DC were mixed with the BD powder (weight/weight) using an amalgamator before mixing with the BD liquid. For experimental BD + 5% DC and BD + 10% DC, 665 mg of BD powder and 35 mg of DC powder, and 630 mg of BD powder and 70 mg of DC, respectively, were weighed and were placed into the pre-capsule of BD and mixed with a vibrator. Then, in both cases, five drops of BD liquid were added, and the mixed were vibrated at 4000 rotations per minute for 30 s. The materials were put into the molds and stored 24 h in an incubator at 100% humidity for setting. They were then removed from the mold and sterilized with ultraviolet light.

The antibiofilm activity of the materials over time was evaluated after 1 and 4 weeks. For the direct antibiofilm test, 10 samples per group (group 1: BD; group 2: BD + 5% DC; group 3: BD + 10% DC) and period of evaluation (1 and 4 weeks) were prepared. To determine biofilm inhibition, 4 samples were obtained per group and time.

Antimicrobial efficacy against polymicrobial biofilms

Dentin specimen preparation and infection

Eighty sterile dentin radicular specimens (4 × 4 × 0.7 mm) obtained from 40 single-rooted non-carious teeth were used as substrate for polymicrobial biofilms, as previously reported [29]. Ten dentin blocks per group and period of evaluation (1 and 4 weeks) were obtained. For dentin infection, microbial samples were collected from infected root canals of single-rooted teeth of three volunteers following a previously described method [31], then mixed in tryptic soy broth (TSB). After anaerobic incubation at 37 °C for 24 h, cell density was adjusted in a spectrophotometer to a concentration of approximately 3.0×10^7 colony-forming units per milliliter in TSB. The wells of 24-well microtiter plates were inoculated with 200 µL of the microbial suspension and 1.8 mL of sterile TSB. The sterile dentine blocks were submerged in the inoculated wells and incubated for 3 weeks at 37 °C in an anaerobic atmosphere. The TSB was refreshed once a week. Two additional dentin samples per group / time were processed for observation with scanning electron microscopy to confirm the growth of biofilms (Fig. 1).

Modified direct contact test

To test the antimicrobial activity of materials against polymicrobial biofilms, a modified DCT was used [32]. The dentin blocks with the formed biofilm were washed with 0.9% saline solution for 1 min to eliminate planktonic bacteria, then randomly allocated to the groups ($n = 10$ group/time). Afterwards, the discs of the material from each study group were placed on each infected dentin block — the

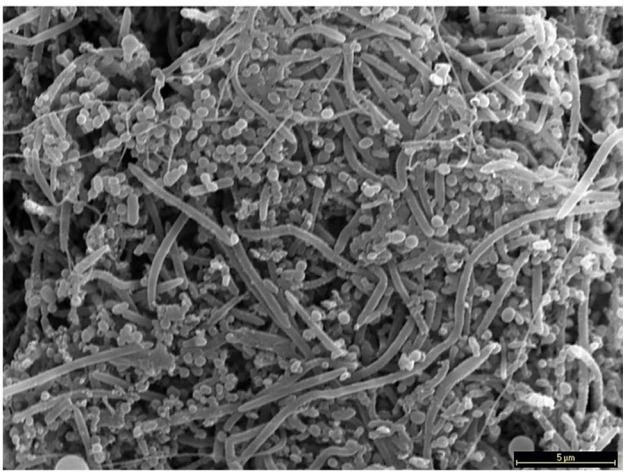

Fig. 1 Representative scanning microscopy image of polymicrobial biofilm grown on dentin samples after 3 weeks

surfaces permitting close contact between dentin and material — in a personalized mold containing 300 µl of TSB in the wells of a 24 microtiter plate, which was incubated for 1 or 4 weeks at 37 °C under anaerobic conditions. Every 3 days, 100 µl of TSB was added to each mold to avoid desiccation. Ten infected dentin blocks without material, per period of evaluation, served as the control group. After each contact time, the dentin samples were placed in Eppendorf tubes with 200 µL of TSB, vortexed for 10 s, and sonicated for 10 min, to ensure recovery of the biofilms.

The microbial viability of the recovered microbiological suspensions was evaluated by means of the ATP assay and flow cytometry (FACS) [33, 34].

For the ATP assay, 100 µL of microbial suspension was added to 100 µL of BacTiter-Glo reagent (BacTiter-Glo; Promega, Madison, WI), in a 96-well opaque plate (Greiner, Monroe, NC), followed by incubation at room temperature for 5 min. The luminescence produced was measured with a luminometer (GloMax E6521, Promega) and expressed as an absolute value and as the percentage (%) reduction of relative light units (RLUs) in each group with respect to the control using the formula: (1-[$RLUs_{test}$/mean $RLUs_{control}$]) / 100.

For flow cytometry, 100 µl of the microbial suspension was introduced into a glass tube with 1 µl of isotonic solution. The suspension was labeled with the microbial viability kit (LIVE/DEAD, BacLight; Invitrogen, Eugene, OR) to assess the integrity of the cytoplasmic membrane. The kit contains two fluorescent nucleic acid dyes with different capacities to penetrate bacterial cells. Syto 9 is a green-fluorescent stain allowing the identification of live and dead microorganisms. Propidium iodide (PI) is a red dye that penetrates only cells with damaged membranes. After labeling the microbial suspension with 100 µL of the fluorochromes according to the manufacturer's instructions, the tube was placed in the FACSCanto flow system (FACSCanto II cytometer, Becton, Dickinson, Madrid, Spain), and the results were analyzed with the software of the cytometer (FACSDiva Version 6.1.3., Becton, Dickinson), which made it possible to obtain a graph of two-dimensional points (*dop-plot* or *contour-plot*) representing the different cell populations within the microbiological sample, with damaged membranes (dead) or undamaged ones (live). Forward and side scatter gates were established to exclude debris. In all cases, 50,000 events were evaluated.

Results were expressed as an absolute value of damaged cell membranes (dead cells) per mL and % increment of death with respect to the control.

Biofilm inhibition formation test (CLSM)

To determine the capacity of the materials to inhibit biofilm formation, 24 samples of material ($n = 4$ per group/time) were exposed to 1.8 mL of sterile TSB and 200 µL of the polymicrobial suspension in a 24-microtiter plate; they were incubated at 37 °C in an anaerobic atmosphere on a rocking table (model Swing Sw 8 10,000–00,015; OVAN, Badalona, Spain) for 1 or 4 weeks. The TSB was refreshed every 2 days.

After each incubation period, the samples were rinsed and stained with Syto 9/PI (LIVE/DEAD, BacLight; Invitrogen, Eugene, OR) for 15 min as previously reported [35]. They were rinsed with saline solution, mounted on a 60 l-Dish (Ibidi, Martinsried, Germany) with the mounting oil (BacLight; Invitrogen) and directly observed using an inverted confocal laser scanning microscope (Leica TCS-SP5 II, Leica Microsystems, Mannheim, Germany). The respective excitation/emission wavelengths were 494/518 nm for Syto9 and 536/617 nm for PI. Five microscopic confocal volumes (stacks) from random areas were acquired from each sample using the 40 × oil lens, 1 µm stepsize, and a format of 512 × 512 pixels. Each picture represented an area of 387 × 387 µm. Scanning was performed from the top of the biofilm to the dentine surface. For quantification purposes, *bio*Image_L software was used (http://www.bioimageL.com/get_bioimage_L) [36]. The parameters evaluated in each group were the total biovolume (μm^3) and % of red-stained population (dead cells).

Statistical analysis

Before each statistical analysis, the normality of the data was evaluated by means of the Shapiro–Wilk test and the equality of the variances with the Levene test. An ANOVA test and Duncan's post-hoc test, to show groupings, were performed when the data followed a Gaussian distribution and variances were equal. An ANOVA with Welch

correction was applied, followed by the Games-Howell test when variances were unequal. In these cases, a Student's *t* test was used for comparison between times. If the data were not Gaussian, global comparison was performed by the Kruskal–Wallis test, and comparison between 1 and 4 weeks by the Mann–Whitney test. Data on the percentage of dead cells were subjected to logit transformation (Ln(p/(1-p)) before global comparison by ANOVA and Duncan's post-hoc tests to show groupings. Comparisons between 1 and 4 weeks for this variable were determined with the Student's *t* test. The statistical analysis was performed by means of SPSS 23.0 (IBM Corp, Armonk, NY). In all cases, a value of $p < 0.05$ was considered significant.

Results

The results obtained with both the ATP test and flow cytometry showed that BD alone and combined with 5% and 10% DC had antibiofilm activity, with respect to the control, in the two evaluated times ($p < 0.001$) (Tables 1 and 2). Comparison between groups in terms of % reduction RLUs showed a tendency to increase the antimicrobial effect, both over time and depending on the DC concentration. These results are consistent with the values obtained by flow cytometry, where the effect is concentration-dependent but only significant in the short term (1 week) and with the addition of 10% DC to BD.

A total of 120 CLSM operative fields (3D stacks) were evaluated (20 stack/group/period). Mean values of total biovolume (μm^3) and % of dead cells after each period are given in Table 3. No significant differences were obtained regarding the total biovolume (μm^3) between groups at either of the evaluated times. However, a tendency of decreased biovolume according to the concentration of DC was observed. Taking into account the % of dead cells, significant differences were seen for BD + 10% DC with respect to the other groups at 1 week that disappeared over time. Yet BD alone and combined with 5% DC showed significantly higher % values at 4 weeks. Representative images of the treated biofilms can be found in Fig. 2.

Table 1 Antimicrobial activity of Biodentine (BD) alone and modified with diclofenac (DC) against polymicrobial biofilms by ATP assay. Mean (standard deviation). $n = 10$/group/time

Groups	Relative light units (RLUs)			RLUs % reduction	
	1 week	4 weeks	Comparison[¥] *p* value	1 week	4 weeks
BD	90,368.50 (37,982.70) [a]	50,296.10 (43,605.82) [a]	0.042	57.33 (17.93)	69.40 (26.53)
BD + 5% DC	92,020.80 (47,442.36) [a]	60,887.40 (40,371.21) [a]	0.131	56.55 (22.40)	62.95 (24.56)
BD + 10% DC	64,977.80 (36,871.45) [a]	32,209.80 (18,841.84) [a]	0.026	69.32 (17.41)	80.40 (11.46)
Control	211,811.40 (84,413.55) [b]	164,368.00 (31,330.05) [b]	0.123		
Comparison *p* value*	< 0.001	< 0.001			

*Global comparison determined by ANOVA. Read vertically, the same superscript letters show no statistically significant differences determined by Duncan's test

¥ Comparison between 1 and 4 weeks determined by Student's *t* test

Table 2 Antibiofilm activity of Biodentine (BD) alone and modified with diclofenac (DC) against polymicrobial biofilms by flow cytometry. Mean (SD). $n = 10$/group/time)

Groups	Death cells/ml			Death % increment	
	1 week*	4 weeks**	Comparison[¥] *p* value	1 week	4 weeks
BD	23,468.70 (8720.15) [a]	18,816.60 (2510.67) [a]	0.135	50.49 (55.92)	79.64 (23.97)
BD + 5% DC	23,202.10 (3051.41) [a]	17,548.30 (6564.17) [a]	0.029	48.78 (19.57)	67.53 (62.67)
BD + 10% DC	29,678.70 (5856.51) [b]	21,158.40 (2500.2) [a]	0.001	90.32 (37.55)	102 (23.87)
Control	15,594.30 (2430.06) [c]	10,483.60 (2336.4) [b]	< 0.001		
Comparison *p* value	< 0.001	< 0.001			

*Global comparison determined by ANOVA test. Read vertically, the same superscript letters show no statistically significant differences determined by Duncan's test. ** Global comparison determined by ANOVA test with Welch's correction. Read vertically, the same superscript letters show no statistically significant differences determined by Games-Howell test. ¥Comparison between 1 and 4 weeks determined by Student's *t* test

Table 3 Antibiofilm activity of Biodentine (BD) alone and modified with diclofenac (DC) by CSLM. Mean (SD). ($n = 20$ *stacks*/group/time)

Groups	Total biovolume (um^3)			% dead cells		
	1 week *	4 weeks *	Comparison[¥] *p* value	1 week **	4 weeks **	Comparison[¥¥] *p* value
BD	11,785.9 (12,909.25)	69,130.6 (140,141.30)	0.841	32.07 (25.41) [a]	59.80 (20.99)	0.001
BD + 5% DC	8732.95 (9755.66)	55,787.65 (99,894.67)	0.341	39.73 (21.27) [a]	66.77 (22.25)	< 0.001
BD + 10% DC	9896.4 (34,245.85)	34,245.85 (49,233.33)	0.174	54.77 (29.84) [b]	71.97 (16.90)	0.227
Comparison *p value*	0.976	0.836		0.007	0.187	

[*] Global comparison by Kruskall -Wallis test. [¥] Comparison between 1 and 4 weeks determined by Mann–Whitney test. ** Global comparison by ANOVA test. Previously data were subjected to logit transformation (Ln(p/(1-p)). Read vertically, the same superscript letters show no statistically significant differences by Duncan test. [¥¥]Comparison between 1 and 4 weeks determined by Student's *t* test

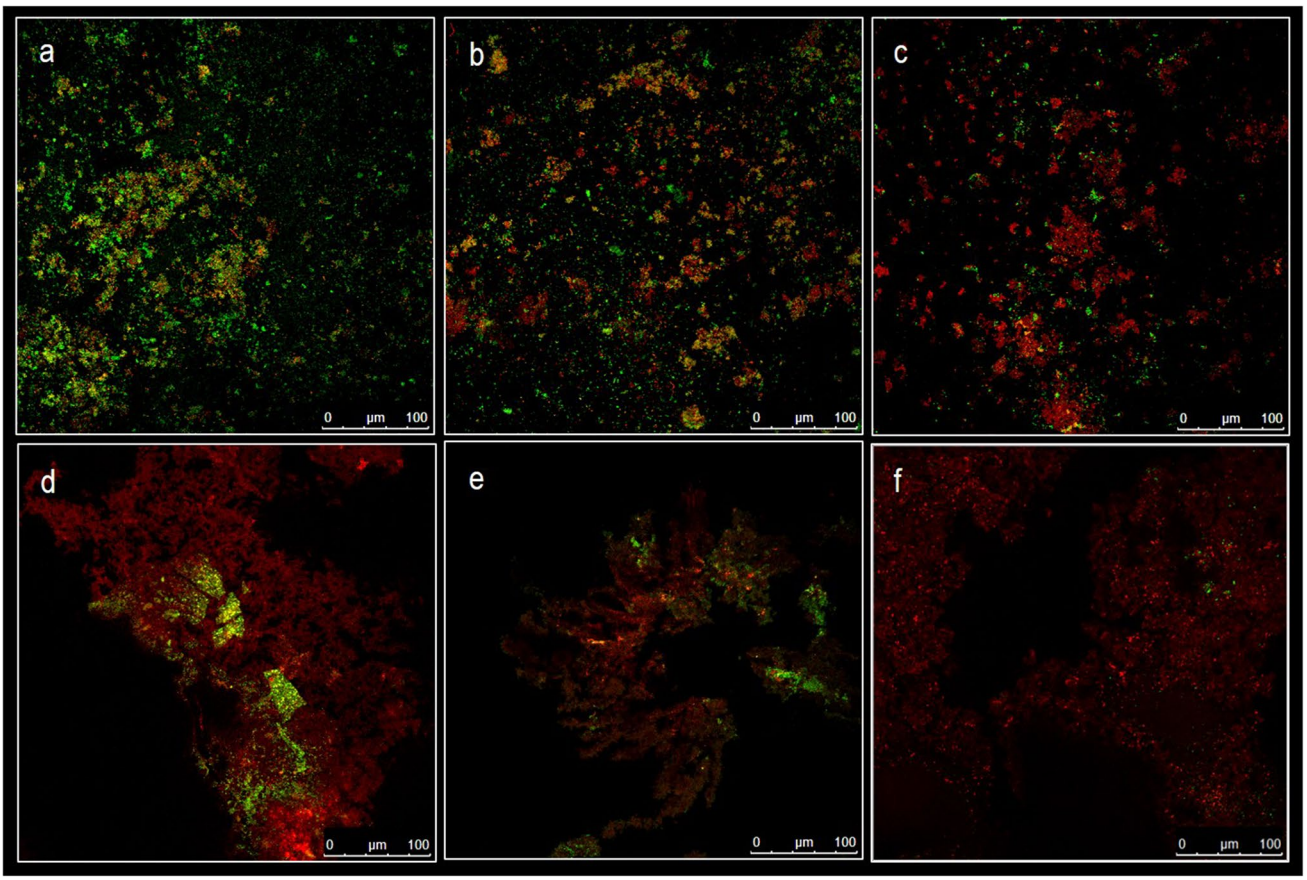

Fig. 2 Representative confocal laser scanning microscopy images of the different study groups. **a** BD 1 week; **b** BD + 5% DC 1 week; **c** BD + 10% DC 1 week; **d** BD 4 weeks; **e** BD + 5% DC 4 weeks **f** BD + 10% DC 4 weeks

Discussion

The antimicrobial activity of materials used in VPT and non-vital procedures is a desirable property, as the presence of residual microorganisms after these treatments could interfere with healing, and therefore with the success of a treatment [1, 25]. The antimicrobial activity of BD has been mainly investigated against planktonic bacteria using the diffusion agar test [12–14, 16, 17, 19, 21–23] or dilution methods [10, 11, 23]. Nevertheless, the efficacy against this mode of microbial growth may be greater than when it is part of a biofilm [37]. Furthermore, the antibiofilm efficacy of BD over time has not been sufficiently investigated.

In this study, a mature polymicrobial biofilm model was developed from microbiological samples of infected teeth to simulate infected dentin in vivo. Biofilm formation was performed in human dentin, which provides for adequate standardization, infection, and manipulation of the specimens. In addition, dentin substrate for the formation of biofilms is more suited to the clinical situation, since it contains dentin tubules and gives information on the buffering capacity of dentin [38]. The biofilms were formed during 3 weeks, at which point they are considered mature, and therefore offer greater resistance to antimicrobial treatments than a young biofilm [37].

To evaluate direct antibiofilm activity over time, a modified TCD was used [32]. It is a quantitative and reproducible method that can be easily standardized; it simulates contact between biofilm microorganism growth in dentin and the materials applied. In addition, it resolves the limitations of the agar diffusion test, a method considered inappropriate because it depends on the solubility of the material in the medium [23, 39].

Cell viability was determined using three quantitative, real-time, fast, and reproducible methods with high sensitivity and specificity [33, 34]. The ATP is the chemical form of energy of all living cells, and therefore its quantification estimates the viable microbial population in a sample [33]. Flow cytometry provides multiparametric information on each individual cell within a heterogeneous population, emitting fluorescence when cells are marked with fluorochromes, thus allowing for the discrimination of microorganisms with damaged versus intact membranes [33, 40]. The staining of the samples and observation with CLSM furthermore make it possible to obtain, in situ, the proportion of viable and non-viable bacteria in a three-dimensional image without disturbing the cells attached to the substrate [36]. Additionally, these techniques enable one to discriminate the viable but non-culturable cell population that cannot be detected using traditional cultures [40, 41].

In this study, after 7 days of contact with biofilms, BD alone produced a reduction in microbial viability — around 50% with respect to the control — according to both the ATP test and cytometry (57.33% reduction of the ULRs and 50.49% increase in dead cells, respectively). Only one study [25] to date has evaluated the antibiofilm efficacy of BD, using DCT after 1 week of exposure, with dentin as a substrate for the formation of mature multispecies biofilms. The results, obtained by CLSM analysis, showed a lower antimicrobial efficacy (30.35% death) than those obtained in this study. It has been suggested that the decrease in pH over time [18] and the buffering effect of dentin [25] could be responsible for a limited effect on microorganisms in biofilms. After 4 weeks of contact in this study, however, data obtained by BD with both tests showed a tendency of increased effectiveness. To our knowledge, the antibiofilm effect of BD has not been previously determined for this exposure period. The effect was confirmed by CLSM analysis. BD at 1 week exerted a low capacity to inhibit polymicrobial biofilm growth (32.07% dead cells). These results agree with the ones obtained by Farrugia et al. [18], who likewise report a limited inhibitory effect of multispecies biofilm formation on BD samples at 7 days (29% dead cells). They attributed the low antibiofilm efficacy of BD to the pH reduction over time. Yet in the present study, data indicate a significant increment of the % dead cells at 4 weeks, close to double (59.80% of microbial biofilm cells in BD samples were dead).

The findings of this study could be related with an increase in alkalinizing conditions during the hydration of BD over time. Recent studies report that BD maintains its ability to alkalize the medium in early and late phases of hydration, with a significant increase in pH at 14 days that became saturated at 28 days [10, 23]. This could be an indirect effect, however. Although an alkaline pH in the microenvironment plays an important role in the inhibition of bacterial viability [42], a significant correlation between the release of free calcium and silica ions and the antibacterial effect of BD has been reported [10, 42, 43] and directly related with antibacterial efficacy. The high release of calcium in BD is attributed to the presence of calcium silicate, calcium chloride and calcium carbonate in its composition [10, 44]. An increase in pH — detrimental for bacterial viability — could occur due to the release of calcium and silica ions from bioceramics [42, 45], while the ions released might lead to bacterial membrane depolarization, by binding positively charged calcium and silica ions to negatively charged bacterial membrane receptors, resulting in cell lysis [42]. In this sense, silica released into the solution at high pH may directly inhibit bacterial viability by acting as a surfactant at solid–liquid interfaces and by interfering with the cellular integrity of bacteria [42, 43].

Some studies suggest that the mechanism of bacterial killing through other bioactive or tricalcium silicate materials depends on the release of ions from the material and the presence of dentin [43, 46]. Even though dentin has shown an inhibitory effect on the antibacterial activity of several endodontic disinfecting agents [38], previous studies report that dentin can facilitate the killing of *E. faecalis* by MTA and Bioaggregate [46], and Bioactive glass (BAG) [43]. These investigations suggest that dentin-triggered material dissolution, causing elevated local pH and ion levels, would exert an indirect effect by promoting Ca/P precipitations, which might interfere with the cellular integrity of bacteria. A recently published study reports that EndoSequence BC Sealer, a CS-based material, exhibited significant antimicrobial capacity in the presence of dentin for up to 2 weeks on an 8-week-old E. *faecalis* biofilm [47].

The incorporation of DC in BD led to a tendency of increased antibiofilm activity after direct contact. This increase was dependent on the concentration and the exposure time, an effect that was more evident after 1 week and with the addition of 10% DC, although the trend was maintained at 4 weeks. Such results coincide with those obtained in CLSM analysis, where efficacy increased with time and DC concentration. Previous investigations have reported an increase in the antimicrobial activity of CH paste with the addition of DC, without altering pH [30]. The antimicrobial efficacy of DC is attributed to different mechanisms, including impairment of membrane activity [27], inhibition of bacterial DNA synthesis [26], antiplasmid activity [28], alteration in gene-encoding transport/binding proteins, and down-regulation of efflux pumps [48]. In turn, Gubler et al. [45] showed that the pH generated in an aqueous environment by silica-based bioactive glasses is affected by the sodium content in the glass. It is furthermore possible that the addition of DC to BD could cause greater alkalinity in the medium given its sodium content.

Recent research has demonstrated that BD exerts anti-inflammatory activity by controlling pro-inflammatory factor secretion and decreasing inflammatory cell recruitment [49, 50]. The addition of DC could heighten the anti-inflammatory and antimicrobial activity overtime of BD. Its incorporation into other CS materials might also be beneficial, to ensure or increase these properties. Care should be taken, however, as a modification of the original product may imply changes in other characteristics, e.g., setting time, hydration capacity, handling properties or physical strength. More studies are required in these specific areas.

Conclusion

Biodentine, over time, showed antimicrobial and antibiofilm efficacy on polymicrobial biofilms. The addition of 5% and 10% DC to Biodentine enhanced this effect, in a concentration- and time-dependent manner.

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Declarations

Ethics approval The procedures and study protocol described here were approved by the Ethics Committee of the University of Granada, Spain (no. 1076 CEIH/2020). All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional Review Board and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent to participate Informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare no competing interests.

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