UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

PRISCILA AMANDA FRANCISCO

INVESTIGAÇÃO DOS PERFIS PROTEÔMICO E MICROBIOLÓGICO E DA SUSCETIBILIDADE ANTIMICROBIANA DE BACTÉRIAS ESPECÍFICAS EM INFECÇÕES ENDODÔNTICAS

INVESTIGATION OF THE PROTEOMIC AND MICROBIOLOGICAL PROFILES AND ANTIMICROBIAL SUSCEPTIBILITY OF SPECIFIC BACTERIA IN ENDODONTIC INFECTIONS

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Clínica Odontológica, na Área de Endodontia.

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Clinical Dentistry, in Endodontics Area.

Orientadora: Profa. Dra. Brenda Paula Figueiredo de Almeida Gomes

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RESUMO

O papel microbiano na infecção intracanal, pode ser inferido pela liberação de substâncias detectadas por análises metaproteômicas. Além disso, as proteínas humanas da resposta imunológica podem igualmente ser rastreadas. Considerando os efeitos negativos das ações patológicas da microbiota intracanal, pode ser necessária em casos restritos a prescrição suplementar antibiótica no tratamento das infecções endodônticas. Assim, os objetivos deste estudo foram: 1- caracterizar por espectrometria de massas o proteoma de 60 canais radiculares de dentes com diferentes situações endodônticas, como: a) polpa vital (PV); b) infecção primária (IP) e c) infecção secundária/persistente (IS); 2- investigar a susceptibilidade antimicrobiana de *E. faecalis* e *S. mutans* obtidos de canais radiculares afetados por IS. Foram selecionados 20 pacientes com PV e saúde periapical, 20 pacientes com periodontite apical e IP e 20 pacientes com periodontite apical e IS. Amostras do conteúdo do canal radicular foram coletadas e processadas por LC-ESI-MS/MS. Os espectros gerados foram pesquisados em bancos de dados de proteínas humanas e microbianas (Swiss PROT e TREMBL) através do software Proteome Discoverer 1.3, algoritmo SEQUEST e ferramentas UniProtKB. Cepas de *E. faecalis* e *S. mutans* foram recuperadas durante 20 casos de retratamentos endodônticos por meio de técnicas de cultura e confirmadas por sequenciamento do gene 16S do rRNA. Esses isolados tiveram sua susceptibilidade antimicrobiana testada por meio do sistema *E-test* contra diversos agentes microbianos. As leituras dos pontos de corte da concentração inibitória mínima (MIC) foram feitas no ponto de intersecção entre o halo de inibição em forma de elipse e a fita do *E-test*. A suscetibilidade ou resistência foi determinada de acordo com guias de interpretação. As MICs dos antibióticos que inibiram 50% e 90% das cepas foram calculadas e expressas como MIC_{50} e MIC_{90}, respectivamente. Por meio de LC-ESI-MS / MS, 2950; 3871 e 2548 diferentes números de acessos humanos e 3425; 5609 e 3544 microbianos, foram revelados a partir de amostras de canais radiculares da PV; IP com lesão periapical; e IS com lesão periapical, respectivamente. Proteínas membros das vias de inflamação e regulação óssea como: MAPK, NF-κβ, MCSF-1 e RANKL, foram encontradas em todas as situações endodônticas, enquanto proteína quinase dependente de cálcio/calmodulina tipo 1D, membro 11 da superfamília do ligante do fator de necrose tumoral o membro 11A da superfamília do fator de necrose tumoral foram encontrados apenas em casos de infecção. Várias proteínas microbianas estiveram presentes em todas as situações endodônticas, principalmente referindo-se à adesão celular, que variou entre simbiose ou formas parasitárias. Formação de biofilme, liberação de toxina e moléculas

de invasão celular também foram encontradas. Quanto a susceptibilidade microbiana, 37 cepas de *E. faecalis* e 7 de *S. mutans* foram isoladas. Todos *E. faecalis* foram sensíveis à amoxicilina, amoxicilina + clavulanato, moxifloxacina e vancomicina. Taxas mais altas (75 - 84%) de eficácia antimicrobiana também foram encontradas para ciprofloxacina, cloranfenicol e doxiciclina. Azitromicina (45,9%) e eritromicina (56,8%) apresentaram taxas intermediárias de suscetibilidade, enquanto 21,6%, 24,3% e 24,3% das cepas de *E. faecalis* expressaram resistência à doxiciclina, rifampicina e tetraciclina, respectivamente. Três agentes antimicrobianos foram totalmente eficazes contra *S. mutans*, amoxicilina, amoxicilina + clavulanato e tetraciclina. Além disso, seis cepas foram consideradas sensíveis à benzilpenicilina, clindamicina, eritromicina e vancomicina. Azitromicina e cloranfenicol apresentaram os resultados menos favoráveis, com suscetibilidade de 28,6% e 14,3%, respectivamente. Amoxicilina, amoxicilina + ácido clavulanato e moxifloxacina apresentaram o menor MIC_{90} para ambas as cepas. Concluindo, proteínas humanas e microbianas foram identificadas em todas as condições endodônticas. A IP continha um número maior de proteínas do que as demais situações endodônticas. Membros importantes das vias de inflamação e reabsorção óssea foram encontrados, enquanto muitas proteínas microbianas detectadas foram relacionadas à adesão em simbiose aos tecidos do hospedeiro e atividades patogênicas, tais como: formação de biofilme, liberação de toxina, proteólise e invasão celular. As cepas de *E. faecalis* foram totalmente suscetíveis à amoxicilina, amoxicilina + clavulanato, moxifloxacina e vancomicina. Enquanto todos os *S. mutans* isolados foram suscetíveis à amoxicilina, amoxicilina + clavulanato e tetraciclina. Além disso, a MIC_{90} mostrou que 1mg / ml de amoxicilina ou amoxicilina + ácido clavulanato, 0,75mg / ml de moxifloxacina e 3mg / ml de vancomicina seriam suficientes para inibir pelo menos 90% de ambas as cepas.

Palavras-chave: endodontia, microbiota, necrose pulpar, polpa dental, espectrometria de massa, proteoma, testes de sensibilidade microbiana.

ABSTRACT

Microbial role in the intracanal infection can be inferred by its release of substances detected by meta-proteomic analyses. Furthermore, human proteins from the immunological response can equally be screened by proteomic technology. Considering the negative effects of the intracanal microbiota pathological actions, it is sometimes necessary the prescription of antibiotic supplementation in restrict cases in the treatment of endodontic infections. Thus, the aims of this study were: 1- to characterize by Mass Spectrometry the proteome of 60 root canals of teeth with different endodontic situation, as: a) vital pulp (VP); b) primary infection (PI) and c) secondary/persistent infection (SI); 2- to investigate the antimicrobial susceptibility of *E. faecalis* and *S. mutans* obtained from root canals affected by SI. Twenty patients with VP and periapical heath, 20 patients with apical periodontitis and PI and 20 patients with apical periodontitis and SI were selected. Samples from the root canal content were collected and processed by LC-ESI-MS/MS. The spectra generated were searched against human and microbial protein databases (Swiss PROT and TREMBL) using Proteome Discoverer 1.3 software, SEQUEST algorithm and UniProtKB tools. Strains of *E. faecalis* and *S. mutans* were recovered during 20 cases of endodontic retreatments through culture technics and confirmed by 16S rRNA gene sequencing. These isolates had their antimicrobial susceptibility tested through the E-test system against diverse antimicrobial agents. Minimum inhibitory concentration (MIC) breakpoints readings were done at the point of intersection between the ellipse-shaped inhibition halo and the E-test tape. Susceptibility or resistance was determined following interpretation guides. The MICs of antibiotics that inhibited 50% and 90% of the strains were calculated and expressed as MIC_{50} and MIC_{90}, respectively. Through LC-ESI-MS/MS, 2950; 3871 and 2548 human and 3425; 5609 and 3544 microbial different accession numbers, were revealed from the root canal samples of vital pulp; primary infection with periapical lesion; and secondary infection with periapical lesion, respectively. Protein's member of inflammation and bone regulations pathways as: MAPK, NF-κβ, MCSF-1 and RANKL, were encountered in all endodontic situations, while Calcium/calmodulin-dependent protein kinase type 1D, Tumor necrosis factor ligand superfamily member 11 and the Tumor necrosis factor receptor superfamily member 11A were found only at infections cases. Several microbial functions were present in all endodontic situations specially relating to cell adhesion, which varied between symbiosis or parasitic forms. Biofilm formation, toxin release and cell invasion molecules were also found. Thirty-seven strains of *E. faecalis* and seven of *S. mutans* were obtained. All *E. faecalis* isolates were susceptible to amoxicillin, amoxicillin

+ clavulanate, moxifloxacin and vancomycin. Higher rates (75 – 84%) of antimicrobial effectiveness have also been found for ciprofloxacin, chloramphenicol and doxycycline. Azithromycin (45.9%) and erythromycin (56.8%) showed intermediate rates of susceptibility, while 21.6 %, 24.3% and 24.3% of the *E. faecalis* strains expressed resistance to doxycycline, rifampicin and tetracycline, respectively. Three antimicrobial agents were totally effective against *S. mutans,* amoxicillin, amoxicillin + clavulanate and tetracycline. Besides, six strains were found to be susceptible to benzylpenicillin, clindamycin, erythromycin and vancomycin. Azithromycin and chloramphenicol showed the less favorable results, with susceptibility rates of 28.6% and 14.3%, respectively. Amoxicillin, amoxicillin + clavulanate acid and moxifloxacin had the lowest MIC^{90} for both strains. In conclusion, human and microbial proteins were identified in all endodontic conditions. PI contained a greater number of proteins than the others endodontic situations. Important inflammation and bone resorption pathways members were encountered, whilst many detected microbial proteins were related to symbiont adhesion to host tissues and pathogenic activities, such as: biofilm formation, toxin release, proteolysis, and cell invasion. *E. faecalis* strains were found to be totally susceptible to amoxicillin, amoxicillin + clavulanate, moxifloxacin, and vancomycin. While all *S. mutans* isolates were susceptible to amoxicillin, amoxicillin + clavulanate, and tetracycline. Furthermore, MIC_{90} showed that 1mg/ml of amoxicillin or amoxicillin + clavulanate acid, 0.75mg/ml of moxifloxacin, and 3mg/ml of vancomycin would be enough to inhibit at least 90% of both strains.

Keywords: endodontics, microbiota, pulp necrosis, dental pulp, mass spectrometry, proteome, microbial sensitivity tests.

SUMÁRIO

1 INTRODUÇÃO

As infecções endodônticas são a principal causa de periodontite apical (Kakehashi et al., 1965). A composição microbiana dos casos de infecções primárias é um reflexo das condições que o meio ambiente oferece para atender às necessidades nutricionais e físico-químicas dos microrganismos colonizantes. Estudos mostram que esta infecção é heterogênea, porém constituída predominantemente de cepas Gram-negativas anaeróbicas (Gomes et al., 2004, 2006a; Herrera et al., 2015).

Portanto, o principal objetivo do tratamento endodôntico é eliminar ou reduzir drasticamente o conteúdo bacteriano intracanal. Quando esse objetivo falha, temos a periodontite apical pós-tratamento, que é causada pela persistência da comunidade bacteriana dentro do sistema de canal radicular (Molander et al., 1998; Sundqvist et al., 1998; Rôças et al., 2004; Endo et al., 2013). As bactérias frequentemente encontradas em canais radiculares de dentes com insucesso endodôntico usando a técnica de cultura são predominantemente Gram-positivas, incluindo cocos (por exemplo, *Enterococcus* spp., *Streptococcus* spp.) e bastonetes (por exemplo, *Actinomyces* e *Propionibacterium*) (Molander et al., 1998; Sundqvist et al., 1998; Hancock et al., 2001; Pinheiro et al., 2003a; Gomes et al., 2006b; Delboni et al., 2017).

Por meio de métodos de identificação microbiana moleculares, em adição as tradicionais técnicas de cultura, diversas espécies foram relacionadas as infecções endodônticas (Munson et al., 2002; Sedgley et al., 2005b; Siqueira e Rôças, 2005a, 2005b; Gomes et al., 2006a, 2006b; Sakamoto et al., 2006; Vianna et al., 2008; Subramanian and Mickel, 2009; Endo et al., 2013). Métodos de biologia molecular independente de cultura apresentam vantagens sobre os procedimentos de identificação bacteriana baseados em características fenotípicas, como maior sensibilidade e especificidade e capacidade de identificar bactérias não cultiváveis (Munson et al., 2002; Siqueira and Rôças, 2005a, 2005b), gerando resultados mais confiáveis em relação ao conteúdo microbiano.

Embora os métodos de identificação microbiológica tenham fornecido uma grande lista de espécies bacterianas encontradas em infecções endodônticas, há limitações na obtenção de informações sobre funções expressas por comunidades microbianas in situ (Maron et al., 2007). Tão importante quanto saber quem está causando a periodontite apical, é entender como eles estão gerando os danos e quais são as respostas do hospedeiro (Provenzano et al., 2016).

Análises metaproteômicas das substâncias produzidas e liberadas por membros de

associações bacterianas multi-espécies podem permitir inferências sobre seu comportamento e papel na patogênese das infecções intracanais. Não apenas proteínas microbianas, mas igualmente humanas, da resposta imunológica, podem ser rastreadas por tecnologia proteômica (Pennington et al., 1997). Portanto, a proteômica pode ser importante para demonstrar a interação entre a infecção microbiana dentro do canal radicular e a defesa do hospedeiro nos tecidos perirradiculares. Vários estudos examinaram a proteômica de sítios orais (Siqueira et al., 2008; Salih et al., 2010; Siqueira and Dawes, 2011; Silva-Boghossian et al., 2013; Delecrode et al., 2015; Moffa et al., 2015; Yaprak et al., 2018), enquanto poucos estudaram especificamente a composição proteica em casos de infecções endodônticas (Nandakumar et al., 2009; Provenzano et al., 2013, 2016; Francisco et al., 2019, Loureiro et al. 2020).

Levando-se em conta os efeitos negativos da combinação entre a ação patológica da microbiota intracanal e a resposta inflamatória do hospedeiro, torna-se por vezes necessária a complementação medicamentosa no tratamento das infecções endodôntica. Todavia, a antibioticoterapia sistêmica não é comumente empregada no tratamento de lesões periapicais crônicas associadas à falha endodôntica, no entanto, em casos de infecções sistêmicas e risco de endocardite bacteriana, esta abordagem pode representar um complemento oportuno a terapia endodôntica convencional (Leblanc, 1990). Cabe ressaltar que os antibióticos devem ser usados com restrição, pois podem causar, entre outros efeitos deletérios, o desenvolvimento de resistência bacteriana aos agentes antimicrobianos (Skucaite et al., 2010; Gomes et al., 2011; Gyssens, 2018).

Como reconhecido anteriormente, a composição da microbiota associada à infecção secundária/persistente é diferente em número e diversidade de espécies quando comparada à infecção primária. Dentre as bactérias anaeróbias facultativas Gram-positivas prevalentes neste ambiente, Enterococcus spp. (Hancock et al., 2001; Gomes et al., 2006b; Ozbek et al., 2009; Wang et al., 2012) e Streptococcus spp. são comumente detectadas (Rôças et al., 2008; Antunes et al., 2015; Lima et al., 2020b).

Enterococcus faecalis contém uma série de mecanismos de virulência e resistência que dificultam a sua erradicação dos canais radiculares, tais mecanismos resultam de alterações fisiológicas e estruturais nas células bacterianas, servindo como estratégias de sobrevivência às diferentes classes de antibióticos (Endo et al., 2014). A relevância do estudo da resistência antimicrobiana dessa bactéria reside no fato de que *E. faecalis* é considerada um indutor crítico de endocardite infecciosa, uma das quatro mais comuns síndromes de infecção com risco de vida (Baddour et al., 2015). *E. faecalis* adquiriu resistência ao longo do tempo à

antimicrobianos, como clindamicina, eritromicina, tetraciclina, cloranfenicol e vancomicina (Pinheiro et al., 2003b; Endo et al., 2014; Barbosa-Ribeiro et al., 2016). Já em estudo mais recente, verificou-se que sua suscetibilidade permanece para amoxicilina + clavulanato, amoxicilina e benzilpenicilina (Barbosa-Ribeiro et al., 2016).

Streptococcus mutans pertence ao grupo dos estreptococos viridans. Embora seja considerado um habitante comum da microbiota oral, incluindo os canais radiculares associados à infecção secundária ou persistente, este pode representar outro patógeno de endocardite infecciosa (Süzük et al., 2016). Os componentes estruturais dos estreptococos viridans são importantes mediadores de virulência e adesão dessas bactérias, sendo detectados com relevante frequência em tecidos cardíacos (Abranches et al., 2009; Oliveira et al., 2019). Poucos estudos na literatura monitoraram a susceptibilidade antimicrobiana de *S. mutans* isolados de casos de falha endodôntica (Skucaite et al., 2010; Al-Ahmad et al., 2014; Łysakowska et al., 2016).

Observando a literatura, notamos falta de trabalhos que analisam como de fato os patógenos endodônticos agem ativando o sistema imune e consequentemente levando a agressões ao hospedeiro, apesar de vários deles contemplarem a composição da microbiota. Apenas cinco trabalhos investigaram a proteômica de canais radiculares (Nandakumar et al., 2009; Provenzano et al., 2013, 2016; Francisco et al., 2019; Loureiro et a. 2020), porém nenhum apresentou um grupo controle de dentes não extraídos com a polpa e periápice saudáveis.

Ainda, tão importante quanto conhecer os mecanismos da infecção e da inflamação endodôntica, é possuir ferramentas para controlá-los. Nesse sentido a acurada terapia antibiótica auxiliar terá papel fundamental na recuperação e manutenção da saúde do paciente em casos de infecções sistêmicas e necessidade de profilaxia antibiótica frente ao insucesso endodôntico. Nas infecções provenientes do insucesso, são poucos estudos que verificaram a suscetibilidade de bactérias como *Enterococcus faecalis* e *Streptococcus mutans*, simultaneamente. Ambas as espécies apresentam fatores de virulências relacionados a doenças sistêmicas.

Desta forma, os objetivos do presente estudo serão: 1- identificar e caracterizar perfis proteômicos de canais radiculares de dentes com: a) polpa vital; b) infecção primária com periodontite periapical e c) infecção secundária/persistente com periodontite periapical, e 2 - observar a resistência de *E. faecalis* e *S. mutans* obtidos de canais radiculares afetados por infecção secundária/persistente à agentes antimicrobianos.

2 ARTIGOS

2.1 Artigo: Endodontic and periapical proteomic parameters in apical periodontitis or health situations

Artigo redigido de acordo com as normas para publicação no periódico *International Endodontic Journal.*

ABSTRACT

Introduction: Microbial behavior and role in the intracanal infection can be inferred by their release of substances detected by meta-proteomic analyses. Furthermore, human proteins from the immunological response can equally be screened by proteomic technology. Thus, this study aimed to characterize by Mass Spectrometry the proteome of 60 root canals of teeth with different endodontic situations, as: a) vital pulp; b) primary infection and c) secondary/persistent infection. **Methodology:** Twenty patients with pulp and periapical health, 20 patients with apical periodontitis and primary infection, and 20 patients with apical periodontitis and need for endodontic retreatment were selected. Samples from the root canal content were collected and processed by Capillary Nano-Flow Liquid Chromatography and Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS). The acquired MS/MS spectra generated were separately searched against human and microbial protein databases (Swiss PROT and TREMBL) for all samples using Proteome Discoverer 1.3 software, SEQUEST algorithm, and UniProtKB tools. **Results:** Through LC-ESI-MS/MS, 2950; 3871 and 2548 human and 3425; 5609 and 3544 microbial different accession numbers, were revealed from the root canal samples of three different endodontic situations, vital pulp; primary infection with periapical lesion; and secondary infection with periapical lesion, respectively. Protein's members of inflammation and bone regulations pathways as: MAPK, NF-κβ, MCSF-1, and RANKL, were encountered in all endodontic situations, while Calcium/calmodulin-dependent protein kinase type 1D, Tumor necrosis factor ligand superfamily member 11, and the Tumor necrosis factor receptor superfamily member 11A were found only at infections cases. Several microbial proteins were present in all endodontic situations specially relating to cell adhesion, which varied between symbiosis or parasitic forms. Biofilm formation, proteolysis, toxin release, and cell invasion molecules were also found. **Conclusion:** In conclusion, human and microbial proteins were identified in all endodontic conditions. Primary infection contained a greater number of proteins than the other endodontic situations. Important inflammation and bone resorption pathways members

were encountered, whilst many detected microbial proteins were related to symbiont adhesion to host tissues and pathogenic activities, such as: biofilm formation, toxin release, proteolysis, and cell invasion.

Keywords: primary infection, secondary/persistent infection, vital pulp, mass spectrometry, metaproteome.

INTRODUCTION

Endodontic infections are polymicrobial in nature and are the primary cause of apical periodontitis (Kakehashi *et al.* 1965). The microbial composition of cases of primary infections reflects the conditions that the environment offers to meet the nutritional and physicochemical requirements of the colonizing microorganisms. Studies show that this infection is heterogeneous but consisting predominantly of Gram-negative anaerobic strains (Gomes et al. 2004, Gomes *et al*. 2006; Gomes *et al*. 2008; Herrera *et al.* 2015).

The major goal of endodontic treatment is to eliminate or reduce drastically the intracanal bacterial content. When this objective fails, revealed by the persistence of the bacterial community within the root canal system, we have post-treatment apical periodontitis. (Sundqvist *et al.* 1998, Molander *et al.* 1998, Rôças *et at.* 2004, Endo *et al.* 2013). The bacteria most frequently found in root canals of teeth with endodontic failure by using culture technique are predominantly Gram-positive, including cocci (e.g., *Enterococcus* spp., *Streptococcus* spp.) and rods (e.g., *Actinomyces* and *Propionibacterium*) (Molander *et al.* 1998, Sundqvist *et al.* 1998, Hancock *et al.* 2001, Pinheiro *et al.* 2003, Gomes *et al.* 2004, Delboni *et al.* 2017).

Microorganism identification through molecular methods has been widely applied to dentistry research (Munson *et al.* 2002, Sedgley *et al.* 2005, Siqueira & Rôças, 2005ab, Gomes *et al.* 2006ab, Sakamoto *et al.* 2006, Vianna *et al.* 2008, Subramanian & Mickel 2009, Endo *et al.* 2013). Culture-independent molecular biology methods show great sensitivity and specificity and the ability to identify unculturable bacteria (Munson *et al.* 2002, Siqueira & Rôças 2005ab). Despite the vast list of microorganisms molecularly identified, there are limitations in obtaining information on functions expressed by microbial communities *in situ* (Maron *et al.* 2007).

In periapical periodontitis, whether resulting from a primary or secondary/persistent infection, certain components of the bacterial structure known as virulence factors may function by stimulating inflammatory and immunological responses

(Amersfoort *et al*., 2003). These factors can activate and remain within macrophages for a long time, inducing the release of proteins like pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α (Amersfoort *et al*., 2003). Besides, they can activate the complement system (Ginsburg, 2002), and be involved in biofilm formation and tooth adhesion due to its adsorptive action to hydroxyapatite (Fabretti *et al*., 2006). Several works in the literature reveal the periapical condition in case of endodontic disease (Rechenberg *et al*. 2014, Herrera *et al*. 2015, Barreiros *et al*. 2018), however, there are no studies that overview the biological parameters of periapical health associated with vital pulp, serving as an important control for cases with a periapical lesion.

Microbial behavior and role in the intracanal infection can be inferred by their release of substances detected by meta-proteomic analyses. Furthermore, human proteins from the immunological response can equally be screened by proteomic technology (Pennington *et al.* 1997). Diverse studies have examined the proteomics of diverse oral sites (Siqueira et al. 2008, Salih et al. 2010, Siqueira & Dawes 2011, Silva-Boghossian et al. 2013, Delecrode et al. 2015, Moffa et al. 2015, Aboodi et al. 2016, Yaprak et al. 2017), but only a few have studied the protein composition in cases of endodontic infections (Nandakumar et al. 2009, Provenzano et al. 2013, 2016, Francisco et al. 2019).

Although several studies contemplate the composition of the endodontic microbiota, few have analyzed how these pathogens act by activating the immune system and consequently leading to aggressions to the host. Currently, five studies have investigated the proteomics of root canals (Nandakumar et al., 2009; Provenzano et al., 2013, 2016; Francisco et al., 2019; Loureiro et a. 2020), but none presented a control group of teeth not extracted with healthy pulp and periapex. Thus, the present study will aim to identify and characterize particular proteome profiles of root canals of teeth with a) vital pulp; b) primary infection with periapical periodontitis and c) secondary/persistent infection with periapical periodontitis, by using two-dimensional capillary nano-flow liquid chromatography and electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS).

MATERIALS AND METHODS

Patient Selection

Only cases of teeth needing endodontic treatment or re-treatment were selected from the records of the Piracicaba Dental School Department of Endodontics. All patients signed an informed consent form prepared following the rules of the Research Ethics Committee of the Piracicaba Dental School - UNICAMP (CEP/FOP-UNICAMP No

119/2015). It should be stressed that all the patients involved in the research received adequate endodontic treatment or re-treatment.

After clinical evaluation, 60 teeth were included in this study, being divided into 3 groups:

a) 20 patients with vital pulp and periapical health;

b) 20 patients with apical periodontitis and primary infection and;

c) 20 patients with apical periodontitis and need for endodontic retreatment.

Factors that could interfere with the root canal microbiota were excluded, namely: endodontic treatment less than 2 years, use of antifungals and antibiotics (<3 months), periodontal disease, teeth with root fracture, teeth allowing neither absolute isolation nor the introduction of a paper point to the working length, teeth with pulp chamber in contact with oral environment, and teeth associated to spontaneous pain.

Sample Collection

The method followed for root canal collection was previously described in detail by Gomes *et al.* (2004, 2011) and Barbosa-Ribeiro *et al.* (2016). The teeth were isolated with a rubber dam, with their crown and surrounding structures being disinfected with 30% hydrogen peroxide for 30 seconds, followed by 2.5% sodium hypochlorite for the same period and then inactivated with 5% sodium thiosulfate. Disinfection of the external surfaces of the crown was checked by taking a swab sample from the crown surface and streaking it onto blood agar plates, which were then incubated aerobically and anaerobically.

Under anesthesia (2% lidocaine with 1:100,000 epinephrine), a two-stage access preparation was performed. The access cavity was made without the use of water spray, but under manual irrigation with sterile saline and by using sterile high-speed diamond bur. This first stage was performed to promote a major removal of contaminants. In the second stage, before entering the pulp chamber, the access cavity was disinfected according to the decontamination protocol described above. Disinfection of the internal surface of the access cavity was checked as previously described, and all procedures were performed aseptically. Root-filling materials were removed when present by using Reciproc R25 files (VDW, Munich, Germany) along the working length (which had been determined with pre-operative radiography), according to the manufacturer's instructions in a crown-down technique, with no chemical solvent.

Before collection of the root canal sample, a K-file #20 (Dentsply Maillefer, Ballaigues, Switzerland) was used to confirm the working length (previously estimated by

radiographs) with an apex locator (Novapex; Forum Technologies, Rishon le-Zion, Israel). A sterile paper point (Dentsply-Maillefer, Ballaigues, Switzerland) was then introduced one millimeter beyond the actual length of the root canal and retained in position for 60 seconds for sampling. To assist the collection in case of a dry canal, sterile saline solution was inserted into the root canal before sampling. The samples were frozen at −80°C for further analysis.

Mass Spectrometry

Part of the methods used in this study was previously described in detail by Siqueira *et al.* (2008) and Heller *et al.* (2016).

The 60 samples collected were processed separately. Before liquid chromatography and mass spectrometry, all samples were treated with 4 M urea, 10 mM DTT in 50 mM NH4HCO3, pH 8.0, at 50°C for 1 hour to denature the proteins and reduce disulfide bonds, followed by alkylation with 10 mM iodoacetic acid in the dark and at room temperature for 1 hour. Samples were submitted to in-solution trypsin digestion (5% w/w) for 16 hours at 37°C prior to LC-ESI-MS/MS. This approach ensures reliable identification and characterization of the root canal content of teeth with vital pulp, primary infection with periapical periodontitis, and secondary/persistent infection with periapical periodontitis.

The proposed work involving mass spectrometric analyses was performed by using in-house Capillary Nano-Flow Liquid Chromatography and Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS) (Thermo-Scientific, San Jose, CA, USA). Briefly, mass spectrometric analyses were carried out with an in-line liquid chromatography containing capillary cation-exchange and C18 columns linked to a mass spectrometer with electrospray ionization in a survey scan in the range of m/z values 390-2000 tandem MS/MS. All samples obtained by in-solution trypsinization were dried by rotary evaporation and re-suspended in 25 µL of 97.5% H2O/2.4% acetonitrile/0.1% formic acid (or another buffer) and then were submitted to cation-exchange chromatography, followed by reversed-phase LC-ESI-MS/MS. The nano-flow LC yielded a linear 85-minute gradient ranging from 5% to 55% of solvent B at a flow rate of 110 nL/min. Electrospray voltage and ion transfer capillary temperature were 3.0 kV and 250 °C, respectively. This long-period gradient facilitated peptide/protein separation and consequently improved identification.

Bioinformatics - Database Searches and Identification by Mass Spectrometry

The acquired MS/MS spectra were separately searched against specific human and microbial protein databases (Swiss PROT, Swiss Institute of Bioinformatics, Geneva,

Switzerland, http://ca.expasy.org) for all samples and using Proteome Discoverer 1.3 software and SEQUEST algorithm. Parameter XCorr was used to validate the existence of peptides within the sample. XCorr is a value computed from cross-correlation of the experimental MS/MS spectrum vs. candidate peptides in the database and which reveals how closely the real spectrum relates to candidate peptides. The SEQUEST score for filtering putative criteria applied to MS/MS spectra was: XCorr score > 1.9, 2.2 or 3.5 for Z = 1, 2, and 3, respectively, for full tryptic peptides and XCorr score = 2.0 and 2.6 for Z = 2 and 3, respectively, for partial tryptic peptide. Sequence-reversed protein databases were used as decoys to evaluate the false-positive rate during the search. Furthermore, each result was judged and validated by manual inspection of the MS/MS spectra to confirm and ensure that fragment ions (e.g. a, b, and y-ions) were above the background and that abundant fragments have been assigned by the search. Gene Ontology Annotation for function category (biological or molecular) was obtained by using UniProtKB tools and database (http://www.uniprot.org). The proteins within the same group were analyzed together.

Statistical Analyses

The results obtained with the tests were statistically analyzed by descriptive statistical analysis.

RESULTS

Through LC-ESI-MS/MS, 2950; 3871 and 2548 human and 3425; 5609 and 3544 microbial different accession numbers, were revealed from the root canal samples of three different endodontic situations, these being vital pulp; primary infection with periapical lesion; and secondary infection with periapical lesion, respectively.

Human proteins

Unique *Human sapiens* proteins from the vital pulp, primary infection, and secondary infection, that were annotated in the Gene Ontology database from UniProtKB, were tabulated and analyzed regarding their biological functions. Most human proteins identified were involved in cellular process, biological regulation, developmental process, metabolic process, localization, and response to stimulus.

The biological process from the human proteins found in each different situation studied and that may be involved in periapical inflammation and bone resorption, as inflammatory response and osteoclastogenesis were tabulated (Tables 1-2).

Nineteen inflammatory response proteins were found in all group cases. Twenty, 41 and 29 were exclusive to vital pulp, primary and secondary/persistent infections.

Concerning the infection groups, Interleukin-1 receptor type 1 (IL-1R-1), Calcium/calmodulin-dependent protein kinase type 1D (CKLiK), and Tumor necrosis factor receptor superfamily member 11A (TNFRSF11A/RANK) were found only in primary infections. While Macrophage colony-stimulating factor-1 (MCSF-1) and Tumor necrosis factor ligand superfamily member 11 (TNFSF11/RANKL) specifically in secondary/persistent infections (Table 1).

Finally, two proteins related to osteoclastogenesis were encountered in all situations: Ephrin type-A receptor 2 and SH3 and PX domain-containing protein 2A (Table 2).

Microbial proteins

In general, the highest number of microbial proteins belonged to the following categories: cellular process, interspecies interaction between organisms (pathogenicity), metabolic process, and biological regulation.

The microorganisms set that appeared more often (at least 9 times) with different proteins diverged between the studied groups. In the vital pulp group, the most prevalent microorganisms were: *Candida dubliniensis* (20/20), *Candida albicans* (20/20), *Granulicatella elegans* (15/20), *Rothia mucilaginosa* (14/20), *Gardnerella vaginalis* (13/20), *Streptococcus mitis* (13/20), *Veillonella parvula* (12/20), and *Dialister* spp. (9/20).

Whereas, in the condition of primary infection with the presence of periapical injury, the most frequent species were: *Candida dubliniensis* (20/20), *Candida albicans* (20/20), *Rothia mucilaginosa* (20/20), *Streptococcus mitis* (20/20), *Streptococcus sanguinis* (20/20), *Dialister* spp. (20/20)., *Gardnerella vaginalis* (20/20), *Veillonella parvula* (17/20), *Bacteroides fragilis* (15/20), *Helicobacter pylori* (15/20), *Rothia dentocariosa* (15/20), *Haemophilus influenzae* (14/20), *Klebsiella pneumoniae* (14/20), *Enterobacter asburiae* (13/20), *Hafnia alvei* (12/20), *Treponema denticola* (12/20), *Acinetobacter calcoaceticus* (10/20), *Pseudomonas aeruginosa* (10/20), *Fusobacterium periodonticum* (9/20), *Streptococcus anginosus* (9/20), *Tannerella forsythia* (9/20), *Acinetobacter baumannii* (8/20), *Capnocytophaga ochracea* (8/20), *Escherichia coli* (8/20), *Prevotella melaninogenica* (8/20).

Lastly, the microorganisms most found in secondary infection with the presence of periapical lesion were: *Candida dubliniensis* (20/20), *Candida albicans* (20/20), *Streptococcus mitis* (17/20), *Gardnerella vaginalis* (11/20), *Streptococcus sanguinis* (10/20),

Rothia mucilaginosa (9/20), and *Rothia dentocariosa* (8/20).

Unique proteins with adhesion, proteolysis, and pathogenic biological functions were studied in each endodontic situation. *Candida albicans* proteins as pH-responsive protein 1 and Hyphal wall protein 2 were found in all cases in adhesion to symbiont to host. Moreover, diverse proteins from *Acinetobacter baumannii; Atopobium rimae; Candida albicans; Candida dubliniensis; Chromobacterium violaceum; Enterobacter asburiae; Escherichia coli; Gemella haemolysans; Granulicatella elegans; Klebsiella aerogenes; Streptococcus milleri; Streptococcus mitis; Streptococcus sanguinis;* and *Treponema denticola* presented functions of cell adhesion not in symbiont to the host (Table 3).

The ability to hydrolyze proteins was found in all cases, but only at TrEMBL/ UniProtKB. Three proteins that were common to all of them were: G5 domain-containing protein, IgA-specific serine endopeptidase, and Toxin CdiA (Table 4).

Regarding pathogenic biological functions, all situations showed microorganisms with this sort of function, with a higher volume of proteins coming from primary infections. Six microbial proteins were present in all endodontic situations: Hep/Hag repeat protein, S-layer protein, 16S rRNA endonuclease CdiA, Putative adhesin, Hemolysin, Hemagglutinin domain protein, and YadA domain protein (Table 5).

DISCUSSION

Investigations of microbial contents and the host's response to the actions inferred by the former are allowed by metagenomics and metaproteomics studies that deal with all the genes and proteins identified in complex communities. In this exploration, diverse endodontic situations as vital pulp, primary infections, and secondary or persistent infections (both infections with radiographic confirmation of periapical lesions) were analyzed concerning their human and microbial proteomics profiles. Substantial protein loads were found in all endodontic conditions. Most of them, human or microbial, have been associated with housekeeping functions, such as cellular process, biological regulation, metabolic process, and developmental process. Notably, several different humans and microbial proteins related to injury response and pathogenicity were encountered.

Human proteins

Through the mass spectrometry method by LC-ESI-MS/MS, 2950; 3871, and 2548 accession numbers were found in 20 cases of each situation, vital pulp, primary infections, and secondary/persistent infections, respectively. A consortium of entries

significantly bigger them the found by previous endodontic studies (Provenzano *et al.* 2013, 2016, Francisco *et al.* 2019).

Proteins that may be involved in periapical inflammation and bone resorption, as inflammatory response and osteoclastogenesis were selected for analysis. Interestingly, while the primary infection was ahead in numbers of entries obtained in the UniProtKB database, vital pulp and secondary infection showed similar amounts. Though, it must be considered that the protein's activity depends on binding to other specific molecules, for instance: viruses or bacteria, marking them for posterior destruction; or other proteins, since the majority of proteins interact with others for proper function (Phizicky and Fields, 1995). In other words, the fact that a specific protein is present at a certain site does not guarantee its function alone. Free fatty acid receptor 3, a protein annotated by Gene Ontology as an immune response molecule found in this work, may have 13 different biological functions depending on their binding to other proteins (according to the UniProtKB database).

Diverse immune proteins were equally found in the three studied groups, but due to the qualitative screening characteristic of this investigation, the levels of these molecules are unknown. Fibroblasts, odontoblasts, inflammatory cells, nerve fibers, and other members within the pulp can express chemical mediators allowing these cells to respond to stimulus (Killough *et al.* 2009, Renard *et al.* 2016, Yumoto *et al.* 2018, Zhan *et al.* 2020). Despite the vast network of possible producers of the immune response, it could be plausible to suppose that in cases of vital pulp fewer molecules mediating the immune response would be found. However, recently, Loureiro *et al.* (2020) in a metaproteome analysis of different endodontic situations, verified that the normal pulp, recovered from extracted teeth, owned various proteins linked to inflammatory response, such as immunoglobulin heavy constant alpha, immunoglobulin heavy constant gamma, trafficking protein particle complex subunit 8, cadherin-3, fibronectin type-III domain-containing protein 3A, serine/threonine-protein kinase 32A, WD repeat domain phosphoinositide-interacting protein 3, amongst many others. Equally, in this work, numerous proteins were detected in normal pulp, some similar to the above, as fibronectin, serine/threonine-protein kinase, and WD repeat domain were found.

Another point that must be highlighted, although all the pulp vital samples collected were from integrate teeth, some degree of injury may have been caused by the high-speed bur generating immediate response. It has been already shown in rat pulpits models that the Notch 2 protein, observed in this endodontic group, can be expressed after LPS or mechanical stimulation (Ma *et al.* 2016).

Primary endodontic infections are well known for their polymicrobial etiology

(Gomes *et al.* 2004, Rôças & Siqueira, 2012). LPS, a Gram-negative by-product of the bacterial cell wall, is considered one of the major factors involved in the inflammation response (Schein and H. Schilder, 1975), presenting its foremost level in primary infections (Gomes *et al.* 2012). LPS binding to Toll-like receptor 4 (TRL-4) provides the activation of p38 mitogen-activated protein kinase (p38MAPK) (common to many inflammatory cytokines) and NF-κβ transcription factor (central to several immune and inflammatory responses), these pathways are linked to the production of interleukin (IL)-1beta, tumor necrosis factor-alpha, prostaglandin E2, and IL-6 and -10 (Stashenko *et al.* 1998, Garcia de Aquino *et al.* 2009). Overall, many inflammatory characters were encountered in primary infection samples, between them it can be cited: Chemokine XC receptor 1, Interleukin; IL-1; IL-6; IL-17; IL-18, Prostaglandin E2. TLR4, fundamental for p38MAPK and NF-κβ activation, was not found. On the other hand, some inflammation pathway participants were detected, an example is the IL-1 receptor that after binding to interleukin-1 associates mediates interleukin-1-dependent activation of NF-κβ, MAPK, and other pathways (Tominaga *et al.* 2000). Another finding that can be cited is NF-κβ, which is a transcription factor present in almost all cell types and is the endpoint of a series of signal transduction events that are initiated by a vast array of stimuli related to many biological processes such as inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis (Beinke *et al.* 2004). The introduction of new targets to inflammation events is important for further quantitative investigations.

Calcium/calmodulin-dependent protein kinase type 1D (CKLiK), was exclusively found at primary infections samples, this protein kinase is mainly expressed by human polymorphonuclear leukocytes (PMNs), as neutrophilic and eosinophilic granulocytes, which has an important participation in host defense against invading microorganisms (Haslett *et al.* 1989). CKLiK is regulated by Ca^{++} and calmodulin and can be activated through IL-8 binding transducing chemokine-induced signals regulating human granulocyte functions (Verploegen *et al.* 2000). To this day there is no study of this protein regarding periodontal tissues.

As in endodontics, the presence of a primary or secondary infection has repercussions on the bone tissues adjacent to the affected tooth, it is important to comment on proteins related to bone regulation by osteoclasts. Concerning secondary/persistent infection, macrophage colony-stimulating factor-1 (MCSF-1), was only observed in this endodontic situation. MCSF-1 and receptor activator of NF-kappa-B (RANKL) signaling, are important pathways for osteoclast differentiation and activation (Li *et al.* 2017, Adamapoulos, 2018). It has been proven a long time ago that MCSF-1 in association with RANKL collaborates to the

differentiation of synovial macrophages into osteoclasts, in cases of inflammatory arthritis (Danks *et al.* 2002). In the endodontics field, the results provided from a study about periapical lesions, such as apical granulomas; radicular cysts; and dentigerous cysts, showed that the expression of MCSF-1 was significantly higher in cysts than in apical granulomas (Weber *et al.* 2019).

RANKL is also known as Tumor necrosis factor ligand superfamily member 11 (TNFSF11). RANKL can bind and activate a TNFR-related protein as the Tumor necrosis factor receptor superfamily member 11A (TNFRSF11A/RANK). RANKL/RANK signaling axis plays an essential role in osteoclastogenesis (Kong *et al.* 1999, Li *et al.* 2000). In mice, it was observed that overexpression of RANKL resulted in severe osteoporosis while its deletion led to severe osteopetrosis (Kong *et al.* 1999, Mizuno *et al.* 2002). Both TNFSF11(RANKL) and TNFRSF11A(RANK) were found in this study, but not together, the former was in the primary infection group and the latter in the secondary infection group.

Additionally, Ephrin type-A receptor 2 (EphA2) was detected in all situations. Regardless of the presence or absence of inflammation, bone is a dynamic tissue that remodels continuously during life (Frost, 1964). Ephrin signaling can mediate the communication between osteoclast and osteoblast. At the beginning phase of bone remodeling, EphrinA2 anchored on osteoclast membranes and signaling through the EphA2 expressed on osteoblast, can cause bone resorption and simultaneously suppresses osteoblastogenesis (Irie *et al.* 2009).

Microbial Proteins

Just like done for human proteins and for more accurate evaluation, reviewed (Swiss Prot) rather than unreviewed (TrEMBL) UniProt Knowledgebase was prioritized for discussion. That means that preferably protein manually annotated and reviewed within information extracted from literature and curator-evaluated computational analysis were considered. Differing from humans, microbial proteins lack manual annotation, which reduced the set of studied functions, as well as the diversity of the microbiota found in each case. Eight, 11, and 9 pathogenic related proteins (and their associated microorganism), from vital pulp, primary infection, and secondary/persistent infection, respectively, had their functions previously manually annotated.

Considering that in endodontics most microbial studies are done in cases of infection, it could be expected that in the vital pulp of pristine teeth, without carious lesions or periodontal involvement, there would be no microorganisms. However, Widmer *et al.* (2018)

showed, through high-throughput DNA sequencing, that all pulp tissue sampled by their study had bacterial genetic material, and an average of 343 taxa was found per sample, 12 of them were common to all teeth, belonged to: *Ralstonia, Acinetobacter, Staphylococcus, Micrococcus, Burkholderia,* and *Corynebacterium.* In the present investigation, an average of 171 different accession numbers was recovered from vital pulp samples, from this set most of the proteins came from *C. dubliniensis, C. albicans, Dialister sp., G. elegans, R. mucilaginosa, G. vaginalis, S. mitis,* and *V. parvula.* Although many microbial proteins from this endodontic situation were linked to pathologic function by automatic annotation (TrEMBL) only eight had their function validated by literature.

In primary endodontic infection, a greater average of 280 accession numbers was observed, followed by 177 in secondary/persistent infection, where the diminished microbial population is explained by the challenges in surviving the effects of previous root canal treatment and persist in filled root canals and dentinal tubules where the interactions with other microorganism and the supply of body fluid nutrients are presumably reduced (Gomes *et al.* 2008).

The most prevalent species in primary infection were: *C. dubliniensis, C. albicans, R. mucilaginosa, S. mitis, S. sanguinis, Dialister sp., G. vaginalis, V. parvula, B. fragilis, H. pylori, R. dentocariosa, H. influenzae, K. pneumoniae, E. asburiae, H. alvei, T. denticola, A. calcoaceticus, P. aeruginosa, F. periodonticum, S. anginosus,* and *T. forsythia*. And in secondary / persistent infection were: *C. dubliniensis, C. albicans, S. mitis, G. vaginalis, S. sanguinis, R. mucilaginosa,* and *R. dentocariosa*. These are slightly contrasting results with the existing literature, according to a literature review on this topic, the genera of putative endodontic pathogens commonly associated with endodontic infections are *Bacteroides; Treponema; Prevotella; Porphyromonas; Fusobacterium; Peptostreptococcus; Streptococcus; Eubacterium; Campylobacter;* and *Actinomyces*, in primary infection, and *Enterococcus; Actinomyces; Streptococcus; Candida; Propionibacterium; Staphylococcus;* and *Pseudomonas*, in secondary/persistent infection (Siqueira, 2002).

Many of the identified and reviewed pathogenic proteins were categorized as adhesins. Amongst this group *C. albicans* was the most prevalent being present in all endodontic situations and presenting various proteins, two of them were common to every situation, pH-responsive protein 1 (PHR1) and hyphal wall protein 2 (Hwp2). Furthermore, Agglutinin-like protein 9 (ALS9) and MFS antiporter QDR2 (QDR2) were found only in vital pulp cases, Probable GPI-anchored adhesin-like 28 protein (PAG28) was present in vital pulp and primary infection, while transcriptional regulatory protein UME6 (UME6) was only

observed in primary infections. It is relevant to state that *C. albicans* is the most studied human fungal symbiont, that can transit between commensalism to parasitism within the human host, the polymorphic feature of this species is a key virulence factor of its transition (Jabobsen *et al.* 2012, Hall and Noverr 2017). Conversion to parasitism in *C. albicans* happens under periods of trauma, dysbiosis, and immune suppression, *C. albicans* morphogenesis induced by abiotic and biotic cues, results in activation and induction of a proinflammatory response (Moyes *et al.* 2010). PHR1 and PHR2 proteins play a crucial role in the cell wall assembly, being differently regulated by extracellular pH. PHR1 is expressed when ambient pH is 5.5 or higher. It was suggested that PHR1 is not required for the induction of hyphal development but plays a key role in the maintenance of hyphal growth (Younes *et al.* 2011). Hwp2 is a cell wall GPI-anchored cell wall protein, shown to be is necessary for proper oxidative stress tolerance, adhesion, and biofilm formation (Younes *et al.* 2011). Both PHR1 and Hwp2 presented GO functions of symbiont to host (UniProtKB), still, it was not produced any literature about these proteins in endodontic or periodontitis topics. Also, QDR2, ALS9, PAG28, and UMEG already had their adhesion or biofilm development capability analyzed before (Fox *et al.* 2013, Garcia-Sherman *et al.* 2014, Shah *et al.* 2014, Huang *et al.* 2019), but none of these investigations were on root canal or periapical lesion environments.

Also representing the adhesion category were found *Acinetobacter baumannii* (vital pulp) and *Escherichia coli* (infections), which are considered usual components of endodontic infections (Britto *et al.* 2007), and even more, the *Acinetobacter* genera was considered to be a part of the dominant community of pristine and health dental pulp tissue (Widmer *et al.* 2018). The adhesin Ata autotransporter is considered a virulence determinant of *A. baumannii*, which may promote biofilm formation and bound to many extracellular matrix/basal membrane members, such as collagen types I, II, III, IV and V and laminin (Bentacor *et al.* 2012). *Escherichia coli* can compose commensal and pathogenic strains, the Yeej gene was proved to be a member of ~40% to 96% completely sequenced *E. coli* genomes, importantly the same study demonstrated that YeeJ may promote biofilm formation in different settings through exposition at the cell surface (Martinez-Gil *et al.* 2017).

Various other identified proteins were probably involved in the pathogenic process, although only a few had their functions reviewed on the GO database (Swiss Prot). No prevalent Swiss Prot accession code protein was linked to proteolysis, in this study. However, in the literature, it was demonstrated that some oral microbial proteases and peptidases as in *Porphyromonas gingivalis*, can degrade host proteins (*e.g.* extracellular

matrix and hemoglobin) to increase the accessibility of nutrients, and apart from that, extracellular *P. gingivalis* peptidases can also release small peptides and free amino acids that benefits other oral bacteria within the microbial consortium (Dashper *et al.* 2004, Ruggiero *et al.* 2013). Another study showed that as well, where an association of enzymatic activities from different microbial species increased the offer of nutrients and proliferation of the microbiome (Bradshaw *et al.* 1994). Contrastingly, in TrEMBL UniProt database several proteins had proteolysis functions, and three of them were present in all sampled situations: G5 domain-containing protein, IgA-specific serine endopeptidase, and Toxin CdiA.

Besides, vital pulp contained two additional reviewed pathogenic proteins that were present equally in primary and secondary/persistent infections. Both, 16S rRNA endonuclease CdiA - *Enterobacter cloacae subsp. Cloacae* and Hemolysin (Shla) - *Serratia marcescens*, own toxic activities but targeted at different cells. CdiA toxin is involved in the growth inhibition of closely related neighboring target bacteria (Beck *et al.* 2014). Whilst Shla can form pores in erythrocytes, fibroblasts, and epithelial cells with possible hemolytic and cytotoxic activity (Hertle, 2000,2005).

Finally, among the infection groups, pathogenic *Candida albicans* proteins Zinc cluster transcription factor CZF1, Histidine protein kinase SLN1, and Serine/threonine-protein kinase CST20 were found. These proteins are required for hyphal formation and virulence through the penetration of fungal cells into host tissue (Leberer *et al.* 1996, Brown *et al.* 1999, Yamada-Obake *et al.* 1999). Furthermore, were encountered Translocated intimin receptor Tir from *E. coli*, a protein that is required for efficient pedestal formation in host cells during infection (de Groot *et al.* 2011), and Flagellin B from *Helicobacter mustelae*, important for motility and virulence (Josenhans *et al.* 1995). Still, none of these two were studied in oral environments.

Final considerations

In the present study a series of human proteins identified as potential causes of inflammatory processes and osteoclastogenesis were found, among them can be cited Notch2, NF-κβ, Receptor IL-1, CKLiK, MCFS-1, RANK, RANKL and EphA2.

Regarding microbial proteins of interest, were found members of pathogenic functions as adhesion from *Candida albicans* (PHR1, Hwp2, QDR2, ALS9, PAG28, UMEG), *Acinetobacter baumannii* (Adhesin Ata autotransporter) and *Escherichia coli* (HAP2 and YeeJ). Proteolytic agents have also been encountered in *Gemella haemolysans* (G5 domain-containing protein), *Haemophilus influenzae* (IgA-specific serine endopeptidase), and

Klebsiella aerogenes (Toxin CdiA). Furthermore, *Enterobacter cloacae subsp. Cloacae* (16S rRNA endonuclease CdiA) and *Serratia marcescens* (Shla) showed toxicity functions.

The identification of endodontic disease biomarkers may open borders for further qualitative and quantitative studies, based on the proteomic profile revealed by the present and previous analyzes (Nandakumar et al., 2009; Provenzano et al., 2013, 2016; Francisco et al., 2019; Loureiro et a. 2020). Whereas may also allow the improvement of infection treatments by acting on predetermined targets.

CONCLUSION

In conclusion, human and microbial proteins were identified in vital pulp, primary and secondary/persistent endodontic infection. Primary infection contained a greater number of proteins than the other endodontic conditions. Important inflammation and bone resorption pathways members were encountered, whilst many detected microbial proteins were related to symbiont adhesion to host tissues and pathogenic activities, such as: biofilm formation, toxin release, proteolysis, and cell invasion.

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TABLES

Table 1. Human proteins linked to inflammatory response of each different situation studied, according to GO UniProtKB.

(continue)

Accession number	Protein names	VP	PI	SI
Q8N4X5	Actin filament-associated protein 1-like 2		x	x
Q08828	Adenylate cyclase type 1		x	x
P40145	Adenylate cyclase type 8	x	x	x
P04083	Annexin A1 (Annexin I)	x	x	
Q4KMQ2	Anoctamin-6		x	
O00203	AP-3 complex subunit beta-1		x	
P02652	Apolipoprotein A-II (Apo-AII)		x	
Q08211	ATP-dependent RNA helicase A			x
O75882	Attractin (DPPT-L)		x	
Q8WV28	B-cell linker protein			x
P41182	B-cell lymphoma 6 protein (BCL-6)		x	
P22004	Bone morphogenetic protein 6 (BMP-6)		x	
Q8IU85	Calcium/calmodulin-dependent protein kinase type 1D		x	
O15444	C-C motif chemokine 25	x		
Q8NHW4	C-C motif chemokine 4-like	x		
P16070	CD44 antigen (CDw44)	x	x	
O43866	CD5 antigen-like		x	
P46094	Chemokine XC receptor 1		x	
Q99788	Chemokine-like receptor 1			x
P00451	Coagulation factor VIII	x	x	x
P01024	Complement C3	x		
P0C0L4	Complement C4-A	x	x	
P0C0L5	Complement C4-B	x	x	x
P01031	Complement C5	x	x	x
Q9BXN2	C-type lectin domain family 7 member A	x		
Q9HBI6	Cytochrome P450 4F11			x
Q5VWQ8	Disabled homolog 2-interacting protein	x	x	
P78325	Disintegrin and metalloproteinase domain-containing protein 8	x	x	
Q96J02	E3 ubiquitin-protein ligase Itchy homolog	x		
Q5D1E8	Endoribonuclease ZC3H12A		x	

Table 1. Human proteins linked to inflammatory response of each different situation studied, according to GO UniProtKB.

(continuation)

P29317	Ephrin type-A receptor 2	x	x	x
P00533	Epidermal growth factor receptor		x	x
P02751	Fibronectin	x	x	x
P21462	fMet-Leu-Phe receptor (fMLP receptor)		x	x
Q16666	Gamma-interferon-inducible protein 16 (Ifi-16)	x		x
P36269	Glutathione hydrolase 5 proenzyme		x	
P19367	Hexokinase-1	x		
Q01362	High affinity immunoglobulin epsilon receptor subunit beta	x		
Q9H3N8	Histamine H4 receptor (H4R)		x	
Q9UKV0	Histone deacetylase 9 (HD9)			x
A1A4Y4	Immunity-related GTPase family M protein			x
O15111	Inhibitor of nuclear factor kappa-B kinase subunit alpha	x		
P20701	Integrin alpha-L (CD11 antigen-like family member A)	x	x	
P05556	Integrin beta-1		x	
P05107	Integrin beta-2	x		
P18564	Integrin beta-6		x	x
Q14624	Inter-alpha-trypsin inhibitor heavy chain H4		x	
P05362	Intercellular adhesion molecule 1 (ICAM-1)	x	x	x
P15260	Interferon gamma receptor 1 (IFN-gamma receptor 1)		x	x
Q9NPH3	Interleukin-1 receptor accessory protein (IL-1RAcP)		x	
P14778	Interleukin-1 receptor type 1 (IL-1R-1)		x	
Q8NFR9	Interleukin-17 receptor E (IL-17RE)		x	
Q16552	Interleukin-17A (IL-17A)	x		x
Q13478	Interleukin-18 receptor 1 (IL-18R-1)		x	x
P24394	Interleukin-4 receptor subunit alpha (IL-4R-alpha)			x
P08887	Interleukin-6 receptor subunit alpha (IL-6R-alpha)		x	
P08779	Keratin, type I cytoskeletal 16	x		
O60449	Lymphocyte antigen 75 (Ly-75)		x	
O15054	Lysine-specific demethylase 6B		x	
O15054	Lysine-specific demethylase 6B			x
P09603	Macrophage colony-stimulating factor 1			x
P34810	Macrosialin (Gp110)	x	x	x
Q16820	Meprin A subunit beta			x

Table 1. Human proteins linked to inflammatory response of each different situation studied, according to GO UniProtKB.

(continuation)

P33076	MHC class II transactivator (CIITA)	x	x	
P10636	Microtubule-associated protein tau		x	
Q32MK0	Myosin light chain kinase 3			x
Q9C000	NACHT, LRR and PYD domains-containing protein 1		x	x
Q96P20	NACHT, LRR and PYD domains-containing protein 3	x		x
Q96MN2	NACHT, LRR and PYD domains-containing protein 4			x
P59044	NACHT, LRR and PYD domains-containing protein 6	x	x	
Q7RTR0	NACHT, LRR and PYD domains-containing protein 9	x	x	x
P49279	Natural resistance-associated macrophage protein 1 (NRAMP 1)		x	
Q04721	Neurogenic locus notch homolog protein 2 (Notch 2)	x		x
Q8NET5	NFAT activation molecule 1		x	
P35228	Nitric oxide synthase, inducible		x	
Q9NPP4	NLR family CARD domain-containing protein 4		x	
Q00653	Nuclear factor NF-kappa-B p100 subunit		x	
Q00653	Nuclear factor NF-kappa-B p100 subunit	x		
P19838	Nuclear factor NF-kappa-B p105 subunit	x	x	x
Q12968	Nuclear factor of activated T-cells, cytoplasmic 3		x	
Q14934	Nuclear factor of activated T-cells, cytoplasmic 4		x	
Q6P4R8	Nuclear factor related to kappa-B-binding protein	x	x	x
Q96EE3	Nucleoporin SEH1			x
Q9Y239	Nucleotide-binding oligomerization domain-containing protein 1			x
O95497	Pantetheinase			x
P48736	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit gamma		x	
P28799	Progranulin (PGRN) (Acrogranin)	x		
O43586	Proline-serine-threonine phosphatase-interacting protein 1	x		
Q07954	Prolow-density lipoprotein receptor-related protein 1	x	x	x
P43119	Prostacyclin receptor (Prostaglandin I2 receptor)	x		
P34995	Prostaglandin E2 receptor EP1 subtype	x	x	x
P35408	Prostaglandin E2 receptor EP4 subtype	x	x	x
P35354	Prostaglandin G/H synthase 2	x		
Q9UKK3	Protein mono-ADP-ribosyltransferase PARP4	x	x	x
Q70J99	Protein unc-13 homolog D	x	x	x

Table 1. Human proteins linked to inflammatory response of each different situation studied, according to GO UniProtKB.

(continuation)

P55085	Proteinase-activated receptor 2 (PAR-2)			x
P16109	P-selectin (CD62 antigen-like family member P)			x
Q8IV20	Purine nucleoside phosphorylase LACC1	x		
O95267	RAS guanyl-releasing protein 1	x	x	
Q06330	Recombining binding protein suppressor of hairless (CBF-1)			x
Q9NQC3	Reticulon-4 (Foocen) (Neurite outgrowth inhibitor)			x
O75676	Ribosomal protein S6 kinase alpha-4 (S6K-alpha-4)		x	
O75582	Ribosomal protein S6 kinase alpha-5 (S6K-alpha-5)		x	
Q15139	Serine/threonine-protein kinase D1	x		
Q9BZZ2	Sialoadhesin (Sialic acid-binding Ig-like lectin 1)	x	x	x
Q8IWY4	Signal peptide, CUB and EGF-like domain-containing protein 1	x	x	
Q99835	Smoothened homolog (SMO) (Protein Gx)	x		
Q15858	Sodium channel protein type 9 subunit alpha	x	x	
Q9NY15	Stabilin-1		x	
P30203	T-cell differentiation antigen CD6			x
P40200	T-cell surface protein tactile			x
Q9NSU2	Three-prime repair exonuclease 1		x	x
P07996	Thrombospondin-1 (Glycoprotein G)		x	
Q86XR7	TIR domain-containing adapter molecule 2 (TICAM-2)			x
Q7L0X0	TLR4 interactor with leucine rich repeats		x	
Q15025	TNFAIP3-interacting protein 1		x	
Q15399	Toll-like receptor 1	x	x	
O60603	Toll-like receptor 2	x		x
O15455	Toll-like receptor 3	x		x
Q9NYK1	Toll-like receptor 7		x	
Q9NR97	Toll-like receptor 8			x
P23771	Trans-acting T-cell-specific transcription factor GATA-3		x	
Q01201	Transcription factor RelB (I-Rel)	x		
Q12986	Transcriptional repressor NF-X1	x	x	x
Q9UP52	Transferrin receptor protein 2 (TfR2)	x	x	x
P21580	Tumor necrosis factor alpha-induced protein 3			x
P20333	Tumor necrosis factor receptor superfamily member 1B			x
P98066	Tumor necrosis factor-inducible gene 6 protein (TSG-6)		x	

Table 1. Human proteins linked to inflammatory response of each different situation studied, according to GO UniProtKB.

(conclusion)

P30530	Tyrosine-protein kinase receptor UFO			x
Q13488	V-type proton ATPase 116 kDa subunit a3			x
Q9BRX9	WD repeat domain-containing protein 83		x	

Legend: VP: Vital pulp, PI: Primary infection, SI: Secondary/persistent infection.

Table 2. Human proteins linked to osteoclastogenesis found in each different situation studied, according to GO UniProtKB.

Proteins name	VP	PI	SI
Catenin beta-1		x	
Ephrin type-A receptor 2	x	x	x
Forkhead box protein P1		x	
Lactoylglutathione lyase	x		
Leucine-rich repeat serine/threonine-protein kinase 1		x	
Macrophage colony-stimulating factor 1 (CSF-1)			x
Platelet-activating factor acetylhydrolase IB subunit alpha	x		
Protein strawberry notch homolog 2		x	
Proto-oncogene tyrosine-protein kinase Src (p60-Src)		x	x
Serotransferrin (Transferrin) (Beta-1 metal-binding globulin)	x		
SH3 and PX domain-containing protein 2A	x	x	x
Sorting nexin-10	x		
Tumor necrosis factor ligand superfamily member 11		x	
Tumor necrosis factor receptor superfamily member 11A			x
V-type proton ATPase 116 kDa subunit a3		x	

Legend: VP: Vital pulp, PI: Primary infection, SI: Secondary/persistent infection.

Table 3. Microbial proteins from each different situation studied linked to adhesion according to GO UniProtKB. Proteins in bold are manually annotated (Swiss Prot – UniProt).

Proteins name	Organism	VP	PI	SI
Adhesin Ata autotransporter	***Acinetobacter baumannii***	x		x
LPXTG-motif cell wall anchor domain protein	*Atopobium rimae*		x	
Candida_ALS_N domain-containing protein	*Candida albicans*	x	x	x
Hyphal wall protein 2 (GPI-anchored protein 8)	***Candida albicans***	x	x	x
pH-responsive protein 1 (pH-regulated protein 1)	***Candida albicans***	x	x	x
Probable GPI-anchored adhesin-like protein PGA28	***Candida albicans***	x	x	
Agglutinin-like protein	*Candida albicans*		x	
Agglutinin-like protein 9 (Adhesin 9)	***Candida albicans***	x		
ALS7 protein (Fragment)	*Candida albicans*		x	
MFS antiporter QDR2	***Candida albicans***	x		
Transcriptional regulatory protein UME6	***Candida albicans***		x	
Agglutinin-like protein, putative	*Candida dubliniensis*	x	x	x
Flagellar hook-associated protein 2 (HAP2)	***Escherichia coli***		x	
Uncharacterized protein YeeJ	***Escherichia coli***		x	x
Uncharacterized protein (Fragment)	*Gemella haemolysans*		x	
Manganese ABC transporter substrate-binding lipoprotein	*Granulicatella elegans*	x	x	
Putative molybdenum cofactor biosynthesis protein	*Hafnia alvei*			x
Flagellar hook-associated protein 2 (HAP2)	*Klebsiella aerogenes*		x	
Pilin isopeptide linkage domain protein (Fragment)	*Streptococcus anginosus*			x
Putative collagen adhesin	*Streptococcus milleri*		x	
Pilin isopeptide linkage domain protein	*Streptococcus mitis*		x	x
Cell wall surface anchor family protein	*Streptococcus sanguinis*		x	
Collagen-binding protein A	*Streptococcus sanguinis*		x	
LPXTG cell wall surface protein	*Streptococcus sanguinis*	x		

Legend: VP: Vital pulp, PI: Primary infection, SI: Secondary/persistent infection.

Table 4. Microbial proteins from each different situation studied linked to proteolysis according to GO UniProtKB. Proteins in bold are manually annotated (Swiss Prot – UniProt).

(continue)

Proteins name	**Organism**	**VP**	**PI**	**SI**
Signal peptide peptidase SppA, 67K type	*Alloprevotella tannerae*			x
tRNA N6-adenosine threonyl carbamoyl transferase	*Atopobium rimae*		x	
ABC transporter, ATP-binding protein	*Bacteroides fragilis*			x
Tricorn protease homolog	*Bacteroides fragilis*		x	
CULLIN_2 domain-containing protein	*Candida dubliniensis*			x
Mitochondrial inner membrane protease	*Candida dubliniensis*		x	
Ubiquitinyl hydrolase 1	*Candida dubliniensis*	x		
Putative phage prohead protease, HK97 family	*Dialister invisus*	x		
Isoaspartyl dipeptidase	*Enterobacter hormaechei*		x	
Putative D-alanyl-D-alanine carboxypeptidase/D-alanyl-D-alanine-endopeptidase	*Gardnerella vaginalis*		x	
Subtilisin-like serine protease	*Gardnerella vaginalis*		x	
Uncharacterized protein	*Gardnerella vaginalis*		x	
Subtilisin-like serine protease	*Gardnerella vaginalis*	x		
Uncharacterized protein	*Gardnerella vaginalis*			x
G5 domain-containing protein	*Gemella haemolysans*	x	x	x
IgA-specific serine endopeptidase	*Haemophilus influenzae*	x	x	x
Peptidase_S9 domain-containing protein	*Hafnia alvei*	x		x
Toxin CdiA	*Klebsiella aerogenes*	x	x	x
Cysteine protease domain, YopT-type	*Klebsiella pneumoniae*		x	
Cell wall-binding repeat protein	*Lachnoanaerobaculum saburreum*	x	x	
TldD/PmbA family protein	*Neisseria elongata*		x	
Serine-type D-Ala-D-Ala carboxypeptidase	*Parvimonas micra*		x	
Papain family cysteine protease	*Peptostreptococcus anaerobius*	x		
Interpain A	*Prevotella intermedia*		x	
Peptidase, M48 family	*Prevotella melaninogenica*	x		
D-alanyl-D-alanine carboxypeptidase/D-alanyl-D-alanine-endopeptidase-like protein	*Propionibacterium namnetense*			x
Signal peptidase I	*Rothia mucilaginosa*		x	
Uncharacterized protein	*Schaalia odontolytica*		x	

Table 4. Microbial proteins from each different situation studied linked to proteolysis according to GO UniProtKB. Proteins in bold are manually annotated (Swiss Prot – UniProt).

(conclusion)

Proteins name	**Organism**	**VP**	**PI**	**SI**
Endopeptidase La	*Streptococcus anginosus*			x
C5a peptidase	*Streptococcus gordonii*		x	x
Zinc metalloproteinase B	*Streptococcus gordonii*		x	
Zinc metalloproteinase C	*Streptococcus gordonii*			x
Gram-positive signal peptide protein, YSIRK family	*Streptococcus mitis*			x
IgA-specific serine endopeptidase	*Streptococcus mitis*		x	
Immunoglobulin A1 protease	*Streptococcus mitis*			x
M26 IgA1-specific Metallo-endopeptidase C-terminal domain protein	*Streptococcus mitis*	x		x
Immunoglobulin A1 protease	*Streptococcus pneumoniae*	x		
IgA-specific serine endopeptidase	*Streptococcus sanguinis*			x

Legend: VP: Vital pulp, PI: Primary infection, SI: Secondary/persistent infection.

Table 5. Microbial proteins from each different situation studied linked to pathogenesis according to GO UniProtKB. Proteins in bold are manually annotated (Swiss Prot – UniProt).

(continue)

Proteins name	**Organism**	**VP**	**PI**	**SI**
Hep/Hag repeat protein	*Acinetobacter baumannii*	x	x	x
Extracellular matrix protein adhesin A	*Aggregatibacter actinomycetemcomitans*		x	x
Histidine protein kinase SLN1	***Candida albicans***		x	x
Serine/threonine-protein kinase CST20	***Candida albicans***			x
Zinc cluster transcription factor CZF1	***Candida albicans***		x	
Probable hemagglutinin	*Chromobacterium violaceum*		x	x
SLH domain-containing protein	*Dialister hominis*			x
Uncharacterized protein	*Dialister hominis*	x		
SLH domain-containing protein	*Dialister invisus*	x	x	
Hep_Hag superfamily protein	*Dialister micraerophilus*		x	
Uncharacterized protein	*Dialister micraerophilus*		x	
Hep/Hag repeat protein	*Dialister microaerophilus*	x	x	x
S-layer protein	*Dialister sp.*	x	x	x
SLH domain-containing protein	*Dialister sp.*		x	
Uncharacterized protein	*Dialister sp.*		x	
SLH domain-containing protein	*Dialister succinatiphilus*	x		
Uncharacterized protein	*Dialister succinatiphilus*		x	x
Hep/Hag repeat protein	*Eikenella corrodens*	x		
16S rRNA endonuclease CdiA	***Enterobacter cloacae subsp. cloacae***	x	x	x
YadA domain-containing protein	*Enterobacter lignolyticus*	x		
Translocated intimin receptor Tir	***Escherichia coli***		x	
Hemagglutinin (Fragment)	*Fusobacterium periodonticum*	x		x
Chromosome segregation ATPase	*Gardnerella vaginalis*		x	
M protein repeat protein	*Gardnerella vaginalis*		x	
M protein repeat protein	*Gardnerella vaginalis*	x		
Putative ATP synthase F1, delta subunit	*Gemella haemolysans*		x	x
Adhesin	*Haemophilus influenzae*	x		x

Table 5. Microbial proteins from each different situation studied linked to pathogenesis according to GO UniProtKB. Proteins in bold are manually annotated (Swiss Prot – UniProt).

(conclusion)

Proteins name	**Organism**	**VP**	**PI**	**SI**
Hsf-like protein	*Haemophilus influenzae*	x		
Hep/Hag repeat protein	*Haemophilus parainfluenzae*	x	x	
Uncharacterized protein	*Haemophilus parainfluenzae*			x
Uncharacterized protein	*Haemophilus somnus*	x	x	x
Flagellin B (Flagellin N)	***Helicobacter mustelae***			x
VacA (Fragment)	*Helicobacter pylori*		x	
Klebicin D activity protein	*Klebsiella oxytoca*	x		
Putative adhesin	*Neisseria lactamica*	x		
Putative adhesin	*Neisseria lactamica*		x	x
YadA-like domain protein	*Neisseria lactamica*		x	
Hep/Hag repeat protein	*Neisseria mucosa*		x	
Type I secretion target GGX repeat	*Neisseria mucosa*		x	
Hep/Hag repeat protein	*Neisseria polysaccharea*		x	x
Hep/Hag repeat protein	*Neisseria sicca*	x	x	x
Hep/Hag repeat protein (Fragment)	*Neisseria subflava*	x		
Hemolysin	***Proteus mirabilis***			x
Hemolysin	***Serratia marcescens***	x	x	
Zinc metalloproteinase C	*Streptococcus gordonii*			x
Hemagluttinin domain protein	*Veillonella parvula*			x
Hep/Hag repeat protein	*Veillonella parvula*			x
YadA domain protein	*Veillonella parvula*			x
Hemagluttinin domain protein	*Veillonella parvula*	x	x	
Hep/Hag repeat protein	*Veillonella parvula*	x	x	
SLH domain-containing protein	*Veillonella parvula*	x	x	
YadA domain protein	*Veillonella parvula*	x	x	

Legend: VP: Vital pulp, PI: Primary infection, SI: Secondary/persistent infection.

2.2 Artigo: Investigation of the antimicrobial susceptibility of *Enterococcus faecalis* and *Streptococcus mutans* in cases of endodontic failure

Artigo submetido para publicação no periódico *International Endodontic Journal.*

ABSTRACT

Introduction: Antibiotic prophylaxis and life-threatening systemic infections are strict cases in which ancillary systemic antibiotic therapy may be necessary in endodontics. Therefore, antimicrobial susceptibility monitoring is important for an accurate drug prescription. This study aimed to assess the antimicrobial susceptibility of *E. faecalis* and *S. mutans* obtained from root canals affected by secondary/persistent infection. **Methodology:** *E. faecalis* and *S. mutans* strains were recovered during 20 cases of endodontic retreatments by means of culture technique and confirmed by 16S rRNA gene sequencing. These isolates had their antimicrobial susceptibility tested through the E-test system, in which diverse antimicrobial agents were tested. Minimum inhibitory concentration (MIC) breakpoints readings were made at the point of intersection between the elliptical inhibition halo and the E-test tape. Susceptibility or resistance was determined following interpretation guides. The MICs of antibiotics inhibiting 50% and 90% of the strains were expressed as MIC_{50} and MIC_{90}. **Results:** Thirty-seven strains of *E. faecalis* and seven of *S. mutans* were obtained. All *E. faecalis* isolates were 100% susceptible to amoxicillin, amoxicillin + clavulanate, moxifloxacin, and vancomycin. Higher rates (75 to 84%) of antimicrobial effectiveness have also been found for ciprofloxacin, chloramphenicol, and doxycycline. *E. faecalis* strains showed intermediate rates of susceptibility to azithromycin (45.9%) and erythromycin (56.8%) as well as resistance to doxycycline, rifampicin and tetracycline (respectively, 21.6 %, 24.3% and 24.3%). Three antimicrobial agents were totally effective against *S. mutans*, amoxicillin, amoxicillin + clavulanate, and tetracycline. Besides, six strains were found to be susceptible to benzylpenicillin, clindamycin, erythromycin, and vancomycin. Azithromycin and chloramphenicol showed less favourable results, with susceptibility rates of 28.6% and 14.3%, respectively. Amoxicillin, amoxicillin + clavulanate acid, and moxifloxacin had the lowest MIC_{90} for both strains. **Conclusions:** *E. faecalis* strains were susceptible to amoxicillin, amoxicillin + clavulanate, moxifloxacin, and vancomycin. Whereas all *S. mutans* isolates were susceptible to amoxicillin, amoxicillin + clavulanate, and tetracycline. Furthermore, MIC_{90} showed that 1mg/ml of amoxicillin or amoxicillin + clavulanate acid, 0.75mg/ml of moxifloxacin, and 3mg/ml of vancomycin would be enough to inhibit at least

90% of both strains.

Keywords: endodontics, microbiota, e-test, antimicrobial susceptibility, endodontic failure

INTRODUCTION

Systemic antibiotic therapy is not commonly used to treat chronic periapical lesions associated with endodontic failure. However, in life-threatening systemic infections and risk of bacterial endocarditis, this approach may represent a timely complement to conventional endodontic treatment (Leblanc 1990). Nevertheless, antibiotics should be used with restriction as they can cause, among other deleterious effects, the development of bacterial resistance to antimicrobial agents (Skucaite *et al.* 2010, Gomes *et al.* 2011, Gyssens 2018).

The microbiota composition associated with secondary/persistent infection is different than that associated with primary infection in terms of number and diversity of species. Gram-positive facultative anaerobic bacteria are prevalent in this environment (Molander *et al.* 1998, Pinheiro *et al.* 2003a b), with *Enterococcus* spp. (Hancock *et al.* 2001, Gomes *et al.* 2006, Ozbek *et al.* 2009, Wang *et al.* 2012) and *Streptococcus* spp. (Rôças *et al.* 2008, Antunes *et al.* 2015, Lima *et al.* 2020a) being usually detected. These species are expected to have a greater ability to survive after chemical-mechanical preparation, intracanal medication, and root canal filling, that is, harsh situations and a restricted nutritional environment where interrelationships are minimal (Sundqvist *et al.* 1998).

Enterococcus faecalis contains a series of virulence and resistance mechanisms, making difficult its eradication from the root canals. These mechanisms result from physiological and structural changes in bacterial cells, serving as strategies for the survival of different antibiotics classes (Endo *et al.* 2014). The relevance of the study of this bacterium´s antimicrobial resistance lies in the fact that *E. faecalis* is considered a critical prompter of infectious endocarditis, one of the four most common life-threatening infectious syndromes (Baddour *et al.* 2015). *E. faecalis* acquired resistance over time to antimicrobials such as clindamycin, erythromycin, tetracycline, chloramphenicol, and vancomycin (Pinheiro *et al.* 2003b, Endo *et al.* 2014, Barbosa-Ribeiro *et al.* 2016). Although, a more recent study found that its susceptibility to amoxicillin + clavulanate, amoxicillin, and benzylpenicillin remains (Barbosa-Ribeiro *et al.* 2016).

Streptococcus mutans belongs to the viridans streptococcus group. Besides considered a common inhabitant of the oral microbiota, including the root canals associated

with secondary or persistent infections, *S. mutans* can represent another infectious endocarditis pathogen (Süzük *et al.* 2016). Structural components of viridans streptococci are essential mediators of virulence and adhesion of these bacteria, and these have been detected with relevant frequency in cardiac tissues (Abranches *et al.* 2009, Oliveira *et al.* 2019). There are a few studies in the literature monitoring the antimicrobial susceptibility of *S. mutans* isolated from cases of endodontic failure (Skucaite *et al.* 2010, Al-Ahmad *et al.* 2014, Łysakowska *et al.* 2016). Nevertheless, its study is vital given the indication, when appropriate, of effective antibiotic prophylaxis and drug treatment in addition to the conventional endodontic treatment.

The knowledge of bacterial susceptibility allows an accurate choice of effective antibiotic therapies in the face of an already installed systemic infection or need for antibiotic prophylaxis in in cases of endodontic failure. Besides, it enables monitoring the increased resistance of important endodontic pathogens to different concentrations and types of antibiotics. Thus, the present study aims to assess the resistance of *E. faecalis* and *S. mutans* obtained from root canals affected by secondary/persistent infection to antimicrobial agents.

MATERIAL AND METHODS

Patient Selection

Cases of teeth needing endodontic retreatment and with presence of periapical radiolucent lesion were selected from the records of the Piracicaba Dental School, Department of Endodontics. All patients signed an informed consent form prepared under the rules of the Research Ethics Committee of the Piracicaba Dental School - UNICAMP (CEP/FOP-UNICAMP No 119/2015). The registry of cases included in this study can be found in the Brazilian Clinical Trials Registry (ReBEC; UTN U1111-1238-5402).

After clinical evaluation and radiographic evaluation, a total of 20 teeth were included in this study.

Exclusion criteria were: endodontic treatment less than two years, use of antifungals and antibiotics (<3 months), presence of periodontal disease, teeth with root fracture, teeth allowing neither absolute isolation nor the introduction of paper points along the working length, teeth with pulp chamber in contact with the oral environment, and teeth associated with spontaneous pain.

Endodontic procedures and sample collection

The method followed for root canal collection was previously described in detail

by Pinheiro *et al.* (2004), Gomes *et al.* (2011), and Barbosa-Ribeiro *et al.* (2016). The teeth were isolated with a rubber dam and the desinfection protocol of the operatory field, crown and surrounding structures was performed with 30% H_2O_2 (v/v) for 30 seconds, followed by 2.5% NaOCl (30 seconds) and subsequent neutralisation with 5% sodium thiosulfate. A swab sample from both internal and external surfaces of the crown and surrounding structures was taken to confirm the disinfection protocol.

The swab sample was streaked onto a plate containing 5% defibrinated sheep blood and fastidious anaerobe agar (FAA – LAB M, Heywood, Lancashire, UK) before being incubated anaerobically and aerobically, respectively, for up to 14 days. Next, DNA extraction from the swab and PCR analysis using universal bacterial primers were performed. If any positive cultures or the presence of DNA amplification products were obtained, then the patient was excluded from the study.

Endodontic retreatment was performed under high magnification using a dental operating microscope (DF Vasconcellos S/A, São Paulo, SP, Brazil). All procedures were performed under strict aseptic conditions.

A two-stage access preparation was performed under anaesthesia (2% lidocaine with 1:100,000 epinephrine). The access cavity was made without water spray but under manual irrigation with sterile saline and by using sterile high-speed diamond bur. This first stage aimed to promote a significant removal of contaminants. In the second stage, before entering the pulp chamber, the access cavity was disinfected according to the decontamination protocol described above. Disinfection of the access cavity's internal surface was checked as previously described, and all procedures were performed aseptically. Root-filling materials were removed using Reciproc R25 files (VDW, Munich, Germany) along the working length (which had been determined on pre-operative radiography) in a crown-down technique with no chemical solvent.

Before collection of the root canal samples, a K-file #20 (Dentsply Maillefer, Ballaigues, Switzerland) was used to confirm the working length (previously estimated by radiographs) together with an apex locator (Novapex; Forum Technologies, Rishon le-Zion, Israel). Three sterile paper points (Dentsply-Maillefer, Ballaigues, Switzerland), one at a time, were then introduced in the entire length of the root canal and retained in position for 60 seconds for sampling. After, the cones were transferred to previously sterilised Eppendorf tubes, containing 1.0 mL of the pre-reduced transport medium VMGA III. Sterile saline solution was inserted into the root canal before sampling to assist the collection in case of a dry canal. The samples were plated within two hours of collection.

Culture technique

The vials containing VMGA were stirred inside the laminar flow cabinet for 1 minute to facilitate the microorganisms' dispersion. Fifty μL of each sample were inoculated into plates containing:

• Difco ™ m-Enterococcus Agar (BD, Franklin Lakes, NJ, USA), incubated in 10% CO^2 at 37 ° C, for up to 14 days, to allow detection of *Enterococcus faecalis*.

• Difco ™ Mitis Salivarius Agar (BD, Franklin Lakes, NJ, USA), plus 1% potassium tellurite (Difco Laboratories, Detroit, MC, USA) and 0.2U bacitracin (Sigma Chemical, St Louis, MO, US) incubated in 10% CO^2 at 37 °C for up to 14 days to allow detection of *Streptococcus mutans*.

Spaced strains were selected and collected from each plate, with a maximum of eight strains collected per plate/patient.

16S rRNA gene sequencing for isolates confirmation

Microorganisms presumably identified as *E. faecalis* or *S. mutans* had their DNA extracted and purified by using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Subsequently to the amplification of the 16S rRNA gene (forward primer: 5'GAG AGT TTG ATY MTG GCT CAG -3'; reverse primer: 5' - GAA GGA GGT GWT CCA RCC GCA – 3') (Invitrogen, São Paulo, SP, Brazil), the purified product (QIAquick® Gel Extraction, Qiagen) at a concentration of 40ng/uL was subjected to sequencing with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). CLC Genomics Workbench software v6.5 (Qiagen) was first used to analyse the sequences obtained for the quality and similarity of nucleotides. Those considered of good quality were aligned by using the 6 Mega software (Molecular Evolutionary Genetics Analysis) and later confronted with the sequences deposited in GenBank by using Basic Local Alignment Tool program for Nucleotide (BLASTn). A confidence level of 99-100% similarity to *E. faecalis* and *S. mutans* was established to reach the sequences' maximum quality level.

Antimicrobial susceptibility test (E-test)

The isolated clinical strains of *Enterococcus faecalis* and *Streptococcus mutans* had their antimicrobial susceptibility tested through the E-test system (BioMérieux SA, Marcy-l'Etoile, France). The antimicrobial agents tested were: amoxicillin (AC), amoxicillin + clavulanic acid (XL), azithromycin (AZ), benzylpenicillin (PG), ciprofloxacin (CI), clindamycin (CM), chloramphenicol (CL), doxycycline (DC), erythromycin (EM), gentamicin

(GM), metronidazole (MZ), moxifloxacin (MX), rifampicin (RI), tetracycline (TC) and vancomycin (VA).

To prepare the inoculum of previously selected strains of *E. faecalis* and *S. mutans*, after 24 hours of incubation on brain heart infusion (BHI, Oxoid, Basingstoke, UK) + 5% of defibrinated sheep blood, the bacterial colonies were transferred to BHI liquid medium and agitated to achieve a turbidity equivalent to the McFarland standard 0.5 (NEFELOBAC, PROBAC, São Paulo, SP, Brazil) that was verified with a spectrophotometer (FEMTO 432, Marconi, São Paulo, SP, Brazil) at a wavelength of 800 nm. Plates containing 4 mm thickness of BHI + 5% of defibrinated sheep blood were used for strain streaking. Sowing was made evenly on the plate's entire length by using a sterile swab moistened with the bacterial suspension. After the plates were dried (10 to 15 minutes), the E-test tapes were distributed on them with the aid of sterile forceps for each substance to be tested. The plates were immediately incubated in a 10% CO^2 oven at 37ºC and the reading was performed after 24 and 48 hours of incubation. The minimum inhibitory concentration (MIC) values were determined at the point of intersection between the elliptical inhibition halo and the E-test tape, following preferably the interpretation guide of the Clinical and Laboratory Standards Institute (CLSI 2020), complemented by the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2020) and two previous works from Fass (1993) and Mather *et al.* (2002) (Table 1). According to Fass (1993), Mather *et al.* (2002), CLSI (2020), or EUCAST (2020), isolates with a MIC less than or equal to the breakpoints were treated as susceptible, whereas those with a MIC higher than the breakpoints were treated as resistant. The MICs of antibiotics inhibiting 50% and 90% of the strains were calculated and expressed as MIC_{50} and MIC_{90}, respectively. The experiment was carried out in triplicate.

RESULTS

Among the 20 cases of endodontic failure and after 16S rRNA sequencing, 37 strains of eight cases were confirmed as *Enterococcus faecalis*, whereas seven strains of one case were found to be *Streptococcus mutans*.

Different susceptibility patterns were found for *E. faecalis.* All isolates were 100% susceptible to amoxicillin, amoxicillin + clavulanate, moxifloxacin, and vancomycin. Higher rates of antimicrobial effectiveness were also found for ciprofloxacin (75.7%), chloramphenicol (83.8%), doxycycline (76.3%), and tetracycline (75,7%). On the other hand, 45.9% and 56.8% of the *E. faecalis* strains showed intermediate susceptibility rates to azithromycin and erythromycin, whereas 21.6 %, 24.3%, and 24.3% of the *E. faecalis*

expressed resistance to doxycycline, rifampicin, and tetracycline, respectively. From these resistant isolates, eight were mutually resistant to doxycycline and tetracycline. The MIC range of each antibiotic, MIC_{50} and $MIC_{90,}$ are listed in Table 2.

Three antimicrobial agents were 100% effective against *S. mutans*, namely, amoxicillin, amoxicillin + clavulanate, and tetracycline. Besides, six strains were susceptible to benzylpenicillin, clindamycin, erythromycin, and vancomycin. Azithromycin and chloramphenicol showed less favourable results, with a susceptibility rate of 28.6% and 14.3%, respectively. One isolate was resistant to five antimicrobials (azithromycin, benzylpenicillin, clindamycin, erythromycin, and vancomycin). MIC range, $MIC_{50,}$ and MIC_{90} for *S. mutans* strains are shown in Table 3.

DISCUSSION

Antibiotics in endodontics

Supplementary antibiotic therapy, in addition to local procedures (endodontic treatment and drainage), may be recommended and monitored carefully in strict cases to avoid the evolution of multidrug-resistant bacteria (Siqueira 2002, Cassini *et al.* 2019, Alzahrani *et al.* 2020). Relevant use of antibiotics includes the presence of systemic involvement, thus avoiding the spread of infection through interstitial and tissue spaces into other body sites (Segura-Egea *et al.* 2017, 2018), and antibiotic prophylaxis, which should be restricted to medically compromised patients at risk of health complications arising from the effects of bacteremia during odontogenic treatments (Pallasch 1994, Segura-Egea *et al.* 2017). Individuals with certain pre-existing heart defects are at risk of developing infective endocarditis when bacteremia occurs (Richey *et al.* 2008). Concerningly, some bacteria frequently found in endodontic infections, such as *Enterococcus faecalis* and *Streptococcus mutans* (Delboni *et al.* 2017, Lima *et al.* 2020a), have been shown a link to infective endocarditis. This probably happened because these two species may express collagen-binding proteins (CBPs), which were associated with the development of infective endocarditis (Singh *et al.* 2010, Otsugu *et al.* 2017). In the current research, both *E. faecalis* and *S. mutans* were detected in cases of persistent/secondary infections, thus revealing the importance of evaluating the antimicrobial susceptibility of these strains to different antibiotic agents.

Enterococcus faecalis

According to worldwide studies that evaluated antibiotics prescription in dental practice, penicillin, particularly amoxicillin (alone or with clavulanic acid), is the most common choice when antibiotic therapy is used (Koyuncuoglu *et al.* 2017, Germack *et al.* 2017). In the present study on persistence/secondary endodontic infection, all *E. faecalis* strains tested were susceptible to amoxicillin and amoxicillin with clavulanate, a finding corroborated by several other studies in the literature (Pinheiro *et al.* 2003b, 2004, Lins *et al.* 2013, Al-Ahmad *et al.* 2014, Barbosa-Ribeiro *et al.* 2016, Anderson *et al.* 2018). On the other hand, high rates (up to 72%) of *E. faecalis* resistance to penicillin had been already found in primary and secondary infections (Dahlén *et al.* 2000, Khemaleelakul *et al.* 2002, Baumgartner & Xia 2003, Dumani *et al.* 2012). Benzylpenicillin, not present in the most recent versions of CLSI and EUCAST breakpoint tables for interpretation of the MIC, was also investigated and showed a higher MIC than that of amoxicillin alone or with clavulanate acid, thus agreeing with previous antimicrobial monitoring (Pinheiro *et al.* 2003b, 2004). The question of whether penicillin should be the first choice of antibiotics in endodontic infections still remains, and more investigations are needed, especially comparing isolates from primary and persistent/ secondary infections. Besides, Macy & Ngor (2013) found that approximately 8% of the population have a history of penicillin allergy, which contraindicates this class of antimicrobials in these situations.

Azithromycin and erythromycin, two examples of macrolide antibiotics expected to be effective against a variety of aerobic and anaerobic Gram-positive and Gram-negative bacteria (Segura-Egea *et al.* 2017), revealed the same susceptibility rate of 43.2% for *E. faecalis* isolates. A further work reported a cross-resistance between these two antimicrobials (Fass 1993), which would explain this finding. Furthermore, 45.9% and 56.8% of the strains had intermediate susceptibility to azithromycin and erythromycin, respectively, and 10.9% were resistant to azithromycin. These results agree with several previous reports as even lower susceptibility was encountered for these agents (Pinheiro *et al.* 2003b, 2004, Barbosa-Ribeiro *et al.* 2016, Łysakowska *et al.* 2016, Saffari *et al.* 2018). Diversely, Skucaite *et al.* (2010) found that most of the enterococci tested by them (90%) were intermediate sensitive to erythromycin, suggesting a greater efficacy of this medicament.

In individuals allergic to penicillin and at risk for endocarditis during endodontic retreatment, it is possible to assume based on exposed data, that azithromycin or erythromycin are not good candidates for antibiotic prophylaxis. Clindamycin, the antibiotic most commonly prescribed in penicillin-allergic patients, also has the same contraindication. Its

ineffectiveness against enterococci has been proven for a long time (NCCLS 2000). Clindamycin and gentamicin, which according to CLSI are not clinically effective against *E. faecalis* (except at high concentrations) and should not be reported as sensitive, were also tested for investigative purposes and showed microbial inhibition ranges of 3-64 mg/mL and 1-12 mg/mL, respectively. Similarly, metronidazole, which is only active against anaerobes and should not be used alone to treat endodontic infections (Baumgartner & Xia 2003), showed no effect on *E. faecalis* strains.

Doxycycline is an active derivative of tetracycline, and as a result, cross-resistance may occur for the same resistant bacteria (Pinheiro *et al.* 2004). Quite similar to Pinheiro et al. (2004), almost all strains resistant to tetracycline were also resistant to doxycycline, except for a strain considered intermediately susceptible to doxycycline and resistant to tetracycline. Also, in this study, 24.3% of *E. faecalis* isolates were resistant to tetracycline. The efficacy of this antibiotic varies largely, with bacterial resistance levels ranging from 10 to 75% (Pinheiro *et al.* 2004, Sedgley *et al.* 2005, Reynaud Af Geijersstam *et al.* 2006, Skucaite *et al.* 2010, Lins *et al.* 2013, Barbosa-Ribeiro *et al.* 2016, Łysakowska *et al.* 2016, Saffari *et al.* 2018), not exactly in chronological order of publication. For instance, Skucaite *et al.* (2010) investigated *E. faecalis* resistance in primary and secondary endodontic infections together, reporting that 30% of the strains were resistant to tetracycline. Some years later, however, Barbosa-Ribeiro et al. (2016) and Saffari et al. (2018) found, 10% and 55% of resistance in strains exclusively found in endodontic failure. Overall, resistance to tetracyclines has diminished their clinical value.

A rifampicin MIC≥1 mg/ml was necessary, to eliminate 90% of *E. faecalis* strains, corroborating with Reynaud Af Geijersstam *et al.* (2006). Furthermore, 56.8% of the isolates were susceptible to rifampicin, and four *ones* were not affected by the highest concentration of the same antibiotic. Significant levels of rifampicin resistance, such as 50% (Anderson *et al.* 2018), 65% (Barbosa-Ribeiro *et al.* 2016), and 81% (Saffari *et al.* 2018), have been recently reported. In the 2020 CLSI edition, rifampicin alone is not recommended for antimicrobial therapy. In general, chloramphenicol shows valuable antimicrobial action (≥95%) against *E. faecalis* from endodontic infections (Pinheiro *et al.* 2004, Barbosa-Ribeiro *et al.* 2016, Łysakowska *et al.* 2016), but the present study found a slightly lower efficacy of 83.8%. Nevertheless, due to the concern over the association with the use of chloramphenicol and the development of aplastic anemia and other blood dyscrasias (Strom 2005), the choice of a safer drug is desired.

Among the tested *E. faecalis* isolates, vancomycin was 100% active, with a MIC_{90} of 3 mg/ml. This result was confirmed by numerous investigations where no resistance to vancomycin was observed over time (Dahlén *et al.* 2000, Pinheiro *et al.* 2004, Reynaud Af Geijersstam *et al.* 2006, Barbosa-Ribeiro *et al.* 2016, Vidana *et al.* 2016, Łysakowska *et al.* 2016, Saffari *et al.* 2018). Quite the contrary, Dumani *et al.* (2012), using an agar dilution method to evaluate antimicrobial activity, found only one enterococcal strain susceptible to vancomycin, between the 18 investigated from primary and secondary infections. Considering the serious threat posed by resistant nosocomial infectious agents, such as vancomycin-resistant enterococci, represent to the current healthcare practices (Raza *et al.* 2018), the use of vancomycin in endodontic infections should be done with extreme caution to avoid growing drug resistance despite its favourable success rate.

Finally, drugs of the fluoroquinolone group (e.g. ciprofloxacin and moxifloxacin) were analysed in relation to *E. faecalis* isolates. Ciprofloxacin was effective against 75.7% of the strains and moxifloxacin against 100%. This improved action of moxifloxacin may be explained by its expanded spectrum of activity compared to ciprofloxacin, which has a limited antimicrobial effect contra Gram-positive organisms (Fass 1993, Andersson & MacGowan 2003). Recent studies showed levels of resistance to ciprofloxacin ranging from 50% (Łysakowska et al. 2016) to 68% (Saffari et al. 2018) for *E. faecalis* in endodontic infections. Moreover, Pinheiro *et al.* (2004) suggested the use of moxifloxacin as an alternative therapy for penicillin-allergic patients. Similar to this study, they encountered that *E. faecalis* was susceptible to this antimicrobial in all cases, a finding also supported by some other authors regarding *E. faecalis* infections (Fass 1993, Mather *et al.* 2002, Pinheiro *et al.* 2004, Tasaka *et al.* 2013, Al-Ahmad *et al.* 2014, Barbosa-Ribeiro *et al.* 2016).

Streptococcus mutans

Streptococcus spp. is a well-known oral bacteria commonly found in primary or secondary/persistent infections (Pinheiro *et al.* 2003a, Rôças *et al.* 2008, Antunes *et al.* 2015, Lima *et al.* 2020a b). In secondary/persistent infections, Rôças *et al.* (2008) used a molecular method of microbial identification and detected Streptococcus species in 47% of the cases at a concentration level of approximately 10^5 bacteria. In this study, were used and only 5% of the cases contained specifically *Streptococcus mutans*, which was expected due to the low sensitivity of culture methods compared to molecular ones. In symptomatic primary endodontic infections, Nóbrega et al. (2016) used non-specific culture medium for *S. mutans* and reported its presence in 10% of the cases.

The justification for the study of antimicrobials directly against this species lies in its previously reported pathogenic potential for extra-oral infections such as infective endocarditis, hemorrhagic stroke, and cerebral microbleeds (Otsugu *et al.* 2017, Inenaga *et al.* 2018). *S. mutans* has an invasive character into endothelial and epithelial cells, which occurs through the expression of collagen-binding proteins, such as Cnm and Cbm (Sato *et al.* 2004, Nomura *et al.* 2012). Importantly, in a recently published work, Lima *et al.* (2020a) verified, in primary and secondary/persistent infections, that all the *S. mutans* strains analysed expressed Cbm genes, thus indicating a risk for bacteremia when CBP positive *S. mutans* is present in root canal infections.

A few authors researched the antimicrobial susceptibility of *Streptococcus spp.* in root canals teeth with from primary or secondary/persistent infection (Skucaite *et al.* 2010, Al-Ahmad *et al.* 2014, Łysakowska *et al.* 2016) or oral environments (Pasquantonio *et al.* 2012, Süzük *et al.* 2016). With regard to the penicillin group, this study found that all the seven strains tested were susceptible to both amoxicillin and amoxicillin + clavulanate and one was resistant to benzylpenicillin. Also, Łysakowska *et al.* (2016) and Al-Ahmad *et al.* (2014) reported no resistance to penicillins. In contrast, susceptibility levels of 86%, 83%, and 66% were observed in other studies (Skucaite *et al.* 2010, Pasquantonio *et al.* 2012, Süzük *et al.* 2016).

In this study, the lowest active antibiotics against *S. mutans* were azithromycin (28.6%) and chloramphenicol (14.3%). Erythromycin resistance was found in 14% of the present isolates and 30% and 35% of prior tests (Skucaite *et al.* 2010, Łysakowska *et al.* 2016).

Clindamycin and vancomycin were equally effective, with 85.7% of the strains being susceptible to them. Likewise, previous works found that the streptococcal susceptibility to clindamycin was ≥80% (Skucaite *et al.* 2010, Pasquantonio *et al.* 2012, Al-Ahmad *et al.* 2014, Łysakowska *et al.* 2016). Anterior data have no record of vancomycin resistance (Skucaite *et al.* 2010, Pasquantonio *et al.* 2012, Al-Ahmad *et al.* 2014, Süzük *et al.* 2016, Łysakowska *et al.* 2016). As for tetracycline, Skucaite *et al.* (2010) and Pasquantonio *et al.* (2012) verified 42% and 34% rates of resistance in streptococci, whereas no resistance was detected in Al-Ahmad *et al.* (2014) and this investigation. Curiously, the same *S. mutans* strain was resistant to most of the antibiotics tested, except to amoxicillin, amoxicillin + clavulanate, and tetracycline.

In addition to these, other antibiotics not listed in the CSLI and EUCAST guidelines or other articles have been tested for research purposes. Ciprofloxacin and

doxycycline showed ranges of 0.38 to 1.5 mg/ml and 0.75 to 2 mg/ml of concentration against the collected *S. mutans*. Metronidazole showed no effect against this species. Meanwhile, rifampicin, gentamicin, and moxifloxacin had MIC_{90} values of 24 mg/ml, 4 mg/ml, and 0.38 mg/ml, respectively.

Final considerations

It is established in the literature that secondary/ persistent endodontic infections are polymicrobial (Francisco *et al.* 2019). Because of this, studies assessing the effect of antimicrobials against different pathogens are necessary.

In this study, we observed that amoxicillin alone or in combination with clavulanate is still a good option for antibiotic prophylaxis or even to treat systemic infections supposedly caused by *E. faecalis* and *S. mutans*, from secondary/ persistent infections. The present results support that 1mg/ml of one or another antibacterial agent is still enough to eliminate 100% of both strains. However, if the patient has a history of penicillin allergy, a new drug therapy must be installed. Usually, clindamycin or azithromycin are the antibiotics of choice in these cases (Ahmadi *et al.* 2021). However, this work has proven that they are not effective agents against bacteria recovered from failed endodontically-treated canals, meaning that the protocol regarding their use should be reviewed in dentistry.

The use of vancomycin could be considered as an effective alternative for patients who are allergic to penicillin, for prevention or treatment of endocarditis caused by viridans streptococci as well as enterococci, in combination with an aminoglycoside (Pinheiro *et al.* 2004).

Although the results showed that one strain of *S. mutans* was resistant to vancomycin, it is important to remember that this data were obtained through the comparison with the MIC breakpoints of CLSI (2020), in which the only information on vancomycin was that susceptible strains should be inhibited at a concentration $\leq$ 1mg/ml. The vancomycin-resistant *S. mutans* needed 2mg/ml to be inhibited, which is less than the MIC_{90} for *E. faecalis* (3 mg/ml). Besides, moxifloxacin exhibited the lowest MIC_{90} for the two analysed species; a dose of 0.75mg/ml would be sufficient and indicates that this antibiotic should be further studied in endodontics.

CONCLUSION

All *E. faecalis* strains were 100% susceptible to amoxicillin, amoxicillin + clavulanate, moxifloxacin, and vancomycin. In comparison, all *S. mutans* isolates were susceptible to amoxicillin, amoxicillin + clavulanate, and tetracycline. Furthermore, MIC_{90} showed that 1mg/ml of amoxicillin or amoxicillin + clavulanate, 0.75mg/ml of moxifloxacin, and 3mg/ml of vancomycin would be enough to inhibit at least 90% of both strains.

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Table 1. Minimum inhibitory concentration breakpoint values (mg/mL) for E*nterococcus faecalis* and *Streptococcus mutans* according to the respective antimicrobial agent.

Antimicrobial agents	MIC breakpoints for *E. faecalis*				MIC breakpoints for *S. mutans*			
	S	**I**	**R**	**RF**	**S**	**I**	**R**	**RF**
Amoxicillin (AC)	≤ 8	-	≥ 16	CLSI	≤ 0.5	-	>2	EUCAST
Amoxicillin + clavulanate (XL)	≤ 8	-	≥ 16	CLSI	≤ 0.5	-	>2	EUCAST
Azithromycin (AZ)	≤ 2	-	≥ 8	Fass	≤ 0.5	1	≥ 2	CLSI
Benzylpenicillin (PG)	NL	NL	NL	None	≤0.25	-	>2	EUCAST
Ciprofloxacin (CI)	≤ 1	2	≥ 4	CLSI	NL	NL	NL	None
Clindamycin (CM)	NL	NL	NL	None	≤0.25	0.5	≥ 1	CLSI
Chloramphenicol (CL)	≤ 8	16	≥ 32	CLSI	≤ 4	8	≥ 16	CLSI
Doxycycline (DC)	≤ 4	8	≥ 16	CLSI	NL	NL	NL	None
Erythromycin (EM)	≤ 0.5	1–4	≥ 8	CLSI	≤0.25	0.5	≥ 1	CLSI
Gentamicin (GM)	NL	NL	NL	None	NL	NL	NL	None
Metronidazole (MZ)	NL	NL	NL	None	NL	NL	NL	None
Moxifloxacin (MX)	≤ 2	-	≥ 8	Mather	NL	NL	NL	None
Rifampicin (RI)	≤ 1	2	≥ 4	CLSI	NL	NL	NL	None
Tetracycline (TC)	≤ 4	8	≥ 16	CLSI	≤ 2	4	≥ 8	CLSI
Vancomycin (VA)	≤ 4	8–16	≥ 32	CLSI	≤ 1	-	-	CLSI

Legends: S: Susceptible; I: Intermediate; R: Resistant; RF: Reference; CLSI: Clinical and Laboratory Standards Institute, 2020; EUCAST: European Committee on Antimicrobial Susceptibility Testing, 2020; Fass: Fass 1993; Mather: Mather et al. 2002; NL: Not listed in any guide or publication.

Table 2. Antimicrobial susceptibility of antimicrobial agents (mg/mL) against *Enterococcus faecalis* strains (N = 37) collected from teeth with endodontic failure.

Antimicrobial agent	Susceptibility rate (%)			MIC		
	S	I	R	Range	MIC_{50}	MIC_{90}
AC	100	___	___	0.38 – 1.4	0.75	1
XL	100	___	___	0.25 – 1	0.75	1
AZ	43.2	45.9	10.9	0.75 – 8	3	6
PG	x	x	x	1.5 – 4	2	3
CI	75.7	24.3	___	0.25 – 2	0.75	2
CM	x	x	x	3 – 64	32	48
CL	83.8	16.2	___	3 – 16	6	12
DC	76.3	2.1	21.6	0.064 – 64	0.75	48
EM	43.2	56.8	___	0.125 – 3	1.5	3
GM	x	x	x	1 – 12	6	8
MZ	x	x	x	0	-	-
MX	100	___	___	0.19 – 0.75	0.38	0.75
RI	56.8	18.9	24.3	0.38 – 8	4	8
TC	75.7	___	24.3	0.125 – 96	25	64
VA	100	___	___	1.5 – 3	2	3

Legends: S: Susceptible; I: Intermediate; R: Resistant; x: No classification.

Table 3. Antimicrobial susceptibility of antimicrobial agents (mg/mL) against *Streptococcus mutans* strains (N = 7) collected from teeth with endodontic failure.

Antimicrobial agent	Susceptibility rate (%)			MIC		
	S	I	R	Range	MIC_{50}	MIC_{90}
AC	100	-	-	0.064 – 0.5	0.064	0.5
XL	100	-	-	0.064 – 0.5	0.064	0.5
AZ	28.6	57.1	14.3	0.5 – 8	0.75	8
PG	85.7	-	14.3	0.032 – 3	0.032	3
CI	x	x	x	0.38 – 1.5	0.38	1.5
CM	85.7	-	14.3	0.047 – 12	0.064	12
CL	14.3	85.7	-	4 – 12’	8	12
DC	x	x	x	0.75 – 2	1.5	2
EM	85.7	-	14.3	0.125 – 3	0.19	3
GM	x	x	x	1.5 – 4	3	4
MZ	x	x	x	0	-	-
MX	x	x	x	0.124 – 0.38	0.125	0.38
RI	x	x	x	0.094 – 24	0.094	24
TC	100	-	-	0.5 – 1.5	1	1.5
VA	85.7	-	14.3	1 – 2	1	2

Legends: S: Susceptible; I: Intermediate; R: Resistant; x: No classification.

3 DISCUSSÃO

Na investigação realizada no artigo 1 diversas situações endodônticas como polpa vital, infecções primárias e infecções secundárias ou persistentes (ambas infecções com confirmação radiográfica de lesões periapicais) foram analisadas quanto aos seus perfis proteômicos humanos e microbianos. Cargas substanciais de proteínas foram encontradas em todas as condições endodônticas. A maioria delas, humana ou microbiana, foram associadas a funções de manutenção, como processo celular, regulação biológica, processo metabólico e processo de desenvolvimento. Notavelmente, várias proteínas humanas e microbianas diferentes relacionadas à resposta a lesões e patogenicidade foram encontradas. Já no artigo 2, foi monitorada a suscetibilidade antimicrobiana de *E. faecalis* e *S. mutans*, duas espécies encontradas nos casos de infecção secundária/persistente no presente estudo e frequentemente detectadas no insucesso endodôntico de trabalhos anteriores (Delboni et al., 2017; Lima et al., 2020b). De forma preocupante, tanto *E. faecalis* quanto *S. mutans* já apresentaram associação positiva com o desenvolvimento de endocardite infecciosa (EI), explicada pela presença e expressão de proteínas ligantes de colágeno (CBPs) em ambas as bactérias (Singh et al., 2010; Otsugu et al., 2017).

No artigo 1, proteínas humanas com funções que podem estar envolvidas na inflamação periapical e reabsorção óssea, como resposta inflamatória e osteoclastogênese, foram selecionadas para análise. Diversas proteínas imunes foram igualmente encontradas nos três grupos estudados. Neste trabalho foram detectadas inúmeras proteínas na polpa normal, como fibronectina, serina/treonina - proteína quinase e domínio de repetição WD. Deve-se considerar ainda que algum grau de lesão pode ter sido causado pela broca de alta rotação gerando resposta inflamatória imediata na polpa vital. Foi demonstrado previamente, em modelos de pulpite em ratos, que a proteína Notch 2, observada nesta particularidade endodôntica, pode ser expressa após a estimulação bacteriana do LPS ou estimulação mecânica (Ma et al., 2016).

No geral, muitos caracteres inflamatórios foram encontrados nas amostras de infecção primária, entre eles podem ser citados: receptor 1 de quimiocina XC, interleucinas; IL-1; IL-6; IL-17; IL-18, prostaglandina E2. Alguns participantes da via de inflamação foram detectados, um exemplo é o receptor de IL-1 que, após a ligação a interleucina-1 associada, medeia a ativação dependente de interleucina-1 de NF-κβ, MAPK e outras vias (Tominaga et al., 2000). Outro achado que pode ser citado é o NF-κβ, que é um fator de transcrição presente em quase todos os tipos de células e é o ponto final de uma série de eventos de transdução de sinal que são iniciados por uma vasta gama de estímulos relacionados a muitos processos

biológicos bem como inflamação, imunidade, diferenciação, crescimento celular, tumorigênese e apoptose (Beinke et al., 2004). Além disso a proteína quinase dependente de cálcio/calmodulina tipo 1D (CKLiK), foi encontrada exclusivamente em amostras de infecções primárias, esta proteína quinase é expressa principalmente por leucócitos polimorfonucleares humanos, que tem importante participação na defesa do hospedeiro contra invasão de microrganismos (Haslett et al., 1989).

Como na endodontia a presença de infecção primária ou secundária repercute nos tecidos ósseos adjacentes ao dente afetado, é importante comentar as proteínas relacionadas à regulação óssea pelos osteoclastos. Em relação à infecção secundária/persistente, o fator estimulador de colônias de macrófagos-1 (MCSF-1), só foi observado nesta situação endodôntica. MCSF-1 e o ativador do receptor de sinalização de NF-κβ (RANKL) são vias importantes para a diferenciação e ativação de osteoclastos (Li et al., 2017; Adamopoulos, 2018). Na área de endodontia, os resultados provenientes de um estudo sobre lesões periapicais, tais como: granulomas apicais; cistos radiculares; e cistos dentígeros, mostraram que a expressão de MCSF-1 foi significativamente maior nos cistos do que nos granulomas apicais (Weber et al., 2019). O eixo de sinalização RANKL/RANK desempenha um papel essencial na osteoclastogênese (Kong et al., 1999; Li et al., 2000). Ambos TNFSF11 (RANKL) e TNFRSF11A (RANK) foram encontrados neste estudo, mas não juntos, o primeiro estava no grupo de infecção primária e o último no grupo de infecção secundária. Ainda, o receptor 2 de Efrina tipo A (EphA2) foi detectado em todas as situações.

Assim como feito para as proteínas humanas e para uma avaliação mais precisa, o banco de dados UniProt revisado (Swiss Prot) em vez do não revisado (TrEMBL) foi priorizado. Muitas das proteínas patogênicas identificadas e revisadas foram categorizadas como adesinas. Dentre este grupo, *C. albicans* foi mais prevalente, estando presente em todas as situações endodônticas e apresentando várias proteínas, duas delas comuns a todas as situações, a proteína 1 responsiva ao pH (PHR1) e a proteína 2 da parede hifal (Hwp2). Além disso, a proteína semelhante à aglutinina 9 (ALS9) e o antiporter MFS QDR2 (QDR2) foram encontrados apenas em casos de polpa vital. A proteína 28 semelhante à adesina ancorada por GPI (PAG28) estava presente na polpa vital e infecção primária, enquanto a proteína reguladora transcricional UME6 (UME6) só foi observada em infecções primárias. É relevante informar que *C. albicans* é o fungo simbionte humano mais estudado, que pode transitar entre comensalismo para parasitismo dentro do hospedeiro humano, a característica polimórfica desta espécie é um fator de virulência chave de sua transição (Jacobsen et al., 2012; Hall and Noverr, 2017). A conversão para parasitismo em *C. albicans* ocorre em

períodos de trauma, disbiose e supressão imunológica, a morfogênese de *C. albicans* induzida por fatores abióticos e bióticos, resulta na ativação e indução de uma resposta pró-inflamatória (Moyes et al., 2010). As proteínas PHR1 e PHR2 desempenham um papel crucial na montagem da parede celular, sendo reguladas de forma diferente pelo pH extracelular. PHR1 é expresso quando o pH ambiente é 5,5 ou superior. Foi sugerido que o PHR1 não é necessário para a indução do desenvolvimento das hifas, mas desempenha um papel fundamental na manutenção do crescimento dessas (Younes et al., 2011). Hwp2 é uma proteína da parede celular marcada por GPI, que se mostrou necessária para a tolerância adequada ao estresse oxidativo, adesão e formação de biofilme (Younes et al., 2011).

Também representando a categoria de adesão, foram encontrados *Acinetobacter baumannii* (polpa vital) e *Escherichia coli* (infecções), que são considerados componentes usuais de infecções endodônticas (Brito et al., 2007), além disso o gênero *Acinetobacter* foi considerado parte da comunidade dominante de tecido pulpar dentário puro e saudável (Widmer et al., 2018). O autotransportador adesina Ata, é considerado um determinante de virulência de *A. baumannii*, que pode promover a formação de biofilme e ligar-se a diversos membros da matriz extracelular/membrana basal, como colágenos tipos I, II, III, IV e V e laminina (Bentancor et al., 2012). *Escherichia coli* pode compor cepas comensais e patogênicas, o gene Yeej provou ser um membro de ~ 40 % a 96% dos genomas de *E. coli* completamente sequenciados (Martinez-Gil et al., 2017).

Várias outras proteínas identificadas provavelmente estiveram envolvidas no processo patogênico, embora apenas algumas tenham suas funções revisadas no banco de dados GO (Swiss Prot). Neste estudo, nenhuma proteína prevalente do código de acesso Swiss Prot foi associada à proteólise. No entanto, na literatura foi demonstrado que algumas proteases e peptidases microbianas orais, como em *Porphyromonas gingivalis*, são capazes de degradar proteínas do hospedeiro (por exemplo, matriz extracelular e hemoglobina) para aumentar a acessibilidade de nutrientes, e além disso, as peptidases extracelulares de *P. gingivalis* podem também liberar pequenos peptídeos e aminoácidos livres que beneficiam outras bactérias orais dentro do consórcio microbiano (Dashper et al., 2004; Ruggiero et al., 2013). Em contraste, na base de dados TrEMBL UniProt várias proteínas tinham funções de proteólise, e três delas estavam presentes em todas as situações amostradas: proteína contendo o domínio G5, serina endopeptidase específica para IgA e toxina CdiA

Ademais, a polpa vital continha duas proteínas patogênicas revisadas adicionais que estavam presentes igualmente em infecções primárias e secundárias/persistentes. Ambas, 16S rRNA endonuclease CdiA - *Enterobacter cloacae subsp. cloacae* e hemolisina (Shla) -

Serratia marcescens, possuiam atividades tóxicas, mas dirigida a diferentes células (Hertle, 2000, 2005; Beck et al., 2014).

Finalmente, entre os grupos de infecção, proteínas patogênicas de *Candida albicans* como: fator de transcrição do agrupamento de zinco CZF1, proteína quinase de histidina SLN1 e proteína quinase CST20 de serina/treonina foram encontradas. Estas proteínas são necessárias para a formação de hifas e virulência através da penetração de células fúngicas no tecido do hospedeiro (Leberer et al., 1996; Brown et al., 1999; Yamada-Okabe et al., 1999). Ainda mais, foram encontrados o receptor Tir da intimina translocado de *E. coli*, uma proteína necessária para a formação de pedestal eficiente em células hospedeiras durante a infecção (De Groot et al., 2011) e a flagelina B de *Helicobacter mustelae*, importante para a motilidade e virulência (Josenhans et al., 1995).

Reconhecidas as proteínas responsáveis pelas respostas inflamatórias e os microrganismos com funções causadoras de processos patogênicos na endodontia, faz-se necessária a instalação de um protocolo de tratamento capaz de controlar os efeitos deletérios da microbiota nos tecidos do hospedeiro. É reconhecido que para debelar tais efeitos, os tratamentos mais eficientes na endodontia são: a desinfecção dos canais radiculares e a drenagem em caso de abscessos agudos. Contudo, quando da presença de infecção sistêmica com possível risco à vida e a necessidade de profilaxia antibiótica em pacientes portadores de doenças pré-existentes cardíacas torna-se primordial a utilização de uma antibioticoterapia eficiente.

Quando necessária a utilização de antibióticos no insucesso endodôntico é preciso levar em consideração as espécies mais prevalentes nesse meio ambiente. O presente artigo 2 e outros trabalhos (Delboni et al. 2017, Lima et al., 2020b) confirmaram a presença de *E. faecalis* e *S. mutans* nas infecções secundárias/persistentes. Todavia, nos resultados do artigo 1 foi encontrada apenas uma proteína revisada (em 1 caso entre 20 coletados) de resistência a medicamentos nos casos de infecção secundária/persistente, a proteína multirresistente a drogas CDR2, pertencente a *Candida albicans.* Nesta condição, a espécie pertencente ao reino Fungi era resistente a antifúngicos. De acordo com Xu e colaboradores (2007), a deleção do gene CDR2 torna as cepas de *C. albicans* mais susceptíveis ao fluconazol, indicando a participação do CDR2 em processos de resistência na espécie. Em relação a proteínas sem revisão na literatura três espécies (*Acinetobacter pittii*, *Pseudomonas aeruginosa*, e *Klebsiella pneumoniae*) foram eletronicamente anotadas com atividade da beta-lactamase. Francisco et at. (2019) verificaram, estudando a proteômica do insucesso endodôntico, que enzimas associadas à resistência aos antibióticos, incluindo proteína de resistência a múltiplas drogas

MdtB, beta-lactamase e regulador transcricional de genes associados com resistência a drogas (isto é, tipo AsnC, tipo LacL e Tipo QseB), estavam sendo produzidos por espécies dos canais radiculares estudados no momento dos procedimentos de coleta.

No estudo do artigo 2 sobre antibioticoterapia em infecção endodôntica persistente/secundária, todas as cepas de *E. faecalis* testadas foram suscetíveis à amoxicilina e amoxicilina com clavulanato, concordando com vários trabalhos na literatura (Gomes et al., 1994; Siqueira and Rôças, 2005a; Siqueira Jr e Rôças, 2007; Siqueira e Rôças, 2009; Barbosa-Ribeiro et al., 2016; Süzük et al., 2016; Saffari et al., 2018). Por outro lado, altas taxas, de até 72%, de resistência de *E. faecalis* à penicilina já foram documentadas em infecções primárias e secundárias (Dahlén et al., 2000; Khemaleelakul et al., 2002; Baumgartner and Xia, 2003; Dumani et al., 2012). Além disso, Macy e Ngor (2013) constataram que aproximadamente 8% da população tem história de alergia à penicilina, o que contraindica essa classe de antimicrobianos nessas situações.

Azitromicina e eritromicina, revelaram a mesma taxa de suscetibilidade de 43,2% para *E. faecalis* isolados. Ainda, 45,9% e 56,8% das cepas apresentaram suscetibilidade intermediária à azitromicina e eritromicina, respectivamente, e 10,9% foram resistentes à azitromicina. Esses resultados estão de acordo com vários relatórios anteriores, onde uma suscetibilidade ainda menor foi encontrada para esses agentes (Pinheiro et al., 2003b, 2004; Barbosa-Ribeiro et al., 2016; Łysakowska et al., 2016; Saffari et al., 2018). Em discrepância, Skucaite et al., (2010), verificaram que a maioria dos enterococos testados por eles (90%) eram intermediariamente sensíveis à eritromicina, sugerindo uma maior eficácia desse medicamento.

Considerando a necessidade de profilaxia antibiótica em indivíduos alérgicos à penicilina e com risco de endocardite durante o retratamento endodôntico, é possível supor, com base nos dados expostos, que azitromicina ou eritromicina não são bons candidatos. Além disso, a clindamicina, o antibiótico mais comumente prescrito em pacientes alérgicos à penicilina, segue a mesma contraindicação. Sua ineficácia contra enterococos foi comprovada por muito tempo (NCCLS, 2000).

Bastante semelhante a Pinheiro et al. (2004), quase todas as cepas resistentes à tetraciclina também foram resistentes à doxiciclina, com a exceção de uma cepa considerada intermediariamente suscetível à doxiciclina e resistente à tetraciclina. Além disso, neste estudo, 24,3% dos isolados de *E. faecalis* eram resistentes à tetraciclina. A eficácia deste antibiótico diverge amplamente, com níveis de resistência bacteriana variando de 10 a 75% (Pinheiro et al., 2004; Sedgley et al., 2005a; Reynaud Af Geijersstam et al., 2006; Skucaite et

al., 2010; Lins et al., 2013; Barbosa-Ribeiro et al., 2016; Łysakowska et al., 2016; Saffari et al., 2018. No geral, a resistência às tetraciclinas diminuiu seu valor clínico.

Para eliminar 90% das cepas de *E. faecalis*, foi necessária uma MIC≥1 mg/ml de rifampicina, corroborando com Reynaud Af Geijersstam et al. (2006). Além disso, 56,8% dos isolados foram suscetíveis à rifampicina e quatro *E. faecalis* não foram afetados pela concentração mais alta do mesmo antibiótico. Em geral, o cloranfenicol mostra ação antimicrobiana importante (≥95%) em *E. faecalis* de infecções endodônticas (Pinheiro et al., 2004; Barbosa-Ribeiro et al., 2016; Łysakowska et al., 2016), neste estudo uma eficácia ligeiramente inferior de 83,8% foi indicado. No entanto, devido à preocupação com a associação do uso de cloranfenicol e o desenvolvimento de anemia aplástica e outras discrasias sanguíneas (Strom, 2005), a escolha de um medicamento mais seguro é desejada.

Entre os isolados de *E. faecalis* testados, à vancomicina foi 100% ativa, com uma MIC_{90} de 3 mg/ml. Este resultado foi confirmado por inúmeras investigações onde nenhuma resistência à vancomicina foi observada ao longo do tempo (Dahlén et al., 2000; Pinheiro et al., 2004; Reynaud Af Geijersstam et al., 2006; Barbosa-Ribeiro et al., 2016; Łysakowska et al., 2016; Vidana et al., 2016; Saffari et al., 2018). Pelo contrário, Dumani et al. (2012) encontraram apenas uma cepa enterocócica suscetível à vancomicina, entre 18 investigadas de infecções primárias e secundárias.

Quanto ao grupo das fluoroquinolonas, a ciprofloxacina foi eficaz contra 75,7% das cepas de *E. faecalis* e a moxifloxacina contra 100%. Trabalhos recentes anteriores mostraram níveis de 50% (Łysakowska et al., 2016) e 68% (Saffari et al., 2018) de resistência de *E. faecalis* à ciprofloxacina em infecções endodônticas. Ademais, Pinheiro et al. (2004), sugeriram a moxifloxacina como uma terapia alternativa para pacientes alérgicos à penicilina, pois como neste estudo eles encontraram que *E. faecalis* era suscetível a este antimicrobiano em todos os casos.

Streptococcus spp. é uma bactéria oral bem conhecida, comumente encontrada na infecção primária ou secundária/persistente (Pinheiro et al., 2003a; Rôças et al., 2008; Antunes et al., 2015; Lima et al., 2020a, 2020b). Neste estudo, por meio de técnicas de cultura, apenas 5% dos casos continham especificamente *Streptococcus mutans*, o que é esperado devido à baixa sensibilidade da cultura em relação aos métodos moleculares. A justificativa para o estudo de antimicrobianos diretamente contra esta espécie está em seu potencial patogênico relatado anteriormente para infecções extra-orais, como endocardite infecciosa, acidente vascular cerebral hemorrágico e micro sangramento cerebral (Otsugu et al., 2017; Inenaga et al., 2018).

Começando com o grupo da penicilina, este estudo descobriu que todas as 7 cepas testadas eram suscetíveis à amoxicilina e amoxicilina + clavulanato e uma era resistente à benzilpenicilina. De acordo, Łysakowska et al. (2016) e Al-Ahmad et al. (2014) não relataram resistência à penicilina, enquanto níveis de suscetibilidade de 86%, 83% e 66% foram observados em outros estudos (Skucaite et al., 2010; Pasquantonio et al., 2012; Süzük et al., 2016).

Os antibióticos menos ativos contra *S. mutans* neste estudo foram azitromicina (28,6%) e cloranfenicol (14,3%). A resistência à eritromicina foi encontrada em 14% dos isolados atuais e em 30% e 35% de testes anteriores (Skucaite et al., 2010; Łysakowska et al., 2016). Clindamicina e vancomicina foram igualmente eficazes, com 85,7% das cepas sendo suscetíveis a elas. Da mesma forma, trabalhos anteriores descobriram que a susceptibilidade dos estreptococos à clindamicina era ≥80% (Skucaite et al., 2010; Pasquantonio et al., 2012; Al-Ahmad et al., 2014; Łysakowska et al., 2016). Em relação à tetraciclina, Skucaite et al. (2010) e Pasquantonio et al. (2012) verificaram taxas de 42% e 34% de resistência em estreptococos, enquanto nenhuma resistência foi detectada em Al-Ahmad et al. (2014) e este monitoramento. Curiosamente, a mesma cepa de *S. mutans* foi resistente à maioria dos antibióticos testados, exceto para amoxicilina, amoxicilina + clavulanato e tetraciclina.

Neste estudo observamos que a amoxicilina isolada ou em combinação com clavulanato ainda é uma opção adequada para profilaxia antibiótica ou mesmo para tratar infecções sistêmicas supostamente causadas por *E. faecalis* e *S. mutans*, vindos de infecções endodônticas secundárias/persistentes. Os presentes resultados suportam que 1mg/ml de um ou o outro agente ainda é suficiente para eliminar 100% de ambas as cepas. No entanto, se o paciente apresentar histórico de alergia à penicilina, uma nova terapia medicamentosa deve ser instalada.

O uso de vancomicina pode ser considerado. Embora os resultados tenham mostrado que uma cepa de *S. mutans* era resistente à vancomicina, é importante lembrar que esses dados foram obtidos por meio da comparação com os pontos de corte da MIC do CLSI (2020), onde a única informação sobre a vancomicina era que cepas suscetíveis deveriam ser inibidas por uma concentração menor ou igual a 1mg/ml. O *S. mutans* resistente à vancomicina precisava de 2 mg/ml para ser inibido, que é menor do que o M_{90} para *E. faecalis* (3 mg/ml). Além disso, a moxifloxacina exibiu o menor M_{90} para as duas espécies analisadas, uma dose de 0,75mg/ml seria suficiente e indica que esse antibiótico deve ser mais estudado em endodontia.

Ambos os artigos produzidos nessa tese de doutorado trouxeram informações inovadoras para a endodontia, preenchendo lacunas na literatura nos temas especificados. No artigo 1, foi encontrada uma série de proteínas humanas identificadas como potenciais causadoras de processos infecciosos e da osteoclastogênese, como: Notch2, NF-κβ, Receptor IL-1, CKLiK, MCFS-1, RANK, RANKL e EphA2. Em relação às proteínas microbianas de interesse, foram encontrados membros com funções patogênicas como adesão, agentes proteolíticos, proteínas com funções de toxicidade e invasão celular. Ademais, nunca antes foram comparadas infecções endodônticas com um grupo controle de polpa vital sob as mesmas condições de coleta. Os resultados encontrados contribuíram para a formação de bancos de dados proteômicos na endodontia, bem como indicaram novos candidatos a biomarcadores de doenças que poderão ser estudados posteriormente.

Quanto ao artigo 2, o monitoramento antimicrobiano de cepas simultâneas no insucesso endodôntico é escasso. Esse estudo permitiu a identificação de antibióticos em suas concentrações eficazes para a eliminação de cepas de *Enterococcus faecalis* e *Streptococcus mutans*, ao mesmo tempo, de canais radiculares com infecção secundária/persistente. A importância da erradicação dessas espécies na endodontia é explicada pelas suas associações a infecções sistêmicas que trazem riscos a vida, como por exemplo, a endocardite infecciosa.

4 CONCLUSÃO

A partir dos resultados obtidos foi possível concluir que:

- Proteínas humanas e microbianas foram identificadas na polpa vital, infecção endodôntica primária e secundária/persistente. A infecção primária continha um número maior de proteínas do que as outras doenças endodônticas. Membros importantes das vias de inflamação e reabsorção óssea foram encontrados, enquanto muitas proteínas microbianas detectadas foram relacionadas à adesão de simbiontes aos tecidos do hospedeiro e atividades patogênicas, tais como: formação de biofilme, liberação de toxina, proteólise e invasão celular. Os estudos de caracterização proteômica abrem fronteiras para a investigação de biomarcadores de doenças, além de permitir o aprimoramento dos tratamentos de infecção e inflamação por meio da atuação em alvos específicos.

- As cepas de *E. faecalis* foram totalmente suscetíveis à amoxicilina, amoxicilina + clavulanato, moxifloxacina e vancomicina. Enquanto todos os isolados de *S. mutans* foram sensíveis à amoxicilina, amoxicilina + clavulanato e tetraciclina. Além disso, MIC_{90} mostrou que 1mg/ml de amoxicilina ou amoxicilina + clavulanato, 0,75mg/ml de moxifloxacina e 3mg/ml de vancomicina seriam suficientes para inibir pelo menos 90% de ambas as cepas.

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APÊNDICES

Apêndice 1 – Termo de consentimento livre e esclarecido

UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

TERMO DE CONSENTIMENTO LIVRE ESCLARECIDO

As informações contidas neste documento foram fornecidas pela orientadora Profª. Drª. Brenda Paula Figueiredo de Almeida Gomes e/ou pelos pesquisadores Augusto Rodrigues Lima, Eloá Cristina Bícego Pereira, Priscila Amanda Francisco e Rafaela Casadei Chapola, com o objetivo de convidar e registrar por escrito, que o indivíduo participante da pesquisa, autoriza sua participação, com pleno conhecimento dos procedimentos e riscos a que se submeterá por livre arbítrio e sem qualquer coação.

I. TÍTULO DA PESQUISA

"Investigação microbiológica e de seus fatores de virulência nas diferentes patologias pulpares e perirradiculares e sua interação com o sistema imunológico inato".

II. OBJETIVO GERAL

Avaliar a influência das bactérias e seus produtos tóxicos na indução de inflamação/infecção em dentes. E também avaliar o efeito do tratamento de canal na redução e/ou eliminação desse conteúdo em canais radiculares, objetivando analisar o efeito das diferentes formas de tratamento utilizadas.

Visa também investigar a resistência/suscetibilidade de bactérias a antibióticos usados rotineiramente em endodontia

III. JUSTIFICATIVA

1. Este trabalho visa investigar a presença bacteriana em casos de abscesso periapical agudo, abscesso periapical crônico, dentes necrosados de pacientes com comprometimento periodontal e casos de insucesso do tratamento endodôntico. Serão recrutados também pacientes com dentes com polpa viva que tenham sidos encaminhados para tratamento endodôntico por indicação protética, assim como casos de inflamação da polpa (pulpite irreversível) que servirá de controle. A realização desta pesquisa servirá para avaliar o efeito do tratamento na redução destas bactérias.

2. Investigar o conteúdo tóxico de bactérias, avaliando a contribuição destas moléculas no desenvolvimento de dor e presença de lesões no ápice do dente.

3. Pesquisas realizadas em nosso laboratório alertaram sobre o aumento da resistência à antibióticos de determinadas bactérias encontradas em canais radiculares de dentes com insucesso do tratamento endodôntico. Desta forma, é necessário um acompanhamento da resistência aos antibióticos destas bactérias.

IV. PROCEDIMENTO DO EXPERIMENTO

Seleção dos pacientes

Inicialmente serão analisadas cerca de 300 fichas dos pacientes cadastrados na Área de Endodontia da Faculdade de Odontologia de Piracicaba (FOP/UNICAMP) encaminhados aos Cursos de Atualização e Especialização. Ao final, serão selecionados 120 voluntários com idade entre 18 e 60 anos, de ambos os sexos e independente de raça. Os pacientes serão divididos em grupos de acordo com a condição do dente. Serão excluídos voluntários que trataram o dente a menos de 2 anos; que tenham usado antifúngicos e antibióticos em menos de 3 meses; dentes com fratura na raiz; dentes com extensa destruição coronária; dificuldade de acesso ao canal radicular; e dentes sem restauração, seja temporária ou definitiva.

Exame clínico (anamnese e exame físico)

Serão anotados dados pessoais dos pacientes tais como idade e gênero, história médica e dentária.

Aspectos clínicos e radiográficos

Ao iniciar o tratamento, durante o exame clínico inicial serão registradas informações sobre o dente, onde será verificada a presença/ausência de dor, natureza da dor, dor à percussão (horizontal e vertical) e à palpação. Também serão anotados dados tais como: presença/ausência de edema (inchaço), fístulas, mobilidade dental e bolsa periodontal. Todas essas características clínicas serão anotadas na ficha clínica de cada paciente.

Coleta de amostras

As coletas clínicas do canal radicular e bolsas periodontais serão realizadas na Clínica de Especialização da FOP/UNICAMP, e as amostras processadas no Laboratório de Microbiologia Endodôntica.

Serão utilizadas técnicas assépticas e instrumentos esterilizados e livre de endotoxinas. Haverá a remoção de cáries e restaurações defeituosas, realização do isolamento absoluto para não haver infiltração de saliva durante o procedimento.

Antes do atendimento, será realizada a descontaminação do rosto do paciente com clorexidina gel 2% e anestesia local na região do dente envolvido. Após a abertura do dente, será utilizado o localizador apical (Novapex, Forum Technologies, Rishon le-zion, Israel) para confirmar se o canal está totalmente acessível para o tratamento e obter o comprimento da raíz.

Para as coletas das amostras, cones de papel absorventes estéreis e apirogênicos serão introduzidos no dente, permanecendo nesta posição por 60 segundos.

A instrumentação do dente será realizada por limas acionadas a motor e manuais, possibilitando posterior obturação do canal radicular. Serão utilizadas substâncias químicas rotineiramente utilizadas na endodontia para instrumentação dos dentes. Em alguns grupos serão colocados medicação intracanal durante 30 dias. Posteriormente, o dente será obturado e selado com restauração defintiva.

Para a coleta periodontal um cone será introduzido até o fundo da bolsa periodontal permanecendo por 1 minuto. Em seguida, esse cone será transferido para frascos de vidro apirogênicos e armazenados em freezer -20 °C.

V. DESCONFORTO OU RISCOS ESPERADOS

A pesquisa tentará minimizar ao máximo possíveis desconfortos, principalmente aquele relacionado à dor pós-operatória, através de um monitoramento contínuo dos pacientes e a utilização de materiais e técnicas adequadas e seguras. Independentemente do grupo, todos os pacientes receberão um tratamento seguro e eficaz, para evitar dor e desconforto durante e após o procedimento clínico.

O tratamento será convencional, com a aplicação de anestesia local, isolamento absoluto, utilização de instrumentos estéreis e a utilização de equipamentos de proteção individual (óculos de proteção, máscaras, luvas, etc), tanto para o paciente quanto para o pesquisador.

Os instrumentos e substâncias químicas utilizadas são constantemente testados e aprovados em pesquisas científicas publicadas na área. Serão adotadas ainda, algumas combinações de medicação intracanal, principalmente nos casos onde se espera maior resistência bacteriana, a fim de combater a infecção dos canais radiculares.

Os procedimentos não acarretam risco de morte para os pacientes, assim como também não exige despesas extras, já que se trata de atendimento convencional realizado diariamente nesta Faculdade. O processo é usualmente sem dor e, caso haja dor, esta não será devido à coleta de amostra e sim referente à inflamação/infecção nos canais radiculares e/ou tecidos perirradiculares. Os procedimentos desenvolvidos nesse trabalho estão em acordo com os tratamentos indicados e realizados atualmente na área de Endodontia da FOP/UNICAMP.

Se houver dor fora dos dias marcados para o tratamento, o paciente receberá uma assistência imediata dos responsáveis pela pesquisa, assim o mesmo deverá entrar em contato através dos telefones locais descritos a seguir:

(0xx19) 2106 5215 (laboratório de Endodontia)
(0xx19) 98309-6747 (celular da pesquisador Augusto Rodrigues Lima).
(0xx16) 98140-1221 (celular do pesquisadora Eloá Cristina Bícego Pereira).
(0xx19) 98157-5250 (celular do pesquisadora Priscila Amanda Francisco).
(0xx19) 99183-8578 (celular da pesquisadora Rafaela Casadei Chapola).

O paciente também poderá ser atendido no Plantão de Emergência da FOP-UNICAMP, que funciona normalmente de segunda à sexta-feira, de 8:00 às 12:00 hs e de 13:30 às 17:30 hs.

Para qualquer informação ou esclarecimento sobre o tratamento, o paciente poderá entrar em contato com os pesquisadores através dos telefones citados acima, e-mail: augusto_rl@hotmail.com, eloacristina13@hotmail.com, priscilaafrancisco@gmail.com, rchapola@gmail.com e bpgomes@fop.unicamp.br, assim como no endereço Rua: Regente Feijó, n° 639, centro.

O paciente tem toda a liberdade de pedir esclarecimentos sobre a metodologia antes e durante a pesquisa, podendo ou não concordar em participar da mesma. Caso recuse, seu tratamento será prosseguido normalmente, se o mesmo demonstrar interesse. Uma cópia deste termo (TCLE) será entregue ao voluntário, portanto o mesmo terá em mãos telefones e endereços para contato.

Apesar dos resultados serem divulgados publicamente para fins acadêmicos e científicos, será preservada a privacidade do indivíduo quanto aos dados confidenciais que possam a ser envolvidos na pesquisa.

VI. BENEFÍCIOS DO EXPERIMENTO

Os voluntários receberão um tratamento mais eficaz e seguro, com o conhecimento das bactérias e das causas da inflamação/infecção dos dentes.

VII. Possibilidades de inclusão em grupo controle ou placebo

Também será realizado o tratamento de pacientes que apresentam indicação protética em dentes vivos (com o objetivo de instalar pinos na raiz do dente), para verificar os níveis quando o dente está sadio. Nesta situação a indicação estará bem adequada, não havendo recrutamento extra de voluntários, pois os pacientes já estarão encaminhados para a realização do tratamento endodôntico.

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VIII. Métodos alternativos para obtenção da informação ou tratamento da condição
Não se aplica métodos alternativos, porque se torna necessário realizar o tratamento de canal para a obtenção das amostras.

IX. INFORMAÇÕES
O voluntário tem a garantia de que receberá respostas a qualquer pergunta ou esclarecimento a respeito dos procedimentos, riscos, benefícios e outros assuntos relacionados à pesquisa.
Todos os dados coletados e qualquer informação referente a este estudo permanecerá confidencial, assegurando proteção à imagem, sigilo e respeitando valores sociais, culturais, morais, éticos e religiosos. Os resultados desta pesquisa serão divulgados apenas para fins científicos, porém a identidade dos voluntários não será divulgada.
Não haverá necessidade de deslocamentos ou procedimentos adicionais para coleta de amostra, além das necessárias para o tratamento endodôntico convencional (04 sessões). Não há previsão de ressarcimento, pois não há risco previsível pela participação na pesquisa.

X. RETIRADA DO CONSENTIMENTO
O voluntário tem a liberdade de retirar seu consentimento a qualquer momento e deixar de participar do estudo e assim do grupo de amostra, este não será penalizado e não haverá prejuízo ao seu tratamento, o qual será prosseguido normalmente.

Eu, _____, certifico que tendo lido as informações acima e suficientemente esclarecido (a) de todos os itens pela Profª. Drª. Brenda Paula Figueiredo de Almeida Gomes, Augusto Rodrigues Lima, Eloá Cristina Bícego Pereira, Priscila Amanda Francisco e Rafaela Casadei Chapola, estou plenamente de acordo com a realização do experimento. Assim, eu autorizo a execução da pesquisa, exposta acima, em mim.

Piracicaba, _____/_____/_____.

Nome: , RG:

Assinatura: _____

ATENÇÃO: A sua participação em qualquer tipo de pesquisa é voluntária. Os pesquisadores garantem a entrega de uma segunda via para o sujeito da pesquisa (ou responsável). Em caso de dúvida quanto aos seus direitos como voluntário da pesquisa, escreva para o Comitê de Ética em Pesquisa da FOP – UNICAMP, endereçado a Av. Limeira, 901 – Caixa Postal 52, CEP 13414-903, fone (19) 2106-5349, e-mail cep@fop.unicamp.br, na cidade de Piracicaba/SP.

_____
Assinatura do Pesquisador

Todas as páginas do TCLE serão rubricadas pelo sujeito da pesquisa (ou responsável) e pesquisador.

Apêndice 2 – Biografia da autora

Priscila Amanda Francisco, nascida em Limeira, São Paulo, Brasil.

2009 - 2013	Graduação em Odontologia na Faculdade de Odontologia de Araraquara – UNESP, Brasil
2011 - 2013	Iniciação Científica no Departamento de Odontologia Restauradora, Área de Endodontia na Faculdade de Odontologia de Araraquara – UNESP, Brasil, com bolsa Fapesp (Processo 11/22783-1)
2015 - 2017	Especialização em Endodontia na Faculdade de Odontologia de Piracicaba – UNICAMP, Brasil
2015 - 2017	Mestrado em Clínica Odontológica, Área de Endodontia, na Faculdade de Odontologia de Piracicaba – UNICAMP, Brasil, com bolsa Fapesp (Processo 15/19215-2)
2016 - 2017	Estágio durante o Mestrado no Laboratório de Bioquímica da University of Western Ontario, Canadá, com bolsa Fapesp (Processo 16/19743-1)
2017 - 2021	Doutorado em Clínica Odontológica, Área de Endodontia, na Faculdade de Odontologia de Piracicaba – UNICAMP, Brasil, com bolsa Fapesp (Processo 17/16516-7)
2019 - 2020	Estágio durante o Doutorado no Laboratório de Bioquímica da University of Saskatchewan, Canadá, com bolsa Fapesp (Processo 18/21553-1)

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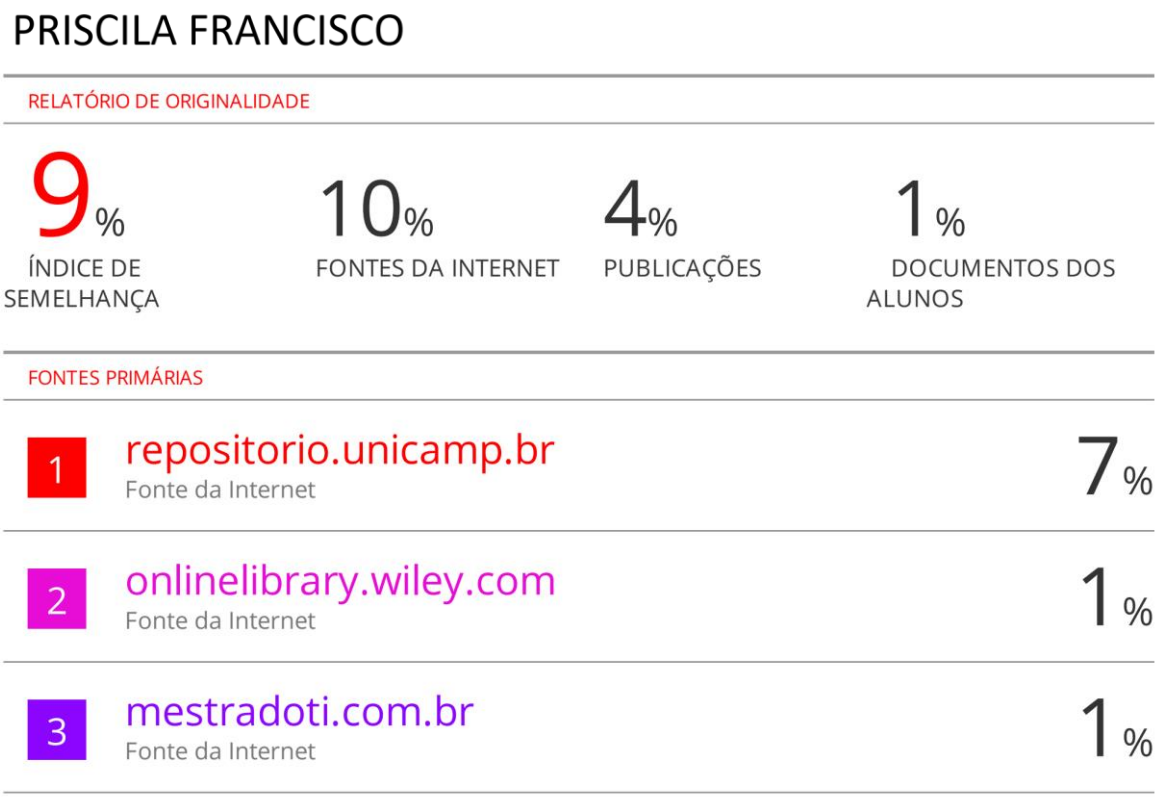
ANEXOS

Anexo 1 – Verificação de originalidade e prevenção de plágio



PRISCILA FRANCISCO

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Anexo 2 – Aprovação do Comitê de Ética em Pesquisa

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Investigação microbiológica e de seus fatores de virulência nas diferentes patologias pulpares e perirradiculares e sua interação com o sistema imunológico inato**", CAAE **48374615.7.0000.5418**, dos pesquisadores **Brenda Paula Figueiredo de Almeida Gomes, Priscila Amanda Francisco, Augusto Rodrigues Lima, Pedro Ivo da Graça Fagundes, João Carlos Leme Junior, Eloá Cristina Bícego Pereira e Rafaela Casadei Chapola**, satisfaz as exigências das resoluções específicas sobre ética em pesquisa com seres humanos do Conselho Nacional de Saúde – Ministério da Saúde e foi aprovado por este comitê em 21/10/2015 (projeto original), em 30/01/2018 (primeira emenda) e em 07/05/2018 (segunda emenda).

The Research Ethics Committee of the Piracicaba Dental School of the University of Campinas (FOP-UNICAMP) certifies that research project "**Microbiological and virulence factors investigation in different pulp and periradicular pathologies and their interaction with innate immune system**", CAAE **48374615.7.0000.5418**, of the researcher's **Brenda Paula Figueiredo de Almeida Gomes, Priscila Amanda Francisco, Augusto Rodrigues Lima, Pedro Ivo da Graça Fagundes, João Carlos Leme Junior, Eloá Cristina Bícego Pereira and Rafaela Casadei Chapola**, meets the requirements of the specific resolutions on ethics in research with human beings of the National Health Council - Ministry of Health, and was approved by this committee on 10/21/2015 (original project), on 01/30/2018 (first amendment) and on 05/07/2018 (second amendment).

Profa. Fernanda Miori Pascon
Vice Coordenador
CEP/FOP/UNICAMP

Prof. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo e a lista de autores aparecem como fornecidos pelos pesquisadores, sem qualquer edição.
Notice: The title and the list of researchers of the project appears as provided by the authors, without editing.

Anexo 3 – Comprovante de submissão do artigo 2

Submission Confirmation

Print

Thank you for your submission

Submitted to
International Endodontic Journal

Manuscript ID
IEJ-21-00277

Title
Investigation of the antimicrobial susceptibility of *Enterococcus faecalis* and *Streptococcus mutans* in cases of endodontic failure

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Date Submitted
18-Apr-2021