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Long, Jiahao ; Kreft, Jan-Ulrich; Camilleri, Josette

DOI:
10.1016/j.jdent.2020.103283

Document Version
Peer reviewed version

Citation for published version (Harvard):
Long, J, Kreft, J-U & Camilleri, J 2020, 'Antimicrobial and ultrastructural properties of root canal filling materials exposed to bacterial challenge', *Journal of Dentistry*, vol. 93, 103283, pp. 1-7. https://doi.org/10.1016/j.jdent.2020.103283

Antimicrobial and ultrastructural properties of root canal filling materials exposed to bacterial challenge

[1]Long J, [1]Kreft JU, [2]Camilleri J

[1]Institute of Microbiology and Infection & Centre for Computational Biology & School of Biosciences, University of Birmingham, Birmingham, United Kingdom; [2]School of Dentistry, Institute of Clinical Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom

Key words: Endodontic infection, root canal therapy, dental materials, surface change, biofilms, antimicrobial properties

Corresponding authors

Dr J. Camilleri
School of Dentistry,
Institute of Clinical Sciences,
College of Medical and Dental Sciences,
University of Birmingham,
5, Mill Pool way
Edgbaston B5 7EG
Birmingham,
United Kingdom

Dr J.-U. Kreft
School of Biosciences &
Institute of Microbiology and Infection &
Centre for Computational Biology,
University of Birmingham,
Edgbaston, B15 2TT
Birmingham
United Kingdom

Abstract

Introduction: Chemo-mechanical preparation of the root canal leaves behind viable bacteria which can lead to treatment failure. Materials used inside the root canal should possess antimicrobial properties and also resist disintegration in the presence of biofilm. **Methods:** Gutta-percha, three root canal sealers (Pulp Canal Sealer, AH Plus and BioRoot RCS) and materials used to make posts (a metal and a resin) were evaluated. Their antimicrobial activity against *Enterococcus faecalis* in direct contact was assessed by scanning electron microscopy and live-dead staining using confocal microscopy over a period of eight weeks. The materials' structural integrity was assessed by scanning electron microscopy.

Results: The antimicrobial activity of the materials varied. The metal alloy posts as well as BioRoot RCS sealer did not allow any biofilm accumulation; but gutta-percha, Pulp Canal Sealer and resin from fibre-reinforced posts encouraged thick biofilm accumulation. Microstructural changes were observed in AH Plus (washout) and BioRoot (crystal deposition) in contact with biofilm. The Pulp Canal and BioRoot RCS sealers exhibited a modified ion leaching pattern in contact with microbial loaded media.

Conclusions: The microbial challenge affected the material microstructure in some of the materials tested and allowed biofilm accumulation. Although clinical success depends on a number of factors, materials that are structurally sound and exhibit antimicrobial properties are preferable for endodontic therapy and tooth restoration involving entry in the root canal.

1. Introduction

Root canal therapy is necessary when the defence mechanisms of the dental pulp break down and the pulp needs to be removed. Apical periodontitis is caused by bacteria therefore, root canal therapy is aimed at elimination of micro-organisms to prevent microleakage. Failure of root canal therapy is usually caused by reinfection of the root canal space after root canal obturation. Coronal microleakage allows micro-organisms access to the root canal causing apical infection. *E. faecalis* strains isolated from saliva, the pulp chamber, and the root canal were similar, suggesting that coronal microleakage is a cause of endodontic failure (1). Regardless of treatment and attempts to eradicate infection, residual microorganisms can remain within the tooth and also in the periapical region leading to secondary endodontic infections (2). Although *Enterococcus faecalis* is commonly associated with failed root canal therapy (3-5), other micro-organisms are implicated with endodontic treatment failure. The intraradicular bacterial community associated with failed endodontic treatment varied significantly in composition from tooth to tooth. Persistent intraradicular infections were present in all root-filled teeth (6).

Root canal sealers and additional clinical steps minimise the chance of secondary infection (5). Endodontic microorganisms have a high affinity to root canal filling materials and sealers, especially to gutta-percha (6). Biofilm formation on these materials has been suggested to lead to the persistence of microorganisms in root canals (7). This study assessed the microstructure and antimicrobial properties of a number of materials used within the root canal during contact with endodontic microbiota.

2. Materials and methods

The materials used in this study included gutta-percha (Obtura Spartan, Algonquin, Canada), the main core material used for obturation and three sealers with different compositions. The sealers were Pulp Canal Sealer (Kerr Italia S.r.l., Salerno, Italy) a zinc oxide eugenol-based sealer, AH Plus (Dentsply DeTrey, Konstanz, Germany) which is an epoxy resin-based and BioRoot RCS (Septodont, Saint Maur-des-Fosses, France), a hydraulic calcium silicate-based sealer. An alloy based on cobalt-chromium which is the component of metal posts and an epoxy resin (Angelus, Londrina, Brazil) making up fibre-reinforced posts were also evaluated.

To have a comparable surface area for bacterial contact, all materials were formed into discs 9 to 11 mm in diameter and approximately 2 mm high, except for the resin that was supplied 6 mm in diameter and 3 mm high. The gutta-percha was melted at 200°C and compacted into the moulds when still hot. The sealers were mixed according to the manufacturers' instructions and compacted into moulds of the same size and incubated at 37°C until set. The metal post alloy was cast directly into the disc shape and the resin was obtained from the manufacturer as long cylinders and cut into shorter discs.

2.1 Strain, media and growth conditions

Enterococcus faecalis strain ATCC 29212 was grown in brain heart infusion and sucrose (BHIS) broth or agar media at 37°C. BHIS contained (in g/L) brain infusion solids (12.5), beef heart infusion solids (5.0), proteose peptone (10.0), NaCl (5.0), D-glucose (2.0), Na_2HPO_4 (2.5) and sucrose (10); with a final pH of 7.4. An *E. faecalis* stock culture was plated on BHIS agar and grown overnight. A colony from this plate was streaked on a fresh plate and incubated overnight before inoculating into the BHI broth. The material discs were sterilised by immersing in 70% ethanol for 1 min, then retrieved and allowed to dry. The discs were placed in 24-well plates (Corning Costar #3527 24-well, clear polystyrene, flat-bottom multiwell plates, 15.6 mm diameter and 3.4 mL well volume), and 2 mL of BHIS medium was added into the wells. Wells were inoculated with 20 µL of *E. faecalis* overnight culture before sealing with a gas permeable membrane (Breathe-Easy medical grade polyurethane membrane with FDA-approved acrylic adhesive for standard multiwell plates, Sigma-Aldrich) and placing the lid on the microtitre plates to decrease evaporation during the long incubation times. All the 24-

well plates were incubated at 37°C in the dark without shaking to simulate the stagnant root canal. Testing was carried out after 0, 1, 2, 4 and 8 weeks.

2.2 Surface morphology

The material cylinders were retrieved after incubating with *E. faecalis* for different time intervals, and washed in phosphate buffered saline (PBS). The bacteria were then fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.3, then dehydrated by successive rinsing in ethanol solutions. A final rinse with hexamethyldisilane (HMDS) was followed by drying. The material discs were mounted on aluminium stubs and sputter coated with gold for electrical conductivity (K550X Sputter Coater). They were imaged with a scanning electron microscope (SEM, Evo MA10, ZEISS) at a working distance of 10.5 mm from the electron gun and the I-probe was set to a current of 20 pA. Material discs which were neither exposed to *E. faecalis* nor media and discs exposed to media for 8 weeks were also prepared and imaged using the scanning electron microscope. In addition, energy dispersive spectroscopy (EDS) was also performed at a working distance of 8.5 mm and with the I probe set to current of 1000 pA.

2.3 Biofilm assessment

Samples were prepared for live/dead staining with SYTO 9 green fluorescent nucleic acid stain (3 µL of a 3.34 mM solution in dimethylsulfoxide, Molecular Probes/Life Technologies), propidium iodide (3 µL of a 20 mM solution in dimethylsulfoxide, Sigma-Aldrich) and 1 mL of filter-sterilised water in a foil-covered 2 mL microcentrifuge tube. The material disks incubated with/without *E. faecalis* were placed into new 24-well plates, with staining solution to cover the surface of the discs and incubated for about 20 minutes in the dark at room temperature. Discs were rinsed with filter-sterilised water to remove excess stain. Samples with a water film were placed under a water dipping, long working distance objective (40 ×/0.80 HCX APO W IMM) of a Leica TCS SP8 confocal system. This microscope was equipped with a Leica DM6 CS5 microscope frame to examine biofilms, count live and dead cells and calculate the percentage of live cells.

2.4 Leachate analyses

At the end of the incubation period of 8 weeks, the leachate was assessed by inductively coupled plasma – optical emission spectroscopy (ICP-OES, Optima 8300, PerkinElmer). Material disks were removed from the wells and the remaining media were sampled and diluted in 2% nitric acid to obtain concentrations from 0.5 to 50 ppm. The diluted samples were filtered through a 0.22 µm nitrocellulose membrane filter (Merck Millipore Ltd., Tullagreen, Cork, Ireland) before injecting into the ICP-OES. Leachates of materials not in contact with *E. faecalis* were also assessed and thus the effect of bacterial colonization on leaching of the elements was investigated. ICP-OES was performed to detect the presence of calcium, silicon, zirconium, tungsten and zinc.

3. Results

3.1 Surface morphology

The surface characteristics and attachment of cells after 8 weeks of exposure to *E. faecalis* were compared to fresh materials by scanning electron microscopy. The fresh materials are shown in Figure 1 and materials incubated in medium for 8 weeks without bacteria in Figure 2. The surface of the test materials varied. The fresh gutta-percha and AH Plus were mostly flat, but the gutta-percha had irregular porosities. The BioRoot and the Pulp Canal Sealer exhibited a particulate surface which was moderately rough. The metal and resin post materials were very rough. The Co-Cr alloy had been sandblasted, which caused the rough surface. The resin was also very rough with glass particles evident all over the surface. After exposure to the media without *E. faecalis* for 8 weeks (Figure 2) , the material surfaces were not modified, except for slight changes in the sealers. The AH Plus and Pulp Canal sealer were slightly smoother while the BioRoot appeared more particulate due to crystal deposition on its surface.

Gutta-percha exposed to *E. faecalis* (Figure 3) accumulated biofilm on the surface after 1 week, which increased at 4 weeks and diminished over the next 4 weeks. The root canal sealers demonstrated different antimicrobial properties. The Pulp Canal Sealer exhibited a thick biofilm over its surface for a 4-week period. The AH Plus surface was smooth and a sparse biofilm adherence was only visible at 1 week. However, the exposure to media and biofilm changed the surface microstructure considerably. Initially, the matrix was washed out leaving the radiopacifier particles on the surface. At later time points, the surface was completely smooth indicating the loss of the radiopacifier particles. This was independent of the biofilm since it was also evident in the control without bacteria (Figure 2). Exposure of BioRoot RCS to *E. faecalis* biofilm resulted in a change in material microstructure as there was evidence of matrix wear and deposits of globular crystals on the BioRoot surface. No biofilm adhesion was evident throughout the 8-week period of testing, indicating the antimicrobial activity of this sealer.

The Co-Cr did not change after exposure to media (Figure 2) or bacteria (Figure 3) for 8 weeks. Sporadic *E. faecalis* cells were seen on the surface of samples from 1 to 4 weeks. By

8 weeks, bacteria were practically absent. The surface of the resin was very rough with and without the media and *E. faecalis* contact. Over a period of 4 weeks, the resin encouraged bacterial biofilm formation on its surface, increasing in amount and thickness.

The EDS analyses are shown in Table 1. BioRoot exhibited a high calcium content while both gutta-percha and Pulp Canal Sealer had a high zinc content with the latter also containing silver. Zirconium from zirconium oxide, which is used as radiopacifier, was present in both BioRoot and AH Plus sealers. The latter also contained tungsten, which is also a radiopacifier, forming part of calcium tungstate in AH Plus sealer. The metal cylinders contained cobalt and chromium. The resin post material was mostly organic but contained Si and Ca. The presence of silica and calcium in the resin post material suggests the presence of a glass.

3.2 Biofilm assessment

No attached bacterial cells were identified for the BioRoot RCS and the Co-Cr metal post materials. For the other materials, the numbers of live and dead cells attached to the surface decreased over time but the fraction of dead cells showed no clear trend apart from AH Plus where it increased consistently (Table 2). The gutta-percha showed high cell numbers initially but then fewer cells were left at 8 weeks. The Pulp Canal Sealer initially had mostly live bacteria, which died after further incubation, both dead and live cells disappeared by 8 weeks. AH Plus also had initially mostly live bacteria, which disappeared without much change in the number of dead cells. For the resin post material, half of the cells were dead in week 1 and the count of both live and dead cells decreased from weeks 2 to 8.

3.3 Leachate analyses

The results of leachate analyses by ICP-OES are in Table 3. The gutta-percha, metal alloy post and resin fibre post were mostly the same in the presence or absence of bacteria. The Pulp Canal Sealer leached higher amounts of zinc into solution when in contact with *E. faecalis*. The calcium ion leaching of BioRoot was reduced in the presence of bacteria.

A summary of the findings for all tests is shown in Table 4.

4. Discussion

The current research was proposed as elimination of microorganisms from the root canal after chemo-mechanical preparation has not been possible. Failure of root canal therapy is caused by a number of microorganisms and *E. faecalis* has been used in the current study as a typical microbe colonizing the root canal space. It has specific features like virulence factors, for example, lytic enzymes, aggregation factors and surface projections (5, 9) that help it to bind to the host cell and express proteins to compete with other bacterial strains and avoid the host response (10). It also adheres well to surfaces, which may be an important factor in its virulence (11). A recent review of the literature indicated that *E. faecalis* is more highly correlated with persistent intraradicular infections than in primary untreated chronic periapical periodontitis (12). Thus, it is used in *in vitro* models to be able to assess and compare antimicrobial characteristics of materials.

Both antimicrobial characteristics and stability to microbial degradation were assessed. Material degradation by bacterial action leads to failure. This has been shown for the resin-based root canal obturation system Resilon where the polycaprolactone was shown to degrade when in contact with bacteria and by-products (13-16). This degradation led to clinical failure (17, 18). There was complete degradation despite the radiographic evidence of adequate obturation (19). In the current study, materials used in root canal therapy, namely gutta-percha and three sealers with different chemistries, were selected. None of these materials have been tested for degradation regardless the long-standing clinical use. Furthermore, two types of post material were also investigated. The metal posts were sandblasted as is common in clinical practice. The resin used was the same as that making up fibre-reinforced posts. Posts are placed inside root canals thus are also subject to bacterial contamination and breakdown.

Scanning electron microscopy allowed both the visualization of the biofilm and material degradation. To distinguish material degradation by biofilms or by leaching in the presence of media, micrographs were obtained for freshly set materials and during 8 weeks of exposure to media and biofilms or only media. The disadvantage of this method is the necessary fixing and drying of the specimens which was deleterious to hydraulic calcium silicate cements (20, 21). Processing affected the microstructure of BioRoot RCS as surface

carbonation was evident in the scanning electron micrographs. Imaging of the materials before and after immersion in media without biofilm exposure enabled the assessment of the effects of the media on the materials. All the sealers tested showed microstructural changes when exposed to the media, similar to a previous study where exposure of sealers to physiological solutions and media used for cytology changed the microstructure of the sealers (22).

The antimicrobial properties of the test materials were investigated using SEM as well as live-dead staining. For live-dead staining, the surface areas of the materials were standardized to make cell counting reliable. The leachate analyses before and after exposure to the bacteria were performed to investigate leaching which may affect the biofilm. The only fully antimicrobial material was BioRoot RCS while AH Plus allows sporadic bacterial growth but does not encourage biofilm formation. The Pulp Canal Sealer encouraged biofilm formation thus is not recommended as a root canal sealer. The lack of biofilm formation on AH Plus could be due to its smooth surface microstructure thus discouraging the accumulation of biofilm. The Pulp Canal Sealer also had biofilm dissolution after 4 weeks of incubation. This could be related to the release of zinc into solution which was identified by leachate analyses. The release of zinc is also responsible for the antimicrobial properties shown by the gutta-percha cones. The release of zinc from the Pulp Canal Sealer was enhanced by incubation with *E. faecalis*.

All the sealers showed micro-structural changes after 8 weeks of media exposure. Thus, long term percolation can lead to sealer dissolution with resultant microleakage exacerbated if the sealer is not antimicrobial. The release of high levels of calcium by the BioRoot is in accordance with previous studies (23) and accounts for the material's antimicrobial properties. The antimicrobial properties of BioRoot have also been shown in previous research and tested using alternative techniques (24). In the current study, a reduction in calcium leaching was shown when BioRoot RCS was in contact with biofilm for 8 weeks.

The resin that is used in fibre-reinforced posts allowed for a thick accumulation of biofilm unlike the metal post. Both exhibited a rough microstructure but the resin is not

recommended for use due to its susceptibility to biofilm accumulation thus risking treatment failure.

Although the materials tested in the current study exhibited biofilm accumulation and the sealers also some structural instability, it should be noted that these materials have been in successful clinical use for many years. The success of root canal therapy is multifactorial, with the chemo-mechanical root canal preparation playing a significant role. The structural integrity and biofilm accumulation capacity of the obturating materials and the posts are other factors that contribute to the clinical success.

Conclusions

Microstructural and chemical changes to the materials were observed when subjected to microbial challenge. The gutta-percha, Pulp Canal Sealer and both post materials exhibited minimal or no structural changes. AH Plus showed structural changes with washout while BioRoot exhibited crystal growth on its surface. All materials tested allowed biofilm formation at various stages except the metal alloy posts and BioRoot RCS, which exhibited highly antimicrobial properties. The structural integrity and antimicrobial properties are important for all materials used in the root canal space.

Acknowledgements

The authors would like to thank Ms Lygia Madi (Angelus) for arranging for the preparation of the standard resin cylinders and Mr Jianguo Liu (SEM facility), Dr Nina Vyas, Mr Christopher Stark (ICP-OES), Ms Kim Summers, Ms Helen McNeil, Dr Alessandro Di Maio (Biosciences Advanced Light Microscopy facility), Dr Rachel Sammons, Mr Philip Murphy and Dr Sarah Kuene for their assistence.

Credit author statement

Jiahao Long: Investigation; Formal analysis; Methodology; Writing - original draft;

Jan Kreft: Formal analysis; Methodology; Supervision; Validation; Writing - review & editing.

Josette Camilleri: Conceptualization; Formal analysis; Methodology; Supervision; Validation; Writing - review & editing.

Declaration

The authors declare no conflict of interest.

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Table 1. Elemental composition of material surfaces by mass (%), measured by EDS.

Material	Si	Ca	Zn	Zr	W	Ba	Co	Cr	Ag
Gutta-percha	0.5	0.0	52.5	0.0	0.0	2.6	0.0	0.0	0.0
Pulp Canal Sealer	0.0	0.0	33.0	0.0	0.0	0.0	0.0	0.0	15.7
AH Plus	0.9	2.9	0.0	13.6	14.3	0.0	0.0	0.0	0.0
BioRoot RCS	5.4	29.2	0.0	17.8	0.0	0.0	0.0	0.0	0.0
Metal alloy post	1.2	0.0	0.0	0.0	5.3	0.0	51.0	22.7	0.0
Resin fibre post	16.0	9.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 2. The counts of live and dead cells, as well as the percentage of dead cells on the surface of endodontic materials imaged in the confocal laser scanning microscope after live-dead staining. No cells were found on BioRoot RCS and the metal alloy post

Week	Gutta-percha			Pulp Canal Sealer			AH Plus			Resin post		
	Live	Dead	Fraction dead %	Live	Dead	Fraction dead %	Live	Dead	Fraction dead %	Live	Dead	Fraction dead %
1	969	355	27	519	89	15	1125	57	5	335	361	52
2	193	15	7	182	191	51	308	40	11	340	261	43
4	249	71	22	133	259	66	68	73	52	60	118	66
8	8	6	43	16	29	64	7	10	59	40	26	39

Table 3. Elements leached from the materials into the media measured by ICP-OES. The concentrations of elements when incubated for 8 weeks in the presence of *E. faecalis* minus concentrations after 8 weeks without the bacteria. Negative numbers mean the leaching was reduced in the presence of bacteria.

Material	**Elements present after exposure to *E. faecalis* (ppm)**				
	Ca	**Si**	**Zr**	**W**	**Zn**
Gutta-percha	/	-0.9	/	/	-6.9
Pulp Canal Sealer	/	/	/	/	930
AH Plus	8.7	1.1	0.0	0.0	/
BioRoot RCS	-6500	-31	0.0	/	/
Metal alloy post	/	-3.4	/	2.7	/
Resin fibre post	7.6	2.4	/	/	/

Table 4. Summary of results by material and recommendation.

Material	SEM-biofilm	SEM-structure	Live-dead staining	ICP-OES	Conclusion
Gutta-percha	Thick biofilm initially, decreasing later; flat surface	Minimal changes in structure	Initially biofilm present; mixture of live and dead cells, both decreasing with time	No leaching	Biofilm growth. Recommended microbial elimination prior to use
Pulp Canal Sealer	Thick biofilm initially, then decreasing; rough surface	Minimal changes in structure	Initially biofilm present; live cells decreasing while dead cells increasing transiently	Leaching of Zn in presence of bacteria	Biofilm growth. Recommended microbial elimination prior to use
AH Plus	Only few cells and only initially; flat surface	Initially surface changes with exposure of radiopacifier, then washout	Initially many live cells, then decreasing without dead cells increasing	No leaching	Initial biofilm formation and microstructural changes in media. Recommended microbial elimination prior to use. Avoid when washout is likely
BioRoot RCS	No bacterial growth on surface; rough surface	Crystal depostion on the material surface	No cells found	Leaching of Ca and Si in the absence of bacteria	Recommended due to lack of biofilms
Metal alloy post	A few cells attached; rough surface	No strucutral changes	No cells found	No leaching	Recommended due to lack of biofilms
Resin fibre post	Biofilm present; rough surface	No structural changes	Live and dead cells initially, declining after week 2	No leaching	Biofilm growth. Recommended microbial elimination prior to use

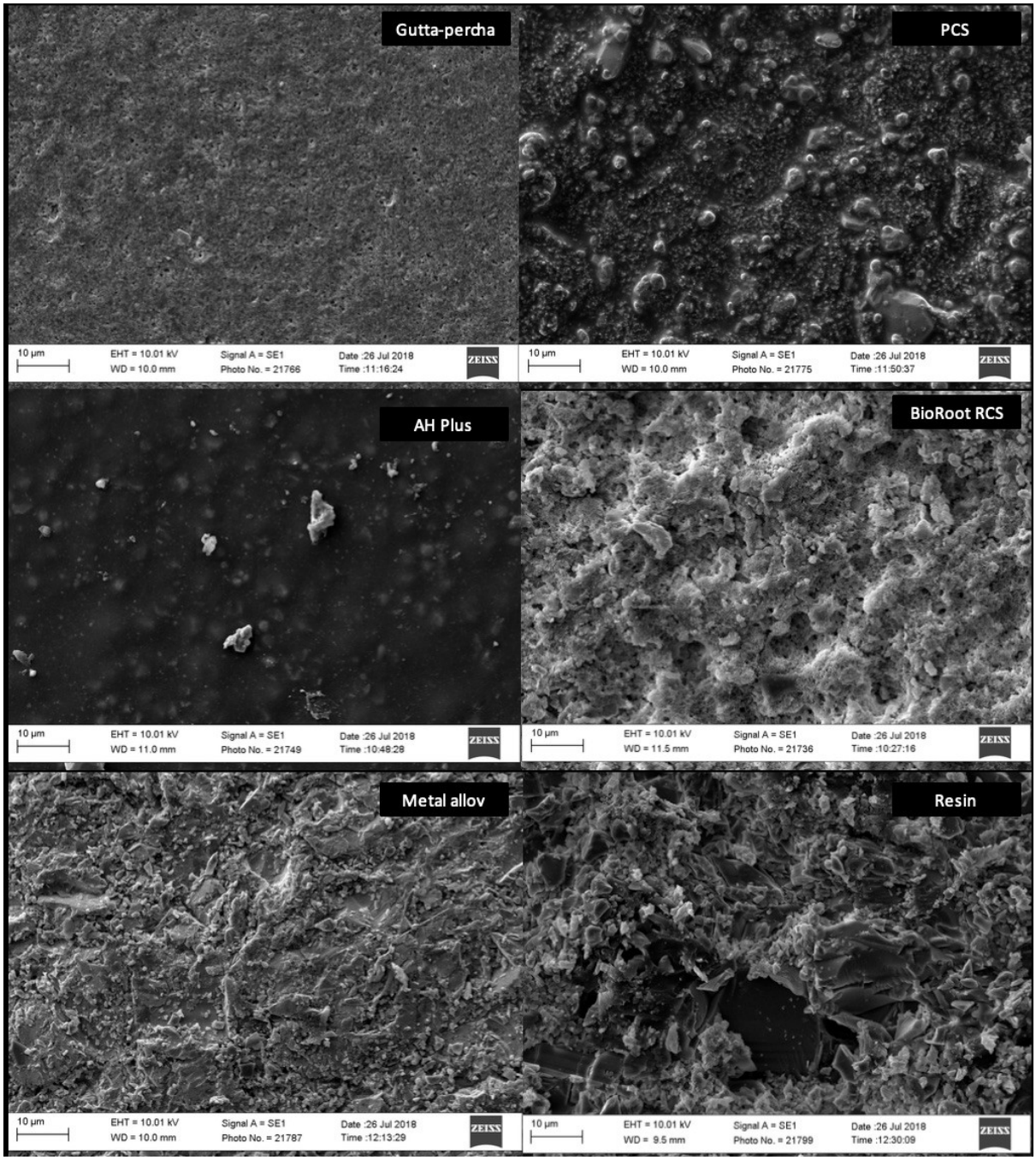

Figure 1: Surface microstructure of all materials, freshly prepared and before contamination with *E. faecalis*.

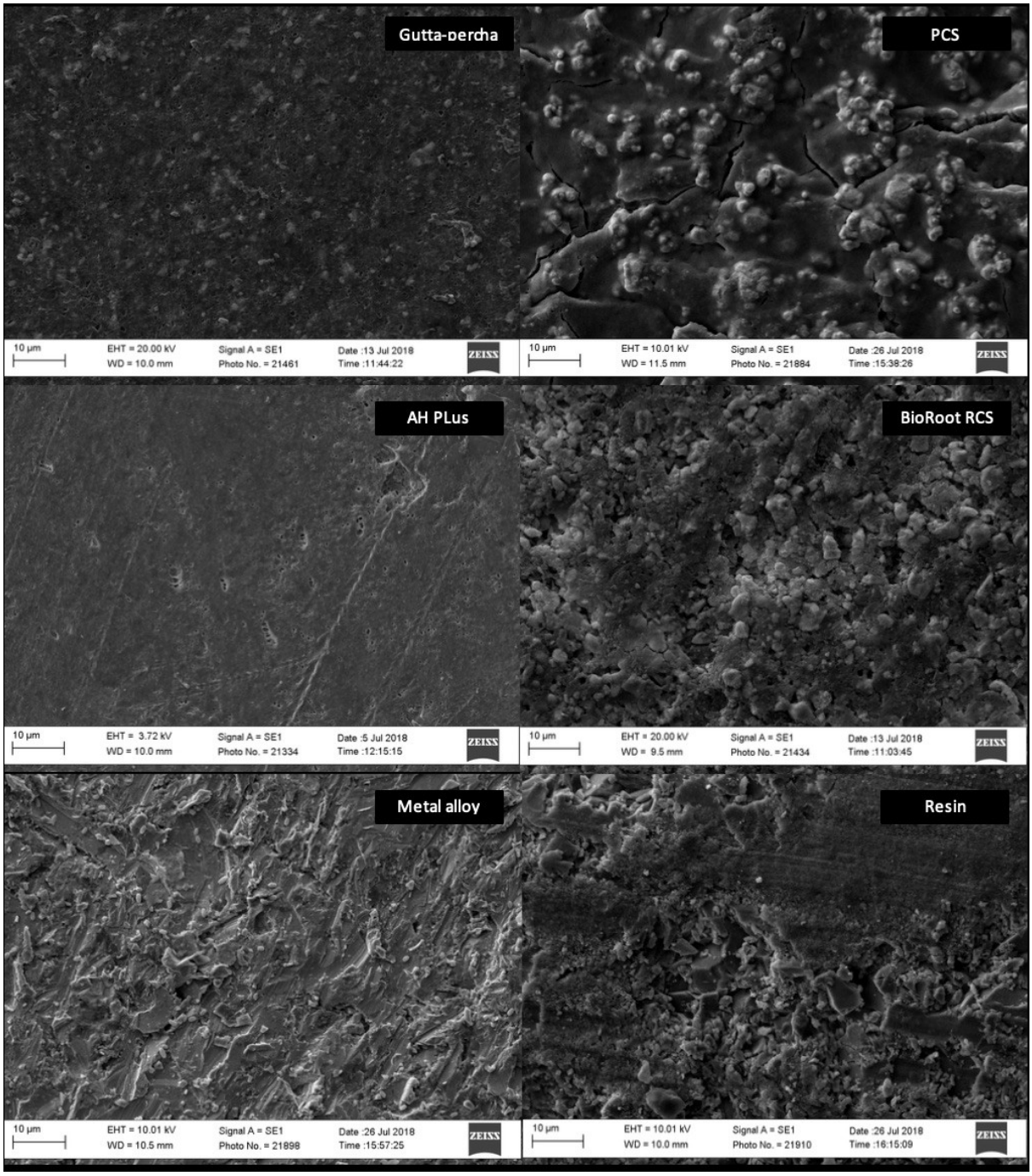

Figure 2: Surface microstructure of all materials, incubated in sterile medium for 8 weeks.

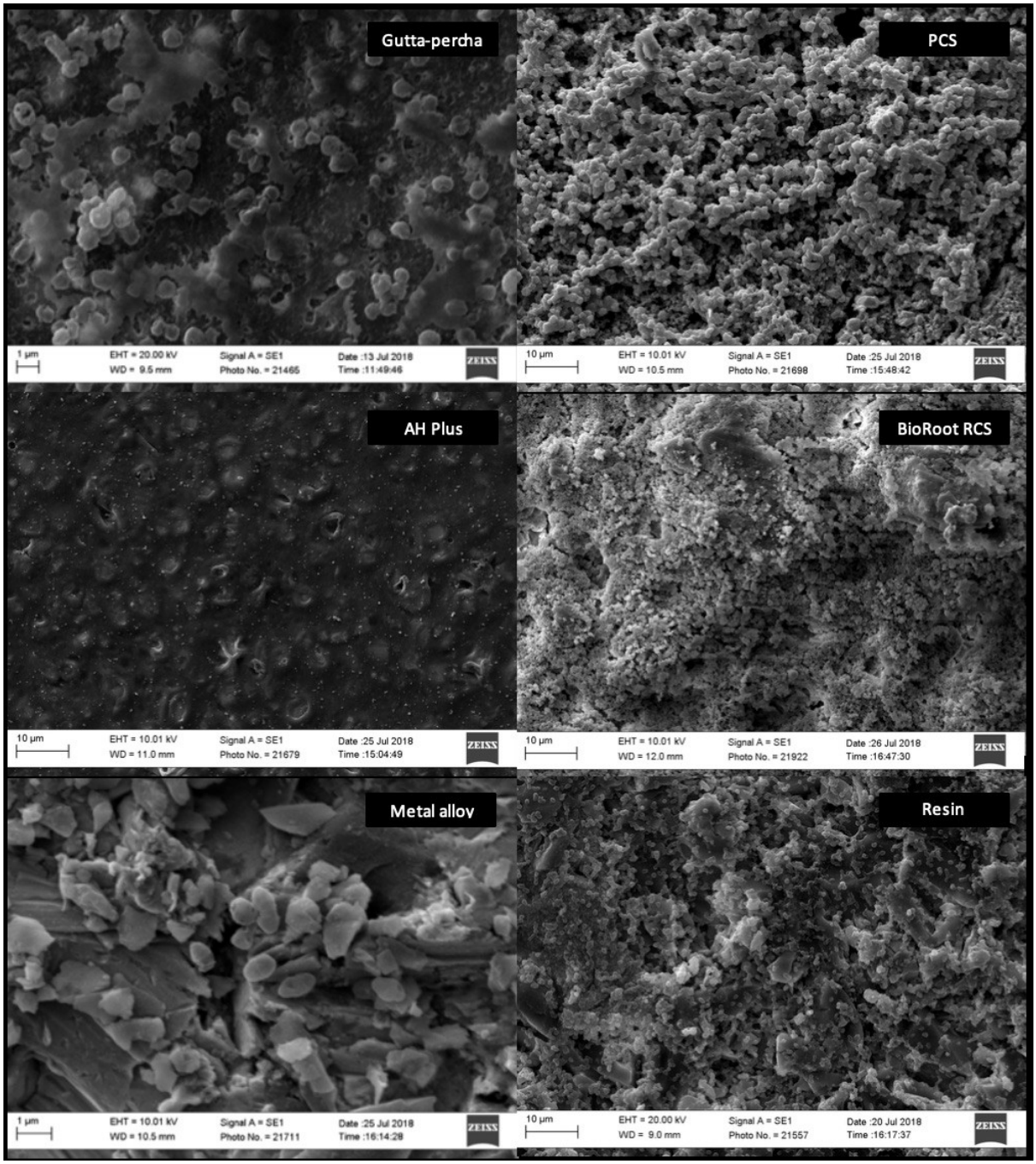

Figure 3: Surface microstructure of all materials, incubated in medium inoculated with *E. faecalis* for 4 weeks.