Research, Society and Development, v. 11, n. 1, e55511123849, 2022
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Baccor, *in vitro* formation of biofilm in broken endodontic files in radicular canals

Baccor, formação *in vitro* de biofilmes em limas endodônticas fraturadas em canais radiculares

Baccor, entrenamiento in vitro de biofilm en limas endodónticas fracturadas en canales raíces

Received: 11/24/2021 | Reviewed: 11/30/2021 | Accept: 01/13/2022 | Published: 01/15/2022

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Abstract
The aim of this study to evaluate the biofilm formation of sulfate-reducing bacteria from two microbial species, *Desulfovibrio desulfuricans* (oral and environmental strain) and *Desulfovibrio fairfieldensis*, in root canals with fractured endodontic files *in vitro* and the biocorrosive changes that these strains are capable of promoting on the metal surface of endodontic files. Fourteen teeth were included with fractured # 90 Kerr files and inoculants of *Desulfovibrio desulfuricans* strains and *Desulfovibrio fairfieldensis* in a modified Postgate E culture medium. The inoculated teeth were evaluated at 28, 41, 51 and 477 days. The biofilm was evaluated through scanning electron microscopy (SEM) and confocal laser scanning microscope with the fluorophore the Live / Dead® kit. For the 477 day, chemical pickling followed, with subsequent evaluation of the metallic surface of endodontic files in the SEM. In the biofilm analyzes of 28, 51 and 477 days, a mixed biofilm was observed, with a predominance of living cells and areas of corrosion along the entire metallic surface of the file for the last time, contrasting the metallic surface of the control groups. The SRB showed cellular activity both on the metal surface of the file and on the dentin surface of the root canal at the deepest levels of the root canal, apical and medium, promoting biocorrosion along the metal surface of endodontic files.
Keywords: *Desulfovibrio*; Microbiota; Sulfur-reducing bacteria; Endodontic.

Resumo
O objetivo deste estudo foi avaliar a formação de biofilme de bactérias redutoras de sulfato de duas espécies microbianas, *Desulfovibrio desulfuricans* (cepa oral e ambiental) e *Desulfovibrio fairfieldensis*, em canais radiculares com limas endodônticas fraturadas *in vitro* e as alterações biocorrosivas que essas cepas são capazes de promover na superfície metálica de limas endodônticas. Quatorze dentes foram incluídos com limas Kerr # 90 fraturadas e inoculantes com cepas *Desulfovibrio desulfuricans* e *Desulfovibrio fairfieldensis* em meio de cultura Postgate E modificado. Os dentes inoculados foram avaliados aos 28, 41, 51 e 477 dias. O biofilme foi avaliado por meio de microscopia eletrônica de varredura (MEV) e microscopia confocal de varredura a laser com o fluoróforo kit Live / Dead®. Após 477 dias, seguiu-se a decapagem química, com posterior avaliação da superfície metálica das limas endodônticas no MEV. Nas análises do biofilme de 28, 51 e 477 dias, observou-se um biofilme misto, com predomínio de células vivas e áreas de corrosão ao longo de toda a superfície metálica da lima pela última vez, contrastando com a superfície metálica dos grupos controle. As BRS apresentaram atividade celular tanto na

superfície metálica da lima quanto na superfície dentinária do canal radicular nos níveis mais profundos do canal radicular, apical e médio, promovendo biocorrosão ao longo da superfície metálica das limas endodônticas.
Palavras-chave: *Desulfovibrio*; Microbiota; Bactérias redutoras de enxofre; Endodontia.

Resumen
El objetivo de este estudio es evaluar la formación de biopelículas de bacterias reductoras de sulfato de dos especies microbianas, Desulfovibrio desulfuricans (cepa oral y ambiental) y Desulfovibrio fairfieldensis, en conductos radiculares con limas endodónticas fracturadas in vitro y los cambios biocorrosivos que estas cepas son capaces de la promoción en la superficie metálica de las limas endodónticas. Se incluyeron catorce dientes con limas Kerr # 90 fracturadas e inoculantes de cepas de Desulfovibrio desulfuricans y Desulfovibrio fairfieldensis en un medio de cultivo Postgate E modificado. Los dientes inoculados se evaluaron a los 28, 41, 51 y 477 días. La biopelícula se evaluó mediante microscopía electrónica de barrido (SEM) y microscopio de barrido láser confocal con el kit de fluoróforo Live / Dead®. Para el día 477, siguió el decapado químico, con la evaluación posterior de la superficie metálica de las limas endodónticas en el SEM. En el biofilm analizado de 28, 51 y 477 días se observó un biofilm mixto, con predominio de células vivas y áreas de corrosión a lo largo de toda la superficie metálica de la lima por última vez, contrastando la superficie metálica de los grupos control. El SRB mostró actividad celular tanto en la superficie metálica de la lima como en la superficie dentinaria del conducto radicular en los niveles más profundos del conducto radicular, apical y medio, promoviendo la biocorrosión a lo largo de la superficie metálica de las limas endodónticas.
Palabras clave: *Desulfovibrio*; Microbiota; Bacterias reductoras de azufre; Endodoncia.

1. Introduction

Different techniques are used in the removal of fractured endodontic instruments in root canals, such as hemostatic forceps for apprehending the fragment, overtaking the fragment for subsequent traction with Kerr files, ultrasound and even the use of cyanoacrylate for adhesion of the fragment with another instrument. in an attempt to achieve success in removing the lime fragment (Gaffney, et al., 1981, Coutinho, et al., 1998, Nehme, 1999, De Oliveira, 2003, Suter, et al., 2005). However, no technique is completely safe, which can lead to perforations, false canals, destruction of the tooth root or a reduction in tooth root resistance (Bahcall, et al., 2005, Terauchi, et al., 2006). In order to assist in the removal of fractured instruments from the inside of the root canal, BACCOR was developed. A biopharmaceutical based on the action of Sulfate-Reducing Bacteria (SRB) with the power to promote biocorrosion in manual endodontic files (Heggendorn, et al., 2015).

Biocorrosion or microbiologically influenced corrosion (MIC) is a type of corrosion in which microorganisms actively participate in this process, initiating or accelerating the electrochemical reaction of metallic dissolution. A fundamental step in this process is the capacity of biofilm formation by microorganisms, where, through their metabolism, they induce and maintain the oxygen concentration gradient, accelerating the corrosion process through the reduction of oxygen and the release of their metabolic products (Videla, 2003, Larry & Hamilton, 2007).

In this context, it is essential to evaluate the *in vitro* action of BACCOR in the conditions closest to the clinical conditions to be applied, with endodontic files fractured in root canals. Therefore, the present study aimed To evaluate the biofilm formation of sulfate-reducing bacteria from two microbial species, *Desulfovibrio desulfuricans* (oral and environmental strain) and *Desulfovibrio fairfieldensis*, in root canals with fractured endodontic files *in vitro* and the biocorrosive changes that these strains are capable of promoting on the metal surface of endodontic files through the use of fluorescence microscopy and scanning electron microscopy techniques.

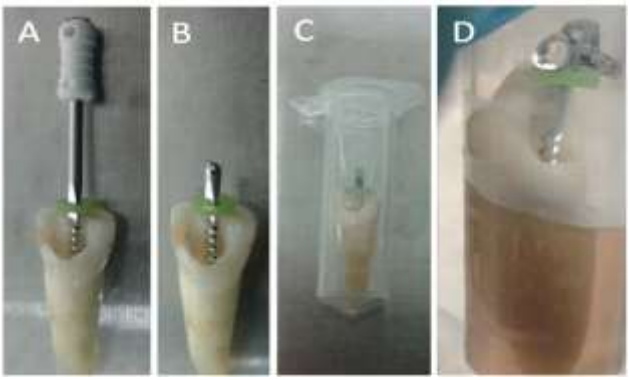
2. Methodology

The study was approved by the Research Ethics Committee of the Federal University of Rio de Janeiro through the consubstantiated opinion of CEP: CAAE 01500258000-09.

The teeth were removed from the 10% formaldehyde solution, washed and dipped in distilled water. After drying, the teeth were accessed in the coronary region and instrumented in the apical third (3 mm apical) (Step-back): Basic dilation to the

apical limit with a file that adjusted to the anatomical diameter of the apical region, followed successive dilations 1 mm below the apex, limited by the diameter of the root canal; Scheduling, with a programmed 1-millimetre indentation for the use of a file with a higher caliber than the file previously used, with only the apical third (3 mm) being prepared; Expansion of the middle third with a wide drill no. 3 and instrumentation with a Kerr file n.º 80 and check whether the expansion achieved in the previous step was sufficient to adopt a Kerr file type 90 or 100 in the middle third (K-File colorinox®; Dentsply Maillefer; REF A 012D 031 902 00; Lot: 1306001790). The Kerr type 90 endodontic files were then rotated clockwise with apical pressure in the middle third of the root canal. Once the endodontic file had been jammed in the root canal, the middle part of the endodontic file was sectioned. Subsequently, they were adapted in a 2 ml Eppendorf microtube, sealed and autoclaved for 15 minutes. After sterilization, a Postgate E culture medium was added to the microtube in the outer apical region of the root, plus Agar-agar, 15g / l., in order to fix the tooth inside the microtube after solidification of the medium and indicate bacterial confinement within the root canal, since, in case of contamination of external regions of the root, the culture medium will indicate this contamination through the change of colour and formation of iron sulfide (Figure 1).

Figure 1. Specimens: Interlocking of the file (A) with posterior section of the intermediate part of the endodontic file (B); adaptation of the tooth into the Eppendorf tube (C) and insertion of the Postgate E culture medium plus agar-agar in the external root region of the tooth (D).



Source: From the authors.

Previously the inoculation of BACCOR, the washing of innocuous bacteria was carried out, centrifuged at 10,000 g / 05 min. for the recovery of bacterial cells, discarding the supernatant. Subsequently, the precipitate from each sample was resuspended with Postgate E culture medium without Agar-agar. A 1.0 mL aliquot of each bacterial inoculum was removed to determine the cell concentration using the most likely number (MLN) technique and then follow the inoculation of 20 µl of each inoculum into the root canal in the respective previously prepared teeth and divided according to Table 1, incubated at 30 °C in an oven, for later observation in the SEM. After the incubation period of each group (Table 1), the formation of iron sulfide in the external region of the tooth root resulting from bacterial growth in the Postgate E culture medium was evaluated, plus Agar-agar, located in the external region of the radicular tooth portion.

Table 1. Distribution of the evaluation test in root canals of biocorrosion and biofilm formation in endodontic files

GROUP	Inoculated Strain	Culture Medium	Cultivation Time days	Analysis sequence - Microscopic technique
D1	Oral *Desulfovibrio desulfuricans*	Postgate medium without agar-agar	41	Biofilm Fixation MEV
D2	Environmental *Desulfovibrio desulfuricans*			
D3	*Desulfovibrio fairfieldensis*			
D4	Without bacterial inoculum			
D5	**Oral *Desulfovibrio desulfuricans***	**Postgate medium without agar-agar**	**28**	**MCVL Biofilm Fixation MEV**
D6 - Control 1	**Without inoculum**	**Without inoculum**		
D7 - Control 2	**Without inoculum**	**Postgate medium without agar-agar**		
D8	**Environmental *Desulfovibrio desulfuricans***	**Postgate medium without agar-agar**	**51**	
D9	***Desulfovibrio fairfieldensis***	**Postgate medium without agar-agar**		
D10 – Control 3	Without inoculum	Postgate medium without agar-agar	477	Chemical pickling ME V MCVL Chemical pickling MEV
D11- Control 4	Without inoculum	Without Culture Medium		
D12	Environmental *Desulfovibrio desulfuricans*	Postgate medium without agar-agar		
D13	Oral *Desulfovibrio desulfuricans*			
D14	*Desulfovibrio fairfieldensis*			
D15	New endodontic file, without specific treatment			

Source: From the authors.

2.1 Biofilm fixation

After the incubation period, the teeth with the inoculum, followed for the fixation of the biological material, the groups were kept for 4 hs in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) prepared with Milli- Q. After 4 h, they were removed from this solution and washed three times in 0.1M cacodylate buffer, each washing time being 15 minutes. Then they were dehydrated in an increasing series of 15 minutes each, in 30, 50, 70 and 100% alcohol. Finally, the essential drying point of the material was carried out in critical point equipment (Leica Microsystems; Leica EM CPDO30). The purpose of this procedure was to replace all residual biofilm liquid with liquid CO_2, which at 31 °C and 73 atm of pressure quickly convert to gaseous CO_2. After the drying time, the teeth were included in acrylic resin and sectioned longitudinally with a carborundum disc with the aid of a micro grinder (Black & Decker, RT 650) in order to induce a longitudinal fracture of the tooth without reaching the conduit root region, thus forming two longitudinal areas and releasing the endodontic file from the conduit region without affecting the structure of the biofilm. Subsequently, the teeth inoculated with the files were separated into groups according to Table 1.

In groups, D1, D2, D3 and D4, the root faces with root canal and endodontic files were metallized with subsequent sputtering coated in gold (15 nm thick) and examined for bacterial biofilm and surface changes using scanning electron microscopy (MEV-FEI-Inspect-S50) (Table 1).

To analyze the possible biofilm formed on the endodontic files and in the root canal, the laser scanning confocal microscope (MCVL) (ZEISS - LSM 710 / LSM 710 NLO and Confocul 3) was used in groups D5 to D9, and the images obtained were analyzed and processed with the software ZEN 2009 (ZEISS). The fluorophore used was the Live / Dead® kit as instructed by the manufacturer (FilmTracerTM Live / Dead® Biofilm Viability Kits, InvitrogenTM), a fluorophore capable of identifying living and dead cells in a mixed population, composed of SYTO® 9, which marks live and dead cells fluorescent green, and Propidium Iodide, which marks dead cells fluorescent red, penetrating only bacteria with damaged membrane, overlapping SYTO® 9. According to the manufacturer's instructions, the fluorophore was prepared following the immersion of each sample in the fluorophore, following an incubation period of 15 minutes in a dark environment and then observed in the MCVL. Subsequently, these groups continued to fix the biofilm and analyze it in the SEM (Zeiss - EVO LS10).

After 477 cultivation, the endodontic files of groups D10-D15 were observed, using the same protocol for observing cell viability in the MCVL with the fluorophore kit Live / Dead®. Subsequently, the endodontic files were subjected to the chemical pickling process to remove corrosion products and impurities present on the metallic surface, thus facilitating the observation of possible Pitts and/or corrosion areas. The specimens were immersed for 20 min. at 60 °C in 10% Nitric Acid solution (HNO_3), washed with 70 alcohol (hydrated ethyl alcohol 70° INPM) and acetone and dried with hot air. Then the groups were observed in the SEM.

3. Results

The cell concentration obtained by the MLN technique in the inoculants of the biocorrosive assay in the teeth in vitro is shown in Table 2.

Table 2. The cellular concentration of biocorrosive assay inoculants in in vitro teeth.

Sample	Cell concentration mln/ml
Environmental *D. Desulfuricans*	$3,5 \times 10^8$
Oral *D. Desulfuricans*	$1,1 \times 10^3$
D. fairfieldensis	$1,2 \times 10^{10}$

MLN / ml: Most likely number per ml. Source: From the authors.

After the 41-day incubation period, each group was assessed macroscopically with the interruption of the biocorrosion assay. Group D1, the tooth with inoculation of oral *Desulfovibrio desulfuricans*, showed darkening in the cementum near the apical foramen (Figure 2). However, after the critical point procedure of the material, this darkening of the apical region was no longer present. The other groups did not present any colour changes or other types of abnormalities in the coronary or radicular tooth portions.

Figure 2. Group with inoculation of oral *Desulfovibrio desulfuricans*, palatine (A) and vestibular (B) without changes and apical region (C, D) with alteration of color in cementum (red arrow). Tooth sectioned after preparation (E).

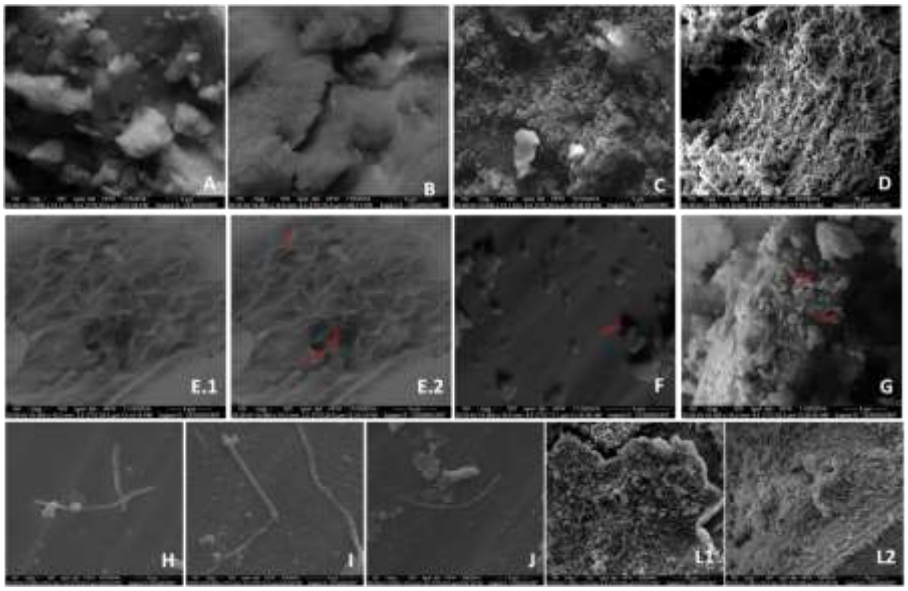
Source: From the authors.

3.1 Scanning electron microscopy

All images presented a smear layer of different intensities, making it challenging to identify biofilms or bacteria. However, group D1 revealed an area suggestive of biofilm on the surface of the endodontic file (Figure 3D), presenting a structure deposited on the different surface of the control sample, D4, only with smear-layer (Figures 3A, 3B and 3C). Group D3, on the other hand, suggested the presence of *Desulfovibrio desulfuricans* in the dentin of the sample. It was possible to verify a suggestive layer of exopolysaccharide (EPS) of the biofilm, mixed with smear-layer overlaid with bacteria in some images (Figures 3E.1, 3E.2), which may indicate that the smear-layer impaired, hiding, a large part of the bacteria. It was also possible to verify the suggestive presence of bacteria in other fields, with similar measurements between the different areas

(Figures 3F, 3G). However, such images suggested a deposit on the cells, which made visualization difficult. On the surface of the endodontic file in group D2, the images suggested the presence of EPS and bacteria in figures 3H, 3I and 3J, in addition to visualizing the rupture of the biofilm, demonstrating the metallic structure of the file below that point (Figures 3L.1 and 3L.2).

Figure 3. Scanning electron microscopy analysis. Control group, D4, with intense presence of smear layer (A, B) on the surface of the endodontic file and (C) in the root canal; group inoculated with oral *D. desulfuricans*, D1, with the presence of biofilm (D) on the surface of the endodontic file; group inoculated with *D. desulfuricans fairfieldensis*, D3, indicating the presence of bacteria on the surface of the endodontic file with 1,478 µm, 1,625 µm, 1,492 µm (E.1, E.2), 1,750 µm (F), 1,628 µm and 1,581 µm (G) and group D2, with environmental *D. desulfuricans* suggesting EPS and bacteria (H, I and J) and rupture of biofilm (L1 and L2) on the file surface.

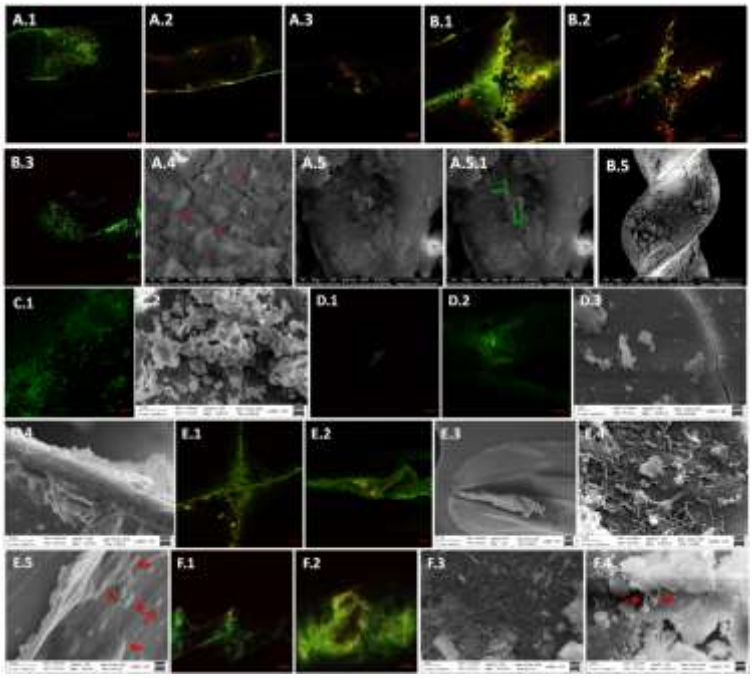


Source: From the authors.

3.2 Biofilm Analysis Association - MCVL and SEM

In group D5, with 28 days of biofilm formation, it was possible to visualize a mixed biofilm with dead and living cells along the entire root canal of the dentinal surface, being corroborated with the findings in the SEM, with traces of the bacterial presence on the dentinal surface. Such as the presence of slime layer in some areas and in the apical third in addition to the presence of SRB in other areas (Figure 4). On the other hand, in the endodontic file, the presence of a biofilm formed by dead and living cells throughout the instrument was demonstrated. In a smaller magnitude in the SEM, it was possible to verify that the biofilm deposits on the surface of the file were concentrated between the cutting edges (Figure 4B.5), very similar to the images of the biofilm in the MCVL (Figures 4B.1 and 4B.2). This corroborates with the previous findings that the biofilm would be mixed with the smear layer, since the highest concentration of smear layer is always found between the cutting edges of the instrument.

Figure 4. Analysis of Biofilms of 28 and 51 days - Correlation between laser confocal microscopy and SEM. Dentinic face (A) and endodontic file (B). Analysis of the MCVL of Group D5: the middle third of the root canal, with the presence of active biofilm (A.1) and a mixture of dead and living cells in another area of the middle third (A.2) and apical third (A.3). In the endodontic file, in the active part next to the intermediate section (B1), region close to the center of the active part (B.2) and section of the penetration guide of the endodontic file (B.3) demonstrating biofilm formed by dead and living cells . In SEM, presence of nanowire (A.4) (red arrow) and in the apical third of the dentin surface, presence of bacteria (A.5 and A.5.1) followed by the distribution of the biofilm with smear layer association (B. 5). Control samples, Group D6 (C.1 and C.2) and D7 (D.1, D.2, D.3 and D.4). The surface of the endodontic file showing laser shine due to the presence of the smear layer (C and D.1) and dentin face, with smear layer shine when the laser is focused (D.2). Analyze in SEM with a large presence of smear layer on the dentin surface (C.2 and D.3) and the endodontic file (D.4). Groups D8 (E.1 and E.2) and group D9 (F.1 and F.2) with biofilm on the surface of the endodontic file (E.1 and F.1) and in the root canal in the apical thirds of the tooth (E .2 and F.2). SEM analysis revealed a compact smear layer structure (E.3) observed in the MCVL with the presence of active biofilm (E.2). Presence of bacteria on the entire surface of the root canal (E.4 and F.3) and on the surface of the endodontic file (red arrows) (E.5 and F.4).



Source: From the authors.

The control groups showed no difference between their treatments, with the presence of only culture medium, Group D7, and without any type of inoculum, Group D6. However, in both control groups, a natural smear layer fluorescence was observed when the laser was applied, both on the dentin surface, root canal, and on the endodontic file, between 488 and 529 nm (Excitation Length and Emission Length) of λ of wave, which covers the same emission / excitation frequency of the SYTO® 9 fluorophore, but with a lower fluorescence intensity when compared to samples with active biofilm. The SEM analysis showed a strong presence of amorphous crystals, compatible with smear layer on the dentin surface (Figures 4.C.2 and D.3) and in the endodontic file (Figure 4.D.4).

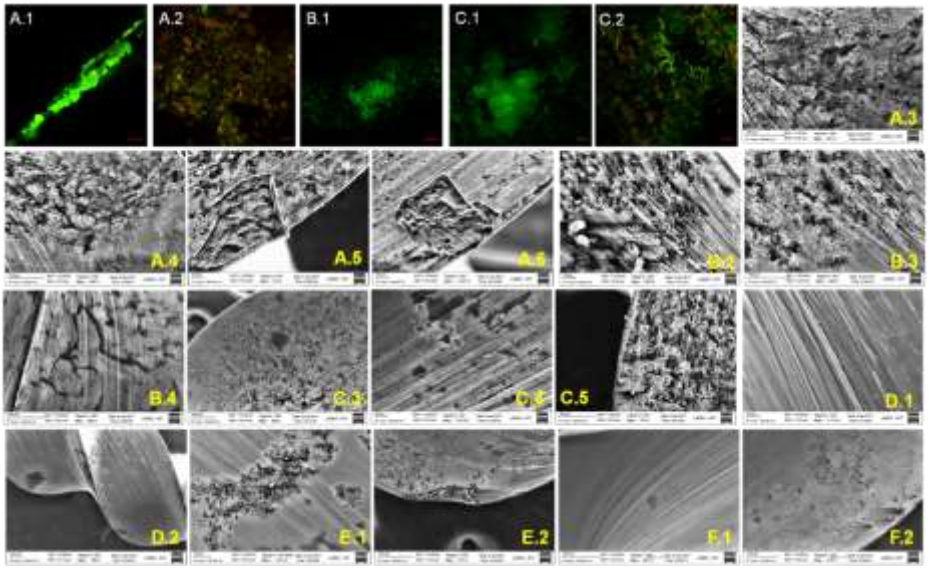
In groups, D8 and D9, with a biofilm with 51 days of formation, a biofilm composed of dead and living cells was also observed (Figure 4). Group D8 presented mixed biofilm, with a predominance of live cells both in the root canal and on the surface of the endodontic file (Figures 4.E.1 and 4.E.2). It was also possible to compare a smear layer agglomerate in the apical

portion of the root with a mixed biofilm in its center with the SEM analysis, the same structure in greater magnitude (Figure 4.E.3) showing a SRB biofilm in smaller magnitude (Figure 4.E.4). In the endodontic file (Figure 4.E.5), the SEM image suggested a structure covering the cells on the metallic surface. The same situation was repeated in the analysis of the D9 group, with a mixed biofilm in the MCVL presenting a structured biofilm in the SEM.

3.3 Association of Biofilm Analysis with Corrosion Areas - MCVL and SEM

After 477 days, it was possible to verify active biofilms with a predominance of live cells in groups D12, D13 and D14. The subsequent analysis in the SEM showed corrosion along the metallic surface of the Group D12, D13 and D14 files of different magnitudes, shapes and location of corrosive attack. It was possible to verify the group D12 file (Figures 5.A.5 and A.6) the area of corrosion associated with a pullout area. It was also possible to verify regions of corrosion well delimited with areas of the integrity of the metallic surface as in Figure 5.A.4 and B.2, where there is clearly a border, making it possible to match these areas with biofilm in the previous stage.

Figure 5. Analysis of Biofilms and corrosion areas in endodontic files - Correlation between laser confocal microscopy and SEM. MCVL analysis: Group 12, with an inoculum of environmental *D. desulfuricans* (A.1 and A.2) showing an active biofilm on the cut edge of the endodontic file and a mixed biofilm in the region between the cutting edges; Group D13, with inoculum of *D. Desulfuricans* Oral, (B.1) and Group D14, with an inoculum of *D. fairfieldensis*, (C.1 and C.2) demonstrating active biofilms with the predominance of live cells. Analysis in SEM: Group 12 (A.3, A.4, A.5 and A.6), 13 (B.2, B.3 and B.4), 14 (C.3, C.4 and C .5) with a strong presence of corrosion along the metal surface, it is also possible to check pullout areas associated with corrosion on the cutting edge of the file (A.5 and A.6); Group 15, control sample, with a smooth surface with only machining grooves (D.1) and with defects arising from machining in addition to the presence of particles and dirt from the file manufacture (D.2). Group D11, control, sample only placed on the tooth (E.1 and E.2) with the presence of corrosion in some areas similar to the results of Group D10 (F.1, and F.2).



Source: From the authors.

Control samples Group D10 and D11 showed areas suggestive of corrosion (Figures 5.E.1, 5.E.2 and 5.F.2), with less intensity and extent of attack when compared to comparing groups with bacterial inoculation (Group D12, D13 and D14), and whole areas without corrosive attack (Figure 5.F.1) similar to the group of new files (Group D15), without the period of exposure in the root canal.

4. Discussion

The process of bacterial adhesion and biofilm formation and its morphology can vary depending on the species, the surface composition, and environmental factors (Clark, et al., 2007, Trinidad, et al., 2010). Therefore, it the important of trying to analyze the closest possible conditions to BACCOR *in vitro*. Regarding the difference in surface composition, SRB were able to form biofilm both on the metallic surface and on the inner surface of the root canal. The data suggested an association of smear layer with the formation of SRB biofilm, leading to the belief that the first can support bacterial adhesion, both on the dentin surface and the metal surface of the endodontic file. Regarding time, SRB remained viable at all times used for 28, 41, 51 and 477 days, in addition to presenting bacterial adhesion on the metal surface of the endodontic file in the shortest incubation time, 28 days.

In addition to proving the formation of MIC by SRB, this study demonstrates that SRB remains viable within the root canal for a long period. Previously, the presence of SRB in the oral cavity, on dentinal surfaces, in saliva had already been identified (Heggendorn, et al., 2013 and 2014). Therefore, in addition to making it possible to prove SRB biocorrosion in endodontic files, such findings open the door to a possible hypothesis of SRB infection in the root canal in cases of communication between the root canal and the oral cavity in patients who have such bacteria in their microflora. oral. Not far away, Jorand et al. (2014) demonstrated biofilm of *desulfovibrio fairfieldenses* in titanium coupons used in dental implants.

Trinidad et al. (2010) reported that the conditioning for observation in SEM, such as fixation, dehydration, and conductive coating, can distort the sample, collapsing the EPS matrices and remaining only proteinaceous structures. This fact may answer the difference found between some groups regarding the morphological difference of biofilm and bacterial cells and the number of cells found. In group D8, it was possible to verify a structure deposited on the SRB on the metallic surface of the file (Figure 4.E.5) different from the fields found in the same group on the face of the tooth root canal (Figure 4.E.4), which can be related to sample preparation.

Clark et al. (2007) detected in the SEM the presence of numerous filaments that linked the SRB to each other in the biofilm, similar to those found in our samples (Figures 3 and 4). The authors argued that such protein filaments are essential structures in the maturation of the SRB biofilm, indicating that *Desulfovibrio Vulgaris* is based more on protein filaments than on the exopolysaccharide matrix for the fixation and initial formation of the biofilm (Clark, et al., 2007). Later, Remoundaki et al. (2008) stated that solid structures in the form of rods could be bacterial cells encapsulated by zinc and iron sulfides. The authors described through the SEM-EDX a SRB encapsulation, shaped like a 10μm rod, by a cloudy fog corresponding to the deposition of metallic sulfides on the surface of the cell wall and or the area adjacent to the bacterial cell (Remoundaki et al., 2008). Based on the findings of this author, during the analysis of the images obtained in the SEM, morphological differences were identified between the SRB cells, between different samples and in the same sample, with different sizes being identified between the cells (unpublished data).

The process of biocorrosion of iron by SRB must be seen as a phenomenon of rupture of the passive film of steel by the corrosive metabolites that SRB release in the environment. This process can be intensified by the presence of chloride ions, associated with biogenic sulfides, released by SRB, causing synergy and an increase in the speed of attack on the metal (Videla, 2003, Larry & Hamilton 2007). The corrosion caused by SRB is predominantly located, predominantly by pirates, with corrosion products with little adherence, dark coloring, and a characteristic odor of hydrogen sulfide (Videla, 2003, Lopes, et al., 2006). This biocorrosive process would be desirable in endodontic files fractures within the root canals since they would facilitate the detachment of the endodontic file from the root canal walls. Therefore, the proof of cell viability of SRB in all inoculated SRB groups, both on the metal surface of the file, suggesting the possibility of MIC, as well as on the root canal wall, in the apical region and the middle third of the root canal, indicates that the SRB would not be immobilized only in the access area of the inoculum, but managed to pass through the fractured endodontic file barrier, reaching apical regions of the

root canal as well as the active tip of the fractured endodontic file in the root canal.

In previous analyzes, the biocorrosion power of BACCOR was proven, with strains of *D. desulfuricas* and *D. fairfieldensis*, in Kerr-type endodontic files embedded in cross-sectioned acrylic resin with 28 days of incubation. The pattern of corrosion in the work of Heggendorn, et al. (2015) was similar to those presented in this work: in areas of the interface (edge) between files and embedded resin (Heggendorn, et al., 2015) that would correspond to the edge areas of cutting endodontic files embedded in teeth. It was also noticed in areas of pre-existing structural defects where the biocorrosive process was able to act (Heggendorn, et al., 2015), sealing the cutting edges with pullout where the area of corrosion was identified in group D12.

Control samples D10 and D11 suggested areas of abiotic corrosion without the presence of SRB. Such a phenomenon arises from the exposure of the culture medium in the endodontic file (Group D10) in the root canal; the culture medium alone would promote abiotic corrosion. The group D11, without any treatment, only the exposure of the endodontic file in the root canal was able to encourage corrosion in some areas, such fact can be related to residual moisture in the dentine, previously treated with sodium hypochlorite and even by the simple presence of oxygen, even if in reduced concentration. However, when comparing the control groups D10 and D11 with the groups inoculated with SRB (D12, D13 and D14), the biocorrosion provided by SRB were able to act to a great extent, suggesting a more aggressive attack on the metallic surface when compared to abiotic corrosion in the control samples D10 and D11, were part of the surface was shown to be integral.

Trinidad et al. (2010) recommend a combination of several techniques when investigating biofilms. The increase in knowledge about biofilms is based on the scanning electron microscope and the scanning laser confocal microscopy, which complement each other in the knowledge of the biofilm ultrastructure and the cell viability in the development of biofilm (Trinidad, et al., 2010). In fact, the application of these two methodologies led to analyzes that, if applied in isolation, the conclusions presented in this work would not be reached. MCVL and SEM made it possible to check and analyze the cellular activity of the SRB and the biofilm structure, respectively, for periods of up to 28, 41, 58 and 477 days of cultivation throughout the entire root canal with the fractured endodontic file in its interior.

5. Conclusion

The SRB showed cellular activity both on the metal surface of the file and on the dentin surface of the root canal at the deepest levels of the root canal, apical and medium, promoting biocorrosion along the metal surface of endodontic files. This corrosion was shown to be diffuse along the surface of the endodontic file, also showing areas of corrosion on the cutting edges of the instrument, which would be what is desired to facilitate the detachment of the instrument when fractured inside the root canal. In parallel, the SRB were able to form biofilm on the dentinal surface of the root canals, with no changes observed on these surfaces.

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Acknowledgments

This work was supported by the Coordination for the Improvement of Higher Education (CAPES), Research Support Foundation of the State of Rio de Janeiro (FAPERJ), National Council for Scientific and Technological Development (CNPq).

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