Original Research Article

Formation of a mature biofilm of Enterococcus faecalis in root canal and its treatment using gold nanorods

ABSTRACT

Aim: The aim of this study was to develop a mature biofilm of *Enterococcus faecalis* in roots using two methods: a Modified Drip Flow Reactor (MDFR) and with Static Method. After this, we eliminated the formed biofilm with a gold nanorods solution and a gold nanorods hydrogel applying photothermal therapy. **Methodology:** Different modifications were done to a commercial Drip Flow Reactor (DFR) to develop a mature biofilm of 5 and 10 days. The biofilm formed with the static method was for a time of 24 and 48 hours. The formed biofilm was observed using scanning electron microscopy (SEM). For the disinfection process, a gold nanorods (AuNR´s) solution and nanorods hydrogel (Ch-AuNR´s) plus photothermal therapy (PPTT) was tested. **Results**: At 10 days of inoculation a mature biofilm of *E. faecalis* was observed with total coverage of the examined surfaces. CFU were counted after the disinfection and a statistically significant difference ($p>0.05$) was found in the groups compared with the control group. **Conclusion:** The proposed MDFR and a static method both can produce a mature biofilm in the root canal. Ch-AuNR´s and the solution with PPTT might be useful for root canal treatment as a coadjutant in treatment; however, it requires more research for it to be used in endodontic therapy.

Key Words

Biofilm, Endodontics, *Enterococcus faecalis*, Root canal, Lasers, Photothermal Therapy, Nanoparticles.

1. INTRODUCTION

One of the challenges and main goal of endodontic treatment is the management for elimination of microorganisms, which are the cause of apical inflammatory lesions. Bacterial elimination is achieved by mechanical and chemical action; mechanics refers to the use of instruments within the canal such as files for disorganization of the biofilm and chemical through the antibacterial action of irrigating solutions. Most endodontic studies are based on bacterial elimination since, for the success of a root canal treatment; the bacteria must be eliminated in the highest possible percentage[1]. Microorganisms create a biofilm which is defined as an organized structure consisting of bacterial cells attached in an exopolymeric matrix on a surface, biofilm is characterized by the immobilization on a surface, cell-cell interactions, formation of microcolonies and excretion of extracellular polymers (EPS). The biofilm is currently described as a bacterial community immersed in a liquid medium, characterized by one or more bacteria that are attached to a substrate or surface and embedded in an extracellular matrix produced by them, and shows an altered phenotype in the degree of cell proliferation or the expression of their genes[2]. This matrix makes it difficult for disinfectant agents to penetrate the biofilm, limiting its effectiveness to the surface layer. The bacteria in the biofilm are up to 1000 times more resistant than the corresponding bacteria in planktonic form[3, 4, 5].

E. faecalis is one the main microorganisms responsible for the periapical disease and the failure of endodontic treatment. *E. faecalis* is able to adhere and form biofilm in dentin and invade dentinal tubules, it resists different environmental conditions. Even though mechanical preparation removes tissue, biofilm, and infected dentin, because of the complexity of the anatomy of the root canal system many sections of the canal may remain without instrumentation and the irrigants as well are incapable of penetrating entirely to all the root canal system[6, 7].

Torabinejad et al. (2005) presented a systematic review about the outcomes for success and failure of endodontic therapy, the conclusion was that 92 to 98% of teeth without periapical lesions remain free of disease after root canal therapy,

74 to 86% of teeth with apical lesions completely heal after initial treatment or retreatment, but there is still a probability of failure because of periapical disease of 14% - 26%. In addition, similar data shows that 91 to 97% of teeth that have had root canal treatment remain functional over time[8, 9]. Treatment failures are mainly caused by persistent infection, microorganisms participating in the primary infection managed to survive the intracanal antimicrobial procedures and remained inside the root canal system, for this reason the aim for various studies is the research for new materials including irrigants and intracanal dressings for the elimination of microorganisms for new protocols for disinfection.

Sodium hypochlorite (NaOCl) is considered the gold standard in terms of bacterial elimination. This is due to the mechanism of action of this solution, which can promote cellular biosynthetic changes, alteration in cellular metabolism and destruction of phospholipids. With a 2.5% concentration it can eliminate 100% of *E. faecalis* in 5 minutes[10, 11]. Sodium hypochlorite is a very caustic and nonspecific agent, it is cytotoxic to all cells; consequently, recent studies attempt to evaluate the efficacy of new root canal irrigants or coadjutants against biofilms, but the ideal has not yet been found since intra and extra-root biofilms are very resistant. *E. faecalis* has many virulence factors such as lytic enzymes, cytolysin, aggregating substances, pheromones, and lipoteichoic acid, which contribute to its survival in the hostile environment of the root canal. In addition, the ability of this bacterium to form biofilms provides it with an ecological advantage of greater resistance to antibacterial activity[11, 12, 13].

Nanomaterials, especially metal nanoparticles (NPs), are emerging materials that have demonstrated bactericidal and biofilm-destroying activities, based on the release of metal ions. Gold nanorods (AuNRs) are ideal for biomedical use in vivo because of their penetration into tissues[14-16]. Biocompatibility applications of NPs with hydrogels include tissue engineering, drug release control, and medical and biological sensors. Sámano-Valencia et al., Perez-Diaz et al. and Bermúdez-Jiménez et al. have reported a characterization and production of chitosan hydrogels together with AgNPs and AuNPs concluding

that these hydrogels have had positive results, demonstrating antibacterial activity against biofilm[18-20].

Positive antimicrobial and antibiotic results have been observed with the addition of nanoparticles in different dental materials. This has encouraged new technologies such as the use of nanoparticles for disinfection of root canals during endodontic therapy, being used as intracanal medicament or as irrigants. Other methods are the use of laser (Photo Thermal Therapy, PPTT) together with nanoparticles, the use of laser and sodium hypochlorite, or the use of individual laser in the root canal, PIPS therapy[21-22].

Gold nanoparticles can be manufactured in various forms, such as nanospheres, nanorods, nanocubes, nanoboxes, and nanostars. In biomedical applications, the most widely used AuNPs are nanospheres and nanorods. Recently, AuNP have been used together with diode lasers to evaluate the antibacterial activity and it has been concluded in studies that laser with AuNPs kills 80% of bacteria compared to laser or AuNP individually, which have shown a reduction of 70%[22, 23].

R. C. D. Swimberghe T., Coenye, R. J. G., De Moor ; published a systematic review to present an overview of laboratory root canal biofilm model systems described in the literature, since there is a variety of systems for laboratory biofilm formation in vitro that have been developed but there is substantial variation in the methodology like in the experimental parameters, and as such, it is difficult to compare results between different studies. None of the reported laboratory endodontic biofilm models has ever been validated. This demonstrates the need for a more standardized approach and a validated endodontic biofilm model. They identified 77 articles that fitted the inclusion criteria. The authors mention various factors for to consider when creating a biofilm[24-29].

For root canal biofilm both models have been used. In contrast to a static biofilm model, a continuous flow over the biofilm induces shear forces that cause additional stress to the biofilm. These forces result in biofilms that are more rigid and homogenous. However, inside the root canal, no such flow conditions are

present. None of the models has ever been validated. This demonstrates the need for a more standardized approach and a validated endodontic biofilm model. In the absence of such a validated model, it seems wise to select experimental parameters that reflect the in vivo situation as much as possible[2, 24, 25].

In this study two models for making biofilm of *E. faecalis* were used, a Modified Drip Flow Reactor (MDFR) and a static model. Since the development and validation of reproducible and clinical laboratory models for the study of biofilms are not stable yet, this study aimed to develop an *Enterococcus faecalis* biofilm on human roots using a static and a dynamic method (Modified Drip Flow Reactor (MDFR)); as well to use photothermal therapy with a gold nanorods solution and a hydrogel to eliminate the formed biofilm.

2. MATERIAL AND METHODS

2.1 Roots preparation

Human anterior teeth with straight and single roots were selected; they were recollected from extractions of patients with periodontal disease or extractions for orthodontic reasons.

2.1.1 Root preparation for biofilm formation with the DMFR

The roots were disinfected with 5.25% sodium hypochlorite for 2 minutes; they were standardized to 11 mm with low-speed diamond discs. Root canals were instrumented using NaOCl 5.25% in between files. (Dentsply, Switzerland). For the elimination of the smear layer, they were taken to an ultrasonic bath with 17% EDTA for 4 minutes, then with 2.5% sodium hypochlorite for another 4 minutes, finally they were rinse three times with distilled water. They were sterilized at 121 °C for 20 minutes on sterilization bags.

2.1.2 Root preparation for biofilm formation with Static method

The roots were disinfected with 5.25% sodium hypochlorite for 24 hours; all roots were standardized at 14 mm and the crown was sectioned off using a

diamond disc. Patency of apical foramina was established with size #10 and # 15 k files (Dentsply, Switzerland). Root canals were instrumented with ProTaper Next files (X1, X2, X3), irrigating with NaOCl 5.25% in between files. After the roots were prepared for the bacterial colonization, they were vertically sectioned along the mid-sagittal plane into 2 halves. The smear layer was removed by an ultrasonic bath with 17% EDTA for 4 minutes, then with 2.5% sodium hypochlorite for another 4 minutes, they were rinsed three times with distilled water and sterilized at 121 °C for 20 minutes on sterilization bags.

2.2 Bacterial strain and culture conditions

Enterococcus faecalis (ATCC 29212) was grown in Trypticase Soy Broth (TSB, BD DIFCO, Sparks MD, USA) at 37 °C, cultures were incubated overnight in aerobic conditions. The final inoculum was prepared to reach a final concentration of $1.5x10^8$ cells/mL adjusted to 0.5 of the McFarland scale. This step was repeated for the experimental phase to replace the inoculum in the continuous flow system every 24 h for 5 and 10 days. Gram staining was employed every 24 h to verify the culture purity.

2.2.1 *Enterococcus faecalis* biofilm formation in root canal by Modified Drip Flow Reactor

The commercial reactor DRIP FLOW (DFR 110, BioSurface Technologies Corporation, EEUU) forms a biofilm in glass slides. In this study it was modified for it to form biofilm in dentin surfaces like dental roots. Our modified and laboratory manufactured design of the Drip Flow Reactor (MDFR) has six chambers for the placement of each root individually, where the *E. faecalis* biofilm can be formed in the root canal. The roots were placed one in each chamber and a 40-gauge needle was placed inside the main feeding input of the MDFR, 2 mm from the main access of the root canal of each root and anchored in each reactor chamber (Figure 1a). Following, the MDFR with the roots already in place were sterilized in an autoclave at 121°C for 20 minutes. The MDFR (Figure 1b) was mounted inside a sterile chamber and in the inferior portion; a magnetic stirrer was placed at 37 °C. Over the magnetic stir plate, a

sterile plastic recipient with a fresh nutrition media for *E. faecalis* was placed, this growth media had the characteristics mentioned in section 2.2. This culture media was connected by a tube, to a plastic chamber, which contained a regulation drip and a purge system. From this plastic chamber, there were six hoses with independent drip regulators. These hoses were connected to the MDFR of each chamber to the main feeding input that was connected to the root canal (Figure 1c). After 5 and 10 days, biofilm was completed, and the roots were washed with sterile saline solution to eliminate not adhered bacteria on roots. This group of roots was selected for the elimination of biofilm with a nanorods solution and diode laser.

2.2.2. *Enterococcus faecalis* biofilm by Static Method

After the roots were sterilized, we randomly selected the roots for them to be placed in groups. Each group was made in triplicate. The sterilized roots were transferred to previously sterilized Petri dishes; we added a wet bed of paper, to maintain humidity in the incubation oven. Each root was inoculated with 10 μL adjusted to 0.5 McFarland scale. The plates with the specimens were incubated at 37 °C for 24 hours, for a 24 h biofilm development. After incubation the roots were rinsed with sterile water to remove the culture medium and nonadherent bacteria, they were placed in new sterile Petri dishes. This group of roots was selected for the elimination of biofilm with a nanorods gel and diode laser (Figure 2.)

3. SEM observation of *Enterococcus faecalis* biofilm

3.1 Preparation of the samples for SEM Observation

After the biofilm was formed in the teeth, the roots were prepared by fixing and placing the samples in 2% glutaraldehyde (SIGMA-ALDRICH) for 1 hour, then placed in refrigeration for 24 hours. After 24 hours, dehydration by alcohol solutions (20%, 40%, 60%, 80%, 90%, and 95%) was carried out for 10 minutes each to be finally stored in absolute alcohol (JALMEK) until it was taken to critical point drying, gold plating and finally the reading to the SEM (JEOL JSM-6510 scanning electron microscope) at different magnifications.

4. Synthesis of AuNR´s

The seed solution was composed of 2.5 mL of $HAuCl_4$ (5×10^{-4} M) mixed with 2.5 mL of 0.2 M cetyltrimethylammonium bromide (CTAB). To this mixture, 0.60 mL of an ice-cold solution of $NaBH_4$ (0.010 M) was added and was left under magnetic stirring for 1 hr., resulting in a brown solution. Meanwhile, another AuNR growth solution was prepared. 5 mL of CTAB (0.20 M) was added to a precipitation glass under a magnetic stirring base and minimum heat, then 0.725 mL of $AgNO_3$ (0.0040 M) also 5 mL of $HAuCl_4$ (1×10^{-3} M). After gently mixing, 130 µL of ascorbic acid (0.0788 M) was added as a reducing agent, which changed the color of the solution from a dark yellow to a colorless solution. As a final step for the formation of the AuNR´s, 12 µL of the first seed solution was added to the second growth solution between 27° and 30°. The solution gradually changed to a pink-violet color in 15–30 min. The solution was taken to the spectrophotometer, and it should be around 520nm to 540nm in the first curve and 790nm to 830 nm in the second with an absorbance of 1 to 1.2. After checking these parameters, the solution was centrifuged at 12,000 rpm for 30 min to remove the unreacted reagents. The solution of nanorods was named AuNR´s and the hydrogel was named Ch-AuNR´s.

4.1 Characterization of AuNRs

The VIS-NIR absorption spectrum was obtained using a CHEMUSB4- VIS-NIR spectrophotometer (Ocean Optics, FL). The solution was taken to the spectrophotometer for it to be tested to be around 520 nm to 540 nm in the first plasmon and 790 nm to 830 nm in the second with an absorbance of 1 to 1.2.

4.2 Preparation of hydrogels

The preparation of the hydrogels was based on the methods reported elsewhere[18, 19, 20] with some modifications for the gel to be usable on root canals. The hydrogel was formed with chitosan and the nanorods gel was prepared by mixing 0.8 ml of acetic acid in 19.2 ml of the gold nanorods dispersion; Then, 0.175 g of chitosan and was added and mixed by magnetic stirring until the hydrogel was unified.

5. Elimination of *E. faecalis* Biofilm

5.1. Elimination of *E. faecalis* Biofilm with Gold Nanorods Solution and Photothermal Therapy

After biofilm formation with the DMFR, three groups were analyzed: group 1 (positive control): biofilm without treatment; group 2 (NaOCl 5.25%): we placed 3 mL of the irrigant solution, 2 mm before the root apex; group 3 (gold nanorods solution plus photothermal therapy, AuNRs PPTT): we placed 3 mL of gold nanorods dispersion at 128 μg/mL and they were irradiated with 810 nm NIR laser for 20 minutes to compare them with the positive control using colony-forming-units (CFU). The microbial count technique by serial dilution consisted in diluting the sample in dilution factors to ten, then seed 0.1 mL of the dilutions in an agar plaque, spread the inoculum in a homogeneous form all over the surface of the plaque with a sterile glass dipstick using L seeding technique, subsequently, the plaques were placed in an incubator at 37 ºC for 24 h. After the disinfection, teeth were placed in a nutrient broth for 24 hours at 37 ºC in a 10 mL tube for each group and “vortex” vibration was applied in each group. Each blood agar plaque from the different groups was labeled with the treatment code, the dilution factor and the date. Once the incubation period was over, the plaques were taken from the incubator, and random plaques were selected to perform the count and a blinded assessment was done, each plaque was assigned a random number by a researcher outside the investigation. The identification label was covered, and the information was cast in a datasheet. Once this step was over, the plaques count was done and the CFU number per plaque was calculated. Subsequently, the conversion of the number of colonies per ml was made, using the following formula: (# of colonies in the plaque X dilution factor) / 0.1 mL = # of colonies per ml.

5.2 Elimination of *E. faecalis* Biofilm with Gold Nanorods Gel and Photothermal Therapy

After biofilm formation with the static method was done, roots were randomly selected and were distributed in groups. Each group was analyzed in triplicate. Group 1: positive control, Sterile water; Group 2: negative control, NaClO

5.25%; Group 3: Ch:AuNR´s Hydrogel with photothetermal therapy. In group 3, which is the study group, the Ch:AuNR´s gel was placed in each root and the diode laser at 800nm/200mW was directed at the root canal in 30 seconds intervals for 2 minutes, they were rinsed with sterile water. A sterile paper point was placed in the canal for 30 seconds and then placed in microtubes with physiological solution (phosphates and nutrition medium). All tubes were incubated at 37 °C for 24 hours. After this time the microbial count technique by serial dilution was done, consisting in diluting the sample in dilution factors to ten, then seed 100 µL of the dilutions in an agar plaque, spread the inoculum in a homogeneous form all over the surface of the plaque with a sterile glass dipstick using L seeding technique, subsequently, the plaques were placed at 37 ºC for 24 h. Colony-forming units were the method used to evaluate the elimination of microorganisms in each group, using the equation: CFU = No. CFU x dilution factor / 0.1ml to be able to apply a LOG10 to have a uniform result between the groups.

6. RESULTS

6.1 SEM Observation after 5 days of inoculation with MDFR

In the negative control a clean dentin can be observed, obliterated dentinal tubules, and no traces of residues or microorganisms (Figures 3a and 3b), demonstrating a good cleaning process of the extracted pieces. After 5 days of inoculation, total coverage of biofilm was demonstrated over the examined surfaces in cervical third, medium third, and apical third on each tooth; no morphological differences were noticed among the 5 teeth inoculated in the DMFR. A polymeric extracellular matrix and *E. faecalis* aggregates were observed on the higher magnification, resulting in a tridimensional heterogeneity structure, microcolonies with communication canals, and structures with a seta shape corresponding to a mature biofilm with cells locked in a polymeric extracellular matrix (Figures 3c and 3d).

7.2 SEM Observation after 10 days of inoculation WITH MDFR

SEM images of root canals after 10 days of inoculation show a thicker biofilm and also show the presence of isolated units of *E. faecalis*, thus demonstrating that the use of the MDFR promotes a possible second colonization in root canals (Figures 3e and 3f).

7.3 SEM Observation after 24 and 48 hours of inoculation with static method

The SEM observation was done with a magnification of 1000X and in 2000x, in the mid and in the apical third of the canal. In the samples where the inoculation was for 24 hours, it was found several isolated colonies of *E. faecalis*, is found as a primary biofilm. In the 48 hours inoculation, we observed as well a primary biofilm but we observed more colonies than that obtained in 24 hours; it was observed *E. faecalis* in the dentinal tubes. In all the samples dentinal debris was observed.

7.4 Treatment *E. faecalis* biofilm with gold nanorods solution with photothermal therapy,

The colony-forming units (CFU) were counted, prior recognition of the strain by Gram staining. A difference was found when compared group 2 (191 ± 18.2 CFU) and group 3 (188.6 ± 26.7) with the control group (337.3 ± 2.82 CFU) but no difference was found when groups 2 and 3 were compared among them.

7.5 Treatment of *E. faecalis* biofilm with gold nanorods gel with photothermal therapy

The results in Group 1 (Ch-AUNR´s) were obtained by performing serial dilutions of each specimen. The count of the UFC´s was done for each agar plate for the correspondent dilution. Group 2 was an inhibition control group using NaClO 5.25% for the specimens. Group 3 was the growing control group; sterile water was used for these samples.

7. DISCUSSION

Nowadays most endodontic studies recreate biofilm to search for new novel therapies to help eliminate *E. faecalis* and biofilms. The modification done to the MDFR was done, to be able to form biofilm on dentin. Tolker-Nielsen and Molin noticed that each microbial biofilm community is unique, but some structural attributes can be considered universal as is the case of this study which by structural features was accomplished to obtain a mature biofilm, it was found embedded bacteria on an extracellular polymeric matrix and an adherence to a dentinal surface on a complex tridimensional structure. Many factors can influence biofilm formation, from the nutrients in the medium composition, the variations of the pH values from the medium, temperature, environment, fluid conditions, etc.. Each of these factors were under our control when the biofilm was formed, this way it was assured a correct form for developing biofilm with the MDFR as well with the static method. Bacteria can form biofilm preferably in high shearing environments (for example fast fluid environments); a study on *E. faecalis* biofilm under a fluid system inducing stress over the bacteria concluded a formation of a more resistant biofilm, which is why it was decided to place a magnetic agitator inside the biofilm-forming system (MDFR). All the system models presented have been demonstrated to provide useful information in the biofilm process. Thus, from all the factors previously mentioned, adjustments must be done to get them closer to the conditions that occur in vivo to obtain better results[26].

In this study, a 5 and 10-day period of inoculation biofilm was created with the MDFR, it was observed on the SEM the establishment of a mature biofilm over all dentinal surfaces, with a highly organized structure, compared to what was reported in a third phase Biofilm or a final phase development. It is defined that once the Biofilm has reached maturity, some cells are released from the exopolysaccharides matrix so they will be able to colonize new surfaces, finishing this way with the biofilm formation process and its development[30].

Dynamic models as the Drip Flow Reactor offer the advantage of more closely replicating certain in vivo conditions, their disadvantages frequently come in the form of higher costs and difficulty of use, it is important to have in mind that when the entire tooth is exposed to the inoculum, biofilm growths on the external tooth surfaces, which can cause bias in the studies[25]. With the static method this factor can be manipulated, in the static biofilm model for this study

the roots were split longitudinally into halves for this way to apply the *E. faecalis* inoculum only in the root canal and for extracanal biofilm not to be formed, all the other superficies of the root were not contaminated expecting biofilm to not be formed. This model of static biofilm model is probably more alike to a biofilm in vivo because in contrast to a continuous flow system it induces shear forces that cause additional stress to the biofilm. These forces result in biofilms that are more rigid and homogenous as already said, however, inside the root canal, no such flow conditions are present[24, 25].

With both methods the biofilm was formed; with the DMFR the biofilm was a mature biofilm, it was formed for 5 and 10 days in contrast with the static method that was 24 hours and 48 hours biofilm, that a primary biofilm was formed, but if more days had passed, we would have found a more mature biofilm as well. It is reported that mature biofilm can also be formed in static method[26].

Biofilm was formed in this study with the MDFR and with the static method also it was proposed to prove the effect of elimination of a Ch-AuNR´s hydrogel and a solution applying photothermal therapy with a diode laser (810nm). It was proposed a solution and a gel because the solution could be used as an irrigant alongside with NaOCl or replacing it and the hydrogel could be used as a coadjutant of the NaOCl.

Comparing with our study the (ChAuNR´s hydrogel and the solution with PPTT) the difference of the treatments was that they used 810 nm and at a power of 200 mW for 30 s, using a tip at 1 mm before the working length; In our study, the same parameters were used but a photosensitizer was not applied and the tip for the laser in the root canal was not used, the laser pointer was directed over roots. In most studies with positive results the laser is used at a power of 1 W or 2 W, the power used in this study was 200 mW, but more time was applied when using the laser.

Bermudez, C. et al. used the gold nanorods gel with photothermal therapy for bacterial elimination in periodontal pockets, they measured the temperature with a FLIR infrared camera, which gave them a maximum increase result of 10 ° C

in to 2 min with laser at a wavelength of 830nm[20]. Based on these authors, this work was carried out modifying the methodology for this study, in which the gel was tested in human roots. There are still no studies that use these nanorods hydrogel with laser therapy in the root canal.

Although the intensity of the laser decreases exponentially in the deeper layers of the tissue, the hydroxyapatite prisms and the dentinal tubules can act as light conductors, so that despite the weakened laser light, the bactericidal effect is maintained. The standard settings commonly applied with a diode laser are 2.5 W in cut or intermittent mode. In the literature, it is reported that the safety threshold for the temperature increase on the root surface is within the range of 7 ° C to 10 ° C. Otherwise, ankylosis can occur as a result of thermal trauma in the roots periodontal tissues.

The solution of the AuNR´s had positive results, but the application time of the laser was 20 minutes which an in vivo situation wouldn´t be possible, it could not replace the NaOCl as an irrigant but as a perspective, testing it with less laser time and with positive results it can be used a replacement of the sodium hypochlorite. The results of the study by Bermudez et al., which used the ChAuNR´s hydrogel and laser in tissues, agree with our results. They obtained positive results, in the bacterial elimination of *E. faecalis* and in our study also positive results were found regarding the bacterial elimination. The difference between studies was the substrates used, they used membranes, and, in this study, human dental roots were used. When using the root canal as a substrate, a level of difficulty is found when it comes to total microorganism elimination because of the anatomy of the root canal system, it has isthmus and accessory canals where *E. faecalis* can be trapped or hidden in these spaces. The results between studies do agree in which there is an elimination of *E. faecalis* but in a major percentage when it comes to eliminating it from a membrane substratum.

CONCLUSIONS

The modification done in this study to the DFR standardized reactor is a method capable of forming *E*. *faecalis* biofilm in the root canal in a mature phase with the proper characteristics. The biofilm formed with the static method was as well

a correct methodology and is more alike at how biofilm is formed in the root canal, being capable of manipulating the conditions. Our results suggested that the solution of AuNRs and Ch:AuRNs hydrogel with PPTT might be useful for root canal treatment as a solution or as a coadjutant respectively in treatment, however, it requires more research for it to be used in endodontic therapy.

Ethical Approval

The protocol was approved by the Ethics Committee of the School of Stomatology of the Universidad Autonoma de San Luis Potosí (CEI-FE-009-020).

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

REFERENCES

1. Yoo A, Rossi-Fedele G, Kidd SP, Rogers AH, Zilm PS. Association between Extracellular Material and Biofilm Formation in Response to Sodium Hypochlorite by Clinical Isolates of *Enterococcus faecalis*. Journal of Endodontics. 2018 Feb;44(2):269 (1. Yoo A & 44(2):269–73.)–73.
2. Gonzalez AM, Corpus E, Cohenca, N. Continuous drip flow system to develop biofilm of *E. faecalis* under anaerobic conditions. ScientificWorldJournal, 2014. 2014: p. 706189
3. Yang Y, Shen Y, Wang Z, Huang X, Maezono H, Ma J. Evaluation of the Susceptibility of Multispecies Biofilms in Dentinal Tubules to Disinfecting Solutions. Journal of Endodontics. 2016 Aug;42(8):1246–50

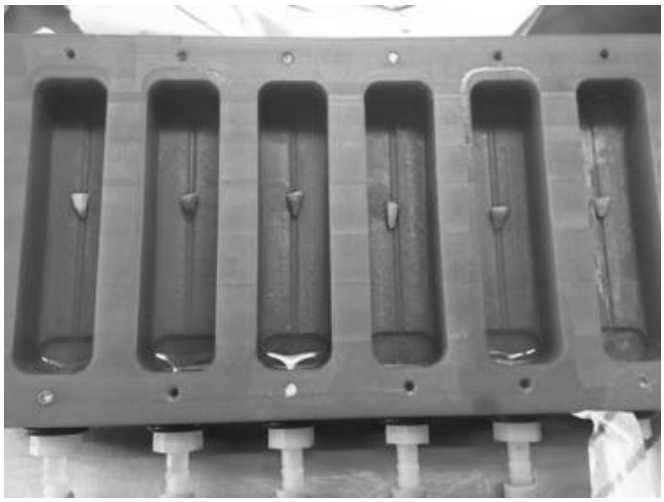
4. Choi Y-S, Kim C, Moon J-H, Lee J-Y. Removal and killing of multispecies endodontic biofilms by N -acetylcysteine. Brazilian Journal of Microbiology. 2018 Jan;49(1):184–8.
5. Niazy AA. Lux quorum sensing system and biofilm formation of oral microflora: A short review article. The Saudi Dental Journal. 2021 Mar;33(3):116–23.
6. Siqueira JF, Rôças I.N. Present status and future directions in endodontic microbiology: Endodontic microbiology: present and future. Endod Topics. 2014 May;30(1):3–22.
7. Pladisai P, Ampornaramveth RS, Chivatxaranukul P. Effectiveness of Different Disinfection Protocols on the Reduction of Bacteria in *Enterococcus faecalis* Biofilm in Teeth with Large Root Canals. Journal of Endodontics. 2016 Mar;42(3):460–4.
8. Torabinejad M, Kutsenko D, Machnick TK, Ismail A, Newton CW. Levels of Evidence for the Outcome of Nonsurgical Endodontic Treatment. Journal of Endodontics. 2005 Sep;31(9):637–46.
9. Ng Y-L, Mann V, Rahbaran S, Lewsey J, Gulabivala K. Outcome of primary root canal treatment: systematic review of the literature – Part 2. Influence of clinical factors. Int Endod J. 2007 Oct 11;0(0):071011095702005.
10. Tawakoli PN, Ragnarsson KT, Rechenberg DK, Mohn D, Zehnder M. Effect of endodontic irrigants on biofilm matrix polysaccharides. Int Endod J. 2017 Feb;50(2):153–60.
11. Marion JJC, Manhães FC, Bajo H, Duque TM. Efficiency of different concentrations of sodium hypochlorite during endodontic treatment. Literature review. Dental Press Endod. 2012 Oct-Dec;2(4):32-7.
12. Chau NPT, Chung NH, Jeon JG. Relationships between the antibacterial activity of sodium hypochlorite and treatment time and biofilm age in early *Enterococcus faecalis* biofilms. Int Endod J. 2015 Aug;48(8):782–9.
13. Ricucci D, Siqueira JF. Biofilms and Apical Periodontitis: Study of Prevalence and Association with Clinical and Histopathologic Findings. Journal of Endodontics. 2010 Aug;36(8):1277–88.
14. Kandaswamy E, Nagendrababu V, Deivanayagam K. Antimicrobial effect of nanoparticles in endodontics. In: Nanobiomaterials in Dentistry

[Internet]. Elsevier; 2016 [cited 2021 May 12]. p. 161–86. Available from: https://linkinghub.elsevier.com/retrieve/pii/B9780323428675000151

15. Samiei M, Farjami A, Dizaj SM, Lotfipour F. Nanoparticles for antimicrobial purposes in Endodontics: A systematic review of in vitro studies. Materials Science and Engineering: C. 2016 Jan;58:1269–78.
16. Shrestha A, Kishen A. Antibacterial Nanoparticles in Endodontics: A Review. Journal of Endodontics. 2016 Oct;42(10):1417–26.
17. Jhajharia K, Mehta L, Parolia A, Shetty Kv. Biofilm in endodontics: A review. J Int Soc Prevent Communit Dent. 2015;5(1):1
18. Samano-Valencia C, Martinez-Castanon G, Martinez-Gutierrez F, Ruiz F., Toro-Vazquez J, Morales-Rueda J, Nino Martinez N. Characterization and biocompatibility of chitosan gels with silver and gold nanoparticles. Journal of Nanomaterials, 2014.
19. Pérez-Díaz M, Alvarado-Gomez E, Magaña-Aquino M, Sánchez-Sánchez R, Velasquillo C, Gonzalez C, and cols. Anti-biofilm activity of chitosan gels formulated with silver nanoparticles and their cytotoxic effect on human fibroblasts. Materials Science and Engineering: C. 2016 Mar;60:317–23.
20. Bermúdez-Jiménez C, Niño-Martínez N, Patiño-Marín N, Martínez-Gutiérrez F, Ruiz F, Bach H, and cols. Effective control of biofilms by photothermal therapy using a gold nanorod hydrogel. J Biomed Mater Res. 2020 Feb;108(2):333–42.
21. Bansode PV, Pathak S, Wavdhane M, Khedgikar S, Gite S. Laser In Endodontics-A Review Article. IOSR JDMS. mayo de 2017;16(04):18-2
22. Afkhami F, Akbari S, Chiniforush N. *Entrococcus faecalis* Elimination in Root Canals Using Silver Nanoparticles, Photodynamic Therapy, Diode Laser, or Laser-activated Nanoparticles: An In Vitro Study. Journal of Endodontics. febrero de 2017;43(2):279-82.
23. De Almeida J, Cechella B, Bernardi A, de Lima Pimenta A, Felippe W. Effectiveness of nanoparticles solutions and conventional endodontic irrigants against *Enterococcus faecalis* biofilm. Indian J Dent Res. 2018;29(3):347.

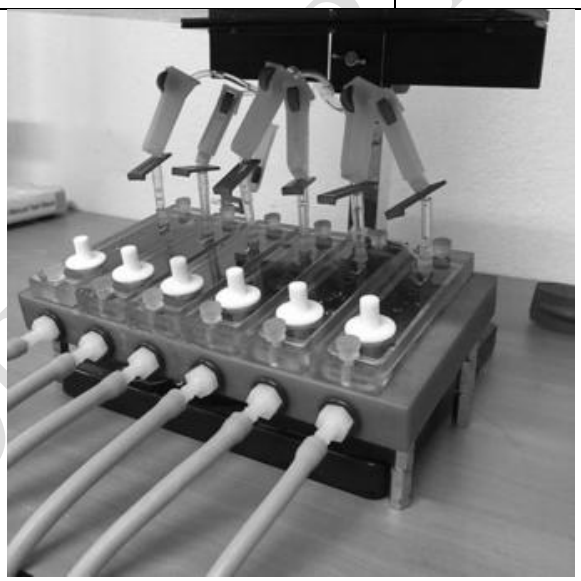
24. Swimberghe RCD, Coenye T, De Moor RJG, Meire MA. Biofilm model systems for root canal disinfection: a literature review. Int Endod J. 2019 May;52(5):604–28.
25. Guzmán-Soto I, McTiernan C, Gonzalez-Gomez M, Ross A, Gupta K, Suuronen EJ, and cols. Mimicking biofilm formation and development: Recent progress in in vitro and in vivo biofilm models. iScience. 2021 May;24(5):102443.
26. Peterson SB, Irie Y, Borlee BR, Murakami K, Harrison JJ, Colvin KM, and cols. Different Methods for Culturing Biofilms In Vitro. In: Bjarnsholt T, Jensen PØ, Moser C, Høiby N, editors. Biofilm Infections [Internet]. New York, NY: Springer New York; 2011 [cited 2021 Nov 4]. p. 251–66. Available from: http://link.springer.com/10.1007/978-1-4419-6084-9_15
27. Merritt JH, Kadouri DE, O'Toole GA. Growing and Analyzing Static Biofilms. In: Coico R, Kowalik T, Quarles J, Stevenson B, Taylor R, editors. Current Protocols in Microbiology [Internet]. Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2005 [cited 2021 Nov 4]. p. mc01b01s00. Available from: https://onlinelibrary.wiley.com/doi/10.1002/9780471729259.mc01b01s00
28. Schwartz K, Stephenson R, Hernandez M, Jambang N, Boles B. The Use of Drip Flow and Rotating Disk Reactors for Staphylococcus aureus Biofilm Analysis. JoVE. 2010 Dec 27;(46):2470.
29. Goeres DM, Parker AE, Walker DK, Meier K, Lorenz LA, Buckingham-Meyer K. Drip flow reactor method exhibits excellent reproducibility based on a 10-laboratory collaborative study. Journal of Microbiological Methods. 2020 Jul;174:105963.
30. Nikoobakht and M. A. El-Sayed, "Preparation and growth mechanism of gold nanorods (NRs) using seed-mediated growth method," Chemistry of Materials, vol. 15, no. 10, pp. 1957– 1962, 2003.

1. Figures



a)

b)



c)

Figure 1. a) Drip flow reactor (MDFR) of 6 chambers, the root inside of each chamber for the biofilm formation in the root canal. b) The biofilm forming system was mounted inside a sterile chamber, the MDFR was in the inferior portion and a magnetic stirrer was placed at 37°C. Over the magnetic agitator a sterile plastic recipient was placed with fresh growth medium of *E. faecalis* under slow agitation. c) The MDFR was connected with hoses coming from the superior portion of the system to the main feeding entrance of each MDFR chamber and feeding the root canal with continuous slow flow the residual fluid of the chambers were heading to the output hoses to a container.

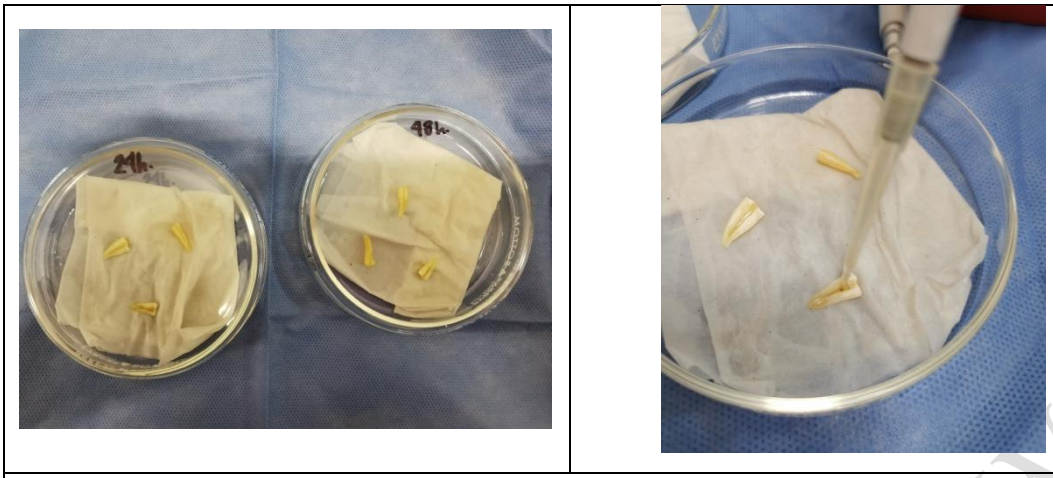
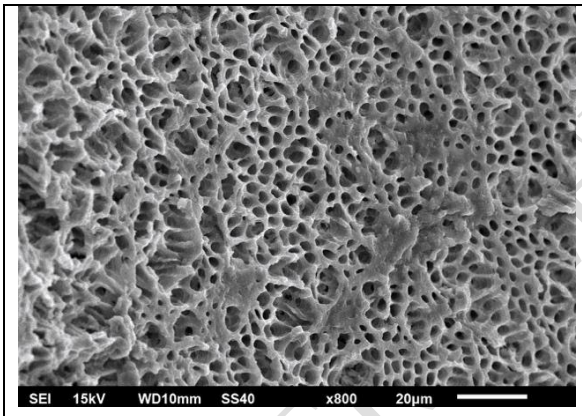


Figure 2. a) This image shows how the biofilm of 24- and 48-hours biofilm with static method was done. The roots were placed in Petri plaques and image 1. b shows how the inoculum of *E. faecalis* was placed in the root canal.



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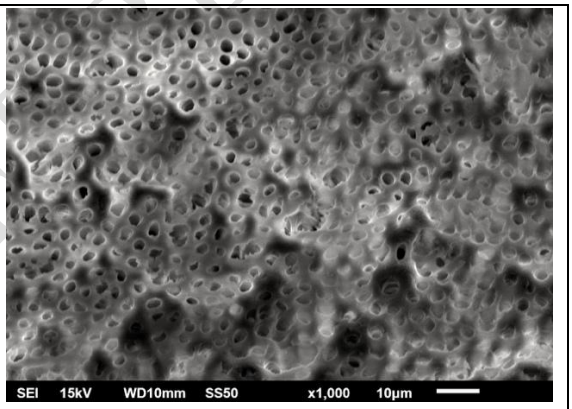

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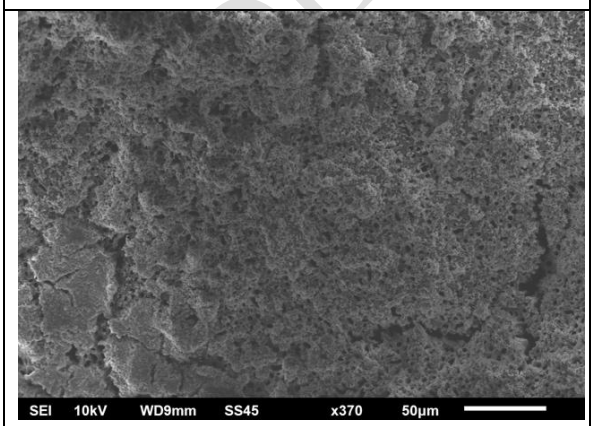

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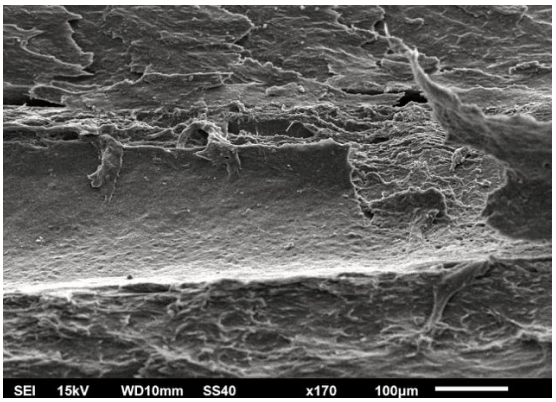

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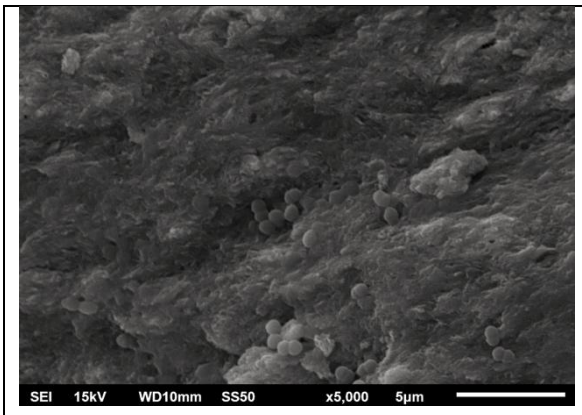

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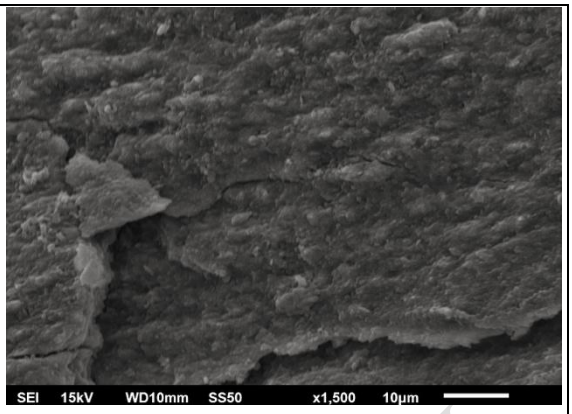
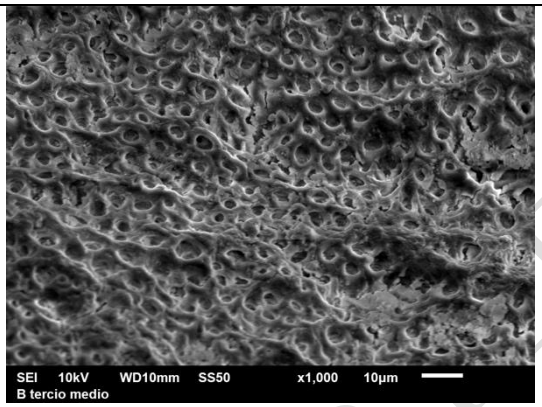


f)

Figure 3. 6.1 SEM Observation of biofilm after a)-d) 5 and e)-f) 10 days of inoculation with MDFR



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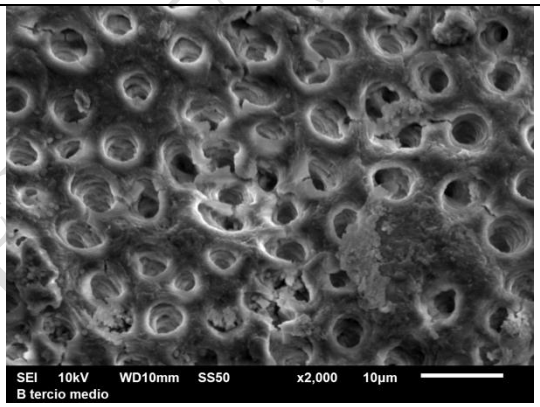

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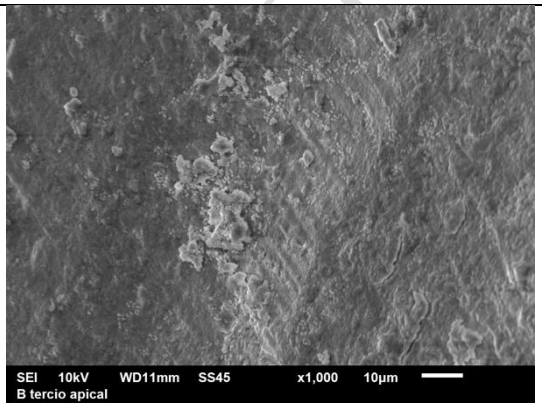

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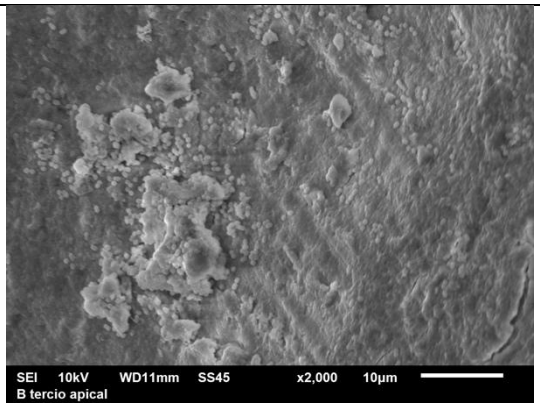
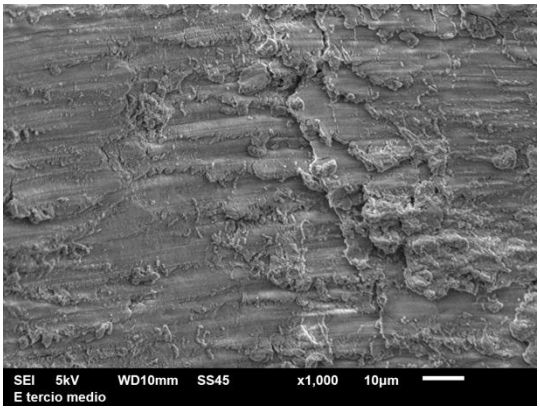


d)

Figure 4. These images showed a 24 hours *E. faecalis* biofilm. In a) and b) is observed the dentinal tubes filled with *E. faecalis*. c) *E. faecalis* can be observed in colonies forming a layer. This is the primary biofilm.



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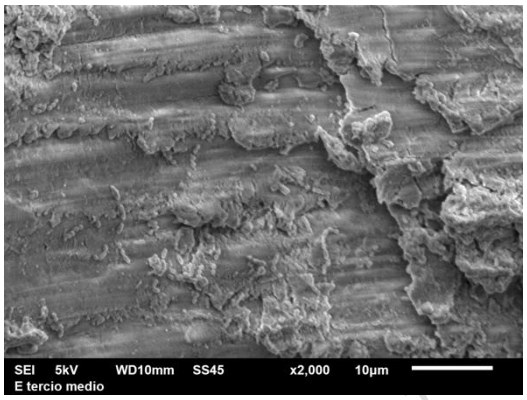

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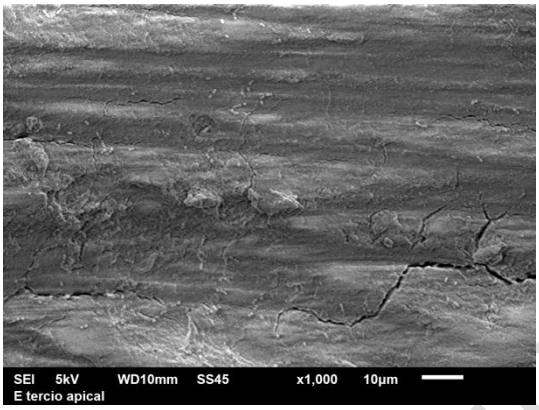

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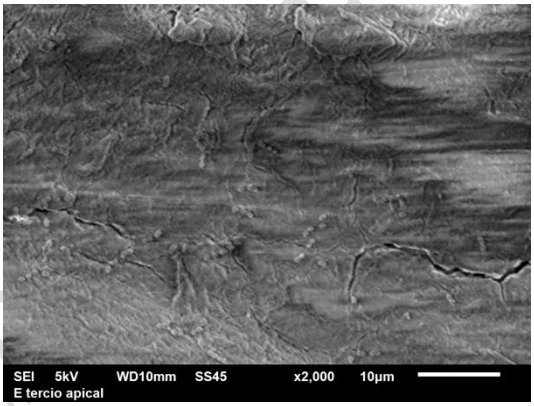

d)

Figure 5. These images show a 48 hours *E. faecalis* biofilm. a) and b) *E. faecalis* is in several colonies in the mid-third. c) It can be observed in the apical third that *E. faecalis* is in isolated colonies, less than in the third mid.