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MICROBIOTA BACTERIANA ASOCIADA A CONDUCTO RADICULAR CON DIAGNÓSTICO DE PERIODONTITIS APICAL CRÓNICA PERSISTENTE Y ROL DE NANOPARTÍCULAS DE COBRE COMO NUEVO ANTIMICROBIANO ENDODÓNTICO

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"El que se enamora de la práctica sin ciencia es como el marino que sube al navío sin timón ni brújula, sin saber con certeza hacia donde va"

Leonardo da Vinci (1452-1519)

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RESUMEN

A nivel mundial se describe una alta prevalencia de periodontitis apical crónica persistente en dientes obturados con lesión apical, asociada a persistencia bacteriana, que va desde 40 % a 61 %. En Chile, la frecuencia de fracasos de tratamientos endodónticos, se informa en 55 %;sin embargo, estos resultados se basan sólo en parámetros clínicos, sin ninguna evidencia microbiológica. Los protocolos de medicación utilizados en endodoncia en Chile se basan en formulaciones diseñadas y utilizadas en el extranjero, para otras poblaciones del mundo. Las bacterias aisladas de conductos radiculares pueden establecerse en biopelículas intraconducto en los túbulos dentinarios, y la captación y penetración de los agentes antimicrobianos en ellos son elementos que hay que tener en consideración en los resultados terapéuticos. Las nanopartículas metálicas son potencialmente útiles dentro de este contexto, ya que por sus características físico-químicas podrían penetrar en la biopelícula, actuando como antimicrobianos a una concentración muy pequeña. El objetivo de esta tesis fue determinar la composición de la microbiota bacteriana asociada a conductos radiculares con diagnóstico deperiodontitis apical crónica persistente y evaluar el rol de nanopartículas de cobre (NPCu) como un nuevo agente antimicrobiano intraconducto. De un universo de 250 piezas dentarias diagnosticadas con periodontitis apical crónica persistente en el periodo de julio a noviembre de 2015, se obtuvo 24 muestras desde pacientes que cumplieron con los criterios de inclusión del estudio. Las características clínicas más relevantes fueron registradas. Se procedió a extraer el ADN bacteriano y a amplificar las regiones variables V3 y V4 del gen ARNr 16S. El producto de amplificación fue secuenciado mediante *Illumina MiSeq System*. Posteriormente se procesaron los datos utilizando la aplicación bioinformática *Quantitative Insights into Microbial Ecology* (QIIME). Las lecturas no quiméricas representativas se agruparon en unidades taxonómicas operacionales (OTUs) utilizando un umbral de identidad de 97% y se clasificaron taxonómicamente mediante coincidencias con secuencias en la base de datos Greengenes (versión gg_13_5). El porcentaje de cobertura se estimó mediante el método estimador de cobertura no paramétrico de Good; Alfa diversidad se evaluó con un estimador de riqueza Chao1; y el índice de diversidad de Shannon se

calculó a través de la herramienta bioinformática Mothur. Las estructuras de comunidades microbianas en diferentes muestras se compararon utilizando UniFrac basado en la relación filogenética de lecturas representativas de diferentes muestras, y las distancias UniFrac ponderadas se utilizaron para construir un análisis de coordenadas principales. Desde todas las muestras se aislaron e identificaron las especies prevalentes cultivables a través de sistemas bioquímicos como API® Rapid ID 32 A (BioMerieux, Marcy-l'Etoile, Francia) y API® 20E (BioMerieux, Marcy-l'Etoile, Francia). Finalmente, se corroboró la identificacion de cada especie bacteriana mediante secuenciación del producto de amplificación con reacción en cadena de la polimerasa convencional para el gen ARNr16S empleando los partidores *P0/P6* o *rrs*. Posteriormente, alas cepas identificadas se les determinó el comportamiento frente antimicrobianos de uso común en endodoncia y a NPCu tanto en su forma planctónica como en tres modelos *ex vivo* de biopelícula formado sobre paredes dentinarias tanto con técnicasrecuento de unidades formadoras de colonia y microscopía confocal. Por último, se relacionó la diversidad bacteriana y el comportamiento de las cepas frente a los antimicrobianos con los parámetros clínicos de los pacientes.

El análisis de coordenadas principales PCoA indicó una separación entre individuos con historia clínica ASA I y ASA II-III basada en la relación filogenética de la comunidad bacteriana presente en cada muestra. Las estimaciones con el índice Chao1 no fueron diferentes entre los grupos, excepto en lo que respecta a la historia clínica, donde ASA II-III presentó mayor estimación de riqueza, así como mayor índice de diversidad de Shannon. El nivel de PAI 5 aumentó el índice de diversidad de Shannon en comparación con PAI 4 y este índice se redujo en pacientes sintomáticos. En todos los grupos, *Proteobacteria* fue el phylum más abundante seguido de *Bacteroidetes.* Sin duda, la familia de mayor abundancia fue *Pseudomonadaceae,* seguida de pequeñas variaciones en la abundancia de otros grupos taxonómicos dependiendo de la historia del paciente. Se identificaron 16 cepas aisladas en condiciones aeróbicas, 10 de las cuales correspondieron a *Pseudomonas* spp. y otras 6 cepas correspondientes a los géneros *Streptococcus* y *Staphylococcus*. Por otro lado, se identificaron 15 cepas aisladas en condiciones anaeróbicas, 6 de las cuales correspondieron a anaerobios estrictos, principalmente *Propionibacterium* spp. y otras 10 cepas anaerobias

facultativas correspondientes principalmente al género *Streptococcus* spp. y *Staphylococcus* spp. Se obtuvo para todas las cepas la media geométrica de las concentraciones mínimas inhibitorias (CMI) siendo de amoxicilina 27,44 µg/mL, de amoxicilina/ácido clavulánico 10,63 µg/mL, de tetraciclina 2,25 µg/mL, de claritromicina 59,7 µg/mL, de eritromicina 65,6 µg/mLy de metronidazol 114,03 µg/mL. A las 31 cepas bacterianas identificadas se les determinó la CMI y la concentracion mínima bactericida (CMB) de nanoalambres de cobre (NWCu) y NPCu, utilizando como control de nanopartículas de Oxido de Zinc (NPZnO). Los valores de CMI y CMB estuvieron en el intervalo de 100 y >2500 µg/mL y 250 y >2.500 µg/mL, respectivamente. Sobre la base de valores medios, la actividad antibacteriana de las tres nanoestructuras en orden ascendente fue NPCu, NWCu y NPZnO. NPCu mostraron buena actividad antimicrobiana a las concentraciones ensayadas, en comparación con otros informes de literatura. Al probar un primer modelo *ex vivo* de biopelícula aeróbica no madura multiespecie, tratada con CuNP, con la técnica de recuento en placa, se presentan diferencias significativas entre la aplicación de CuNP como medicación y como irrigante, comportándose de igual manera con la medicación convencional de hidróxido de calcio, a 1 y 7 días. En los ensayos de evaluación de viabilidad celular con microscopía confocal sobre un segundo modelo de biopelícula aeróbica no madura multiespecie, tratada con NWCu a una concentración equivalente a 6xCMI (1500 µg/mL) para *E. faecalis* , se observó que a mayor tiempo de exposición a las NWCu existió una disminución de la viabilidad. Con un tercer modelo *ex vivo* de biopelícula, en este caso, anaeróbica madura y multiespecie, se determinó que NPCu, tienen mayor actividad antimicrobiana comparada con otros sistemas de irrigación endodóntica.

Se concluye que la microbiota bacteriana asociada a conductos radiculares con diagnóstico de periodontitis apical crónica persistente en el ámbito local es diversa, y evidencia altos niveles de resistencia a los antibacterianos de uso común en el tratamiento endodóntico. Los resultados sugieren que la constitución bacteriana de la periodontitis apical crónica persistente está en relación con al menos dos características clínicas de los pacientes. Finalmente, las nanopartículas de cobre (NPCu) pueden ser una alternativa de tratamiento más efectiva que los antibacterianos intraconducto convencionales empleados actualmente en el tratamiento endodóntico.

ABSTRACT

A high prevalence of persistent chronic apical periodontitis (CAP) in filled teeth with apical lesion associated with bacterial persistence, ranging from 40% to 61%, is reported worldwide. In Chile it has been reported that the frequency of endodontic failures is 55%. However, these results are based only on clinical parameters, without providing any microbiological evidence. The medication protocols used in root canal treatments in Chile are based on foreign formulations, designed and tested in other and for other populations of the world. Bacteria isolated from root canals can create intracanal biofilms in the dentinal tubules, and the uptake and penetration of antimicrobial agents into them should be taken into consideration while evaluating therapeutic results. Metallic nanoparticles are potentially useful in this context, because their physical and chemical characteristics have the potential to penetrate biofilm, acting as antimicrobials in a very small concentration. The purpose of this thesis was to determine the composition of bacterial microbiota found in root canals associated with diagnosis of persistent chronic apical periodontitis and to evaluate the role of copper nanoparticles (CuNP) as a new intracanal antimicrobial agent. From a total of 250 teeth diagnosed with persistent CAP from July to November 2015, 24 samples were obtained from patients who met the inclusion criteria of the study. The most relevant clinical characteristics were registered. Bacterial DNA was extracted and the V3 and V4 variable regions of the 16S rRNA gene were amplified. Amplification was sequenced using the Illumina MiSeq System. Data were subsequently processed using Quantitative Insights into Microbial Ecology (QIIME) bioinformatics software. The representative non-chimera readings were pooled into operational taxonomic units (OTUs) using a 97% identity threshold and were taxonomically classified by coincidence with sequences in the Greengenes database (version gg_13_5). The percentage of coverage was estimated using Good's nonparametric coverage estimator method; Alpha diversity was assessed with *Chao 1* richness estimator; and the Shannon diversity index was calculated using Mothur bioinformatics tool. Structures of microbial communities in different samples were compared using UniFrac-based phylogenetic relationships of representative readings from different samples, and weighted UniFrac distances were

used to perform a major coordinate analysis. From all samples, specimens were then isolated and identified for cultivable species via biochemical systems such as Rapid ID 32 A (BioMerieux, Marcy-l'Eileile, France) and API 20E (BioMerieux, Marcy-l'Etoile, France). Finally, the identification of each species was confirmed by sequencing the amplification with conventional Polymerase Chain Reaction for the RNAr16S gene using P0P6 or *rrs*. Subsequently, these identified strains were determined the behavior against antimicrobials commonly used in endodontics and CuNPs, both in their planktonic form and in three *ex vivo* models of biofilm formed on dentin walls, by means of colony forming units count and confocal microscopy. Finally, bacterial diversity and the behavior of strains against antimicrobial agents were related to the clinical parameters of the patients.

The principal coordinate analysis (PCoA) indicated a separation between individuals with ASA I and ASA II-III clinical history based on the phylogenetic relationship of the root canal bacterial community. Estimates with the *Chao 1* index were not different between the groups, except for medical history, where ASA II-III had the highest richness estimate as well as the highest Shannon diversity index. The level of PAI 5 increased the diversity index of Shannon compared to PAI 4, and this index was reduced in symptomatic patients. In all groups, *Proteobacteria* was the most abundant phylum followed by *Bacteroidetes*. Unquestionably, *Pseudomonadaceae* was the most abundant family, followed by small variations in the abundance of other taxonomic groups depending on the history of the patient. Sixteen strains isolated in aerobic conditions were identified, 10 of which corresponded to *Pseudomonas* spp. and another 6 strains corresponded to the group of *Streptococcus* spp. and *Staphylococcus* spp. On the other hand, 15 strains isolated in anaerobic conditions were identified, 6 of which corresponded to strict anaerobes mainly *Propionibacterium* spp. and another 10 facultative anaerobic strains corresponding mainly to the group of *Streptococcus* spp. and *Staphylococcus* spp. A geometric mean of the minimum inhibitory concentrations (MIC) was obtained for all strains, amoxycillin being 27.44 µg/mL, amoxicillin/clavulanate 10.63 µg/mL, tertacycline 2.25 µg/mL, clarithromycin 59,7 µg/mL, erythromycin 65.6 µg/mL and metronidazole 114.03 µg/mL. MIC and minimum bactericidal concentrations (MBC) were determined for the 31 bacterial strains tested and exposed to cooper

nanowires (CuNW), CuNP; and a zinc oxide nanoparticles (ZnONP) control was used, based on three repeated determinations. MIC and MBC values ranged from 100 µg/mL to> 2500 µg/mL and 250 µg/mL to> 2,500 µg/mL, respectively. On the basis of mean values, the activity of the three nanostructures in ascending order was CuNP, CuNW and ZnONP. CuNP showed good antimicrobial activity at the concentrations tested, compared to other reports. When testing a first *ex vivo* model of non-mature multispecies aerobic biofilm treated with CuNP, with the plaque-counting technique, there are significant differences between the application of CuNW as medication and as irrigant, an effect similar to the one obtained with conventional medication of Calcium Hydroxide, at 1 and 7 days. In the evaluation of cell viability with confocal microscopy, on a second multispecies non-mature aerobic biofilm model, treated with CuNW at a concentration of 6xMIC (1500 µg/mL) to *E. faecalis*, it was observed that the longer the exposure to CuNW the greater the decrease in viability. With a third ex vivo biofilm model, in this case, anaerobic, mature and multispecies, it was determined that CuNPs have higher antimicrobial activity compared to other endodontic irrigation systems.

It is concluded that the bacterial microbiota in root canals with an associated diagnosis of persistent CAP at country level is diverse and shows high resistance to antimicrobial agents commonly used in endodontic treatment. The results suggest that the bacterial constitution of persistent chronic apical periodontitis is related to at least two clinical characteristics of the patients. Finally, copper nanoparticles (CuNP) could be a more effective treatment alternative than the conventional intracanal antibacterial agents currently used in endodontic treatments.

CAPITULO I: INTRODUCCIÓN

La pulpa es un tejido que se encuentra ocupando la cámara y el sistema de conductos radiculares dentro de las piezas dentarias, manteniéndose aséptica en condiciones de salud. Las vías a través de las cuales los microorganismos alcanzan a la pulpa dental son múltiples y finalmente les permiten invadir, colonizar y producir la necrosis pulpar, lo que se traduce en una inflamación de los tejidos del periodonto apical[1,2]. Las bacterias son el principal agente etiológico de la periodontitis apical, tanto de su presentación primaria como del cuadro secundario o persistente luego de una obturación radicular[2]. El cuadro primario es causado por microorganismos que inicialmente invaden y colonizan el tejido pulpar produciendo una necrosis por licuefacción y que es la causa del proceso de inflamación del tejido periapical. Este tipo de infecciones es de naturaleza polimicrobiana. Técnicas de identificación fenotípicas como las últimas técnicas moleculares, mucho más exactas, indican que aproximadamente un 70% de las bacterias son anaerobias estrictas o microaerófilas. *Peptostreptococcus* spp., *Fusobacterium* spp., *Prevotella* spp. y *Porphyromonas* spp son miembros comunes en infecciones primarias[3-6], describiéndose para un mismo conducto que la microbiota apical es más diversa que aquella microbiota coronal, por lo tanto hay variabilidad intraconducto[7].

La presentación secundaria o persistente puede ser causada por bacterias de la infección primaria y que fueron capaces de resistir los procedimientos endodónticos y la privación de nutrientes, o también por contaminación intra o post tratamiento[8,9]. Un informe de muestras de periodontitis apical crónica persistente destacó la alta prevalencia de especies de *Pseudomonas* spp. así como *Burkholderiales* spp., asociando su presencia a contaminación por filtración desde la saliva al conducto radicular. También informó que diferentes raíces de un mismo diente con infección secundaria muestra baja similitud en la composición bacteriana, mientras que una muestra equivalente obtenida de la infección primaria contenía poblaciones casi idénticas[10]. La periodontitis apical crónica persistente es considerada una infección polimicrobiana, pero más reducida en especies que el cuadro primario. A la fecha se han encontrado tres o más especies de bacterias Gram negativas y Gram positivas

anaerobias estrictas y una a dos especies de anaerobias facultativas Gram positivas por conducto[3,6,11]. Las especies que establecen una infección persistente del conducto radicular expresan rasgos fenotípicos que comparten en común como la capacidad de penetrar e invadir la dentina, un patrón de crecimiento cohesivo, la resistencia a los antimicrobianos utilizados en el tratamiento de endodoncia, así como capacidad de sobrevivir a períodos de carencia de nutrientes y de evadir la respuesta inmune del hospedero[6,10].

Enterococcus faecalis, cocácea Gram positiva anaerobia facultativa, ha sido por más de 30 años asociada como principal responsable de los fracasos endodónticos por causa infecciosa[1,2]. Sin embargo, algunas publicaciones actuales ponen en duda esta aseveración, ya que informan que otras especies bacterianas[6,9,11-14] pueden ser patógenos predominantes asociados con infecciones endodónticas secundarias. Las variaciones pueden deberse a enfoques metodológicos diferentes para el muestreo y detección, diferentes condiciones clínicas o las distintas localidades geográficas de los sujetos estudiados[2]. Es así que, aunque se sigue utilizando dicha especie, principalmente en estado planctónico, como parámetro o estándar de comparación en diversos estudios, actualmente es cuestionable, ya que no se asemeja a la composición bacteriana real informada en este cuadro clínico en la actualidad. Los ensayos para probar nuevas moléculas antibacterianas deben realizarse en modelos de biopelícula multiespecie, lo mas parecido sal conducto radicular *in vivo*, para obtener información de su eficacia en condiciones lo más cercanamente posible de la situación clínica[15].

Estudios moleculares que comparan la microbiota de infecciones primarias en pacientes que residen en diferentes lugares geográficos han revelado diferencias significativas en la prevalencia de algunos patógenos[10,11]. Diversos análisis han confirmado que algunas especies son más frecuentes en algunas regiones a nivel mundial demostrando que los perfiles de la comunidad bacteriana pueden tener un patrón relacionado con la geografía donde habita el hospedador, como la etnia, la dieta y otros factores como la tasa de consumo de antibioticos de los habitantes. En otras palabras, a pesar de la variabilidad interindividual en la estructura de las comunidades bacterianas, la microbiota de endodoncia de los individuos que residen en la misma región son más similares entre ellos en comparación con individuos de lugares

distantes[16,17]. No hay estudios comparativos de casos de periodontitis apical crónica persistente en distintas poblaciones a nivel mundial. Pero si se comparan los reportes de este específico cuadro clínico, realizados principalmente en Alemania, Estados Unidos y Brasil, se pueden apreciar diferencias, en cuanto a la composición taxonómica de las comunidades bacterianas[4-6,11,12,18-22]. También se ha informado diferencias en los perfiles de susceptibilidad a los antibióticos de los aislados bacterianos orales[23]. El escenario se torna aún más complejo si consideramos que se informa la presencia de biopelículas intrarradiculares en 80% de las infecciones primarias y 74% de los conductos ya tratados endodónticamente[15,24,25].

Uno de los objetivos fundamentales del tratamiento radicular es desinfectar el conducto radicular[2], para reducir la carga bacteriana a niveles compatibles con la reparación del tejido perirradicular, mediante la instrumentación quimiomecánica y la medicación intraconducto. No obstante, la evidencia científica nos muestra que aun no se ha encontrado un compuesto capaz de eliminar totalmente las bacterias que colonizan el sistema de conductos radiculares luego de una necrosis pulpar. La anatomía compleja de estos conductos, permite que actúen como reservorio para los microorganismos persistentes[26], donde los medicamentos e irrigantes, el sistema inmune y los procesos de reparación no pueden llegar para ejercer su acción antibacteriana[14,24,25].

La estructura morfológica de la biopelícula de la periodontítis apical varía de un caso a otro y no se identifica un patrón único para las infecciones endodónticas, sin embargo, biopelículas maduras se asocian con procesos patológicos de larga data[24]. Se ha informado que bacterias en la fase de crecimiento exponencial son las más sensibles a la medicación intraconducto; sin embargo, en una fase de deprivación de nutrientes son aún más resistentes[27]. Esta condición podría presentarse en los casos de periodontitis apical crónica persistente donde las bacterias remanentes pueden permanecer largos periodos dentro de los túbulos dentinarios en estados de privación. Otro factor importante es el tiempo de administración del antibacteriano en el conducto, que también podría determinar su grado de efectividad y la posible aparición de cepas bacterianas resistentes[28]. Los protocolos endodónticos actuales indican que el intervalo de tiempo mínimo utilizado para medicar en endodoncia es de 10-14 días, aunque

periodos de 2 a 3 semanas también son usados. Además, si los signos y síntomas se mantienen, entonces puede utilizarse un período de tiempo de medicación más prolongado[29-31]. Está descrito que las moléculas antimicrobianas pueden inducir la selección de microrganismos persistentes que son tolerantes a multidrogas y por otro lado se ha descrito un papel fundamental de estos microrganismos en la tolerancia a antibióticos dentro de biopelículas en modelos *in vitro*, lo que sugiere que además podrían ser responsables de las infecciones crónicas[26]. Es así que bacterias residuales son más frecuentes y abundantes en ramificaciones, istmos y túbulos dentinarios cuando los conductos radiculares fueron tratados sin un medicamento intraconducto. Por lo tanto, el uso de un agente antibacteriano entre sesiones se sugiere como necesario para maximizar la reducción bacteriana antes de la obturación[31]. Así, un antimicrobiano de contacto podría minimizar los tiempos de exposión.

Contextualizando, mundialmente se describe una alta prevalencia de periodontitis apical crónica persistente asociada a persistencia bacteriana, que va desde 40 % a 61 %[32-41]. En Chile, una tesis desarrollada por Barriga y col.[42] (2004) investigó los factores de fracaso de tratamiento endodóntico, en pacientes atendidos en la Facultad de Odontología de la Universidad de Concepción e indicó que 55 % de los fracasos era de origen endodóntico, de los cuales 88% se atribuye a causa bacteriana, residual o remanente del tratamiento original. No existen informes de la microbiota asociada a los cuadros de periodontitis apical crónica persistente a nivel local. Los tratamiento que en Chile se aplican, derivan de protocolos endodónticos realizados en el extranjero, los que pudiesen no ser efectivos porque la microbiota asociada a estos cuadros clínicos podría ser diferente. Es asi que buscar alternativas a los agentes antibacterianos utilizados en endodoncia, considerando todo el contexto microbiológico se torna fundamental para el éxito del tratamiento[2,43].

CAPITULO II: ANTECEDENTES GENERALES

II.I COBRE COMO AGENTE ANTIBACTERIANO

El cobre es un metal esencial para el metabolismo de las células y corresponde a un oligoelemento presente en la mayoría de los organismos, con más de 30 tipos de proteínas que lo contienen[44]. A su vez, en los sistemas biológicos y a bajas concentraciones, el cobre se comporta como un micronutriente fundamental para la mayoría de los organismos, debido al rol que cumple como cofactor en asociación con diversas enzimas involucradas en reacciones de óxido-reducción, como la citocromo oxidasa y la superóxido dismutasa[45]. Su actividad antimicrobiana es reconocida mundialmente y ha sido recientemente registrado en la Agencia de Protección Ambiental de EE.UU. como el primer material antimicrobiano sólido[44]. Ya en el año 2200 A.C. se utilizó para esterilizar heridas y agua potable y en el siglo XIX se observó que los trabajadores del cobre eran inmunes al cólera, generalizándose su uso en el siglo XX[44,46]. Gracias a sus propiedades fisicoquímicas se utiliza frecuentemente, incorporándolo en la fabricación de cables, cañerías y artículos electrónicos[47].

Respecto de sus actividad antibacteriana, las bacterias mueren rápidamente sobre superficies metálicas de cobre y el término "muerte por contacto" ha sido acuñado para este proceso. En algunos casos se ha observado que la muerte por contacto se produce a una velocidad de al menos 7 a 8 logarítmos por hora y, además, generalmente no se recupera microorganismos vivos de las superficies de cobre después de una incubación prolongada, lo que varía según el medio de exposición[46]. En relación a su intensidad de acción bactericida, se reconoce que existiría una relación directa con la concentración de éste, observándose un efecto máximo (99,9 %) para el cobre metálico, actividad que se mantiene en aleaciones que contengan al menos 70 % de cobre[44]. El mecanismo de acción se fundamenta principalmente en la capacidad del cobre para ceder y captar electrones, facilitando la generación de especies de oxígeno reactivo (ROS, por *reactive oxygen species*) en una reacción de tipo Fenton, las que a su vez pueden conducir a una mayor producción de radicales hidroxilos tóxicos[46]. Estos radicales participan en una serie de reacciones perjudiciales para las macromoléculas celulares, alterando la estructura de las proteínas, lo que se traduce en la pérdida de la

función proteica[48]. El cobre puede competir con otros iones metálicos, como el hierro de grupos de enzimas deshidratasas[49], y además, puede peroxidar los ácidos grasos de la membrana, provocando un aumento de la permeabilidad y la lisis celular[50]. Finalmente se describe que puede desnaturalizar el ADN[51] e inhibir la formación de biopelículas[48].

Un estudio realizado por Warnes SL y col. (2012), acerca de la tranferencia horizontal de genes de resistencia antibiótica en superficies abióticas elaboradas con aleaciones metálicas, demostró que en superficies de cobre, además de la muerte rápida de cepas de *Escherichia coli* y *Klebsiella pneumoniae* resistentes a antibióticos se produjo la degradación del ADN plasmídico y del ADN genómico, lo que tiene una implicancia en la prevención de la propagación de la infecciones y la transferencia horizontal de genes. En condiciones húmedas o secas de material contaminado con cepas de *E. coli* y *K. pneumoniae* que portaban genes de resistencia como $bla_{CTX\text{-}M\text{-}15}$ y $bla_{NDM\text{-}1}$, respectivamente, los patógenos murieron rápidamente en superficies de cobre a temperatura ambiente (21°C)[51]. Se informó que al exponer bacterias sobre superficies secas de cobre, las células sufrieron daños extensos en la membrana externa a los pocos minutos. Además, las células extraídas mostraron pérdida de la integridad celular. El contacto por pocos minutos con superficies metálicas de cobre no resultó en un aumento de las tasas de mutación o lesiones del ADN bacteriano[50].

Chile es uno de los países con mayor reserva de cobre en el mundo y aun no hay mucha evidencia del uso de este material en el área odontológica.

Sin embargo, el cobre al formar parte natural de ciertas estructuras bacterianas, posee mecanismos de tolerancia al cobre iónico, que permiten mantener su correcto nivel al interior de las células, descritos en diversos géneros bacterianos, incluyendo los que componen el grupo de patógenos incluidos en el acrónimo ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* y *Enterobacter* spp.)[47].Estos mecanismos de tolerancia al cobre van desde el secuestro extracelular de iones, impermeabilidad de las membranas bacterianas externas e internas, proteínas de barrido de cobre parecidas a las metalotioneínas (*metallothionein-like copper-scavenging proteins*) a expulsión activa de cobre a partir de la célula[47].

Existen estudios donde se observó una disminución de la susceptibilidad a cobre

iónico y a antimicrobianos de última generación en *K. pneumoniae* y *A. baumannii*[52] y una asociación estadísticamente significativa entre mayores niveles de tolerancia a cobre iónico y la multi-resistencia a antimicrobianos en *Enterococcus* spp.[53]. Por lo tanto es importante considerar la potencial selección de estos fenotipos donde se utilice cobre como agente antimicrobiano. Esto se debe a que se ha descrito que la presencia de mayores grados de tolerancia a cobre iónico otorgaría una mayor sobrevida bacteriana en superficies cobrizadas[47] lo que se explica debido a que la actividad antimicrobiana del metal es mediada por la liberación de iones de cobre[50]. Es por ello que esto apunta a la posibilidad de selección de cepas multi-resistentes, para lo cual sería sumamente relevante realizar a futuro ensayos de supervivencia de cepas, sobretodo del tipo *Enterococcus* spp. a superficies de cobre.

II.II NANOPARTÍCULASANTIMICROBIANAS

Las enfermedades infecciosas son una amenaza mundial para la salud humana y el uso excesivo e indebido de antibióticos ha seleccionado microorganismos resistentes a los antimicrobianos que pueden desafiar el tratamiento clínico. La búsqueda de agentes antimicrobianos seguros y alternativos para tratar las infecciones producidas por tales microorganismos, es una tarea encomendada a la comunidad científica y el nacimiento de la nanotecnología se vislumbra como una oportunidad.

Durante las dos últimas décadas diversas nanoestructuras metálicas, conocidas comúnmente como nanopartículas metálicas, se han convertido en una posible alternativa para controlar microorganismos resistentes a las diversas clases de antibióticos. Su vasta gama de propiedades fisicoquímicas permite que dichas nanopartículas actúen como agentes antimicrobianos a través de diversos mecanismos[54,55]. En odontología se describe el uso de nanopartículas antimicrobianas como componentes de revestimientos de dispositivos protésicos, agentes de aplicación tópica y dentro de los materiales dentales de operatoria, pero aún hay muy pocos reportes acerca de su aplicación como medicación en Endodoncia[56]. Las nanopartículas tienen un diámetro≤100 nm y se las encuentra de diversas formas, ya sea cilíndricas, circulares, en alambre, etc. con una longitud que va desde 5-100 nm hasta micrómetros en algunos casos[43]. Cualquiera sea su naturaleza, ya sea metálicos o poliméricos, con

un área de superficie activa, reactividad química y actividad biológica, son a menudo, radicalmente diferentes a las de partículas de un tamaño mayor. Estas características permiten que las nanopartículas interactúen estrechamente con las membranas microbianas y, por lo tanto, su efecto antimicrobiano no se debe exclusivamente a la liberación de iones metálicos[56]. Las nanopartículas son potencialmente útiles dentro de este contexto, ya que es posible alterar sus propiedades físicas y químicas. Este conocimiento es importante desde la perspectiva del uso seguro de los nanomateriales[57,58]. Ha sido claramente demostrada una relación inversa entre el tamaño de las nanopartículas y la actividad antimicrobiana, donde las partículas en el rango de tamaño de 1-10 nm tienen una mayor actividad antimicrobiana en comparación con partículas de mayor tamaño. En bacterias Gram-negativas se ha informado que las nanopartículas actuaron principalmente en el intervalo de 1-10 nm[58]. Se adhieren a la superficie de la membrana celular y perturban drásticamente funciones como la permeabilidad y la respiración celular. Son capaces de penetrar dentro de la bacteria y causar más daños por posible interacción con azufre y compuestos que contienen fósforo, tales como el ADN, y además liberan iones, los cuales adicionalmente contribuyen en el efecto bactericida de las nanopartículas[57]. Sin embargo, las más pequeñas son más tóxicas que las partículas más grandes, y más aún cuando se oxidan[59].

Se sugiere que las bacterias son mucho menos propensas a adquirir resistencia a las nanopartículas de metal en comparación a antibióticos convencionales, debido a que los metales pueden actuar sobre una amplia gama de blancos microbianos, y tendrían que ocurrir muchas mutaciones para que los microorganismos resistan su actividad[56]. Sin embargo, aunque existe un estudio que menciona que los mecanismos de transferencia horizontal de genes podrían estar operando en el ámbito de resistencia a nanopartículas, solo infiere que la diferencia en la actividad antimicrobiana de nanopartículas de plata frente a diferentes especies y a su vez entre cepas de una misma especie, podría deberse a islas genómicas que se adquieren a través de transferencia horizontal de genes, que estén codificando enzimas para resistir la actividad antimicrobiana de las nanopartículas de plata, no hay datos concluyentes al respecto[60].

La forma de la nanoestructura también puede afectar a la actividad de las nanopartículas, como se ha demostrado contra cepas de *E. coli*[61]. Nanoplacas de plata triangulares mostraron la mayor actividad biocida en comparación con las nanopartículas esféricas y con forma de varilla. Las diferencias parecen explicarse por la proporción de las facetas activas en las nanopartículas de diferentes formas[55,61].

II.III NANOPARTÍCULAS DE COBRE

Las propiedades antimicrobianas de nanopartículas de cobre (NPCu)[54-56,62] son unas de las más descritas, en comparación con los antibióticos tradicionales. Las nanopartículas son eficaces a concentraciones 1000 veces menores a las que se necesitan para ejercer la actividad antibacteriana de los antibióticos. Cepas de patógenos periodontales *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Prevotella intermedia* y *Aggregatibacter actinomycetencomitans* fueron susceptibles a las nanopartículas de plata y óxido de cobre en condiciones anaeróbicas, con concentraciones mínimas bactericidas en el rango de 250 a 2500 µg/ml[63]. Mas aun, el cobre es más barato que la plata, es fácilmente miscible con polímeros y relativamente estable química y físicamente[62].

Si bien el cobre puede tener un modo de acción similar a la plata, se cree que existe un comportamiento diferente frente a cepas específicas[64]. La clave está en la acción del cobre sobre grupos-SH de las enzimas microbianas. Se ha demostrado que las nanopartículas de cobre tienen mayor afinidad que las nanopartículas de plata, a las aminas y los grupos carboxilo de las proteínas, presentes en una alta densidad, en la superficie de cepas de *Bacillus subtilis,* lo que se traduce en una actividad antimicrobiana superior. Los resultados de este trabajo refuerzan el hallazgo de que NPCu actúan mejor sobre cepas de *B. subtilis* en comparación a las nanopartículas de plata[65], no así sobre cepas de *Staphylococcus aureus* y *E. coli*[64]*.* Las NPCu en suspensión muestran actividad contra una variedad de patógenos bacterianos, incluyendo *S. aureus* resistentes a meticilina (SAMR) y *E. coli*, con concentraciones mínimas inhibitorias (CMI) que oscilan entre 0,1 y 5,0 mg/ml.

El óxido de cobre (I) u óxido cuproso (Cu_2O) es un polvo de color rojo y también se puede producir como nanopartículas. Actividad similar a la del óxido de cobre(II) u óxido

cúprico (CuO), ambos han demostrado ser efectivo como antimicrobiano contra una serie de cepas de bacterias de distintas especies[62]. Se estudiaron los daños causados por NPCu esféricas caracterizadas por su diámetro (13 ± 3 nm) con un máximo de 254 nm, sobre las cepas *S. aureus* ATCC 24213 y *Pseudomonas aeruginosa* ATCC 27833. Estas nanopartículas redujeron la viabilidad celular, con una CMI de 500 y 700 ppm para *S. aureus* y *P. aeruginosa*, respectivamente. Los resultados indicaron que las nanopartículas de cobre provocaron una cascada de señalización intracelular que produjo estrés oxidativo, lo que condujo a la generación de ROS[66]. Por otro lado, las aplicaciones de estos materiales están a menudo limitadas por su tendencia a oxidarse. Se informa que NPCu mediante un simple método de poliol, con un buen control sobre el tamaño de partícula, mostraron una buena resistencia a la oxidación en solución, una citotoxicidad limitada y excelentes propiedades antibacterianas[67]. Una tesis reciente informa que luego de la oxidación de nanopartículas de cobre, se produce una estructura tridimensional de esta, que generaría daño sobre la membrana de *Cándida albicans* de forma mecánica, lo que mantendría sus propiedades antimicrobianas disminuyendo además su toxicidad[68]. No hay reportes hasta el momento de dicha acción sobre bacterias.

En cuanto a la bioseguridad, un estudio comparó la toxicidad y acumulación de NPCu en los eritrocitos y los diferentes tejidos en la trucha arco iris (*Oncorhynchus mykiss*). Los resultados de toxicidad *in vitro* indican que el efecto es menor con el tratamiento de las células con nanopartículas de cobre y es más marcado en las células tratadas con $CuSO_4$. Estos datos permiten aseverar que las nanopartículas de cobre son menos tóxicas que en su tamaño a macroescala[69]. Otro reporte señala que la toxicidad de cobre para las células A6 de *Xenopus laevis* (rana de garras africana) depende de la forma tridimensional de la nanopartícula de cobre y del estado de la célula, de modo que los efectos tóxicos se produjeron más tempranamente para células en etapas de diferenciación, que para células en etapas de proliferación[70].

La eficacia terapéutica de las nanopartículas antibacterianas requiere la optimización de sus características físicas, químicas y biológicas, teniendo en cuenta los factores específicos del tejido en el sitio de la infección y el método para administrar las nanopartículas de manera eficaz en el tejido diana. Las estrategias de tratamiento

basadas en nanopartículas tienen el potencial de mejorar la eficacia antibacteriana de los agentes antibacterianos utilizados en endodoncia, mediante modificaciones de la superficie que proporcionarán la oportunidad de suministrar fármacos y/o productos químicos al sitio de infección con el fin de interactuar selectivamente con la biopelícula. En términos de toxicidad de nanopartículas, y su eficacia terapéutica requiere la optimización de sus características físicas, químicas y biológicas, teniendo en cuenta los factores específicos del tejido en el sitio de la infección y el método para administrar las nanopartículas de manera segura y efectiva en el tejido diana[71]. En odontología, el creciente interés en el campo de la nanotecnología, justifica una investigación sólida basada en colaboraciones científicas y clínicas para enfatizar el potencial de las nanopartículas en la endodoncia clínica en un futuro próximo ya que se describe escasamente el uso de NPCu[62,63] a nivel general y no hay reportes acerca de su aplicación como antimicrobiano en Endodoncia[70].

II.IV NANOPARTÍCULAS EN ENDODONCIA

Se han incorporado nanopartículas en materiales o adhesivos dentales para inhibir el crecimiento bacteriano a través de distintos mecanismos, pero no hay reportes del uso de nanopartículas de cobre en endodoncia[71,72]. Se ha informado una prometedora actividad antibacteriana de nanoparticulas de plata[73] y nanopartículas de óxido de magnesio, tanto en estudios *in vitro* como *ex vivo* de conductos radidulares. En comparación con la solución convencional de NaOCl (5,25%), las nanopartículas de óxido de magnesio (5 mg/L) mostraron un efecto estadísticamente significativo a largo plazo en la eliminación de *Enterococcus faecalis* adherido a la dentina del conducto radicular[74]. En Chile, la investigación de Sierra y col. (2013) estableció un punto de partida para el uso clínico del cobre, específicamente para la desinfección del sistema de conductos radiculares en el que se comparó los medicamentos usados comúnmente en endodoncia y sulfato de cobre (CuSO4) pentahidratado, el que tuvo mejor actividad antibacteriana a bajas concentraciones (1.5-12 µg/mL) que la clorhexidina (5-10 µg/mL)[75]. Con el objetivo de conocer el efecto *ex vivo* de CuSO4 sobre la especie *E. faecalis*, treinta y seis conductos radiculares de dientes humanos extraídos fueron inoculados con la cepa *E. faecalis* ATCC 29212. En los conductos medicados con

CuSO4, el recuento de bacterias se redujo 6 logaritmos en base 10 después de 4 días y se mantuvo como tal sin cambio estadísticamente significativo hasta el día 10. Estos resultados corroboraron su poder antibacteriano en un modelo intraconducto[76]. Por lo tanto, las propiedades antibacterianas del cobre a escala nanométrica también podrían tener aplicación en endodoncia, ya que el ambiente en el conducto radicular, que sirve de nicho ecológico para los microorganismos, es una superficie formada por pequeños túbulos dentinarios, de 1-5 μm de diámetro, que van desde el límite pulpar hasta el esmalte en corona y cemento en raíz, donde la dentina, segundo tejido más duro del cuerpo, conforma el mayor volumen del órgano dentario. La dentina está formada por una matriz colágena calcificada con un 70% de materia inorgánica (principalmente cristales de hidroxiapatita), 18% de materia orgánica (principalmente fibras colágenas tipo I y proteínas) y 12% de agua[77]. Es en estos túbulos donde se alojan y se establecen las bacterias formando la biopelícula endodóntica[24], y es aquí donde la nanopartícula de cobre puede jugar un rol como nuevo agente antibacteriano, ya que el cobre al ser considerado un antibacteriano de contacto en superficies de cobre, su tiempo de utilización podría reducirse al mínimo necesario.

CAPÍTULO III: HIPOTESIS, OBJETIVO GENERAL Y OBJETIVOS ESPECÍFICOS

HIPOTESIS

1. La microbiota bacteriana asociada a conductos radiculares con diagnóstico de periodontitis apical crónica persistente en el ámbito local es diversa, y posee elevados niveles de resistencia a los antibacterianos de uso común en el tratamiento endodóntico, lo que está en directa relación con al menos una característica clínica del paciente.

2. Las nanopartículas de cobre constituyen una alternativa de tratamiento más efectiva que los antibacterianos intraconducto convencionales que se emplean actualmente en el tratamiento endodóntico.

OBJETIVO GENERAL

Determinar y caracterizar la microbiota bacteriana asociada a conductos radiculares con diagnóstico de periodontitis apical crónica persistente en pacientes chilenos y evaluar el rol potencial de nanopartículas de cobre, como un innovador agente antimicrobiano intraconducto, relacionando los hallazgos con los antecedentes clínicos de los pacientes.

OBJETIVOS ESPECÍFICOS

1. Determinar la composición y diversidad bacteriana de la microbiota asociada a conductos radiculares con diagnóstico de periodontitis apical crónica persistente.

2. Determinar la actividad antibacteriana de los principales antimicrobianos de uso común en odontología y nanopartículas sobre cepas de las especies bacterianas prevalentes cultivables aisladas desde conductos radiculares con diagnóstico de periodontitis apical crónica persistente.

3. Evaluar la actividad antibacteriana de nanopartículas de cobre sobre una biopelícula endodóntica artificial constituida por cepas de las especies clínicas cultivables prevalentes aisladas desde conductos radiculares con diagnóstico de periodontitis apical crónica persistente.

4. Relacionar la diversidad bacteriana y el comportamiento de cepas frente alos antimicrobianos con los parámetros clínicos de los pacientes.

CAPÍTULO IV: METAGENOMICS STUDY OF BACTERIAL MICROBIOTA IN PERSISTENT ENDODONTIC INFECTIONS USING NEXT-GENERATION SEQUENCING.

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Abstract

Aim To determine the bacterial microbiota in root canals associated with persistent apical periodontitis and their relationship with the clinical characteristics of patients using next-generation sequencing (NGS).

Methodology Bacterial samples from root canals associated with teeth having persistent apical periodontitis were taken from 24 patients undergoing root canal retreatment. Bacterial DNA was extracted, and V3-V4 variable regions of the 16S rRNA gene were amplified. The amplification was deep sequenced by Illumina technology to establish the metagenetic relationships among the bacterial species identified. The composition and diversity of microbial communities in the root canal and their relationships with clinical features were analysed. Using parametric and nonparametric tests accounting for differences between the patient characteristics and microbial data, performed statistical analysis.

Results A total of 86 different OTUs were identified and Good's nonparametric coverage estimator method indicated that 99.9 ± 0.00001% diversity was recovered per sample. The largest number of bacteria belonged to the phylum *Proteobacteria*. According to the medical history from the American Society of Anesthesiologists (ASA)

Classification System, ASA II-III had higher richness estimates and distinct phylogenetic relationships compared to ASA I individuals ($p<0.05$). Periapical index (PAI) score 5 was associated with increased microbiota diversity in comparison to PAI score 4, and this index was reduced in symptomatic patients.

Conclusions Based on the findings of this study, it is possible to suggest a close relationship between several clinical features and higher microbiota diversity with persistent endodontic infection. This work allows provides a better understanding on how microbial communities interact with their host and vice versa.

Introduction

A variety of bacterial species has been reported to be a present in the root canals of teeth associated with the primary stages of periapical inflammation. Various identification techniques have shown that approximately 70% of these bacteria are obligate anaerobes or microaerophiles. Species of *Peptostreptococcus*, *Fusobacterium*, *Prevotella*, and *Porphyromonas* are common in primary infections (Gomes *et al.* 2008, Siqueira *et al.* 2011, Hsiao *et al.* 2012, Hong *et al.* 2013). Moreover, it been reported that the microbial community present in combined endodontic-periodontal lesions is complex and more diverse than previously thought (Gomes *et al.* 2015).

Persistent infection of periapical tissues associated with root filled teeth can be caused by microorganisms present in the primary canal infection, which were somehow able to withstand the root canal procedures and periods of nutrient deprivation (Lewis 2010, Aw 2016). Bacterial species that cause persistent infection of the root canal have common phenotypic traits, such as the ability to penetrate and invade dentine, a cohesive growth pattern, a resistance to antimicrobials used in root canal treatment, and the ability to survive nutrient deprivation and evade the host's immune response (Jungermann *et al.* 2011).

It has been reported that persistent endodontic infections include a high prevalence of *Pseudomonas* spp. and *Burkholderia* spp. (Siqueira *et al.* 2011, Anderson *et al.* 2013) and that these contaminating microorganisms would come from the saliva and pass into the root canal. Moreover, persistent apical periodontitis associated with root filled teeth

is a polymicrobial infection but contains many fewer species than the primary infection (Chugal *et al.* 2011). However, other studies have reported there is great diversity in secondary infection, suggesting no differences between the infection states (Hong *et al.* 2013)

Enterococcus faecalis, a Gram-positive anaerobic facultative bacterium, has been considered for many years as the main species responsible for post treatment endodontic diseases (Siqueira & Rôças 2004). However, several reports cast doubt on this assertion and suggest that other species may be predominant pathogens in secondary canal infections (Chugal *et al.* 2011, Anderson *et al.* 2012, Hong *et al.* 2013, Tennert *et al.* 2014). These differences may be due to different clinical conditions or different geographical locations of the subjects studied and most importantly, the diverse methodological approaches used (Anderson *et al.* 2012, Hong *et al.* 2013, Tennert *et al.* 2014). Molecular studies comparing the microbiota of primary infections in patients residing in different geographic locations have revealed significant differences in the prevalence of certain pathogens. Some bacterial species are more prevalent in particular countries, demonstrating that bacterial community profiles may have a pattern related to the geographical location where the host lives, as well as other factors such as ethnicity, diet and other lifestyle aspects (Tzanetakis *et al.* 2015). In other words, despite inter-individual variability in the structure of bacterial communities, the root canal microbiota of individuals residing in the same region are more similar to each other in comparison with individuals from distant locations (Baumgartner *et al*. 2004, Machado *et al*. 2007, Siqueira *et al*. 2008). There are no comparative studies of persistent apical periodontitis in different populations worldwide, but differences in terms of composition and prevalence of species in studies conducted mainly in Germany, the United States and Brazil can be observed (Rôças *et al*. 2008, Anderson *et al*. 2012, Tennert *et al*. 2014). Next-generation sequencing (NGS) has improved the performance and quality of studies related to elucidating the microbiomes in a diagnostic and ecological context, helping to reveal the bacterial composition and its relationship with the host. Among a wide range of technologies, Illumina sequencing has the lowest rate of sequencing errors and cost per sample (Di Bella *et al.* 2013).

Therefore, knowing the microbiota involved in persistent apical periodontitis and its

possible relationships with several clinical features is essential for the use of new therapeutic options that may ensure successful treatment. The objective of this study was to determine through a metagenetics approach the composition and bacterial diversity of the microbiota associated with root canals diagnosed with persistent apical periodontitis and the relationships with several clinical features, according the medical history from the American Society of Anesthesiologists, ASA Classification System (American Society of Anesthesiologists 2014), whose description is detailed in the American Association of Endodontic Case Difficulty Assessment Form and Guidelines (American Association of Endodontic 2010).

Materials and methods

Study population

This study was developed following the general principles of the Code of Ethics established in “The Belmont Report" (Ethical Principles and Guidelines for the Protection of Human Subjects of Research 1979). Consequently, subjects signed an informed consent included in the protocol approved by the Ethics Committee of the School of Dentistry at University of Concepción, Chile (C.I.Y.B. Nº04/15). The initial unit of analysis and experimental design (Fig. 1) consisted of a sample of 24 root canals diagnosed with persistent apical periodontitis, which were obtained from 24 patients older than 18 years who were receiving treatment at the Postgraduate Endodontics Unit at the School of Dentistry, University of Concepcion, Chile.

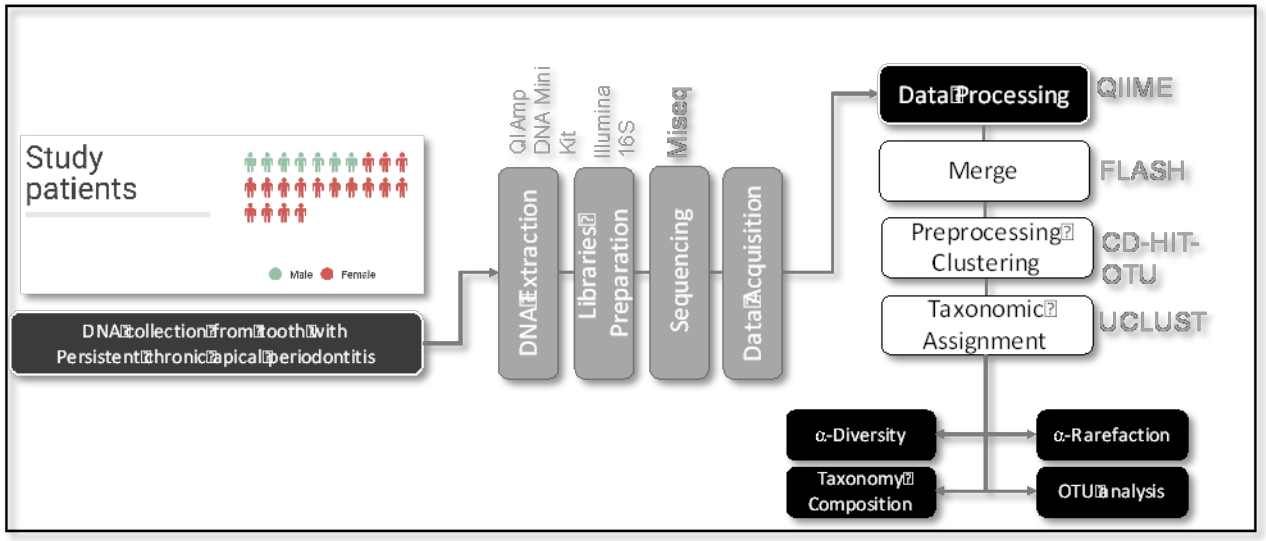

Figure 1. Experimental design and workflow.

Teeth root filled more than 2 years before the study and had evidence of periapical disease (widening of the apical periodontal ligament space or radiolucent lesion) by means of radiological examination were included. Patients were excluded if they had received antibiotic treatment in the previous 3 months, had teeth that could not be isolated with a rubber dam, teeth that had coronal leakage associated to an inadequate coronal restoration, and/or teeth that were associated with periodontal disease which is defined as infections of the structures around the teeth, which include the gums, periodontal ligament and alveolar bone. Prior to the procedure, clinical characteristics that could be correlated with microbiological findings, such as age, diet, ethnicity, oral hygiene, eating habits, use and abuse of substances, associated diseases (diabetes mellitus, hypertension, etc.), size of the periapical lesion according to the PAI index (Tarcin *et al*. 2015) and quality of root filling (Vengerfeldt *et al*. 2014) were registered for each patient and are described in Table 1.

Table 1. List of the clinical history characteristics assigned to the patients included in this study

	Patient clinical history								
Patient	**GENDER**	**AGE**	**Medical History**	**Symptoms**	**PAI INDEX (1-5)**	**Tooth Location**	**Adequacy of previous filling**	**Working length**	**Follow-up period (Years)**
1	Male	≤ 30 years	ASA I	Symptomatic	*4*	Maxillary	Inadequate	Short	≤ 10
2	Female	> 30 years	ASA I	Symptomatic	5	Mandibular	Inadequate	Short	≤ 10
3	Male	≤ 30 years	ASA I	Symptomatic	5	Maxillary	Inadequate	Short	≤ 10
4	Female	> 30 years	ASA II	Symptomatic	4	Maxillary	Adequate	CDC	>10 ≤20
5	Male	> 30 years	ASA I	Symptomatic	4	Maxillary	Adequate	Short	≤ 10
6	Female	≤ 30 years	ASA I	Symptomatic	*4*	Maxillary	Adequate	CDC	≤ 10
7	Female	> 30 years	ASA I	Symptomatic	4	Maxillary	Inadequate	Short	≤ 10
8	Female	> 30 years	ASA II	Asymptomatic	5	Mandibular	Adequate	Short	>10 ≤20
9	Female	> 30 years	ASA I	Symptomatic	*4*	Maxillary	Adequate	CDC	≤ 10
10	Female	> 30 years	ASA I	Asymptomatic	*3*	Mandibular	Inadequate	Short	≤ 10
11	Female	> 60 years	ASA II	Asymptomatic	4	Maxillary	Inadequate	Short	>20
12	Male	> 30 years	ASA II	Symptomatic	5	Maxillary	Adequate	Overfilling	>10 ≤20
13	Female	≤ 30 years	ASA I	Asymptomatic	5	Mandibular	Adequate	Short	≤ 10
14	Female	> 30 years	ASA II	Symptomatic	3	Mandibular	Adequate	Short	>10 ≤20
15	Female	> 60 years	ASA II	Symptomatic	4	Mandibular	Inadequate	Short	≤ 10
16	Male	> 30 years	ASA II	Asymptomatic	5	Maxillary	Adequate	CDC	>20
17	Female	> 30 years	ASA II	Asymptomatic	5	Maxillary	Adequate	CDC	≤ 10
18	Female	> 30 years	ASA I	Asymptomatic	3	Mandibular	Adequate	CDC	≤ 10
19	Female	> 30 years	ASA I	Symptomatic	4	Mandibular	Adequate	CDC	≤ 10
20	Female	> 60 years	ASA II	Asymptomatic	3	Maxillary	Adequate	Short	≤ 10
21	Female	> 60 years	ASA II	Asymptomatic	5	Mandibular	Inadequate	Short	≤ 10
22	Male	> 60 years	ASA III	Symptomatic	5	Maxillary	Adequate	CDC	≤ 10
23	Female	≤ 30 years	ASA I	Symptomatic	4	Maxillary	Adequate	CDC	≤ 10
24	Male	≤ 30 years	ASA I	Asymptomatic	3	Maxillary	Adequate	CDC	≤ 10

Abbreviations: ASA - Physical status classification system according to American Society of Anesthesiologists; PAI index: Periapical index

CDC: Cementary dentine constriction

Root canal samples

Samples were obtained from the root canals by an endodontist, under strict aseptic conditions (Schirrmeister *et al*. 2009) with modifications (Tzanetakis *et al.* 2015). Briefly, the tooth was cleaned with pumice and isolated with a rubber dam. The tooth and the operating field were decontaminated and disinfected with a 30% hydrogen peroxide solution (H_2O_2) and then with 2.5% sodium hypochlorite solution (NaOCl). The access cavity was prepared with a high-speed sterile carbide bur, and before the pulp exposed, the cleaning of the tooth and the rubber dam was repeated as previously described. The NaOCl solution was quenched with 5% sodium thiosulfate in order to avoid interference with the process of taking bacteriological samples. To control the sterility of the operating field, samples of the disinfected tooth crown were taken with two pellets of cotton and transferred to a tube containing reduced transport fluid (RTF). If growth occurred or was detected after 72 h of anaerobic incubation, the sample of the root canal was excluded from the study.

Gutta-percha in the coronal canal was removed using Gates-Glidden burs (Dentsply Sirona, Ballaigues, Switzerland). The apical portion of the root canal filling was removed with K- and/or H-type files and ProTaper retreatment files (Dentsply Sirona). No solvent was used. All root filling material removed from the apical portion of the canals was transferred into a tube containing 0.75 mL RTF. Radiographs were used to ensure that the entire root filling had been removed. Apical patency was obtained and the working length established with the help of an electronic apex locator (Propex PIXI Dentsply Sirona) and subsequent radiographic control. Canal walls were filed gently again at full working length with K files until size 30 (Dentsply Sirona), using sterile saline solution as the irrigant. After use, the active portion of each instrument was stirred in a cryotube with 0.75 mL RTF to obtain the dentine debris. After using size 30 hand files, 0.2 mL of sterile saline solution (Difco, Detroit, MI, USA) were placed into the root canal with a 27G endodontic irrigation syringe with a needle (Monoject^{TM}, Covidien Mansfield, MA, USA), and the contents of the root canal were absorbed into four consecutive sterile paper points. Each paper point was held in place within the canal at working length for one minute and transferred into the same tube with 0.75 mL RTF in which the active

portions of the files had been rinsed. Both samples, 0.75 RTF with root filling material removed from the apical portion and 0.75 RTF where active portions of the files had been rinsed and paper points were placed, were processed within the next three hours. Then, root canal treatment of each tooth was completed using the appropriated canal instrumentation, disinfection and root filling.

DNA extraction

Genomic DNA from the sample kept in RTF, both from the gutta-percha remnants and from the canal walls of the apical third, was extracted using a QIAamp® DNA Mini Kit (Qiagen, GmbH, Hilden Germany) according to the manufacturer's instructions. DNA quantitation was performed using a NanoDrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) to ensure that the quantity and quality were within the required parameters to obtain an amount of 20 µL with a minimum concentration of 1 ng/µL and a density higher than 1.8 from an OD260 reading. Quality was assessed by performing conventional PCR with primers P0/P6 for bacterial 16S rRNA (Di Cello *et al.* 1997). For high-throughput sequencing, V3 and V4 variable regions of the 16S rRNA gene were amplified using primers (Klindworth *et al*. 2013) according to the Illumina 16S Metagenomic Sequencing Library Preparation instructions. Briefly, libraries for each of the 24 samples were prepared using the limited PCR cycle and adding Illumina sequencing adapters for amplification of the amplicon and Nextera XT (Illumina, San Diego, USA) barcode indices. Samples were pooled in equal proportions and paired-end sequenced for 300 cycles in a MiSeq Platform (Macrogen, Seoul, Korea). PhiX viral DNA was added to the sample pool as a control for focusing, template building, and phasing, thus mitigating possible problems arising from the sequencing of a low diversity library such as 16S rRNA.

Metabarcoding Analysis

Paired-end reads were demultiplexed, and Illumina adapters were removed by Macrogen's in-house pipeline before being reported in FASTQ format. The paired-end sequences for each sample were merged using FLASH (Magoč & Salzberg, 2011) with a minimum overlap threshold of ten base pairs. Data were further processed using the Quantitative Insights into Microbial Ecology (QIIME) bioinformatics pipeline. The resulting sets of merged reads were pre-processed to trim low-quality and chimeric

reads, and general denoising was applied using CD-HIT-OUT (Fu *et al*. 2012). The remaining representative, non-chimeric reads were clustered into operational taxonomic units (OTUs) using a 97% identity threshold. The reads were taxonomically classified by matching against sequences in the Greengenes database (version gg_13_5). Matching was performed in QIIME using the UCLUST algorithm (Edgar 2010). Samples were rarified, and coverage percentage was estimated by Good's nonparametric coverage estimator method; alpha diversity was evaluated with a bias-corrected Chao1 richness estimator; and the Shannon diversity index was calculated through Mothur (Schloss *et al*. 2009). Microbial community structures in different samples were compared using UniFrac based on the phylogenetic relationships of representative reads from different samples, and the weighted UniFrac distances were used to perform a principal coordinate analysis.

Statistical Analysis

Data from alpha diversity indices were screened for normality and were Box-Cox transformed to assist normality standards. Differences between the different patient characteristics (descriptors) were tested with a two-way ANOVA, followed by a post hoc Tukey's HSD test when applicable. The levels ASA II and ASA III of the descriptor "Medical history" were evaluated as ASA II-III, and the level overfilling of the descriptor "Working length" was eliminated from analysis due to the reduced number of individuals. All statistical analyses were performed using the JMP 9.0 software package (SAS Institute Inc., Cary, NC, USA).

Results

Sequencing

Merged paired-end reads by FLASH resulted in 7,296,106 reads distributed across the 24 samples (Supplementary material: Table 2). After filtering for quality and ambiguities, Illumina MiSeq sequencing returned a total of 2,248,552 reads that were further evaluated. A total of 86 different Operational Taxonomic Units (OTUs) were identified from the 24 samples, and Good's nonparametric coverage estimator method indicated that 99.9 ± 0.00001% diversity was recovered per sample. All samples reached a saturation plateau, as shown by the rarefaction curves constructed from OTUs (Fig. 2a).

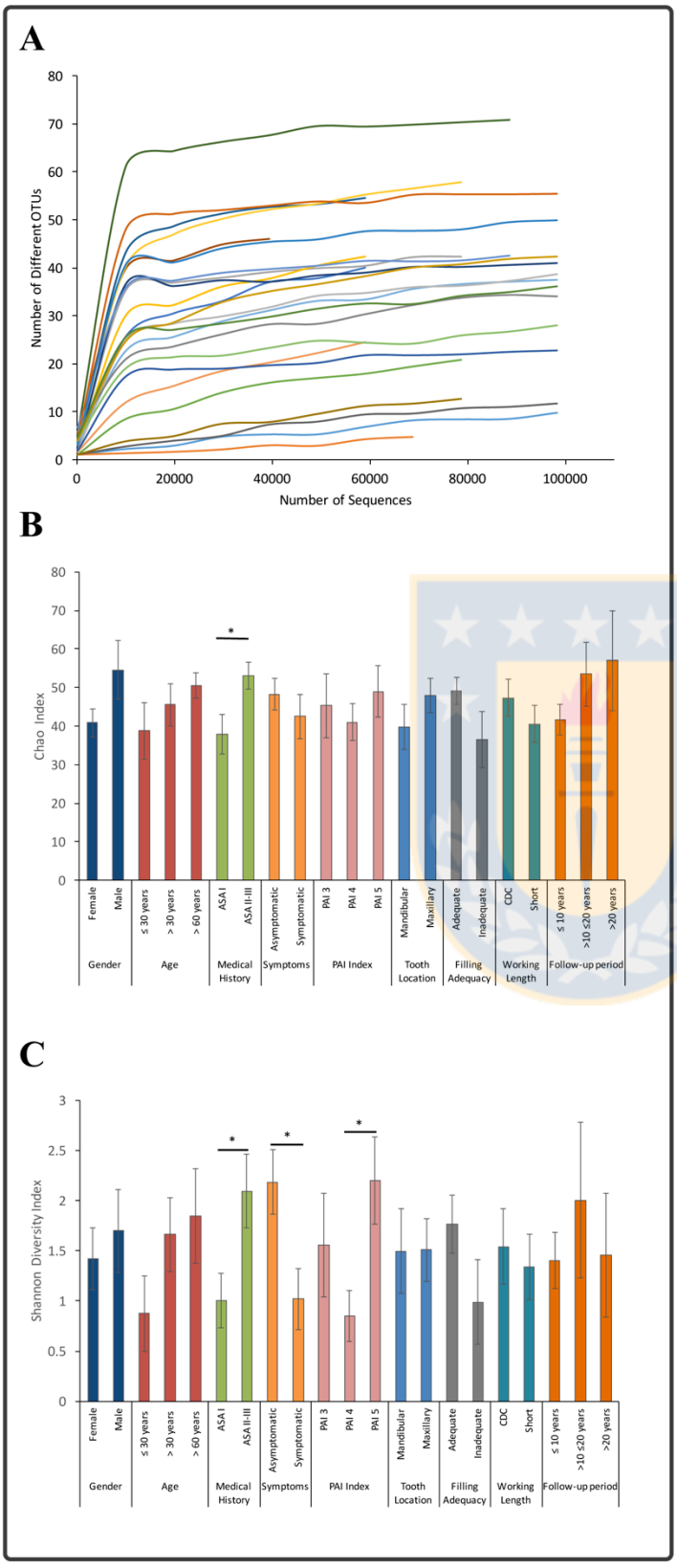

Figure 2. Alpha diversity of bacterial communities in root canals diagnosed with persistent chronic apical periodontitis. (a) Rarefaction curves of microbial communities from the 24 samples and a summary of the patients' descriptors with sample codes and categories; (b) the Chao1 richness estimator; (c) the Shannon diversity index. Columns represent the average values for each group. Error bars indicate standard deviation, and asterisks indicate significant differences between groups with horizontal black lines (two-way ANOVA; Tukey's HSD, $P<0.05$).

The sampling quality was excellent, which is expressed in the asymptotic curves, which means, the probability of adding a new species to the final samples reaches zero. Therefore, the sequencing results contained most of the bacterial species present.

Richness and Diversity

Chao1 richness estimates were not different between groups, except regarding medical history, where ASA II-III patients had a higher richness estimate (Fig. 2b) and a higher Shannon diversity index (Fig. 2c). The PAI level 5 increased the Shannon diversity index in comparison to PAI 4, and this index was reduced in symptomatic patients.

Principal coordinate analysis indicated a separation between individuals with ASA I and ASA II-III medical histories based on the phylogenetic relationships of the root canal bacterial communities (Fig. 3).

Microbiota taxonomical composition

In all patients, *Proteobacteria* was the most abundant phyla, followed by *Bacteroidetes* (Fig. 4a). Some differences were observed between individuals less than 30 years old compared to older patients who had a greater abundance of less represented phyla such as *Actinobacteria* or *Tenericutes*. The differences between ASA I and ASA II-III patients were notable, with the latter having a higher abundance of *Bacteroidetes*, *Firmicutes* and *Tenericutes*, than ASA I. Patients with any symptomology also exhibited different microbiota abundances because in the symptomatic patients, a greater abundance of Proteobacteria was observed compared to the asymptomatic patients, who had a higher abundance of *Bacteroidetes*. Unquestionably, the most abundant family of bacteria was *Pseudomonadaceae*, followed by minor variations in abundance by other taxonomic groups depending on the different descriptors (Fig. 4b).

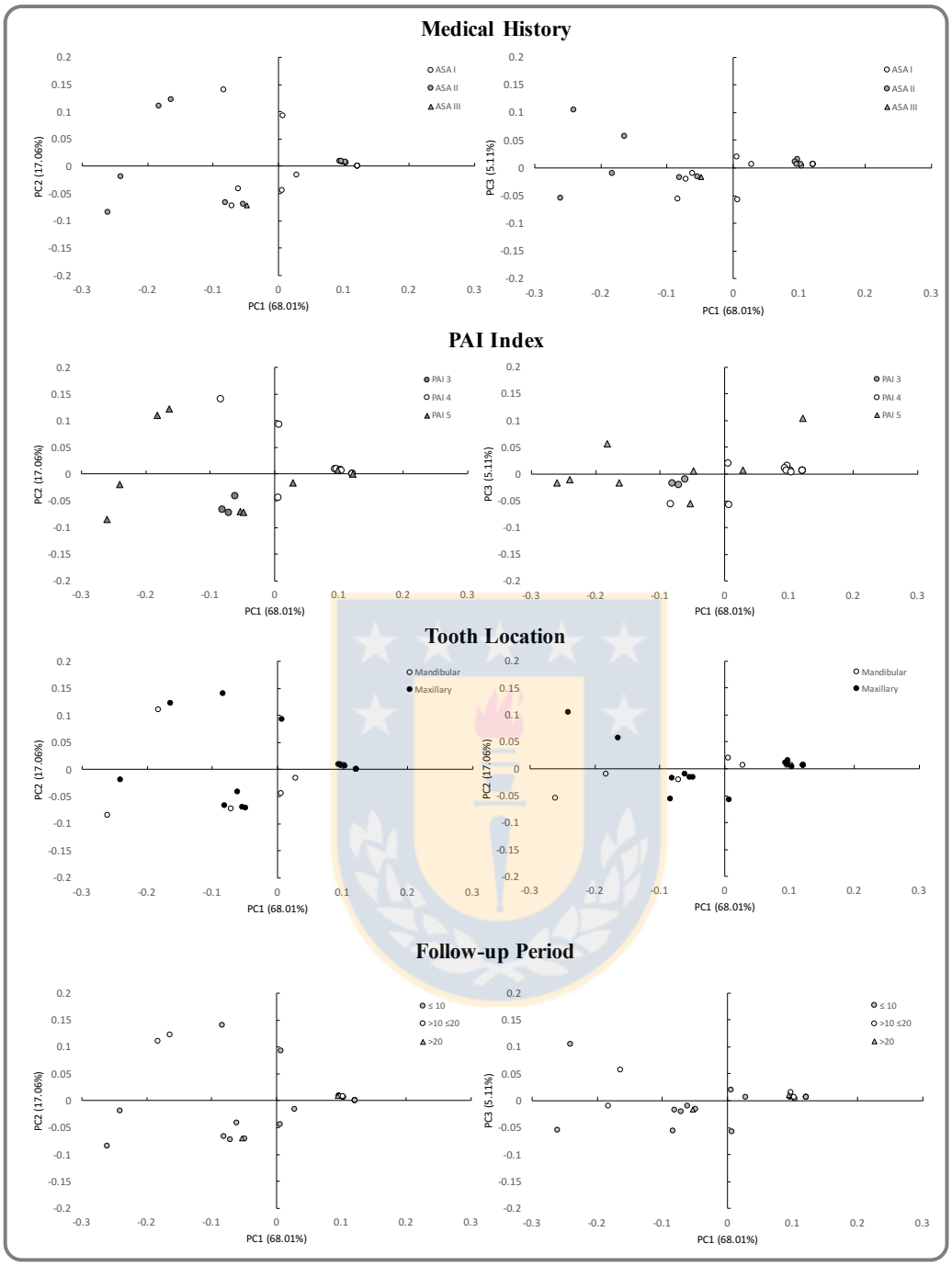

Figure 3. Principal coordinate analysis score plot representing the phylogenetic relationships between root canals diagnosed with persistent chronic apical periodontitis according to patients' medical history, PAI index, tooth location and follow-up period. Beta diversity was calculated for all samples using the UniFrac metric for the V3 and V4 regions of the 16S rRNA gene, and a principal coordinate analysis was performed based on weighted UniFrac distances. PC1, PC2 and PC3 are principal coordinates 1, 2 and 3, respectively.

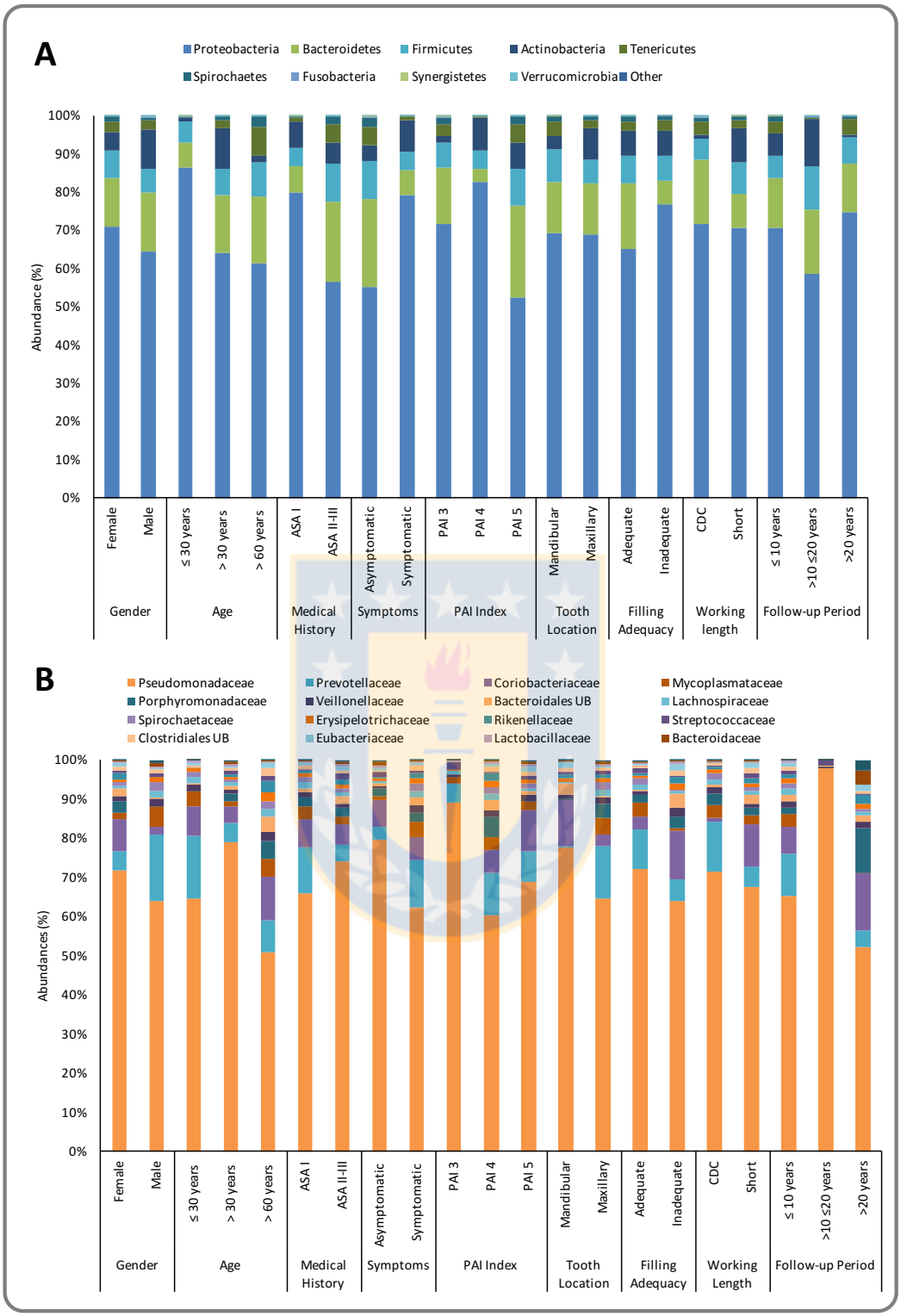

Figure 4. Taxonomical composition of (a) phyla and (b) families of bacterial communities found in the canal roots diagnosed with persistent chronic apical periodontitis of 24 patients, grouped by descriptors (see Table 1 for descriptor details).

Discussion

Currently, high-throughput sequencing technologies allow the evaluation of bacterial compositions in a more accurate way, allowing a better understanding of the role of prokaryotes in the pathogenesis of apical periodontitis. In this study, amplicon sequencing of the V3 and V4 hypervariable regions of the 16S rRNA gene was used to assess taxonomic diversity in persistent apical periodontitis and elucidate clinical patterns in community structure with the Illumina MiSeq sequencing by synthesis. In the same context, many previous studies had been performed by barcoded multiplex sequencing using 454 pyrosequencing (Santos *et al*. 2011, Siqueira *et al*. 2011, Hong *et al*. 2013, Anderson *et al*. 2013, Tzanetakis *et al*. 2015). However, while it is an improvement over other methods to evaluate bacterial composition, 454 pyrosequencing is limited by a relatively high reading error rate. In contrast, the Illumina sequencing platform has a substantially lower error rate than the 454 pyrosequencing platform, and the reading length is deterministic rather than random (Gloor *et al*. 2010, Diaz *et al*. 2012, Di Bella *et al*. 2013). Therefore, the Illumina platform is not susceptible to misinterpreting the number of nucleotides in homopolymer regions. In next-generation sequencing platform technologies, bases are inferred from light intensity signals, a process commonly referred to as base-calling. This type of base-call error is more pronounced in pyrosequencing (Gloor *et al*. 2010, Diaz *et al*. 2012).

In this study, the phylum with the highest number of bacteria was *Proteobacteria*, in agreement with previous reports (Siqueira *et al.* 2011, 2016, Saber *et al.* 2012,). It should be noted that these three reports are from individuals residing on the American continent. Other reports developed in Asia, Europe or Africa have reported higher numbers of bacteria belonging to the phyla *Bacteroidetes* (Hong *et al*. 2013, Tzanetakis *et al*. 2015) and *Firmicutes* (Santos *et al*. 2011, Hsiao *et al.* 2012, Anderson *et al.* 2013, Vengerfeldt *et al.* 2014). However, a direct relationship between geographic location and taxonomic composition cannot be established since other factors may influence the observed composition, such as the type of NGS tool used. Only one report has been published with the Illumina MiSeq system, and the results obtained in relation to the taxonomic composition are consistent with the present results (Siqueira *et al.* 2016).

Although there are many studies that report the composition of the microbiota

associated with persistent endodontic lesions, few used NGS, and only one study involved a Latin American population (Siqueira *et al.* 2016). If the composition of the bacterial community in cases of persistent apical periodontitis reported in other populations is compared with the present findings in Latin America, there is agreement over the low reports of *E. faecalis* found in the Asian population, with only 0.7% of the cases reporting *E. faecalis* (Hong *et al.* 2013); in an African population, with 17.5% cases reported (Anderson *et al*. 2013); and in a European population, with 33% cases reported (Vengerfeldt *et al.* 2014). In Latin America, the highest value was reported, with 40% of the cases reporting *E. faecalis* (Siqueira *et al.* 2016), very similar to the present study, and both in relatively low abundance. The exclusion of teeth with coronal leakage associated to an inadequate coronal restoration, could be the cause of the low number of reports of this bacterial species, as has been mentioned by other reports (Hong *et al.* 2013).

In the present study, *Pseudomonas* spp. was observed in all samples and at a high percentage in some cases. Indeed, a high prevalence of *Pseudomonas* spp., as well as Burkholderiales, has been reported in samples of secondary endodontic infections (Chugal *et al.* 2011). Using denaturing gradient gel electrophoresis (DGGE), a technique widely used for the analysis of communities before the appearance of NGS, the results indicated that *Burkholderia* is a prevalent genus. Both genera are closely related, and the latter was previously classified as part of the genus *Pseudomonas* (Wang *et al*. 2012). A limitation of DGGE is the possible migration of bands of different species to the same position in the gel, thereby altering its sequencing and subsequent phylogenetic analysis. All these results corroborate the concept that NGS is a much more sensitive and accurate technique to describe microbial communities (Gloor *et al*. 2010, Diaz *et al*. 2012).

Next generation sequencing studies of primary endodontic infection that were not exposed to the oral cavity by a previously initiated therapy or a root treatment have reported that the presence of *Pseudomonas* spp. is limited to 0.1% (Saber *et al.* 2012). Since cases with coronal leakage associated to an inadequate coronal restoration were excluded, the presence of *Pseudomonas* spp. in the present study could be due to the possibility of contamination during the first endodontic treatment. A recent report on the

use of rubber dams concluded that less than half of the samples of 1,490 American dentists routinely used isolation during root treatment (Lawson *et al.* 2015). Although there is no information whether rubber dam was used in the initial treatment, one of the reasons for the high prevalence of *Pseudomonas* spp. could be that it is a consequence of contamination during the root canal treatment. Another report using MiSeq agrees with the present findings that *Pseudomonas* spp. is one of the most prevalent genera in canals associated with post-treatment apical periodontitis (Siqueira *et al.* 2016). Although teeth with satisfactory coronal restorations and without direct exposure of the root filling to the oral cavity were also included, the technique for obtaining the samples in the previous study was different when as it used cryopulverization. However, this sample was half the size of the present study, and the researchers did not analyse the clinical data of the patients (Siqueira *et al.* 2016).

In relation to the clinical data, the results of the current study reveal that the Shannon diversity index was significantly reduced in symptomatic patients. This result agrees with previously report that observed a significantly greater diversity in symptomatic infections than in asymptomatic infections; however, this previous report is related to primary endodontic infections (Santos *et al.* 2011). Compared to reports of secondary infections, the diversity of asymptomatic cases appeared marginally higher than that of symptomatic cases, although these differences were not significant (Anderson *et al.* 2013). This relationship may be due mainly to the fact that host defences, in the case of chronic asymptomatic lesions, are in equilibrium with a low virulence of the microorganisms, where a specific humoural and non-specific cellular response of low intensity contributes to a marked vascular reaction with the production of granulation tissue, enhancing a greater bacterial diversity. Moreover, another report of pyrosequencing reported a mismatch between the phylogenetic diversity index and the Shannon diversity index, where the phylogenetic diversity index agrees with results of the present study with higher diversity in asymptomatic cases; the results do not match in the case of the Shannon diversity index, showing higher diversity in symptomatic cases. However, that previous report suggested, symptomatic and asymptomatic infections were not significantly different, except for Proteobacteria (Tzanetakis *et al*. 2015). However, the most current reports of NGS in persistent apical periodontitis only

mention the presence of apical radiolucency versus specimens with normal periapical states but did not perform an analysis in relation to the associated microbiota (Vengerfeldt *et al.* 2014).

There is an association between endodontic pathosis and cardiovascular diseases in reports with low levels of bias (Khalighinejad *et al.* 2016), unlike that which occurs in the case of diabetes mellitus, where there are no studies with low levels of bias that explore the association of this disease with endodontic pathosis. However, the results of a previous report suggest that there may be a moderate risk and correlation between several systemic diseases and endodontic pathosis and suggests new studies are required to provide better evidence (Khalighinejad *et al*. 2016). All NGS studies performed to date include only systemically healthy patients but do not specify their classification according to the American Society of Anesthesiologists (ASA) Classification System (American Society of Anesthesiologists 2014). Moreover, none of these studies relate bacterial composition to the patient's systemic state. The present study describes a relationship between the presence of a systemic disease and the composition of the microbiota, with a significant difference of the bacterial composition in ASA I patients in comparison to the ASA II and III patients, where the latter groups had higher richness estimates and different phylogenetic compositions. Again, these differences may be due mainly to the fact that host defences are altered in patients with systemic diseases. On the other hand, it has been reported that systemic levels of C-reactive proteins, IL-6, and fibrinogen are influenced by the presence of persistent apical periodontitis in hypertensive patients, which, besides generating interest from a scientific point of view, has an impact on public health (Rashmi *et al*. 2017). Increasing scientific research in this area can determine this association.

It is important to consider the limitations of the present study. The sampling method is always a problem because it is impossible to recover all the microbiological contents of the root canal. Many of the microorganisms colonize the dentinal tubules, a specific niche for several species, which differ from species found in the lumen of the accessory or main canals. These specific microorganisms reside deeply within dentinal tubules, and are probably not recovered from samples obtained with paper points. The use of paper points to obtain samples of the root canal is still widely accepted. However,

researchers all agree with these limitations as to the scope and depth of the sampling (Tzanetakis *et al.* 2015).

Cryopulverization has been mentioned as an alternative sampling technique. But it is not an alternative for the sampling technique in cases of conventional root canal retreatment. Moreover, it is not mentioned whether the root apical portion is maintained in any medium at -20 °C, which increases bacterial viability and thus decreases the probability of DNA degradation. It has been reported that 10% skim milk solution or DMSO are better cryoprotective agents than the widely used 15% glycerol solution (Cody et al, 2008, Kerckhof *et al*. 2014).

Finally, as metagenomic approaches are refined, the focus of microbiome research will shift towards how bacteria interact with their host and vice versa. The incorporation of metabolomics, nutrients, host genomes and other metadata will allow the production of an a detailed picture of the interactions between the microbiome and its host, and as dysbiotic changes occur, these approaches will allow a better understanding of the aetiopathogenesis of periapical disease.

Conclusion

Through a metagenomic approach, the composition and bacterial diversity of the microbiota associated with root canals diagnosed with persistent apical periodontitis was diverse, with Proteobacteria as the most abundant phyla, followed by Bacteroidetes, showing concordance with previous studies conducted in other near geographical locations regarding microbiota taxonomical composition with the same technique. The results suggests that relationships between several clinical features and a greater biodiversity of the microbiota could be associated with persistent endodontic infection, specifically between individuals categorized as ASA II-III and Periapical index 5 and asymptomatic patients with higher richness and diversity. Further proteomic and metabolomic analyses of the interaction of persistent endodontic microbiota are needed to improve understanding of the microbial function of endodontic pathogenesis.

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SUPPORTING INFORMATION

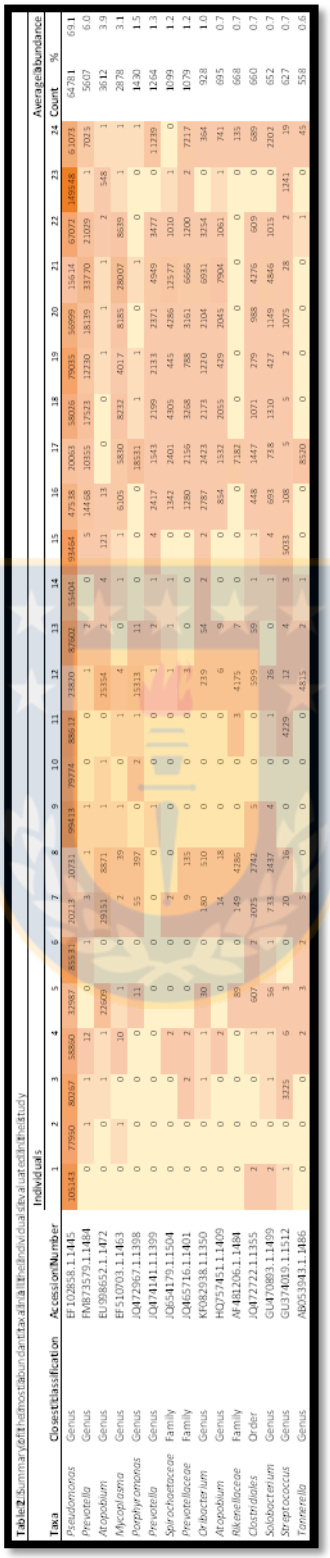

Table 2 Summary of the most abundant taxa in the individuals evaluated in this study.

Taxa	Closest classification	Accession Number	Individuals																					Average abundance	
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	Count	%
Pseudomonas	Genus	EF102858.1.1445	106148	77950	80267	58850	32987	85331	20213	10731	94618	79774	88617	23820	87802	55404	93464	47538	20063	58026	79035	56999	15614	67072	149148	61073	64781	69.1
Prevotella	Genus	FM873579.1.1484	0	1	1	12	0	1	3	1	1	0	0	1	2	0	5	14468	10355	17523	12230	18139	33770	21029	1	7015	5607	6.0
Atopobium	Genus	EU908652.1.1472	0	0	1	1	27609	0	29151	8871	1	1	0	21354	2	4	121	13	0	0	1	1	1	2	548	1	3612	3.9
Mycoplasma	Genus	EF510703.1.1463	0	1	0	10	1	0	2	39	1	0	1	4	0	1	1	6105	5830	8232	4017	8185	29007	8639	1	1	2878	3.1
Porphyromonas	Genus	JQ472967.1.1398	0	0	0	0	11	0	55	397	0	2	1	13113	11	0	0	0	18531	1	1	1	0	1	0	1	1430	1.5
Prevotella	Genus	JQ474141.1.1399	0	0	0	0	0	0	0	0	1	0	0	1	2	1	4	2417	1543	2199	2133	2371	4949	3477	0	11239	1264	1.3
Sporichthyaceae	Family	JQ654179.1.1504	0	0	0	2	0	0	7	0	0	0	0	1	1	1	0	1342	2401	4305	445	4286	12577	1010	1	0	1099	1.2
Prevotellaceae	Family	JQ465716.1.1401	0	0	2	2	0	0	9	135	0	0	0	3	0	0	0	1280	2156	3268	788	3161	6666	1200	2	7317	1079	1.2
Oribacterium	Genus	KF082938.1.1350	0	0	1	1	30	0	180	510	0	0	0	219	54	2	2	2787	2423	2173	1220	2104	6931	3254	0	364	928	1.0
Atopobium	Genus	HQ757451.1.1409	0	0	0	2	0	0	14	18	0	0	0	6	9	0	0	854	1532	2055	429	2045	7904	1061	1	741	695	0.7
Rikenellaceae	Family	AF481206.1.1484	0	0	0	0	89	0	149	4286	0	0	3	4175	7	0	0	0	7182	0	0	0	0	0	0	135	668	0.7
Clostridiales	Order	JQ472722.1.1355	2	0	0	1	607	2	2075	2742	5	0	0	599	39	1	1	448	1447	1071	279	988	4276	609	0	689	660	0.7
Solobacterium	Genus	GU470893.1.1499	2	0	1	1	56	1	733	2437	4	0	1	26	0	1	4	693	718	1330	427	1149	4846	1015	0	2202	652	0.7
Streptococcus	Genus	GU370019.1.1512	1	0	3235	6	3	0	20	36	0	0	4229	12	4	3	5033	108	5	5	2	1075	38	2	1241	19	627	0.7
Tannerella	Genus	AB053941.1.1486	0	0	0	2	3	2	3	0	0	0	0	4815	2	1	0	0	8520	0	0	0	0	1	0	45	558	0.6

CAPÍTULO V: ANTIMICROBIAL ACTIVITY OF ORAL ANTIBIOTICS AND COPPER NANOPARTICLES AGAINST ENDODONTIC PATHOGENS

Manuscrito a enviar a *Australian Endodontic Journal*

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Abstract

In recent years there has been an increase in the number multiple-drug resistant microorganisms, and the field of oral microbiology has also been affected by this phenomenon. In this context, copper nanoparticles can become a viable alternative for the treatment of endodontic infections as they have proved to be effective to use in infections caused by resistant microorganisms, and to avoid the appearance of resistance produced by the use and abuse of antibacterial agents. The purpose of this study was to determine the behavior of prevalent and cultivable bacterial strains, isolated from persistent endodontic infections, against the main antimicrobial agents commonly used in dentistry and to evaluate the effect of copper nanostructures on the susceptibility and inactivation capacity of strains. Tests of susceptibility to antibiotics commonly used in dentistry were evaluated by determining minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Additionally, the activity of copper nanoparticles (CuNPs) and copper nanowires (CuNWs) over thirty-one strains isolated from aerobic and anaerobic conditions from 24 teeth diagnosed with persistent endodontic infection was also evaluated. Bacterial killing kinetics was carried out to examine the dynamics of antimicrobial activity of the nanostructures that showed a better antibacterial effect. All Gram-positive facultative anaerobic isolates, 7.8% were resistant to amoxicillin and tetracycline, and 70.3% to erythromycin and clarithromycin; and of all strict anaerobic isolates, 85.7% were resistant to metronidazole. Copper

nanoparticles had the lowest MIC and MBC values with 179.3 µg/mL and 440.6 µg/mL, respectively. Death trials with CuNPs showed a significant decrease in the growth of all the species tested in 4 h, reaching 100% in 2 h for *P. acnes*, within 3 h for *Propionibacterium acidifaciens*, and within 4 h for *Streptococcus constellatus*. The use of antimicrobial nanoparticles may be a viable alternative for endodontic treatment, in the search for a higher success rate and a lower number of resistant strains.

Introduction

Persistent endodontic infections can be caused by microorganisms involved in the primary infection, which are somehow able to resist endodontic disinfection procedures and the subsequent lack of nutrients (1). *Enterococcus faecalis* has been considered as the main responsible for endodontic failures for many years. In addition, it has been used as a model to test various antibacterial agents for endodontic use (2,3). However, some current studies question this assertion and report that other species may be predominant pathogens associated with secondary endodontic infections. Differences may be due to varying methodological approaches for sampling and detection, different clinical conditions, or differences in the geographical origin of the subjects studied (4-7). Persistent endodontic infections are considered polymicrobial infections involving fewer species than the primary infection (4). A high prevalence of *Pseudomonas spp*. as well as *Burkholderiales spp.* has been observed, associating their presence to contamination by filtration from saliva to the root canal (8). *Propionibacterium acnes* and *Staphylococcus epidermidis* isolated from refractory endodontic lesions are considered opportunistic pathogens, acquired during endodontic treatment (9). Now, irrespective of the species of oral bacterial isolates, differences have been reported regarding their profiles of sensitivity to antibiotics. These differences have been associated with geographical origin, where Latin American countries have shown lower susceptibility to antibiotics commonly used in dentistry (10). In the case of strains of endodontic origin, a sharp increase in the antimicrobial resistance of anaerobes isolated from primary endodontic infections over a period of time has been observed (11). The selection of resistant strains due to the use and abuse of antimicrobial agents is currently generating public health concern in many countries (12,13), and the oral microbiome could be acting as a reservoir of genes involved in antibiotic resistance (14,15).

There is currently a growing interest in the use of copper nanoparticles as antimicrobials to control various infections, including those affecting the oral cavity, since on the one hand, they possess biocidal properties and anti-adhesive capabilities against biofilms and, on the other, they could also be used as an alternative to prevent the emergence of superbacteria due to antibiotic resistance. Regarding endodontic treatment, it is in the dentinal tubules where bacteria establish and form biofilm. Here is where CuNPs can play a role as an antibacterial agent. Due to their extremely small size, CuNPs able to reach where other antimicrobials cannot, and since copper possesses contact antibacterial properties, its application time could be reduced to the minimum necessary (16,17).

Materials and methods

Twenty-four samples were taken from teeth diagnosed as previously treated for apical periodontitis. They were obtained from 24 patients older than 18 years, who received treatment at the Endodontic Unit. Inclusion criteria included teeth that were treated more than two years ago and that showed radiographic evidence of periapical disease (widened apical periodontal space, or periapical radiolucency). Exclusion criteria included patients who had been receiving antibiotic treatment for at least three months before the study, teeth that could not be completely isolated with a rubber dam, with leakage in dental crowns and/or with associated periodontal pathology. This study was carried out according to the protocol approved by the Ethics Committee of the School of Dentistry of Universidad de Concepción. Subjects agreed to participate in the study by signing an informed consent (C.I.Y.B. No. 04/15).

Isolation and identification of strains

Samples were collected with strict asepsis by a single trained operator, as described by Schirrmeister *et al*. (18). Three sterile paper tips were placed at the working length to obtain the sample from the canal. The paper tips were transferred to a 1.5 ml cryotube containing 1 mL of reduced transport fluid broth (RTF) for further culture. All samples were seeded under aerobic and anaerobic conditions. In the samples sown directly and in which a confluent development was obtained, up to 5 dilutions were made to obtain isolated colonies. Under aerobic conditions, each sample was seeded on BHI agar (Oxoid Ltd., Basingstoke, UK) for 48 h, at 37°C. From each bacterial plate, different

representative pure colonies were isolated according to their macroscopic differences (shape, color, appearance, elevation, etc.). Each colony was initially characterized microscopically according to its Gram stain, shape, and group; in addition, the ability to produce catalysis and oxidation was tested. Under anaerobic conditions, each sample was cultivated as described by Gomes *et al*. (11). Plates were incubated at 37°C under anaerobic conditions in a GENbox 2.5 L anaerobic jar, (Biomerieux, Marcy-l'Etoile, France), with a Genbox anaerobiosis sachet generator (Biomerieux, Marcy-l'Etoile, France) for a maximum of 14 days. From each bacterial plate, different representative pure colonies were isolated according to their macroscopic differences (shape, color, appearance, elevation, etc.), and incubated in broth Anaerobe Basal Broth (Oxoid Ltd., Basingstoke, UK). The strains were then grown in an aerobic environment to separate strict anaerobes from facultative anaerobes. Strict and facultative aerobic strains of Enterobacteriaceae and other nonfermenting Gram-negative bacilli were initially identified by means of a biochemical scale. Identification was then corroborated by the use of the API 20E biochemical identification kit (BioMerieux, Marcy-l'Etoile, France). Strict and facultative aerobic strains, Gram-positive cocci strains, were further characterized according to their ability to grow in NaCl and perform hemolysis. Strains presumed to be strict anaerobes were subjected to identification using the Rapid ID 32A biochemical identification kit (BioMerieux, Marcy-l'Etoile, France). Finally, identification of species was corroborated by sequencing (19).

Susceptibility profile

Tests on aerobic microorganisms were performed according to the recommendations given by CLSI (M100-S27) and the modifications suggested by Kuriyama *et al*. (20,21). For tests of nanoparticles and anaerobic strains, CLSI recommendations were taken as reference and modifications were made as described by Vargas-Reus *et al*. (22). Amoxicillin (AMX), amoxicillin/clavulante (AMC), tetracycline (TET), clarithromycin (CLR), erythromycin (ERY) and metronidazole (MTZ) were tested (10). MIC was determined for all strains by the E-test technique (Biomerieux, Marcy-l'Etoile, France)(10). MIC cut-off points to indicate resistance to antibiotics were determined from the twenty-seventh informative supplement of the Performance Standards for Antimicrobial Susceptibility Tests (M100-S27), issued by the Clinical & Laboratory

Standards Institute (CLSI) in January 2017. For infrequent bacteria, CLSI document M45-Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria(20) was consulted. CuNPs were manufactured and provided by the Laboratory of Hybrid and Polymer Materials (LMHP) of the Department of Materials Engineering (DIMAT) School of Engineering, Universidad de Concepción. Two types of copper nanostructures, copper nanoparticles (CuNPs) and copper nanowires (CuNWs) were used. In addition Zinc Oxide Nanoparticles (ZnONPs) were included as control.

Determination of bacterial killing kinetics with CuNPs

Bacterial killing kinetics was determined for a representative strain of each species by means of the plate count technique. Dilutions of 2500, 1000, 500, 250 and 100 µg/mL of the nanostructure that showed the best antibacterial behavior were used. Sampling times were 0, 1, 2, 3 and 4 hours. Three repeated experiments were performed on three different times (21).

Statistical analysis

Data were entered into Microsoft Excel sheets for analysis. Nonparametric tests (Kruskal-Wallis ANOVA) were performed. To get a significant p value <0.05 was considered difference. SPSS software (SPSS, Chicago, USA, version 23) was used.

Results

Isolation and molecular identification of species

Isolation of bacteria from aerobic incubation conditions: 16 bacterial colonies were selected from this culture condition according to their macroscopic differences (shape, color, appearance, etc.).

Ten strains were initially classified as *Pseudomonas* by means of microscopic and biochemical analyses (Table 1). The other six strains were classified as cocci or coccobacillary, all Gram-positive, presumably of the genus *Staphylococcus* spp., *Streptococcus* spp. and *Enterococcus* spp. After the sequencing, a summary was made with the species; percentages of similarity and accession number were registered. This is shown in table 2.

Table 1:Identification of Gram-negative strains isolated under aerobic conditions.

	% **Identification according API 20E**	**16S rRNA Sequencing** % **Identification according NCBI**
1	*Pseudomonas fluorescens/putida*(85%)	*Pseudomonas putida* (99% KM079616.1)
2	*Pseudomonas fluorescens/putida*(85%)	*Pseudomonas putida* (98%KM079616.1)
3	*Pseudomonas fluorescens/putida*(85%)	*Pseudomonas putida* (98% KM079616.1)
4	*Pseudomonas fluorescens/putida*(85%)	*Pseudomonas putida* (99% KM079616.1)
5	*Pseudomonas fluorescens/putida*(85%)	*Pseudomonas putida* (97% KM079616.1)
6	*Pseudomonas fluorescens/putida*(90.4%)	*Pseudomonas fulva* (97% EU855189.1)
7	*Pseudomonas fluorescens/putida*(90.4%)	*Pseudomonas putida* (99% KM079616.1)
8	*Pseudomonas fluorescens/putida*(75.1%)	*Pseudomonas putida* (99% KM079616.1)
9	*Pseudomonas fluorescens/putida*(75.1%)	*Pseudomonas putida* (98% KM079616.1)
10	*Pseudomonas fluorescens/putida*(75.1%)	*Pseudomonas fulva* (97% KP292608.1)

Table 2: Identification of Gram-positive strains isolated under aerobic conditions.

	16S rRNA Sequencing % **Identification according NCBI**	
1	*Staphylococcus warneri*	(99% KY608138.1)
2	*Enterococcus faecalis*	(99% KT260534.1)
3	*Staphylococcus haemolyticus*	(99% MF157599.1)
4	*Leuconostoc mesenteroides*	(97% KC108669.1)
5	*Enterococcus faecalis*	(99% EU708623.1)
6	*Enterococcus faecalis*	(97% KU324904.1)

All the strains were sequenced over 1000 base pairs (bp). A good quality of the sequencing was obtained, observing separate peaks, with Signal G above 200. Most bacteria showed a similarity of 98% to 99% with each other, consequently they were considered as part of the same species. The lower percentage found ranged from 95% to 97%; bacteria in this range were considered to belong to the same genus.

Isolation of bacteria from anaerobic incubation conditions: in this case there was no bacterial growth in all the samples. Fifteen colonies were isolated under anaerobic conditions. Of the 15 colonies, 14 corresponded to Gram-positive bacteria and only one strain was Gram negative. Six strains identified using the Rapid ID 32A identification kit (BioMerieux, Marcy-l'Etoile, France) were classified as strict anaerobes (Table 3). According to the microscopic and biochemical analyses, of the nine facultative anaerobic strains (Table 4), seven were classified mainly as cocci or coccobacillary-shaped, all Gram positive, presumably of the genus *Staphylococcus* spp., *Streptococcus* spp. and *Enterococcus* spp., a Gram negative bacillary strain and a Gram positive bacillary strain were not classified in any of the groups.

Table 3: Identification of Gram-positive strict anaerobic strains isolated under anaerobic conditions.

	% Identification according RAPID ID 32A	**16S rRNA Sequencing % Identification according NCBI**
1	*Propionibacterium acnes* (99,9%)	*Propionibacterium acnes* (98% KP944184.1)
2	*Actinomyces viscosus* (85,9%) *Actinomyces naeslundii* (13,7%)	*Propionibacterium acnes* (98% CP003195.1)
3	*Propionibacterium acnes* (99.9%)	*Propionibacterium sp.* (99% KM507346.1)
4	*Clostridium bifermentans* (94.2%)	*Propionibacterium acnes* (98% KP944184.1)
5	*Bifidobacterium adolescentes* (92,1%)	*Bifidobacterium dentium* (99% LC071795.1)
6	*Propionibacterium spp* (99%)	*Propionibacterium acidifaciens* (99% LT223673.1)

Finally, all the strains were identified by sequencing and, in the same way as the strains isolated from aerobic conditions, all were sequenced on 1000 base pairs. A good quality of the sequencing was obtained. Most strains showed a similarity of 99% to 100%, consequently they were considered as part of the same species. The lower percentage found ranged from 95% to 97%, bacteria in this range were considered to belong to the same genus. Identification of *Klebsiella oxytoca* and *Propionibacterium acnes* isolated from anaerobic conditions stands out. A summary was made with the species; percentages of similarity and accession number were register, which can be observed in tables 3 and 4.

Table 4: Identification of facultative anaerobic strains isolated under anaerobic conditions.

	16S rRNA Sequencing	
	% Identification according NCBI	
1	*Staphylococcus capitis*	(98% KT719505.1)
2	*Enterococcus faecalis*	(99% KJ725203.1)
3	*Klebsiella oxytoca*	(99% KC462193.1)
4	*Enterococcus faecalis*	(98% KJ803877.1)
5	*Streptococcus constellatus*	(98% KC569555.1)
6	*Streptococcus constellatus*	(99% JX993443.1)
7	*Enterococcus faecalis*	(99% KT260534.1)
8	*Enterococcus faecalis*	(99% KT260534.1)
9	*Propionibacterium acnes*	(98% KX096287.1)

Susceptibility profile

The summary of the results of MIC for more common antibiotics used in Endodontics obtained by E-test (Biomerieux, Marcy-l'Etoile, France) in agar diffusion method is presented in tables 5, 6 and 7.

For the only Gram-negative strain of the *Enterobacteriaceae* group, *Klebsiella oxytoca*, MIC of 32 µg/mL, 64 µg/mL y 32 µg/mL were observed for AMX, CLR and ERY, respectively. The strain was considered resistant to these three antibiotics, remaining susceptible to AMC and TET.

Table 5: Antimicrobial activity of clinically important antibiotics against *Pseudomonas* spp. strains.

	Strain	Species	AMX*	AMC*	TET*	CLR*	ERY*
1	6.1	*Pseudomonas putida*	24	16	3	48	96
2	6.2	*Pseudomonas putida*	32	24	3	48	64
3	9.1	*Pseudomonas putida*	32	24	4	32	64
4	9.3	*Pseudomonas putida*	32	16	3	32	48
5	13.2	*Pseudomonas putida*	24	16	3	32	64
6	14.1	*Pseudomonas fulva*	32	24	3	24	32
7	14.2	*Pseudomonas putida*	32	16	3	32	48
8	15.1	*Pseudomonas putida*	32	24	3	32	32
9	15.2	*Pseudomonas putida*	32	16	4	48	48
10	14.3	*Pseudomonas fulva*	6	6	1	24	24
		Resistant	10	10	0	10	10
		Susceptible	0	0	10	0	0
		% Susceptibility strains	0	0	100	0	0

*: µg/mL; (AMX) amoxicillin; (AMC)amoxicillin/clavulante; (TET) tetracycline, (CLR) clarithromycin, (ERY) erythromycin

Table 6: Antimicrobial activity of clinically important antibiotics against Gram-positive facultative anaerobic strains.

	Strain	Species	AMX*	AMC*	TET*	CLR*	ERY*
1	9.2	*Staphylococcus warneri*	0.75	0.25	0.75	**256**	**256**
2	15.3	*Leuconostoc mesenteroides*	0.094	0.064	**8**	0.16	0.16
3	11.1	*Enterococcus faecalis*	0.38	0.25	0.5	**>256**	**>256**
4	11.2	*Enterococcus faecalis*	0.25	0.25	0.5	**128**	**96**
5	11.3	*Enterococcus faecalis*	0.25	0.25	0.5	**128**	**96**
6	C1	*Enterococcus faecalis*	0.19	0.19	0.5	**32**	**16**
7	C3	*Enterococcus faecalis*	**48**	1	2	**96**	**48**
8	E1	*Enterococcus faecalis*	0.25	0.25	0.75	**32**	**64**
9	E2	*Enterococcus faecalis*	0.125	0.125	0.75	**>256**	**>256**
10	B2	*Staphylococcus capitis*	0.064	0.064	1	0.094	0.064
11	13.1	*Staphylococcus haemolyticus*	0.75	0.5	0.38	**48**	**96**
12	D1	*Streptococcus constellatus*	0.125	0.125	2	0.032	0.032
13	D2	*Streptococcus constellatus*	0.125	0.125	2	0.047	0.032
		Resistant	1	0	1	9	9
		Susceptible	12	13	12	4	4
		% Susceptibility strains	92.3	100	92.3	30.7	30.7

*: µg/mL; (AMX) amoxicillin; (AMC)amoxicillin/clavulante; (TET) tetracycline, (CLR) clarithromycin, (ERY) erythromycin.

Table 7: Antimicrobial activity of clinically important antibiotics against strict anaerobic strains.

	Strain	Species	AMX*	AMC*	TET*	CLR*	ERY*	MTZ*
1	A1	*Propionibacterium acnes*	0.016	0.016	0.5	0.016	0.016	**>256**
2	A2	*Propionibacterium acnes*	0.032	0.032	0.38	0.032	0.064	**>256**
3	E3	*Propionibacterium acnes*	0.016	0.016	0.75	0.016	0.016	**>256**
4	G1	*Propionibacterium acnes*	0.016	0.016	1	0.016	0.032	**->256**
5	F2	*Propionibacterium acidifaciens*	0.016	0.016	0.5	0.016	0.023	**>256**
6	B1	*Propionibacterium sp.*	0.23	0.016	0.5	0.023	0.032	**>256**
7	F1	*Bifidobacterium dentium*	0.094	0.064	0.75	0.016	0.016	2
		Resistant	0	0	0	0	0	6
		Susceptible	7	7	7	7	7	1
		% Susceptibility strains	100	100	100	100	100	85.7

*: µg/mL; (AMX) amoxicillin; (AMC)amoxicillin/clavulante; (TET) tetracycline, (CLR) clarithromycin, (ERY) erythromycin; (MTZ) metronidazole

CuNPs obtained ranged between 20 and 40 nm. Most of the structures had well-defined decahedron shape (mostly spherical). The low-resolution image shows the low dispersion in size presented by the CuNPs. Table 8 shows MIC and MBC determinations for the 31 bacterial strains tested when exposed to CuNWs, CuNPs; an NPZnO control was used, based on three repeated determinations. MIC and MBC values were in the range of 100 µg/mL to >2500 µg/mL to 250 µg/mL to >2.500 µg/mL, respectively.

Table 8: Antimicrobial activity of Copper Nanostructures against Gram-negative strains.

Strain	Species	CuNWs		CuNPs		ZnONPs	
		MIC*	MBC*	MIC*	MBC*	MIC*	MBC*
6.1	*Pseudomonas putida*	>2500	>2500	2500	2500	2500	>2500
6.2	*Pseudomonas putida*	>2500	>2500	2500	2500	2500	>2500
9.1	*Pseudomonas putida*	>2500	>2500	2500	2500	2500	>2500
9.3	*Pseudomonas putida*	>2500	>2500	2500	2500	2500	>2500
13.2	*Pseudomonas putida*	>2500	>2500	2500	2500	1000	2500
14.1	*Pseudomonas fulva*	>2500	>2500	1000	>2500	>2500	>2500
14.2	*Pseudomonas putida*	>2500	>2500	2500	2500	2500	>2500
15.1	*Pseudomonas putida*	>2500	>2500	500	>2500	500	>2500
15.2	*Pseudomonas putida*	>2500	>2500	2500	2500	2500	>2500
14.3	*Pseudomonas fulva*	>2500	>2500	1000	2500	2500	>2500
C2	*Klebsiella oxytoca*	2500	>2500	1000	2500	2500	>2500
Geometric mean		>2500	>2500	1682,1	>2500	1987,1	>2500

*: µg/mL; (CuNWs) cooper nanowires; (CuNPs) cooper nanoparticles;(ZnONPs) zinc oxide nanoparticles, (MIC) minimum inhibitory concentration, (MBC) minimum bactericidal concentration.

Table 9: Antimicrobial activity of Copper Nanostructures against Gram-positive strains.

Strain	Species	CuNWs		CuNPs		ZnONPs	
		MIC*	MBC*	MIC*	MBC*	MIC*	MBC*
9.2	*Staphylococcus warneri*	>2500	>2500	500	1000	1000	2500
15.3	*Leuconostoc mesenteroides*	500	1000	250	500	1000	2500
11.1	*Enterococcus faecalis*	500	1000	250	500	1000	2500
11.2	*Enterococcus faecalis*	1000	>2500	250	1000	500	2500
11.3	*Enterococcus faecalis*	1000	>2500	250	1000	500	2500
C1	*Enterococcus faecalis*	100	500	100	500	250	1000
C3	*Enterococcus faecalis*	1000	>2500	250	1000	1000	2500
E1	*Enterococcus faecalis*	100	500	100	500	250	1000
E2	*Enterococcus faecalis*	100	500	100	500	250	1000
B2	*Staphylococcus capitis*	500	1000	250	500	1000	2500
13.1	*Staphylococcus haemolyticus*	500	1000	250	500	1000	2500
D1	*Streptococcus constellatus*	1000	2500	250	500	1000	2500
D2	*Streptococcus constellatus*	1000	2500	250	500	500	2500
A1	*Propionibacterium acnes*	250	500	100	250	250	1000
A2	*Propionibacterium acnes*	250	500	100	250	250	1000
E3	*Propionibacterium acnes*	250	500	100	250	500	1000
G1	*Propionibacterium acnes*	250	500	100	250	250	1000
F2	*P. acidifaciens*	500	1000	100	250	250	1000
B1	*Propionibacterium sp.*	500	1000	250	500	500	2500
F1	*Bifidobacterium dentium*	500	1000	250	500	500	2500
	Geometric mean	440,6	1032,8	179,3	482,9	500	1732,8

*: µg/mL; (CuNWs) cooper nanowires; (CuNPs) cooper nanoparticles; (ZnONPs) zinc oxide nanoparticles, (MIC) minimum inhibitory concentration, (MBC) minimum bactericidal concentration.

Bacterial killing kinetics with CuNPs

The seven strains tested with CuNPs during a period of 4 h showed a very fast dose-dependent bactericidal effect (Figure 1 to 4). Bacterial reduction was significant and reached 100% in several cases; 1 h for *Propionibacterium acidifaciens* (Figure 1) within2 h for *Propionibacterium acnes* (Figure 2), and within 3 h for *Streptococcus constellatus* (Figure 3).

All species responded clearly to the dose tested, and in the case of *Propionibacterium acnes* and *Propionibacterium acidifaciens* the statistical analysis indicated that there were significant differences in the antimicrobial activity of the five concentrations of nanoparticles tested ($P < 0.05$ comparing the higher dose of 2500 µg/mL with the dose of 100 µg/mL). However, for the other five species evaluated, significant differences were also found between the concentrations tested ($P<0.05$). *Propionibacterium acidifaciens* was the most sensitive strain in the 1-h exposure to 100 µg/mL, followed by *Streptococcus constellatus*. Only in the latter, there was reduction of three logarithms in that period of time.

Staphylococcus warneri (Figure 4B) was the least susceptible strain in the 4-h exposure to 2500 µg/mL, followed by *Staphylococcus haemolyticus* (Figure 4A); both strains showing a reduction of three logarithms at 3 hours.

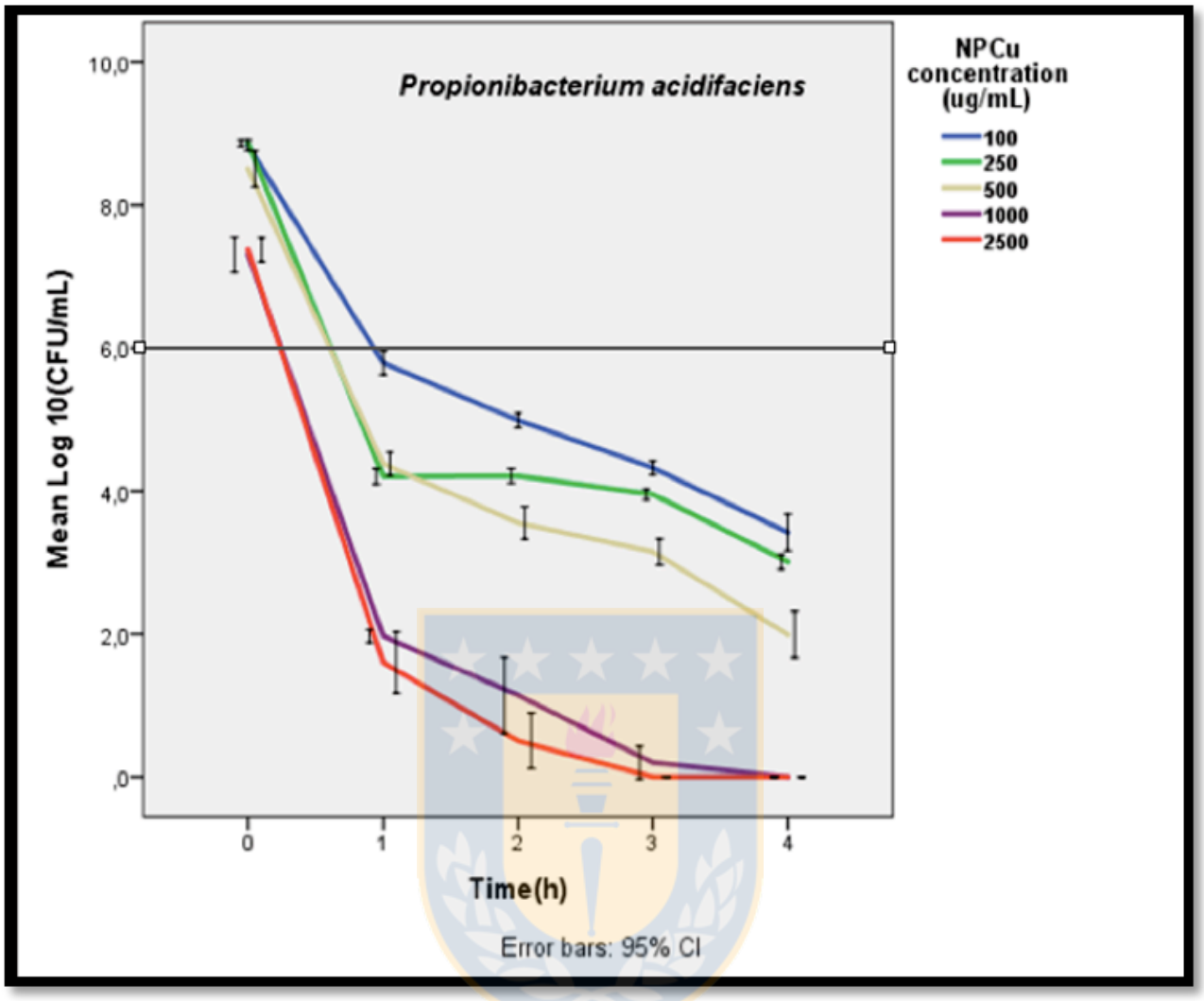

Figure 1: Decrease in the bacterial count of *Propionibacterium acidifiens* by incubation time, exposed to different concentrations of NPCu.

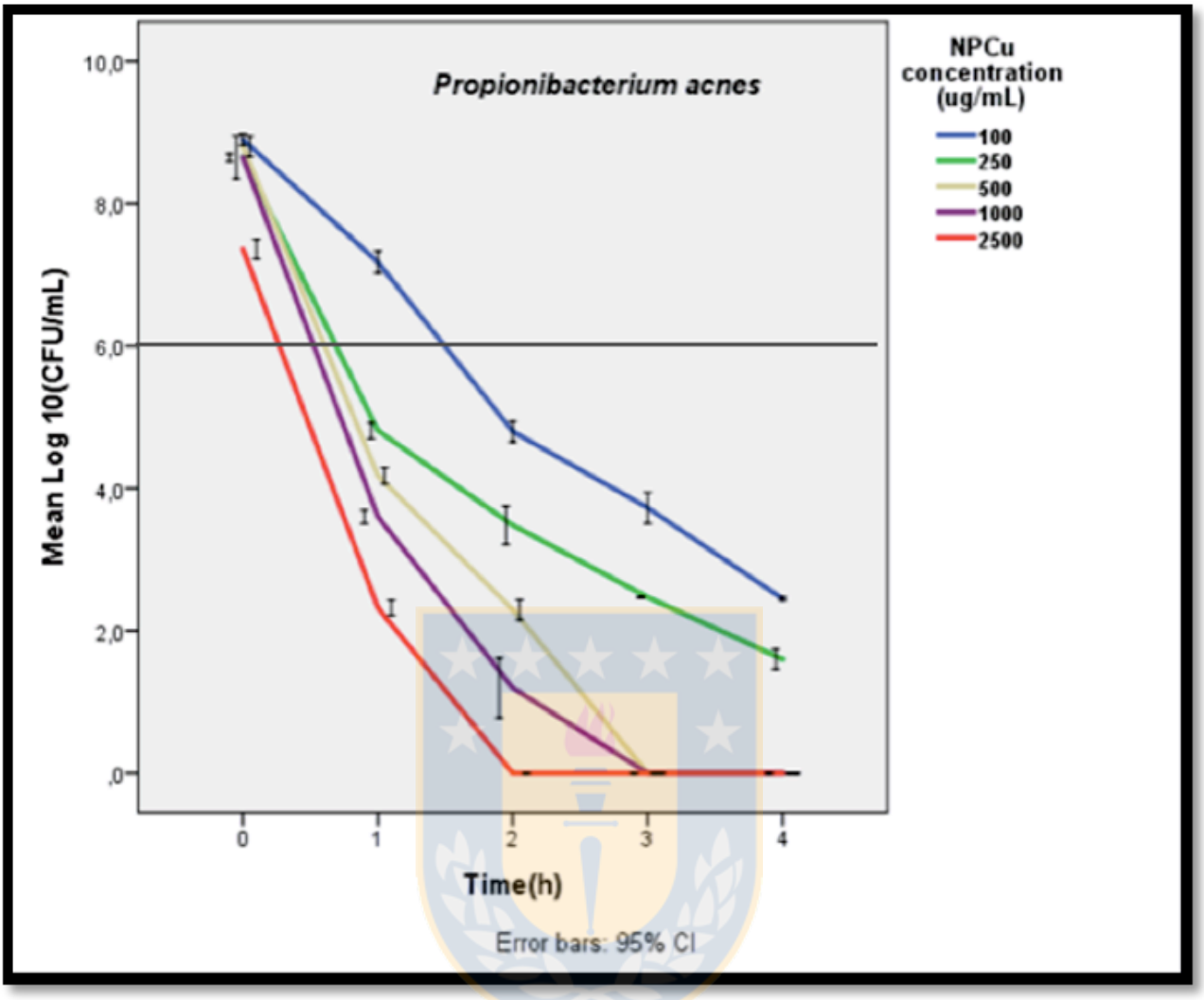

Figure 2: Decrease in the bacterial count of *Propionibacterium acidifiens* by incubation time, exposed to different concentrations of NPCu.

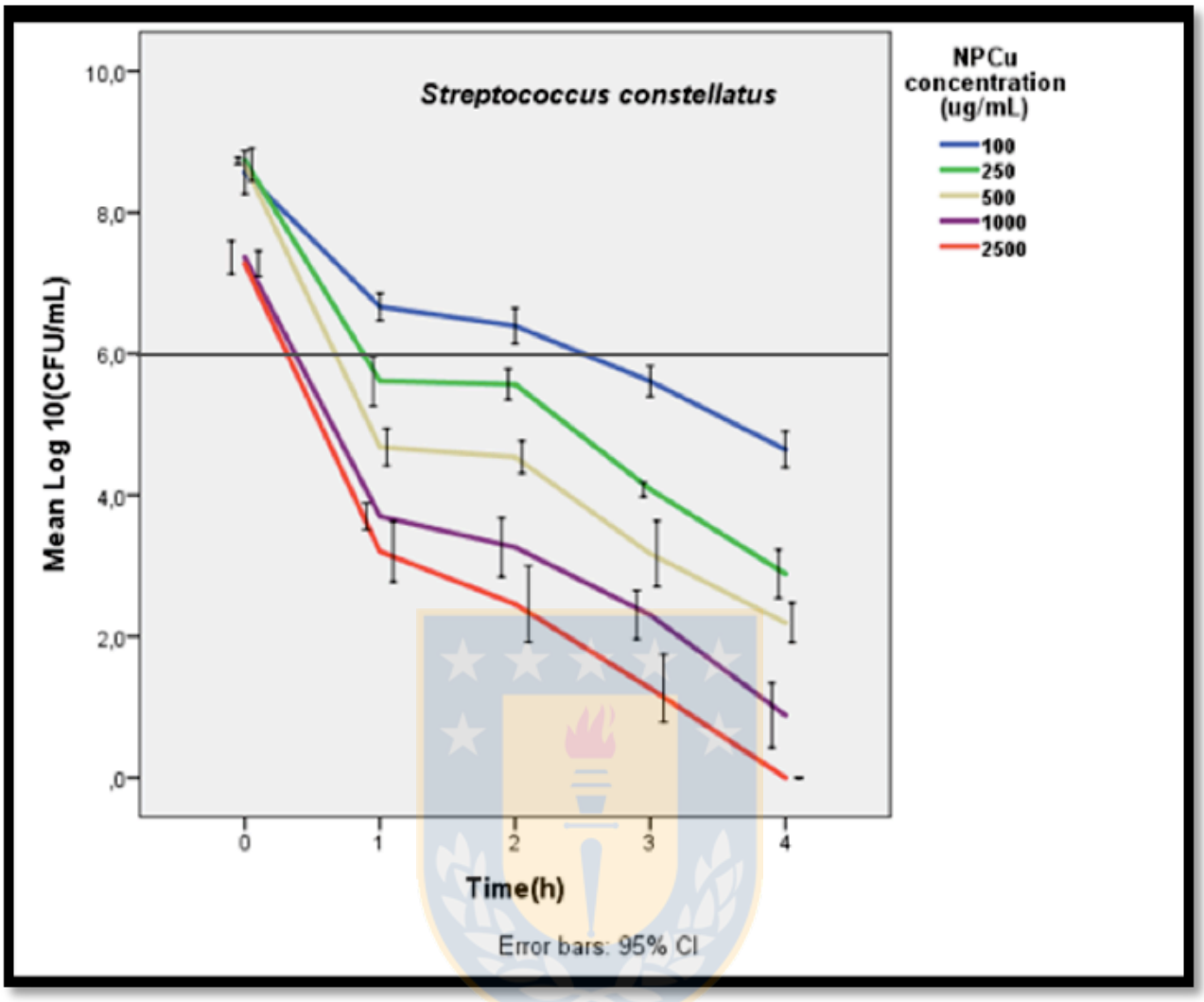

Figure 3: Decrease in the bacterial count of *Streptococcus constellatus* by incubation time, exposed to different concentrations of NPCu.

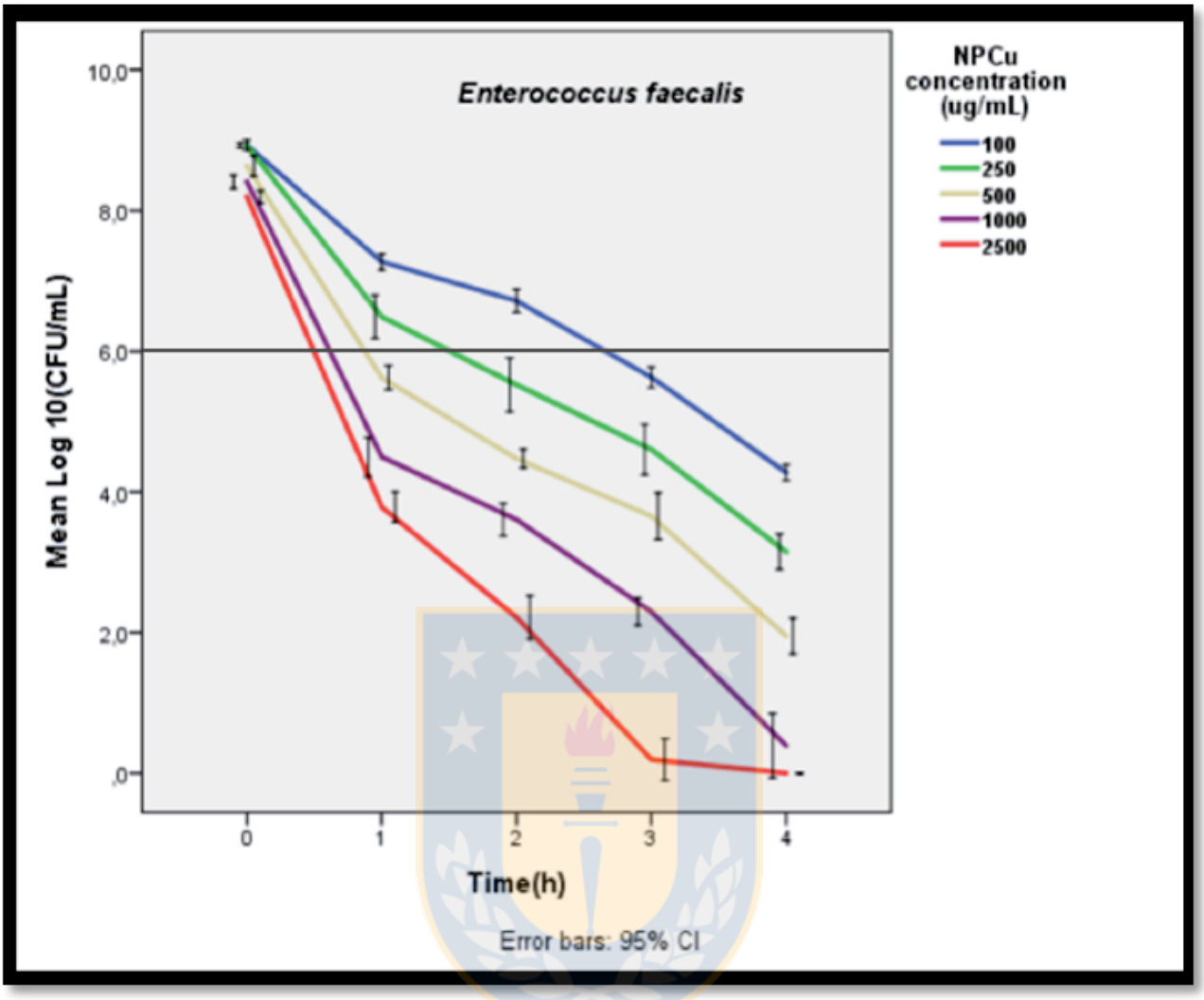

Figure 4: Decrease in the bacterial count of *Enterococcus faecalis* by incubation time, exposed to different concentrations of NPCu.

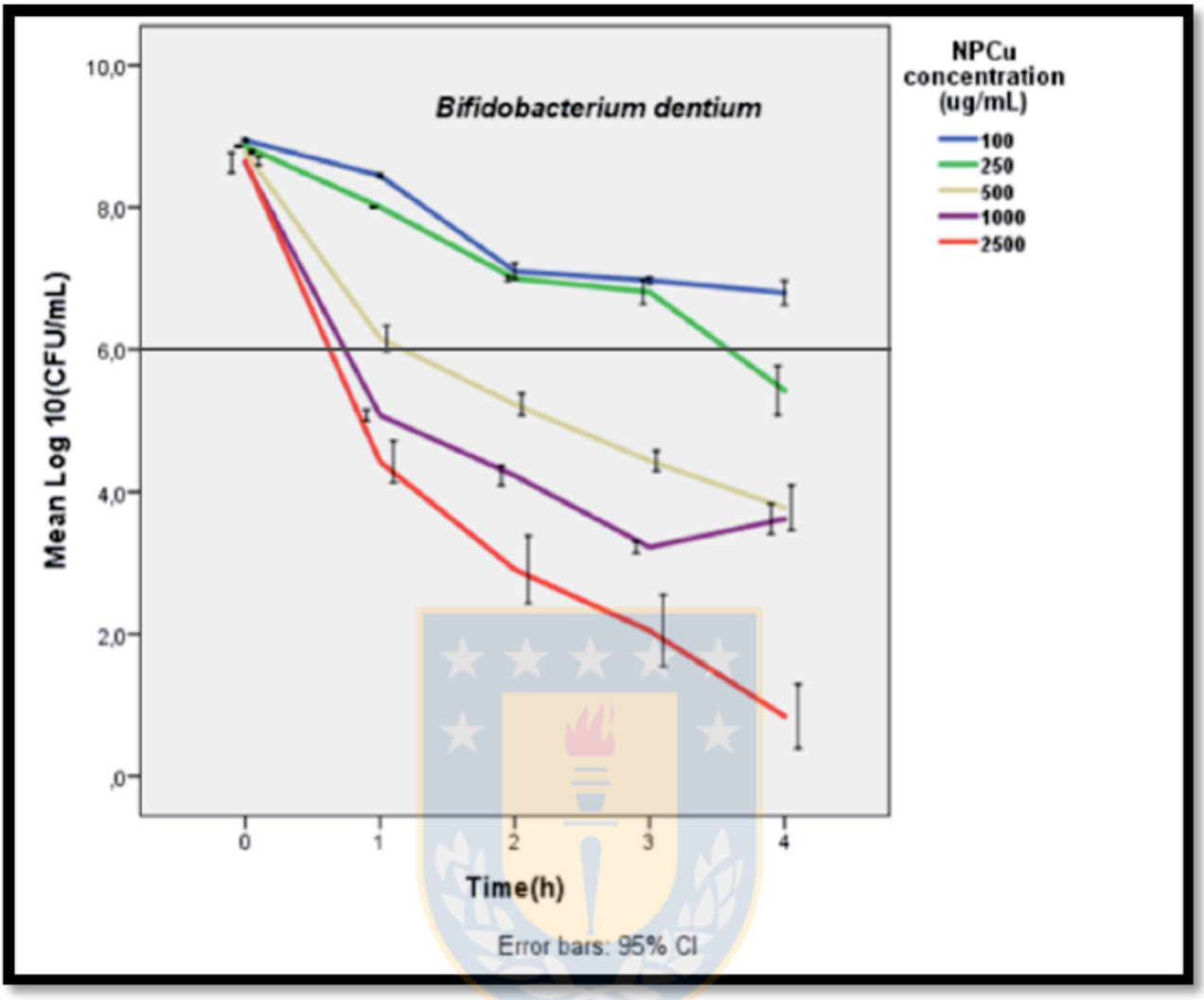

Figure 5: Decrease in the bacterial count of *Bifidobacterium dentium* by incubation time, exposed to different concentrations of NPCu.

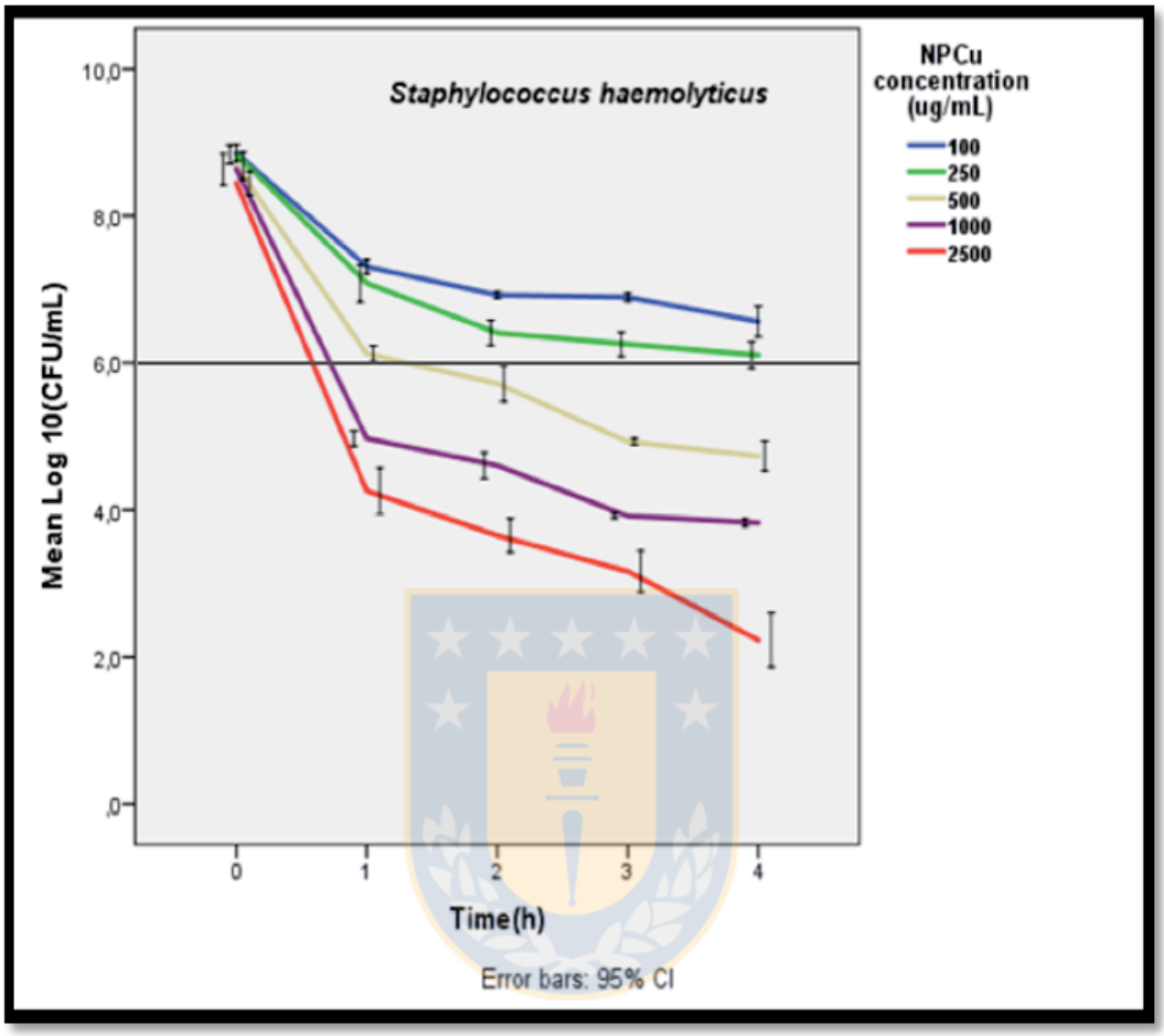

Figure 6: Decrease in the bacterial count of *Staphylococcus haemolyticus* by incubation time, exposed to different concentrations of NPCu.

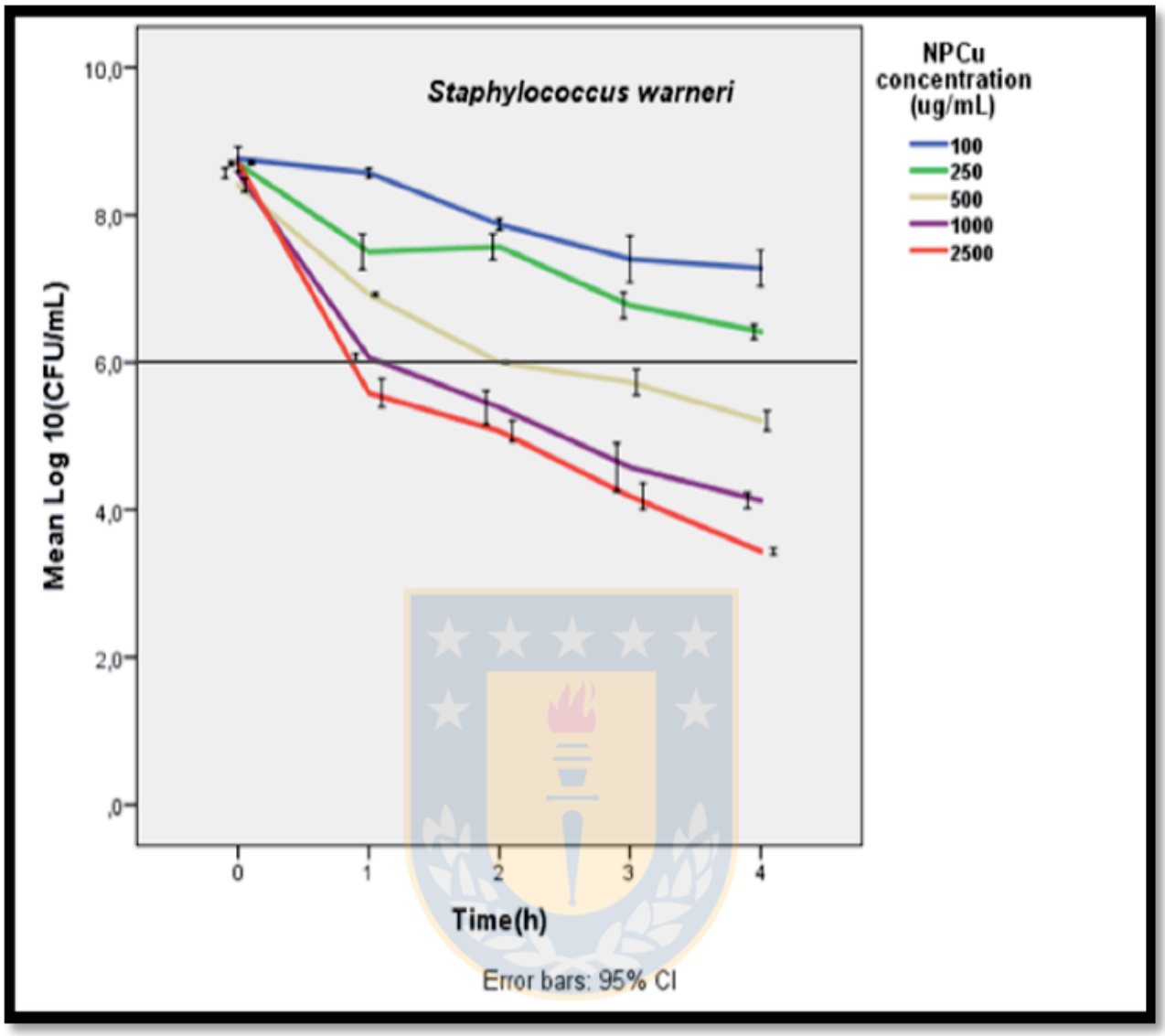

Figure 7: Decrease in the bacterial count of *Staphylococcus warneri* by incubation time, exposed to different concentrations of NPCu.

Discussion

Recent metagenomic studies of persistent endodontic infections have reported only a moderate presence of *streptococci* and *enterococci*, such as *E. faecalis*, contradicting what was indicated years ago, mainly with basic molecular techniques or culture, where they were identified and listed as the most prevalent species in this pathology (4-9,24). Current studies report the presence of other prevalent genera including *Lactobacilli*, *Actinomyces*, *Peptostreptococci*, *Pseudoramibacter* and *Propionibacterium* (1). The prevalence of Gram-positive cocci has been attributed to their ability to adhere and invade the dentinal tubules, to withstand prolonged lack of nutrients, resist most intracanal medications and tolerate pH levels of up to 11.5, making their elimination a much more difficult endeavor (25). However, it should be mentioned that although metagenomic studies are more accurate in terms of describing bacterial microbiota composition (7), said composition will not necessarily correspond to culture studies generated from the same samples. Recoverability is dependent on the ability of the methodologies described so far to grow different species (26). The metagenomic report derived from these samples (unpublished data) (27) suggests that *Pseudomonas* spp. stands out as the prevalent genus, and indicates that they are followed by *Prevotella* spp., *Atopobium* spp. and *Mycoplasmas* spp. Our findings show that *Pseudomonas spp*. had a high percentage in the culture, but other species such as *E. faecalis* and *P. acnes* are also observed, the latter does not agree with the metagenomic report. Therefore, in this type of trials, prevalence results from culture studies cannot be extrapolated, and therefore, these limitations must be taken into account when determining the prevalence of species in taxonomic composition.

In endodontics, biochemical tests and PCR Polymerase Chain Reaction with specific primers for housekeeping genes are mainly used for the identification of clinical strains at species level (28). However, 16S rRNA sequencing has been used as a frequent test for identification, especially when working with multiple strains isolated from clinical samples (9,24). This study identified 31 strains isolated from persistent endodontic infections, initially with biochemical tests and then confirmed through 16S rRNA sequencing; 51.6% of the strains obtained 99% identity with sequences, 35.4 % of

strains obtained 98% identity, and 13% of the strains 97% identity, all with reference in GenBank. There were variations in the initial identification with biochemical tests and in the results of the sequencing, which could be due to two reasons. The first is that systems such as API 20E and Rapid ID 32A (BioMerieux, Marcy-l'Etoile, France) have a limited database of species, and therefore, they are able to identify only some of the samples at 100% at species level; those that are not included in the database are identified with relative accuracy only at genus level. The other reason is that despite the fact that 16S rRNA sequencing is currently considered as the most important target of study in bacterial ecology, its use for the description of bacterial diversity is limited by the presence of variability in the number of copies in the bacterial genomes (29). Information on the number of copies of 16S rRNA allows to obtain alternative estimates of the composition of the bacterial community, that is, having a variability in terms of classification at species level and only being able to assert the presence in 100% of the identified bacterial genus; subsequently, complementing the sequencing with other types of genes, such as Housekeeping genes, could be an alternative so that identification at species level can reach 100% identity (30).

Fifteen strains were isolated from anaerobic conditions, of these 6 could be recovered only from this condition, therefore they were classified as strict anaerobes, mainly of the genus *Propionibacterium spp*. Strains of the genus *Propionibacterium spp*. are commonly detected in the oral cavity, especially in carious dentin, with *Propionibacterium acnes* being the most frequently reported species (29). This species is described as a strict anaerobe, however, it has been reported that some strains of *P. acnes* have the ability to grow in microaerophilic conditions, which probably contributes to the success of this organism to transfer itself to the anoxic environment of the deepest systemic infections, which could explain the isolation of this species from aerobic conditions (31).

P. acnes is the most prevalent species in primary endodontic infections with a history or clinical evidence of communication with the oral environment, while it is absent in lesions without communication with the oral environment (9). In addition, there is evidence that *P. acnes* isolates of refractory endodontic infections, with or without periapical abscesses, are probably nosocomial infections that occur during root canal

treatment (32). This could explain the possible cause of the isolation of *Propionibacterium* spp. strains.

Pseudomonas spp. has been reported as a prevalent genus in the microbiota of persistent endodontic lesions, associating its presence with contamination by filtration from the saliva to the root canal (7, 8). Eight strains of *Pseudomonas putida* and two *Pseudomonas fulva* isolated in this report belong, are considered strict aerobes, however, their oxygen requirement can be changed under anaerobic conditions using an alternative electron acceptor, such as nitrate. The anoxic environment generated after canal filling and crown sealing could favor the emergence of this bacterium as it can use the available oxygen, creating favorable conditions for facultative and strict anaerobic species. On the other hand, the components of the sealing materials could become the necessary source of nitrogen and favor the persistence of these bacteria inside the canals (33,34).

The multi-drug resistance of *Pseudomonas spp*. is due to numerous intrinsic or acquired mechanisms, such as a decrease in the permeability of the outer membrane, the production of beta-lactamases and the presence of multidrug efflux pumps. *Pseudomonas spp*. has been reported to be resistant to sulfamethoxazole, erythromycin, amoxicillin, ampicillin, chloramphenicol, trimethoprim, rifampicin and ceftazidime, as well as colistin and tetracycline. Resistance to multiple drugs, up to 13 antibiotics (65% of *Pseudomonas spp*. resistant to between 8 to 13 antibiotics) was found, which was produced by practically all the known mechanisms of antimicrobial resistance (34). Our results show the following values: *Pseudomonas spp*. MIC50 for 32 µg/mL AMX, MIC50 for 16 µg/mL AMC, MIC50 for 3 µg/mL TET, MIC50 for 32 µg/mL CLR, MIC50 for 48 µg/mL ERY, reflecting high levels of resistance to the most commonly used antibiotics for the treatment of odontogenic infections. There are no reports of antimicrobial susceptibility of *Pseudomonas spp*. isolated from endodontic pathologies.

Regarding the profiles of sensitivity to antibiotics of dental clinical importance for facultative anaerobic strains, our report showed a high percentage of susceptibility to AMX, AMC and TET, which fell to 30.7% susceptibility to CLR and ERY. In seven *E. faecalis* strains, with a MIC50 for 0.25 µg/mL AMX, a strain with a 48 µg/mL MIC was

isolated for AMX. All *E. faecalis* strains were susceptible to AMC and TET, and there were no strains susceptible to CLR and ERY. A study conducted on Brazilian population agrees with our findings regarding the percentage of susceptibility (23). Research on German population indicates that of 6 strains of *E. faecalis*, only one was resistant to AMX, and two to TET. Studies further concluded that facultative anaerobic bacterial species were resistant to a series of clinically relevant antibiotics (36). A study conducted on Polish population showed a low percentage of *E. faecalis* strains susceptible to ERY (28). Results of our study suggest that the genus *Staphylococcus spp*. showed low susceptibility to CLR and ERY. There are no reports on the susceptibility of strains of the genus *Staphylococcus spp*. isolated from endodontic infections. In the case of anaerobes isolated from primary endodontic infections, strains of endodontic origin, an increase in antimicrobial resistance was observed over time (11). *Propionibacterium spp*. is susceptible to most of the antibiotics used in dental treatments, however it is highly resistant to MTZ (24,28,35), in agreement with the findings of our report. This is highly worrying because MTZ is one of the few options to treat infections caused by anaerobes. The presence of the *ermC*, *tetM* and *tetW* genes has been reported in 6,10 and 7 of 24 cases of asymptomatic apical periodontitis, respectively, which explains the low levels of susceptibility to these antibiotic groups, reason why it is suggested to research the presence and expression of these and other resistance genes in future studies.

Based on the MIC and MBC values obtained, the antimicrobial activity of the three nanostructures tested against the 31 species of bacteria associated with persistent endodontic infections was, in descending order, CuNP> CuNW> ZnONP. Regarding the susceptibility of Gram negative strains exposed to CuNPs, a very high MIC50 was observed in comparison to previous reports, where the values of 312 µg/mL of and 325 µg/mL are 4 to 5 times lower. However, differences lie mainly in the fact that our report included clinical aerobic strains and Cu was used in its pure state and not in an oxidative state, such as CuO or Cu2O. The previous report was carried out with anaerobic and American Type Control Culture (ATCC) strains, which are usually very susceptible to antimicrobial tests (22). Regarding the susceptibility of Gram-positive strains exposed to copper nanostructures, we observed a MIC50 of 250 µg/mL and a MBC50 of 500 µg/mL.

There are no previous reports of MIC/MBC of CuNPs on aerobic strains of oral origin. In reports on clinical strains isolated from other human pathologies, MBC values above 500 µg/mL have been observed (37).

The bactericidal effect of copper nanoparticles on all the species studied in this research suggest that it would be death by contact, implying that the release of ions in the local environment is necessary for optimal antimicrobial activity (22,37). Copper nanostructures that cause death by contact are potentially useful to tackle the emerging resistance to conventional antimicrobial agents, since in a short time the action of simultaneous antimicrobial mechanisms makes the development of resistance to these nanoparticles very unlikely (38).

In conclusion, the phenomenon of drug resistance is currently a global public health concern (12) due to the emergence of resistant strains by the use and abuse of antimicrobial agents. These new strains have gained control of an ecological niche previously occupied by sensitive ones. This is precisely what happens in the course of the treatment of chronic infections, which will eventually leave clinicians without effective antimicrobial agents (13). It is necessary to continuously monitor the susceptibility profiles of bacteria isolated from the oral microbiome, to know their behavior, and to search for strategies that avoid the selection of resistance, as the use of alternative antimicrobial agents such as copper nanoparticles. The results of this study suggest low levels of susceptibility of strains isolated from persistent endodontic infections to antibiotics of regular use in the field of dentistry and demonstrate that copper nanoparticles can be a viable alternative for disinfection for the treatment of endodontic infections.

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CAPÍTULO VI: ANTI-BIOFILM POTENTIAL OF COPPER NANOPARTICLES (CUNPS) AGAINST AN AEROBIC MULTISPECIES *EX VIVO* MODEL

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Abstract

Endodontic treatment aims to reduce the amount of bacteria by means of irrigation and medication with antimicrobial agents, keeping levels of harmful microorganisms low in order to favor healing. However, treating all the surfaces of the canal system is difficult due to its anatomical complexity, which in some cases may result in endodontic failure. Copper has antimicrobial properties against different microorganisms. It is for that reason that to evaluate its action at nanoscale could offer an alternative for disinfection during endodontic treatments. Objective: The aim of this study was to evaluate the antibacterial activity of copper nanoparticles (CuNPs) on an *ex vivo* aerobic biofilm. Materials and Methods: On an *ex vivo* aerobic biofilm model by means of plaque count, to evaluate the antibacterial activity of CuNPs compared to the antibacterial activity of calcium hydroxide [$Ca(OH)_2$].Results: There were significant differences between the $Ca(OH)_2$ and CuNPs groups as intracanal medication in comparison with the CuNPs groups as irrigation solution. An increase in the count of the group exposed to 7 days of $Ca(OH)_2$ was observed, compared to the group exposed to $Ca(OH)_2$ for 1 day. These findings differ from what was observed with CuNPs in the same periods of time.

Antibacterial activity of CuNPs was observed on a multispecies aerobic biofilm,

detecting an immediate action and an over time effect, gradually reaching their highest efficacy on day 7 after application. A higher count in the $Ca(OH)_2$ group exposed for 7 days compared to the group exposed for 1 day was observed, which differs from the results obtained with CuNPs during the same measured times. Conclusion: The latter raises the possibility of the emergence of $Ca(OH)_2$ resistant strains, and supports the use of CuNPs as alternative intracanal medication.

Introduction

One of the main causes of endodontic failure is the persistence of microorganisms in the root canal system [33]. It is well documented that microorganisms possess and develop resistance against disinfectant agents and endodontic drugs, which further complicates the treatment of the root canal system[9]. The problem increases because, during infection, microorganisms form biofilms, making them 1,000 times more difficult to eliminate [10,22].

It has long been reported that secondary endodontic infections show a high prevalence of *Enterococcus faecalis*, as they are able to persist after root canal treatment. Due to its excellent bactericidal action, Calcium Hydroxide [$Ca(OH)_2$] is the antimicrobial agent of choice for intraradicular medication. A central aspect related to the antimicrobial activity of $Ca(OH)_2$ is its high pH, close to 12.5. However, some studies have demonstrated the survival of *E. faecalis* even in this alkaline environment [2,12].

Copper, a trace element essential for life, is involved in a wide range of processes and has been used by humans for more than 10,000 years. Recently, copper has become a focus of scientific interest due to its antimicrobial properties and its reported low toxicity in humans. Combined pharmacological complexes based on copper have been shown to be more effective as antibacterial, antifungal and antiviral agents [8,23].

The mechanism by which copper acts produces a bacteriostatic [15] or bactericidal effect, which has been directly related to its concentration. The maximum reported effect has been for copper metal (99.9%) and these results have been observed in alloys containing at least 70% copper [8,23,25]. However, due to the anatomy of the canal system, which has very small ecological niches such as the dentinal tubules, where antimicrobial agents cannot reach, nanotechnology appears as an alternative to increase the success

rate of treatments and endodontic retreatments [17].

Nanoparticles originate from a metal at macrometric size. They have a diameter smaller than 100 nm with different and improved properties with respect to the original metal [16]. ShivShankar *et al.*[29] report that copper nanoparticles (CuNPs) have various potential applications in electronics, optics and medicine. CuNPs have been used in the manufacture of conductive films, lubrication, nanofluids, and as a potent antimicrobial agent. In addition to controlling the growth of yeasts and fungi, CuNPs have also been shown to be effective against Gram-positive and Gram-negative bacteria. The antimicrobial activity of the nanoparticles depends on their size. To achieve maximum antibacterial activity, they must be synthesized to a size that allows greater contact of the nanostructure with the bacterial surface, showing a more effective antimicrobial action than at their normal size, covering a broad bacterial spectrum, including multi-drug resistant microorganisms [16].

Kruk *et al*. [19] synthesized CuNPs at a concentration of 300 ppm through the reduction of copper salt. The morphology and structure of the nanoparticles were observed using scanning electron microscopy, obtaining average diameters of 50 nm. They were then tested against different microorganisms showing high antimicrobial activity against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA).

Avinash *et al*. [16] reported that CuNPs are able to penetrate through the bacterial cell wall causing cellular damage. After entering the cell, nanoparticles indirectly affect cellular DNA or protein synthesis, inactivate their enzymes and promote the generation of hydrogen peroxide. Finally, nanoparticles interact with –SH groups (atoms of hydrogen), which lead to the denaturation of proteins. All these elements make the possibility of selecting resistant strains extremely low.

Finding a new antibacterial agent as an alternative for use in endodontic treatment is essential to reduce the resistance of microorganisms. Unfortunately, it is well known that the systematic application of high doses of an antimicrobial agent leads to the selection of strains that produce higher levels of persistent bacteria. This is precisely what happens in the treatment of chronic infections, which is becoming a serious threat since in the near future we may find ourselves deprived of effective antimicrobial agents [18-20].

Materials and methods

This project was carried out in accordance with the general principles of the Code of Ethics established in the "Belmont Report", supported by the signing of an informed consent, approved by the Ethics Committee of the School of Dentistry of Universidad de Concepción, for the use of isolated clinical strains from persistent chronic apical periodontitis for an *ex vivo* biofilm model. (C.I.Y.B. No. 04/15).

Selection of the sample

Eighty roots from extracted teeth with type I canals according to the Weine classification were selected for the study. Samples had slight moderate curves (less than 20°) according to Schneider, with a minimum of 9 millimeters in length. They were clinically permeable, with complete apical closure and stored in physiological serum [17]. To measure the root curvatures, dental x-rays were taken and angular measurements were performed using VistaScan software (Dürr Dental®, Stutgart, Germany).

Development of an *ex vivo* model of aerobic artificial biofilm on root canal surface

Eighty root canals, from extracted teeth, with type I canal according to the Weine classification were prepared using reciprocant instrumentation technique with 25/08 Wave OnePrimary file (Dentsply- Maillefer®, Ballaigues, Switzerland) and constant irrigation of sodium hypochlorite 5,25% according to manufacturer's instructions. After instrumentation, canals were irrigated with a solution of 10% EDTA for 3 minutes and finally 5.25% NaOCL to completely remove dentinal mud. The roots were dried with sterile gauze and covered on the outer surface with 2 layers of nail polish (taking care not to block the entrance to the canal) to avoid external contamination of the roots. Subsequently, samples were taken individually into test tubes with a buffered saline solution, then autoclaved for 30 minutes at 121°C. Efficacy of sterilization was confirmed using the protocol proposed by Javidi [17].

Bacterial strains *Streptococcus mutans* ATCC 25175 (American Type Culture Collection) and the isolated root canal strain, *E. faecalis* UDEC 6.1 confirmed by Polymerase Chain Reaction (PCR), were cultured aerobically at 37°C for 24 hours on brain heart infusion agar (BHA, Merck Millipore, Darmstadt, Germany). A colony of each

strain was separately inoculated in 5 mL of BHI broth to ensure pure culture. After 18 to 20 hours of incubation at 37°C, the suspension of each strain was adjusted with Oxoid turbidimeter (Fisher Scientific Company, Ottawa, Canada) until a turbidity equivalent to McFarland 0.5 was achieved (1.5-2 x 10^8 CFU/mL). A 500 µL aliquot of each adjusted suspension was deposited in each of the tubes containing the dental specimens. The tubes with the samples were incubated at 37°C for 48 hours. After incubation, samples were washed gently 2 times with 1 mL of PBS to remove unbound bacteria. Roots were divided into 9 groups: group 1 was negative control group (no treatment), groups 2 to 6 were treated with CuNPs for 1, 10, 30, 60 minutes, 1 day and 7 days, respectively; groups 7 and 8 were the positive control treated with calcium hydroxide (UltraCal® XS) for 1 day and 7 days.

Dressing and incubation of the roots

Medication was applied and samples were taken from each tube according to the respective medication period. CuNPs were obtained by a new technique known as arc discharge in controlled atmosphere (DARC-AC). In this technique no stabilizing agent is used so the surface of these nanoparticles is not passivated. Passivation of the metal surface causes the electrical, optical and even antimicrobial properties to worsen because electronic or ionic exchange with the medium is not possible due to stabilization. The obtained particles had a size that ranged between 20 and 40 nm, as seen in Figure 1. Most of the structures had well-defined decahedral (mostly spherical) shapes. The low resolution image shows the low dispersion in size of the nanoparticles.

Because nanoparticles do not dissolve but disperse in liquid, propylene glycol was used at the concentration of ¼ of the highest Minimum Inhibitory Concentration (MIC) for both strains as a dispersing medium. A dispersion with a concentration equal to MIC was prepared for *Enterococcus faecalis* UDEC 6.1, corresponding to 256 µg/mL, greater than the MIC for *Streptococcus mutans* ATCC 25175, which was determined at 125 µg/mL.

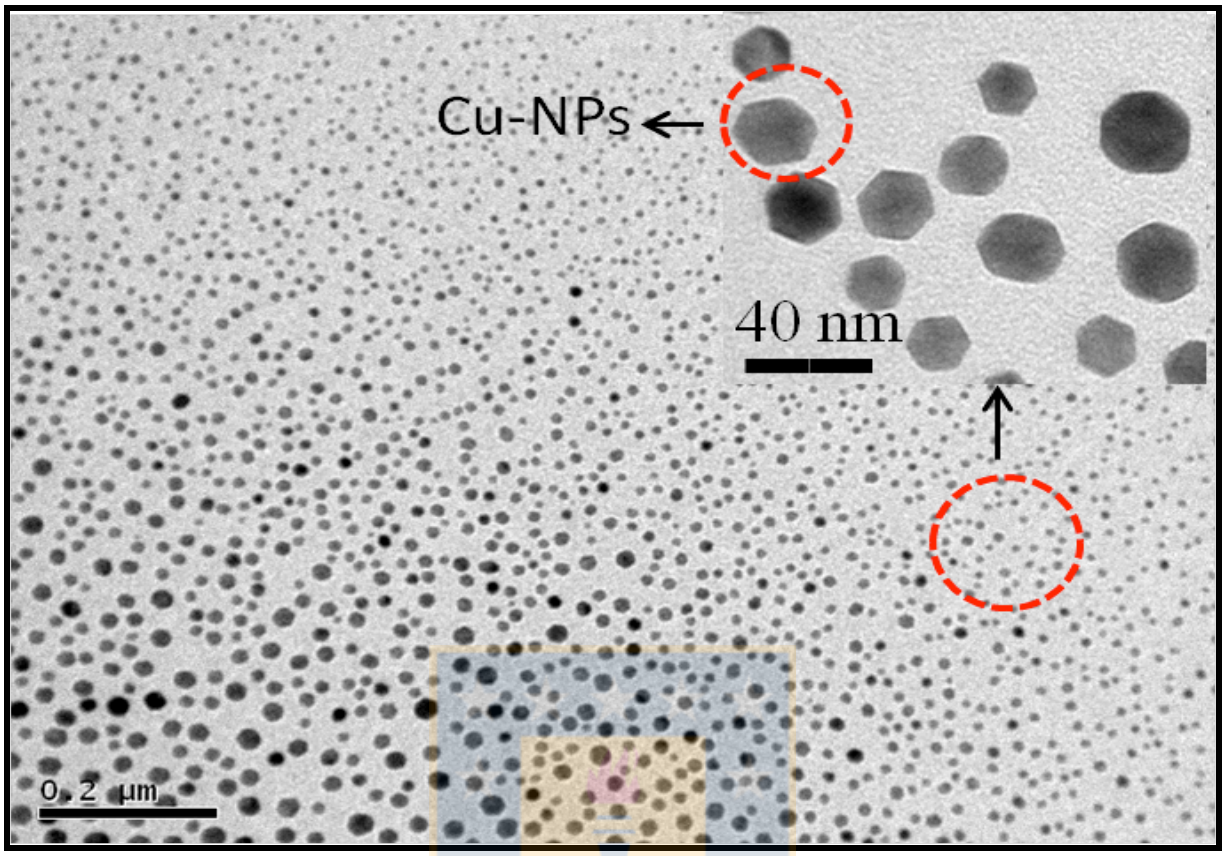

Figure 1. Copper nanoparticles obtained by arc discharge in controlled atmosphere

Plaque count

Following the respective incubation times in the laminar flow chamber (Thermo Scientific®, Marietta, Ohio, USA), samples were taken and evaluated by counting colony-forming unit (CFU) in triplicate, according to Javidi's protocol [17]. To verify that there was no contamination, colonies were taken randomly on day 7 and observed a microscope with a 100x magnification and immersion objective.

Statistical analysis

To compare the activity of antibacterial agents, non-parametric ANOVA (Kruskal-Wallis test) and the Bonferroni multiple comparison test were performed. Level of significance of 5%. All statistical analysis was carried out using the InfoStat ® program.

Results

From the descriptive point of view, all the groups showed antimicrobial activity since a decrease of more than 3 logarithms was observed in the count in comparison with the control group.

Table 1: UFC count per Log_{10} group

Treatment	n	Mean	S.D.	Min	Max
1 min CuNP	27	8.E+07	4.E+07	6.E+04	1.E+08
10 min CuNP	27	8.E+07	4.E+07	6.E+04	1.E+08
30 min CuNP	27	8.E+07	4.E+07	3.E+05	1.E+08
60 min CuNP	27	9.E+07	3.E+07	3.E+06	1.E+08
1 day $Ca(OH)_2$	27	7.E+01	2.E+02	0.E+00	1.E+03
1 day CuNP	27	3.E+04	4.E+04	2.E+03	1.E+05
7 days $Ca(OH)_2$	27	3.E+03	6.E+03	0.E+00	3.E+04
7 days CuNP	27	4.E+03	8.E+03	0.E+00	3.E+04
Control	27	1.E+08	0.E+00	1.E+08	1.E+08

The Kruskal-Wallis non-parametric test was performed. There were significant differences in mean per treatment ($p<0.0001$). Additionally, the Bonferroni multiple comparisons test was performed and significant differences were obtained in mean per treatment ($p<0.0001$; ANOVA). The groups with different letters (A, B and C) showed statistically significant differences. Groups with a common letter were not significantly different ($p>0.05$).

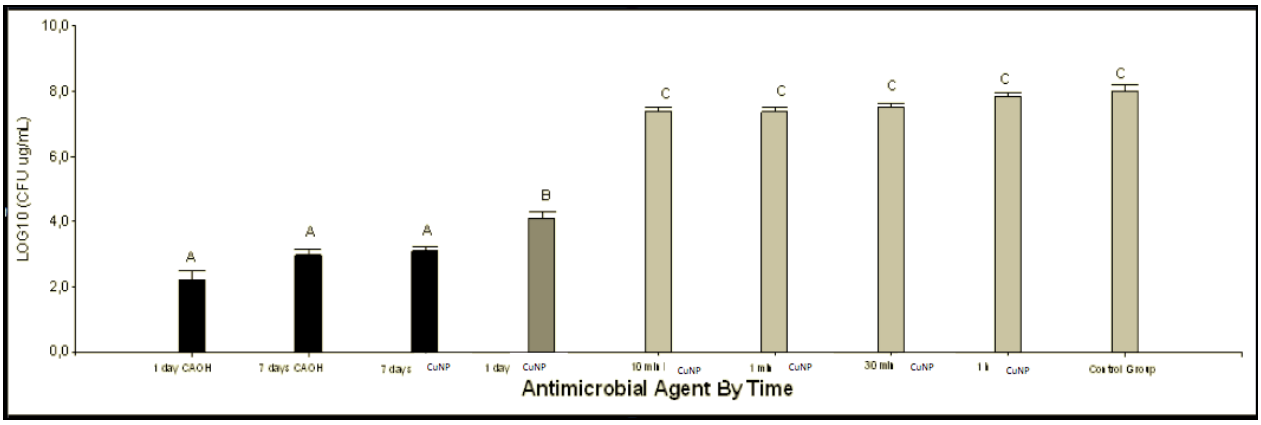

Figure 2. UFC count in $\log_{10}$ per group and statistically significant differences between groups A, B and C. ($p<0.0001$; ANOVA).

DISCUSSION

Bacterial resistance is a constant concern in the failure of root treatments, hence the importance of studying new antimicrobial agents that can be used as coadjuvants and/or alternatives in endodontic therapy [7]. Copper has multiple properties, among which antimicrobial contact and anti-contact action avoids the appearance of resistant microorganisms. CuNPs improve the properties of copper as pure metal, which is a great advantage, as they can access tiny dentinal tubules with an average size of 5µm, in which the endodontic biofilm is housed [16]. The present report showed antimicrobial activity in all the tested groups, since a decrease of more than 3 logarithms was observed in all the pre-established times, reason why from the microbiological point of view, antimicrobials behaved as bactericidal agents. Statistically significant differences were obtained between samples exposed to one day $Ca(OH)_2$, 7 days of $Ca(OH)_2$, 7 days of CuNPs and 1 day of CuNPs, compared to samples exposed to CuNPs at 1 hour or less. This reinforces the idea that CuNPs could be an alternative treatment as a medication and not as an adjunct in the irrigation of canals, at least in the concentration used in this study. This had already been described for other antimicrobial nanostructures, whose behavior was better as medication than as an irrigant [29]. However, these results, observed from a descriptive point of view, are in contrast to those reported by Javidi *et al*. Although, they are not comparable since the present results are expressed in the logarithm of the counts, unit of measurement accepted for

this type of tests [17].

The role of $Ca(OH)_2$ as one of the most used intracanal drugs in endodontic treatments has been well documented to date [24]. In the present report it is relevant to emphasize its greater antimicrobial efficacy at day 1 compared to day 7 of medication. This contrasts with most of the existing literature that suggest that the peak of action is reached between 7 and 10 days after its application [14]. This could be related to the fact that the $Ca(OH)_2$ used in this study (UltraCal® XS) is a commercial preparation that uses propylene glycol as transport medium; it is less soluble and does not need replacement, besides it maintains pH values stable in a range of 12.07 to 12.78. The latter does not occur in other types of $Ca(OH)_2$ preparations, which would account for the prolonged selection pressure within the canal [3,28]. There is scientific evidence that suggest resistance of strains of *E. faecalis* to pure $Ca(OH)_2$ [13,30,31], but further clinical studies are necessary [3]. However, this result would reinforce the idea that resistance could be due to prolonged exposures to a potent antimicrobial agent, resulting in the selection of resistant strains that, over time, gain the ecological niche in the root canal [20]. It has been reported that *E. faecalis* is capable of producing physiological and genetic changes in response to changes in its environment, which favors its survival [2,32].

Our results suggest that there are no significant differences between the action of CuNPs at 7 days and $Ca(OH)_2$ at 1 day and at 7 days of exposure, but there are statistically significant differences of these groups with the samples exposed to 1 day of CuNPs. We assume that the antibacterial action was lower in the groups that were exposed only minutes because the MIC used for CuNP in this *ex vivo* study was lower, compared to the average MIC for the same species in other *in vitro* studies (*E. faecalis* 31.25µg / ml [1,4,26,33] , *S. mutans* 48µg/ml) [11,27]. When using *in vitro* MICs in *ex vivo* or *in vivo* tests, there are other factors that affect the efficiency of the antimicrobial agent (biofilm, interaction with other tissues, pH, oxygen, etc.) [28]. Therefore, we suggest to carry out assays with 2 and 4 times the CMI for CuNPs in addition to increasing the number of bacterial species, to include anaerobes in a mature biofilm, since this will allow to obtain more conclusive results, closer to the *in vivo* environment of the root canal system [2]. This would give CuNPs greater antimicrobial efficacy over time [5].

In this study we failed to establish statistically significant differences between CuNPs

and $Ca(OH)_2$ at 7 days of treatment resulting in very similar counts of colony forming units. And although the count was a larger log on the 1-day exposure to CuNPs compared to the 7-day exposure, viewed from the point of perspective of resistance selection, it is an excellent result compared to $Ca(OH)_2$. Jose *et al.* [18] report CuNPs anticancer activity. This is due to their potential for degradation of DNA, which is in direct relation with their low potential for selection of resistant strains. CuNPs degrade DNA with oxygen mediation, even in the absence of any external agent such as hydrogen peroxide or ascorbate. This makes CuNPs excellent candidates for targeted therapy. The use of CuNPs as a therapeutic agent could in particular be advantageous because the human organism has an efficient system to metabolize copper, since it is a micronutrient, thus, the produced residual copper can be handled easily.

In addition, this potential DNA degradation and the cytotoxic effect of CuNPs can be used in the formulation of anticancer drugs by chemically modifying copper [16]. We cannot fail to consider the toxicity of CuNPs, which is based especially on its cytotoxic potential [6]. Liu *et al*. [21], in an in vitro study, suggest that in order to perform metal nanoparticle toxicity tests, the released dose (i.e. the mass of nanoparticles per volume of suspension) should be considered instead of taking into account the administered dose alone (initial concentration in mass of nanoparticles). From this, they conclude that *in vitro* dose-response results depend on complex toxicodynamic reactions, which include nanoparticle/cell association-relation, activation of the response pathways of cells involved in the uptake of nanoparticles, and multiple physical-chemical parameters that influence the sedimentation and internalization of these. It is suggested to perform studies to evaluate the barrier capacity of apical constriction, in an *ex vivo* or *in vivo* model, in relation to the cytoxicity of CuNPs on the periapical tissues.

CONCLUSION

In the present study it was possible to observe the antibacterial activity of CuNPs on a biofilm of *E. faecalis* and *S. mutans*. It was possible to detect an immediate action and an over time effect, gradually reaching their highest efficacy on day 7 after application. The latter shows the potential use of CuNPs as intracanal medication. It is important to emphasize the action of calcium hydroxide from a commercial preparation, which was

higher on day 1 compared to day 7; this could suggest shorter medication periods with superior effectiveness in root canal treatments, reducing selection pressure within the root canal system.

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CAPÍTULO VII: SYNTHESIS OF COPPER NANOWIRES AND THEIR ANTIMICROBIAL ACTIVITY ON STRAINS ISOLATED PERSISTENT ENDODONTIC INFECTIONS

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Abstract

Copper nanowires, Cu-NWs may have a good antimicrobial effect in endodontic treatment. The objective of this work was to synthesize, characterize and evaluate the antibacterial activity of Cu-NWs on strains obtained from human root canal. A wide distribution of Cu-NWs diameters from 30 to 90 nm was obtained with lengths ranging from 5 to 40 μm. Structural analysis of Cu-NWs showed crystalline planes, which corresponded to Cu, with preferential growth in the direction [110]. The geometric mean MICs was of 289.30 μg/mL, with a MIC_{50} of 256 μg/mL and a MIC_{90} of 512 μg/mL for Cu-NWs. Cellular viability of a biofilm tends to decrease the longer it is exposed to Cu-NWs. Synthesized and characterized Cu-NWs have a good antimicrobial effect against clinical strains used in the present study and has a potential to be used for disinfection of the root canal system.

Keywords: Copper nanowires, Antibacterial activity, Endodontic, *Enterococcus faecalis.*

1. Introduction.

There is a growing interest in the application of nanostructures as antimicrobial agents to treat various oral infections because of their biocidal and anti-adhesive properties against biofilms. The use of antimicrobial nanostructures as components of coatings of prosthetic devices, topical application medications and dental surgical materials has been previously described in dental literature, but there are still very few reports on their application as medication in Endodontics.[1, 2] Nanostructures are particles with a variety of shapes and a size inferior to 100 nm may be made of metal or polymers.[3] They have an active surface area, chemical reactivity and biological activity, and their shapes are often very different from those of larger-size particles. These characteristics allow nanostructures to interact closely with microbial membranes and, therefore, their antimicrobial effect is not exclusively due to the release of metal ions.[2] Nanostructures are potentially useful within this context, since it is possible to modify their physical and chemical properties to increase their activity against many microorganisms.[4]

It has been demonstrated that when nanostructures adhere to the surface of the microbial cell membrane, they drastically disrupt its functions, such as cell permeability and respiration. They are able to penetrate into the bacterium and cause further damage by possible interactions with sulfur and phosphorus-containing compounds such as DNA. They also release ions, which will additionally contribute to the bactericidal effect of nanostructures.[4] However, the smaller particles, around 10-20 nm, are more toxic than the larger ones, and more so when they oxidize.[5] It is suggested that bacteria are much less likely to acquire resistance to metal nanostructures than to conventional antibiotics. This is because metals can act on a wide range of microbial targets, and microorganisms would have to undergo many mutations to resist the antimicrobial activity of metals.[2] The shape of the nanostructure can also affect the activity of microorganisms, as it has been demonstrated against strains of *Escherichia coli*, where triangular silver nanoplates showed the highest biocidal activity compared to spherical nanostructures. The differences seem to be explained by the proportion of active facets among nanostructures with different shapes.[6]

Copper is an essential metal for the metabolism of all cells and its antimicrobial activity has been documented worldwide. In relation to the intensity of its bactericidal action, it is

accepted that there would be a direct relation with its concentration; a maximum effect (99.9%) for metallic copper has been observed.[7] The mechanism of action is based on the ability of copper to yield and capture electrons, facilitating the generation of radical oxygen species (ROS), which in turn can lead to increased production of toxic hydroxyl radicals.[8] These radicals take part in a series of reactions that damage cell macromolecules, altering the structure of proteins, which results in loss of protein function.[9] Copper may compete with other metal ions, such as iron from dehydratase enzymes,[10] and may also peroxidate membrane fatty acids, resulting in increased permeability and cell lysis.[11] It has also been reported that it can denature Desoxiribonucleic Acid (DNA)[12] and inhibit biofilm formation.[9] It has been shown that on copper surfaces, in addition to the rapid killing of antibiotic resistant strains, degradation of plasmid DNA and genomic DNA has occurred, which has an implication in preventing the spread of infections and horizontal gene transfer.[12] Therefore it is very important to know the methodology of synthesis and characterization of nanostructures. Consequently, the antibacterial properties of copper nanostructures may also have application in endodontics, since the environment in the root canal, which serves as an ecological niche for microorganisms, is a surface formed by small dentinal tubules of 1-5 µm in diameter.[13,14] It is in these tubules where persistent bacteria, resistant to multiple antimicrobials, are established, forming an endodontic biofilm.[15] Therefore, this would be the place where Cu nanostructures may play a role as a new antibacterial agent.

The antimicrobial properties of copper nanostructures are among the most described in other areas of medicine.[16] Compared with traditional antibiotics, nanostructures are effective at 1000-fold lower concentrations. Strains of periodontal pathogens were susceptible to silver and cooper nanostructures under anaerobic conditions, with minimal bactericidal concentrations in the range of 250 to 2500 µg/mL.[17] However, copper is cheaper than silver, readily miscible with polymers, and relatively chemically and physically stable.[16] Another study reinforces the finding that copper nanostructures work best on *Bacillus subtilis* strains than silver nanostructures.[18] A study established a starting point for the clinical use of copper, specifically for the disinfection of the root canal system. Researchers compared the drugs commonly used in endodontics with copper sulphate pentahydrate. The latter showed antimicrobial activity at low

concentrations, being slightly more effective than Chlorhexidine.[19] However, there are no reports of the antibacterial action of copper nanostructures on clinical strains isolated from endodontic pathologies. It is reported that antimicrobials commonly used in endodontics may not be effective in eradicating resistant bacteria,[20] so searching for alternative treatments is relevant. In this study, Cu-NWs were synthesized and characterized to later perform an *in vitro* and *ex vivo* evaluation of their antibacterial activity on clinical strains isolated from the root canal.

2. Experimental Details.

Synthesis and characterization of Cu-NWs: The synthesis of the Cu-NWs was carried out by the hydrothermal method, using a 30 mL autoclave reactor with a 20 mL inert teflon container. This type of synthesis is appropriate for obtaining type 1D nanometric structures like wires or bars, because the reaction system is closed and the temperature of the process is increased above 100 °C generating high pressure inside the system. A precursor molecule is added to interact with the growth seeds, this interaction depends on the energy of the different facets of the seed (a nanoparticle of 2-4 nm). The high pressure of the system then forces preferential growth on the facets of the seed where interaction with the precursor molecule is weak. The process is simple, highly reproducible and crystalline type 1D nanometric structures are obtained.

A typical synthesis is based on the use of 50 mL of $CuCl_2$ (12.5 mmol/L). A 25 mL aliquot of the previous stock solution is taken and 0.0876 g of octadecylamine are added; the remaining 25 mL were added 0.1509 g of hexadecylamine. These last two compounds act as reducing agents and also as preferential growth molecules. The solutions were vortexed vigorously for 5 hours to form a blue emulsion (Figure 1A). The solutions were then transferred to the hydrothermal reactor (Figure 1B) and placed in an oven with programmable temperature at 145 °C for 48 h. After this time, the reactor is cooled slowly to room temperature. The reaction has a conversion of 95% in obtaining Cu-NWs and 5% in obtaining nanoparticles. Separation of NWs was carried out by decantation, and then they were washed repeatedly with absolute ethanol, n-hexane and deionized water (Figure 1C). The wires were suspended in deionized water and a certain amount of hydrazine was added to prevent their oxidation (Figure 1D). When the

wires oxidize their coloration changes from brick red to a dark colored solution as shown in Figure 1C. Hydrazine acts as an antioxidant agent preventing the degradation of the wires.

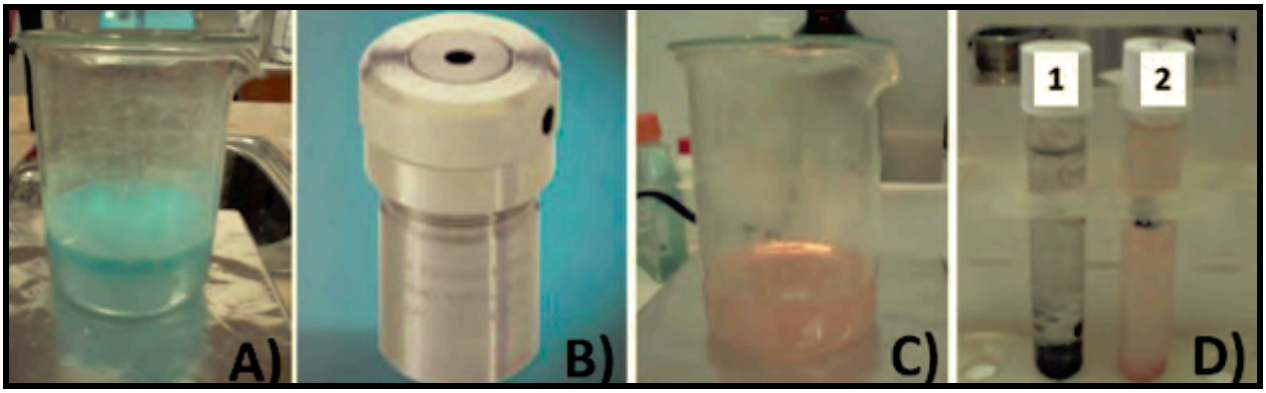

Figure 1. (A) Reaction mixture of $CuCl_2$ and octadecylamine after the homogenization process. (B) Hydrothermal reactor where the above mixture is placed. (C) Nano wires obtained after hydrothermal growth. (D) Comparison of the stability of the nanowires using hydrazine.

Characterization of Cu-NWs: Samples were structurally characterized by X-ray diffraction (XRD) using a Philips X'Pert PW3040 diffractometer (PANalytical, Almelo, the Netherlands) with Cu-Ka radiation. High-resolution transmission electron microscopy (JEM-ARM200F, JEOL, USA) was performed in a probe aberration corrected analytical microscope with a resolution of 0.08 nm. Selected area of electron diffraction was performed in a JEOL 2010F operating at 200 kV (point resolution of 0.19 nm). Scanning electron microscopy (SEM) was carried out using an ultra-high-resolution electron microscope (FEG Hitachi S-5500, UK) (0.4 nm at 30 kV) with a BF/DF Duo-STEM detector and in a FEI-Nanonova 100 FESEM. The UV absorption spectra of colloid particles (0.25%v/v) were analyzed with a spectrophotometer UV (Shimadzu, Kyoto, Japan). Absorption spectra were recorded from 190 to 400 nm, using quartz cells.

Bacterial strains: This study was carried out in accordance with the general principles of the Code of Ethics established in the Belmont Report and was performed in compliance with the protocol approved by the Faculty Ethical Committee (C.I.Y.B N°08/14). Sixteen clinical strains of *E. faecalis* isolated from persistent endodontic infections from a human root canal were used in the study.[20]

Antibacterial activity of Cu-NWs on E. faecalis: The minimum inhibitory concentrations (MICs) of Cu-NWs was determined for each strain according to the guidelines of the CLSI (Clinical and Laboratory Standards Institute) [21,22] using the broth microdilution technique. The culture medium used was Mueller-Hinton broth (MHB) (Oxoid, Sigma-Aldrich, UK.). MIC was determined and decreasing concentrations of the antibacterial agent were established in a microplate starting at 1024 µg/mL, with a final inoculum of 10^7 CFU/mL. Sterile broth and $CuSO_4$ were used as positive and negative controls respectively. Subsequently, the plates were incubated in an oven at 37 °C for 24 h. All strains were assayed in triplicate. The reading was initially performed by visual inspection and the bacterial growth was considered positive in the wells that showed turbidity. Wells that were completely translucent were classified as negative bacterial growth. After visual inspection, 10uL of MTT (tetrazolium salts) were added to facilitate the reading of turbidity, as it is increased if bacteria are metabolically active. The plates were incubated again in the Shaking incubator for 30 min at 37 ºC. After this time, it was possible to corroborate the MIC of Cu-NWs against each strain of *E. faecalis* obtained previously by visual inspection without MTT.

Ex vivo model of aerobic artificial biofilm on root canal surface*.* Twelve extracted roots type I canal according to Weine's classification of root canal morphology were extracted. They had slight to moderate curves (less than 20°) according to Schneider, with a minimum of 9 millimeters in length, clinically permeable, with complete apical closure and stored in sodium chloride (0.9%). [23] To measure the root curvatures, dental x-rays were taken and the angular measurement was performed with VistaScan software (Dürr Dental®, Stutgart, Germany). Canals were instrumented using the Wave-one Primary system (Dentsply Maillefer; Ballaigues, Switzerland) at working length with constant NaOCl irrigation of 5.25% sodium according to the manufacturer's instructions. After instrumentation, canals were irrigated with a 17% EDTA solution (1 min) and then with NaOCl at 5.25% (1min) to completely remove smear layer. The 12 roots were divided longitudinally with a diamond disk. Subsequently, the samples were taken to a buffered saline solution (BSS) to be autoclaved for sterilization for 30 min at 121°C. The efficacy of sterilization was confirmed using the protocol proposed by Javidi *et al*.[23] All specimens were used as support for the formation of aerobic multispecies biofilm as

described by Shresta *et al*. with modifications suggested by Pan *et al*.[24,25]

The bacterial strains *Streptococcus mutans* ATCC 25175 and the clinical strain *E. faecalis* with the highest MIC determined in the previous assay, were cultured aerobically at 37 °C for 24 h on Brain Heart agar (BHA, Merck Millipore, Darmstadt, Germany). A group of 5 to 10 colonies was inoculated into 5 mL of BHI broth. After 18 to 20 h of incubation at 37 °C, the suspension of each strain was adjusted with an Oxoid turbidimeter (Fisher Scientific Company, Ottawa, Canada) to give a turbidity equivalent to 0.5 McFarland standard (1.5-2 x 10^8 CFU/mL). A 500 µL aliquot of *S. mutans* suspension and a 500 µL aliquot of *E. faecalis* suspension were deposited in each well containing the dental samples. Twenty-four samples were deposited in four microplates containing 24 flat-bottomed wells each (TPP, Tissue culture testplate 24) Trasadingen, Switzerland. These were incubated at 37 °C in an orbital shaker Shaking Incubator Litekvo (THZ-100, Shanghai, China) at 100 rpm for 24 h.[22] Afterward, samples were washed 2 times with 1 mL of BBS and then 1 mL of fresh BHI broth was added. The process was repeated at 48 and 72 h, after which the confocal microscopy analysis was performed.

Cu-NWs were dispersed in propylene glycol at a concentration of 0.25MIC for *E. faecalis* (6.25%) as a dispersing medium. A dispersion of 1mg/mL was prepared, corresponding to 6 times the MIC of *E. faecalis* strain. Cu-NWs were added in drops to the samples with a micropipette tip (1000 µL) according to the established times. After this period of interaction between the biofilm and the nanoparticles, samples were washed with 1 mL of sterile deionized water to remove remaining nanoparticles. Sodium chloride (0.9%) was added in drops on the samples with 27G monoject irrigation syringes only for 2 min as a control. After this period of interaction between the biofilm and the sodium chloride, the samples were washed with 1 mL of sterile deionized water to remove the remaining sodium chloride.

Confocal microscopy analysis. The LIVE/DEAD® Baclight™ Bacterial Viability Kit (Invitrogen, Carlsbad, CA) was prepared according to the manufacturer's instructions. Then, samples treated with the different antimicrobials during the established times were stained with 500 µL of the reagent and incubated in the dark for 10 min using aluminum foil (Alusa Foil, Aluhome, Chile). Samples were analyzed with a spectral confocal laser

microscope (LSM 780, ZEISS, Germany). An argon laser was the source of illumination, with excitation of 488 nm and an acquisition spectrum adjusted to the following parameters: for green 490-560 nm and for red 560-639 nm. Images of each sample were obtained using a 25X immersion lens and a 20X lens. From these parameters, images were obtained from the samples irrigated with sodium chloride for 2 min, Cu-NWs for 2 min, Cu-NWs for 6 min and Cu-NWs for 10 min.

***Statistical analysis**:* Optical sections of the biofilm structure were recorded and analyzed using IMARIS software, version 7.5.2, “Measurement Pro module” (Bitplane, USA). It detected green and red zones of different intensities (pixels) and yielded data of the volumes of those zones. The proportion of live/dead bacteria was determined and with this ratio the bacterial viability was compared in the different periods analyzed in this study.

3. Results and Discussión.

The synthesis process used in the preparation of Cu-NWs showed a high conversion greater than 95%, this can be seen in Fig.2B, where a SEM micrograph is shown at low magnification (20 μm). Most studies of nanotechnology in dentistry are not clear in describing the process of obtaining these materials. In this case, the wires are aggregated so their diameter seems to be higher than that reported in the literature.[26] This is because the materials needed more washing to remove the residues of preferential growth molecules. Fig. 2A shows the EDX analysis of the sample, peaks of around 7.9, 9.1 and 1.2 KeV correspond to the Cu (K) and Cu (L) signals, respectively. The latter confirms that the obtained wires are of metallic copper. This evidence is reinforced by the absence of oxygen signals, which rules out the formation of oxides due to the oxidation of the walls of the wires.[27] This demonstrates that hydrazine works quite well in protecting the material.

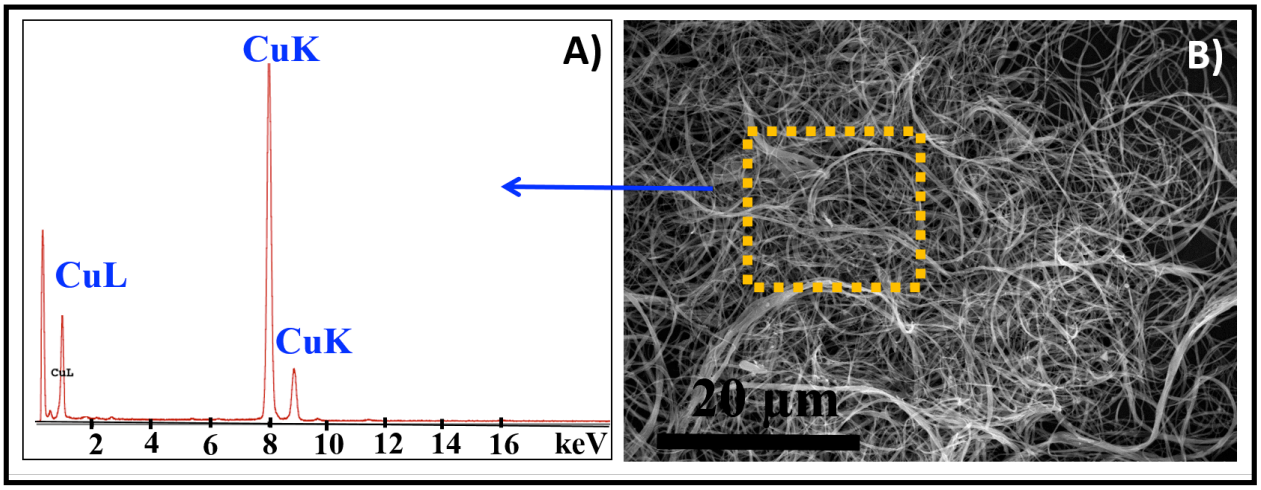

Figure 2. (A) EDX of nanowires obtained via hydrothermal growth. (B) SEM microscopy of copper nanowires at low magnification.

On the other hand, Fig. 3 shows TEM images of nanowires. There is a wide distribution of diameters ranging from 30 to 90 nm; however, all the diameters of the obtained wires are below 100 nm. The length of these varied from 5 to 40 µm. The color contrast observed in the wires corresponds to defects in grain boundary size that are produced by the high pressure of the reaction system; this did not affect the crystallinity of the material.

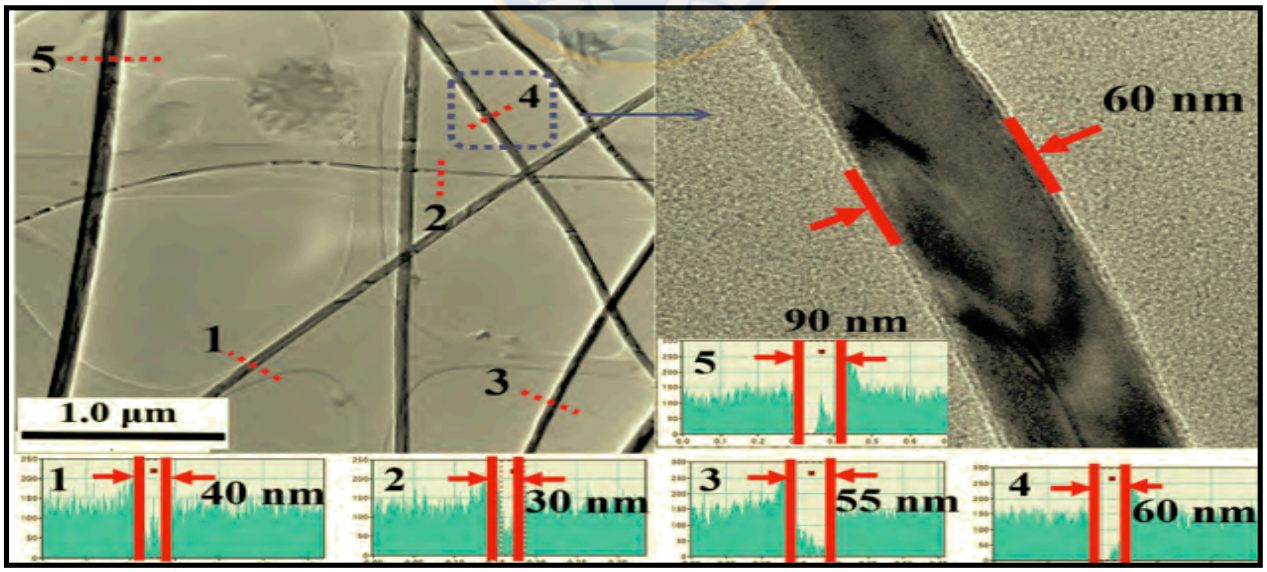

Figure 3. TEM microscopy of copper nanowires and profile diagrams of their diameters.

The measurements of the diameters were made by measuring the contour profile that appears next to the TEM micrograph at a magnification of 1µ. These analyses revealed contour defects, these can be observed as a peak within the red lines of these profiles. If there were no defects, they appear like a peak within the red lines of these profiles. If there were no defects within these lines, there would not be any protrusion like the one shown. There are no studies to date of copper nanowires with antibacterial properties in dentistry.[28] In this paper, we clearly characterize copper nanoparticles with antibacterial potential use in dentistry. Figure 4 shows the structural analysis of copper nanowires. Figures 4D-F corresponds to digital processing using Fast Fourier Transform (FFT).

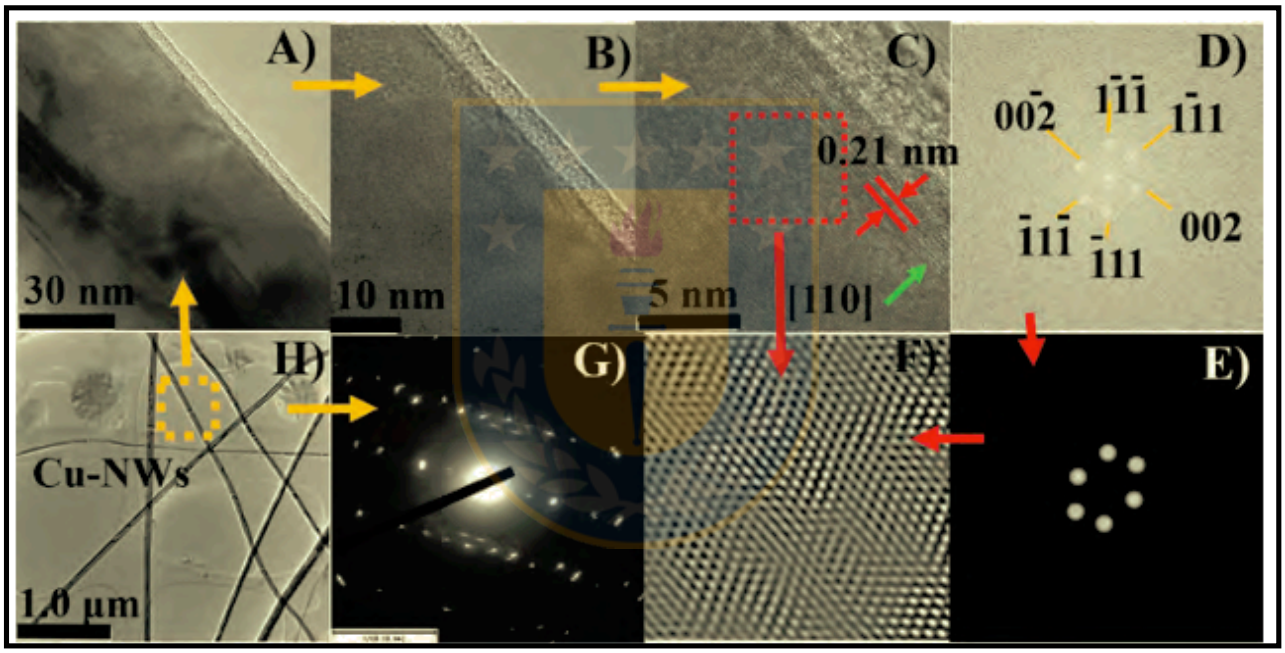

Figure 4. (A-B) High resolution TEM microscopy of the box in (H) where the formation of a nanometric layer of copper oxide is shown. (C) HRTEM microscopy showing the direction of preferential growth and the interplanar distance. (D) Fast Fourier transform (FFTs) of the box in "c", the indexing of the spots indicates that the wires correspond to metallic copper. (E) Mask/Filter applied to the FFTs to filter specific spots and clean the image. (F) Filtered image where the copper atoms are observed. (G) Diffraction pattern of the box in (H) where the crystallinity of the nanowires is shown.

Figures 4A-C are images from high resolution transmission electron microscopes (HRTEM) at different magnifications. In these images, the crystalline planes of the wires showing the preferential growth in the direction can be observed [110]; this also confirms the crystallinity of the sample. As the analysis is performed in solid state and not in liquid state, there is no hydrazide present, so the walls of the wires are covered with a layer of oxide as seen around the wires. Fig. 4H is a low magnification image with an enlarged box showing an image in high resolution. Figure 4G corresponds to electron diffraction; by indexing the spots (white spots) of this diffraction pattern it is shown that the wires have the distinctive FCC cubic structure of this metal. Similarly, the red box in Figure 4C was performed an FFT, which is shown in Figure 4D. The FFT analysis of a high-resolution image corresponds to a diffraction pattern similar to the Figure 4G. The indexing of these spots confirmed that the sample is metallic copper. A mask/filter (Fig 4E) is applied to this process of digitization (Fig. 4D) to eliminate signals that do not correspond to the wires; then after a filtering process Fig 4F is obtained. Fig 4F shows the copper atoms forming a perfect crystal lattice shown in the box in Fig. 4C.

The interplanar distance found for the direction of growth was 0.21 nm, which matches the direction [110]. In summary, Figure 4 demonstrates the type of growth direction of the wires, the crystallinity of these, the exact structure of the wires, and their purity and stability at atomic level. Oxide formation is not counterproductive for studies because it has been demonstrated that both Cu metal and CuO exhibit antifungal and antimicrobial properties. Sometimes the unwashed Cu-NWs look aggregated (Fig. 5A-B). When these are washed, their aggregation decreases (Fig. C-D). Moreover, when the concentration of $CuCl_2$ decreases in relation to the octadecylamine, the aggregation of the nanowires also decreases. In summary, the present study demonstrates the type of growth direction of the wires, the crystallinity of these, the exact structure of the wires, and their purity and stability at atomic level.

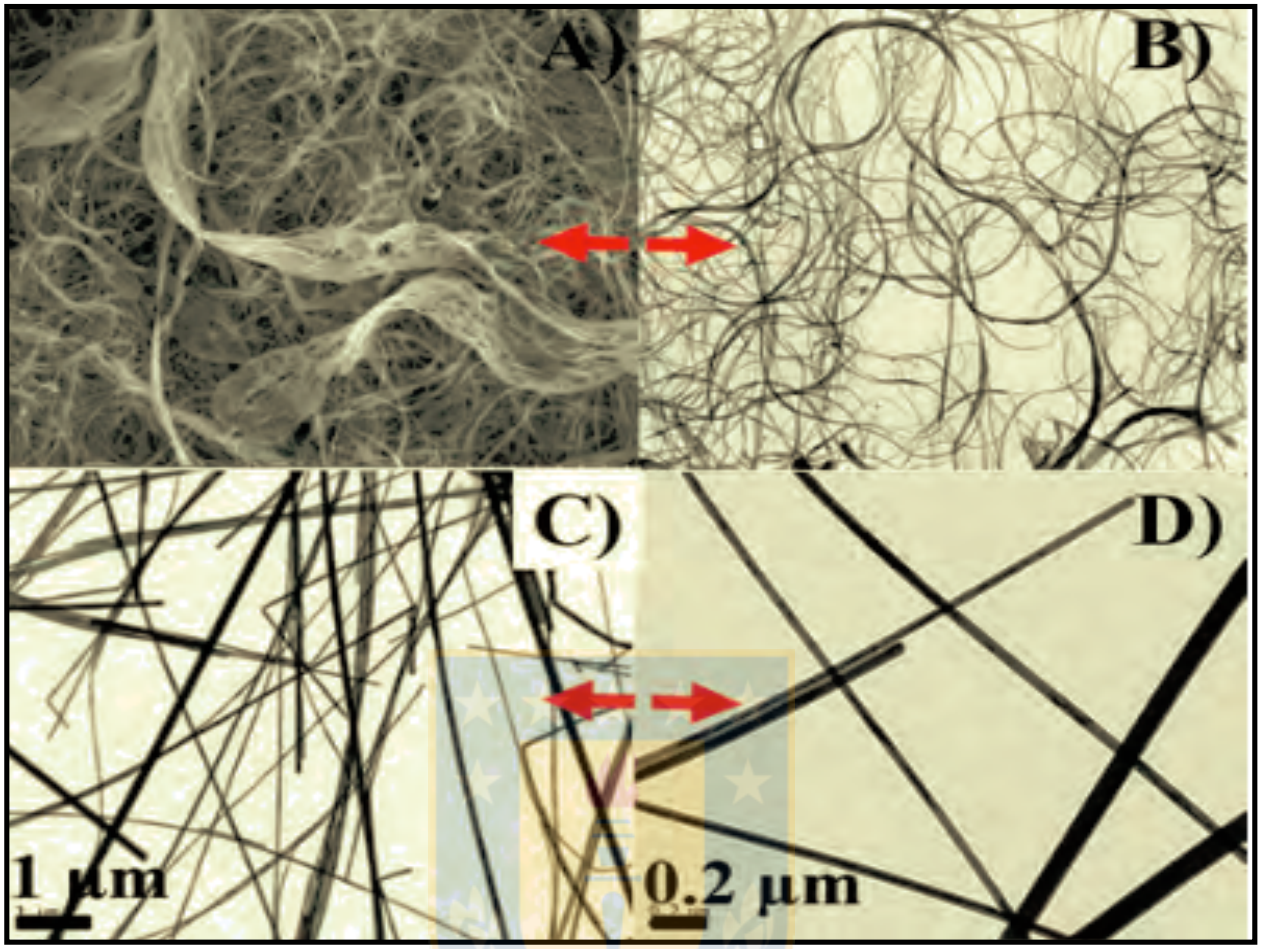

Figure 5. (A) Unwashed Cu-NWs obtained using twice the concentration of $CuCl_2$, the aggregation of the wires forming fibers is observed; the high production obtained is also evidenced. (B) Cu-NWs obtained at low concentration of $CuCl_2$. (C-D) Cu-NWs after the washing process, removal of by-products from the reaction prevents the aggregation of/in the wires.

On the other hand, in the *in vitro* tests, a geometric mean MIC of 289.30 µg/mL was obtained, with an MIC_{50} of 256 µg/mL and an MIC_{90} of 512 µg/mL for the copper nanoparticles. In the *ex vivo* assays, three-dimensional images of each sample were obtained (Fig. 6), and from these, the statistical data for the study of bacterial viability were obtained. From these data, only the total sum of the volumes of each image were used in order to compare the volume of green fluorescence (live bacteria) with the volume of red fluorescence (dead bacteria), a graph was drawn for each of the three samples irrigated with Cu-NWs (Fig. 7) according to the different time periods (2, 6, 10 min).

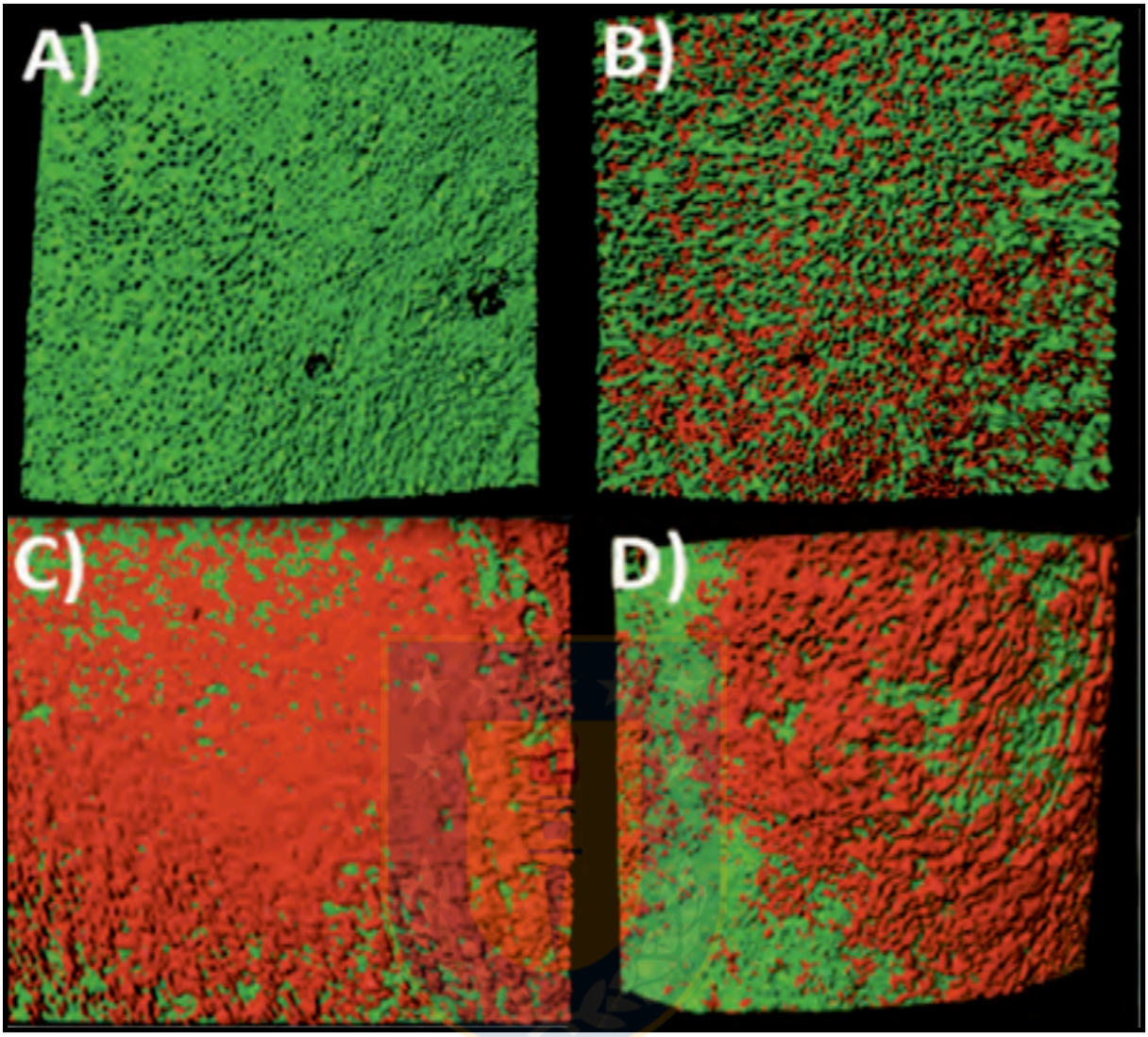

Figure 6. Three-dimensional images of Confocal microscopy. (A) sample H irrigated with sodium chloride (0.9%) for 2 min; (B) Sample I. irrigated with Cu-NWs for 2 min; (C) sample J irrigated with Cu-NWs for 6 min;(D) sample K irrigated with Cu-NWs for 10 min.

These graphs showed that the sample irrigated for 2 min (Fig. 7A) had a larger volume of live bacteria in relation to dead bacteria. In the sample irrigated for 6 min (Fig. 7B), this difference narrowed, but the volume of live bacteria is still larger.

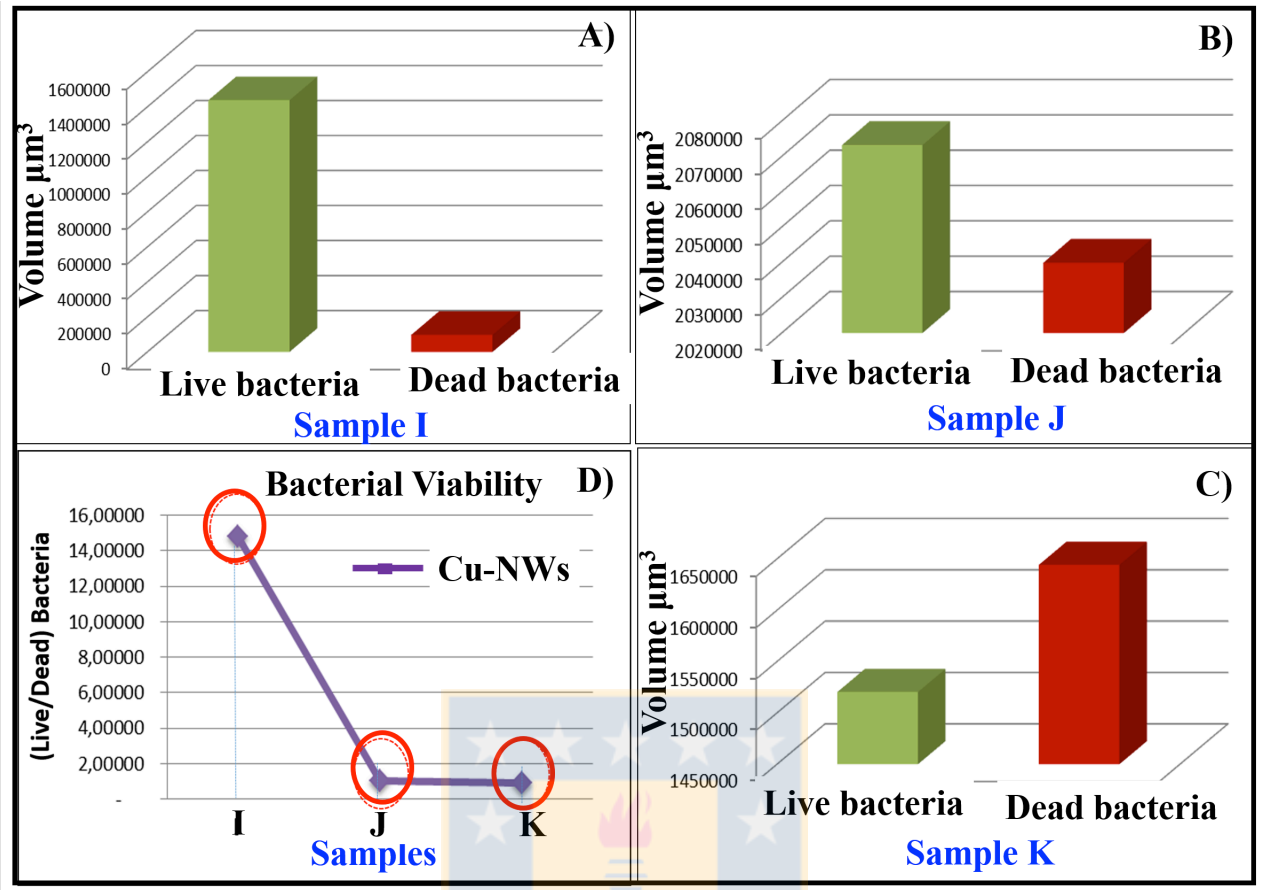

Figure 7. (A) Comparison of volumes of green and red areas of sample I irrigated with Cu-NWs for 2 minutes. (B) Comparison of volumes of green and red areas of sample J irrigated with Cu-NWs for 6 minutes. (C) Comparison of volumes of green and red areas of sample K irrigated with Cu-NWs for 10 minutes. (D) Live/dead ratios of samples irrigated with Cu-NWs at 2 min (I), 6 min (J) and 10 min (K).

Unlike the sample irrigated at 10 min (Fig. 7C), in which the proportion is reversed and the volume of dead bacteria is larger than the volume of live bacteria. In order to describe a trend of the anti-microbial effect of Cu-NWs on an aerobic biofilm, using the values reported above, the proportion of live/dead bacteria was determined, and with this ratio the bacterial viability was compared in the different periods analyzed (Fig. 7D). If this value is higher than 1, the proportion of live bacteria is larger than the proportion of dead bacteria, and if this value is less than 1, the proportion of dead bacteria exceeds that of live bacteria. It was observed that the longer exposure to the Cu-NWs the higher the decrease in viability.

Copper is a known biocide and its effectiveness in disinfection processes has been documented in the literature.[29] In fact, a MIC of $CuSO_4$ for VAN-susceptible *Enterococcus* spp. has been reported in the range of 300µg/mL and of 1000 µg/mL for VAN-resistant *Enterococcus*.[30] For copper at nano-scale, reports on strains of oral origin indicate that MIC of copper nanoparticles (Cu-NPs) have geometric mean values of 312 µg/mL for CuO-NPs and 325 µg/L for CuO_2-NPs;[8] values that coincide with the ones obtained in this study. However, against periodontal pathogens the range is extended to values ranging from 250 to 2500 µg/mL.[17] Values obtained in this study have a geometric mean dilution below the geometric mean of the control used ($CuSO_4$), which is a completely expected value.

Results obtained in *in vitro* studies with other metal nanomaterials have shown a promising antibacterial activity, which focuses on specific bacteria, present mostly in persistent root canal infections.[31,32] An *in vitro* study established a starting point for the potential clinical use of copper in endodontics. In this study drugs commonly used in endodontics and pentahydrated copper sulphate were compared. It was observed that at low concentrations Copper Sulfate has higher antimicrobial activity than Chlorhexidine and $Ca(OH)_2$ [20]. Also, an *ex vivo* study reported that canals treated with pentahydrated copper sulphate showed a reduction of 6 logarithms in the count of colony forming units at day 4.[33] However, now research on copper nanostructures has been gaining attention due to its availability and the great advantages that it has thanks to its antibacterial and antifungal properties.[34]

The trend found in this research coincides with previous studies conducted on copper. In general, a significant antimicrobial activity of Cu-NPs against various microorganisms is described in the literature, without generating a cytotoxic effect on humans. However, its application is limited by rapid oxidation due to air exposure.[34-36] Chatterjee *et al.* described toxic effects for *E. coli* bacteria such as generation of reactive oxygen species, lipid peroxidation, protein oxidation and DNA degradation, leading to bacterial death.[34] Ghasemiman *et al.* evaluated the inhibition of biofilm formation by *Listeria monocytogenes* and *Pseudomonas aeruginosa* on Cu-NPs coated surfaces. They concluded that Cu-NPs inhibited bacterial growth, and that microbial count and microbial biofilm formation decreased on Cu-NPs coated surfaces.[33] Ramazanzadeh *et al.*

compared the antibacterial effects of CuO-NPs and ZnO-NPs on orthodontic brackets populated with *S. mutans*, finding that they had a greater and significant antimicrobial effect than copper nanoparticles.[37]

The results of this study allow to visualize the antibacterial activity of Cu-NWs against an aerobic biofilm, evaluating the presence of live/dead bacteria by fluorescence. It was observed that nanowires killed bacteria (*S. mutans* and *E. faecalis*) without completely extinguishing them. However, cell viability depends on time, because although two min of exposure with Cu-NWs produced cell death, the proportion of live bacteria was larger than the proportion of dead bacteria. This ratio was reversed when the application of Cu-NWs was carried out for 10 min. The irrigation solution with Cu-NWs was not activated, which would also influence bacterial death, because it has been shown that activation increases the elimination of bacteria.[23]

4. Conclusions.

It is concluded that Cu-NWs have a good antibacterial effect against *E. faecalis* strains used in the present study. It is necessary to conduct further studies on multispecies anaerobic biofilms based on *ex vivo* models to continue supporting their possible use as antibacterial agents in endodontics. Exposure of an aerobic biofilm to Cu-NWs causes death of bacterial cells. The cellular viability of a multispecies aerobic biofilm tends to decrease the longer they are exposed to Cu-NWs.

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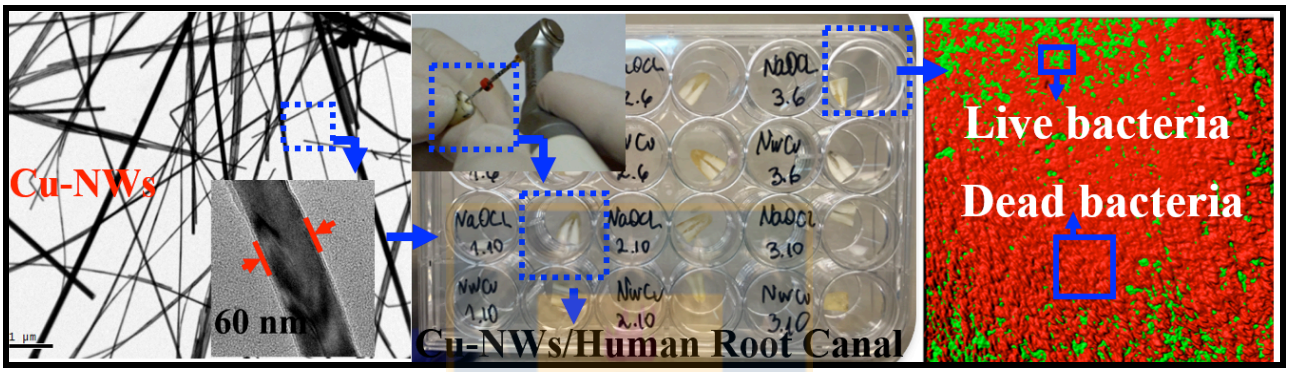
SUPORTING INFORMATION

Graphical Abstract

The application of nanostructures as antimicrobial agents to treat various oral infections it possible due their biocidal and anti-adhesive properties against biofilms. Synthesize, characterization and *in vitro* and *ex vivo* antimicrobial activity of Cu-NWs on strains obtained from human root canal may support a potential use of this Cu-NWs for disinfection in persistent endodontic infections.



CAPÍTULO VIII: DISCUSIÓN

La periodontitis apical crónica persistente es una condición caracterizada por la inflamación y destrucción de los tejidos peri-radiculares donde las bacterias tienen un papel fundamental en su etiología y patogénesis[1-2,78]. Aunque en nuestra comprensión actual, la asociación directa de microorganismos específicos en esta patología no se dilucida completamente, la etiología polimicrobiana y el establecimiento en una biopelícula madura, parecen ser factores muy importantes[78], por lo tanto es muy relevante la búsqueda de nuevas estrategias para la eliminación de tales bacterias[77]. Los microorganismos que residen en istmos y conductos accesorios, así como en los túbulos dentinarios obtienen protección contra los procedimientos de desinfección, y es allí, en ese espacio de 5 µm de diámetro promedio donde la nanotecnología antimicrobiana podría tener alguna aplicación[78-82]. Este complejo tema ha sido el foco de esta investigación, para lograr la resolución de la periodontitis apical crónica persistente.

Con el objetivo de determinar la composición y diversidad bacteriana de la microbiota asociada a conductos radiculares con diagnóstico de periodontitis apical crónica persistente (Objetivo específico 1) se procedió a extraer el ADN bacteriano, desde una muestra tomada a 24 pacientes, secuenciando las regiones hipervariables V3 y V4 del gen ARNr 16S mediante el secuenciador *Illumina MiSeq System*[83], realizando el primer análisis metagenómico reportado bajo esta metodología (Capítulo IV). Actualmente, las tecnologías de secuenciación de alto rendimiento, o de nueva generación, nos permiten evaluar la composición bacteriana de una microbiota de forma más precisa[84]. El gen ARNr 16S tiene nueve regiones hipervariables y la secuenciación Illumina, permite generar un amplicon de 460 pb, un poco más grande que cuando se secuencia otras zonas como en el caso de pirosecuenciación. Como las regiones V3 y V4 estan muy próximas entre sí, se analizan en solo una secuenciacion, mejorando la eficiencia de la asignacion de taxa. La literatura indica que existen estudios previos en este contexto endodóntico; sin embargo, éstos han sido realizados mediante pirosecuenciación 454[6, 12,20]. Si bien la pirosecuenciación 454 es una mejor herramienta para evaluar la composición bacteriana, comparada con técnicas de hibridación de ADN, como la restricción de fragmentos de longitud polimórfica (RFLP) o microarreglos, está limitada

por una tasa de error de lectura relativamente alta. Por el contrario, la plataforma de secuenciación Illumina tiene una tasa de error sustancialmente menor, y la longitud de lectura es determinista en lugar de aleatoria. Por lo tanto, la plataforma Illumina no es susceptible de malinterpretar la cantidad de nucleótidos en las regiones homopolímeras. En las tecnologías de plataforma de secuenciación de próxima generación, las bases se deducen a partir de señales de intensidad de luz, un proceso comúnmente conocido como "*base-calling*". Este tipo de error es más pronunciado en la pirosecuenciación[83, 84].

En cuanto al análisis metagenómico, la combinación de pares (FLASH, Fast Length Adjustment of SHort reads) resultó en 7.296.106 lecturas las que luego de ser filtradas con herramientas bioinformáticas utilizadas previamente[20, 85], la secuenciación devolvió un total de 2.248.552 lecturas (Anexo 1: Tabla 1), que se tradujeron en 86 diferentes unidades taxonómicas operacionales (OTUs), muy por debajo de 538 OTUs obtenidos en el estudio de Siqueira y col. (2016). Si bien la técnica utilizada por ambos estudios fue la misma, difirieron en el tipo de muestreo utilizado, lo que podría dar cuenta de la diferencia de OTUs encontradas. El método de muestreo siempre es una consideración importante, puesto que los microorganismos que colonizan los túbulos dentinarios difieren de aquellos que colonizan el lumen de conductos accesorios o de conductos principales. Los primeros señalados, probablemente no se recuperan de las muestras obtenidas con conos de papel, que aunque sigue siendo un método ampliamente aceptado, están claras las limitaciones en cuanto al alcance y la profundidad del muestreo[20]. Siqueira y col. (2016) menciona la criopulverización como una técnica de muestreo alternativa. Sin embargo, no indican si la porción apical de la raíz se mantiene en algún medio de cultivo a -20 °C, lo que aumentaría la viabilidad bacteriana y por lo tanto disminuye la probabilidad de degradación del ADN[22]. Se ha reportado en otros informes que una solución de leche desnatada al 10 % o DMSO son mejores agentes crioprotectores que la solución de glicerol al 15 %, ampliamente utilizada[86, 87].

El análisis metagenómico mostró una correlación entre los índices de diversidad de Shannon y Simpson, donde las muestras con valor del índice más alto, presentan una mayor biodiversidad del ecosistema (Fig. 1, Anexo I). Tzanetakis y col. (2015) informaron discordancias entre el índice de diversidad filogenética y el índice de

diversidad de Shannon. El índice de Shannon, mide el contenido de información por individuo, en muestras obtenidas al azar proveniente de una comunidad extensa de la que se conoce el número total de especies. Este índice subestima la diversidad específica si la muestra es pequeña. El índice de diversidad de Simpson indica la probabilidad de encontrar dos especies diferentes en dos extracciones sucesivas al azar sin reposición. Este índice le da un peso mayor a las especies abundantes subestimando las especies raras. Que haya concordancia en los índices de diversidad indica mayor robustez de los resultados[20].

Existen hasta hoy, solo 6 reportes, donde analizan muestras desde dientes con periodontitis apical crónica persistente con secuenciación de nueva generación. En dos de estos estudios, ambos en pacientes provenientes de países del continente americano,[22,85] se informa que el phylum con mayor número de bacterias fue *Proteobacteria*, resultado concordante con los obtenidos en esta tesis (Fig. 2, Anexo I). Cabe mencionar, que en los cuatro estudios restantes se incluyeron individuos que residen en los continentes asiático[6], africano[12], europeo[20,21] y los resultados indican que el mayor número de bacterias perteneció a los phyla *Bacteroidetes*[6,20] y *Firmicutes*[12,21]. Sin embargo, no podemos establecer una relación directa entre la ubicación geográfica y la composición taxonómica ya que pueden existir otros factores que estén influyendo en la composición microbiana observada. Entre los factores, se puede encontrar el tipo de herramienta de secuenciación de última generación utilizada. Solo se ha publicado un informe con el sistema Illumina MiSeq utilizado en este estudio, y los resultados obtenidos en relación con la composición taxonómica son consistentes con nuestros resultados[22].

A nivel de género bacteriano, los resultados del análisis metagenómico indicaron como prevalente a *Pseudomonas* spp. seguido de *Prevotella* spp. y *Atopobium* spp. y *Mycoplasmas* spp. (Figure 4, Capítulo IV), con una abundancia alta de *Pseudomonas* spp. en algunas muestras (Table 2, Capítulo IV) lo que concuerda con otro reporte similar[22]. Un estudio encontró, un bajo porcentaje de *P. aeruginosa* se obtuvo en muestras de periodontitis apical crónica persistente utilizando técnicas de PCR y microarreglos con cebadores específicos para dicha especie. Sin embargo no se incorporaron cebadores que amplificaran otras especies de este género[88,89]. Por otro

lado, Chugal y col. (2011) son los primeros que informan una alta prevalencia de *Pseudomonas* spp., así como de *Burkholderiales*, en muestras de infecciones endodónticas secundarias[10]. Wang y col. (2012), usando electroforesis en gel de gradiente desnaturalizante (DGGE), una técnica ampliamente utilizada para el análisis de comunidades, antes de la aparición de la secuenciación de última generación, indicaron que *Burkholderia* spp. es un género prevalente en periodontitis apical crónica persistente. Ambos géneros están estrechamente relacionados, y el último, previamente, había sido clasificado como parte del género *Pseudomonas* spp.[90]. Una limitación de DGGE es la posible migración de bandas de diferentes especies en la misma posición en el gel, alterando así su asignación y posterior análisis filogenético. La secuenciación de última generación es una técnica mucho más sensible y precisa para describir comunidades microbianas[83,84]. Saber y col. (2012), en un estudio metagenómico de lesiones endodónticas persistentes que no fueron expuestas a la cavidad oral, han informado que la presencia de *Pseudomonas* spp. está limitado a menos de 1 %[85]. Dado que los casos con filtración coronaria también se excluyeron en el presente estudio, la presencia de *Pseudomonas* spp. podría deberse a la posibilidad de contaminación durante el primer tratamiento endodóntico. Un informe reciente de Lawson y col. (2015) sobre el uso de diques de goma, mostró que menos de la mitad de 1.490 dentistas estadounidenses utilizaban rutinariamente aislamiento absoluto durante el tratamiento endodóntico[91]. Aunque no tenemos información si se usó un dique de goma en el primer tratamiento, uno de los motivos de la alta prevalencia de *Pseudomonas* spp. podría ser consecuencia de la contaminación durante el tratamiento endodóntico.

Los últimos estudios metagenómicos de infecciones endodónticas persistentes, han reportado presencia relativa de estreptococos y enterococos como *E. faecalis*, contradiciendo lo que se afirmaba años atrás, principalmente con técnicas moleculares básicas o de cultivo, donde figuraba como la especie prevalente de esta patología[1,3,11,16]. Los estudios metagenómicos actuales, concuerdan en reportar una baja prevalencia de *E. faecalis,* así se informa que la prevalencia de esta bacteria sería de 0,7 % en población asiática[6], de 17,5 % en población africana[12] y de 33 % en población europea[21]. En América Latina, se ha informado una prevalencia más alta,

siendo ésta de 40 %[22], valor muy similar al obtenido en esta tesis, ambos con una abundancia relativamente baja por muestra. La exclusión de los dientes con filtración coronaria podría ser la causa del bajo número de informes de esta especie bacteriana, como se ha mencionado en otros reportes[6].

Todo el análisis metagenómico discutido anteriormente, fue concordante con los resultados obtenidos a partir de los ensayos de cultivo (Capítulo V). Mayoritariamente, las colonias bacterianas desarrolladas en las placas de cultivo correspondieron a *Pseudomonas* spp; sin embargo, aparecieron otras especies bacterianas como *P. acnes* y *Streptococcus constelatus,* que no se detectaron a nivel de especie en los análisis metagenómicos, pero sí se encuentran identificadas a nivel de género, familia u orden. No hay estudios hasta hoy que combinen resultados de secuenciación de nueva generación con técnicas de cultivo en endodoncia. Un estudio realizado por Anderson y col. (2013) usando librerías de clones, concluyó que la combinación de los métodos de cultivo y moleculares revelaron nuevos patógenos en patología endodóntica y una gran diversidad de la microbiota en dientes con lesiones persistentes. En dicho reporte, ambos métodos arrojaron resultados diferentes, enfatizando el beneficio de usar ambos metodologías en forma combinada, para la detección de la diversidad microbiana real en la periodontitis apical, lo que concuerda con nuestros hallazgos[14]. Por lo tanto, se hace énfasis en el factor de recuperabilidad, que es dependiente de la capacidad de las metodologías, hasta hoy descritas, para cultivar diferentes especies[81].

En relación a la diversidad encontrada desde las técnicas de cultivo, las 8 cepas de *Pseudomonas putida* y 2 cepas de *Pseudomonas fulva,* aisladas en este reporte (Capítulo VI), son consideradas como bacterias aerobias; sin embargo, su requerimiento de oxígeno puede cambiar bajo condiciones anaeróbicas, usando un aceptor de electrones alternativo, tal como nitrato. El ambiente anóxico generado luego de la obturación radicular y sellado coronal, podría verse favorecido por la utilización del oxigeno disponible por parte de esta bacteria, propiciando condiciones favorables para las especies anaerobias facultativas y estrictas. Por otro lado, los componentes de los materiales de obturación, podrían ser la fuente de nitrógeno necesario, para favorecer la persistencia de esta bacteria dentro de los conductos[92,93].

La identificación a nivel de especie de cepas endodónticas, se realiza principalmente

con pruebas bioquímicas y Reacción de la Polimerasa en Cadena (RPC) con partidores específicos, generalmente, para genes conservados denominados *housekeeping*[94]. Sin embargo, en las últimas décadas, la secuenciación del ARNr 16S se ha incorporado como prueba frecuente para la identificación, sobre todo cuando se estudian múltiples cepas aisladas desde muestras clínicas[95,96]. En esta tesis, inicialmente se hizo una identificación preliminar de las cepas con pruebas bioquímicas y que luego se confirmó a través de secuenciación del ARNr 16S (Table1-4, Capítulo V). Las variaciones entre la identificación inicial (pruebas bioquímicas) y los resultados de la secuenciación pueden deberse a que los sistemas como API 20E y Rapid ID 32 A (BioMerieux, Marcy-l'Etoile, Francia) poseen una base de datos a nivel de especies bacterianas muy limitada y, por lo tanto, identifica solo algunas de ellas a nivel de especie, con una identidad de 100 %, quedando un importante grupo identificadas solo a nivel de género[12]. La otra razón, es que la secuenciación del ARNr 16S con la finalidad de describir la diversidad bacteriana está limitado por la presencia de variabilidad en el número de copias en los genomas bacterianos[97]. La información sobre el número de copias del gen ARNr 16S permite obtener estimaciones alternativas de la composición de la comunidad bacteriana, es decir, tener una variabilidad en cuanto a la clasificación a nivel de especie, lo que nos permite poder aseverar la presencia de una especie solo cuando hay 100 % de identidad[97,98].

En este caso, la diversidad bacteriana intrapaciente recuperada concuerda con los resultados metagenómicos y, además, concuerda con el reporte de Anderson y col. (2013)[14]. Esto significa que en algunos casos, se aislaron hasta 3 especies bacterianas distintas desde un mismo paciente, aunque en la mayoría de ellos solo se aisló una sola especie bacteriana e inclusive en uno de ellos no se logró el asilamiento de bacterias. Cabe recalcar que el criterio de selección de las cepas bacterianas se basó fundamentalmente en las diferentes características fenotípicas presentadas por las colonias bacterianas[14].

El presente estudio aisló 15 cepas desde condiciones anaeróbicas. Solo 6 de ellas resultaron ser anaerobios estrictos, perteneciendo principalmente al género *Propionibacterium* spp. (Capítulo VI). Esta bacteria se detecta comúnmente en la cavidad oral, en especial, en la dentina cariosa, siendo *Propionibacterium acnes* la

especie más comúnmente reportada[96]. Esta especie bacteriana se describe como un anaerobio estricto; sin embargo, se ha reportado para algunas cepas de *P. acnes*, la capacidad de crecer en condiciones microaerofílicas, lo que probablemente contribuya al éxito del organismo para transferirse al ambiente anóxico de las infecciones sistémicas más profundas, lo que explicaría el aislamiento de una cepa de esta especie desde condiciones aeróbicas[97]. *P. acnes* es la especie más prevalente en las infecciones endodónticas primarias con antecedentes o evidencia clínica de comunicación con el medio oral, mientras que está ausente en lesiones sin comunicación con el medio oral[97]. Los aislados de *P. acnes* obtenidos de infecciones endodónticas refractarias, con o sin abscesos periapicales, son probablemente infecciones nosocomiales que ocurren en el momento del tratamiento del conducto radicular[9,97,100]. Entendiendo por infección nosocomial, en el ámbito de la salud, aquella infección que es contraída por pacientes ingresados en cualquier recinto de atención de salud, no sólo en hospitales. Esto podría dar cuenta de una posible causa del aislamiento de cepas de *Propionibacterium* spp. al igual que ocurre con *Pseudomonas* spp. ya discutido previamente.

La actividad antibacteriana de los principales antimicrobianos de uso común en odontología y NPCu sobre las cepas de las especies bacterianas prevalentes cultivables aisladas desde conductos radiculares con diagnóstico de periodontitis apical crónica persistente (Objetivo específico 2, Capítulo VI), indicó que en las bacterias cocáceas Gram positivas el 92,3 % de las cepas fueron susceptibles a tetraciclina y antibióticos betalactámicos de uso odontológico, como AMX, AMC. Sólo 30,7 % fue susceptibles a los macrólidos, tales como CLR y ERY (Table 6, Capítulo VI), resultados que concuerdan con un reporte efectuado en población brasileña[101]. Rocas y col. (2013) señalan, que de 6 cepas de *E. faecalis* aisladas desde lesiones persistentes en una población alemana, solo una fue resistente a AMX y dos cepas a TET, y concluyen además que especies bacterianas anaerobias facultativas eran resistentes frente a una serie de antibióticos clínicamente relevantes[102]. Otro estudio mostró un bajo porcentaje de cepas de *E. faecalis* suceptibles a ERY[94]. El género *Staphylococcus* spp. mostró en nuestro estudio baja suceptibilidad a CLR y ERY; sin embargo, no hay estudios de susceptibilidad de cepas del género *Staphylococcus* spp. aisladas de infecciones

endodónticas.

Las cepas de bacterias anaerobias estrictas fueron susceptibles a los antimicrobianos ensayados, con excepción de MTZ donde 85,7 % presentó resistencia. Se han descrito anaerobios aislados desde infecciones endodónticas primarias, donde se observó un aumento en la resistencia antimicrobiana en el tiempo[105]. *Propionibacterium* spp. es susceptible a la mayoría de los antibioticos de uso odontológico, sin embargo, es altamente resistente a MTZ[94,95,104], lo que concuerda con nuestro reporte, lo que es altamente preocupante debido a que es una de las pocas opciones que tenemos para tratar bacterias anaerobicas. Se ha reportado presencia de genes *erm*C, *tet*M y *tet*W, en 6, 10 y 7 de un total de 24 casos de periodontitis apical primaria asintomática, respectivamente[102], lo que podría estar dando cuenta de los bajos niveles de susceptibilidad a estos grupos de antibioticos, por lo que se sugiere, pesquizar presencia y expresión de estos y otros genes de resistencia en estudios posteriores.

Las cepas del género *Pseudomonas* spp. fueron susceptibles solo a un 20 % de los antibióticos ensayados. No hay estudios previos de susceptibilidad antimicrobiana de *Pseudomonas* spp. aisladas de patologías endodónticas, ya que el reporte de la presencia de esta especie en endodoncia es relativamente reciente[10]. Sin embargo, un estudio realizado a *Pseudomonas putida* de origen ambiental, donde el 46 % de los aislamientos presentaron resistencia adquirida a cefotaxima, La resistencia a múltiples fármacos presentada por cepas de *Pseudomonas* spp. se debe a múltiples mecanismos intrínsecos o adquiridos, como disminución en la permeabilidad de la membrana externa, producción de beta-lactamasas y presencia de bombas de expulsión multidrogas. Se han reportado cepas de *Pseudomonas* spp. resistentes a sulfametoxazol, amoxicilina, ampicilina, cloranfenicol, trimetoprim, rifampicina y ceftazidima, así como a colistín y tetraciclina, encontrando resistencia a múltiples fármacos, inclusive hasta 13 antibióticos (65 % de *Pseudomonas* spp. resistentes a 8 a 13 antibióticos), causados por prácticamente todos los mecanismos conocidos de resistencia antimicrobiana[93]. El fenómeno de la resistencia hoy en día es un tema de salud pública mundial[105] debido a la selección de cepas resistentes por el uso y abuso de agentes antimicrobianos, las cuales ocupan un nicho ecológico, antes colonizado por cepas sensibles. Esto es precisamente lo que sucede en el curso del tratamiento de las

infecciones crónicas, dejando a los clínicos, desprovistos de agentes antimicrobianos eficaces[26].

En relación a la actividad antibacteriana de NPCu (Objetivo específico 2, Capítulo VI) esta fue superior sobre las bacterias Gram positivas, lo que queda demostrado al determinar la media geométrica de la CMI de NPCu, cuyo valor es de 179,3 µg/mL, comparado con la media geométrica de la CMI de1682,1 µg/mL, para las bacterias Gram negativas. El efecto bactericida de las NPCu se verifica con los valores de la media geométrica de la CMB de las NPCu, siendo 482,9 µg/mL para las cepas Gram positivas y >2500 µg/mL para las cepas Gram negativas. No hay reportes previos de CMI/CMB de NPCu sobre cepas aerobias de origen oral. Estos resultados concuerdan con un reporte sobre cepas clínicas aisladas de otras patologías humanas, donde se describen valores de CMB sobre los 500 µg/mL[62]. Otro reporte indica que nanopartículas de cobre y óxido de zinc (tamaño <50 nm) fueron más efectivas contra los patógenos Gram positivos. Las nanopartículas de ZnO tenían una CMI en el rango de 3.125 µg/ml y 6.25 µg/ml contra los patógenos probados[106].

En cuanto a la susceptibilidad de cepas Gram negativas expuestas a NPCu, observamos una media geométrica de las CMI muy elevadas comparadas con el reporte previo de Vargas-Reus y col. (2012)[63], donde los valores de CMI obtenidos son 4 a 5 veces menores, en comparación al presente estudio. Sin embargo, las diferencias radican principalmente en que nuestro reporte son cepas Gram negativas aerobias clínicas y se utilizó Cu en estado puro y no en estado oxidativo, como CuO o Cu_2O. El reporte de Vargas-Reus y col. (2012), se realizó con cepas anaerobias y de la colección American Type Control Culture, ATCC, que suelen ser muy susceptibles a pruebas antimicrobianas[63]. Un reciente estudio que abordó la actividad antibacteriana de NPCu mono dispersas colocadas en un portador de oleilamina mostró valores CMI 10 veces más bajos que las CMI de nanopartículas de plata, con valores de 150 µg/mL para *Staphylococcus aureus*, e incluso a 30 µg/mL para *Escherichia coli*, lo que plantea una acción especie dependiente[107]. Sin embargo, cabe mencionar que existe un reporte que indica que la mayoría de las cepas de *P. aeruginosa* ensayadas, tanto resistentes como susceptibles a carbapenémicos, presentaron niveles similares de tolerancia a cobre ($p = 0,148$), concluyendo este estudio que no habría relación entre la resistencia

a antibióticos y la resistencia a cobre y, que el comportamiento frente a estos agentes podría estar relacionado con la especie[52]. Sin embargo, dicho reporte está en relación a la acción del cobre como sulfato y no como nanopartícula. Avinash y cols. (2014) describen que NPCu son capaces de penetrar a través de la pared celular bacteriana causando daño celular y luego afectarían indirectamente al ADN celular o a la síntesis de proteínas. Además, inactivan sus enzimas y promueven la generación de peróxido de hidrógeno. Finalmente, las nanopartículas interaccionan con grupos -SH (átomos de hidrógeno del grupo tiol) que conducen a la desnaturalización de proteínas[108]. Todos estos múltiples puntos blancos hacen que sea casi nula la incidencia de seleccionar cepas resistentes[109]. Solo el año 2016 aparecen reportes que mencionan las nanopartículas como alternativa de tratamiento en endodoncia, donde la revisión bibliográfica derivada de esta tesis doctoral, hace su aporte[68,110,111].

En relación a los resultados del efecto bactericida de las nanopartículas de cobre sobre todas las especies bacterianas, estudiado mediante curvas de muerte (Figure 1-4, Capítulo VI), éstos sugieren que la muerte bacteriana sería por contacto para bacterias planctónicas, lo que implica que la liberación de iones en el entorno local es necesaria para una actividad antimicrobiana óptima[62,63]. No hay estudios de curvas de muerte de NPCu con bacterias orales. Un estudio realizado por Shrestha y col. (2010) acerca de la acción de NPZnO sobre *E. faecalis* indicó que bacterias planctónicas se eliminaron más rápidamente y a concentraciones más bajas de NPZnO en comparación con bacterias en biopelícula. Ellos sugieren que la inhibición de las bacterias planctónicas dependiente del contacto directo puede ser el principal mecanismo de muerte de estas nanopartículas, mientras que la resistencia a la penetración de las nanopartículas como resultado del exopolisacarido de la biopelícula cargada negativamente podría ser la causa de la necesidad de mayores concentraciones y una mayor duración de contacto requerida para eliminación de bacterias en biopelícula[112].

Por otra parte, el estudio de la actividad antibacteriana de NPCu sobre una biopelícula endodóntica artificial constituida por cepas de las especies clínicas cultivables prevalentes aisladas desde conductos radiculares con diagnóstico de periodontitis apical crónica persistente (Objetivo específico 3) permitió inicialmente, probar un primer modelo de biopelícula artificial sobre conducto radicular con una biopelícula joven

multiespecie aerobia, tratadas con NPCu (250 µg/mL) (Capítulo VI).

El presente reporte mostró actividad bactericida en todos los grupos testeados, ya que se observó una disminución de más de 3 logaritmos en todos los tiempos. (Table 1, Capítulo VI). Se obtuvieron diferencias estadísticamente significativas a los 7 días a NPCu, lo que plantea la idea que NPCu podrían ser una alternativa de tratamiento como medicación. Esto ya se había descrito para otras nanoestructuras antimicrobianas, donde su comportamiento fue mejor como medicación que como irrigante[113]. Los resultados de este estudio se expresaron en logaritmo de los recuentos, considerando que esta unidad de medida es aceptada para este tipo de ensayos, y lo que no permitió compararlos con los de Javidi y col.(2014), a pesar de ser estudios similares entre si[73]. El rol del $Ca(OH)_2$ como uno de los medicamentos intraconducto mas usado en el quehacer de la endodoncia ha sido bien documentado hasta la fecha[30,73]. En los resultados, es relevante destacar una mayor eficacia antimicrobiana de éste al día 1 comparado con el día 7 de medicación, que se contrapone con la mayoría de la literatura existente que señala que su acción antibacteriana mayor se alcanza entre los 7 a 10 días posterior a su exposición[114]. El fenómeno de resistencia podría deberse a exposiciones prolongadas a un antimicrobiano potente, que resultan en la selección de cepas resistentes[26]. Es ahí donde se ha reportado que *E. faecalis* es capaz de generar cambios fisiológicos y genéticos en respuesta a modificaciones en su entorno lo que favorece su supervivencia[8,27,28,30,115] Es importante mencionar que el $Ca(OH)_2$ utilizado en este estudio (UltraCal® XS) al ser menos soluble no necesita recambio y además mantiene el valor de pH estable en un rango de 12.07 a 12.78, lo que no ocurre con otras preparaciones[116].

En una segunda fase (Capítulo VII), un modelo similar al anterior, se trató con nanoalambres de cobre (CuNW) (1250 µg/mL) y visualizó con microscopía confocal con objetivo 25X de inmersión. Existe un reporte que indica que la dentina puede presentar autofluorescencia cuando es observada bajo un microscopio confocal sin utilizar algún reactivo fluorescente[117]. Lucchese y col. (2008)[118] analizaron la distribución de colágeno en dentina coronaria con microscopia confocal laser de barrido. Demostraron que las muestras de dientes con tinción de hematoxilina eosina y sin tinciones presentan una fluorescencia natural o autofluorescencia con un patrón no homogéneo. Así, diferentes

capas de la dentina muestran distintos grados de fluorescencia: predentina y dentina secundaria una excelente resolución, dentina circumpulpar buena resolución y dentina del manto una resolución difusa, por lo tanto, fluorescencia débil. Un análisis comparativo permitió la identificación de autofluorescencia en la matriz colágena[118]. Ambos reportes nos permiten aseverar que el grosor de la muestra juega un rol fundamental en la calidad de las imágenes, para evitar el ruido de la autofluorescencia. En nuestro caso se ajustó la señal de autofluorescencia Figura 3 (Anexo I) de la dentina radicular, corrigiendo los parámetros de adquisición (potencia de láser y sensibilidad de luz) que originó un control negativo eliminando dicha señal. Tampoco fue posible utilizar como variable comparativa el hipoclorito de sodio debido a la ausencia de fluorescencia en las imágenes obtenidas en este último. Existe la necesidad de disminuir la concentración de hipoclorito de sodio debido a la generación de burbujas en superficie (cuando las concentraciones son superiores al 1%) impidiendo su visualización con microscopia confocal. Cuando las concentraciones son mayores al 2% de hipoclorito de sodio se produce desprendimiento y disolución de la biopelícula[119].

Ya que la evidencia actual, sugiere que las pruebas *in vitro* o *ex vivo*, sean realizadas en condiciones de anaerobiosis, con biopelículas multiespecies maduras para simular de una forma lo mas parecida el ambiente intra conducto[15], en una tercera fase se probó un nuevo modelo (Fig. 4-5, Anexo I) con una concentración de 250 µg/mL de tres nanoestructuras, CuNW (Fig.6 Anexo I), NPCu (Fig.7, Anexo I) y NPZnO (Fig. 8, Anexo I) comparados entre si, con una concentración de 1000 µg/mL (Fig. 9, Anexo I). Un estudio informó que una biopelícula de *Pseudomonas aeruginosa*, al ser tratada con NPCu a una concentración de 100 ng/mL reduce en 94 % el volumen de la biomasa, lo que se observa mediante análisis de microscopía confocal, pero sin actividad bactericida. Esto concuerda parcialmente con nuestros resultados ya que se observó una disminución significativa del volumen de la biomasa total (μ^3) por exposición de 250 µg/mL NPCu en comparación con el control (PBS), pero si hubo actividad bactericida. Esto podría deberse a que se utilizó un modelo distinto al utilizado en esta tesis y con una concentración menor[120]. Nuestros resultados indican que no hay diferencias en la disminución de la biomasa total al aumentar la concentración de las nanoestructuras (Fig. 10-13 Anexo I) lo que sugiere nuevamente una rápida acción antibacteriana. Ya se

ha reportado que las NPCu reducen la carga de una biopelícula en > 75 % tomando como variable el tamaño de la nanoparticula, pero no en relación al factor tiempo/concentración[106]. De hecho, nuestro estudio mostró diferencias significativas entre el volumen de la biomasa viable y no viable, expuesta a las nanoestructuras para NPCu observándose un aumento del volumen no viable por sobre el viable considerando todos los tiempos (Fig. 14 Anexo I) y, nuevamente, se corrobora que la exposición a menores concentraciones por mas tiempo muestra una disminución tanto del volumen viable como no viable, con una mayor disminución del biovolumen total al comparar con la exposición de la biopelícula a una mayor concentración por más tiempo. La decisión de que utilizar, pasa necesariamente por la consideración de una mayor citotoxicidad de la NPCu a mayores concentraciones[121].

La cuantificación de la viabilidad muestra claramente una diferencia significativa entre la intensidad de la señal roja para las NPCu en relación al resto de las nanoestructuras y los antimicrobianos testeados (Fig. 16 Anexo I) lo que contrasta con los resultados de Lewis Oscar y col. (2015) que no evidenciaron efecto bactericida, mencionando nuevamente que la posible causa es que ellos utilizaron concentraciones mas bajas que en el presente estudio[120].

La resistencia a la penetración de las nanopartículas por la presencia del exopolisacárido de la biopelícula cargada negativamente podría ser la explicación que se necesita de mayores concentraciones y/o una mayor duración de contacto requerida para eliminación total de bacterias de biopelícula lo que concuerda con Shrestha y col (2010)[112]. Como se mencionó anteriormente, la inhibición de las bacterias planctónicas dependiente del contacto directo puede ser el principal mecanismo de muerte de estas nanopartículas[112]. Este estudio mostró que NPCu poseen propiedades antibiopelícula ya que pueden alterar la arquitectura tridimensional multicapa. Además, el exopolisacárido también podría servir como una barrera química al absorber los radicales libres (ROS) perjudiciales que llegan a la superficie celular, disminuyendo así el su efecto. La mayor reducción de bacterias de la biopelícula por NPCu en comparación con las otras nanoestructuras podría deberse a la mayor producción de ROS por NPCu, que fue capaz de difundirse en la estructura de la biopelícula[112]. Gomes-Filho y col. (2010) han reportado que el hipoclorito de sodio induce una

respuesta inflamatoria moderada en los tejidos periapicales y más aún que la dispersión de nanopartículas de plata son biocompatible en comparación con la solución de hipoclorito de sodio[122]. No hay reportes de respuesta inflamatoria en tejidos periapicales frente a NPCu, por lo que se plantea como un necesario estudio posterior.

Métodos físicos y químicos convencionales de síntesis de nanopartículas de metal pueden ser una posible razón de la toxicidad que presentan, pudiendo superar este problema mediante la síntesis de nanopartículas desde fuentes biológicas. Un intento de establecer nanopartículas metálicas de importancia fisiológica como los mejores candidatos para futuros nano medicamentos, sólo puede tener éxito si estas partículas se sintetizan con una mejor biocompatibilidad y baja o ninguna toxicidad[123]. Hoy se habla de "Nanotecnología verde" y "nanotoxicología" como dos áreas importantes de la investigación de nanomateriales. Un reporte demostró que coloides de nanopartículas de plata sustentadas en biopolímeros, estabilizan las partículas a nanoescala, haciéndola mínimamente tóxica y manteniendo su actividad antimicrobiana, lo que tiene profundas implicaciones biomédicas[123]. Nanopartículas de Cu_2O fueron preparadas, utilizando desechos agrícolas de *Arachis hypogaea* (maní), extractos de hojas que contienen azúcares reductores, que actúan como agente reductor a temperatura ambiente[58]. Otro reporte señala que la morfología de nanopartículas de Cu de 15-30 nm puede ser controlada mediante la regulación de la cantidad de extracto de Aloe vera. Este enfoque ecológico para la síntesis es adecuado para la producción comercial a gran escala y aplicaciones relacionadas con la salud de las nanopartículas de Cu[124,125]. Todo lo anterior refuerza la idea que las nanopartículas metálicas son prometedores nanoantibióticos, debido a sus notables propiedades antimicrobianas[126].

Finalmente, con el objetivo de relacionar la diversidad bacteriana y el comportamiento de cepas frente a los antimicrobianos con los parámetros clínicos de los pacientes (Objetivo específico 4), los resultados del presente estudio muestran que el índice de diversidad de Shannon fue estadísticamente significativo reducido en pacientes sintomáticos (Figure 2, Capítulo IV). Este resultado concuerda con los hallazgos previamente informados por Santos y col. (2011), que observaron una diversidad significativamente mayor en las infecciones sintomáticas que en las infecciones asintomáticas; sin embargo, este informe previo está relacionado con infecciones

endodónticas primarias[19]. En comparación con el informe de Anderson y col. (2013), acerca de infecciones secundarias, concuerda con nuestro reporte donde, la diversidad bacteriana en casos asintomáticos parece ser un poco mayor que la de los casos sintomáticos, aunque estas diferencias para ellos no fueron estadísticamente significativas[12]. Esta relación puede deberse principalmente al hecho de que las defensas del hospedero, en el caso de lesiones asintomáticas crónicas, están en equilibrio con una baja virulencia de los microorganismos, donde una respuesta celular humoral específica y no específica de baja intensidad contribuyen a una marcada vascularización con producción de tejido de granulación, potenciando una mayor diversidad bacteriana. Además, Tzanetakis y col. (2015), empleando pirosecuenciación, informa un desajuste entre el índice de diversidad filogenética y el índice de diversidad de Shannon, donde el índice de diversidad filogenética concuerda con los resultados del presente estudio que muestran una mayor diversidad en casos asintomáticos. Sin embargo, los resultados no coinciden, encontrándose un índice de diversidad de Shannon que demuestra que se presenta una mayor diversidad en los casos sintomáticos. No obstante, estos resultados nuevamente no fueron significativamente diferentes, excepto en el caso de *Proteobacteria*[20]. Sin embargo, los informes más recientes de secuenciación de última generación en periodontitis apical crónica persistente solo mencionan la presencia de radiolucidez apical versus casos con estados periapicales normales, pero no realizan ningún análisis en relación con la microbiota asociada[21].

Existe una asociación entre la patología endodóntica y enfermedades cardiovasculares, en reportes con bajo nivel de sesgo[127], a diferencia de lo que ocurre en el caso de la diabetes mellitus, donde no hay estudios con bajo nivel de sesgo que exploren la asociación de esta enfermedad con patología endodóntica. Los resultados de Khalighinejad y col. (2016) sugieren que puede haber un riesgo moderado y una correlación entre algunas enfermedades sistémicas y la patología endodóntica; sin embargo, recomiendan nuevos y más estudios que brinden una mejor evidencia de esta idea[127]. Todos los estudios metagenómicos realizados hasta la fecha incluyen solo pacientes sistémicamente sanos, pero no especifican su clasificación de acuerdo con el Sistema de Clasificación de la Sociedad Estadounidense de Anestesiólogos (ASA).

Además, ninguno de estos estudios relaciona la composición bacteriana con el estado sistémico del paciente. Los resultados del presente estudio sugieren una relación entre la presencia de una enfermedad sistémica y la composición de la microbiota, con una diferencia significativa de la composición bacteriana en pacientes ASA I en comparación con los pacientes ASA II y III, donde estos dos últimos grupos presentan estimaciones de mayor riqueza y diferentes composiciones filogenéticas. Una vez más, estas diferencias pueden deberse principalmente al hecho de que las defensas del huésped están alteradas en pacientes con enfermedades sistémicas. Por otro lado, se ha informado que los niveles sistémicos de proteínas C-reactivas, IL-6 y fibrinógeno están influenciados por la presencia de periodontitis apical crónica persistente en pacientes hipertensos, lo que, además de generar interés desde un punto de vista científico, es desde la perspectiva de la salud pública[128]. Mayor investigación científica en esta área puede determinar aportes a esta asociación.

Se encontró que de los 24 pacientes, la mitad de ellos fueron catalogados en la clasificación ASA I y la otra mitad en ASA II-III. De los 24 pacientes, la mitad de ellos, no coincidente con la anterior, presentó lesiones sintomáticas versus asintomáticas, y así sucesivamente, muy similar al resto de las variables clínicas estudiadas. Por lo tanto, la alta variabilidad interindividuo, se consideró al momento de establecer solo relaciones descriptivas entre los resultados de ensayos de cultivo con dichos parámetros clínicos. Una posible relación entre el paciente con el tratamiento endodóntico más antiguo, que data de 30 años, y el aislamiento de las dos cepas, *Klebsiella oxytoca* y *E. faecalis*, con CMI de AMX, CLR and ERY con valores muy por sobre los de otras cepas aisladas del mismo estudio (Objetivo 4), lo que podría estar dando cuenta de un fenómeno descrito en endodoncia como persistencia[26,43].

En resumen, esta tesis permitió obtener conocimiento acerca de la composición y diversidad bacteriana de la microbiota asociada a periodontitis apical crónica persistente, concordantemente con lo ya reportado[22]. Además, se describió por primera vez para aislados orales obtenidos en población chilena, la actividad antibacteriana de los antibióticos más comunes utilizados en endodoncia como así también la actividad antibacteriana en modelos de biopelícula de NPCu, resultados que apoyan a la evidencia existente para proponer que las nanopartículas de cobre, son agentes

antimicrobianos potenciales para el uso en patologías endodónticas[43,109,111]. Finalmente, la asociación de las características clínicas con las patologías endodónticas sigue siendo un desafío debido a las innumerables variables en juego[90]; sin embargo, este trabajo aporta con poner en discusión la importancia de realizar dichos análisis.

CONCLUSIONES

1. En los pacientes con periodontitis apical crónica persistente la comunidad bacteriana, es diversa, siendo el phylum predominante *Proteobacteria*, seguido por *Bacteroidetes* y *Firmicutes*.

2. La actividad antibacteriana de los antibioticos de uso regular en el ámbito de la odontología depende de la especie bacteriana estudiada. Siendo las bacterias cocaceas Gram positivas y las anaerobias estrictas, mayoritariamente susceptible. En cambio, las especies del género *Pseudomonas* spp. en su gran mayoría presentaron resistencia a los antibióticos.
   La actividad antibacteriana de nanopartículas de cobre fue 9,4 veces mayor sobre las bacterias Gram positivas respecto a las bacterias Gram negativas. Presentando un efecto bactericida.

3. Nanopartículas de cobre poseen actividad bactericida sobre una biopelícula aerobia multiespecie joven, disminuyendo su viabilidad a pocos minutos de su exposición. Nanopartículas de cobre sobre una biopelícula anaerobia multiespecie madura, tiende a disminuir su volumen a mayor tiempo de exposición a NPCu.

4. Existe relación estadísticamente significativa entre individuos clasificados como ASA II- III, índice periapical 5 y pacientes asintomáticos con mayor riqueza y diversidad.

PROYECCIONES

A medida que se refinan los enfoques metagenómicos, el foco de la investigación del microbioma se desplazará hacia la forma en que las bacterias interactúan con su hospedero y viceversa. La incorporación de estudios de metabolómica del hospedador y otros metadatos permitirá la producción de una imagen increíblemente detallada de las interacciones entre el microbioma y su huésped, y a medida que se produzcan cambios disbióticos, estos enfoques permitirán una mejor comprensión de la etiopatogenia de las infecciones endodónticas persistentes. Se necesitan más análisis proteómicos y metabolómicos de la interacción de la microbiota endodóntica persistente para mejorar nuestra comprensión sobre la función microbiana de la patogénesis endodóntica. Finalmente, como se mencionó anteriormente, una proyección de esta tesis es dar un salto al estudio de la citotoxicidad y biocompatibilidad de una posible presentación comercial de estas nanopartículas, donde ya se obtuvo la data inicial de evaluación de citotoxidad de NPCu y NPZnO sobre *Streptococcus sanguinis*.

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ANEXO I: TABLAS Y FIGURAS

Tabla 1: Combinación de lecturas en las 24 muestras (7.296.106 lecturas por Flash)

Sample Name	Total Bases	Read Count	GC (%)	Q20 (%)	Q30 (%)
1	123.301.038	265.556	51,67	98,32	93,45
2	91.959.564	197.981	51,67	98,36	93,58
3	121.935.039	263.553	52	98,1	92,78
4	100.829.968	218.899	52,32	97,98	92,4
5	98.845.112	218.122	55,67	97,7	91,45
6	104.071.588	224.487	51,69	98,31	93,37
7	119.591.585	268.137	55,92	97,56	91,11
8	95.738.152	213.137	54,23	97,38	90,33
9	113.648.918	244.901	51,68	98,3	93,33
10	102.345.207	220.455	51,68	97,89	91,85
11	146.250.764	316.566	52,08	97,94	92,23
12	182.962.150	404.418	54,35	97,38	90,52
13	171.842.002	375.155	52,39	97,94	92,28
14	91.954.569	198.364	51,79	97,49	90,69
15	150.587.929	328.438	51,86	98,01	92,47
16	121.465.795	267.589	52,22	97,56	91,09
17	153.128.929	334.302	51,84	97,25	90,21
18	145.568.515	315.704	52,17	97,74	91,71
19	171.660.517	373.046	52,36	97,87	92,1
20	157.971.210	343.082	52,3	97,68	91,56
21	192.999.097	422.167	52,42	97,33	90,47
22	158.769.321	344.948	52,09	97,79	91,86
23	246.200.667	556.978	51,99	98,23	93,38
24	174.333.044	380.121	53,07	97,65	91,43

Tabla 2: Cepas aisladas por paciente

Paciente	Cepas	Nombre
1	*Pseudomonas putida*	6.2
2	-	-
3	*Propionibacterium acnes*	A1
4	*Pseudomonas putida*	9.3
5	*Pseudomonas putida*	15.2
6	*Pseudomonas putida*	6.1
7	*Propionibacterium acnes*	A2
8	*Enterococcus faecalis*	11.2
9	*Pseudomona putida*	9.1
10	*Pseudomonas fulva*	14.3
11	*Enterococcus faecalis / Klebsiella oxytoca*	C1 / C2
12	*Streptococcus constellatus*	D2
13	*Staphylococcus haemolyticus/Pseudomonas putida / Streptococcus constellatus*	13.1/13.2 / D1
14	*Pseudomonas fulva / Pseudomonas putida*	14.1 / 14.2
15	*Bifidobacterium dentium/Propionibacterium acidifaciens / Leuconostoc mesenteroides*	F1 / F2 / 15.3
16	*Propionibacterium acnes / Staphylococcus warneri*	G1 / 9.2
17	*Enterococcus faecalis*	E2
18	*Enterococcus faecali*	C3
19	*Enterococcus faecalis*	11.1
20	*Enterococcus faecalis*	E1
21	*Propionibacterium* spp / *Staphylococcus capitis*	B1 / B2
22	*Propionibacterium acnes*	E3
23	*Pseudomonas putida*	15.1
24	*Enterococcus faecalis*	11.3

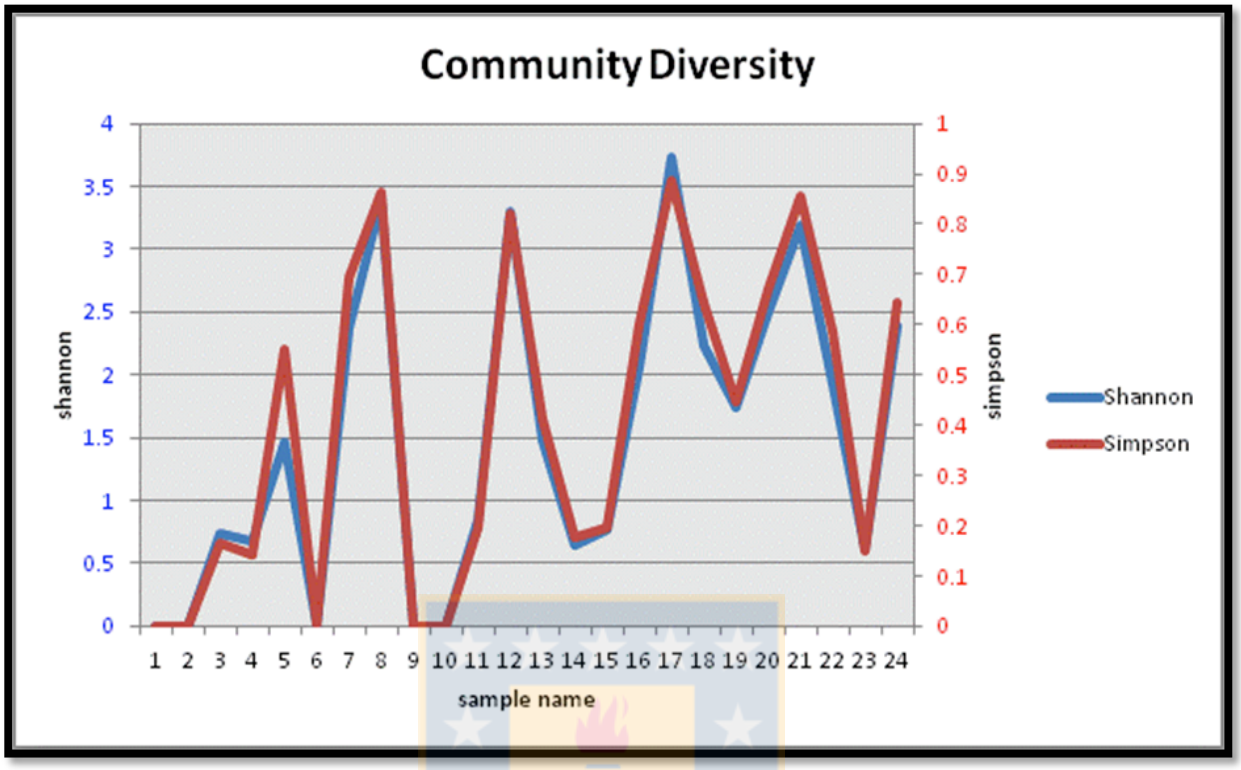

Figura 1: Índice de diversidad de Shannon/Simpson (ANOVA de dos vías, HSD de Tukey P <0,05)

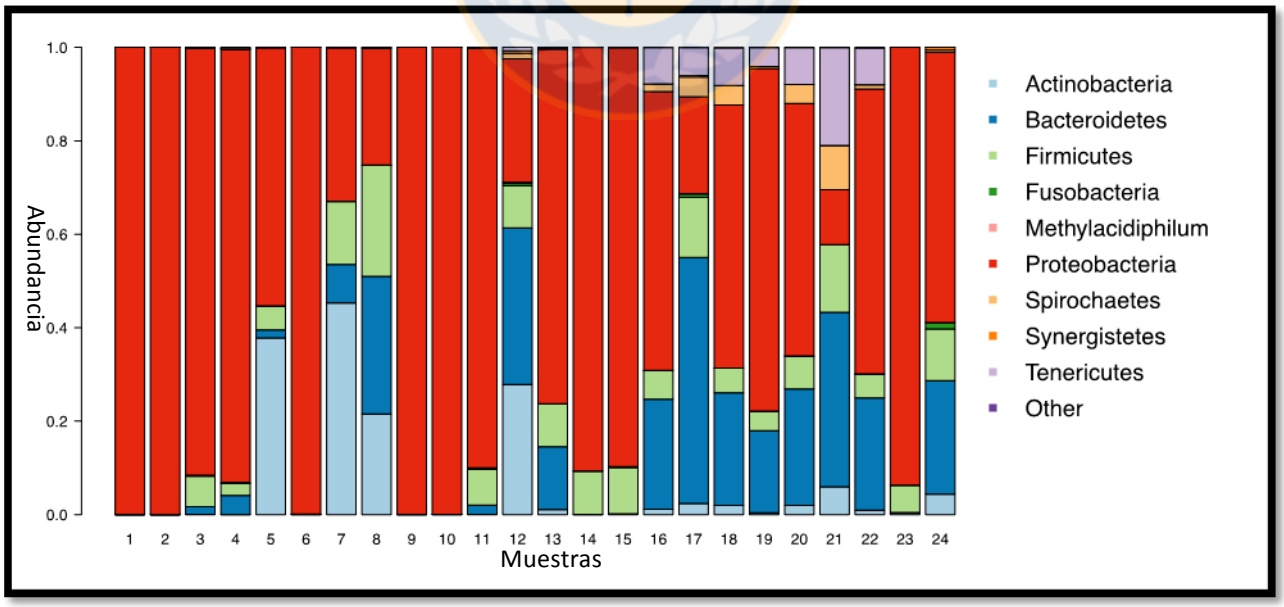

Figura 2: Composición taxonómica a nivel de Phyla por muestra.

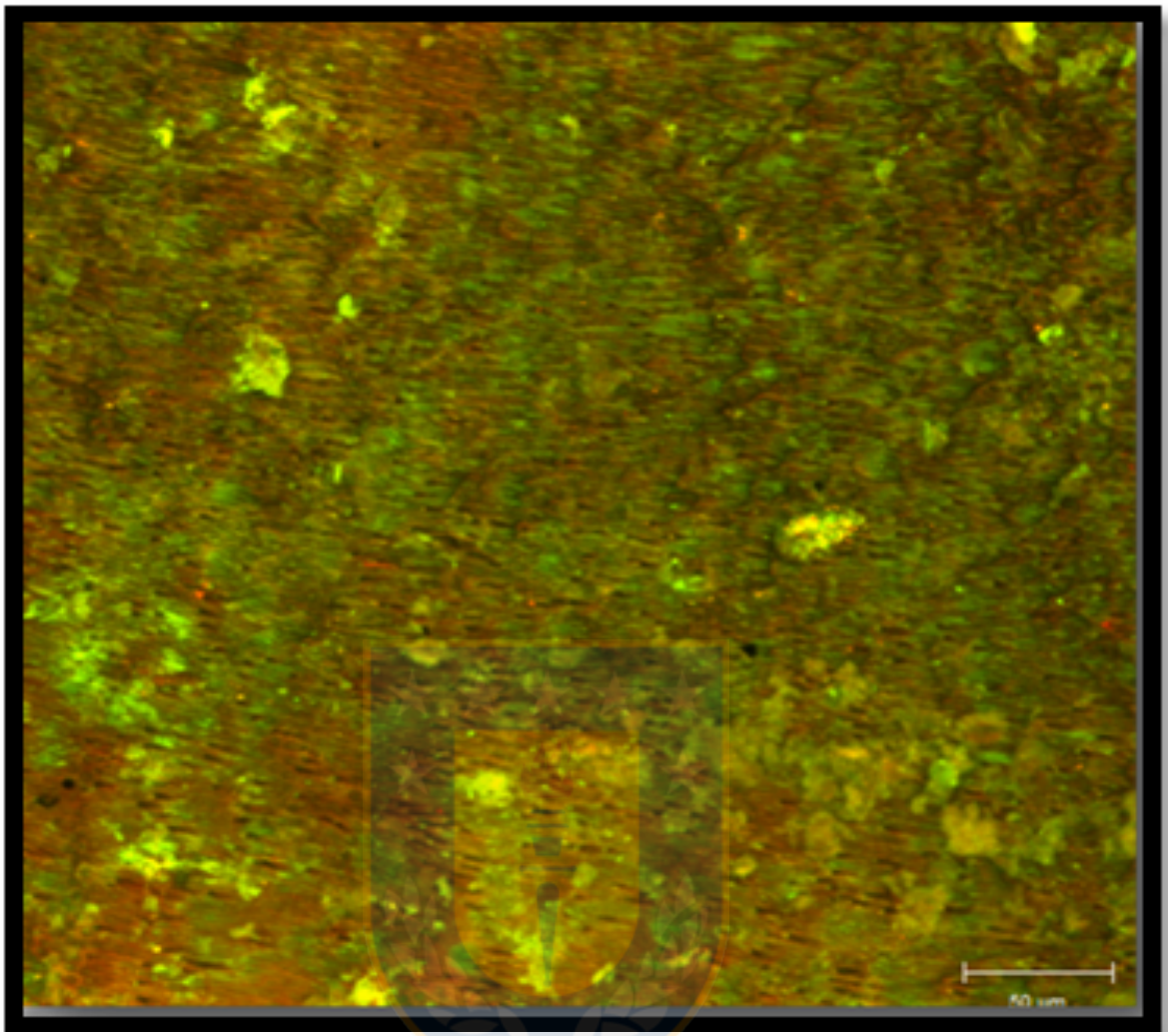

Figura 3. Señal de autofluorescencia de la dentina radicular

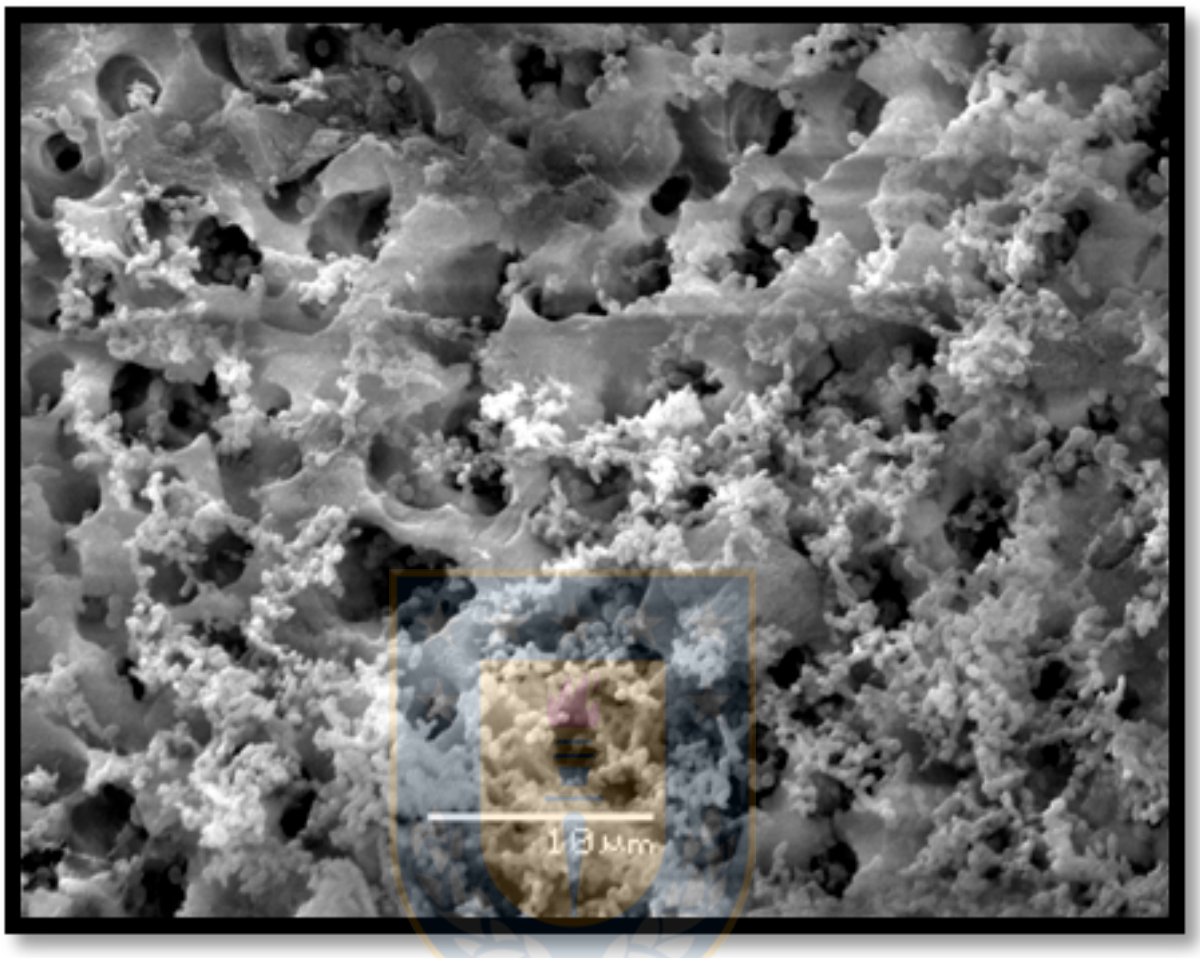

Figura 4: Microscopía Electrónica de Barrido de modelo de biopelícula anaerobia multiespecie madura de 21 días, sobre cortes transversales de las raíces con un grosor no mayor a 0.5 mm.

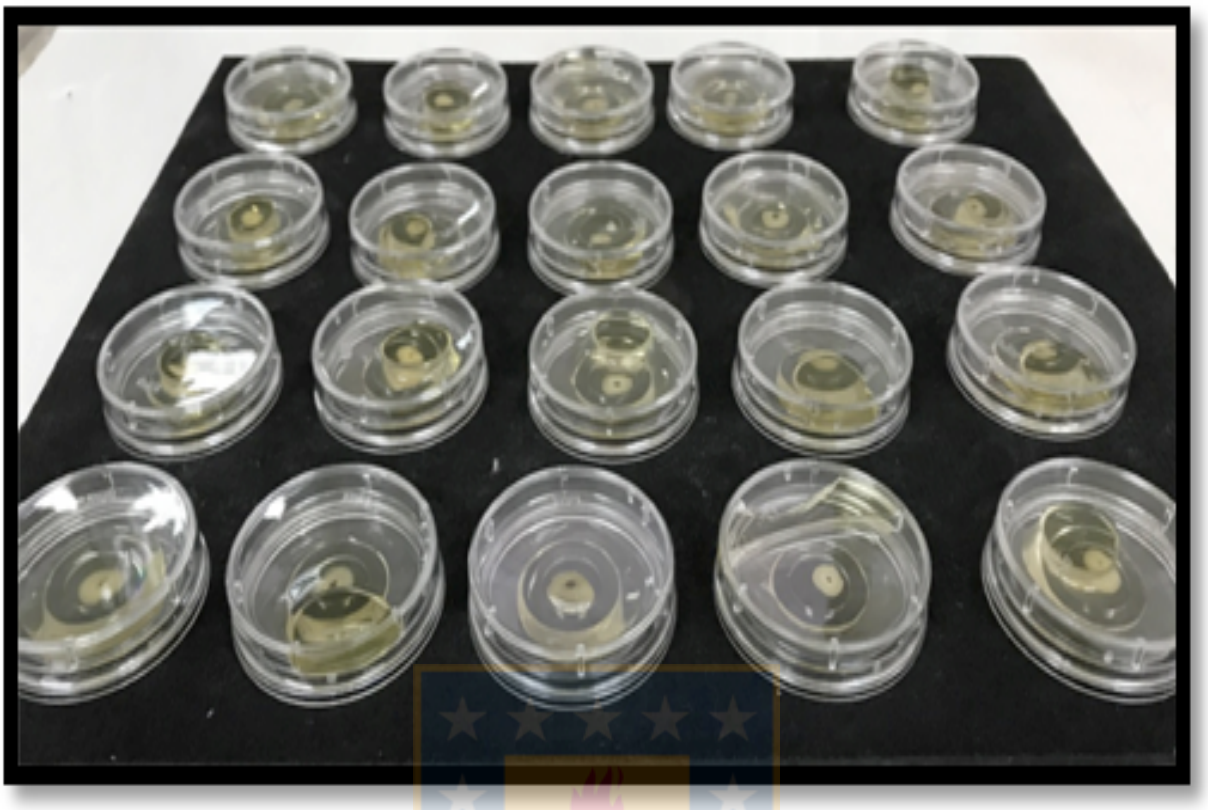

Figura 5: Biopelícula anaerobia multiespecie sobre dientes montados en pocillos para microscopia de fluorescencia WPI modelo Fluorodish, con las cepas *Porphyromonas gingivalis ATCC 33277, Prevotella melaninogénica ATCC 25845, Propionibacterium acnes clínica A1, Enterococcus faecalis clínica C1, Streptococcus constellatus clínica D2 y Enterococcus faecalis ATCC 29212.*

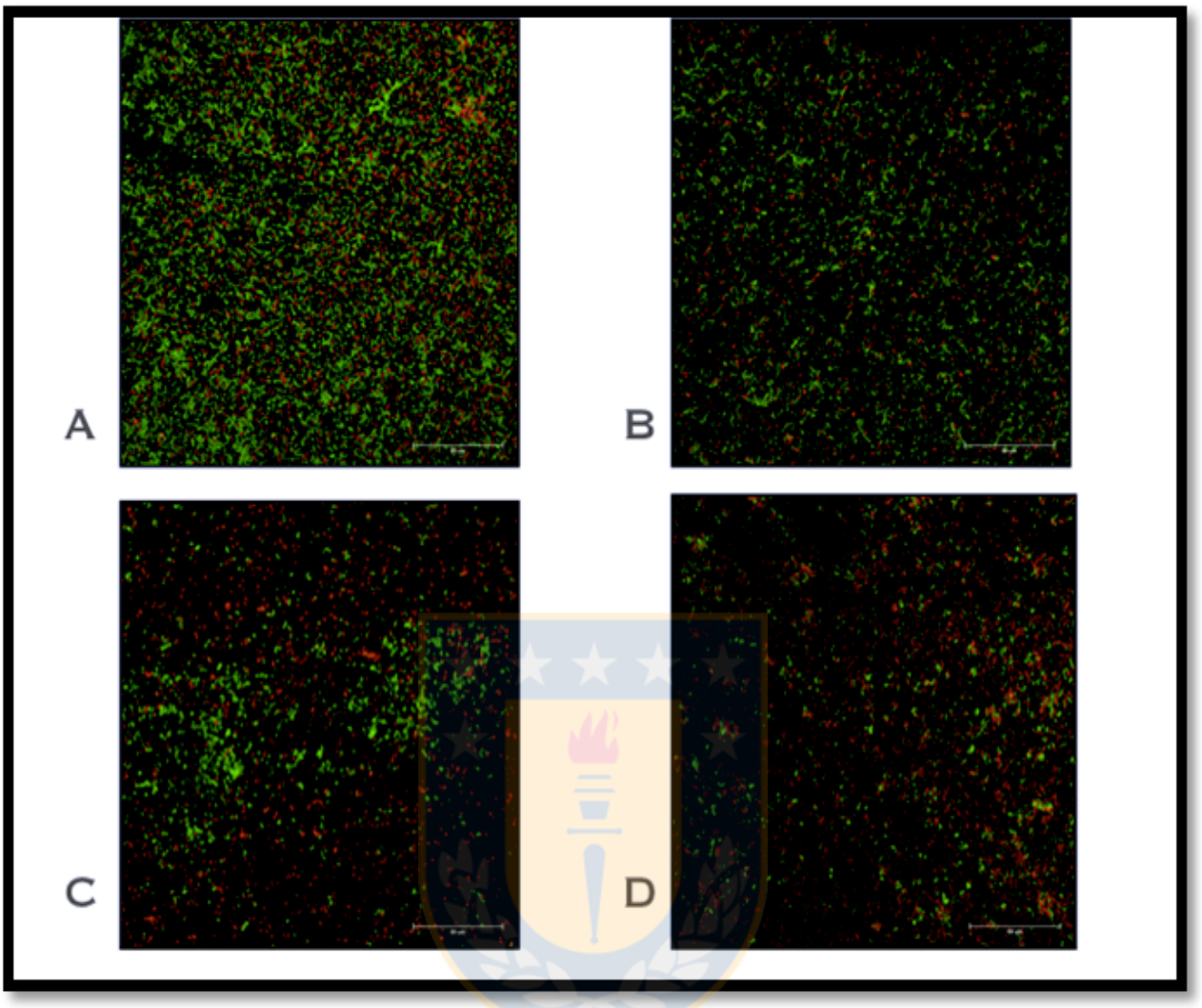

Figura 6: Imágenes Microscopía Confocal CuNW [250µg/mL] (A) 2 min (B) 6 min (C) 10 min(D) CuNW 2 min [1000µg/mL]. El verde indica células viables teñidas con SYTO 9, y el rojo indica células dañadas / muertas teñidas con yoduro de propidio.

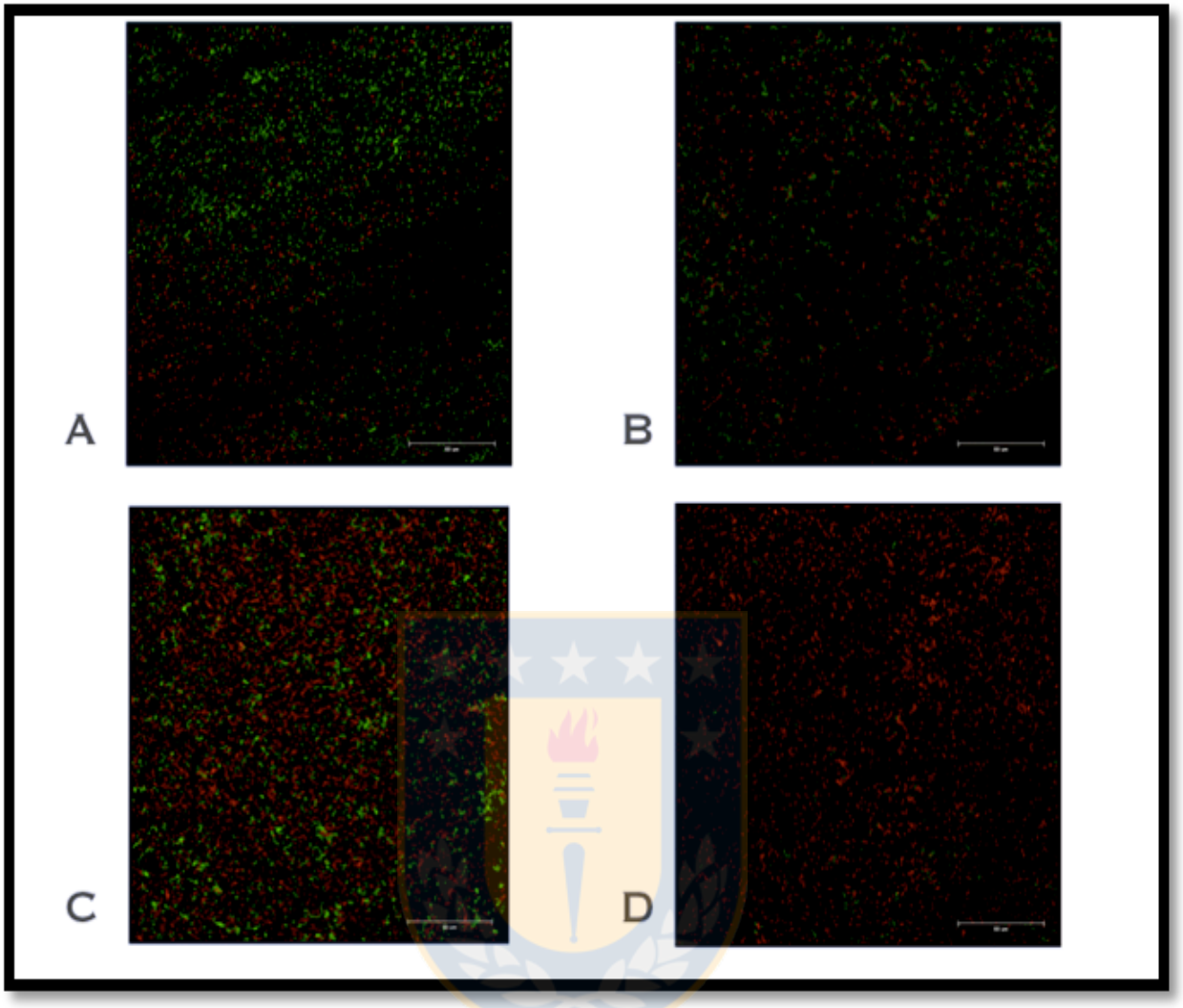

Figura 7: Imágenes Microscopía Confocal NPCu [250µg/mL] (A) 2 min (B) 6 min (C) 10 min (D) NPCu 2 min [1000µg/mL]

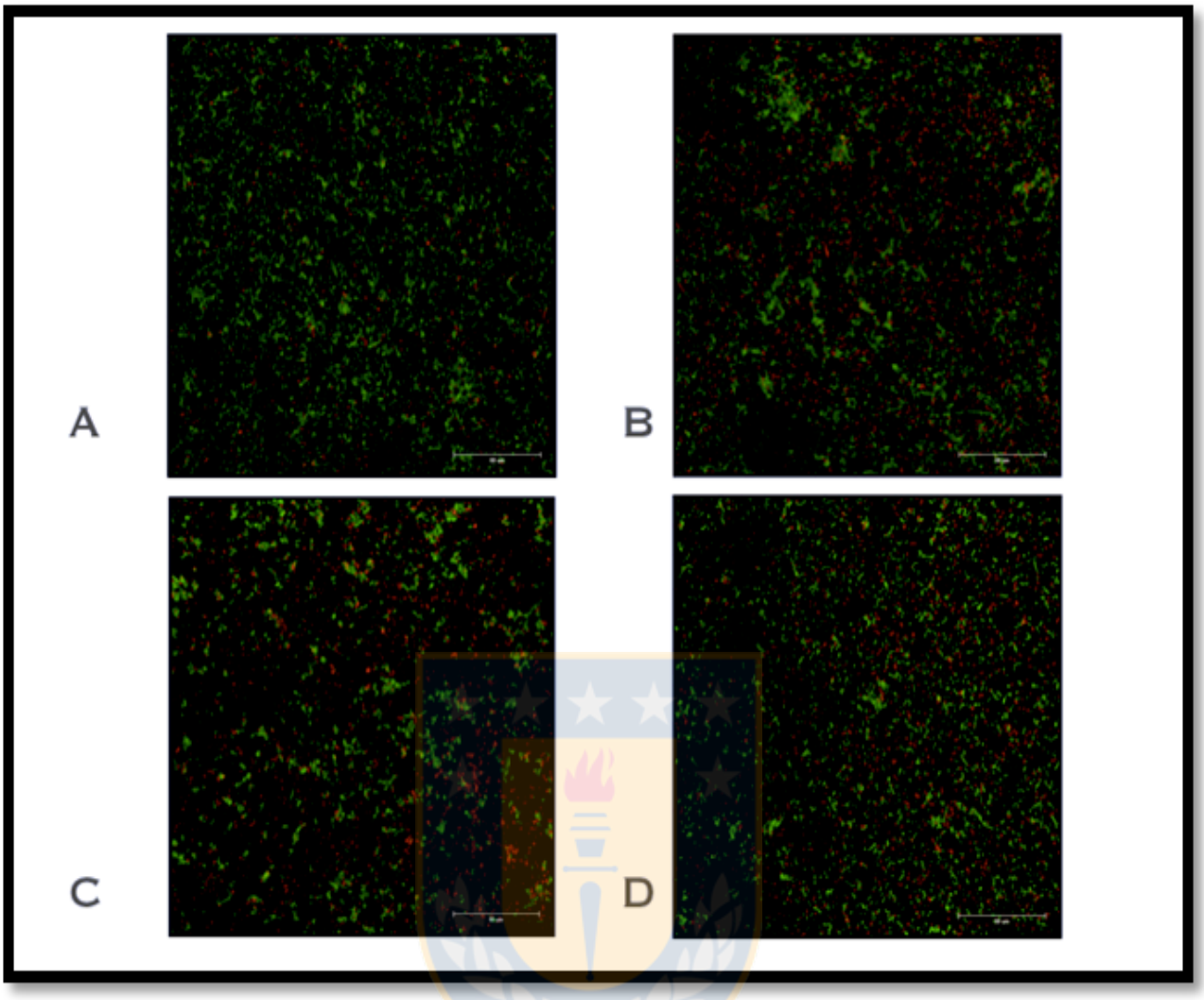

Figura 8: Imágenes Microscopía Confocal NPZnO [250µg/mL] (A) 2 min (B) 6 min (C) 10 min (D) NPZnO 2 min[1000µg/mL]

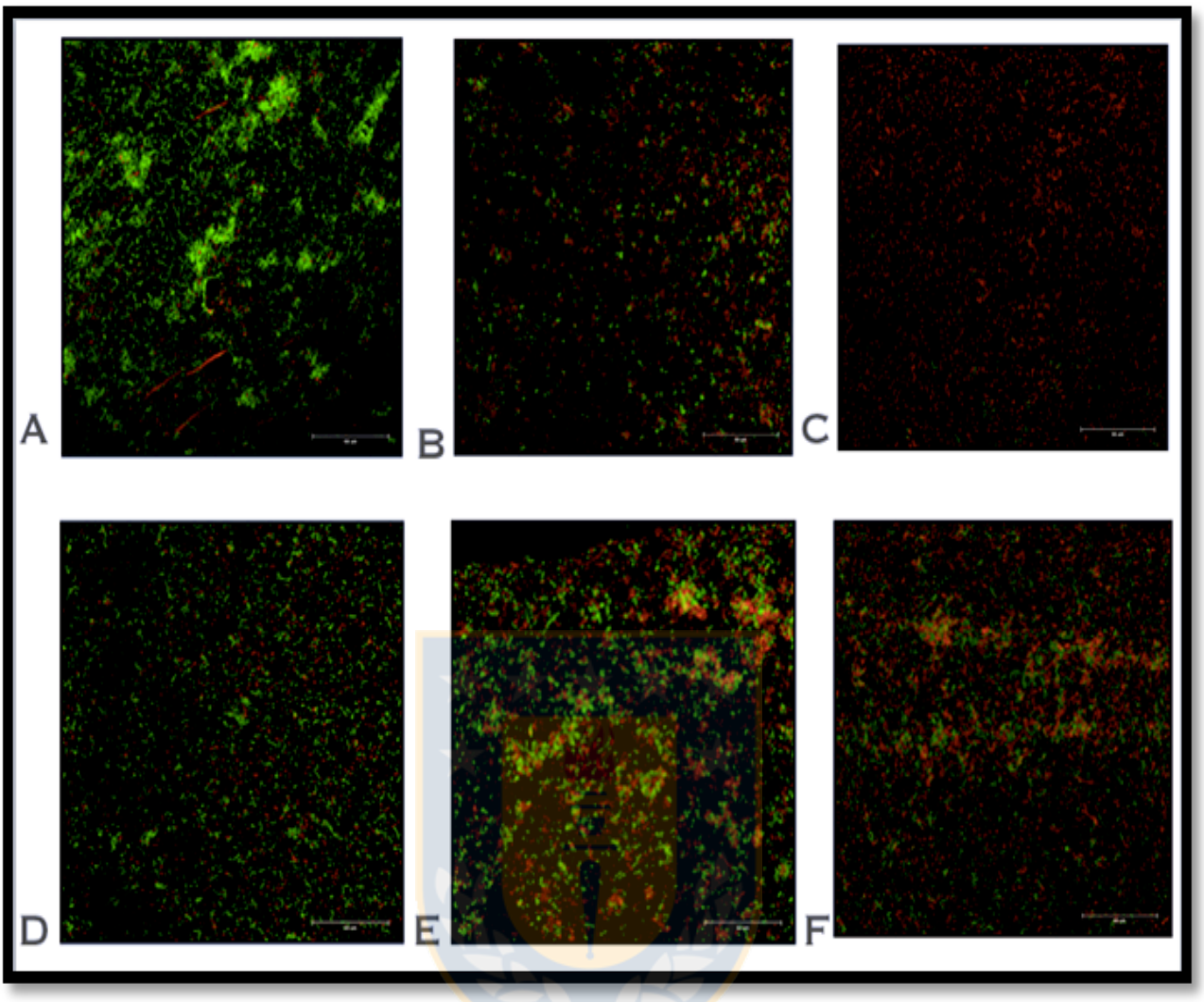

Figura 9: Imágenes Microscopía Confocal (A) Control (B) CuNW 2 min [1000µg/mL] (C) NPCu 2 min [1000µg/mL] (D) NPZnO 2 min [1000µg/mL] (E) CHX 2% 2 min (F) NaOCl 0,5% 2 min.

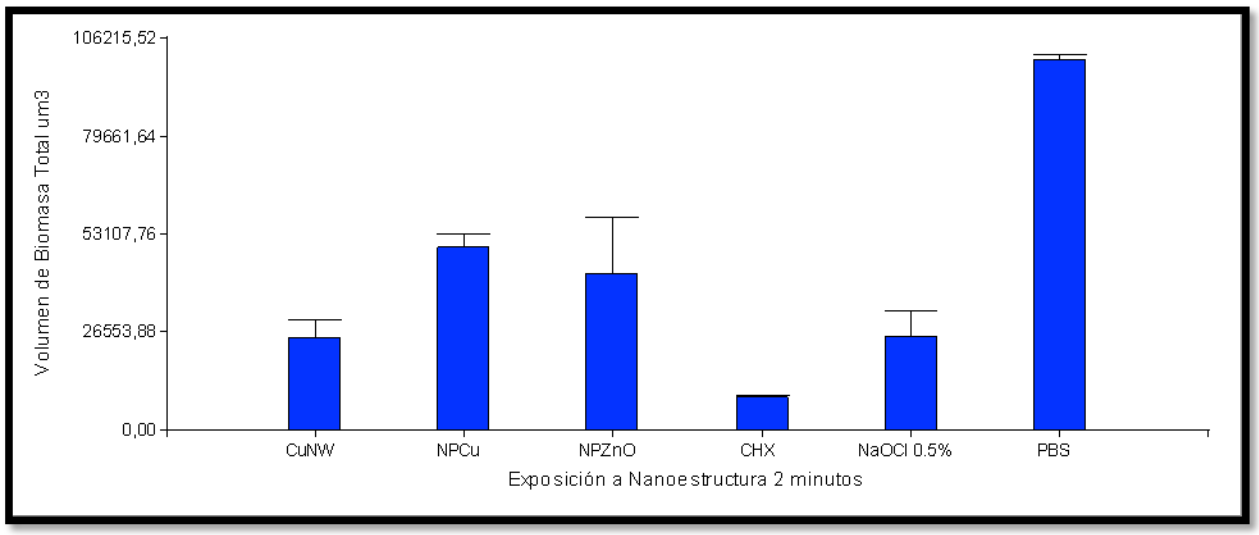

Figura 10: Disminución de la Biomasa total por exposición de nanoestructuras(250µ/mL) por 2 min.

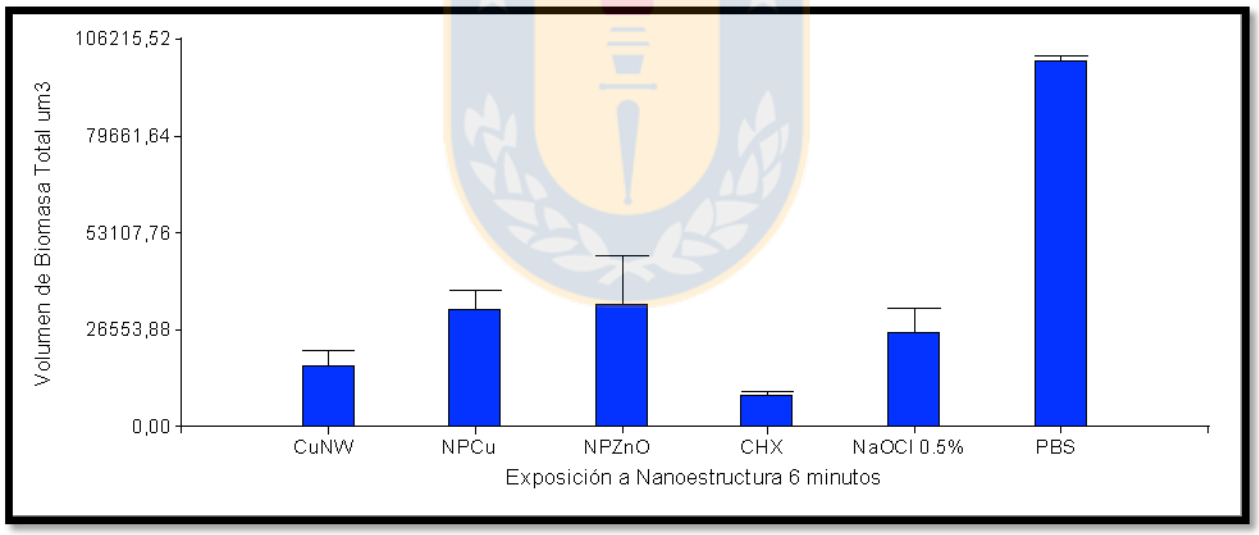

Figura 11: Disminución de la Biomasa total por exposición de nanoestructuras(250µ/mL) por 6 min.

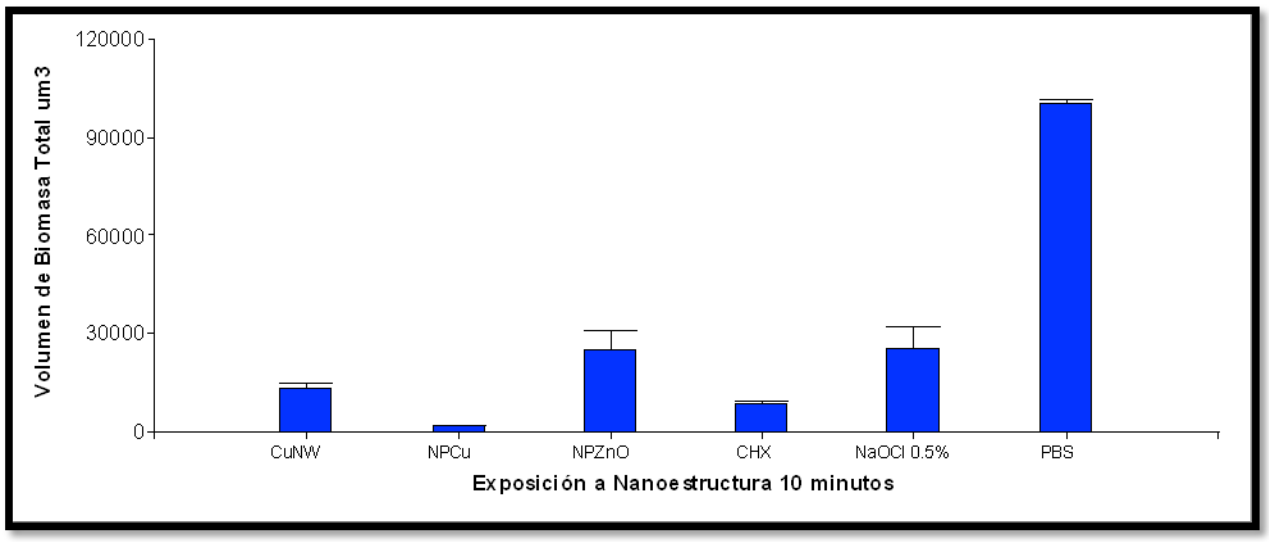

Figura 12: Disminución de la Biomasa total por exposición de nanoestructuras(250µ/mL) por 10 min.

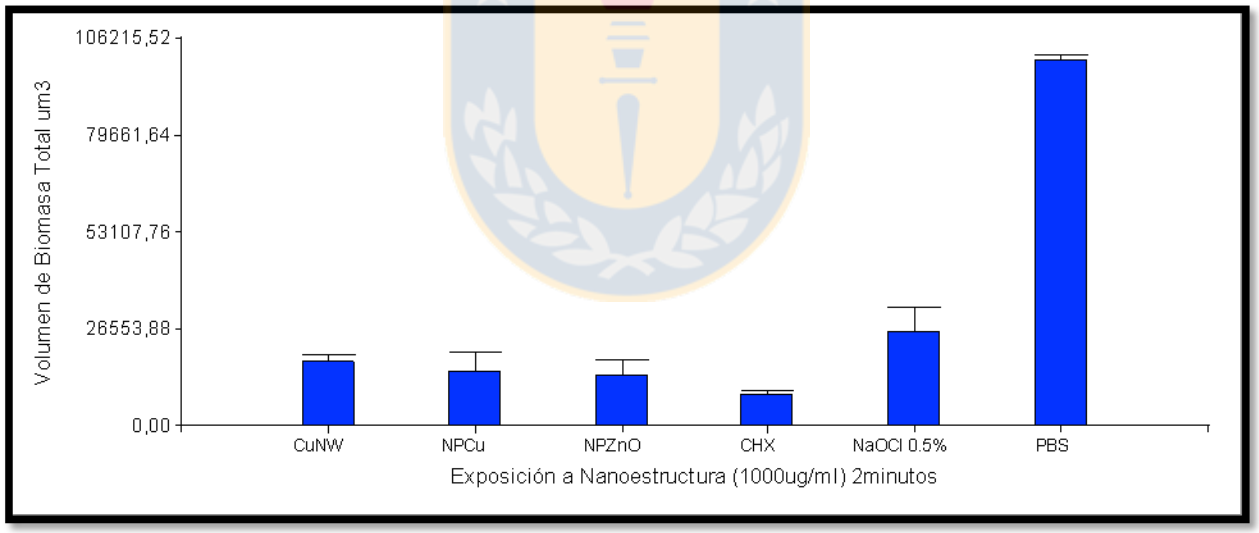

Figura 13: Disminución de la Biomasa total por exposición de nanoestructuras(1000µ/mL) por 2 min.

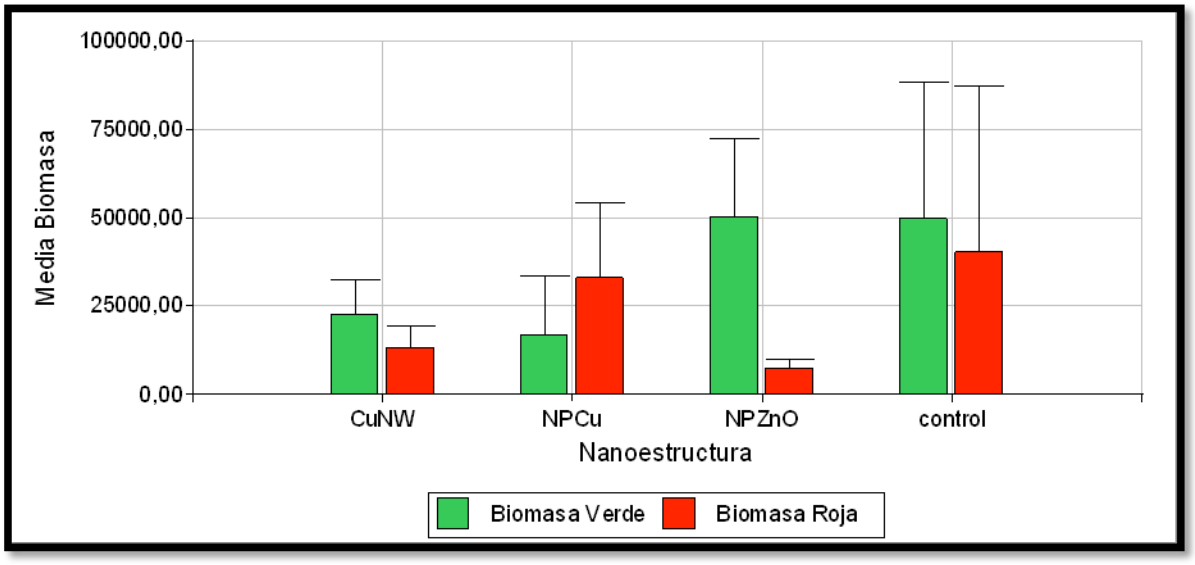

Figura 14: Diferencia de medias del Volumen de la Biomasa verde y Biomasa roja (μm^3) por exposición de nanoestructuras(250 µg/mL) a una biopelícula madura.(Prueba de Kruskal Wallis, P <0,05).

Tabla 3: Medidas de Resumen para el Volumen de la Biomasa verde(Prueba de Kruskal Wallis) p =0,0045

Variable	Nanoestructura	N	Medias	D.E.	Medianas
Biomasa Verde	control	9	49709,64	38712,42	40651,10
Biomasa Verde	CuNW	12	22822,74	9655,61	20858,45
Biomasa Verde	NPCu	12	16542,11	17045,24	11476,90
Biomasa Verde	NPZnO	12	50348,38	21948,14	51090,50

Trat.	Ranks			
NPCu	14,75	A		
CuNW	18,50	A	B	
control	28,00		B	C
NPZnO	32,00			C

Medias con letrasdiferentesindica diferencias significativas (p < 0,05)

Tabla 4: Medidas de Resumen para el Volumen de la Biomasa Roja(Prueba de Kruskal Wallis) p=0,0367

Variable	Nanoestructura	N	Medias	D.E.	Medianas
Biomasa Roja	control	9	40240,04	46944,92	10560,12
Biomasa Roja	CuNW	12	13377,94	5651,85	11765,30
Biomasa Roja	NPCu	12	32777,23	21592,51	35591,40
Biomasa Roja	NPZnO	12	7172,71	2743,82	6797,22

Trat.	Ranks		
NPZnO	14,00	A	
CuNW	24,50	A	
control	25,00	A	
NPCu	29,00		B

Medias con letrasdiferentesindica diferencias significativas (p < 0,05)

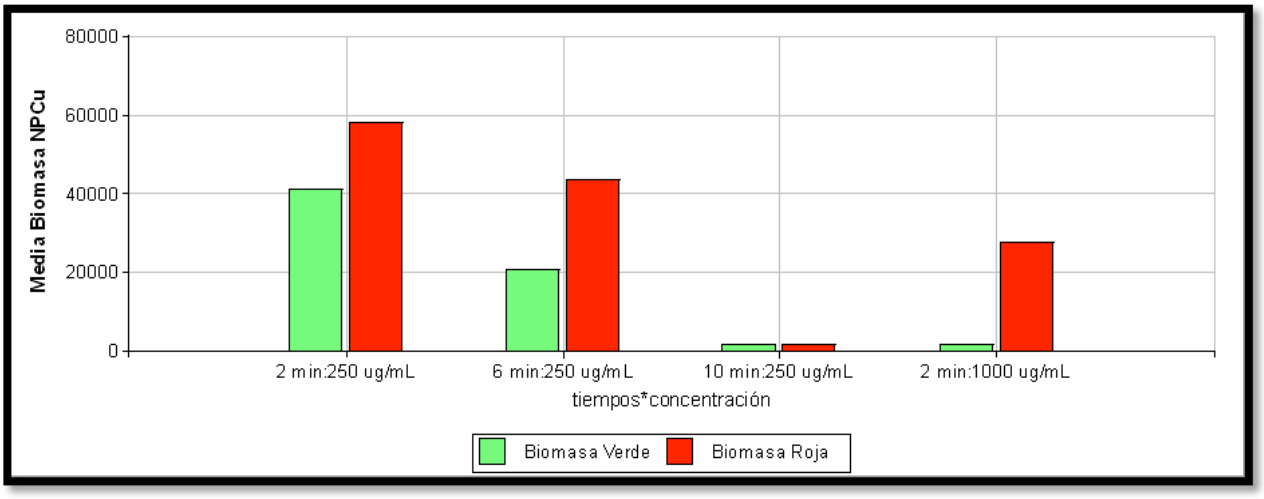

Figura 15: Diferencia de medias del volumen de la Biomasa verde y Biomasa roja por exposición de nanopartículas de cobre a una biopelícula madura en distintos tiempos y concentraciones.

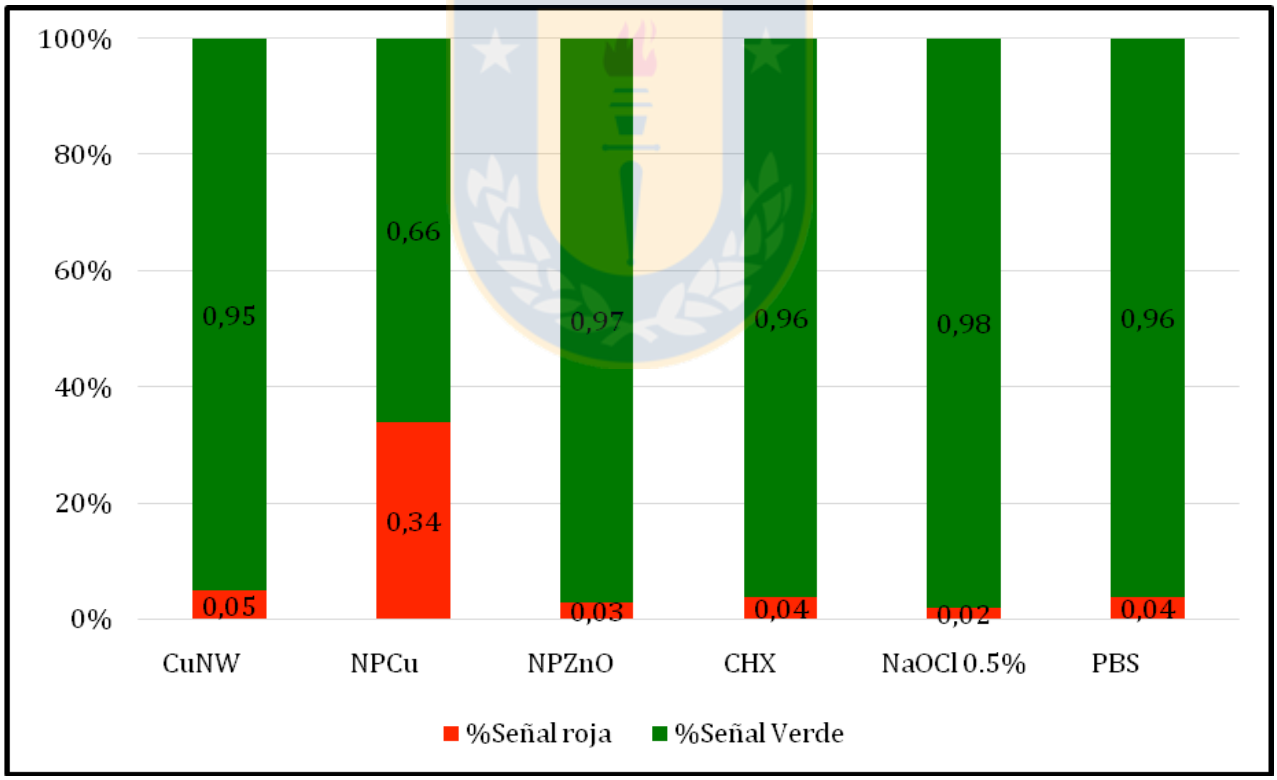

Figura 16: Viabilidad de la biopelícula después de ser tratadas con Nanoestructuras (250 ug/mL) por 10 minutos. La cuantificación de la viabilidad se determinó mediante el porcentaje de la señal promedio entre la señal verde (SYTO 9) y roja (yoduro de propidio) en relación con la señal total capturada.

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