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ORIGINAL ARTICLE

In vitro physicochemical characterization of five root canal sealers and their influence on an *ex vivo* oral multi-species biofilm community

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Abstract

Aim: To evaluate the physicochemical properties of five root canal sealers and assess their effect on an *ex vivo* dental plaque-derived polymicrobial community.

Methodology: Dental plaque-derived microbial communities were exposed to the sealers (AH Plus [AHP], GuttaFlow Bioseal [GFB], Endoseal MTA [ESM], Bio-C sealer [BCS] and BioRoot RCS [BRR]) for 3, 6 and 18 h. The sealers' effect on the biofilm biomass and metabolic activity was quantified using crystal violet (CV) staining and MTT assay, respectively. Biofilm community composition and morphology were assessed by denaturing gradient gel electrophoresis (DGGE), 16S rRNA sequencing and scanning electron microscopy. The ISO6876:2012 specifications were followed to determine the setting time, radiopacity, flowability and solubility. Obturated acrylic teeth were used to assess the sealers' effect on pH. Surface chemical characterization was performed using SEM with coupled energy-dispersive spectroscopy. Data normality was assessed using the Shapiro–Wilk test. One-way ANOVA and Tukey's tests were used to analyze data from setting time, radiopacity, flowability and solubility. Two-way ANOVA and Dunnett's tests were used for the data analysis from CV, MTT and pH. 16S rRNA sequencing data were analyzed for alpha (Shannon index and Chao analysis) and beta diversity (Bray–Curtis dissimilarities). Differences in community composition were evaluated by analysis of similarity ($p < .05$).

Results: The sealers significantly influenced microbial community composition and morphology. All sealers complied with ISO6876:2012 requirements for setting time, radiopacity and flowability. Although only AHP effectively reduced the biofilm biomass, all sealers, except BRR, reduced biofilm metabolic activity.

Conclusion: Despite adequate physical properties, none of the sealers tested prevented biofilm growth. Significant changes in community composition were observed. If observed *in vivo*, these changes could affect intracanal microbial survival, pathogenicity and treatment outcomes.

KEYWORDS
antimicrobial, bacteria, biofilm, endodontic infection, physicochemical properties, root canal sealers

INTRODUCTION

Non-surgical root canal treatment aims to disinfect and tightly seal the root canal space, facilitating apical tissue healing and preventing reinfection. Nonetheless, the complex anatomy of root canals and the endodontic microbiota challenges the success of the therapy (Siqueira & Rocas, 2008). Microbes can evade disinfection by sequestering inside dentinal tubules and anatomic irregularities (Siqueira & Rocas, 2008), forming complex biofilm communities (Neelakantan et al., 2017). Given that bacteria and their by-products are the primary aetiological agents of periapical infections, the inability to eliminate bacteria from the root canal system negatively impacts treatment prognosis and contributes to treatment failure (Hancock et al., 2001; Sundqvist, 1992).

Although chemo-mechanical debridement of the root canal substantially reduces bacterial load, surviving microorganisms remain the leading cause of treatment failure (Siqueira & Rocas, 2008). Given that non-surgical endodontic treatment success rates range from 62% to 96%, effective removal of the periapical infection and microbial burden would appear to affect the outcome (Laukkanen et al., 2019; Siqueira, 2001). Hence, optimized disinfecting strategies can be beneficial endodontic treatment.

After chemo-mechanical debridement, the root canal system is filled using gutta-percha cones and root canal sealers (Kaur et al., 2015). Whilst filling materials can sequester residual microbes inside the canal and reduce their access to nutrients, the antimicrobial compounds in sealers can help limit growth and reduce bacterial burden (Kitagawa et al., 2021; Spangberg & Haapasalo, 2002). Thus, antimicrobial sealers could be an effective strategy to minimize the bacterial load in the root canal system.

Here, we assess the antimicrobial potential of five commercially available endodontic sealers, AH Plus (AHP; Dentsply); GuttaFlow Bioseal (GFB; Coltene); Endoseal MTA (ESM; Maruchi); Bio-C sealer (BCS; Angelus) and BioRoot RCS (BRR; Septodont), on an *ex vivo* multispecies dental plaque-derived microbial community. Additionally, physicochemical properties were determined to inform on potential antimicrobial features of the sealers.

MATERIALS AND METHODS

This manuscript was written according to the Preferred Reporting Items for Laboratory Studies in Endotology (PRILE) 2021 guidelines (Nagendrababu et al., 2021). The PRILE 2021 flowchart (Figure S1) summarizes the key steps.

AH Plus (paste/paste formulation) and BRR (powder/liquid) were each mixed to a homogeneous paste according to the manufacturer's instructions. GFB, ESM and BCS are ready to use formulations. Additional information about the sealers is listed (Table 1). The sample size was determined individually for each analysis, based on previously reported methods. Negative and positive controls were determined as needed and are described separately for each analysis.

Physical and chemical analysis

Setting time, radiopacity, flowability and solubility were evaluated using the ISO 6876:2012 (International Organization for Standardization 2012) standards in two independent experiments. As needed, sealer specimens were incubated at 37 ± 1°C and 90 ± 5% humidity using setting conditions specified by the manufacturers.

To determine setting time, sealers were placed into metallic rings (10 mm × 1 mm; $n = 3$) and subjected to periodic vertical pressure using a 113.4 g Gilmore needle (ASTM-C 266–08). Setting time was recorded from the moment sealer mixing was completed until surface indentations were no longer visible (International Organization for Standardization, 2012).

For radiopacity analysis, sealer specimens (10 × 1 mm; $n = 3$) were prepared and, after their final setting time, radiographed on a digital periapical sensor (Micro Imagem) next to a comparative 2–16-mm aluminium scale (International Organization for Standardization, 2012). For image acquisition, the X GE 1000 unit (General Electric Co.) was set at 70 kVp, 10 mA and 0.10 exposure time. Radiographic density in grayscale values was converted into aluminium equivalent thickness (mmAl) using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

The sealers' flowability was determined by placing 0.05 ml of each sealer at the centre of a glass plate. Next, a second 20 g plate and an additional 100 g weight were placed on top of the sealers. After 10 min, the mean of the specimens' major and minor diameters was measured using a digital calliper (Mitutoyo, MTI Co.) and flow value was determined as the average between the two diameter values.

For solubility analysis, sealer specimens (10 mm × 1.5 mm; $n = 3$) were prepared, and after three

TABLE 1 Root canal sealers, their manufacturer, composition, base and batch number

Root Canal Sealer	Manufacturer	Composition	Sealer base	Batch Number
AH Plus (AHP)	Dentsply	Bisphenol-A epoxy resin, bisphenol F epoxy resin, calcium tungstate, zirconium oxide, silica, iron oxide pigments dibenzyldiamine, amino-adamantane, tricyclodecane-diamine, silicone oil	Epoxy resin-based	349908K
GuttaFlow Bioseal (GFB)	Coltene	Gutta-percha powder, platinum catalyst silicates, polydimethylsiloxane, silicone oils, silver zinc oxide, zirconium dioxide, bioactive glass, colour pigments	Silicon- based	J17328
Bio-C Sealer (BCS)	Angelus	Calcium silicates, calcium aluminate, calcium oxide, zirconium oxide, iron oxide, silicon dioxide and dispersing agent	Calcium silicate-based	47730
Endoseal MTA (ESM)	Maruchi	Calcium silicates, calcium aluminates, calcium aluminoferrite, calcium sulphates, radiopacifier and thickening agents	Calcium silicate-based	CI190315A
BioRoot RCS (BRR)	Septodont	Tricalcium silicate, zirconium oxide, excipients in powder form, calcium chloride and excipients as an aqueous liquid	Calcium silicate-based	B22493

times, their final setting time specimens were weighed on an analytical balance (Denver Instrument Co.). All specimens were placed into containers containing 25 ml of distilled water and incubated at 37°C with 95% relative humidity for 24 h. After incubation, the specimens were dried for 24 h and reweighted. Solubility was determined as the percentage of the specimen's weight loss (the difference between the final and the initial mass).

The pH of each sealer was analysed as described previously by (Pelepenko et al., 2021). Standardized single-rooted acrylic teeth ($n = 5$) were prepared using a Reciproc file (R40—VDW) and obturated using sealers combined with a single matched-taper gutta-percha cone (R40—VDW). Cones were pre-measured to fill the root canal length but end 3 mm short of the apical foramen. Acrylic teeth were immersed in 10 ml of distilled water for 28 days, and water pH was measured with a pH meter (371; Micronal) after 3 h, 24 h and 28 days, in two independent experiments. Non-obturated acrylic teeth immersed in distilled water served as controls.

For surface characterization and elemental analysis, fresh root canal sealer specimens (10 mm × 1 mm, $n = 2$) were incubated for 24 h, dried, desiccated and embedded in resin (Epoxyfix, Struers GmbH, Ballerup, Denmark). Samples were polished with progressively finer diamond discs using an automatic machine (Tegramin20, Struers GmbH) and carbon-coated. Surface morphology and elemental composition were assessed by scanning electron microscopy (SEM) with coupled energy-dispersive spectroscopy (EDS) (JSM-5600/LvJEOL) (Marciano et al., 2016).

Antimicrobial effects of sealers on dental plaque-derived biofilms

Sealer specimens were prepared by placing approximately 30 µl of each fresh sealer onto the bottom of 96-well round-bottom plates (Costar, Corning Inc.) ($n = 5$). Sealer-coated plates were sterilized by UV irradiation for 15 min and incubated at 37 ± 1°C and 90 ± 5% humidity for 24 h, allowing set reactions to occur. Saliva-coated wells were used as growth control ($n = 5$).

We used a previously described supragingival plaque community (Hall et al., 2019), representative of the complex oral microbiota (Edlund et al., 2013; Tian et al., 2010), as a model system to evaluate the antimicrobial potential of the root canal sealers. Following IRB guidelines, supragingival plaque samples from maxillary molars of five healthy volunteers were collected approximately 24 h after tooth brushing. The plaque samples were washed with pre-reduced sterile phosphate-buffered saline (PBS), pH 7.4, and pooled for overnight anaerobic growth in pre-reduced SHI medium, an enriched growth medium developed to support oral-sourced multispecies bacterial communities as *in vitro* biofilms with high reproducibility (Tian et al., 2010), at 37°C (10% H_2, 10% CO_2 and 80% N_2). After overnight growth, the multispecies cultures were frozen in 10% glycerol and served as stock for the experiments described here.

For the experiments described here, the dental plaque stock cultures were used to inoculate fresh modified SHI medium (25% SHI medium and 75% of sterile human saliva) and incubated overnight at 37°C under 5% CO_2

or anaerobically. Overnight cultures were diluted into fresh modified SHI medium to an optical density (OD) at 600 nm of 0.1. Each set root canal sealer-coated well (Costar, Corning Inc.) was inoculated with 200 µl of modified SHI medium containing the dental plaque-derived cultures. Samples were incubated at 37°C for 18 h under 5% CO_2 or anaerobically. Control cultures were grown on saliva-coated wells without sealers. Sterile human saliva (100 µl) was added to wells and coated for 1 h at room temperature with gentle shaking (5s rocking shaker, Reliable Scientific Inc.). Excess saliva was aspirated, and plates were sterilized by UV irradiation for 1 h, to ensure that any potential contamination during the preparation of the plates did not affect our results. After inoculation and incubation, the control biofilms that formed were used as the baseline for biomass formation, metabolic activity, community composition and morphology. All experiments were performed at least two times to ensure technical and biological reproducibility.

Assessment of biofilm biomass and metabolic activity

Biofilm biomass and metabolic activity were evaluated 3-, 6- and 18-h post-incubation under 5% CO_2 at 37°C. Crystal violet was used to determine biomass accumulation on the root canal sealers and saliva control wells, as previously described (de Avila et al., 2015). At 3-, 6- and 18-h post-incubation, the supernatant (planktonic bacteria) was aspirated; the wells ($n = 5$) were washed with PBS once, submerged in 100 µl crystal violet solution and incubated at room temperature for 10 min, followed by three washes with PBS to remove excess crystal violet. The plates were gently shaken for 5 min during the last two PBS washes to ensure the complete removal of residual dye. After the final PBS wash, 100 µl of acidified 95% ethanol was added and the plates were incubated at room temperature on a rocking shaker (Reliable Scientific Inc.) for 10 min. The ethanol solution containing the crystal violet stain retained by the biofilms was transferred to a flat-bottom 96-well plate (Costar, Corning Inc.), and the absorbance at 570 nm was measured. Biofilm metabolic activity was determined using the 3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyltetrazolium bromide (MTT; 0.5 mg/ml in PBS) assay (Sigma) according to the manufacturer's instructions. After the removal of the culture medium, wells were washed twice with PBS and 150 µl of MTT solution were added to each well following a 4-h incubation. After incubation, formazan precipitate was solubilized using 95% ethanol on a rocking shaker (Reliable Scientific Inc.) at room temperature for 10 min. The solutions were then transferred to a flat-bottom 96-well plate (Costar, Corning Inc.), and absorbance was measured at 570 nm using a microplate reader. Uninoculated sealer-coated wells were used as controls for possible background retention or reaction of reagents.

Denaturing gradient gel electrophoresis (DGGE) and DNA Sequencing of excised DGGE bands

After 18 h of incubation under 5% CO_2 and anaerobically, at 37°C, biofilm communities developed onto sealer specimens ($n = 4$) were collected from all wells by flushing their surfaces with 150 µl of sterile PBS. Biofilm DNA was isolated using the DNeasy PowerSoil Kit (QIAGEN). Primers Bac1 with a GC clamp and Bac2 were used to amplify a region approximately 300 base pair, in length, of the 16S ribosomal RNA gene. Amplicons were resolved by denaturing gradient gel electrophoresis (DGGE) as previously described (de Avila et al., 2015). Gels were stained with ethidium bromide, and images were taken with the Molecular Imager Gel Documentation system (Bio-Rad Laboratories Inc.). Bands of interest were excised from the DGGE gel with a sterile razor blade, placed into 1.5-ml tubes containing 15 µl sterile Milli-Q water. The tubes were incubated overnight at 4°C, and 5 µl of the DNA sample was used as a template for re-amplification with universal primers, Bac1 and Bac2. The product was sent to the University of Minnesota Genomic Center (UMGC) for Sanger sequencing. 16S rRNA gene sequence identity was determined by BLAST comparison with the Human Oral Microbiome Database (HOMD).

DNA extraction and sequencing

After biofilm collection ($n = 4$), as described above, genomic DNA was isolated using the DNeasy PowerSoil Kit (QIAGEN) according to the manufacturer's instructions. The DNA concentration was determined using a Nanodrop (Thermo Fisher Scientific), and total genomic DNA (20 µl) was submitted for Illumina MiSeq sequencing of the V3-V4 hypervariable regions of the 16S rRNA as described (Gohl et al., 2016) at the UMGC. DNA extracted from elution buffer was also sequenced, serving as negative contamination control. Sequence processing was done using Mothur software ver. 1.41.1 and a previously published pipeline (Staley et al., 2018). Briefly, trimmed sequences were quality screened and aligned against the SILVA database (ver. 138) (Pruesse et al., 2007). Operational taxonomic units (OTUs) were binned at 99% sequence similarity using the furthest-neighbour algorithm, and taxonomic classifications were made against

TABLE 2 Characterization of root canal sealers' physical properties. The mean and standard deviation of setting time (min), radiopacity (mmAl), flowability (mm) and solubility (%) of experimental sealers.

Root Canal Sealer	Setting time (min)	Radiopacity (mmAl)	Flowability (mm)	Solubility (%)
AH Plus	531.67 ± 16.07^{a}	9.11 ± 0.40^{a}	19.63 ± 0.60^{ab}	0.18 ± 0.33^{a}
GuttaFlow Bioseal	21.67 ± 2.89^{b}	5.41 ± 0.40^{b}	21.17 ± 0.96^{a}	0.08 ± 0.09^{a}
Endoseal MTA	245.67 ± 8.66^{c}	8.57 ± 0.55^{c}	18.33 ± 0.75^{bc}	**−3.47 ± 1.21^{b}**
Bio-C Sealer	318.33 ± 7.64^{d}	4.50 ± 0.47^{d}	30.15 ± 0.88^{d}	**−5.30 ± 1.78^{bc}**
BioRoot RCS	73.33 ± 2.89^{e}	4.74 ± 0.43^{e}	17.32 ± 0.93^{c}	**−6.90 ± 1.46^{c}**

Note: Read vertically; different superscript letters represent statistically significant differences between materials ($p < .05$; one-way ANOVA with Tukey's multiple comparisons test). Results in bold letters do not comply with the ISO 6876:2012 standard.

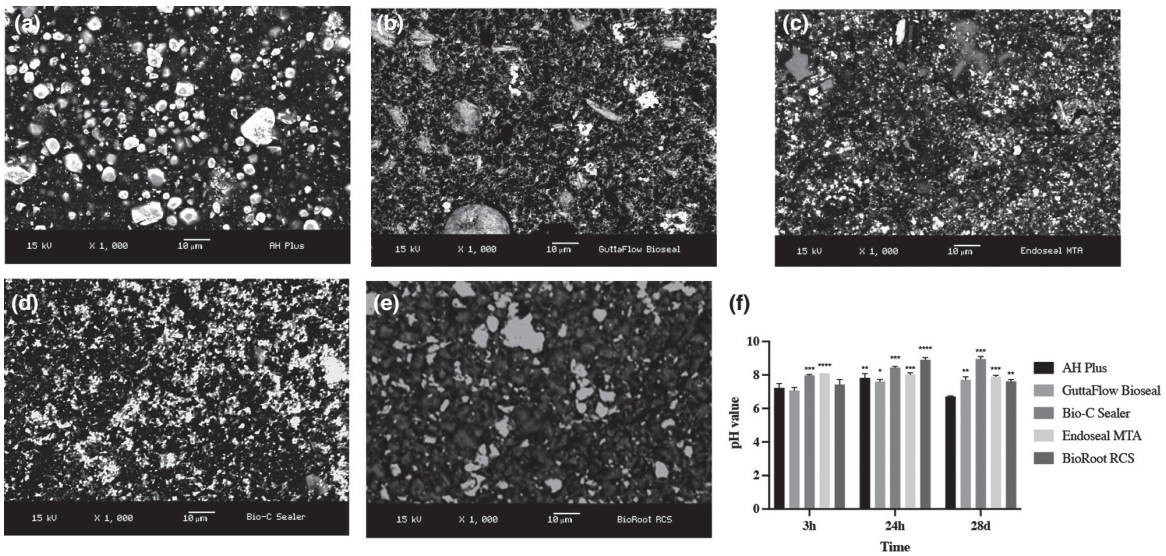

FIGURE 1 Representative backscatter scanning electron micrographs showing root canal sealers' surfaces microstructure at 1000× magnification. All sealers present a regular and compact matrix embedding different-sized particles. (a) AH Plus; (b) GuttaFlow Bioseal; (c) Endoseal MTA; (d) Bio-C Sealer; and (e) BioRoot RCS. pH analysis revealed that calcium silicate-based sealers induced increased alkalinization over time (f) Quantitative measurement (mean and standard deviation) of pH relative to negative control by pH analysis. Different symbols in each column indicate statistically significant differences between tested materials and negative control (*$p < .05$; **$p < .01$; ***$p < .001$, ****$p < .0001$, respectively; two-way ANOVA with Dunnett's multiple comparisons test)

the Ribosomal Database Project (ver.16) (Cole et al., 2009). Raw sequence data were deposited in the Sequence Read Archive under BioProject accession number SRP303009.

Characterization of biofilm morphology

Dentine discs were prepared from bovine single-rooted teeth (Li et al., 2014), sterilized and randomly divided into six groups (five experimental and one control). The discs ($n = 3$) were placed in a 48-well plate (Costar, Corning Inc.), and the root canal spaces were filled with sealers. After 24 h, the sealer-filled specimens were inoculated with 2 µl of plaque-derived overnight cultures at an optical density (OD) at 600 nm of 0.1 in 500 µl of modified SHI medium. Samples were incubated for 18 h at 37°C under 5% CO_2. After incubation, samples were fixed, processed and imaged using a field-emission scanning electron microscope (FE-SEM) (JEOL 6500, JEOL), as described previously (Rudney et al., 2012).

Statistical analysis

GraphPad Prism software (version 8.01, GraphPad) was used for statistical analysis. Sample normality was assessed using the Shapiro–Wilk test. Setting time, radiopacity, flowability and solubility were analysed using one-way ANOVA and Tukey's tests. Two-way ANOVA and Dunnett's tests were used for CV, MTT and pH analysis. Using 16S rRNA sequencing, differences in alpha diversity were calculated using XLSTAT (ver. 17.06, Addinsoft). Shannon index and Chao analysis were calculated using Mothur software. Beta diversity

was calculated using Bray–Curtis dissimilarities and was visualized by principal coordinate analysis (PCoA) in Mothur software. Differences in community composition were evaluated by analysis of similarity. Significance was set at 0.05 or less with a 95% or greater confidence interval.

RESULTS

Physical properties and chemical characteristics of the sealers

All sealers showed setting time, flowability and radiopacity within acceptable ISO6876:2012 specifications (International Organization for Standardization 2012) (Table 2). ESM, BCS and BRR did not satisfy ISO6876:2012 solubility requirements. Nonetheless, all sealers showed comparable volumetric contraction when contained in simulated standardized root canals ($p > .05$, Table 2). GFB showed the best dimensional stability, slightly increasing mass (0.18%) and decreasing volume (−0.67%) during solubility analysis. GFB and AHP volumetric change and solubility, however, were comparable ($p > .05$, Table 2).

When used to obturate standardized simulated root canals, calcium silicate-based sealers induced the highest pH over time (Figure 1f). BCS caused a gradual, statistically significant, increase in pH, reaching the maximum (mean of 8.95) after 28 days of immersion, when compared to the negative control ($p < .0001$). Calcium (Ca), silica (Si) and zirconium (Zr) from all root canal sealers were detected using EDS (Table 3). ESM surface composition was the most complex, containing low levels of detected sulphur (S), aluminium (Al) and titanium (Ti).

Sealers affect biofilm formation and metabolism

Most sealers showed little impact on the biofilm biomass, except for AHP, which showed a ~50% decrease at 18-h post-inoculation ($p < .0001$). Conversely, BRR enhanced biofilm formation by approximately twofold at 3- and 6-h post-inoculation and a ~50% increase by 18h ($p < .01$, Figure 2a). All sealers, except for BRR, decreased MTT reduction activity compared with the control, suggesting a reduction in biofilm metabolic activity or viable cell numbers (Figure 2b). At 18 h, ESM caused the largest decrease in MTT reduction (~68%, $p < .0001$). BRR promoted an increase in MTT reduction at 3- and 6-h post-inoculation, comparable to the control group by 18 h ($p > .05$, Figure 2b).

Sealers affect biofilm community composition

Multiple changes in community composition at 18 h under anaerobic and 5% CO_2 conditions were visualized using DGGE (Figure S2). DGGE bands with similar migration patterns, but different intensities, were excised and Sanger sequenced. Microorganisms enriched on sealers included *Rothia mucilaginosa* (biofilms grown on AHP), *Granulicatella adiacens* (all sealers except BRR), *Peptostreptococcus* (all sealers except AHP) and *Streptococcus gordonii* (only BRR) compared with the control group.

The sealers' effect on community profile was analysed further by sequencing the V3-V4 hypervariable region of the 16S rRNA genes (Figure 3a,b). Community composition of biofilms formed on sealers under both 5% CO_2

TABLE 3 Energy-dispersive spectroscopy chemical characterization of sealer surface

Element	AH Plus	GuttaFlow Bioseal	Endoseal MTA	Bio-C Sealers	BioRoot RCS
Ca	11.3	11.9	7.6	20.8	59.9
Si	17.7	5.3	5.3	4.0	16.1
Zr	22.2	79.2	20.3	45.1	19.2
Fe	27.9	3.2	52.7	29.8	
W	20.6		8.5		
Zn		0.4			
P					
Al			1.3	0.4	
S			3.3		
Ti			1.1		
Cl					4.7

Note: The mean percentage (weight %) of elemental detected on experimental sealers.

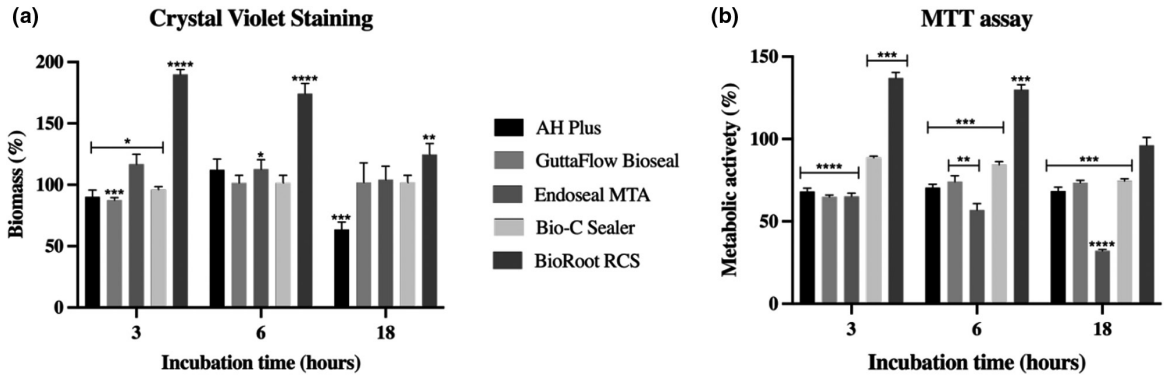

FIGURE 2 Effects of root canal sealers on biofilm biomass and metabolic activity after 3, 6 and 18 h of incubation under 5% CO_2 at 37°C. Except for AH Plus, which induced a ~50% decrease at 18-h post-inoculation, most sealers did not cause impairment on biofilm biomass. Conversely, except for BioRoot RCS, all sealers decreased MTT reduction compared with the control, suggesting a negative impact in biofilm metabolic activity or viable cell numbers (a) Quantitative measurement of crystal violet staining relative to negative control as an indicator of biomass accumulation on root canal sealer specimens. (b) Quantitative measurement of plaque-derived biofilm MTT conversion relative to negative control as an indicator of community metabolic activity. Values were significantly different compared with the control group (*$p < .05$; **$p < .01$; ***$p < .001$, ****$p < .0001$, respectively; two-way ANOVA and Dunnett's multiple comparisons test)

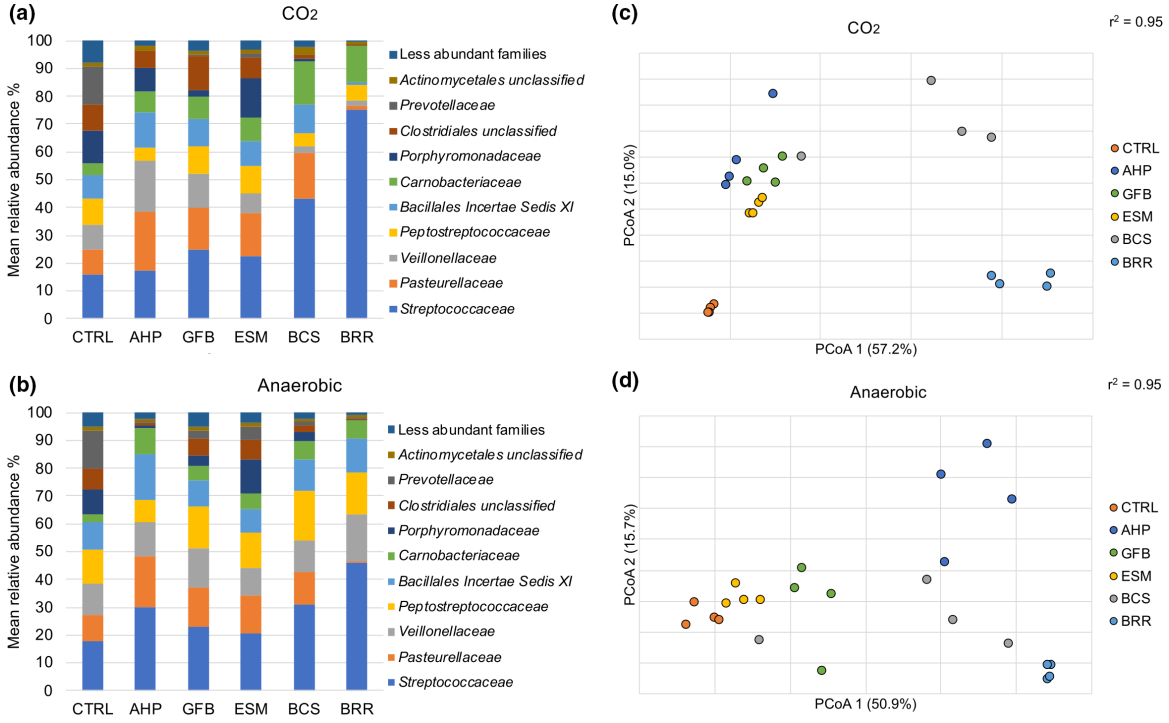

FIGURE 3 Taxonomic and principal coordinate analysis from 16S rRNA gene sequencing. The community composition of biofilms formed on sealers under 5% CO_2 and anaerobic conditions showed significant differences in alpha diversity using the Shannon index and Chao analysis of variance. (a) Relative abundances of families within biofilm communities incubated under 5% CO_2 at 37°C. (b) Relative abundances of families within biofilm communities incubated under anaerobic conditions. (c) Specimens were incubated under 5% CO_2. (d) Specimens were incubated under anaerobic conditions. Negative control (CTRL); AH Plus (AHP); GuttaFlow Bioseal (GFB); Endoseal MTA (ESM); Bio-C Sealer (BCS); BioRoot RCS (BRR)

($p < .05$) and anaerobic ($p < .05$) conditions showed significant differences in alpha diversity using the Shannon index and Chao analysis of variance in rank order: CTRL > ESM > GFB > AHP = BCS > BRR and CTRL > ESM = GFB = BCS ≥ AHP > BRR. BRR exerted the greatest selective pressure on biofilms. Under 5% CO_2, biofilm communities developed over BRR were mostly composed of *Streptococcaceae* family members (~75%), which represented only ~17% of the control community. Conversely, *Porphyromonadaceae*, *Prevotellaceae* and *Clostridiales* family members were highly diminished or absent in BRR samples. *Streptococcaceae* family members comprised

~45% of the BRR biofilm community compared with the control (~30%) in anaerobic conditions. In biofilms grown on BRR, *Prevotellaceae family* members were reduced in prevalence. Community composition was significantly different amongst sealers (Figure 3c,d; ANOSIM $R = .88$ and .85, $p < .001$) under 5% CO_2 and anaerobic conditions, although pairwise differences were not significant (Bonferroni-correct $\alpha = .003$). Sequencing of DNA extracted from the elution buffer control did not return quantifiable amplicon concentrations, confirming there was no contamination during sample processing.

On dentine discs, several distinct morphotypes, including cocci, rods and spirals, were observed in diverse arrangements in FE-SEM images of 18-h control biofilms grown under 5% CO_2, consistent with community richness (Figure 4). In the presence of the sealers, the community richness decreased. In images from the BRR group, structures suggestive of extracellular matrix-like structures are evident (Figure 4, BRR—5000× magnification); the community was predominantly of coccal morphotypes and less diverse than the control biofilm. Other experimental sealers showed at least three distinct morphotypes, suggesting more diverse communities than BRR but less than controls.

DISCUSSION

Microorganisms are the primary aetiologic factors in the pulpal and periapical infections (Siqueira & Rocas, 2008). Given that the therapeutic objectives of root canal therapy include microbial elimination and effective intracanal isolation, we characterized a set of physicochemical properties from five commercially available sealers that we hypothesized could reduce intracanal microbial burden. Sealer compositions dictate physicochemical properties and can contribute to indirect antimicrobial activity by promoting an effective seal of the root canal space (Siqueira & Rocas, 2008). For example, elevated radiopacity in filling materials contributes to the radiographic identification of the obturation limits, a factor shown to influence final endodontic treatment outcome and prognosis (Holland et al., 2007, 2017; Ricucci & Langeland, 1998; Suzuki et al., 2010). Additionally, some sealers can solubilize when challenged by aqueous environments, such as at the interface between foramen and periapical tissues. High sealer solubilization will form voids in the obturation (Sfeir et al., 2021; Urban et al., 2018), which can compromise the isolation of the root canal space—and of the bacteria and their by-products contained within—from the tissues surrounding the tooth. Importantly, some level of solubilization might be essential for the antimicrobial activity of some biomaterials, as it contributes to the release of bioactive antimicrobial molecules (Muñoz-Bonilla & Fernández-García, 2012). So, if sealers are to be used as complementary antimicrobial therapy on root canal infections, minimal solubilization enabling some antimicrobial activity must be balanced against any negative impact on long-term sealing of the root canal space.

The sealers tested here met ISO6876:2012 requirements (International Organization for Standardization 2012) for radiopacity, setting time and flowability, corroborating previous studies (Lim et al., 2020; Tanomaru-Filho et al., 2017; Zordan-Bronzel et al., 2019). However, solubility standards were met only by AHP and GFB. Notably, conventional solubility tests do not simulate clinical reality and can overestimate the solubility of hydrophilic sealers, such as calcium silicate-based sealers (Lim et al., 2020; Zordan-Bronzel et al., 2019). Furthermore, the final drying process in conventional tests may cause water evaporation and, consequently, loss in total mass (Gandolfi et al., 2015) which could explain why ESM, BCS and BRR, failed to meet ISO6876:2012 standards in our experiments. Over time, sealer solubility could negatively affect the isolation of disinfection-resistant microorganisms, potentially compromising long-term treatment success. Thus, we propose that the impact of dimensional stability of hydrated sealers and bacterial microleakage warrants further studies.

Sealers can change their surrounding chemical environment (Baras et al., 2019; Kitagawa et al., 2021), inducing sustained alkalinity, which can also contribute to antimicrobial activity. Our pH analysis showed that only calcium silicate-based sealers sustained high alkalinity over 28 days. The increase in local pH likely results from the calcium silicate setting reaction induced by hydration. Hydroxyl ions promote local environment alkalinization, damage bacterial membranes and cause protein denaturation (Siqueira & Lopes, 1999). As a result, hydroxyl ions can inactivate cytoplasmic enzymes, impairing bacterial metabolism and cellular division (Estrela et al., 1995). Interestingly, amongst the alkalizing sealers, only ESM substantially reduced (~70%) microbial community metabolism relative to control (Figure 2b).

The antimicrobial activity of some calcium silicate-based sealers has also been attributed to their trace metal compositions (Shin et al., 2018). The surface of ESM contained Al, S and Fe as determined by EDS analysis. Oxides of Al and Fe (Al_2O_3, and Fe_2O_3) can damage the Gram-positive bacterial cell wall, increasing the flux of molecules into and out of the cells (Azam et al., 2012; Hajipour et al., 2012). Additionally, surfaces containing sulphur can impair bacterial adherence *in vitro* (Smith et al., 2020). Our data support previous findings suggesting that, in addition to alkalinizing potential, ESM surface chemical composition may also contribute to the sealer's antimicrobial properties (Shin et al., 2018).

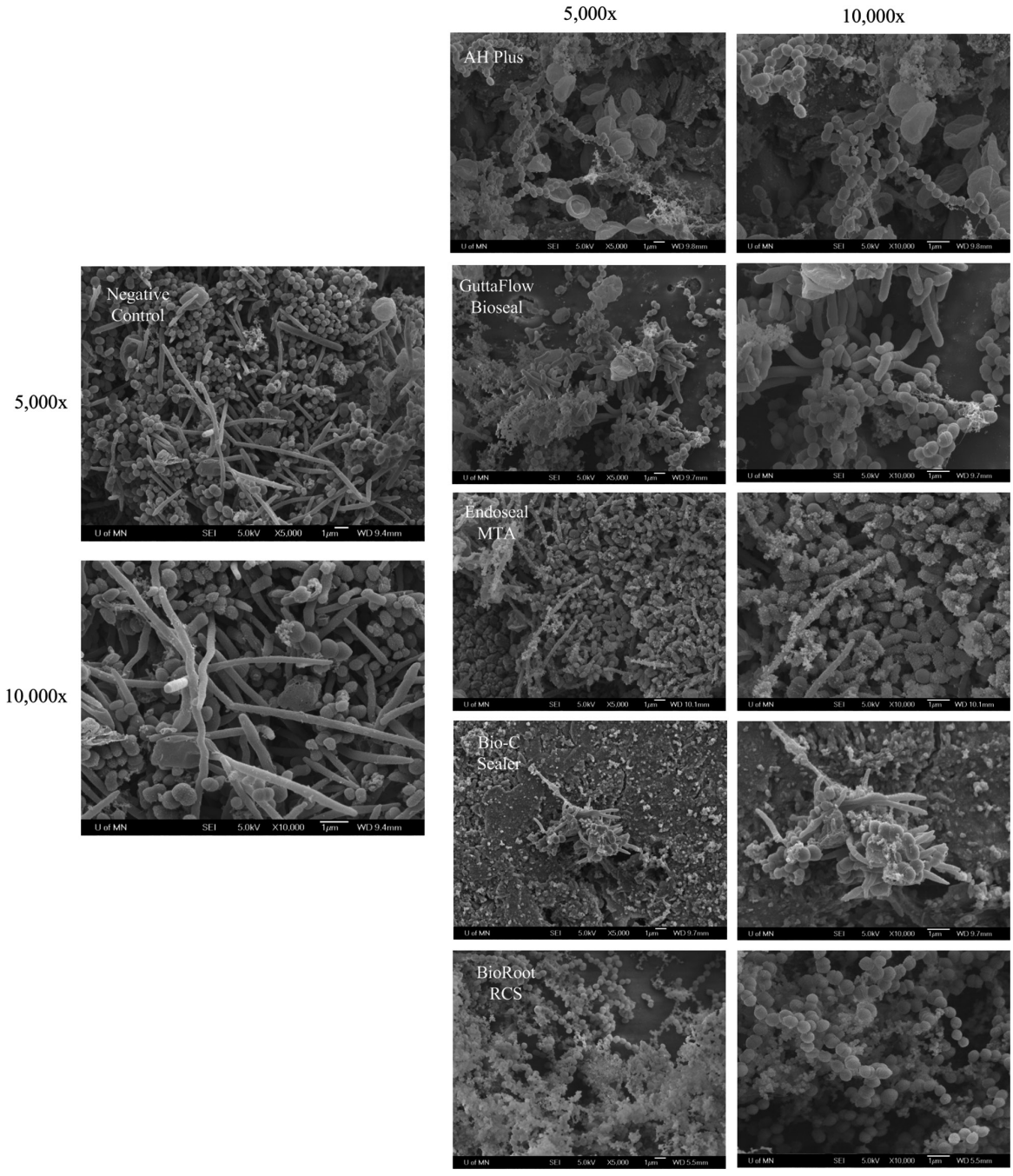

FIGURE 4 Biofilm morphology induced by different root canal sealers and negative control. FE-SEM images of control biofilms (left side images; plaque-inoculated sterile dentine discs) show several distinct morphotypes, including cocci, rods and spirals, in diverse arrangements. In the presence of the sealers (right side images; plaque-inoculated sealer/dentine discs), the community richness decreased. Biofilms were visualized using a field-emission gun scanning electron microscope at 5000 and 10,000× magnification

AH Plus caused minor deficits in biofilm biomass (~37%) and metabolic activity (~32%) (Figure 2), which could be attributed to the elution of unreacted monomers post-polymerization (Zhang et al., 2009). To determine whether AHP leaches potentially bioactive organic components, the surrounding experimental environment was analysed using proton nuclear magnetic resonance (1H NMR). Spectra collected at 3, 6 and 18 h (Figure S3) showed peaks consistent with leached methylenedianiline (listed as dibenzyldiamine on the ingredient list) and bisphenol F (BPF). Both molecules are bioactive antimetabolites that can be antimicrobial (Hąc-Wydro et al., 2019; McQueen & Williams, 1990).

Endodontic infections vary in course amongst individuals, infection stages (vital/necrotic pulp) and types (primary and secondary/persistent), implicating

multiple bacterial genera as endodontic pathogens (Rocas & Siqueira, 2008; Sassone et al., 2008; Tzanetakis et al., 2015). Endodontic microorganisms form biofilms, which adhere to canal walls and dentinal tubules, escaping antimicrobial strategies (Siqueira & Rocas, 2008). Because microbial-induced tooth decay is the most common cause of endodontic infections, we used a supragingival plaque-derived biofilm model (Edlund et al., 2013; Hall et al., 2019; Tian et al., 2010) to assess sealers' antimicrobial properties. All sealers affected biofilm development, reducing biomass and metabolism. We also observed a significant impact on community composition (Figure 3), including changes in the abundance of known endodontic pathogens, such as *R. mucilaginosa* (Yamane et al., 2009), *G. adiacens* (Siqueira & Rocas, 2006) and *Peptostreptococcus* (Rocas & Siqueira, 2008) (Figure S2).

Biofilms grown on BRR were particularly interesting. In 5% CO_2, BRR biofilms were denser, more metabolically active (Figure 2a,b), and showed greater community composition differences compared with the negative control than other sealers. Approximately, 75% of the DNA collected from BRR biofilms mapped to *Streptococcaceae* family members (Figure 3a). Although many *Streptococcaceae* family members, such as *S. gordonii*, are commensal microorganisms (Costalonga & Herzberg, 2014), substantial enrichment may indicate a dysbiotic endodontic community; streptococcal species are commonly found in endodontic infections (Lima et al., 2020). For example, *S. mutans*, commonly associated with dental caries, has been identified in 70% of patients with primary and secondary endodontic infections (Lima et al., 2020). Conversely, BRR biofilms contained reduced or undetectable levels of *Porphyromonadaceae*, *Prevotellaceae* and *Clostridiales* (Figure 3a,b). These families encompass many known endodontic pathogens, including *Porphyromonas gingivalis*, *Prevotella* and *Clostridium* species (Rocas & Siqueira, 2008; Tzanetakis et al., 2015), which are also highly associated with periodontal disease (Costalonga & Herzberg, 2014). Given that microbe in complex biofilm communities has evolved synergistic and antagonistic relationships to facilitate cohabitation, the loss of species from the *Porphyromonadaceae*, *Prevotellaceae* and *Clostridiales* families may affect microorganisms from other families.

We recognize that our *in vitro* studies provide limited simulation of the *in vivo* environment, but they are relevant to the questions we posed. Future studies might build on our work to develop an *ex vivo* root canal model to test the antimicrobial effects of sealers. An ex vivo model would greatly expand our ability to draw clinical inferences.

Using our multiparameter approach, we investigated the antimicrobial effects of five commercially available root canal sealers on *ex vivo* dental plaque-derived multispecies biofilms, containing originally more than 11 families of bacteria (Figure 3). Our results show robust reproducibility of our *ex vivo* dental plaque-derived multispecies biofilm model.

CONCLUSION

Despite adequate physical properties, which might contribute to effective seal and isolation of the root canal space, none of the sealers tested completely prevented a multispecies biofilm development under the conditions tested. Nonetheless, the different root canal sealers significantly affected the biofilm community composition. The impact of the changes in the biofilm community composition on the long-term success of endodontic treatment remains to be investigated.

CONFLICT OF INTEREST

The authors report no conflicts of interest in connection with this article.

ETHICAL APPROVAL

Dental plaque samples were collected according to the University of Minnesota Internal Review Board guidelines.

AUTHOR CONTRIBUTIONS

Conception and design: Flavia M. Saavedra, Mark C. Herzberg, Marina A. Marciano, and Bruno P. Lima. *Data acquisition, analysis, and interpretation*: Flavia M. Saavedra, Lauter E. Pelepenko, William S. Boyle, Anqi Zhang, Christopher Staley, Mark C. Herzberg, Marina A. Marciano, and Bruno P. Lima. *Drafted the manuscript*: Flavia M. Saavedra. *Revised the manuscript*: Flavia M. Saavedra, Lauter E. Pelepenko, William S. Boyle, Anqi Zhang, Christopher Staley, Mark C. Herzberg, Marina A. Marciano, and Bruno P. Lima.

DATA AVAILABILITY STATEMENT

Raw data are available under BioProject accession number SRP303009.

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