UNIVERSIDAD AUTÓNOMA DE SAN LUIS POTOSÍ

FACULTAD DE CIENCIAS QUÍMICAS

POSGRADO EN CIENCIAS FARMACOBIOLÓGICAS

TITULO:

EVALUACIÓN DE LA ACTIVIDAD ANTIMICROBIANA DE CELULASA CON ANTIBIÓTICO SOBRE LA MATRIZ POLIMÉRICA EXTRACELULAR DE BIOPELÍCULA DE *Enterococcus faecalis*

TESIS PARA OBTENER EL GRADO DE MAESTRA EN CIENCIAS FARMACOBIOLÓGICAS

PRESENTA

Q.F.B. SELENE VELÁZQUEZ MORENO

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SAN LUIS POTOSÍ, S.L.P. **AGOSTO, 2019**

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Contenido

Tesis en formato tipo artículo

Resumen

Objetivo: Determinar la actividad antimicrobiana de la celulasa de *Trichoderma reseei* en combinación con Amoxicilina/Clavulanato en la sustancia polimérica extracelular (EPS) de biopelícula (BP) de *E. faecalis* y su citotoxicidad.

Métodos: A partir de diecinueve cepas clínicas de *E. faecalis* aisladas de pacientes con fracaso de tratamiento de conductos; fue seleccionada la de mayor producción de biopelícula y celulosa. La evaluación de la actividad enzimática de la celulasa estudiada se llevó a cabo a diferentes valores de pH, concentración de enzimas y tiempo de hidrólisis a 37 °C; las mejores condiciones de hidrólisis se utilizaron para probar la actividad antimicrobiana en BP formada por el método de microcolonias, así como en BP formada en un reactor de flujo laminar sobre ápices de piezas dentales. Se analizaron cuatro grupos de tratamiento: Control, NaClO al 2.25%, Amoxicilina/Clavulanato y Celulasa con Amoxicilina/Clavulanato. La actividad citotóxica de las soluciones se evaluó mediante ensayos de calceína y homodímero de etidio. Se analizaron micrografías obtenidas por microscopio electrónico de barrido (SEM) para visualizar los efectos de los tratamientos sobre la EPS.

Resultados: De las cepas clínicas estudiadas la mayoría fueron moderadamente productoras de EPS; sólo una de ellas fue alta productora, la cual se utilizó para los estudios posteriores. Una vez identificadas las condiciones ideales de la enzima se procedió a realizar la combinación de celulasa y Amoxicilina/Clavulanato que se comportó como un desestabilizador de EPS con una importante capacidad antimicrobiana; aunque estos efectos fueron menores en comparación con el NaClO. Sin embargo, la viabilidad celular fue mejor que la obtenida con NaClO.

Palabras clave: Antibiótico, *E. faecalis,* Endodoncia, Celulasa, Tratamiento de conductos.

Abstract

Objective: To determine the antimicrobial activity of cellulase from *Trichoderma reseei* (*Hypocrea jecorina*) in combination with Amoxicillin/Clavulanate on the extracellular polymeric substances (EPS) from the *E. faecalis* biofilm and its cytotoxicity.

Methods: From nineteen clinical strains of *E. faecalis* isolated from patients with endodontic failed treatments; one of them was selected with the highest biofilm and cellulose production. The evaluation of enzymatic activity of the studied cellulase was carried out at different pH values, enzyme concentration, and hydrolysis time at 37°C; the best hydrolysis conditions were used to test the antimicrobial activity on biofilm microcolonies and biofilms in apices. Four treatment groups were analyzed: Group 1, Control; Group 2, 2.25% NaClO; Group 3, Amoxicillin/Clavulanate; and Group 4, Cellulase with Amoxicillin/Clavulanate. The cytotoxic activity of the tested solutions was also evaluated by calcein and ethidium homodimer (EthD-1) assays. SEM micrographs allowed visualizing the effects of the treatments.

Results: The combination of Cellulase and Amoxicillin/Clavulanate behaved as an EPS destabilizer with important antimicrobial capacity; although these effects were smaller when compared to NaClO. Nonetheless cell viability was better than that obtained with NaClO.

Conclusions: Formulation improvement and combination of irrigants should not be discarded as potential alternatives for the fight against biofilm-associated infections.

Key words: Antibiotic, *E. faecalis*, Endodontics, Cellulase, Root canal treatment.

Actividad antimicrobiana y citotoxicidad de celulasa y amoxicilina/clavulanato sobre matriz de biopelícula de *Enterococcus faecalis*

1. Introducción

La eliminación de los microorganismos del conducto radicular infectado es una constante preocupación en endodoncia; se han realizado diversas investigaciones para evaluar la eficacia de la instrumentación, irrigantes y medicación. Se ha demostrado la presencia de biopelículas en el foramen apical relacionándola con la persistencia de la periodontitis crónica[1].

La biopelícula (BP) es definida como una población microbiana adherida a un sustrato y rodeada de una matriz (productos extracelulares), la cual confiere resistencia a los agentes antimicrobianos y a la respuesta inmune del hospedero. La resistencia que presentan los microorganismos formadores de BP se ve aumentada en el interior del sistema de conductos ya que su anatomía proporciona zonas de difícil acceso a las soluciones irrigantes y no puede ser removida por la preparación biomecánica, lo que puede conducir a fracasos del tratamiento endodóntico. La bacteria *Enterococcus faecalis* fue encontrada en un rango del 33 al 90% en el análisis de conductos radiculares con infecciones persistentes asociados a infecciones mono o multiespecie que lo involucran[2].

Es por ello que actualmente uno de los objetivos de dicha área de investigación se enfoca en el desarrollo de agentes antimicrobianos con la capacidad de alteración y erradicación de la biopelícula periapical.

2. Materiales y Métodos

Este proyecto fue aprobado por el Comité de Ética en Investigación de la Facultad de Estomatología (folio CEI-FE-014-018) y por el Comité de Ética en Investigación y Docencia de la Facultad de Ciencias Químicas (folio CEID2018-07S).

2.1 Pacientes y cepas.

Las cepas clínicas se aislaron de pacientes con fracaso endodóntico, el estudio incluyó diecinueve cepas clínicas de *E. faecalis*; así como una cepa clínica

de *E. faecalis* aislada de un dispositivo médico y una cepa referencia de *E. faecalis* de la American Type Culture Collection (ATCC) con número 29219.

2.1.1 Ensayo de producción de biopelícula.

Se incubaron cultivos en placas de 96 pozos por 48 h a 37 °C y 110 rpm. Los pozos se lavaron para eliminar las bacterias no adherentes, una vez secas se tiñeron con solución cristal violeta al 0.1%. La densidad óptica se midió a 570 nm con un espectrofotómetro.

2.1.2 Ensayos de producción y detección de celulosa.

Se seleccionaron y cultivaron tres cepas con la mayor producción de BP en cupones de hidroxiapatita por 5 días a 37 °C y 110 rpm. Las muestras fueron analizadas utilizando un espectrofotómetro infrarrojo con Transformada de Fourier y Reflectancia Total Atenuada (ATR-FTIR).

2.2 Producción de biopelícula periapical usando un reactor de flujo laminar.

Los ápices dentales fueron previamente seleccionados como dientes anteriores con raíces de más de 13 mm y curvatura máxima de 20°. Las raíces fueron estandarizadas y decoronadas a 10 mm de longitud. Las muestras fueron sometidas a limpieza por ultrasonido y esterilización.

Las biopelículas se generaron en un reactor de flujo laminar (DFR, por sus siglas en inglés) y un reactor de activación de las cepas del Centro de Control de Enfermedades (CDC). El desarrollo de biopelículas se logró utilizando flujo continuo sobre veintitrés ápices dentales como sustrato. El flujo de bacterias se mantuvo a temperatura ambiente durante 24 h a un flujo laminar continuo de 0.82 mL/min a 25 °C.

2.3 Actividad enzimática.

2.3.1 Ensayo de condiciones óptimas de hidrólisis.

La hidrólisis de celulosa cristalina se realizó a 37 °C con celulasa de *T. reesei* durante 120 min. El efecto del pH se evaluó en el rango 5-8, mientras que la

concentración enzimática en el rango de 10-1000 U/mL. Después de ejecutar la hidrólisis, el sobrenadante fue recuperado y analizado para medir la concentración de azúcares por el método del ácido 3,5-dinitrosalicilico (DNS); la absorbancia se registró con un espectrofotómetro a 575 nm.

2.3.2 Ensayo de hidrólisis de biopelícula periapical.

Tres BP en ápices dentales fueron expuestas por inmersión, diez veces, en 1 mL de solución de celulasa (a 100 U/mL) a pH 5 durante 1 min. El volumen total se recuperó para determinar espectrofotométricamente la concentración de azúcares reductores utilizando el método DNS.

2.4 Ensayos de actividad antibiopelícula.

2.4.1 Ensayo de biopelícula en microcolonias.

Se utilizó agar de tripticaseína de soya (TSA) en donde se colocaron membranas de nylon de 0.2 µm; la inoculación se realizó con suspensión 0.5 McFarland (DO_{600} de 0.08 que equivale aproximadamente a 1×10^8 UFC/mL) de la cepa *E. faecalis* E.F M4. Después de la incubación, las membranas con microcolonias se dividieron en cuatro grupos; un grupo libre de tratamiento (control) y tres grupos de tratamiento: Amoxicilina/Clavulanato (2000 µg/mL/500 µg/mL), celulasa (100 U/mL) y Amoxicilina/Clavulanato (2000 µg/mL/500 µg/mL) + celulasa (100 U/mL). Posteriormente, las membranas se llevaron a diluciones en serie. Se inoculó cada dilución en TSA y el recuento de UFC después de 24 h.

2.4.2 Ensayo de biopelícula periapical.

Veinte biopelículas en ápices dentales se dividieron aleatoriamente en cuatro grupos; se irrigaron a través de una aguja para endodoncia: Grupo I: solución salina al 0.85%, Grupo II: solución de NaClO al 2.25%, Grupo III: Amoxicilina/Clavulanato (2000 µg/mL/500 µg/mL) y Grupo IV: celulasa-amoxicilina/clavulanato (100 U/ml-2000 µg/mL/500 µg/mL). Los Grupos II, III y IV utilizaron un instrumento ultrasónico a frecuencia de 25-30 kHz a 1 mm del ápice durante el último minuto de irrigación.

Tres de los ápices tratados por grupo se llevaron a análisis de recuento de UFC. Se prepararon dos ápices tratados por grupo para el análisis por microscopio electrónico de barrido (SEM) con electrones secundarios (SE).

2.5 Ensayo de viabilidad celular.

Para determinar si los tratamientos con celulasa y amoxicilina/clavulanato fueron ensayos citotóxicos se utilizó el kit de viabilidad/citotoxicidad live/dead para células de mamíferos. Los fibroblastos dérmicos se obtuvieron de piel de cirugías estéticas, con firma previa de carta de consentimiento. Las células estaban en contacto con diferentes condiciones experimentales: celulasa completa, celulasa purificada, Celulasa-Amoxicilina/Clavulanato, Amoxicilina/Clavulanato y NaClO (todas ellas a diferentes concentraciones). Después de 24 h de incubación, los medios fueron reemplazados por un medio con calceína y EthD-1; las células fueron incubadas y se tomaron fotografías utilizando un microscopio de epifluorescencia.

3. Resultados

3.1 Selección de una cepa clínica de E. faecalis.

Las diferentes cepas clínicas se dividieron en tres categorías según su absorbancia; no productores (<0.086 U.A.), productores moderados (0.086-0.258 U.A.) y productores altos de biopelícula (>0.258 U.A.). Dieciocho cepas orales, la cepa aislada de dispositivo médico y la cepa ATCC 29212 resultaron productores moderados de BP; sólo una cepa oral fue catalogada como alta productora. La cepa oral *E. faecalis* E.F M4 (productor alto de BP con 0.280 U.A.) fue seleccionada para estudios posteriores, así como las cepas E.F M10 y E.F M14.

3.1.1 Ensayo de producción de celulosa.

Las tres cepas seleccionadas fueron analizadas por ATR-FTIR para la identificación del enlace glucosídico β(1-4). Las tres muestras de biopelícula mostraron una absorción importante en el rango de número de onda de 1080 a 800 cm^{-1} que cubre las vibraciones de los enlaces C-O, C-O-C del enlace glucosídico

β(1-4) y el carbono anomérico de glucosa. Por tanto, la cepa elegida para ser utilizada para estudios posteriores fue la cepa E.F M4.

3.2 Actividad enzimática.

3.2.1 Mejores condiciones de hidrólisis.

Al comparar la glucosa liberada en función del pH de los grupos de diferentes concentraciones enzimáticas, se observó que las concentraciones de glucosa liberada en los tratamientos de 100 y 1000 U/mL a pH 5 eran significativamente más altas ($p<0.05$). Estos resultados llevaron a determinar que la solución de Celulasa de *T. reesei* a 100 U/mL y pH 5 fueron las mejores condiciones para continuar el estudio.

3.2.2 Ensayo de hidrólisis de biopelículas periapicales.

El ensayo dio lugar a una diferencia estadísticamente significativa para el grupo tratado con celulosa completa; donde la concentración de glucosa liberada fue en promedio de 2.6708 mg/mL en comparación con las concentraciones resultantes en la celulasa purificada y el grupo de solución salina.

3.3 Ensayo de actividad antibiopelícula.

3.3.1 Ensayo de biopelícula de microcolonias.

Se encontró una diferencia estadísticamente significativa cuando se utilizó el tratamiento de celulasa + amoxicilina/clavulanato; donde se logró una reducción logarítmica de 1.6 en referencia al control, lo que representa una reducción del 97.6% de UFC.

3.3.2 Ensayos de biopelícula periapical.

El tratamiento con NaClO al 2.25% mostró una reducción logarítmica de 8.4447 (100% de reducción de UFC); los resultados del SEM-SE pueden corroborar la ausencia de bacterias. En el grupo de irrigación Amoxicilina/Clavulanato se encontró una reducción logarítmica de 0.3020 (50% de reducción de UFC). En el caso del grupo de la combinación Celulasa + Amoxicilina/Clavulanato, la reducción

logarítmica fue de 1.0634 (reducción del 914% de UFC). Las micrografías correspondientes muestran EPS desintegrada, así como la ausencia de microorganismos en la zona.

3.4 Ensayo de viabilidad celular.

Una solución completa de celulasa a 25 U/mL resultó en 72% de células viables, no se encontraron células vivas cuando se utilizaron concentraciones más altas; cuando la celulasa fue purificada mediante diálisis, todas las concentraciones probadas mostraron más del 95% de viabilidad. Los tratamientos con todas las concentraciones de antibiótico analizadas mostraron más del 97% de viabilidad; cuando el antibiótico se combinó con la celulasa total a 50 U/mL la viabilidad alcanzó el 98%. En el tratamiento con NaClO no se encontró alguna célula viva.

4. Discusión y Conclusión

En este estudio todas las cepas clínicas de *E. faecalis* fueron productoras moderadas de BP, sólo una cepa se comportó como productora alta de BP. Estos resultados se correlacionan con las cepas orales reportadas por Anderson *et al.* (2016)[3] y Al-Ahmad (2014)[4]. Las cepas *de E. faecalis* han sido reportadas como productoras de celulosa por Romero-Rodríguez et *al*. (2016)[5] y Navarrete-Olvera *et al.* (2017)[6]; por lo tanto, es probable que la cepa elegida para este estudio fuera productora de celulosa.

La celulasa comercial es una mezcla de tres enzimas que actúan de manera sinérgica; se recomienda realizar la hidrólisis a pH 4.5-5.0 y rango de temperatura de 40 a 50 °C.

En Europa, la amoxicilina fue el antibiótico de primera elección en múltiples encuestas. Aunque es capaz de erradicar las bacterias del conducto radicular en estado planctónico, los resultados del estudio muestran que falla cuando los microorganismos adoptan una forma de BP. En este estudio, la combinación de celulasa y amoxicilina/clavulanato mostró una acción antibiopelícula mejorada erradicando el 90% de las bacterias después de 10 min y 10 mL de irrigación pasiva.

Por lo tanto, se postula que la solución antibiótico-enzima propuesta podría ser más eficaz cuando se combina con instrumentación mecánica o irrigantes como NaClO a concentraciones inferiores a las regulares.

La combinación de antimicrobianos con enzimas puede ser una alternativa eficaz para la lucha contra las infecciones asociadas a la biopelícula. La combinación de celulasa y amoxicilina/clavulanato dio lugar a una importante capacidad antibiopelícula tras la desestabilización de la EPS. Aunque estos efectos fueron menores en comparación con el NaClO, las mejoras de formulación y la combinación de irrigantes no deben descartarse; así como estudios adicionales para determinar la concentración específica de la combinación propuesta y su uso clínico.

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Antimicrobial and cytotoxicity activity of cellulase and amoxicillin/clavulanate on the *Enterococcus faecalis* biofilm matrix

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Running title: Alternative versus *Enterococcus faecalis* biofilm

Key words: Antibiotic, *E. faecalis*, Endodontics, Enzyme, Root canal treatment.

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ABSTRACT

Objective: To determine the antimicrobial activity of cellulase from *Trichoderma reseei* (*Hypocrea jecorina*) in combination with Amoxicillin/Clavulanate on the extracellular polymeric substances (EPS) of the *E. faecalis* biofilm and its cytotoxicity.

Methods: From nineteen clinical strains of *E. faecalis* isolated from patients with endodontic failed treatments; one of them was selected with the highest biofilm and cellulose production (determined after the crystal-violet and FTIR-ATR analyses). The evaluation of enzymatic activity of the studied cellulase was carried out at different pH values, enzyme concentration, and hydrolysis time at 37°C (using the DNS test to quantify the released reducing sugars); the best hydrolysis conditions were used to test the antimicrobial activity on biofilm microcolonies and biofilms in apices after CFU count. Four treatment groups were analyzed: Group 1, Control; Group 2, 2.25% NaClO; Group 3, Amoxicillin/Clavulanate; and Group 4, Cellulase with Amoxicillin/Clavulanate. The cytotoxic activity of the tested solutions was also evaluated by calcein and ethidium homodimer (EthD-1) assays. SEM micrographs allowed visualizing the effects of the treatments.

Results: The combination of Cellulase and Amoxicillin/Clavulanate behaved as an EPS destabilizer with important antimicrobial capacity; although these effects were smaller when compared to NaClO. Nonetheless cell viability was better than that obtained with NaClO.

Conclusions: Formulation improvement and combination of irrigants should not be discarded as potential alternatives for the fight against biofilm-associated infections.

Key words: Antibiotic, *E. faecalis*, Endodontics, Enzyme, Root canal treatment.

INTRODUCTION

The oral cavity is the second most colonized anatomical site in the human body. More than 800 endemic species have been described and only the suitable have the possibility of entering the root canal through carious processes, periodontal disease, trauma, or fractures to develop a pulpal pathology (Tronstad & Sunde 2003, Love 2005, Zambrano *et al.* 2016).

The objective of the endodontic treatment is to reduce the bacterial charge, which is associated to the biofilm on the infectious processes, using a biomechanical process and disinfection with a 0.5-6% NaClO solution followed by obturation (Gómez de Ferraris 2002, Kaplan JB 2010, Niazi *et al.* 2014).

According to the American Association of Endodontics, in 2015 in the United States 22.3 million procedures were performed and 15.1 million of these were associated to root canal infections. Several studies argue that the success of the endodontic treatment lies in the 70-85% range (Imura *et al.* 2007, Molander *et al.* 2007, de Chevigny *et al.* 2008), although Correira-Sousa *et al*. (2015) describes that the prevalence of individuals with at least one root canal treatment is between 41 and 87% and, based on this, the estimated percentage of individuals with apical periodontitis is in the 34-70% range.

It has been demonstrated that periradicular lesions facilitate the persistence of apical periodontitis mainly associated to biofilm formation, which represents a risk health not only because bacteria are no longer reached by antimicrobial agents or the immune system, but because biofilm provides a reservoir of bacteria for chronic infections (Tronstad & Sunde 2003, Khalifa *et al*. 2015). Biofilms are defined as a sessile microbial population adhered to a substrate surrounded by extracellular polymeric substances (EPS) composed of proteins, lipids, nucleic acids, and polysaccharides; joined by intermolecular forces that develop in the biofilm maturation stage (Stewart & Costerton 2001, Ch'ng *et al.* 2018). Biofilms have multiple functions that include: surface adhesion, drying prevention, diffusion barrier, mechanical stabilization, interchange of genes by quorum sensing, immunity protection, and resistance to environmental changes and antimicrobials. In the last sense, biofilms are characterized as being 100 to 1000 times more resistant to antimicrobial agents compared to microorganisms in a planktonic state (Stewart & Costerton 2001, Siqueira *et al.* 2010, Zambrano *et al.*, 2016).

The periapical biofilm in the apical third is clinically important for its difficult eradication through biomechanical preparation, which could cause failures in the endodontic treatment. Some parameters such as temperature and nutrients inflow are important to develop biofilms with specific features. A laminar fluid dynamics in

the root canal produces an intraradicular biofilm characteristic of this flow, however when advancing to the apical foramen a turbulent flow is generated (by the circulation of the periradicular tissues, the anatomy of the cortical bone, and occlusive movements) causing the formation of more solid biofilms (Siqueira *et al.* 2010, Gonzalez-Amaro *et al.* 2014). If the endodontic treatment succeeds in eradicating the entire biofilm, an infection in the extra-radicular of the periapical area would be avoided. Failure rates have been related to the complexity of the anatomy in the root of the tooth, iatrogenies, and resistance of microorganisms (that include *E. faecalis*) to intraconduct medication (Khalifa *et al.* 2015, Siqueira Junior *et al.* 2018, Yamaguchi *et al.* 2018).

E. faecalis is frequently recovered from persistent secondary infections, associated with root canal treatment failures that can result in invasion of the periradicular tissue with subsequent development of abscesses and diffuse infections (Khalifa *et al.* 2015). Approximately 33 to 90% of endodontic failures have been associated to persistent infections of *E. faecalis* or multispecies infections that involve it (Gomes *et al.* 2008, Rôças & Siqueira 2012, Khalifa *et al.* 2015). The persistence of *E. faecalis* can be explained since it has multiple virulence factors (Love 2005, Anderson *et al.* 2016, Ch'ng *et al.* 2018) that include the production of biofilms difficult to eradicate, easy dissemination of resistance genes, and cellulose production in its EPS (Moniri *et al.* 2017, Ch'ng *et al.* 2018). For this persistence, the development of new antimicrobial agents has become primordial.

The components of the biofilm have been studied and it has been determined that the layer surrounding the biofilm is formed by several polymers that generate the EPS, alginate is present in the EPS of *Pseudomonas* and cellulose exists in the EPS biofilms *Enterococcus* and *Salmonella* (Solano *et al.* 2002, Romero-Rodriguez 2016, Navarrete-Olvera 2017). Since the EPS confer protection to the biofilm, its elimination would leave the cells available for eradication in planktonic form; favoring the action of numerous antimicrobials. Thus enzymes, peptides, and other molecules have been proposed for biofilm disintegration (Niazi *et al.* 2014, Wang *et al.* 2017). Cellulose is the most abundant polymer on the planet; being produced by plants, algae, tunicates, and bacteria (Moniri *et al.* 2017). It consists of a network of glucose

molecules, linked by a β (1,4)-glucosidic bond, that are arranged in chains joined by hydrogen bonds to generate microfibrils (Horn *et al.* 2012). In contrast to the vegetable cellulose, the bacterial cellulose has better water-absorption capacity, superior crystallinity when free of hemicellulose and lignin, and high mechanical resistance due to its band-like structure. Cellulose generated by bacteria, such as *E. faecalis*, could provide stability to the biofilm and be a factor that contributes to its persistence after endodontic treatment with NaClO (Solano *et al.* 2002, Moniri *et al.* 2017).

It should be noted that NaClO is the gold standard as endodontic irrigant even though it has disadvantages such as cytotoxicity (Ioannidis *et al.* 2018) and dentine erosion (Galdamez *et al.* 2019), which can cause problems in obturation or rehabilitation of the dental organ (Correr *et al.* 2006, Tartari *et al.* 2016, Abuhaimed & Neel 2017, Ramírez-Bommer *et al.* 2018, Morgan *et al.* 2019).

Therefore, research in this area is currently focused on the development of antimicrobial agents with the ability to alter the EPS of the periapical biofilm in a safely manner for the patient. Using enzymes such as cellulose in combination with antimicrobials is an attractive approach presented in this work as possible alternative or coadjuvant to the treatment with NaClO. Cellulose is an enzyme capable of hydrolyzing cellulose to its monomeric form (Horn *et al.* 2012). Thus, the objective of this work was to determine the enzymatic and antimicrobial activity of cellulase from *Trichoderma reseei* (now *Hypocrea jecorina*) in the EPS of the *E. faecalis* biofilm.

MATERIAL AND METHODS

Patients and strains.

The clinical strains were isolated from patients with endodontic failed treatments, all of them signed a letter of informed consent. The protocol was approved by the School of Dentistry Investigation Ethics Committee and registered with the number CEI-FE-005-1-018. The study included nineteen clinical strains of *E. faecalis* isolated from samples of patients with endodontic failed treatments, as well as one clinical strain of *E. faecalis* isolated from a medical device and a

reference strain of *E. faecalis* from the American Type Culture Collection (ATCC) with number 29219.

Identification and antimicrobial profile.

All twenty clinical strains were previously identified by the API 20 Strep (Biomerieux, France) with a 90% probability of being *E. faecalis*. The sensitivity profile in the planktonic state was carried out with the Kirby Bauer disk diffusion method in accordance to the Clinical Laboratory Standard Institute (CLSI 2019) (Supplementary Tables 1 and 2).

Biofilm plate assay.

The bacterial strains were grown in Trypticase Soy Broth (TSB) overnight at 37°C. The inoculum was standardized per well at 96 CFU/mL. 96-well polystyrene tissue culture plates were filled with 180 µL of fresh TSB and 20 µL of the overnight culture were added to each well. The plates were incubated for 48 h at 37 °C and 110 rpm. The culture medium was discarded and the wells were washed three times with 200 µL of phosphate-buffered saline solution to remove nonadherent bacteria. The plates were air dried and stained with 0.1% crystal violet solution for 10 min. Excess stain was removed by washing three times with 200 µL of sterile, distilled water. The plates were dried for 10 min at 60 °C. 200 µL of absolute alcohol were added to each well for resolubilization of the dye. The optical density was measured at 570 nm with a Multiskan FC Microplate Photometer (Thermo Fisher Scientific, Waltham, MA). All tests were performed in triplicate, whereas the experiments were carried out three times and the mean values determined. The biofilm formation categories were considered according to the Al-Ahmad method (Al-Ahmad *et al.* 2014).

Cellulose production and detection assays.

Three strains with the highest BP production were selected and grown in TSB overnight at 37°C. The inoculum was standardized per well at 96 CFU/mL. Three hydroxyapatite coupons were placed in a 24-well polystyrene tissue culture plate; the culture plate was filled with 1350 µL of TSB and 150 µL of the overnight culture were added to each well. The plates were incubated for 5 days at 37 °C and 110 rpm. The culture medium was discarded and the coupons were washed with phosphate-buffered saline solution to remove nonadherent bacteria; they were subsequently dried for 1 day at 50°C. A coupon was used as control surface in the spectrum, while planktonic cells of the E.F M4 strain (one of the strains isolated from endodontic failed treatments as later described) as comparative sample without EPS.

The samples were analyzed using a Nicolet 6700 / Smart iTRro Fourier-transform infrared spectrometer with attenuated total reflection (FTIR-ATR) (Thermo Fisher Scientific, Waltham, MA), analyzing from 4000 to 700 cm^{-1} with a 4 cm^{-1} resolution of, with controls of planktonic cells and coupons of hydroxyapatite (Fuller, Andaya and McClay, 2018).

Periapical biofilm production by drip flow reactor.

Periapical biofilm production.

The biofilms were generated in a drip flow reactor (DFR) (BioSurface Technologies Corp., Bozeman, MT), which uses laminar flow and a Control Disease Center (CDC) reactor (BioSurface Technologies Corp., Bozeman, MT) assembled as per ASTM E2647-13. Development of biofilms was accomplished using continuous flow over twenty-three dental apices as substrate. The CDC reactor was filled with 800 mL of TSB and it was inoculated with 1 mL of *E. faecalis* (0.5 McFarland solution, optical density of 0.08 at 625 nm) to activate the biofilm genes at room temperature (25 ± 1 °C); stirring was applied for 1 h keeping the Reynolds number at 800-1500. Afterwards, the CDC reactor was attached to a sterile supply of TSB and to the DFR, which included four individual channels at a 10° angle with one dental apice. The flow of bacteria with TSB was maintained at room temperature

for 24 h at a continuous laminar flow rate of 0.82 mL/min (Alvarado-Gomez *et al.* 2018, Watson *et al.* 2018).

Selection and preparation of dental apices.

Dental apices were previously selected as anterior human teeth with roots longer than 13 mm and maximum curvature of 20°. Afterwards the roots were standardized and decoronated using a double-sided diamond disc to 10 mm in length, filed with 10 to 25 K-files (Dentsply Sirona, York, PA) until the apice was opened (Arslan *et al.*, 2014). The samples were subjected to ultrasound cleaning (BioSonic UC50, Coltene/Whaledent Inc., Cuyahoga Falls, OH) with immersion protocols using 17% EDTA (JT Baker Chemical Co., Phillipsburg, NJ) and 5.25% NaClO solutions; followed by sterilization at 121 °C and 15 psi for 20 min (Gonzalez-Amaro *et al.* 2014).

Enzymatic activity.

Optimal hydrolysis conditions assay.

To obtain the values of enzyme concentration, pH, and contact time that favor the enzymatic activity of cellulose; the hydrolysis of crystalline cellulose (Sigmacell 20 µm, Sigma-Aldrich Sc., St. Louis, MO) was performed at 37 °C with cellulase of *Trichoderma reesei* (Sigma-Aldrich Sc., St. Louis, MO) for 120 min. The effect of pH was evaluated in the 5-8 range, while enzyme concentration in the 10-1000 U/mL range. After running the hydrolysis, the supernatant was recovered after centrifugation and analyzed for reducing sugars concentration. For this the 3,5-dinitrosalicylic acid (DNS) method was used; to 50 µL of supernatant, 450 µL of deionized water and 500 µL of 1% DNS reagent were added. The reaction was carried out at 90° C for 15 min to develop the red-brown coloration and finally 167 µL of 40% $KNaC_4H_4O_6 \cdot 4H_2O$ solution was added to stabilize the coloration. After cooling to room temperature, the absorbance was recorded with a spectrophotometer at 575 nm (Miller 1959).

Periapical biofilm hydrolysis assay.

Three biofilms in dental apices were exposed by immersion, ten times, in 1 mL of cellulose solution (at 100 U/mL) at pH 5 for 1 min. The total volume was recovered to determine spectrophotometrically the concentration of reducing sugars using the DNS method mentioned above.

Antibiofilm activity assays.

Microcolony biofilm assay.

A 0.5 McFarland suspension (approximately 1×10^8 CFU/mL) was obtained from a 24 h culture of the *E. faecalis* strain E.F M4. Trypticase soy agar (TSA) was used in Petri dishes on which 0.2 µm nylon filter membranes were placed (Thermo Scientific, Rochester, NY); inoculation was performed with 10 µL of the 0.5 McFarland suspension and the incubation was performed 24 h at 37 ° for the development of microcolony biofilms on the membranes. Following incubation, the microcolony biofilms membranes were divided into four groups; one treatment-free (control group) and three treatment groups: Amoxicillin/Clavulanate (2000 µg/mL/500 µg/mL), Cellulase (100 U/mL), and Amoxicillin/Clavulanate (2000 µg/mL/500 µg/mL) + Cellulase (100 U/mL). Treatments were applied as follows: the microcolony biofilms were placed in a 24-well polystyrene plate (one membrane per well), where 1 mL of particular treatment solution was added and applied for 10 min at 37°C. Afterwards, membranes were rinsed in 1 mL of sterile 0.85% NaCl solution, then each treated membrane was placed in 9 mL of sterile 0.85% NaCl solution; carrying serial dilutions with 0.85% NaCl solution (Alvarado-Gomez *et al.* 2018). 10 µL of each dilution are inoculated in TSA, incubation is performed at 37°C and plate count after 24 h.

Periapical biofilm assay.

Twenty biofilms in dental apices were divided randomly into four groups (n=5) and irrigated through a blunt-ended needle as follows: Group I: 10 mL of 0.85% saline solution, Group II: 10 mL of NaClO solution at 2.25%, Group III: 10 mL of Amoxicillin/Clavulanate (2000 µg/mL/500 µg/mL), and Group IV: 10 mL of Cellulase-

Amoxicillin/Clavulanate (100 U/mL-2000 µg/mL/500 µg/mL) (Arslan *et al.* 2014). Groups II, III, and IV used a soft ultrasonic instrument (tip 15) at frequencies of 25–30 kHz (5-LED, DTE Woodpecker, Guilin, China) in the channel 1 mm from the apice without touching the walls during the final minute of irrigation (Van Der Sluis *et al.* 2007, Arslan *et al.* 2014). The flow rate was 1 mL/min for all groups studied.

Three of the apices treated per group were rinsed in 1 mL of sterile 0.85% NaCl solution, afterwards each treated apice was placed in 9 mL of sterile 0.85% NaCl solution carrying serial dilutions with 0.85% NaCl sterile solution (Alvarado-Gomez *et al.* 2018). Inoculation of 10 µL in triplicate on TSA at 37 °C and plate count after 24 h were performed.

Two apices treated per group were prepared for the SEM-SE analysis: they were washed gently with 0.85% sterile NaCl solution and fixed with 1% solution of Alcian blue dye (Sigma-Aldrich, St. Louis, MO) and 2% glutaraldehyde solution (Sigma-Aldrich, St. Louis, MO) at 4 °C for 24 h. Afterwards the samples were washed with sterile 0.85% NaCl solution to remove excess material. Samples were dehydrated using increasing concentrations of ethanol (JT Baker, Ltd.) in water (from 20 to 95%) for 10 min per solution; ending with absolute ethanol until drying in a critical point dryer (CPD 030 BAL-TEC GmbH, Schalksmühle, Germany). Subsequently, they were sputter coated with gold (Fine Coat Ion Sputter JFC-1100, USA). The treated apices were evaluated with secondary electrons (SE) in SEM (JEOL JSM-6610 LV, JAPAN) (Gonzalez-Amaro *et al.* 2014).

Cell viability assay.

To determine if treatments with cellulase and amoxicillin/clavulanate were cytotoxic, calcein and ethidium homodimer (EthD-1) assays were performed using the live/dead viability/cytotoxicity kit for mammalian cells (Thermo Scientific, Rochester, NY). Dermal fibroblasts were obtained from the skin of aesthetic surgeries, with previous sign of consent letter. Cells were culture in DMEM/F12 medium (Gibco) supplemented with 10% of fetal bovine serum (Gibco, Thermo Scientific, Rochester, NY) and 10% of ampicillin/streptomycin (Gibco, Thermo Scientific, Rochester, NY). Cells were grown until passage 10 and seeded (15000

cells/well) in 96-well culture plates during 24 h. Cells were in contact with different experimental conditions: Control (PBS 1×, Gibco), complete Cellulase (25, 50, 100, 150, and 200 U/mL), purified Cellulase (25, 50, 100, 150, and 200 U/mL), Cellulase-Amoxicillin/Clavulanate (25 U/mL-200 µg/mL/125 µg/mL, 50 U/mL-1000 µg/mL/250 µg/mL, 100 U/mL-2000 µg/mL/500 µg/mL, 150 U/mL-3000 µg/mL/750 µg/mL, and 200 U/mL-4000 µg/mL/1000 µg/mL), Amoxicillin/Clavulanate (200 µg/mL/125 µg/mL, 1000 µg/mL/250 µg/mL, 2000 µg/mL/500 µg/mL, 3000 µg/mL/750 µg/mL, 4000 µg/mL/1000 µg/mL), NaCl (0.4, 0.6, 0.8, 1, and 1.2%), and NaClO (1, 1.5, 2, 2.5, 3, and 3.5%). After 24 h of incubation, media were replaced with fresh medium having 5 µM of calcein and 5 µM of EthD-1, but without the treatments; afterwards the cells were incubated during one hour at 37 °C under 5% of CO_2 and photographs were taken using an epifluorescence microscope (AxioVert A1, Zeiss, Oberkochen, Germany). Live and dead cells were counted using the ImageJ software. The percentages were calculated using GraphPad Prism 6.0 and ANOVA analyses were performed with a Dunnet post hoc test ($p<0.05$ was considered a significant difference).

Statistical analysis.

The differences between groups were analyzed with One-way ANOVA or Kruskal-Wallis and Mann-Whitney tests. The test was performed at a 95% confidence level (p=0.05). All statistical analyses were performed using version 23 of the SPSS software.

RESULTS

Selection of a clinical strain of E. faecalis.

The different clinical strains were divided into three categories according to the results previously presented by Al-Ahmad *et al*. (2014) who defined an OD value < 0.086 U.A. corresponding to non-producers of biofilm, an OD value ranging 0.086-0.258 U.A. was associated to moderate biofilm producers, and an OD value > 0.258 U.A. to high biofilm producers. Figure 1 shows the results of biofilm production by all evaluated strains.

Eighteen oral strains (from the 19 strains isolated from endodontic failed treatments and identified as E.F M1-M19), the strain islolated from a medical device, and the ATCC 29212 strain resulted moderate producers of biofilm; only one oral strain was cataloged as high producer of biofilm. The *E. faecalis* oral strain E.F M4 (high biofilm producer) was selected for further studies (cellulose production assay), as well as the strains E.F M10 and E.F M14.

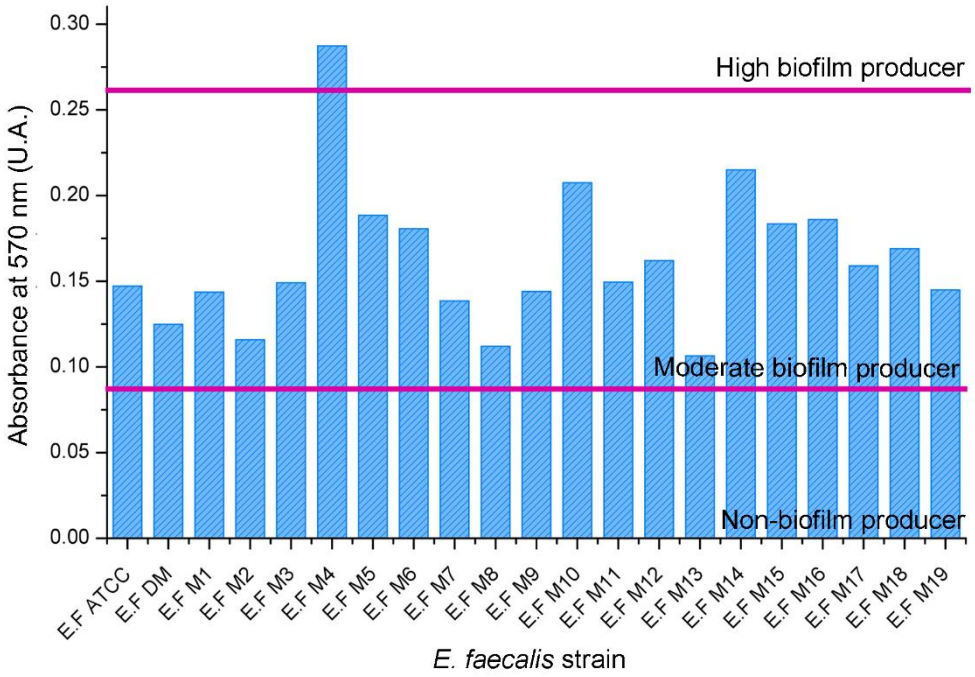

Fig. 1 Biofilm production assay. Levels of biofilm production according to the absorbance recorded from the analysis of different strains of *E. faecalis*; the horizontal lines differentiate the three levels of production.

Cellulose production assay.

The three selected strains having the highest production of BP were analyzed by ATR FTIR for the identification of the β (1-4) glycosidic bond. For comparison purposes, Fig. 2 shows biofilm spectra of the strains E.F M4, E.F M10, and E.F M14; as well as the bacteria in planktonic state of the strain E.F M4. The characteristic vibration bands for cellulose were located at 1054 and 1030 cm^{-1} for C-O bonds), 1160 cm^{-1} for C-O-C, and 898 cm^{-1} for the vibration of the anomeric carbohydrate carbon group. The three biofilm samples showed important absorption in the 1080 to 800 cm^{-1} wavenumber range covering the vibrations of the C-O bonds, C-O-C

belonging to the β (1-4) glycosidic bond, and the anomeric carbon of cellulose; while absorption of the planktonic bacteria sample was clearly decreased. These results corroborate the presence of cellulose in the extracellular polymeric matrix of biofilms. In addition, the region where lipids absorb (3000-2800 cm^{-1}, due to the C-H bonds of membrane lipids) is important for planktonic cells, while it is reduced for the biofilm samples.

Considering the previous results, the strain chosen to be used for further studies was the E.F M4 strain (a clinical strain of *E. faecalis* isolated from an endodontic retreatment).

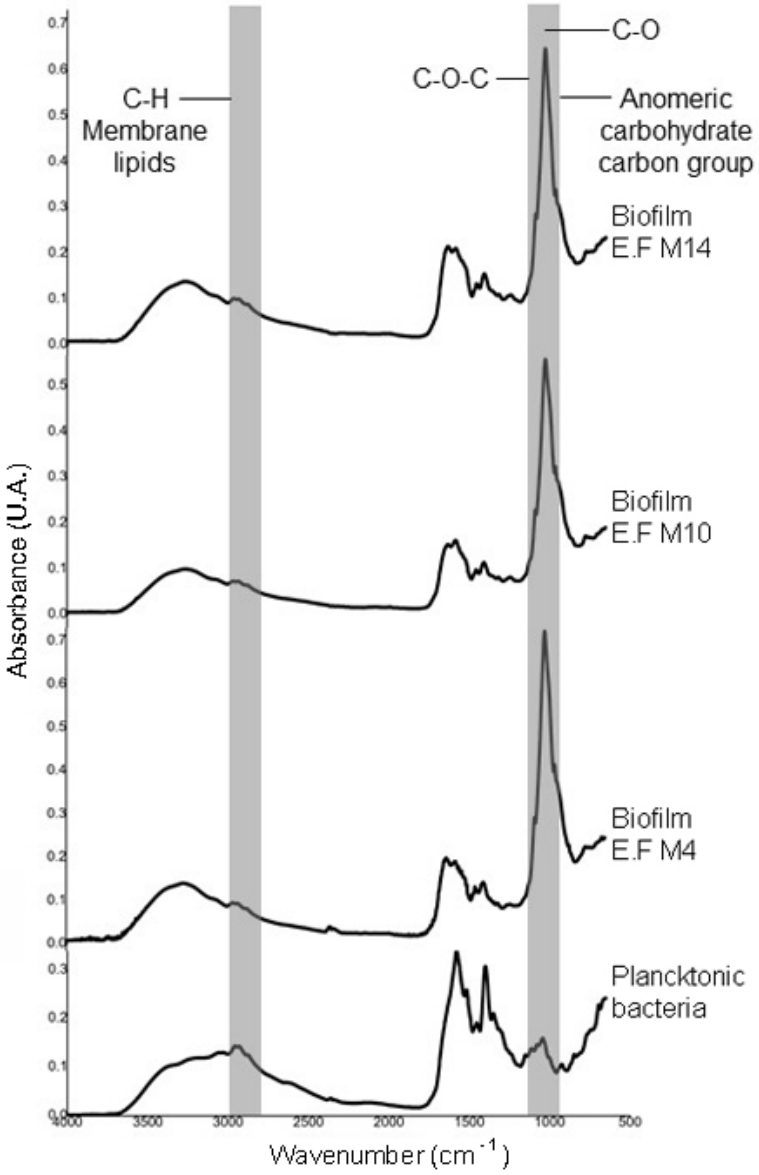

Fig. 2 Infrared biofilm spectra. The characteristic bands of cellulose (gray areas) between 4000-800 cm^{-1} are shown. The difference of vibrations between the spectra from biofilms vs. planktonic cells, is evident mainly in the vibration bands of C-O, C-O-C, carbohydrate anomeric carbon, and C-H from membrane lipids.

Enzymatic activity.

Best hydrolysis conditions.

The best hydrolysis conditions for pH value (pH values tested were 5, 7, and 8) and enzymatic solution concentration (10, 100, and 1000 U/mL) were determined for a Cellulase from *Trichoderma reesei*. The results in concentration of glucose released were consistent between groups of pH (Fig. 3); this is, the lowest enzyme concentration (10 U/mL) generated lower glucose concentration with a statistically significant difference ($p<0.05$) with respect to the medium (100 U/mL) and high (1000 U/mL) concentrations after 120 minutes of reaction at 37°C. Similarly, the medium and high concentrations for each pH value did not generate a significant difference in hydrolysis capacity. When comparing the glucose released as a function of pH from the groups of different enzyme concentrations, it was observed that the glucose concentrations of 100 and 1000 U/mL at pH 5 (790 and 932 mg/mL, respectively) were significantly higher ($p<0.05$) when compared to those obtained for pH values 7 and 8. These results led to determine that the *Trichoderma reesei* Cellulase solution at 100 U/mL and pH 5 were the best conditions to continue the study.

Once the best conditions of enzyme concentration and pH were established, a hydrolysis curve was developed at 37°C for 120 minutes; sampling every 10 min (Fig. 4). It was found that the hydrolytic action steadily increases until 80 min; time where a plateau appears on the curve for the next 40 min.

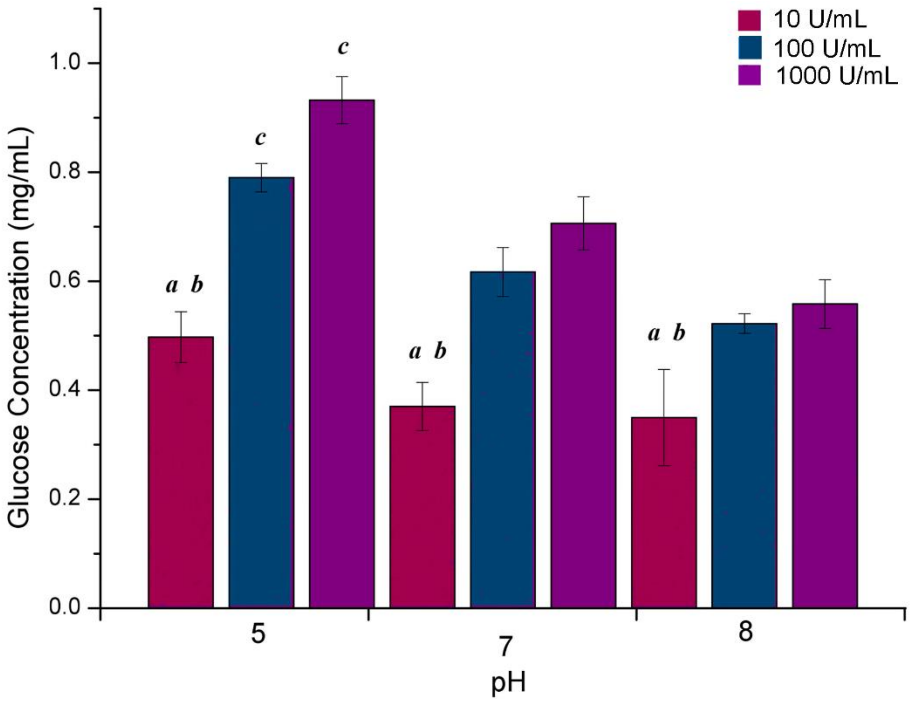

Fig. 3 Cellulose hydrolysis with different enzyme conditions (pH and enzyme concentration). The concentration of glucose released at 10, 100, and 1000 U/mL of enzyme concentration at different pH values (5, 7, and 8) are shown. ***a.*** $p<0.05$ vs. 100 U/mL at the same pH value; ***b.*** $p<0.05$ vs. 1000 U/mL at the same pH value; ***c.*** $p<0.05$ same enzyme concentration vs. different pH values.

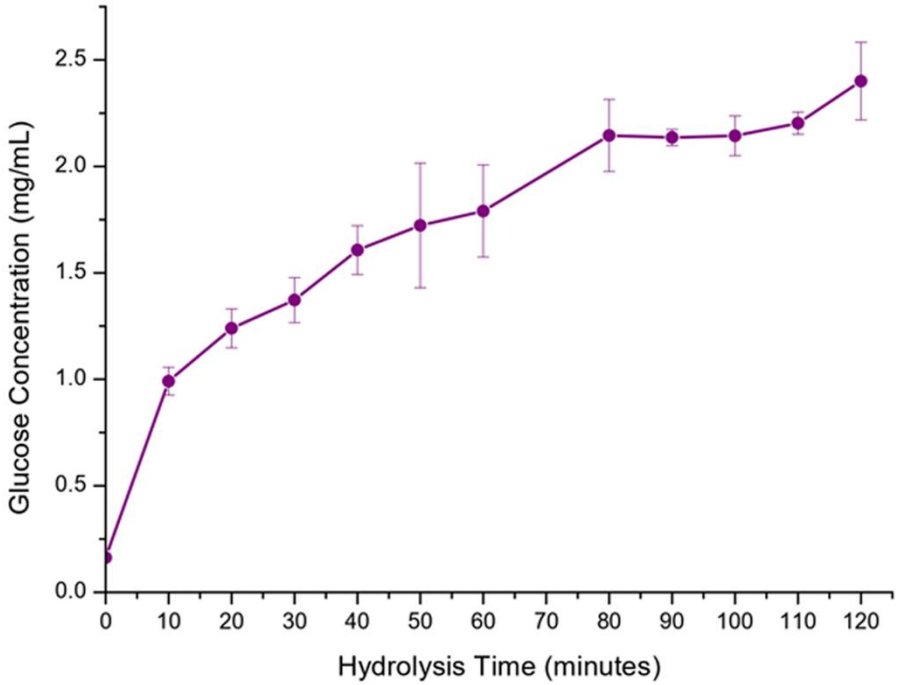

Fig. 4 Kinetics of Cellulose hydrolysis. Evaluated over 120 min at pH 5, 100 U/mL of enzyme concentration, and 37 °C. A rapid increase in glucose concentration is perceived from the first minutes of hydrolysis.

Periapical biofilms hydrolysis assay.

In the hydrolysis assay on periapical biofilms the conditions selected were: enzyme concentration set to 100 U/mL, pH set to 5, and 10 min of reaction (considering the approximate time of clinical irrigation). The hydrolysis assay on periapical biofilms resulted in a statistically significant difference for the group treated with complete cellulose, where the concentration of glucose released was on average 2.6708 mg/mL when compared to the resulting concentrations in the dialyzed (purified) cellulase and the saline solution group (1.6079 and 0.2872 mg/mL, respectively).

Table 1. Glucose released from the hydrolysis of the periapical biofilm exopolymer matrix.

Treatment solution	Glucose concentration (mg/mL) (±SD)
0.85% Saline solution	0.2872 (0.08)***a***
Dialyzed Cellulase 100 U/mL	1.6079 (0.08)***a***
Complete Cellulase 100 U/mL	2.6708 (0.15)***a***

SD = standard deviation***. a.*** $p<0.05$ vs. remaining groups.

Antibiofilm activity assay.

Microcolony biofilm assay.

After exposing the microcolonies biofilms to the treatments solutions: Amoxicillin/Clavulanate (1000 µg/mL/500 µg/mL), Cellulase (100 U/mL), and Cellulase + Amoxicillin/Clavulanate (100 U/mL, 2000 µg/mL/500 µg/mL); the antibiofilm effect of each treatment was verified (Table 2). A statistically significant difference was found when the Cellulase + Amoxicillin/Clavulanate (100 U/mL, 2000 µg/mL/500 µg/mL) treatment was used; where a logarithm reduction of 1.6 was achieved in reference to the control of 0.85% saline solution, which represents 97.6% reduction of CFU.

Table 2. Anti-biofilm solutions evaluation on microcolony biofilm.

Treatment solution	CFU Log Red (±SD)	Percentage reduction (±SD)
Amoxicillin/Clavulanate (2000/500 µg/mL)	0.5789 (0.11)	73.0% (0.50)
Cellulase (100 U/mL)	0.4961 (0.09)	67.6% (0.16)
Cellulase + Amoxicillin/Clavulanate (100 U/mL, 2000/500 µg/mL)	1.6224 (0.11)[a]	97.6% (0.45)

SD = CFU Log Red standard deviation. ***a.*** $p<0.05$ studied treatments vs. control group (Kruskal-Wallis test).

Periapical biofilm assays.

After exposing the periapical biofilms to the studied treatments (Groups I to IV): 0.85% saline solution, 2.25% NaClO solution, Amoxicillin/Clavulanate (2000 µg/mL/500 µg/mL), or Cellulase + Amoxicillin/Clavulanate (100 U/mL, 2000 µg/mL/500 µg/mL); the CFU log reduction was calculated, two treatments showed a statistically significant decrease with respect to the control (Table 3). The 2.25% NaClO treatment showed a logarithmic reduction of 8.4447 (100% of CFU reduction); this agrees with the results of the SEM-SE analyses presented in Fig. 5 where the absence of bacteria can be corroborated. Moreover, upon magnification (marked as F) the presence of microfractures is evident. In the Amoxicillin/Clavulanate (2000 µg/mL/500 µg/mL) irrigated group a logarithmic reduction of 0.3020 was found, which represents 50% of CFU reduction (Table 3) for this group Fig.5 shows the development of mature biofilm in the periapical area, communication pores of smaller diameter (marked as C), and bacteria with EPS coating (marked as D) having a construction of multiple layers. In the case of the group irrigated with the Cellulase + Amoxicillin/Clavulanate (100 U/mL, 2000 µg/mL/500 µg/mL) combination treatment the logarithmic reduction was 1.0634, which is interpreted as a 91.4% reduction of CFU (Table 3). The corresponding micrographs show disintegrated EPS, cellulose microfibrils that could be separating,

and disintegrating are evident (marked E); as well as the absence of microorganisms in the area (Fig. 5). These groups were compared with the logarithmic development of CFU (8.4440) and the micrographies obtained for the control group (Fig. 5), the development of mature biofilm in the periapice allows observing possible extensive communication and nutrition pores (marked as A); moreover bacteria excreting EPS (marked as B) and the multilayer construction can be observed. Based on the ANOVA statistical analysis and the Tukey and Bonferroni post hoc tests, the groups irrigated with NaClO and Cellulase + Amoxicillin/Clavulanate obtained a statistically significant decrease respect to the control, which indicates that the enzyme-antibiotic synergism is effective against the *E. faecalis* periapical biofilm.

Table 3. Anti-biofilm solutions evaluation on periapical biofilm.

Treatment solution	CFU Log Red (±SD)	Percentage reduction (±SD)
2.25% NaClO	8.4440 (0.36) *a*	100% (0.00)
Amoxicillin/Clavulanate (2000/500 µg/mL)	0.3020 (0.01)	50% (0.88)
Cellulase + Amoxicillin/Clavulanate (100 U/mL, 2000/500 µg/mL)	1.0634 (0.02)*a*	91.4% (0.31)

SD = CFU Log Red standard deviation*. **a.*** $p<0.05$ studied treatments vs. control group (Kruskal-Wallis test).

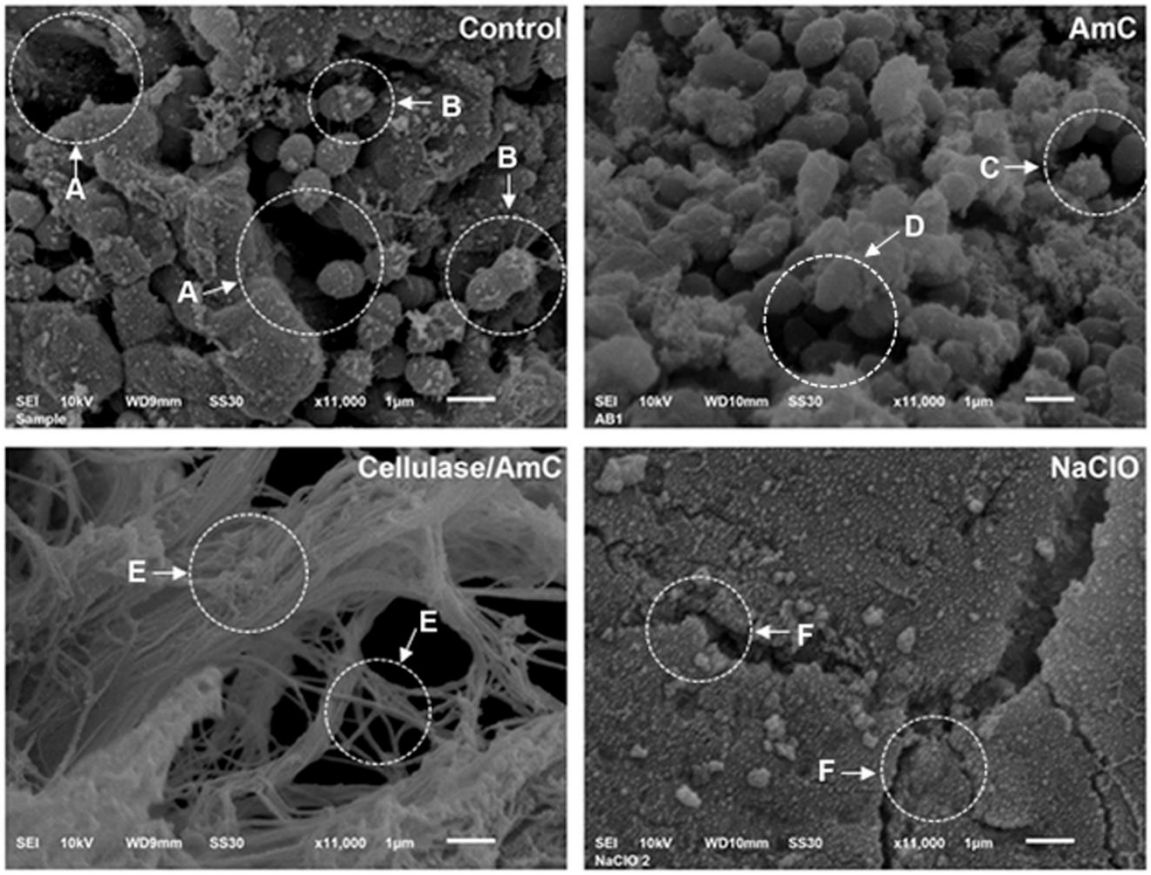

Fig. 5 Periapical biofilms after irrigation. The arrangement of images shows the biofilms by treatment group in rows. The micrographies shows the greatest magnification, with important signs in the structure.

Cell viability assay.

In this work, total cellulase was purified using dialysis membranes. Purified cellulase and low doses of total cellulase allowed dermal fibroblast cell viability. When a total cellulase solution at 25 U/mL was tested 72% of viable cells was achieved, no live cells were found when using higher enzyme concentrations of total cellulase. However, when the cellulase was purified using dialysis, all tested enzyme concentrations exhibited more than 95% of cell viability. Treatments with all the tested antibiotics concentrations showed more than 97% of cell viability; when the antibiotics were combined with total cellulase at 50 U/mL the cell viability reached 98%, but higher enzyme concentrations resulted cytotoxic. In the NaClO treatment no live cell was found (Figures 6 and 7).

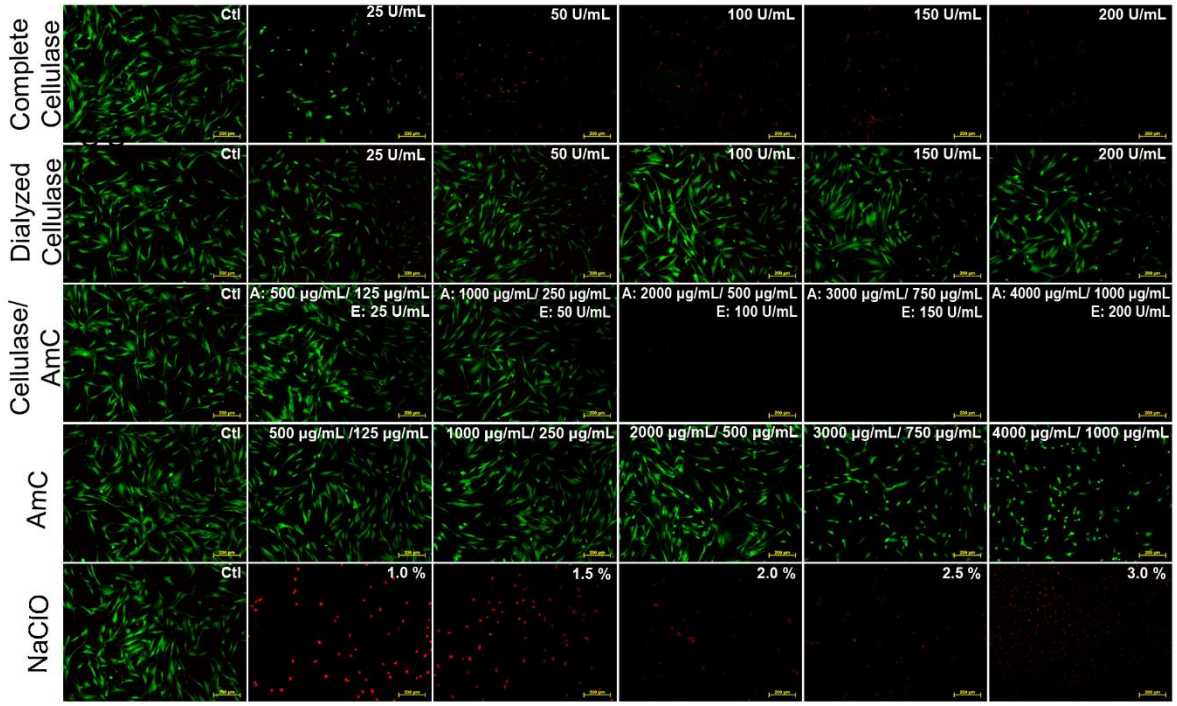

Fig. 6 Viability of dermal fibroblasts to studied solutions. The micrographs show green calcein-positive cells (live) and in red cells positive for EthD-1 (dead). The different treatments are described on the left, while concentrations used are depicted in the corresponding micrograph.

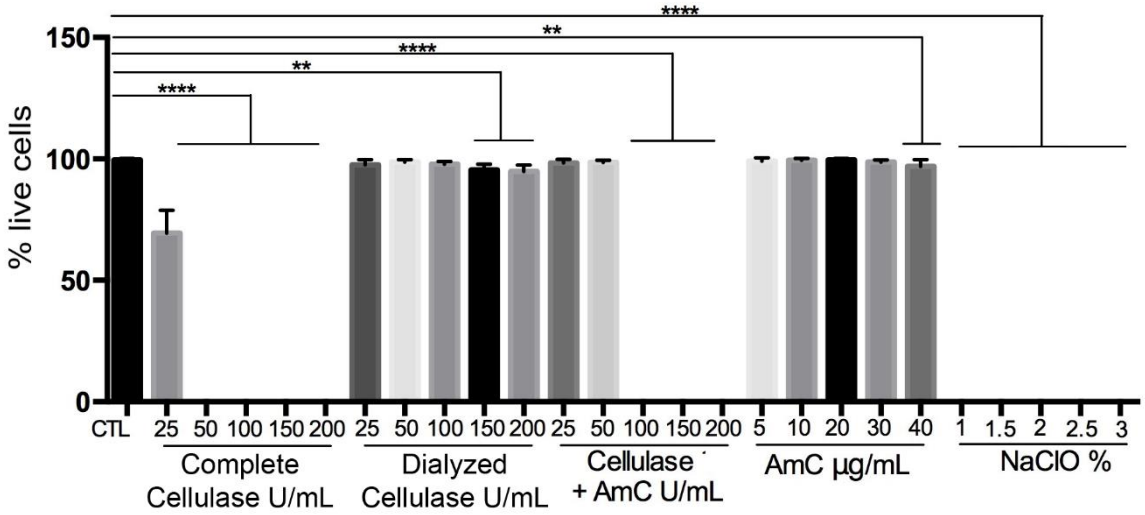

Fig. 7 Percentage of viable cells (positive for calcein). After different experimental solutions conditions. ANOVA was performed with a post hoc Dunnet test. ** p <0.001, **** p <0.00001.

DISCUSSION

Approximately 460 strains of microbial species have been reported in endodontic infections. *E. faecalis* is widely described as one of the main microorganisms isolated and identified in secondary infections or retreatments (Siqueira & Rôças 2009, Rôças & Siqueira 2012) with an incidence fluctuating from 38 to 90% of the reported cases (Gomes *et al.* 2008, Rôças & Siqueira 2012). In this study all the clinical *E. faecalis* strains were moderate producers of biofilm, only one strain behaved as high biofilm producer. These results correlate with the oral strains reported by Anderson *et al.* (2016) where 74% of the studied strains were described as moderate and high biofilm producers. On the other hand, in the study published by Al-Ahmad *et al.* (2014) 33 and 17% of the reported *E. faecalis* strains turned out to be moderate and high biofilm producers, respectively.

Moniri *et al*. (2017) mention that strains of *Gluconacetobacter xylinus*, *Agrobacterium tumefaciens*, *Gluconacetobacter*, *Rhizobium spp*., *Sarcina ventriculi*, and *Salmonella sp.* have been reported as cellulose producers when coding cellulose synthase. *E. faecalis* strains have been reported as cellulose producers by Romero-Rodríguez *et al*. (2016) and Navarrete-Olvera *et al.* (2017). Thus, it is likely that the strain chosen for this study was producer of cellulose as part of the EPS, the peaks at 1000-1050 cm^{-1} were evaluated indicating the presence of the β (1-4) glycosidic bond (Yang *et al.* 2017, Fuller *et al.* 2018). Bacterial cellulose is described as microfibrils secreted from the longitudinal axis of the cell, formed from glucose residues (Moniri *et al.* 2017).

On the other hand, studies of fungal proteins that are classified as 61 families of Glycoside Hydrolases (Harris *et al.* 2010, Langston *et al.* 2011) indicate that these proteins can break the polysaccharide chains using an oxidative mechanism dependent on metal ions and electron donors (Vaaje-Kolstad *et al.* 2010). The hydrolysis of polymers such as cellulose can be accelerated by the addition of a mixture of enzymes. Commercially some of the enzymatic complexes are produced by *Trichoderma reesei* (also known as *Hypocrea jecorina*) or *Asperguillus sp.* The hydrolysis is recommended to be performed at pH 4.5-5.0 and temperature range from 40 to 50°C (Horn *et al.* 2012).

The commercial cellulase is a mixture of three enzymes acting in a synergistic way: endo-1,4-β-glucanases that hydrolyze random bonds in the cellulose chain, β-glucosidases that convert cellobiose in glucose, and exo-1,4-β-glucanases or cellobiohydrolases that attack the cellulose polymer termination. These enzymes are the most abundant components in mixtures of natural and commercial cellulases (Kostylev & Wilson 2012) as in the case of the cellulolytic enzymes produced by *H. jecorina* (Rosgaard *et al.* 2007).

In the industrial realm enzymes are commonly used as biofilm degraders, however this application has not been frequently studied in endodontics. One of the articles published in this area evaluates the antimicrobial action of two enzymes and ultrasound in biofilms of endodontic microorganisms and compared them against NaClO and chlorhexidine; the enzymes showed better performance than the latter being able of disintegrating the EPS of biofilms (Niazi *et al.* 2014). This last result agrees with the present study confirming that cellulase at ideal conditions can disrupt the EPS, hydrolyzing the β (1-4) glycosidic bond from cellulose produced by *E. faecalis*; allowing the bacterial cells to be exposed and facilitate their elimination by antimicrobial substances such as antibiotics.

In the endodontic area, antibiotics are commonly used to prevent infection spread in systemically compromised patients; the risk of antibiotics ineffectiveness in some conditions of the pulp and periapice has led to the use of antibiotics topically, in pulp coatings, and as root canal treatment. Nonetheless limited studies have focused on the use of antibiotics as endodontic irrigants, the majority focused on tetracyclines (Krause *et al.* 2007, Mohammadi & Abbott 2009). However, studies with microorganisms isolated from the root canal showed resistance to this group of drugs (Al-Ahmad *et al.* 2014).

In Europe, several surveys have studied the prescription scheme of antibiotics in the treatment of endodontic disease. Amoxicillin was the antibiotic of first choice in most of the surveys conducted (from 2000 to 2015) by numerous authors (Segura-Egea *et al.* 2017). Amoxicillin is a β-lactam antibiotic, of moderate spectrum, with bacteriolytic activity, effective against the cell wall of Gram-negative bacteria, and more efficient against Gram-negative when compared to penicillin (Slots 2002).

However, amoxicillin is susceptible to degradation by β-lactamase-producing bacteria and that is why it is commonly chosen in combination with clavulanic acid. This combined formulation is one of the recommended treatments for odontogenic infections due to its broad spectrum against Gram positive bacteria and low incidence of resistance (Gomes *et al.* 2011). Although amoxicillin is capable of eradicating root canal bacteria in their planktonic state (Gomes *et al.* 2011), the results of the current study show that it fails when microorganisms adopt a biofilm form. In this study, the combination cellulase and amoxicillin/clavulanate showed an improved antibiofilm action eradicating 90% of the bacteria after 10 minutes and 10 mL of passive irrigation. EPS disintegration facilitates the release of cells layers since intra-radicular biofilms are usually thick and composed of multiple layers of cells as described by Siqueira *et al*. (2010). This can also be observed in the micrographs obtained in the present work (Fig. 6) for the control and amoxicillin/clavulanate groups. Although biofilm eradication was not complete, the biofilm was appropriately debilitated. It is thus postulated that the proposed antibiotic-enzyme solution could be more effective when combined with mechanical instrumentation or irrigants such as NaClO at lower than regular concentrations looking to completely eliminate the biofilm. On the other hand, the total cellulase and amoxicillin/clavulanate combination resulted cytotoxic within the analyzed concentrations; therefore it could be possible to analyze lower concentrations of complete cellulase or replace it with the same purified enzyme to decrease toxicity without sacrificing antimicrobial action. Moreover the idea of proposing an irrigation protocol that combines the proposed antibiotic-enzyme solution with NaClO at lower concentrations would decrease cellular toxicity (Ioannidis *et al.* 2018), and the erosion of the dentine by collagen changes (Correr *et al.* 2006, Tartari *et al.* 2016, Abuhaimed & Neel 2017, Ramírez-Bommer *et al.* 2018, Morgan *et al.* 2019) without scarifying the antimicrobial action.

The present study demonstrates the possibility of using a dual enzyme-antibiotic solution (Cellulase-amoxicillin/clavulanate) for the elimination of *E. faecalis* biofilms, with an important antimicrobial effect and cytotoxicity lower than NaClO

treatment, emphasizing that EPS can be an important attack point to combat the origin of endodontic diseases.

CONCLUSION

The combination of antimicrobials with enzymes can be an efficient alternative for the fight against biofilm-associated infections. Further study of the biofilms composition brings us closer to new treatment alternatives that should be explored. The combination of Cellulase and Amoxicillin/Clavulanate resulted in an important antibiofilm capacity upon EPS destabilization. Although these effects were smaller when compared to NaClO, formulation improvements and combination of irrigants should not be discarded. Additional studies are necessary to determine the specific concentration of the proposed combination and its clinical use, without discarding its use in combination with lower concentrations of NaClO.

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SUPPLEMENTARY MATERIAL

Supplementary table 1. Antimicrobial susceptibility profile of E. faecalis strains. Test Kirby-Bauer (inhibition zone) [a].

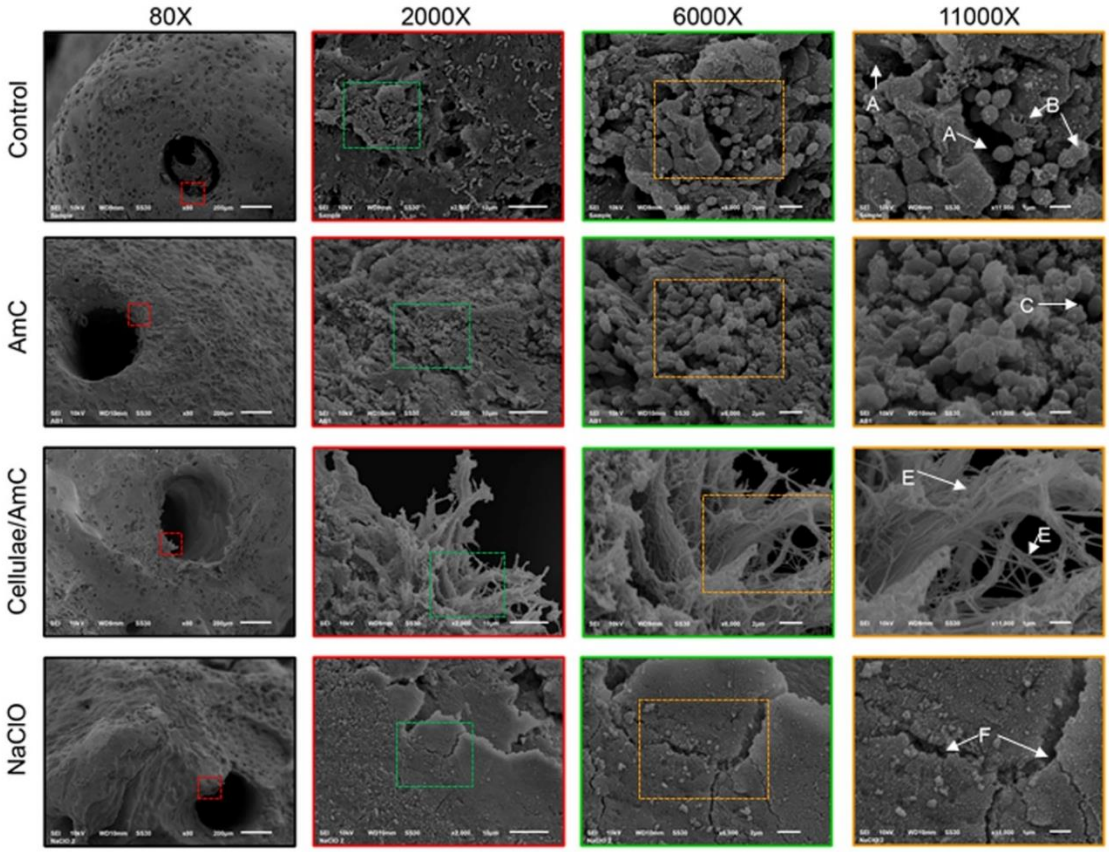
Antibiotic	CLSI Reference (mm)			*E. faecalis* strains																		
	R	I	S	E.F M1	E.F M2	E.F M3	E.F M4	E.F M5	E.F M6	E.F M7	E.F M8	E.F M9	E.F MI0	E.F MI1	E.F MI2	E.F MI3	E.F MI4	E.F MI5	E.F MI6	E.F MI7	E.F MI8	E.F MI9
Amoxicillin/Clavulanate 20/10 µg	≤16		≥17	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Clindamycin 2 µg	≤14	15-20	≥21	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Cephalothin 30 µg	≤14	15-17	≥18	R	I	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Vancomycin 30 µg	≤14	15-16	≥17	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

S= Sensitive, I= Intermedius, R= Resistant.
a=According to CLSI (2019)

Supplementary table 2. Extended antimicrobial susceptibility profile of selected *E. faecalis* strain (Dilution test, MIC) [a]

Antibiotic	CLSI Reference (µg/mL)			Susceptibility profile of E.F M4
	Sensitive	Intermedius	Resistant	
Ampicillin	≤8		≥16	S
High level Gentamicin	≤16	15-16	≥8	S
Ciprofloxacin	≤1	2	≥4	S
Levofloxacin	≤2	4	≥8	S
Erythromycin	≤0.5	1-4	≥8	R
Linezolid	≤2	4	≥8	S
Vancomycin	≤4	8-16	≥32	S
Doxycycline	≤4	8	≥16	R
Tetracycline	≤4	8	≥16	R
Nitrofurantoin	≤32	64	≥128	S

S= Sensitive, I= Intermedius, R= Resistant.
a=According to CLSI (2019)



Supplementary figure 1. Periapical biofilms after irrigation. The arrangement of images shows the biofilms by treatment group in rows, the increasing order from left to right can be observed the magnifications of each group. The color boxes show the amplification zones in the micrograph on the right. The last column shows the greatest magnification, with signs in the structure.

Apéndices