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ORIGINAL ARTICLE

Effect of exposure conditions on chemical properties of materials for surgical endodontic procedures

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Abstract

This study investigated the role of aging and changes in environmental conditions on selected properties of a prototype radiopacified calcium silicate-based cement (TZ-base) with or without incorporation of silver nanoparticles or bioactive glass, and two commercial materials, Biodentine and intermediate restorative material. Materials were immersed in ultrapure water or fetal bovine serum for 28 days and were characterized with scanning electron microscopy and energy dispersive x-ray analysis. Immersion media were either replaced weekly or not replenished at all and were assessed for alkalinity and calcium release after 1, 7, 14, 21, and 28 days; antibacterial effect against 2-day monospecies biofilms; and cytotoxicity by the 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide assay after 1, 7, or 28 days. Alkalinity, calcium release, antibacterial activity, and cell cytotoxicity increased over time when the medium was not changed but decreased with medium replenishment. Immersion in fetal bovine serum resulted in lower alkalinity, less bactericidal properties, and lower cytotoxicity of prototype cements and Biodentine than did water immersion. Biodentine and 20% bioactive glass-containing cement had overall lower alkalinity, calcium release, and antibacterial activity than TZ-base, and Biodentine was less cytotoxic than TZ-base. In conclusion, exposure conditions and cement modifications significantly affected materials' leaching properties. Exposure conditions warrant consideration when evaluating cements' clinical properties.

KEYWORDS

anti-bacterial agents, bioactive glass 45S5, calcium silicate, chemical characterization

INTRODUCTION

Surgical endodontic procedures entail application and direct exposure of materials to host tissue fluids. Materials should remain physically stable but not necessarily inert, as stimulation of healing and antibacterial activity are desirable [1]. The

material may induce transient or more long-lasting changes in the local microenvironment; however, the microenvironment can also affect the material [2]. For hydraulic calcium silicate cements, in vivo conditions may alter their chemical reactivity through consumption of the released calcium hydroxide by the carbon dioxide present in the tissue fluid environment [3].

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TABLE 1 Presentation of test materials, their composition and handling procedure.

Material name	Composition	Handling
TZ-base	<u>Powder</u> : 80% w/w tricalcium silicate cement (CAS No: 12,168–85-3, American Elements), 20% w/w zirconium oxide (2–15 μm particle size, Koch-Light Laboratories) <u>Liquid</u> : ultrapure water	Hand-mixed and spatulated at a 0.35 liquid/powder ratio
TZ-bg10, TZ-bg20	<u>Powder</u> : TZ-base with 10 or 20 % w/w bioactive glass 45S5 (Cas No: 65,997–17-3, Mo-Sci Corporation) replacement in the cementitious phase respectively <u>Liquid</u> : ultrapure water	
TZ-Ag0.5, TZ-Ag1, TZ-Ag2	<u>Powder</u> : TZ-base <u>Liquid</u> : 0.5, 1 or 2 mg/mL silver nanoparticle solution (CAS No: 7440–22-4, < 100 nm particle size, Sigma Aldrich) respectively, following dispersion of silver nanoparticles in ultrapure water [5]	
Biodentine (Septodont)	<u>Powder</u> : Calcium silicates, calcium carbonate, zirconium oxide <u>Liquid</u> : water, calcium chloride, water-soluble polymer	According to the manufacturer
Intermediate restorative material (IRM; Dentsply Sirona)	<u>Powder</u> : Zinc oxide, polymethylmethacrylate <u>Liquid</u> : eugenol, acetic acid	

The effect of environment on surface characteristics and corresponding antibacterial activity of hydraulic calcium silicate cements has been assessed *in vitro* [3–7]. However, despite the long known positive role of alkalinity on their antibacterial potential [8], the effect of clinical environment on antimicrobial properties associated with leaching is still not clear. Furthermore, studies on the antibacterial activity of endodontic cements for root-end filling and perforation repair *per se* are scarce [9].

Current formulations of commercial hydraulic calcium silicate cements appear to have limited antibacterial activity [10]. The bactericidal effect in hydraulic calcium silicate cements is mainly pH-dependent and is therefore considerably reduced over time as alkalinity is neutralized due to local interactions with host tissues [11]. Incorporation of silver nanoparticles could be explored to enhance their antibacterial profile [12]. Silver nanoparticles can damage bacterial cells by interfering with the permeability of the bacterial membrane as well as interacting with bacterial proteins and DNA [13]. Alternatively, bioactive glass formulations could promote release of calcium and phosphate ions and enhance the biological profile of cements [14]. Their incorporation in hydraulic calcium silicate cements could therefore be explored as well [15].

The aim of this study was to investigate the role of exposure conditions (immersion medium and aging period) on selected properties of endodontic cements used for surgical procedures. Furthermore, in order to assess the role of material composition on these properties, silver nanoparticles and bioactive glass were incorporated in prototype hydraulic calcium silicate cements. The null hypotheses were that aging period, immersion medium, and material composition do not affect the leaching characteristics of endodontic cements.

MATERIAL AND METHODS

All chemicals and equipment were purchased from Merck unless stated otherwise. Table 1 presents the test materials.

Material discs (9 mm diameter, 1 mm thickness) were prepared and allowed to set for 24 h at 37°C, 100% relative humidity. Consequently, each material was immersed in tubes containing either 4 mL ultrapure water (water; Elix Essential 5 UV Water Purification System) or fetal bovine serum (F7524) and stored at 37°C. Testing of material leachates was conducted over a 28 day aging period, during which the immersion liquid was either replenished weekly or not changed at all (Figures 1A, 2A, 3A).

For biological assays, samples were additionally prepared inside cell culture inserts (pore size 0.4 mm, 9 mm diameter) and placed immediately in 12-well plates containing 1.8 mL medium at 37°C for 24 h (Figure 2B).

Test conditions per assay are presented in Table 2 as well as in Figures 1–3. Leachates were sterile-filtered (Millex-GV, 0.22 μm) prior to testing.

Chemical analysis

Alkalinity ($n = 9/\text{subgroup}$) was monitored with a pH meter (Sension+ PH3; Hach), and calcium ion release ($n = 9/\text{subgroup}$) with a calcium ion selective electrode connected to a laboratory meter (ION450; Radiometer analytical, Hach). Separate samples were assessed for pH and Ca^{2+} at the following periods: after 1, 7, or 28 days without changing the medium (independent samples); every 7 days for a total of 28 days upon replenishing the medium weekly

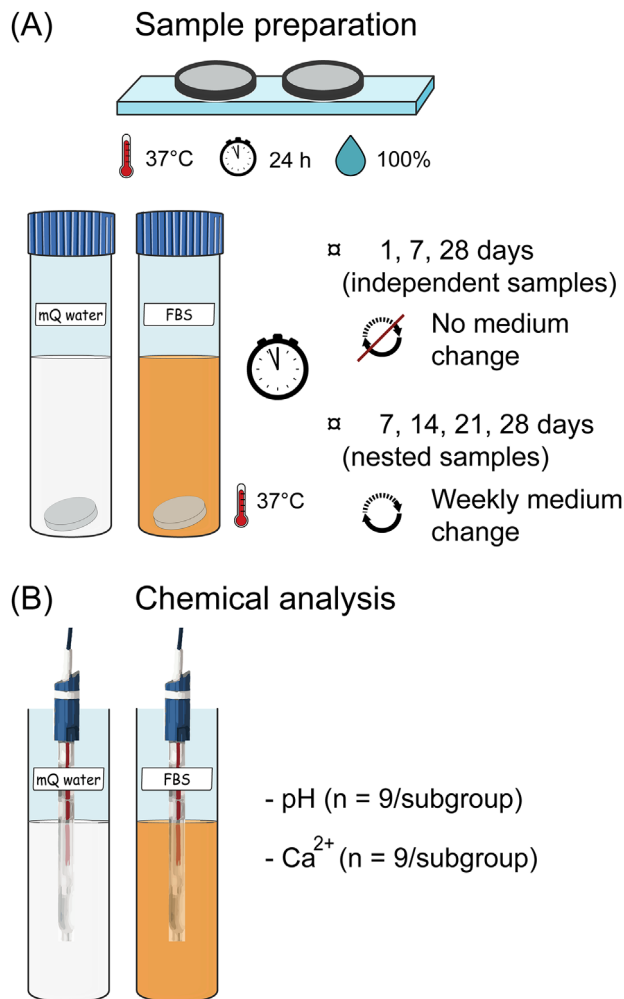


FIGURE 1 Schematic representation of the chemical analysis. (A) Sample preparation for extract acquisition, and (B) testing of material extracts ($n = 9/\text{subgroup}$). The analysis was performed separately for pH and Ca^{2+} . The figure was partially generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license. FBS, fetal bovine serum; mQ, Milli-Q.

(nested specimens) (Figure 1, Table 2). For calibration purposes, three standard solutions were used in the alkalinity assessments (pH 4, 7, 10) and four standard solutions in the calcium release analysis (0.01, 0.1, 1, 10 mM Ca).

Biological assays

All samples were incubated at 37°C, 5% CO_2 , 100% relative humidity. Sterile water and fetal bovine serum served as negative controls. Testing of leachates was done after 1, 7, or 28 days for samples without any medium change (independent samples) and at 7 and 28 days for samples where the medium was refreshed weekly (nested samples) (Figure 2A). Material leachates were additionally obtained using an alternative

preparation method: Materials were placed into a cell culture insert system immediately after mixing for a 24 h aging period (0–24 h leachates) (Figure 2B, Table 2).

Antibacterial activity

Enterococcus faecalis OG1RF ATCC 47077 and *Pseudomonas aeruginosa* ATCC 9027 from frozen stock cultures were incubated overnight in tryptic soy broth. Bacterial inocula (10^8 CFU/mL) were prepared following centrifugation and resuspension of each culture in tryptic soy broth.

Two-day monospecies *E. faecalis* and *P. aeruginosa* biofilms were cultured upon membrane filters (MF-Millipore; 3 mm diameter), following a previously established protocol [16]. Briefly, membranes were placed upon tryptic soy broth agar plates and received 2 μL bacterial inoculum. After 48 h incubation, membranes with biofilm were collected and immersed in 24-well plates containing 500 μL test leachates ($n = 9/\text{subgroup}$).

Following a 24 h overnight incubation, test membranes were retrieved and immersed in tubes containing 1 mL phosphate buffered saline, vortexed, and sonicated. Viable bacterial colonies were calculated upon serial dilution and plating on tryptic soy broth agar plates (Figure 2C).

Cell viability

L929 murine fibroblast cells were cultured in 75 cm^2 flasks in Dulbecco's modified eagle's medium supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin, 2 mM glutamine. At 70%–80% confluence, 10,000 cells were seeded in 96-well plates in 100 μL medium. After 24 h incubation, the medium in the wells was replaced with 100 μL fresh medium and 100 μL test leachate ($n = 9/\text{subgroup}$). Three dilutions of each leachate were also tested. The following day, solutions were aspirated and 100 μL 3-(4,5 dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 0.5 mg/mL) was added to each well. After 3 h, MTT was replaced with 100 μL dimethyl sulfoxide. The optical density of the wells was read in a microplate reader (Synergy H1; BioTek) at 570 nm (Figure 2D).

Scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) analysis

Following a 28 day incubation period in the two media without replenishment (Figure 3A), material specimens ($n = 3/\text{subgroup}$) were retrieved, vacuum desiccated, embedded in epoxy resin, and ground and polished with a series of silicon carbide foils and polishing cloths (Stuers).

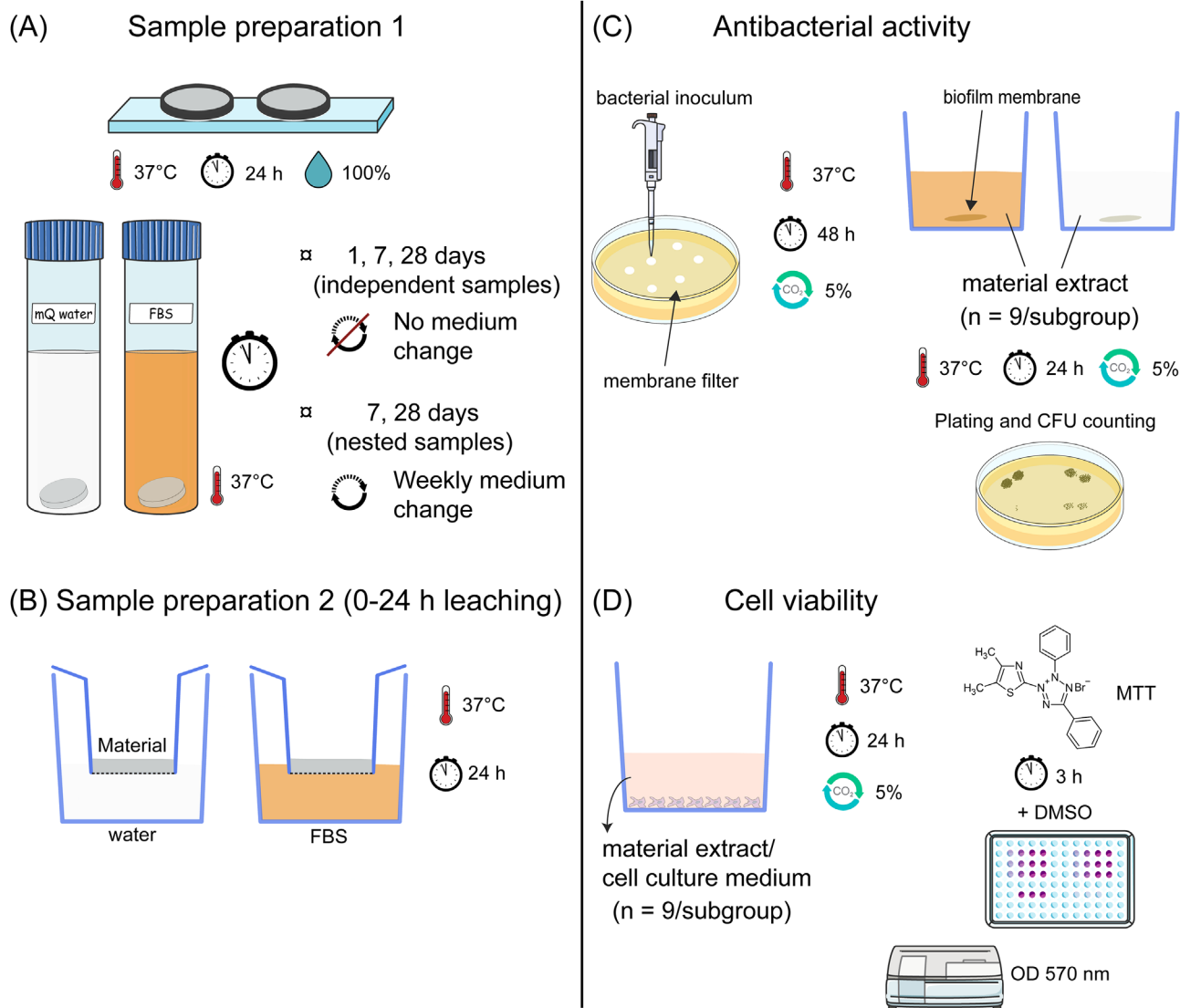


FIGURE 2 Schematic representation of the biological assays. Material extracts were acquired using two different preparation methods. (A) In the first method, extracts were obtained after 1, 7, or 28 days with or without replenishing the medium weekly. (B) In the second method of extract preparation, materials were mixed and immediately placed in cell culture inserts positioned inside 12-well plates containing the respective extract medium for 24 h. (C) For antibacterial activity testing, membrane filters were placed on agar plates and received a bacterial inoculum to cultivate 2-day monospecies biofilms of *Enterococcus faecalis* and *Pseudomonas aeruginosa*. Subsequently, they were exposed to 500 μ L material extract for 24 h and bacterial viability was assessed by aliquots plated on agar plates. (D) For cell viability assessment, an MTT assay was performed using four different concentrations of each material extract. Parts of the figure were generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license. DMSO, dimethylsulfoxide; FBS, fetal bovine serum; mQ, Milli-Q; MTT, 3-(4,5-dimethylthiazolyl-2-yl)32,5-diphenyltetrazolium bromide; OD, optical density.

Finally, they were imaged in backscatter mode with scanning electron microscopy (SEM) (TM4000Plus II; Hitachi). Energy dispersive X-ray (EDX) analyses in random cement areas were also performed (Figure 3B).

Statistical analysis

Power calculations were initially conducted considering a factorial analysis of variance and the sample size used in the

assays accounted overall for an effect size of $f = 0.20$ – 0.28 (α error probability = 0.05, power = 0.95) upon estimations with G*Power 3.1 (Heinrich Heine University).

Regression analyses were performed using Stata/SE 17.0 (StataCorp) to assess the effect of material, immersion medium, and aging period to pH, calcium release, bacterial survival, and cell viability. As data consisted of both repeated measurements within the 28 day period (nested samples) and independent ones, two different models were explored. Additionally, subgroup analyses were conducted, either for

TABLE 2 Exposure conditions of materials per assay prior to testing.

Assay	Aging period prior to testing	Immersion medium
Chemical	<ul style="list-style-type: none"> 1, 7, 28 days without medium replenishment (independent samples) 7, 14, 21, 28 days upon weekly medium change (nested samples) 	<ul style="list-style-type: none"> Ultrapure water Fetal bovine serum
Biological	<ul style="list-style-type: none"> 1, 7, 28 days without medium change (independent samples) 7, 28 days upon weekly medium change (nested samples) 24 h (“0–24 h”) for samples prepared inside cell culture inserts 	
Characterization	28 days without changing medium	
<ul style="list-style-type: none"> Scanning electron microscopy Energy dispersive X-ray analysis 		

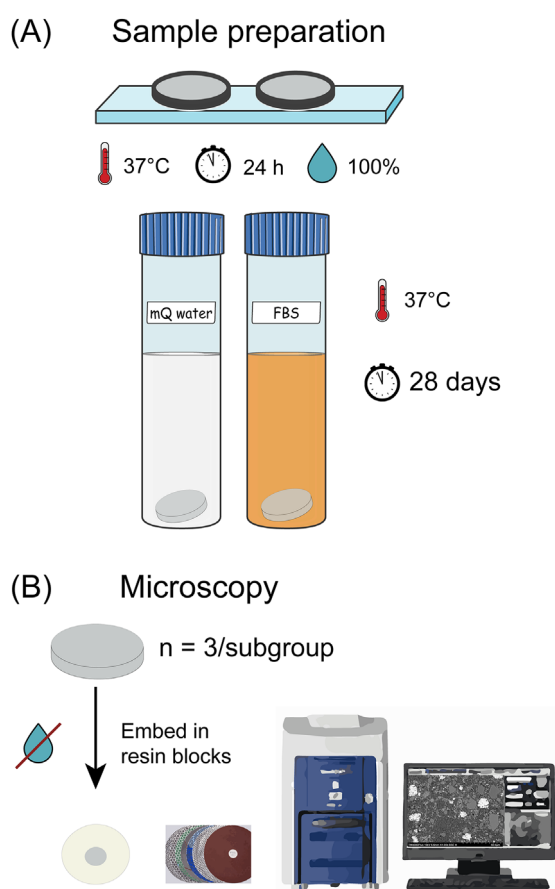


FIGURE 3 Illustrative representation of sample preparation for scanning electron microscopy (SEM) and energy dispersive X-ray analysis. (A) Samples ($n = 3$ /subgroup) underwent a 28-day immersion period in ultrapure water or fetal bovine serum (FBS).

(B) Subsequently, they were retrieved, desiccated, embedded in epoxy resin and imaged in the SEM after grinding and polishing. Figure 3A was partially generated by using pictures from Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license. mQ, Milli-Q.

independent assessment of the effect of exposure conditions on properties of material leachates, or for comparison of the effect of material in the same immersion medium. Specifically for each assay, the following were performed:

- In the chemical analysis, multiple linear regression models were fitted for the independent leachate samples of pH and Ca^{2+} for material specimens where the medium was not refreshed (at 1, 7, 28 days; $n = 378$). In addition, mixed-effects linear regression models were fitted to examine pH or calcium release as outcome variables for the weekly investigation of leachates of nested specimens following medium refreshment (at 7, 14, 21, and 28 days; $n = 126$). For calcium release, subgroup analyses were also conducted for Biodentine ($n = 54$) or for the prototype hydraulic calcium silicate cements ($n = 324$), to assess the effect of the immersion medium in the accumulative leaching conditions separately. The intermediate restorative material (IRM) was not included in any of these analyses due to its different chemical composition.
- In the biological assays, the data from the antibacterial activity assays were logarithmically transformed and expressed as $\log(\text{CFU}+1)/\text{mL}$; the data from cell viability were normalized to the respective negative control values and expressed as relative MTT activity (%). Multiple linear regressions were conducted for the independent leachate measurements, where the medium was not refreshed (at 1, 7, 28 days; $n = 864$ for antibacterial activity and $n = 432$ for cell viability), as well as for the 0–24 h leachates ($n = 288$ for antibacterial activity; $n = 144$ for cell viability). Additionally, mixed-effects linear regression models were fitted for data from nested material specimens (testing after 7 and 28 days upon weekly medium replenishment) for bacterial survival ($n = 288$) and cell viability ($n = 144$) as outcome variables. In all the above regression models for antibacterial assays, the factor “bacterium” was also included as

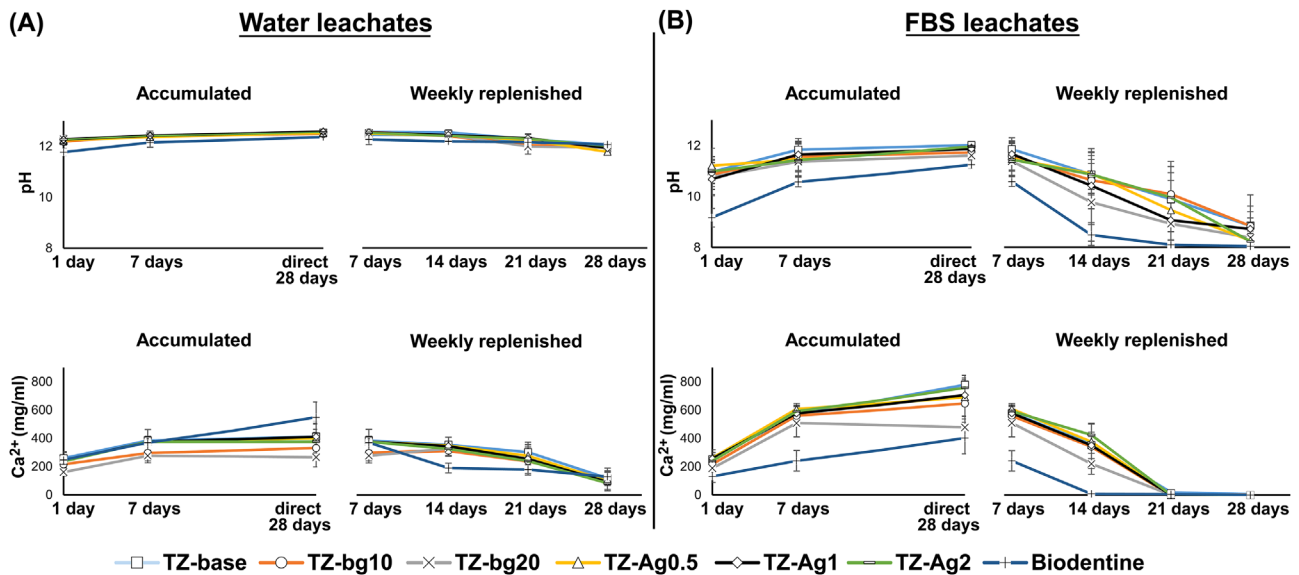


FIGURE 4 Mean pH and calcium release (mg/mL) of materials immersed in (A) water or (B) fetal bovine serum (FBS) over a 28-day evaluation period. Line charts are organized on whether medium was replenished weekly or not (“accumulated”). An overlap in pH values of prototype cements is evident, particularly in water leachates. Test results for Ca^{2+} release were expressed following subtraction of the respective background measurements for pure water and neat fetal bovine solution (FBS). Error bars indicate standard deviation. TZ-base, prototype calcium silicate-based cement; TZ-bg10, TZ-base with 10% bioactive glass; TZ-bg20, TZ-base with 20% bioactive glass; TZ-Ag0.5, TZ-base with 0.5 mg/mL silver nanoparticle solution; TZ-Ag1, TZ-base with 1 mg/mL silver nanoparticle solution; TZ-Ag2, TZ-base with 2 mg/mL silver nanoparticle solution.

a predictor variable. Finally, for antibacterial activity, subgroup analyses were also conducted to compare the effect of each material within the same immersion medium and against the same bacterial species.

Evaluation of assumptions was conducted and in cases where heteroscedasticity of standardized residuals was observed, regressions were performed using robust standard errors. Additionally, the models were further validated by inspecting the QQ-plots of standardized residuals.

RESULTS

Chemical analysis

The data for pH and calcium ion leaching are shown in Figure 4A for water samples and in Figure 4B for fetal bovine serum-immersed materials. Results from the overall regression models are presented in Tables 3 and 4 for independent and nested leachate measurements, respectively.

Aging had a significant effect on pH; alkalinity was increased with aging when the medium was not replenished (Table 3; 7–28 days: mean increase = 0.25, 95% CI [0.16; 0.34]). Alternatively, the alkalinity decreased upon medium replenishment (Table 4; 14–21 days: mean decrease = -0.52, 95% CI [-0.73; -0.31]; 21–28 days: mean decrease = -0.53,

95% CI [-0.72; -0.33]). Exposure to fetal bovine serum statistically significantly reduced the pH of hydraulic calcium silicate cements (Figure 2B). The prototype calcium silicate-based cement (TZ-base; i.e., without inclusions of silver nanoparticles or bioactive glass) had overall higher alkalinity compared to Biodentine and TZ-base with 20% bioactive glass (TZ-bg20).

Aging had a significant effect on calcium release as well, with a similar pattern as seen for alkalinity. The bioactive glass-containing materials and Biodentine leached overall significantly less Ca^{2+} than TZ-base, except for TZ-base with 10% bioactive glass (TZ-bg10) when the medium was changed weekly (Table 4). The immersion medium had a varied effect on the calcium leaching. In the nested samples model, fetal bovine serum immersion led to a decrease in calcium release compared to water. In the accumulative leaching model of independent measurements, subgroup analyses per material revealed that prototype cements leached more calcium ions when immersed in fetal bovine serum than in water. However, for Biodentine, fetal bovine serum significantly reduced the leaching of Ca^{2+} (Table 5; Figure 2).

Antibacterial activity

Results from antibacterial assays are presented in Figure 5A and B for water and fetal bovine serum material leachates,

TABLE 3 Multiple linear regression analyses for independent leachate samples for pH (model 1a) and calcium release (model 2a), with estimated change in pH and calcium release in comparison to the reference group of the predictor variables and their 95% confidence intervals provided.

Variable	Model 1a: pH. Independent samples of accumulative leaching (<i>n</i> = 378)	Model 2a: Calcium release (mg/mL). Independent samples of accumulative leaching (<i>n</i> = 378)
	Coefficient [95% CI]	Coefficient [95% CI]
Material (Ref. TZ-base)	–	–
TZ-bg10	–0.12 [–0.25; 0.01]	–64.47 [–101.65; –27.29]**
TZ-bg20	–0.17 [–0.32; –0.03]*	–132.28 [–167.03; –97.54]***
TZ-Ag0.5	–0.04 [–0.18; 0.10]	–13.8 [–50.42; 22.83]
TZ-Ag1	–0.09 [–0.23; 0.05]	–14.4 [–49.78; 20.98]
TZ-Ag2	–0.06 [–0.2; 0.08]	–13.86 [–51.29; 23.57]
Biodentine	–0.78 [–0.96; 0.60]***	–119.62 [–170.81; –68.43]***
Immersion medium (Ref. Water)	–	–
FBS	–1.07 [–1.15; 0.98]***	136.8 [115.18; 158.43]***
Aging period (Ref. 1 day)	–	–
7 days	0.47 [0.35; 0.59]***	211.83 [190.03; 233.64]***
Direct 28 days	0.72 [0.61; 0.83]***	289.68 [261.25; 318.12]***

TZ-base, prototype calcium silicate-based cement; TZ-bg10, TZ-base with 10% bioactive glass; TZ-bg20, TZ-base with 20% bioactive glass; TZ-Ag0.5, TZ-base with 0.5 mg/mL silver nanoparticle solution; TZ-Ag1, TZ-base with 1 mg/mL silver nanoparticle solution; TZ-Ag2, TZ-base with 2 mg/mL silver nanoparticle solution; FBS, fetal bovine serum.

**P* < 0.05.

***P* < 0.01.

****P* < 0.001.

TABLE 4 Mixed-effects linear regression analyses for nested data for pH (model 1b) and calcium release (model 2b), with coefficients and 95% confidence intervals (CIs).

Variable	Model 1b: pH. Repeated measurements upon weekly medium change (<i>n</i> = 126)	Model 2b: Calcium release (mg/mL). Repeated measurements upon weekly medium change (<i>n</i> = 126)
	Coefficient [95% CI]	Coefficient [95% CI]
Material (Ref. TZ-base)	–	–
TZ-bg10	–0.07 [–0.34; 0.2]	–28.44 [–62.77; 5.89]
TZ-bg20	–0.40 [–0.68; –0.13]**	–51.35 [–85.68; –17.02]**
TZ-Ag0.5	–0.21 [–0.49; 0.06]	–1.74 [–36.07; 32.59]
TZ-Ag1	–0.19 [–0.46; 0.08]	–12.96 [–47.29; 21.37]
TZ-Ag2	–0.25 [–0.52; 0.03]	–6.07 [–40.4; 28.26]
Biodentine	–0.82 [–1.09; –0.54]***	–125.16 [–159.49; –90.83]***
Immersion medium (Ref. Water)	–	–
FBS	–2.25 [–2.4; –2.1]***	–40.78 [–59.23; –22.42]***
Aging period (Ref. 7 days)	–	–
14 days	–0.68 [–0.88; –0.47]***	–131.38 [–156.73; –106.03]***
21 days	–1.20 [–1.40; –1.00]***	–307.97 [–334.71; –281.24]***
28 days	–1.73 [–1.92; –1.54]***	–383.06 [–408.41; –357.71]***

TZ-base, prototype calcium silicate-based cement; TZ-bg10, TZ-base with 10% bioactive glass; TZ-bg20, TZ-base with 20% bioactive glass; TZ-Ag0.5, TZ-base with 0.5 mg/mL silver nanoparticle solution; TZ-Ag1, TZ-base with 1 mg/mL silver nanoparticle solution; TZ-Ag2, TZ-base with 2 mg/mL silver nanoparticle solution; FBS, fetal bovine serum.

**P* < 0.05.

***P* < 0.01.

****P* < 0.001.

TABLE 5 Subgroup multiple linear regression analyses assessing separately the effect of prototype cements and Biodentine in calcium release (outcome variable) for the independent leachate measurements, with linear regression coefficients and confidence intervals as the estimates provided.

Variable	Model 2c: Calcium release (mg/mL). Independent samples of accumulative leaching-subgroup analysis for prototype cements ($n = 324$) Coefficient [95% CI]	Model 2d: Calcium release (mg/mL). Independent samples of accumulative leaching-subgroup analysis for Biodentine ($n = 54$) Coefficient [95% CI]
Material (Ref. TZ-base)	–	Na.
TZ-bg10	–64.47 [–100.55; –28.39]**	Na.
TZ-bg20	–132.28 [–166.25; –98.31]***	Na.
TZ-Ag0.5	–13.8 [–49.46; 21.86]	Na.
TZ-Ag1	–14.4 [–49.17; 20.37]	Na.
TZ-Ag2	–13.86 [–50.01; 22.28]	Na.
Immersion medium (Ref. Water)	–	–
FBS	181.29 [161.38; 201.2]***	–130.12 [–175.52; –84.72]***
Aging period (Ref. 1 day)	–	–
7 days	227.82 [207.2; 248.44]***	115.9 [60.3; 171.5]***
Direct 28 days	290.25 [262.26; 318.23]***	286.31 [230.71; 341.91]***

TZ-base, prototype calcium silicate-based cement; TZ-bg10, TZ-base with 10% bioactive glass; TZ-bg20, TZ-base with 20% bioactive glass; TZ-Ag0.5, TZ-base with 0.5 mg/mL silver nanoparticle solution; TZ-Ag1, TZ-base with 1 mg/mL silver nanoparticle solution; TZ-Ag2, TZ-base with 2 mg/mL silver nanoparticle solution; FBS, fetal bovine serum.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

respectively, as well as in Figure 6 for test leachates extracted in the cell culture inserts (0–24 h). Results from the overall regression analyses are presented Tables 6–8 for independent, nested, and 0–24 h leachate samples, respectively.

Bacterial killing increased throughout time when the medium was not changed (Table 6; 7–28 days: mean decrease $\log(\text{CFU}+1)/\text{mL} = -0.40$, 95% CI [–0.62; –0.18]) and decreased when the medium was refreshed (Table 7). The immersion medium had a significant effect in bacterial survival; fetal bovine serum leachates showed reduced antibacterial activity compared with water leachates. TZ-base was overall significantly more antibacterial than Biodentine in all tested models; TZ-bg10 and TZ-bg20 except for when the medium was replenished; and IRM except for the 0–24 h aging condition (Table 8, Figure 6). Overall, silver nanoparticle-containing prototype cements exhibited similar antimicrobial activity to TZ-base. Subgroup analyses for water samples against *E. faecalis* showed a higher bacterial reduction induced by leachates of TZ-base with 2 mg/mL silver nanoparticle solution (TZ-Ag2) than the other cements investigated, except for the 0–24 h leaching period (Table 9, Figure 6); *E. faecalis* biofilms were overall more resistant to material leachates than *P. aeruginosa* biofilms, except for the 0–24 h leaching period (Table 8).

Cell viability

Data for cell viability are presented in Figure 7A and B for water and fetal bovine serum material leachates respectively; Figure 8 shows normalized data from the MTT assay for the 0–24 h leachates. The outcomes from the overall regression analyses can be found in Tables 6–8. Results from diluted test leachates can be found in Figures S1 and S2.

Cell viability was decreased when the medium was not changed from 1 day onwards (Table 6), until it was eliminated at the direct 28 day period (7–28 days: mean decrease in relative MTT activity = -7.62 , 95% CI [–9.76; –5.47]); refreshing the medium resulted in reduced cytotoxicity at 28 days (Table 7, Figure 7). The immersion medium significantly affected cell viability; fetal bovine serum leachates displayed diminished cytotoxicity values compared to the water leachates, particularly at 28 days upon medium change (Figure 7), but not in the 0–24 h leaching period (Table 8, Figure 8).

Biodentine and IRM had overall a less cytotoxic profile than TZ-base. Modifications in prototype cements did not significantly change the cell viability values compared to TZ-base. In prototype materials, only the 28 day fetal bovine serum leachates upon changing medium weekly reported higher cell viability than the cytotoxicity threshold (70%)

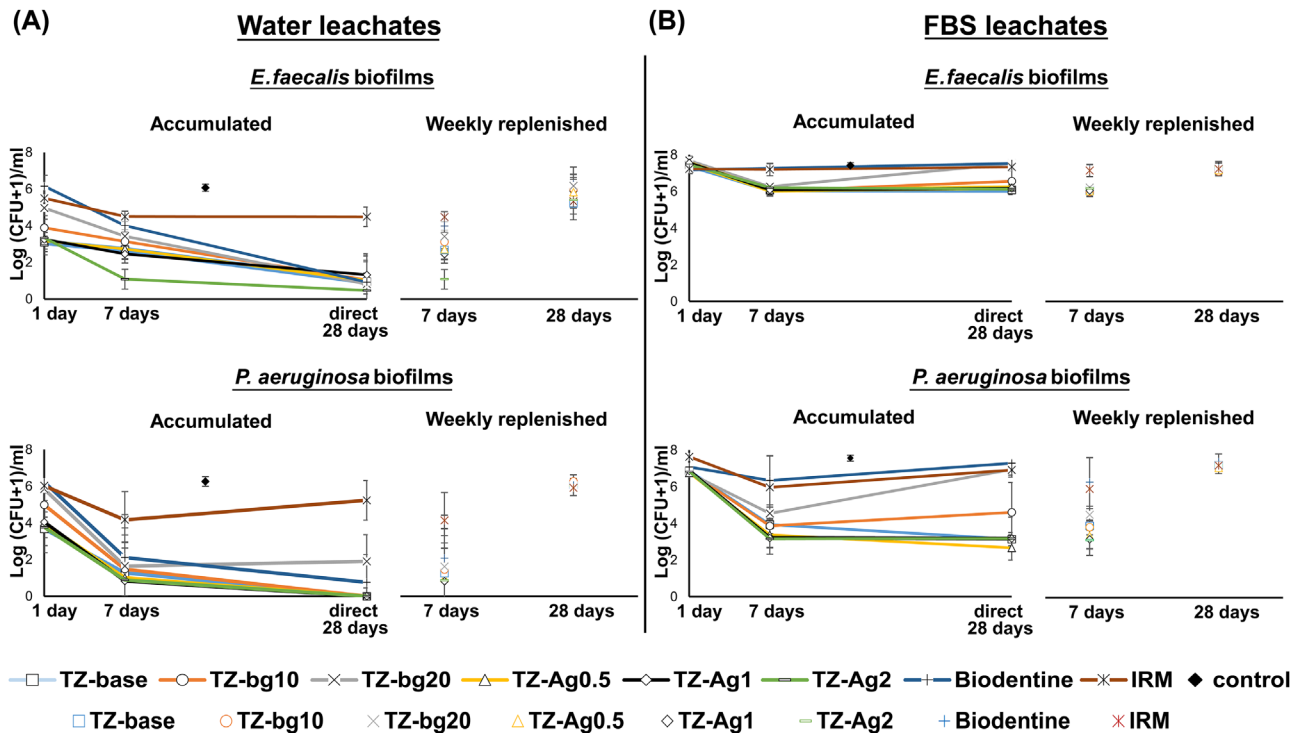


FIGURE 5 Mean bacterial survival with standard deviation following overnight exposure of monospecies biofilms of *Enterococcus faecalis* or *Pseudomonas aeruginosa* to (A) water or (B) fetal bovine serum (FBS) material leachates aged for 1, 7, or 28 days with or without changing the medium weekly. Control corresponds to neat water or FBS respectively in each graph. Line charts correspond to groups without medium replenishment (“accumulated”) and graphs with markers and standard deviation are for groups with weekly medium replenishment. TZ-base, prototype calcium silicate-based cement; TZ-bg10, TZ-base with 10% bioactive glass; TZ-bg20, TZ-base with 20% bioactive glass; TZ-Ag0.5, TZ-base with 0.5 mg/mL silver nanoparticle solution; TZ-Ag1, TZ-base with 1 mg/mL silver nanoparticle solution; TZ-Ag2, TZ-base with 2 mg/mL silver nanoparticle solution; IRM, intermediate restorative material.

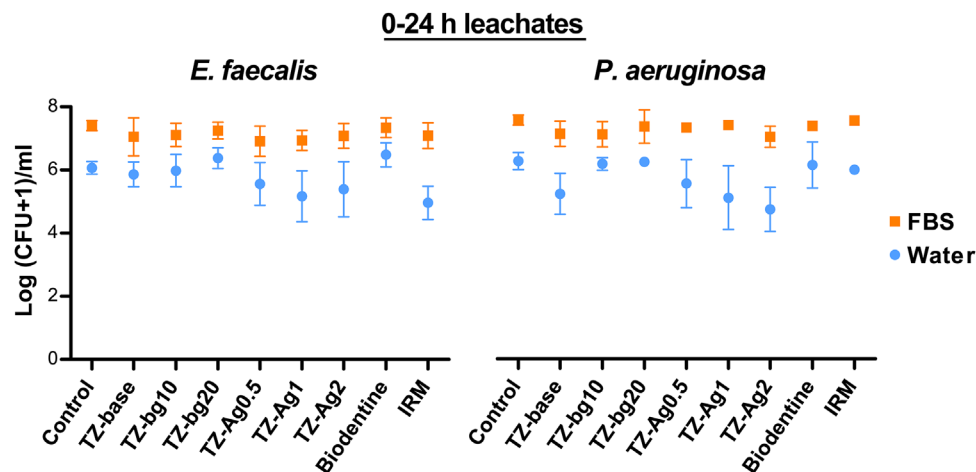


FIGURE 6 Mean log(CFU+1)/mL and standard deviation of *Enterococcus faecalis* or *Pseudomonas aeruginosa* following exposure to water or fetal bovine serum (FBS) material leachates. Leaching of samples prior to testing was performed immediately after material mixing and placement in cell cultures inserts in the presence of medium for 24 h (0–24 h leachates). Antibacterial activity in leachates of FBS was overall significantly reduced in comparison to those of water. TZ-base, prototype calcium silicate-based cement; TZ-bg10, TZ-base with 10% bioactive glass; TZ-bg20, TZ-base with 20% bioactive glass; TZ-Ag0.5, TZ-base with 0.5 mg/mL silver nanoparticle solution; TZ-Ag1, TZ-base with 1 mg/mL silver nanoparticle solution; TZ-Ag2, TZ-base with 2 mg/mL silver nanoparticle solution; IRM, intermediate restorative material.

TABLE 6 Multiple linear regression analyses for independent leachate samples for bacterial survival (LogCFU+1/mL) (model 3a) and cell viability (%) (model 4a), with the factor “Bacterium” only applicable to the model for bacterial survival.

Variable	Model 3a: Bacterial survival (LogCFU+1/mL). Independent samples of accumulative leaching ($n = 864$) Coefficient [95% CI]	Model 4a: Relative MTT activity (%). Independent samples of accumulative leaching ($n = 432$) Coefficient [95% CI]
Material (Ref. TZ-base)	–	–
TZ-bg10	0.39 [0.08; 0.7]*	0.27 [–2.51; 3.05]
TZ-bg20	1.09 [0.75; 1.43]***	0.97 [–2.80; 4.74]
TZ-Ag0.5	–0.06 [–0.36; 0.24]	–1.11 [–3.89; 1.67]
TZ-Ag1	–0.02 [–0.3; 0.25]	1.3 [–1.48; 4.07]
TZ-Ag2	–0.23 [–0.52; 0.06]	0.41 [–2.3; 3.11]
Biodentine	1.47 [1.11; 1.83]***	23.9 [16.5; 31.29]***
IRM	2.25 [1.91; 2.58]***	5.43 [0.19; 10.67]*
Immersion medium (Ref. Water)	–	–
FBS	3.42 [3.25; 3.58]***	2.95 [0.49; 5.4]*
Aging period (Ref. 1 day)	–	–
7 days	–2.00 [–2.19; –1.81]***	–18.19 [–21.72; –14.66]***
Direct 28 days	–2.40 [–2.60; –2.20]***	–25.81 [–28.98; –22.64]***
Bacterium (Ref: <i>Enterococcus faecalis</i>)	–	Na.
<i>Pseudomonas aeruginosa</i>	–0.94 [–1.10; –0.77]***	Na.

TZ-base, prototype calcium silicate-based cement; TZ-bg10, TZ-base with 10% bioactive glass; TZ-bg20, TZ-base with 20% bioactive glass; TZ-Ag0.5, TZ-base with 0.5 mg/mL silver nanoparticle solution; TZ-Ag1, TZ-base with 1 mg/mL silver nanoparticle solution; TZ-Ag2, TZ-base with 2 mg/mL silver nanoparticle solution; IRM, intermediate restorative material; FBS, fetal bovine serum.

* $P < 0.05$.

*** $P < 0.001$.

TABLE 7 Mixed-effects linear regression analyses for nested data for antibacterial activity (LogCFU+1/mL) (model 3b) and cell viability (%) (model 4b), with the predictor variable “Bacterium” applicable only to the model for bacterial survival.

Variable	Model 3b: Bacterial survival (LogCFU+1/mL). Repeated measurements upon weekly medium change ($n = 288$) Coefficient [95% CI]	Model 4b: Relative MTT activity (%). Repeated measurements upon weekly medium change ($n = 144$) Coefficient [95% CI]
Material (Ref. TZ-base)	–	–
TZ-bg10	0.16 [–0.21; 0.54]	–0.02 [–1.82; 1.78]
TZ-bg20	0.33 [–0.05; 0.70]	3.27 [–1.35; 7.88]
TZ-Ag0.5	–0.05 [–0.42; 0.33]	–0.28 [–2.07; 1.52]
TZ-Ag1	–0.09 [–0.47; 0.29]	0.47 [–1.74; 2.67]
TZ-Ag2	–0.3 [–0.68; 0.07]	0.54 [–1.82; 2.91]
Biodentine	0.88 [0.5; 1.25]***	5.82 [0.97; 10.67]*
IRM	0.97 [0.59; 1.34]***	11.75 [3.14; 20.36]**
Immersion medium (Ref. Water)	–	–
FBS	2.16 [1.97; 2.35]***	5.28 [2.47; 8.10]***
Aging period (Ref. 7 days)	–	–
28 days	2.77 [2.58; 2.96]***	64.71 [58.86; 70.55]***
Bacterium (Ref: <i>Enterococcus faecalis</i>)	–	Na.
<i>Pseudomonas aeruginosa</i>	–0.79 [–0.97; –0.60]***	Na.

TZ-base, prototype calcium silicate-based cement; TZ-bg10, TZ-base with 10% bioactive glass; TZ-bg20, TZ-base with 20% bioactive glass; TZ-Ag0.5, TZ-base with 0.5 mg/mL silver nanoparticle solution; TZ-Ag1, TZ-base with 1 mg/mL silver nanoparticle solution; TZ-Ag2, TZ-base with 2 mg/mL silver nanoparticle solution; IRM, intermediate restorative material; FBS, fetal bovine serum.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

TABLE 8 Multiple linear regression analyses for leachate measurements for bacterial survival (LogCFU+1/mL) (model 3c) and cell viability (%) (model 4c) at the 0–24 h leaching period, with the factor “Bacterium” only applicable to the model for bacterial survival.

Variable	Model 3c: Bacterial survival (LogCFU+1/mL). 0–24 h leaching period (n = 288) Coefficient [95% CI]	Model 4c: Relative MTT activity (%). 0–24 h leaching period (n = 144) Coefficient [95% CI]
Material (Ref. TZ-base)	–	–
TZ-bg10	0.28 [0.04; 0.51]*	–7.46 [–16.01; 1.1]
TZ-bg20	0.49 [0.26; 0.72]***	–5.3 [–13.86; 3.26]
TZ-Ag0.5	0.03 [–0.23; 0.29]	0.27 [–8.28; 8.83]
TZ-Ag1	–0.17 [–0.46; 0.13]	–3.51 [–12.07; 5.05]
TZ-Ag2	–0.26 [–0.55; 0.04]	–1.76 [–10.32; 6.80]
Biodentine	0.51 [0.26; 0.76]***	16.22 [7.66; 24.78]***
IRM	0.08 [–0.17; 0.34]	45.28 [36.72; 53.83]***
Immersion medium (Ref. Water)	–	–
FBS	1.51 [1.38; 1.64]***	–2 [–6.28; 2.28]
Bacterium (Ref. <i>Enterococcus faecalis</i>)	–	Na.
<i>Pseudomonas aeruginosa</i>	0.05 [–0.08; 0.18]	Na.

TZ-base, prototype calcium silicate-based cement; TZ-bg10, TZ-base with 10% bioactive glass; TZ-bg20, TZ-base with 20% bioactive glass; TZ-Ag0.5, TZ-base with 0.5 mg/mL silver nanoparticle solution; TZ-Ag1, TZ-base with 1 mg/mL silver nanoparticle solution; TZ-Ag2, TZ-base with 2 mg/mL silver nanoparticle solution; IRM, intermediate restorative material; FBS, fetal bovine serum.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

TABLE 9 Subgroup analyses assessing the effect of material and aging period only in water leachates against *Enterococcus faecalis* (LogCFU+1/mL), with multiple linear regression analyses for independent samples of accumulative leaching (model 3d) or for the 0–24 h leaching period (model 3f), and mixed-effects linear regression analysis for nested data (model 3e).

Variable	Model 3d: Bacterial survival (LogCFU+1/mL). Subgroup analyses for water leachates against <i>E. faecalis</i> biofilms—Independent samples of accumulative leaching (n = 216) Coefficient [95% CI]	Model 3e: Bacterial survival (LogCFU+1/mL). Subgroup analyses for water leachates against <i>E. faecalis</i> biofilms—Repeated measurements upon weekly medium change (n = 72) Coefficient [95% CI]	Model 3f: Bacterial survival (LogCFU+1/mL). Subgroup analyses for water leachates against <i>E. faecalis</i> biofilms—0–24 h leaching period (n = 72) Coefficient [95% CI]
Material (Ref. TZ-Ag2)	–	–	–
TZ-base	0.61 [0.1; 1.12]*	1.32 [0.92; 1.71]***	0.47 [–0.16; 1.11]
TZ-bg10	1.06 [0.61; 1.50]***	1.82 [1.42; 2.21]***	0.60 [–0.08; 1.27]
TZ-bg20	1.44 [0.91; 1.97]***	2.09 [1.69; 2.48]***	0.99 [0.37; 1.61]**
TZ-Ag0.5	0.68 [0.25; 1.11]***	1.45 [1.05; 1.85]***	0.17 [–0.56; 0.91]
TZ-Ag1	0.71 [0.27; 1.15]**	1.23 [0.84; 1.63]***	–0.22 [–1.01; 0.56]
Biodentine	2.07 [1.49; 2.64]***	2.59 [2.20; 2.99]***	1.09 [0.46; 1.72]**
IRM	3.20 [2.72; 3.67]***	2.86 [2.46; 3.25]***	–0.44 [–1.1; 0.23]
Aging period (Ref. 1 day)			Na.
7 days	–1.17 [–1.4; –0.94]***	(Ref. 7 days)	Na.
28 days [#]	–2.79 [–3.16; –2.43]***	2.79 [2.52; 3.06]***	Na.

TZ-base, prototype calcium silicate-based cement; TZ-bg10, TZ-base with 10% bioactive glass; TZ-bg20, TZ-base with 20% bioactive glass; TZ-Ag0.5, TZ-base with 0.5 mg/mL silver nanoparticle solution; TZ-Ag1, TZ-base with 1 mg/mL silver nanoparticle solution; TZ-Ag2, TZ-base with 2 mg/mL silver nanoparticle solution; IRM, intermediate restorative material.

[#]Model 3d: 28 days of accumulative leaching; Model 3e: 28 days upon weekly medium change.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

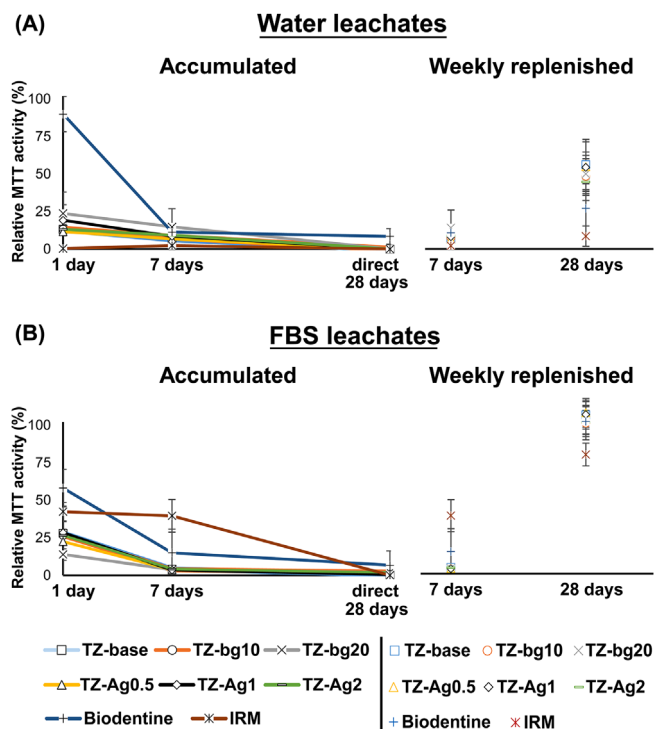


FIGURE 7 Mean relative 3-(4,5 dimethylthiazolyl-2-yl)3,2,5-diphenyltetrazolium bromide (MTT) activity with standard deviation of L929 cell cultures following a 24 h exposure period to (A) water and (B) fetal bovine serum (FBS) material leachates from different aging periods. Graphs are separated depending on whether the medium was replenished weekly or not (accumulated). TZ-base, prototype calcium silicate-based cement; TZ-bg10, TZ-base with 10% bioactive glass; TZ-bg20, TZ-base with 20% bioactive glass; TZ-Ag0.5, TZ-base with 0.5 mg/mL silver nanoparticle solution; TZ-Ag1, TZ-base with 1 mg/mL silver nanoparticle solution; TZ-Ag2, TZ-base with 2 mg/mL silver nanoparticle solution; IRM, intermediate restorative material.

of ISO 10993-5:2009 [17]. Biodentine had values indicating absence of cytotoxicity in 1 day water leachates.

Microscopy

SEM images of prototype cements and Biodentine revealed a hydrated matrix interrupted by particles consisting of calcium, silicon, and oxygen as well as particles rich in zirconium (Figure 9, Figure S3). In the bioactive glass-containing materials, particles rich in silicon, calcium, sodium, phosphate, and oxygen were also observed. Silver was occasionally demonstrated in the EDX scans of silver nanoparticle-containing materials. IRM had a dense matrix interrupted by pores and particles consisting mainly of zinc. In the fetal bovine serum-immersed materials, magnesium, fluorine, and sodium were additionally detected in the EDX scans.

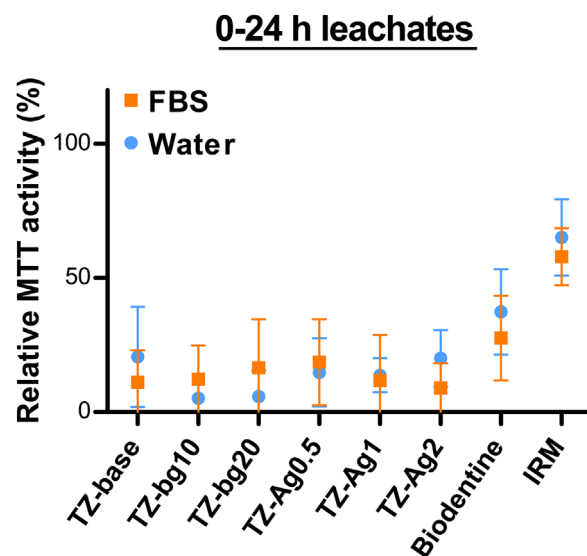


FIGURE 8 Mean relative 3-(4,5 dimethylthiazolyl-2-yl)3,2,5-diphenyltetrazolium bromide (MTT) activity and standard deviation of L929 cell cultures after 24 h exposure to water or fetal bovine serum (FBS) leachates. Test leachates derived from a 24 h extract process from materials placed in cell culture inserts and immersed in well-plates containing the respective medium (0–24 h leachates). No significant differences were observed overall in cell viability between water and FBS leachates. TZ-base, prototype calcium silicate-based cement; TZ-bg10, TZ-base with 10% bioactive glass; TZ-bg20, TZ-base with 20% bioactive glass; TZ-Ag0.5, TZ-base with 0.5 mg/mL silver nanoparticle solution; TZ-Ag1, TZ-base with 1 mg/mL silver nanoparticle solution; TZ-Ag2, TZ-base with 2 mg/mL silver nanoparticle solution; IRM, intermediate restorative material.

DISCUSSION

Hydraulic calcium silicate cements are used for a variety of endodontic procedures, each of which represents different clinical environments [10]. The present study investigated whether exposure conditions as well as material modifications affect selected properties of cements used for surgical endodontic procedures. Our results suggest that fetal bovine serum considerably reduced the antimicrobial potential of the leachable components. The effect of aging depended on the replenishment of the extract medium, having a cumulative effect in leachate reactivity when it was not refreshed or diminishing upon medium change. Biodentine and TZ-bg20 showed attenuated leaching properties compared to TZ-base, which consequently reduced their antibacterial activity and for Biodentine also the cytotoxic potential. The null hypotheses that exposure conditions and material composition do not affect the leaching characteristics of endodontic cements were therefore rejected.

Calcium hydroxide is a by-product of the reaction of tricalcium silicate cement with water; dispersion and consequent release of hydroxyl and calcium ions in the environment may

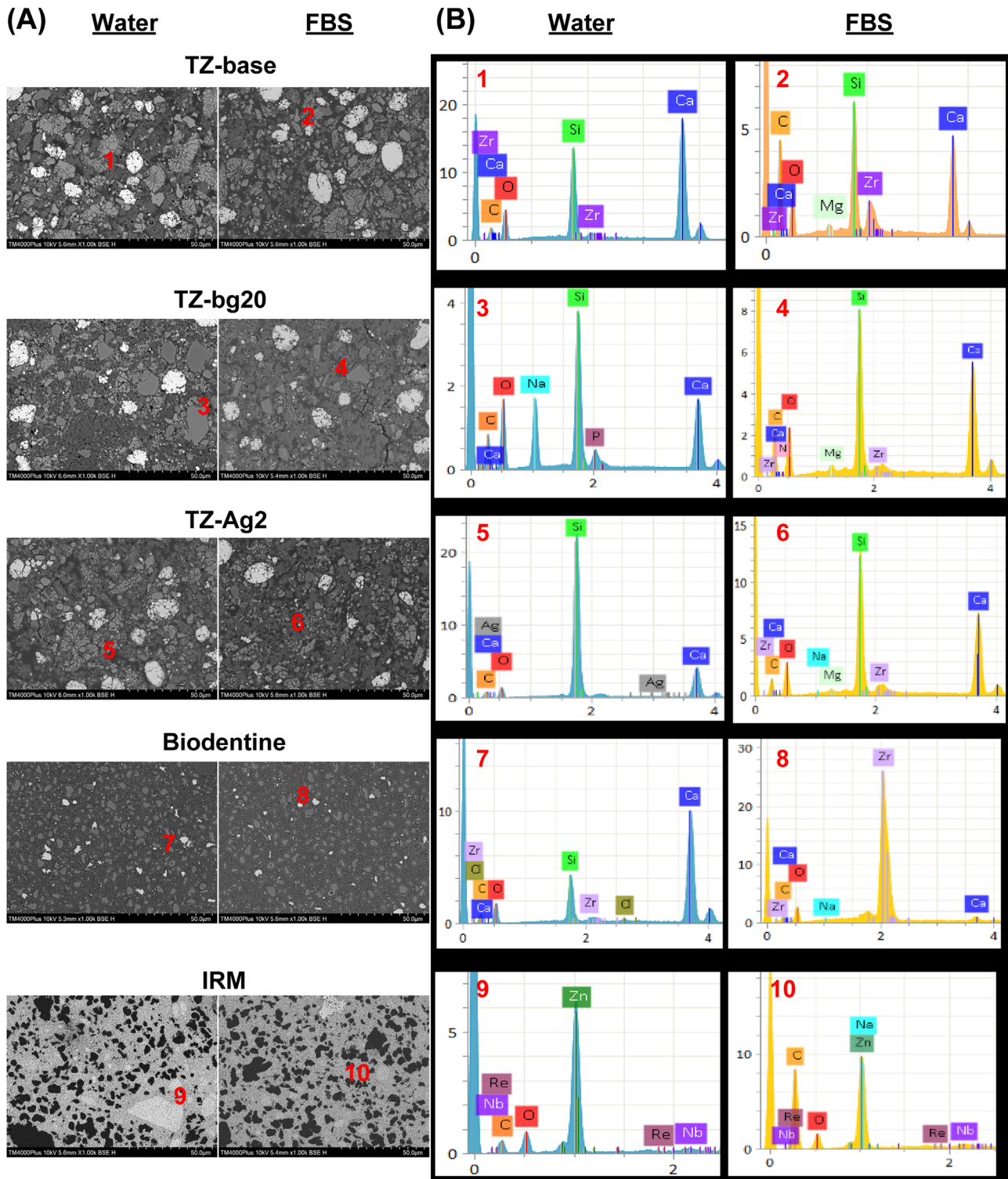


FIGURE 9 (A) Indicative back-scatter scanning electron micrographs of polished sections of five test materials (1000× magnification) after a 28 day exposure period to water or fetal bovine serum (FBS), with (B) accompanying energy-dispersive X-ray (EDX) scans of areas with Arabic numerals in the micrographs. Unhydrated tricalcium silicate cement particles (1), zirconium oxide particles-depicted white in micrographs, as well as bioactive glass particles for TZ-bg20 (3) are spread in a hydrated matrix in prototype cements. Particles of smaller size are observed in Biodentine. Elemental analysis reveals the presence of sodium and magnesium in the material bulk of cements that were immersed in FBS. A universal color coding for elements in the EDX scans is used: C = orange; Ca = blue; Si = light green; Zr = purple; Na = light blue; O = red; P = plum; Mg = turquoise; Ag = grey; Zn = dark green. TZ-base, prototype calcium silicate-based cement; TZ-bg20, TZ-base with 20% bioactive glass; TZ-Ag2, TZ-base with 2 mg/mL silver nanoparticle solution; IRM, intermediate restorative material.

contribute to tissue healing and bacterial elimination [18, 19]. Indirect assays were therefore selected to evaluate leaching characteristics. Water and fetal bovine serum were used to explore the effect of extract medium in these assessments. Water is commonly employed in investigations of hydraulic calcium silicate cements [20] offering an easily standardized environment. Yet it does not represent the clinical situation as host tissue fluids comprise a complex environment rich in ions and proteins [21]. In that perspective, fetal bovine serum may be more clinically relevant [7].

Different incubation periods enabled monitoring a potential plateau in calcium hydroxide release and its relation to the biological activity of the cements. An additional testing period of leaching during the first hours immediately after placement was assessed in biological assays maintaining the same surface area/medium volume ratio. In clinical conditions, blood circulation and tissue fluid drainage may refresh the leachate micro-environment; thus, investigating cement extracts in a closed environment might provide an over-estimation of their effect due to cumulative leaching throughout time [10]. However, the *in vitro* extract refreshment conditions also fail to resemble the clinical case as constant blood supply [22] creates a continuous renewal of tissue fluid, unlike the periodic one-off medium change *in vitro*. As *in vivo* conditions are more complicated, outcomes from laboratory studies such as the present one on long-term material activity can only be extrapolated in respect to the relevant test conditions applied. This was highlighted by implementing the two different protocols of leaching conditions: weekly replenishment of the extract medium and a closed system, both of which were maintained throughout the 28 day evaluation period.

The study was mainly conducted on prototype hydraulic calcium silicate cements with or without incorporation of silver nanoparticles or bioactive glass. Two commercial materials were included for control purposes. Biodentine is a Type 4 hydraulic calcium silicate cement [23], a conventional tricalcium silicate cement with additives to its cementitious (calcium carbonate) and its liquid phase (calcium chloride) [24]. IRM was also tested as a material with different chemistry but similar clinical application [25].

Calcium leaching of prototype cements and Biodentine was affected differently by fetal bovine serum exposure. Prototype cements leached more Ca^{2+} in fetal bovine serum than water in the accumulative leaching model. Increased leaching has been previously reported in contact with blood [26] or in a serum-supplemented physiologic solution [21]. Contrarily, Biodentine released overall less Ca^{2+} in fetal bovine serum. A similar effect has been observed for Biodentine leachates in bicarbonate solution [27] but not in a serum-supplemented physiologic solution [21], possibly due to differences in the extract media. Carbon dioxide present in tissues can facilitate the consumption of leached Ca^{2+} towards carbonation [28]. Additionally, calcium carbonate precipitates upon the

material surface, blocking the pore channels through which calcium is leached [29, 30]. The latter scenario might be more relevant for describing the overall decreased leaching of Biodentine in fetal bovine serum, as observations from prototype hydraulic calcium silicate cements suggest that there is incomplete consumption of calcium via carbonation. Biodentine's hydration is less affected by environmental conditions [31] due to the presence of additives that accelerate the reaction, resulting in a higher percentage of amorphous phase [32] and thus rendering it more stable. This is evidenced also by the sharp reduction in the amount of Ca^{2+} after changing the extract medium.

The decreased leaching of calcium and hydroxyl ions of Biodentine compared to prototype hydraulic calcium silicate cements in fetal bovine serum was also demonstrated by its diminished antibacterial activity. Fetal bovine serum has a buffering capacity, containing proteins that act as weak acids [33]; as leachate alkalinity decreased, so did the bactericidal activity of hydraulic calcium silicate cements. At the same time, serum components may provide better conditions for bacteria to survive. In water, even though hydraulic calcium silicate cements showed relatively high—yet reduced—pH values after 28 days upon changing the medium, the bactericidal potential diminished. It could therefore be postulated that the antibacterial activity is determined by a relatively high alkalization potential. Biodentine showed a lower degree of alkalization particularly after the first day, which was accompanied by reduced bactericidal activity and cytotoxicity. These findings are in accordance with a previous study [19], despite differences with the current investigation in aging media and test methods employed.

The antibacterial effect of hydraulic calcium silicate cements showed a cumulative increase in water leachates when the medium was not refreshed, particularly from 1 to 7 days. In fetal bovine serum, the bactericidal activity peaked at 7 days and did not exhibit further improvement at the 28 day accumulative leaching period. Furthermore, for the bioactive glass-containing materials and Biodentine, the bactericidal activity was even further reduced beyond that time point. The latter findings indicate the dynamics of a constant buffering effect of fetal bovine serum in the antibacterial potential even in static conditions. High concentration of silver nanoparticles (2 mg/mL) induced enhanced antibacterial efficacy in water leachates against *E. faecalis*. *P. aeruginosa* was more susceptible than *E. faecalis* and a potential enhancement of the bactericidal activity from silver nanoparticles after 7 days could therefore not be distinguished. Silver nanoparticles have been previously incorporated in varying concentrations in hydraulic calcium silicate cements [34, 35]. As the reactivity of silver nanoparticles appears to depend on the specific physicochemical characteristics of each formulation [36], different concentrations employed in the literature cannot be directly compared. The silver nanoparticle

concentrations used in the current study were selected with the rationale to enable dispersion of silver nanoparticles to the liquid vehicle of prototype cements [37].

Silver nanoparticles did not increase the antibacterial activity of hydraulic calcium silicate cements in fetal bovine serum leachates. At the same time, the antibacterial effect of IRM was also affected negatively by exposure to fetal bovine serum, despite its different mechanism, namely eugenol released by progressive hydrolysis of the material surface in the presence of aqueous medium [38, 39]. Therefore, it seems that the clinical environment is capable not only of depressing the alkalinity-mediated bactericidal effect of hydraulic calcium silicate cements, but also neutralizing the antibacterial effect of other released constituents.

A recent study showed that the incorporation of 10% or 20% bioactive glass in tricalcium silicate cement does not have a negative impact on bacterial adhesion to the material surface. In fact, TZ-bg20 aged in water enhanced the cement's antibacterial characteristics [5]. Further evaluation of the independent antibacterial leaching of these cements was therefore deemed necessary. Bioactive glass 45S5 has been demonstrated to exhibit more favorable physicochemical characteristics compared to other bioactive glass formulations [40]. It also serves as a filler in a commercial endodontic material [40]. Bioactive glass altered the hydration profile of the tricalcium silicate cement; a more gradual Ca^{2+} release was observed that was overall lower than the unmodified cement. Calcium from bioactive glass particles might not be available in the environment and stay deposited in the cement instead [41]. This may explain the impaired antibacterial potential, which is described to mainly be pH-dependent as in tricalcium silicate cement [13]. Measurement of alkalinity might not serve as a sensitive indicator of the chemical reactivity and the calcium hydroxide release, due to possible saturation of the material leachates. However, this could be better reflected in results from calcium release experiments.

Within the limitations of the current study, it can be concluded that exposure conditions (immersion medium and aging period) significantly affected the materials' leaching properties. Aging of hydraulic calcium silicate cements in a more complex medium than water had a negative effect on their antibacterial properties. Laboratory studies should therefore incorporate such experimental parameters in order to better represent the clinical situation. Modifications in hydraulic calcium silicate cement composition altered the chemical properties of the cements. Biodentine showed reduced antibacterial capacity and cytotoxicity than TZ-base, as a consequence of its modifications from the standard cement/radio-opacifier composition. Addition of 2 mg/mL silver nanoparticles to hydraulic calcium silicate cement improved the bactericidal effect in water leachates, but not in a serum-containing environment.

AUTHOR CONTRIBUTIONS

Conceptualization: Andreas Koutroulis, Håkon Valen, Josette Camilleri, Pia Titterud Sunde; **Methodology:** Andreas Koutroulis, Håkon Valen, Vasileios Kapralos, Josette Camilleri, Pia Titterud Sunde; **Validation:** Andreas Koutroulis, Håkon Valen, Dag Ørstavik, Josette Camilleri, Pia Titterud Sunde; **Formal analysis:** Andreas Koutroulis; **Investigation:** Andreas Koutroulis; **Writing – original draft preparation:** Andreas Koutroulis; **Writing – review and editing:** Håkon Valen, Dag Ørstavik, Vasileios Kapralos, Josette Camilleri, Pia Titterud Sunde; **Supervision:** Håkon Valen, Dag Ørstavik, Josette Camilleri, Pia Titterud Sunde; **Project administration:** Andreas Koutroulis, Håkon Valen, Josette Camilleri, Pia Titterud Sunde; **Funding acquisition:** Andreas Koutroulis, Håkon Valen, Pia Titterud Sunde.

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CONFLICT OF INTEREST STATEMENT

The authors declare no potential conflicts of interest related to this study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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