UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

JOSÉ BURGOS PONCE

Study of phagocytic ability of human macrophages against different strains of *Enterococcus faecalis*

Estudo da capacidade fagocítica de macrófagos humanos frente a diferentes cepas de *Enterococcus faecalis*

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Dedico este trabalho a todos que, de alguma forma, aportaram para sua idealização, realização e finalização, seja física ou moralmente. Com certeza sem a sua ajuda, nada poderia ter sido feito.

“Nenhum homem é uma ilha…” - John Donne.

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"*Um homem da aldeia de Neguá, no litoral da Colômbia, conseguiu subir ao céu. Quando voltou, contou. Disse que tinha contemplado, lá do alto, a vida humana. E disse que somos um mar de fogueirinhas.*

– O mundo é isso – revelou. – Um montão de gente, um mar de fogueirinhas.

Cada pessoa brilha com luz própria entre todas as outras. Não existem duas fogueiras iguais. Existem fogueiras grandes e fogueiras pequenas e fogueiras de todas as cores. Existe gente de luz serena, que nem percebe o vento, e gente de luz louca, que enche o ar de faíscas. Algumas luzes, luzes bobas, não alumiam nem queimam; mas outras incendeiam a vida com tamanha vontade que é impossível olhar para elas sem pestanejar, e quem chegar perto se ascende." – O mundo; livro dos abraços, Eduardo Galeano.

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“I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I—
I took the one less traveled by,
And that has made all the difference.”
From “the road not taken” - Robert L. Frost

RESUMO

Estudo da capacidade fagocítica de macrófagos humanos frente a diferentes cepas de *Enterococcus faecalis*

Enterococcus faecalis (E. faecalis) é um microrganismo presente em lesões endodônticas persistentes, mostrando maior resistência do que outras bactérias ao Hidróxido de Cálcio, um medicamento alcalino que consegue eliminar diversos microrganismos durante o tratamento endodôntico. Assim, os objetivos desse estudo foram: (a) avaliar a resposta de *E. faecalis* isolados de canal radicular, após estresse alcalino, quanto sobrevivência, crescimento, alteração do pH, resistência/susceptibilidade antimicrobiana e formação de biofilme sobre discos de dentina; (b) avaliar a capacidade fagocítica e produção de óxido nítrico (NO) por macrófagos humanos, frente a bactérias *E. faecalis* de canais radiculares, submetidas a estresse alcalino; (c) avaliar a expressão de TLR2 e CD14 na superfície dos macrófagos desafiados com as diferentes cepas bacterianas. As cepas utilizadas foram: ATCC4083 (CANAL 1) e uma cepa clínica, obtida por nós, a partir de uma lesão endodôntica primária (CANAL 2), ambas isoladas de canais radiculares; e ATCC29212 isolada de urina (URINA), utilizada como controle. O estresse alcalino foi obtido através da inoculação das bactérias em meio BHI-alcalino por 4, 24, 48 e 72 horas. As bactérias alcalino-resistentes foram semeadas em ágar, com ou sem troca do meio, e quantificadas por CFU/mL. A susceptibilidade antimicrobiana das diferentes cepas, estressadas ou não (controle), foi determinada pelo Etest; e o biovolume do biofilme foi quantificado microscopicamente. Para avaliar a capacidade fagocítica, macrófagos obtidos a partir de monócitos do sangue periférico foram desafiados com as diferentes cepas, estressadas ou não em meio BHI-alcalino, por 30 minutos, na proporção 5:1 (bactéria/macrófago), e corados com Laranja de Acridina. Foi contado o total de macrófagos com bactérias internalizadas, considerando o número de bactérias internalizadas por célula (<5 e ≥5). A concentração de NO foi medida em sobrenadantes, através da reação de Griess, e a expressão de TLR2 e CD14 pelos macrófagos foi analisada por citometria de fluxo. Os resultados revelaram que *Enterococcus* oriundos de canal radicular foram menos resistentes ao estresse alcalino e mais susceptíveis aos antibióticos testados, do

que as bactérias oriundas de urina. A falta de nutrientes foi um fator determinante para o crescimento bacteriano de todas as cepas. O biovolume dos biofilmes foi semelhante para todas as cepas estudadas, e não foi alterado após exposição ao BHI-alcalino. Na presença de bactérias submetidas ao estresse alcalino, houve um menor número de macrófagos com bactérias internalizadas, em comparação ao controle. No entanto, a produção de NO e a expressão de TLR2 e CD14 não foram alteradas. Independentemente da cepa utilizada e da presença de estresse alcalino, a maioria dos macrófagos apresentavam-se com ≥5 bactérias internalizadas por célula. Na ausência de estresse, as cepas de urina resultaram em maior produção de NO que aquelas oriundas do canal radicular; entretanto, a produção deste gás foi semelhante entre as cepas após estresse alcalino. A partir desses resultados, podemos concluir que bactérias *E. faecalis* de urina diferem daquelas oriundas do canal radicular, principalmente quanto a susceptibilidade/resistência microbiana; assim sugerimos que estudos envolvendo o campo da Endodontia devam ser realizados com cepas oriundas de canal radicular, preferencialmente que de urina. Concluiu-se ainda que um ambiente alcalino associado a falta de nutrientes pode reduzir o crescimento de *E. faecalis*. Adicionalmente, o estresse alcalino pode levar a alterações na estrutura da parede de *E. faecalis*, o que dificulta o seu reconhecimento, reduzindo sua fagocitose, mas não a sua capacidade de ativar a produção de NO, pelos macrófagos. Assim, uma medicação intracanal a base de hidróxido de cálcio associada a restaurações coronais muito bem adaptadas, para se evitar infiltração, é fundamental em tratamentos endodônticos. No entanto, os efeitos do estresse alcalino, nos *Enterococcus* alcalino-resistentes, podem prejudicar sua fagocitose, contribuindo para sua persistência na doença endodôntica.

Palavras-chave: macrófagos. *Enterococcus faecalis*. fagocitose.

ABSTRACT

Study of phagocytic ability of human macrophages against different strains of *Enterococcus faecalis*

Enterococcus faecalis (*E. faecalis*) is an microorganism present in persistent endodontic lesions, with greater resistance than other bacteria to the calcium hydroxide, an alkaline intracanal dressing which eliminate several bacterial species during endodontic treatment. The objectives of this study were: (a) to evaluate the response of *E. faecalis*, isolated from root canal, under alkaline-stress, starvation, antimicrobial resistance/susceptibility and biofilm formation on dentin disks; (b) to evaluate the phagocytic ability and the nitric oxide (NO) concentration of human macrophages against root canal *E. faecalis* isolates submitted to alkaline stress; (c) to evaluate the intensity of TLR2 and CD14 expression on the surface of macrophages challenged with the different bacterial strains. The bacterial strains used were: ATCC 4083 (CANAL 1) and a clinical strain, obtained by us, from a primary endodontic lesion (CANAL 2), both isolated from pulpless teeth; and ATCC29212, isolated from urine (URINE), was a reference for comparison. All strains were inoculated in alkaline-BHI broth for 4, 24, 48 and 72 hours. The alkaline-resistant bacteria were seeded in agar and quantified by CFU/mL. Antimicrobial susceptibility of bacterial strains, stressed or not (control) was determined by the Etest and the biovolume after biofilm formation was quantified by microscopy. To evaluate the phagocytic ability, macrophages obtained by culture of peripheral blood monocyte, were challenged with bacterial strains, stressed or not in BHI-alkaline for 30 minutes at 5:1 ratio (bacteria/macrophages) and stained with Acridine Orange. The total of macrophages with internalized bacteria and also the number of internalized bacteria per cell (<5 and ≥5) were counted. The NO concentration in the supernatants was measured by Griess reaction and the intensity of TLR2 and CD14 expression on the surface of macrophages was also analyzed by flow cytometry. Results shows less resistance to alkaline stress in root canal strains and less resistance to tested antibiotics when compared with urine enterococci. The lack of nutrient was a determining factor for the bacterial growth in all enterococci strains. The biovolume of biofilm formed by all strains were similar, and were not altered after exposure to an alkaline-BHI. In the presence of alkaline-stressed bacteria, there was

a smaller number of macrophages with internalized bacteria, when compared to the control. The NO production or the TLR2 and CD14 expression were not altered. Regardless of the strain or alkaline environment, the number of macrophages that showed ≥5 internalized bacteria per cell was higher. Without an alkaline-stress the NO production results higher in the urine strain, when compared with the root canal strains, however, was not modificated after the exposure of bacteria to alkaline-stress. We conclude that root-canal strains have different features when compared with urine enterococci, with the main differences being evident in their resistance/susceptibility to antibiotics; thus, we suggest that researches with aims directed to interpreting responses to endodontic treatment should be conducted with strains from root-canals. Besides, an alkaline environment associated to a starvation condition can reduce bacterial growth. Additionally, alterations in the structure of bacterial cell wall after alkali-stressing possibly made their recognition difficult, reducing their ability to be phagocytized, but not their ability to activate NO production. Therefore, intracanal medication with calcium hidroxyde dressing and coronal restorations, to prevent infiltration, should be critical in treatments of endodontics infections. However, the impact of alkaline stress, in alkaline-resistant enterococci, can impair the phagocytosis, contributing to their persistence in endodontic disease.

Key words: Macrophages. *Enterococcus faecalis*. Phagocytosis.

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

ATCC	*American type culture collection*
BHI	*Brain Heart Infusion*
BSA	*Bovine Serum Albumin*
CD	*Cluster of differentiation* (agrupamento de diferenciação)
CH	*Calcium hidroxyde*
CO_2	*Carbon dioxide*
DNA	*Deoxyribonucleic acid*
EDTA	*Ethylenediaminetetraacetic acid*
E. faecalis	*Enterococcus faecalis*
ELISA	*Enzyme-linked immunosorbent assay*
EPS	*Exopolysaccharide*
FCS	*Fetal calf serum*
FITC	*Fluorescein Isothiocyanate*
iNOS	*Inducible nitric oxide synthase*
LTA	*Lipoteichoic acid*
MDM	*Monocyte-derived macrophages*
MFI	*Mean fluorescence intensity*
mg	*Milligram*
mL	*Milliliter*
mm	*Millimeter*
M1	*Type I macrophage*
M2	*Type II macrophage*
NADPH	*Nicotinamide-adenine dinucleotide phosphate*
NaOH	*Sodium hydroxide*
nm	*Nanometer*
NO	*Nitric Oxide* (Óxido nítrico)
NOD	*Nucleotide oligomerization domain*
NCCLS	*National Committee for Clinical Laboratory Standards*
P	*Probability*
PAMP	*Pathogen-associated molecular pattern*
PBS	*Phosphate buffered saline*

PCR	*Polymerase chain reaction*
PE	*Phycoerythrin*
RPMI	*Roswell Park Memorial Institute medium*
rRNA	*Ribosomal ribonucleic acid*
TLR	*Toll like receptor* (Receptor tipo *Toll)*
VBNC	*Viable but not cultured*
µM	*Micromolar*
ºC	*Degree Celsius*
%	*Percent*
<	*Less-than*
≥	*Greater than or equal to*
δ	*Delta*
γ	*Gamma*
*	*Asterisk*

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1 Introduction

1 **INTRODUCTION**

Apical periodontitis is an inflammatory process in periapical tissues, associated with the presence of bacteria in necrotic dental pulp (KAKEHASHI et al., 1965; NAIR, 1997; KIRKEVANG et al., 2007), which represent the most frequently diagnosed apical odontogenic diseases in human teeth (SCHULZ et al., 2009). Polymorphonuclear leukocytes, macrophages and lymphocytes are among the cells involved in the process responsible for generating an immune response.

Macrophages are key players in immunological defense and pathological processes, mainly in host response to intracellular bacteria (WEISS; SCHAIBLE, 2015). Macrophages M1 or classically activated macrophages are related to host defense against bacteria and exhibit a pro-inflammatory phenotype; whereas, M2 or alternatively activated macrophages show an immunosuppressive profile, re-establishing homeostasis after inflammation (GORDON, 2003; GORDON; MARTINEZ, 2010). Macrophage phagocytosis is a feature of anti-bacterial host defense, in which bacterial cells are destroyed by the protective action of lysosomal enzymes, creating an environment hostile to pathogens. One of the most powerful microbicidal mechanisms is inducible *nitric oxide synthase* or iNOS, which synthesizes nitric oxide (NO) from arginine and NADPH. To enable them to perform phagocytosis, macrophages are equipped with specialized receptors that bind to pathogen-associated molecular patterns (PAMPs). These are so-called pattern recognition receptors (PRRs), including the Toll-like receptors, C-type-lectin receptors and NOD-like receptors (PROLO et al., 2014).

Macrophages are also important in activating adaptive immunity, by acting as antigen presenting cells to T lymphocytes and in the secretion of several cytokines (ABBAS; LICHTMAN; PILLAI, 2008). Furthermore, in normal periapical tissue, there are a relatively low number of macrophages predominantly distributed around the venous blood vessels (KAWASHIMA et al., 1996). In apical periodontitis, the number of macrophages around the root apex increases significantly during the active period of bone resorption. This number gradually declines when lesion growth decreases and stabilizes. Thus, there are positive relations between the size of lesions and number of macrophages presented (LIN et al., 2000).

Ricucci et al. (2006) evaluated the presence of bacteria and inflammatory cells within the root canal and in apical periodontitis from extracted human teeth without

endodontic treatment. In all cases, bacteria were observed every level of the root, coating the dentinal wall, accompanied by inflammatory infiltrate. In apical lesions, bacteria were also detected in necrotic areas of abscesses and periapical cysts. When granuloma was diagnosed, the bacteria were confined to the root canal. Among the microorganisms isolated from roots with apical periodontitis, the predominant genera were *Staphylococcus, Propionibacterium, Prevotella, Streptococcus, Pseudomonas and Fusobacterium*; showing a combination of strict and facultative anaerobic microbiota (FUJII et al., 2009).

Whereas, in endodontically treated teeth with persistent apical periodontitis, *Enterococcus faecalis (E. faecalis*) was often found associated with unsatisfactory fillings in comparison with teeth that had satisfactory filling (WANG et al., 2012). According to Pinheiro et al. (2012), the prevalence of *E. faecalis* in root canals of teeth that require endodontic retreatment is 50%, although some authors have detected the bacteria in root canals of teeth with primary endodontic infections (MOLANDER et al., 1998; SIQUEIRA; ROÇAS , 2004; RAZAVI et al., 2007).

E. faecalis is a common inhabitant of the gastrointestinal tract, accounting for 80-90% of human infections by enterococcus (JETT; HUYCKE; GILMORE, 1994 FISHER; PHILLIPS, 2009). These bacteria can colonize a variety of sites in humans including the oral cavity, perhaps through foods that appear to act as sources of transient oral colonization (RAZAVI et al., 2007; ITOH et al., 2012). In the oral cavity, the presence of *E. faecalis* in the root-canal is associated with the saliva of patients; and approximately 10-17% of patients undergoing radical endodontic treatment have this organism in the saliva (WANG et al., 2012). These bacteria can be in the planktonic form or associated with microbial biofilm attached to tooth surfaces or interfaces such as dentin and dentinal tubules (PINHEIRO et al., 2012; DAW et al., 2012).

E. faecalis shows greater resistance to calcium hydroxide dressing - the main intracanal medication used during endodontic treatment - than other bacterial cells (EVANS et al., 2002; CHÁVEZ DE PAZ et al., 2003; McHUGH et al., 2004; CHÁVEZ PAZ et al., 2007). Calcium hydroxide has antimicrobial effects on intracanal microbiota, inhibiting the majority of microorganisms, and can penetrate into dentin through the dentinal tubules, increasing the pH of root surfaces (TRONSTAD et al., 1981; McHUGH et al., 2004; LEONARDO, 2005). Furthermore, many authors have suggested that *E. faecalis* resistance to alkaline environments is involved in the

pathogenesis of persistent apical lesions (HANCOCK et al., 2001; ZOLETTI; SIQUEIRA Jr; SANTOS, 2006).

Another challenge in the treatment against infections by *E. faecalis* is the number of mechanisms by which they evade immune defenses, such as capsule synthesis, protein alterations, lipoteichoic acid (LTA), and extrapolysaccharide substances (EPS) (SÜSSMUTH et al., 2000). Nevertheless, not much is known about the potential impact of calcium hydroxide medication and *E. faecalis* interaction on the host immune response. A dysregulation of phagocytosis can be detrimental to the host defense (CARDONE et al., 2013).

Therefore, we investigated the ability of monocyte-derived macrophages (MDM) to phagocytose root-canal *E. faecalis* strains in comparison with a urine strain, either stressed with an alkaline broth, or not, simulating a CH dressing. In addition, NO concentration was measured in supernatants, and the expression of TLR2 and CD14 on the surface of macrophages was analyzed by flow cytometry.

Before this challenge, we evaluated the response of root-canal strains compared with a urine strain, to alkaline pH resistance, starvation, antimicrobial susceptibility, and biofilm formation, under different growth conditions.

2 Articles

2 **ARTICLES**

The articles presented in this thesis were written according to instructions and guidelines for article submission presented in Journal of Endodontics.

2.1 ARTICLE 1 -

Title: Root canal *Enterococcus faecalis* upon in vitro alkaline stress alters pH, antimicrobial susceptibility and growth

Introduction: The aim of the present *in vitro* study was to evaluate the effect of alkaline stress on the survival and response of root-canal strains of *Enterococcus faecalis*, including growth, pH alteration, antimicrobial susceptibility and biofilm formation. **Methods:** Three strains of *E. faecalis,* two isolates from root-canals and one urine isolate (as reference for comparison), were inoculated in alkaline-BHI broth for 4, 24, 48 and 72 hours. A BHI medium with pH of 7.2 was used as control. Afterwards, the alkaline-resistant bacteria were seeded in agar and quantified by CFU/mL. Antimicrobial susceptibility of *E. faecalis* strains against 3 antibiotics was determined by the Etest®. Furthermore, biovolume after biofilm formation from different isolates was quantified by using a confocal scanning laser microscope. Statistical analysis was performed with non-parametric tests. **Results:** All *E. faecalis* strains presented reduced growth at pH 9.5 or higher, when compared with control medium pH ($p<0.05$), while lack of nutrient was a determining factor for bacterial growth in advanced stages. Root-canal strains were more susceptible to antibiotics than the urine strain. When comparing root-canal isolates, the susceptibility to clindamycin was different. The biovolume of biofilms formed by all strains was similar, and were not changed upon exposure to an alkaline medium. **Conclusions:** The association between an alkaline environment and starvation condition can reduce bacterial growth, therefore intracanal medication with CH dressing and coronal restorations to prevent infiltration should be critical in treatments of contamined pulp necrosis.

Key Words

Enterococcus faecalis; Alkaline resistance; antimicrobial resistance; environmental changes; biofilm

Introduction

Enterococcus faecalis (*E. faecalis*) is an enteric Gram-positive, non-spore-forming, catalase-negative, facultative anaerobic bacteria belonging to the lactic acid bacteria group, capable of surviving in hostile environments, including extreme temperatures and pHs (1,2).

It commonly infects the urinary tract, abdomen, bloodstream, endocardium and *in situ* foreign devices. Although few viable enterococci are observed in oral samples from healthy individuals, these bacteria play an important role in chronic endodontic treatment failure (3,4,5,6).

Is well-known that *E. faecalis* probably reaches the oral cavity through foods such as meats and cheese (5). This may explain its presence in caries lesions, apical periodontitis of untreated canals (5,8,9), and persistent periapical lesions after root canal treatment with calcium hydroxide (CH) used as intracanal medication, since *E. faecalis* survives at alkaline pH (9-13). Nowadays, antibiotics are added into endodontic sealers to enhance the antimicrobial effect on *E. faecalis* (14). Systemic antibiotic therapy is infrequently used to treat apical periodontitis (15), however it has specific indications as a prophylactic method for patients at risk for infective endocarditis, and for some periapical abscesses (16).

The response to alkaline stress varies for different bacteria, even for different strains of *E. faecalis* found in the oral cavity (13,17). Understanding the response of root-canal strains, after exposure to alkaline pH, may be important to improve the impact of intracanal medications on endodontic treatments. Adittionally, there are no studies comparing the responses of *E. faecalis* isolated from root-canals and ATCC 29212, a reference ATCC® strain isolated from urine, widely used in endodontic-related research. We evaluated in vitro if survival, growth, pH of the medium, the antibiotic susceptibility and biofilm formation by root-canal enterococcus are altered after alkaline stress. The null hypothesis tested was that alkaline-stressed *E. faecalis* strains would present similarity to non-stressed *E. faecalis* in relation to alkaline pH resistance, antimicrobial susceptibility and capacity in biofilm formation.

Materials and methods

Bacterial strains

The *E. faecalis* bacterial strains ATCC 4083 (CANAL 1), isolated from a pulpless tooth, were obtained from the American Type Culture Collection (ATCC®), and stored at -80°C until use. Another clinical strain (CANAL 2) was isolated from a patient's pulpless tooth presenting primary infection, at the Endodontic Clinic of the authors' University. As reference for comparison, *E. faecalis* strain ATCC 29212 (URINE), isolated from urine, was used, which was obtained from ATCC® and stored at -80°C until use. Previously, the Institutional Ethics Committee of this University approved the study and the patient signed a term of informed consent (Process #CAAE 46839215.0.0000.5417). The isolation protocol followed the Pinheiro *et al.* (10) and Ferreira *et al*. (18) methodology.

The polymerase chain reaction (PCR) with 16s rRNA specific for this species was performed to confirm enterococcal identification of the CANAL 2 strain. The DNA was purified with the QIAamp DNA Mini kit (Qiagen, Valencia, California, USA) according to the manufacturer's guidelines. The sequence of primers for the 16S rRNA gene used to confirm the isolates found *E. faecalis* PCR, as described by Sedgley *et al*. (19): Forward - CCGAGTGCTTGCACTCAATTGG; Reverse - CTCTTATGCCATGCGGCATAAAC; resulting in an amplification fragment containing 138 base pairs.

Culture conditions and pH Resistance of strains by CFU quantification

Innitially, aliquots of each *E. faecalis* strain were taken from storage at -80°C and incubated overnight at 37°C in 4mL of brain–heart infusion (BHI). Subsequently, these bacterial strains were inoculated into test tubes containing neutral BHI (CONTROL) and alkaline-BHI broth, buffered at pH 9.0; 9.5; 10.0; 10.5; 11.0; 11.5 and 12.0 with 5M NaOH using a pH-meter. The strains were incubated at 37°C for 4 hours; growth was assessed by spectrophotometry with the McFarland 0.5 standard, followed by diluting and plating on M-*Enterococcus* agar. After 2 days, the number of colony forming units (CFU/mL) was quantified. As all *E. faecalis* strains presented reduced growth in alkaline-BHI buffered at 9.5 or higher, pH 9.5 was selected to evaluate pH resistance of *E. faecalis* strains incubated at 37°C, for 4, 24, 48 and 72 hours, followed by diluting and plating on M-Enterococcus agar.

Two conditions were evaluated: with medium exchanged every 24 hours, and without renewing culture medium until completing 24, 48 and 72 hours of culture (starvation condition). The same procedure was performed using neutral BHI (CONTROL). Subsequently, dilutions were seeded on M-*Enterococcus* agar and the number of CFU/mL was counted after 48 hours. Each experiment was performed in triplicate for each strain.

pH Alteration

Throughout all evaluations of pH resistance *of E. faecalis* strains, the alkaline-BHI broth was collected on conclusion of every test, and pH was measured by using commercially available colorimetric test strips (Macherey Nagel GmbH & Co. KG). The images obtained from colorimetric strips were compared with the scale provided by the manufacturer by means of Photoshop® Software.

Antimicrobial susceptibility tests

The antimicrobial susceptibility/resistance to ampicillin, clindamycin and amoxicillin plus clavulanate of each *E. faecalis* strain was tested after incubation in alkaline-BHI broth, buffered at pH 9.5 for 4 hours, and determined by the minimal inhibitory concentrations using the Etest® (AB Biodisk, Solna, Sweden). The *E. faecalis* strains were suspended in sterile phosphate-buffered saline (PBS) equivalent to a 0.5 McFarland standard and streaked with a cotton wool swab on the surface of Mueller-Hinton agar plates. To avoid drug interactions, only one strip per plate was placed and incubated at 37°C for 24 h. The same procedure was performed in the CONTROL group after incubation, using neutral BHI. Susceptibility to each antibiotic was interpreted as the intersection of the elliptical zone of growth inhibition reaching around the strip scale, according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS). All the *E. faecalis* strains were tested in duplicate.

Quantification of Biofilm formed on bovine dentin

Dentin disks (made by using a bone trephine 4.2mm in diameter), were obtained from the roots of fully developed bovine central incisors. They were polished to allow better adaptation to the microscope plate. Before sterilization by autoclaving at 121ºC for 20 minutes, the smear-layer was removed from the dentin disks by using

17% ethylenediaminetetraacetic acid (EDTA) solution.

Biofilms were created as described in a previous study (20). Briefly, one dentin disk per well was kept submerged in a 24-well culture plate containing 0.9 mL sterile BHI medium and 0.1 mL inoculum of each *E. faecalis* strain. The culture plates were incubated at 37ºC for 21 days, with complete replacement of the BHI culture medium every 2 days, without the addition of bacteria. Dentin disks with established biofilms were incubated in alkaline-BHI at pH 9.5 for 4 hours; the same procedure was performed using neutral BHI (CONTROL).

Afterwards the dentin disks were placed on a glass slab and stained with 50 mL live/dead reagent (BacLight Bacterial Viability Kit L7012; Molecular Probes, Inc). They were incubated at room temperature for 15 minutes before being analyzed under a confocal scanning laser microscope (Leica TCS-SPE; Leica Microsystems GmbH, Mannheim, Germany) at 40x magnification by using the Leica Application Suite-Advanced Fluorescence software (LAS AF; Leica Microsystems GmbH). The images of 4 fields of each sample were analyzed with BioImage_L software (21) and the total biovolume (μm^3), including live and dead cells (%), was obtained.

Statistical analysis

Kruskal-Wallis and Dunn's multiple comparison tests were used and the level of significance was set at $P < 0.05$. Analyses were performed by the GraphPad Prism version 5.0.

Results

pH Resistance

Aliquots of three *E. faecalis* strains were incubated overnight at 37°C in 4mL of BHI and were then exposed to alkaline-BHI broth (pH: 9.0; 9.5; 10.0; 10.5; 11.0; 11.5; 12.0) for 4 hours. As shown in Figure 1, as from pH 9.5 all alkaline-BHI significantly resulted in a lower bacterial growth, compared with the control. This influence in growth tended to be directly proportional to the increase in alkalinity of the broth.

Figure 2 shows the results of growth after 4, 24; 48 and 72 hours at pH 9.5. Under alkaline stress, the three strains showed significantly reduced growth in the number of CFU/mL, compared with their corresponding control group throughout 4 and 24 hours. After 48 and 72 hours, with medium exchange, bacterial growth was

not significantly reduced in alkaline-BHI when compared with each control, except in CANAL-2 strain/48h. Nevertheless, in periods without renewed culture medium until completing 48 and 72 hours of culture, starvation seemed to be the decisive factor in growth reduction (Figures 2e and 2f), irrespective of alkaline stress.

pH Alteration

The more alkaline the initial culture medium was, the lower was the bacteria ability to decrease the pH (Table 1). Reduction in pH was directly proportional to increase in the time of incubation without any difference between the *E. faecalis* strains for each individual period (Figure 3).

Antimicrobial Susceptibility test

No effect on antibiotic susceptibility/resistance of all enterococcal strains was observed after exposure to 9.5 alkaline-BHI broth. Among the isolates, the root-canal strains showed a different profile when compared with the URINE strain that was resistant to all tested antibiotics. CANAL 2 strain was susceptible only to antibiotics of the penicillin group, while CANAL 1 strain was susceptible to all tested antibiotics (Table 2).

Biofilm analysis by Confocal Scanning Laser Microscopy

Biofilm was formed on dentin substrate in all the *E. faecalis* strains at 21 days. Established biofilm exposure to alkaline-BHI broth for 4 hours did not significantly change the total biovolume (Figure 4) or the percentage of live/dead cells (data not shown). The total biovolume of biofilms was similar among all strains, irrespective of alkaline stress for 4 hours.

Table 1. pH decrease in alkaline-BHI medium after 4h of *E. faecalis* strains incubation. Results are expressed as the mean of pH decrease from three independent experiments.

E. faecalis	Alkaline-BHI						
	Initial pH						
	9	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**
URINE	*7,25*	*8,25*	*9,25*	*9,75*	*10*	*10,75*	*11,25*
CANAL 1	*8,25*	*8,75*	*9,25*	*10,25*	*10,75*	*11*	*11,75*
CANAL 2	*8,5*	*9*	*9,25*	*10,25*	*10,75*	*11,5*	*12*

Table 2. Antibiotic susceptibility test (Etest®) of three *E. faecalis* strains previously cultured for 4 hours in alkaline-BHI medium (pH9.5), and neutral medium (control). S or R indicates that microorganism is clinically susceptible or resistant, respectively, to an specific antibiotic.

	Alkaline stress			Control		
	URINE	CANAL 1	CANAL 2	URINE	CANAL 1	CANAL 2
AMPICILLIN	**R**	**S**	**S**	**R**	**S**	**S**
AMOXICILLIN +CLAVULANATE	**R**	**S**	**S**	**R**	**S**	**S**
CLINDAMYCIN	**R**	**S**	**R**	**R**	**S**	**R**

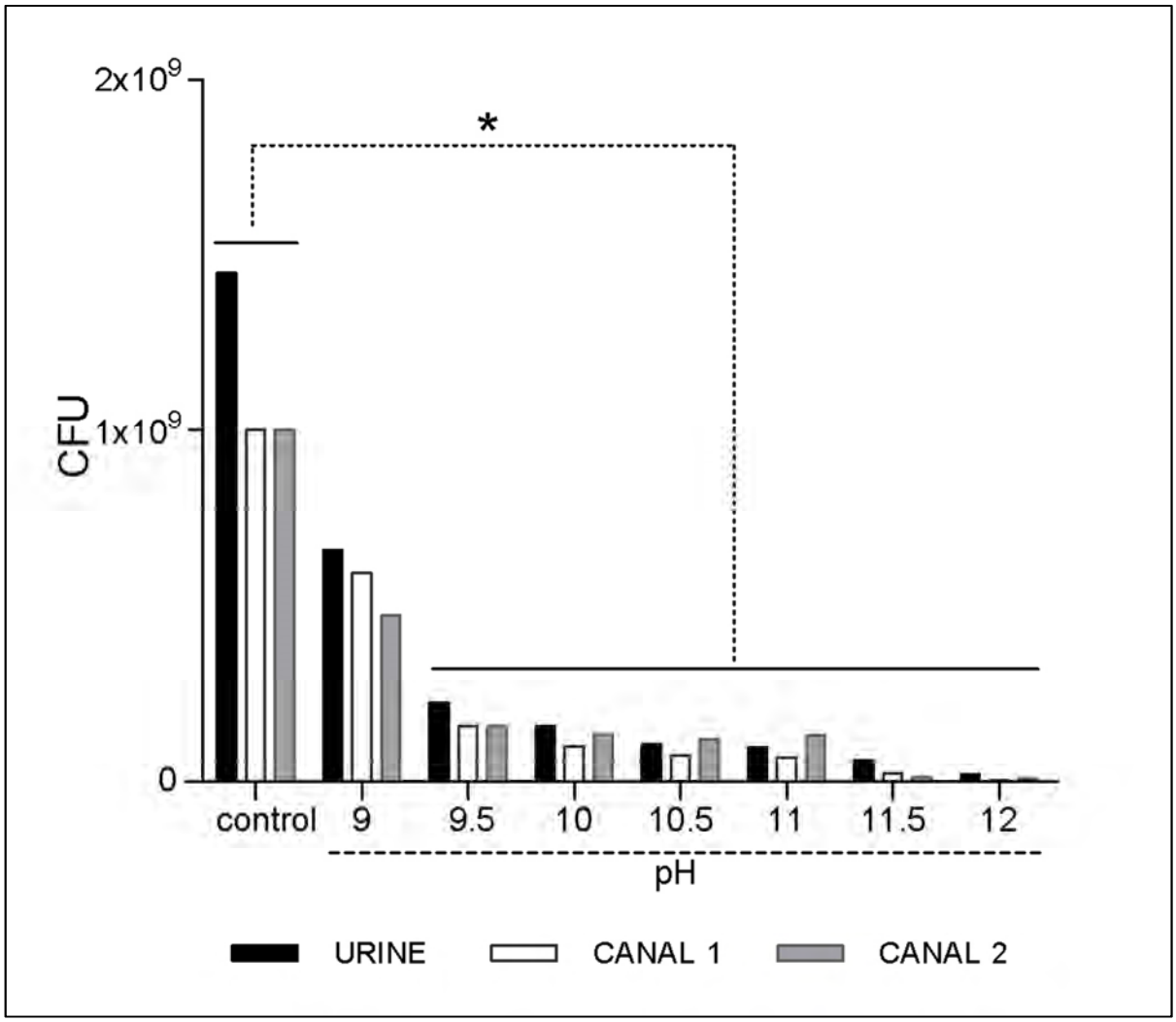

Figure 1. Bacterial growth of three *E. faecalis* strains, measurement of CFU, after alkaline stress of 4 hours in differents alkaline-BHI medium (pH from 9 to 12) and neutral medium (control). This figure shows the mean from three independent experiments. ***** P<0.05.

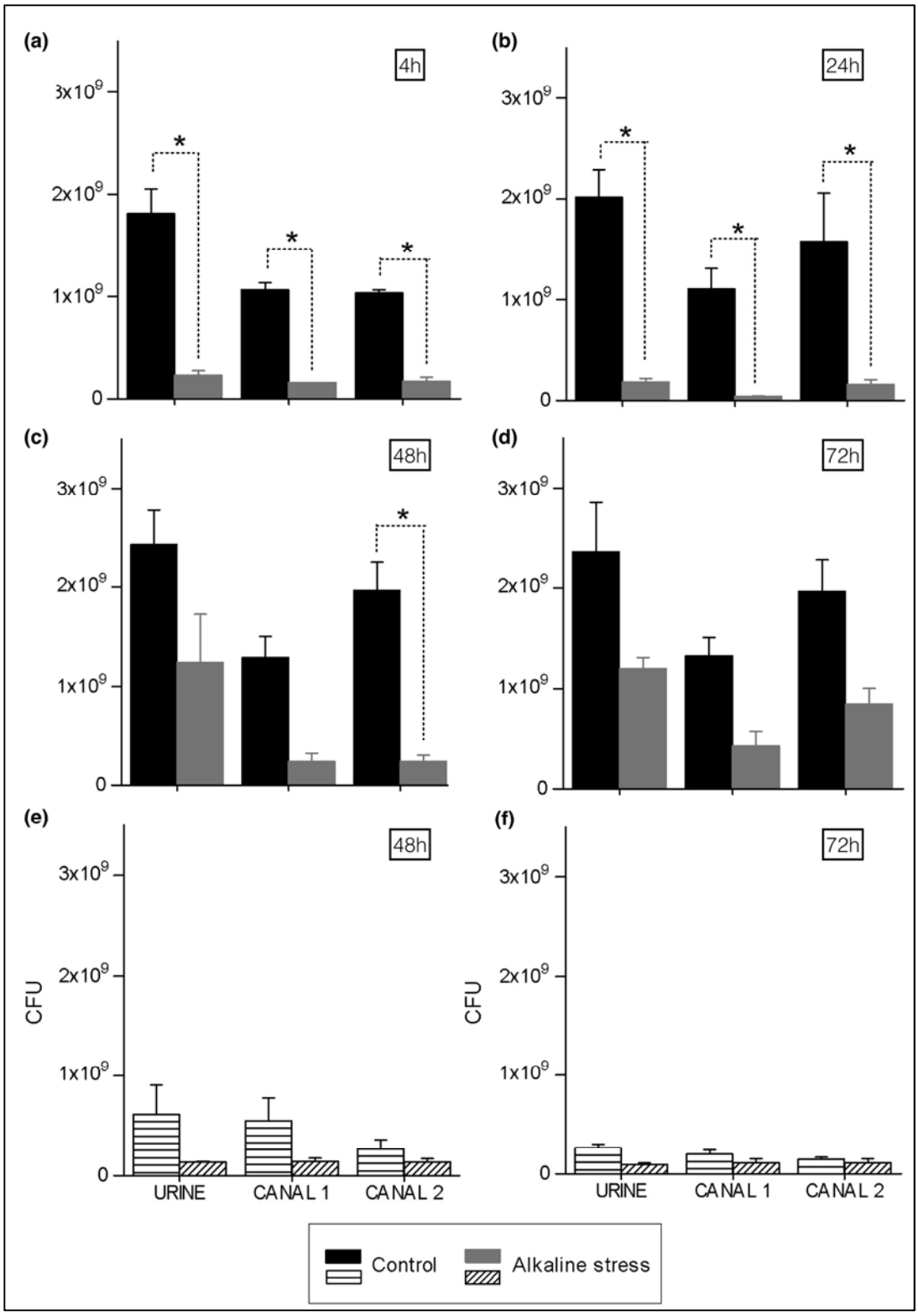

Figure 2. Bacterial growth of three *E. faecalis* strains, after alkaline stress in buffered-BHI of pH 9.5 and neutral medium (control), for 4(a); 24(b); 48(c) and 72(d) hours, by CFU quantification. The culture medium was renewed, or not (e and f), after every 24 hours; This figure shows the mean ±SD from three independent experiments.*P<0.05.

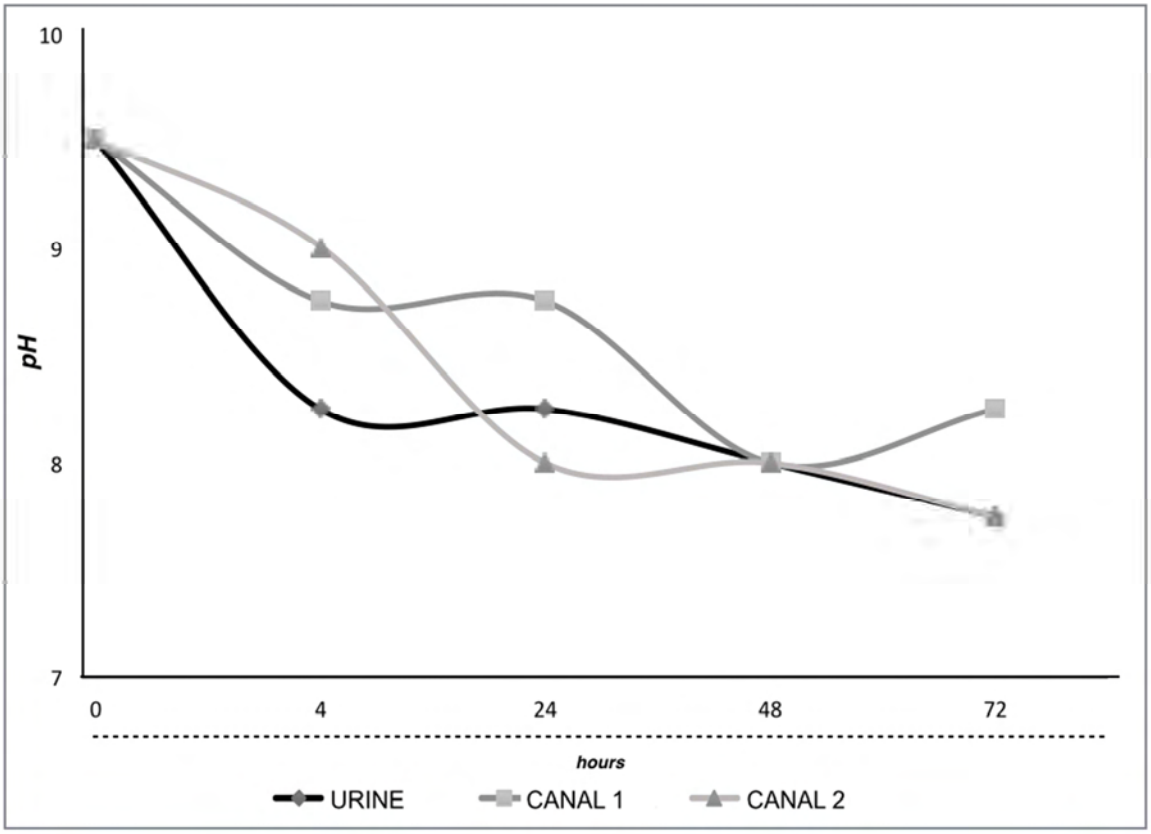

Figure 3. pH decrease after culture of three *E. faecalis* strains in alkaline-BHI medium buffered to pH 9.5, after 4, 24, 48 and 72 hours, without renewed culture medium. The pH was measured by colorimetric test strips. The results represent the mean of three independent experiments.

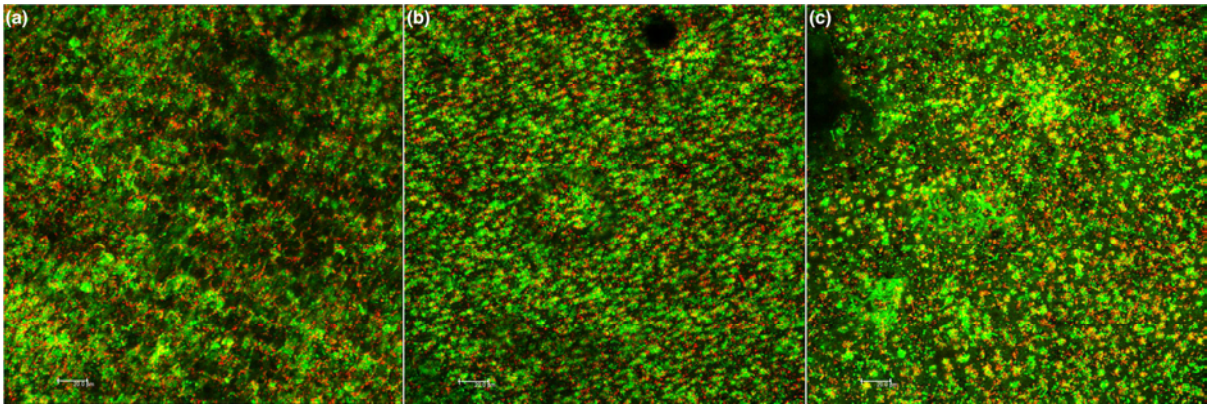

Figure 4. Biofilm formation of three *E. faecalis* strains on bovine dentin substrate at 21 days in BHI medium: (a) URINE; (b) CANAL 1 and (c) CANAL 2. Original magnification, x40. The Live/Dead BacLight reagent stains the viable cells in green and those with damaged membranes in red.

Discussion

Irrespective of environment, bacteria need to maintain their pH against extreme extracellular pH to survive (22,23). Growing in agar buffered to pH 9.0 or higher, the genus *Enterococcus* is the most alkaline-resistant, compared with other bacteria (23,24). Consequently, *E. faecalis* is resistant to killing by CH, an intracanal medication usually used in endodontics (3,4,11,12,13).

In fact, we observed, in the present study, that the pH ≥ 9.5 did not lead to death of all the root-canal bacteria, but resulted in fewer CFU of *E. faecalis* than those who were not stressed by the alkaline pH. This CFU reduction was directly proportional to the increase in alkalinity of the medium. Then, these alkaline environments do not kill all the bacteria in short time intervals and, as expected, pH values lower than 9.5 did not prevent *E. faecalis* ability to grow. If compared with URINE strain, the root-canal strains tended to be a more susceptible to alkaline stress, in regard to growth profile.

Our results also showed decreased bacterial proliferation in starvation periods among all *E. faecalis* strains, without difference among them. *E. faecalis* enters a non-growth state in starvation periods, and is capable of living at low metabolic rates (11,12,13,25). Therefore, the cells in this state may be viable, but can no longer be cultured in medium, probably representing a programmed physiological response to survive during potentially lethal environmental stresses, such as starvation and pH stress (26,27). When they consider the environment favorable, bacteria will reactivate their metabolism (25). Following this reasoning, the survival of bacteria in persistent root canal infections could possibly be conditioned by both resistance to alkaline pH and decreased nutrients, accompanied by changes in gene expression (28,29). We believe that after root-canal treatment, residual bacteria are in a viable but not cultivable state, conditioned by the absence of nutrients and antimicrobial ability of endodontic sealers. However, if nutrient infiltration occurs, bacterial metabolism is reactivated, perhaps resulting in persistent lesions.

One strategy used by *E. faecalis* to survive in alkaline environments is their capacity to lower the medium pH, by pumping protons across the cytoplasmic membrane. The potassium is the major cellular cation enabling enterococci to regulate pH (11,17,24). In the present work, although less effective than the urine

strain, the root-canal *E. faecalis* strains were able to lower the pH. This bacterial mechanism also explains why these microrganisms can survive in alkalinized environments, such as the root canals treated with CH.

Also, these bacteria, after alkaline stress, maintained their capacity to form biofilm on dentin substrate, as observed in other studies (13,30). Thus, 21-day biofilm biovolume was not influenced by alkaline stress or percentage of live/dead cells in biofilm, after the same stress, suggesting increased resistance of biofilms compared with planktonic cultures.

Taken together, our results indicate that the association between an alkaline environment and starvation condition can reduce bacterial growth, therefore intracanal medication with CH dressing and coronal restorations to prevent infiltration should be critical in treatments of pulpal necrosis with apical periodontitis.

Dental pulp removal during root canal treatment prevents the use of systemic antibiotics, nevertheless, antibiotics added to endodontic sealers can enhance their antimicrobial effect by reducing microbial concentration (14). Penicillins have traditionally been recommended as a first-line against most bacteria, with low adverse effects (15,31,32). Our results revealed that only root-canal strains (CANAL 1 and CANAL 2) were susceptible to penicillins in vitro. *E. faecalis* recovered from urine was resistant to antibiotics recommended for dental procedures, suggesting possible differences between strains of the same species obtained from different environments. Moreover, some virulence factors are higher in medical *E. faecalis* strains, when compared with food isolates (33), probably determining less aggressiveness of food isolate strains and possibly root-canal strains, based on their food origin.

Despite *E. faecalis* intrinsic resistance to clindamycin (32), one of the root-canal strains was susceptible to this antibiotic. This could be explained by the participation of a gene associated with resistance to clindamycin, which would be less expressed in animal strains (34), suggesting the possible animal origin of this root-canal strain, differently from the URINE strain that conserved this intrinsic resistance.

With respect to these differences between the bacteria originating root-canal and those of urine, we suggest that researches with aims directed to interpreting responses to endodontic treatment should be conducted with ATCC® strains from root-canals or those isolated from root-canals during endodontic procedures, since the clinical extrapolation of URINE does not fully refine the characteristics and behavior of these bacteria at endodontic level.

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2.2 ARTICLE 2-

Title: Alkaline-stress impairs the phagocytosis of *Enterococcus faecalis* by human macrophages

Introduction: The ability of monocyte-derived macrophages (MDM) to phagocytose and to produce NO was tested against root-canal strains of *E. faecalis* submitted to alkaline stress. **Methods:** *E. faecalis* were stressed with alkaline-BHI broth and incubated in vitro with the cells at 1:5 ratio of cell/bacteria. Phagocytosis was analyzed by fluorescence microscopy using acridine orange stain, and NO concentration was measured in supernatants. The expression of TLR2 and CD14 on the surface of macrophages was also analyzed by flow cytometry. Root-canal strains were compared with urine enterococci. **Results:** The alkaline-stress significantly impairs phagocytosis by MDM of the *E. faecalis* strains analyzed, except in ATCC4083 isolated from a pulpless tooth. However, the NO production and expression of CD14 and TLR2 were unchanged. Comparing different strains, the urine type resulted in higher NO levels than root canal strains. **Conclusion:** Alterations in the structure of bacterial cell wall after alkali-stressing possibly made their recognition difficult, reducing their ability to be phagocytized, but not their ability to activate NO production by MDM. This impaired phagocytosis of *E. faecalis* could contribute to their persistence if they reach the periapical tissues.

Key-words: *Enterococcus faecalis***,** macrophages**,** nitric oxide, Toll-like receptor 2, phagocytosis

Introduction

In apical periodontitis, the predominant genera are *Staphylococcus, Propionibacterium, Prevotella, Streptococcus, Pseudomonas and Fusobacterium*; showing a combination of strict and facultative anaerobic microbiota (1). Whereas, in endodontically treated teeth with persistent apical periodontitis, *Enterococcus faecalis (E. faecalis*) is often found associated with unsatisfactorily filled teeth compared with those with satisfactory filling (2). Some studies have suggested that viable food-borne *E. faecalis,* from products such as cheese and meat, can leak into the pulp chamber space and persist in periapical lesions (3,4,5).

Calcium hydroxide (CH) is the most popular antimicrobial root canal medication with an alkaline pH used to inhibit bacterial growth during endodontic treatment. Alkaline medication may cause an increase in gene transcription and expression associated with an increased cytoskeletal function and decrease in peptoglycan synthesis in bacteria (6,7). Even CH can inactivate LTA (lipoteichoic acid) in gram-positive bacteria, reducing their ability to induce TNF-α in macrophages, resulting in attenuation of the host response and possibly reducing the progress of apical periodontitis, highlighting the beneficial effects of CH in endodontic treatment (8).

Nevertheless, studies have shown that *E. faecalis* can withstand harsh environmental conditions, and has great resistance to direct exposure to CH dressing, which could determine the prevalence of this bacteria in persistent periapical lesions (9,10,11,12). Some enterococci virulence genes are possibly related to persistence endodontic infections (13).

Macrophages are prevalent in endodontic infections and they are the main cells of the immune system that destroy the microorganisms. They express Toll-like receptors, type I transmembrane proteins that recognize bacterial structures and trigger inflammatory signaling pathways, activating distinct transcription factors, such as A nuclear factor-kappa B (14,15,16,17). Macrophages may also express CD14, the well-known surface binding protein for LPS (lipopolysaccharide) recognition, and participate in responses elicited by gram-positive bacteria (18,19). Macrophages also produce reactive species to eliminate the internalized microorganisms. Nitric oxide (NO) is an intermediate reactive nitrogen oxide species, synthesized by nitric oxide synthase activity, an enzyme present in macrophages. Substances generated from the reaction of NO molecules with themselves or with other molecules, such as reactive oxygen species, act on several microorganisms, including *E. faecalis* (20,21,22).

Despite the clinical importance of enterococci in endodontic failure, and given that they are capable of surviving at alkaline levels resulting from application of CH (the most commonly used intracanal dressing), little is known about how the host immune system responds to enterococcal infections, under alkaline stress.

Considering the foregoing, this study evaluated phagocytosis, NO production and intensity of TLR2/CD14 expression in monocyte-derived macrophages (MDM) cultured with alkaline-stressed *E. faecalis*.

Material and methods

This study was approved by Ethics Committee and by the volunteers enrolled. The patients signed a term of free and informed consent (Process #CAAE 46839215.0.0000.5417).

Bacterial strains

The ATCC 4083 (CANAL 1) isolated from a pulpless tooth was obtained from American Type Culture Collection (ATCC®); and another root canal strain from a pulpless tooth presenting primary infection of a patient at the Endodontic Clinic of the authors' University (CANAL 2). As reference for comparison, the *E. faecalis* isolated from urine and obtained from ATCC®, ATCC 29212 (URINE), was used. All the strains were stored at -80°C until use. The isolation protocol used for the CANAL 2 strain was the Pinheiro *et al.* (23) and Ferreira *et al*. (24) methodology; and to confirm their enterococcal identification, polymerase chain reaction (PCR) with 16s rRNA specific for this species was performed, by using the sequence of primers according to Sedgley et al. (25): Forward - CCGAGTGCTTGCACTCAATTGG; Reverse: CTCTTATGCCATGCGGCATAAAC. The PCR reaction resulted in amplification of a fragment containing 138 base pairs. Previously, the DNA was purified with QIAamp DNA Mini kit (Qiagen, Valencia, California, USA) according to the manufacturer's guidelines. Enterococci strains were cultured using brain-heart infusion (BHI) and stored at -80 °C.

Isolation and culture of Human Monocyte-Derived Macrophage (MDM)

Heparinized whole blood (30 mL) was obtained from 15 healthy volunteers and the peripheral blood monocytes were separated using Histopaque 1083 gradients (Sigma-Aldrich Brazil Ltda., São Paulo, Brazil). Immediately, $1x10^6$ cells/mL were counted with a hemocytometer using neutral red, and cultured in a 24-well culture plate, containing sterile spherical glass coverslips (13 mm in diameter). After 2 hours in 5% CO2 at 37ºC, the non-adherent cells were removed by aspiration. Finally, the monocytes were incubated in 1 mL of complete medium (RPMI 1640 + 10% heat-inactivated fetal calf serum [FCS] + 1% penicillin/streptomycin). After 7 days, the viability of MDM was assessed by trypan blue exclusion (>94%).

Phagocytosis assay

The MDM cultured in a 24-well plate were infected with *E. faecalis* at a cell/bacteria ratio of 1:5 at 37ºC for 30 minutes in an atmosphere of 5% CO_2. For this assay, all strains were divided into 2 groups, the first inoculated in neutral BHI (CONTROL), and the second group (ALKALINE-STRESS), with alkaline-BHI broth buffered at pH 9.5 with 5M NaOH, for 4 hours, just prior to the experiment.

After this, the wells were washed three times with sterile phosphate-buffered saline (PBS) to remove the extracellular bacteria. Cells were fixed with 4% paraformaldehyde for 30 minutes; and finally washed two times with sterile PBS. The internalized bacteria were observed using a fluorescent-quenching technique described in a previous study (26). Briefly, 0.05mg/mL of acridine orange was used for 15 minutes to stain the bacteria and the MDM. Immediately, the fluorescence of remaining extracellular bacteria was quenching by crystal violet stain. The coverslips with adherent MDM were carefully removed and placed on glass slides by using a mounting medium (VECTASHIELD®).

The slides were analyzed by Axiostar HBO plus 50/AC Fluorescence Mycroscopy (Carl Zeiss, Germany), and phagocytosed bacteria were quantified considering the number of internalized bacteria per cell (<5 and ≥5), at 100x magnification in 20 randomly selected fields. The results were obtained from the average of blood samples from 15 volunteers.

NO production

MDM were infected as described above, and the supernatants recovered after 24 hours of stimulation with bacteria (CONTROL and ALKALINE STRESS). The NO production was obtained by measuring the nitrite levels by Griess reaction. The absorbance was measured in a spectrophotometer at 540 nm and the values expressed in μM.

Intensity of TLR2 and CD14 expression

The intensity of mean fluorescence intensity (MFI) of TLR2 and CD14 on the surface of the MDM stimulated with *E. faecalis* was quantified by Flow Cytometry. MDM were infected as previously described for 30 minutes. Briefly, after trypsinization and incubation with PBS+BSA 2% for 30 minutes, the cells ($1x10^6$) were labeled with FITC-conjugated mouse anti-human CD14 antibodies (BD

Bioscience, cat number 555397) and PE-conjugated mouse anti-human TLR2 (eBioscience, cat number 12-9922) antibodies at 4ºC for 1 hour. Subsequently, labeled-cells were fixed in paraformaldehyde 4% and analyzed using a FACSCanto II flow cytometer (BD-Becton-Dickson and Company, San Diego, CA, USA) and FlowJo software (TreeStar, Ashland, OR, USA). Aliquots of cells were also seeded on glass slides and visualized with a confocal scanning laser microscope (Leica TCS-SPE; Leica Microsystems GmbH, Mannheim, Germany).

Statistical analysis

Friedman and One-Way ANOVA tests were used, and the level of significance was set at $P < 0.05$. Analysis was performed by the GraphPad Prism version 5.0 program.

Results

Phagocytosis of E. faecalis

Alkaline stress affected the phagocytosis of *E. faecalis* strains by MDM after 30 minutes. Significant reduction in the number of MDM with internalized bacteria was observed in assays performed with CANAL 2 or URINA strains, after stress, when compared with their matched CONTROL (Figure 1A).

Moreover, phagocytosis occurred in different ways for each strain of *E. faecalis*. In the CONTROL group, the number of MDM with internalized bacteria were statistically lower in assays with root-canal strains than those performed with URINE strains.

These differences were not observed in assays of ALKALINE STRESS group (Figure 1A). The green stained-bacteria were easily observed inside the MDM cytoplasm after fluorescent acridine orange staining (Figure 1 B-C). In Figure 1D, the cells tagged with PE-labelled antibody against TLR2 and FITC-labelled antibody against CD14 were clearly visible by immunofluorescence, with marked expression of their surface molecules. Irrespective of the strain used for challenge, a higher percentage of MDM showed ≥5 internalized bacteria per cell than <5 internalized bacteria. Interestingly, this proportion was not altered by alkaline-stress of the enterococci before infection (Figure 2).

NO production

After 24 hours, MDM infected with root-canal strains showed diminished NO production in comparison with those cultured with URINE strains. The alkaline-stressed bacteria showed no change in NO production when compared with their corresponding CONTROL groups (Figure 3).

TLR2 and CD14 expression

Approximately 64.2% of the total number of MDM obtained in our cultures expressed CD14 molecules on their surface, of which 22.8% were also TLR2-positive cells. Among the total sample, 10.4% of MDM were TLR2-positive cells. In every MDM population, the mean fluorescence intensity (MFI) of the respective receptors was similar irrespective of the strain. Moreover, the challenge with alkaline-stressed enterococci did not change the intensity of TLR2 and CD14 expression (MFI) by human macrophages (Figure 4).

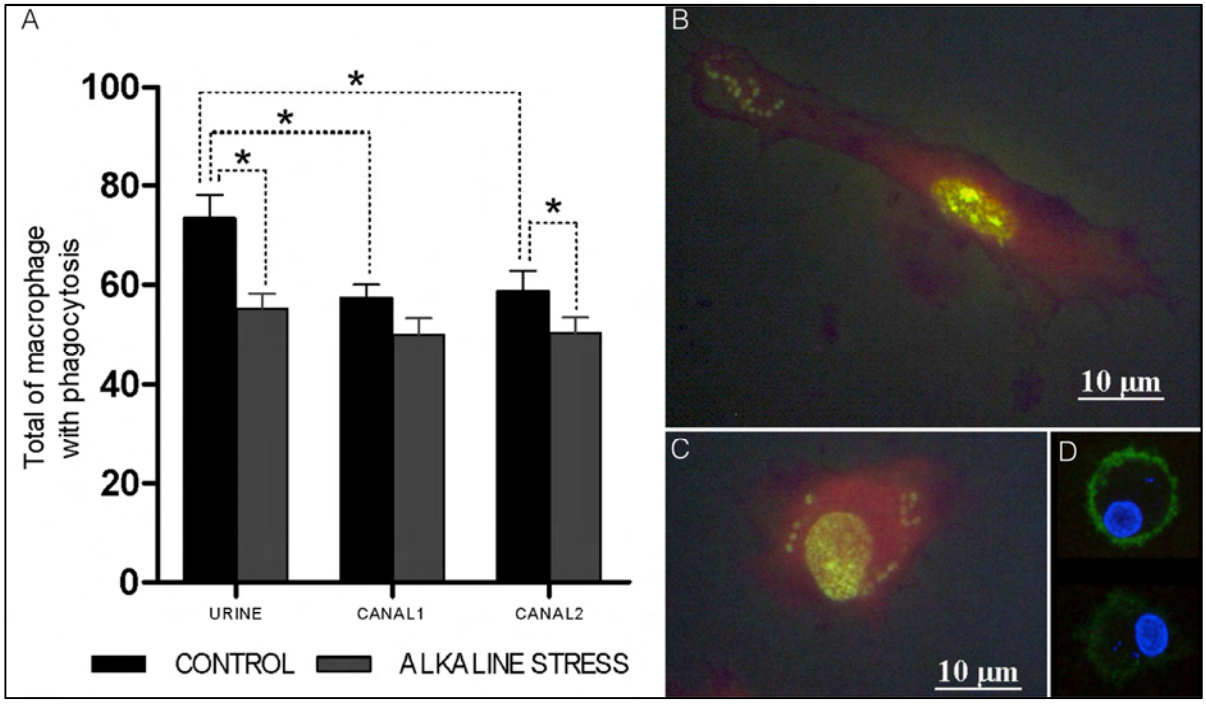

Figure 1. (A) Number of MDM with internalized bacteria after infection with different strains of *E. faecalis*, either stressed (ALKALINE STRESS) or not (CONTROL) with alkaline-BHI. * represents $p<0.05$. (B and C) *E. faecalis* internalized (green fluorescence) by MDM 30 minutes after infection. The stain of extracellular bacteria was quenched by crystal violet stain. (D) MDM labeled with DAPI (nucleus), expressing TLR2-PE and CD14-FITC.

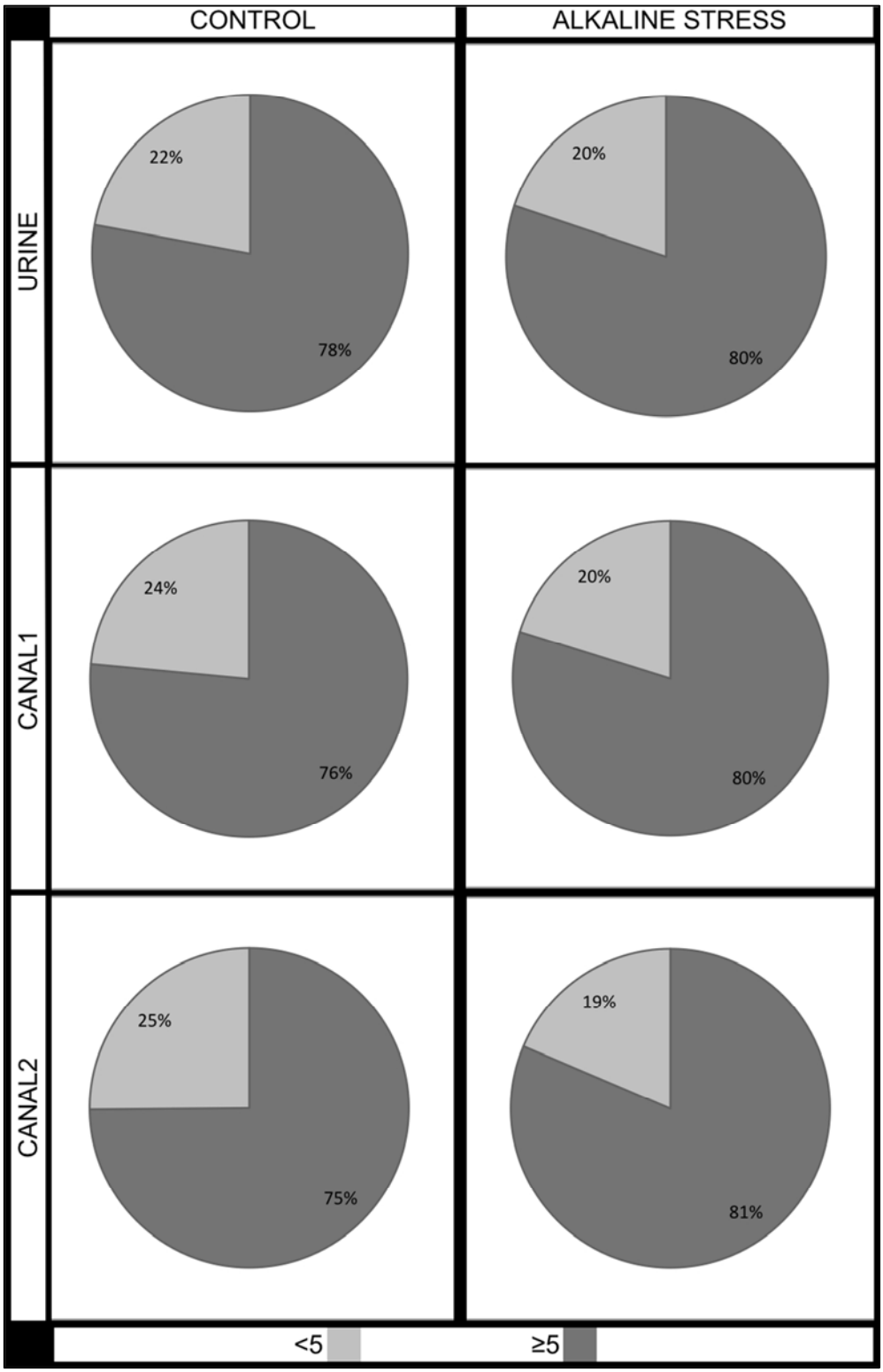

Figure 2. Percentage of MDM presenting <5 or ≥5 internalized bacteria per cell after 30 minutes. The three different strains of *E. faecalis*, either stressed (ALKALINE STRESS) or not (CONTROL) with alkaline-BHI, were quantified at 100x magnification in 20 randomly selected fields.

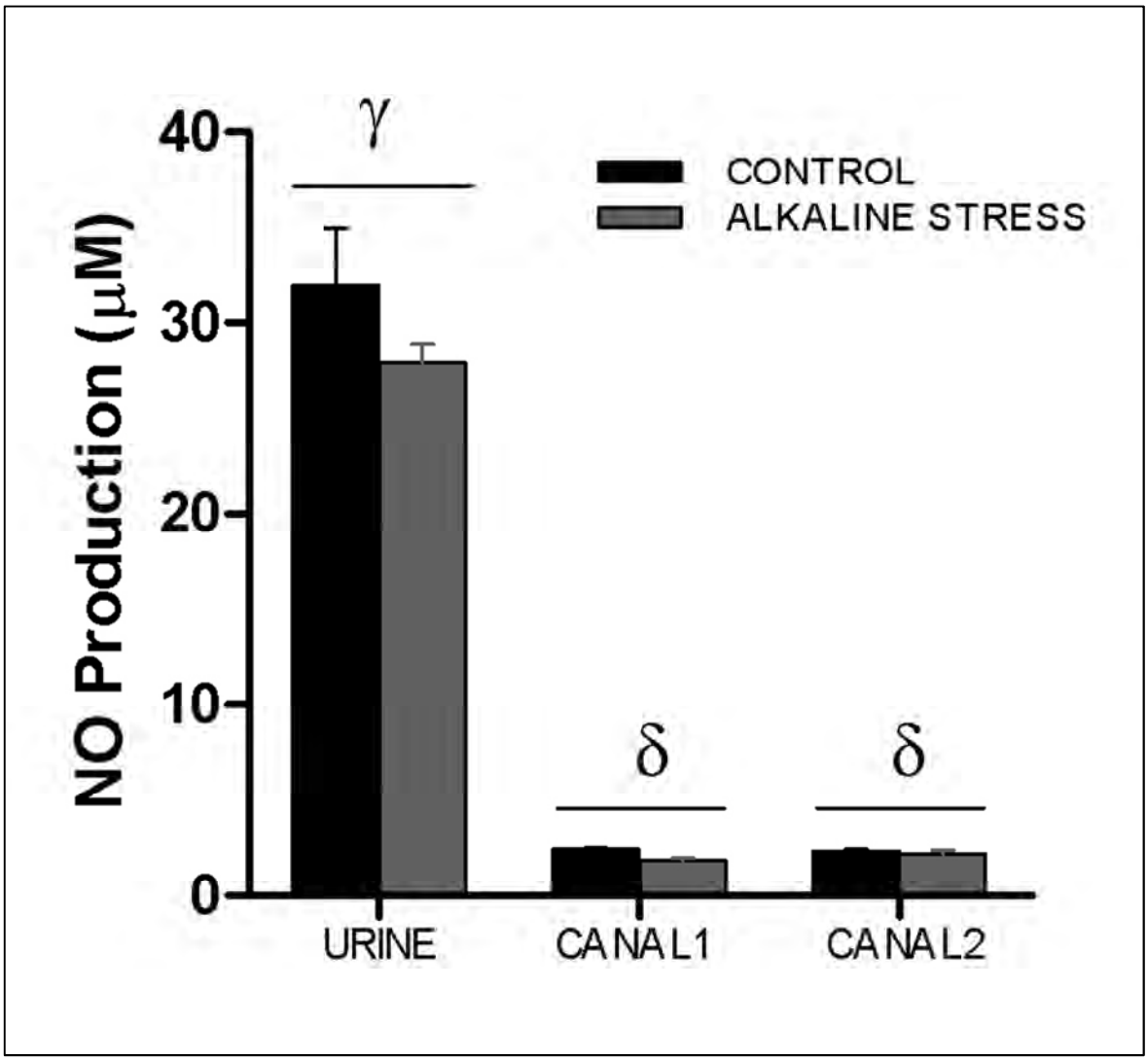

Figure 3. NO production by MDM in response to *E. faecalis* infection for 24 hours. The three strains were either stressed (ALKALINE STRESS) or not (CONTROL) with alkaline broth. Nitrite accumulation was used as an indicator of NO production. Different symbols indicate $p<0.05$.

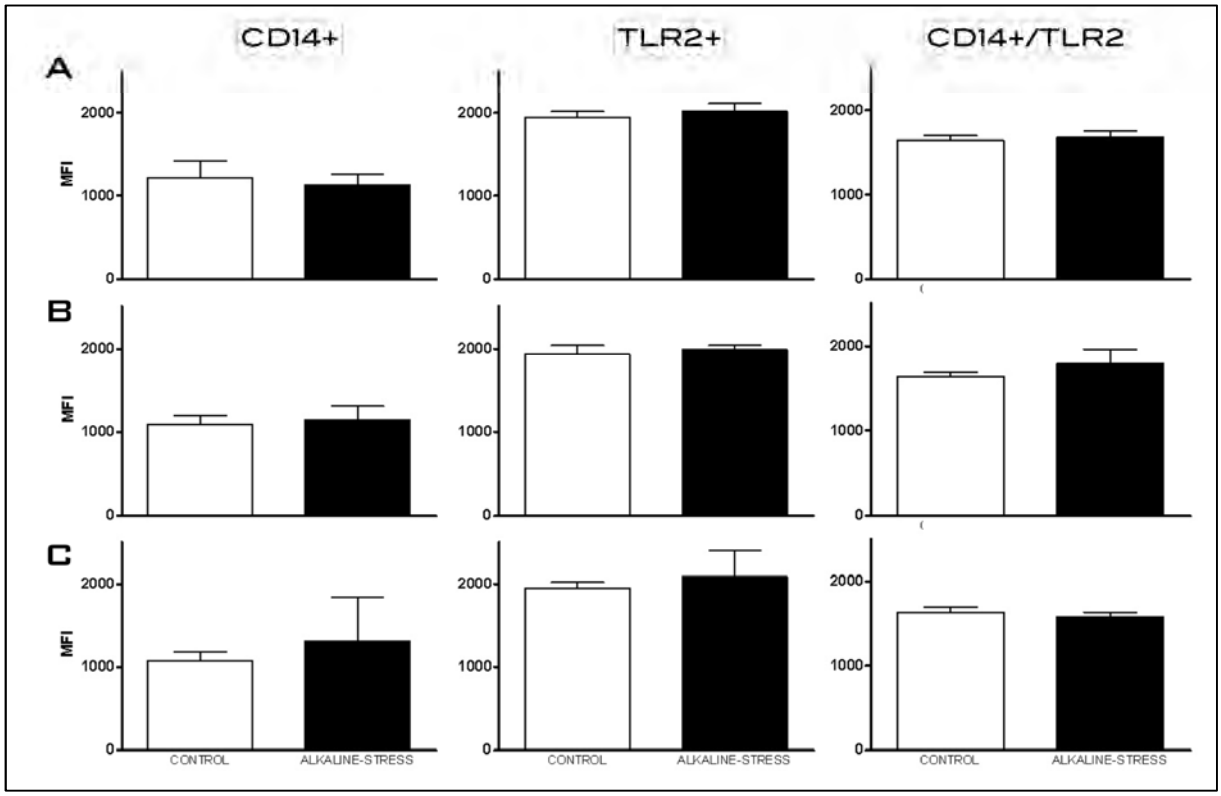

Figure 4. Intensity of TLR2 and CD14 expression in MDM, after stimulation with different strains of *E. faecalis* either stressed (ALKALINE STRESS) or not (CONTROL) with alkaline-BHI: (A) URINE; (B) CANAL 1 and (C) CANAL 2.

Discussion

Some virulence factors are more significantly expressed in a number of isolates; and a trend in the distribution of a specific virulence factor amongst isolates from different sources could indicate a link between pathogenicity and a specific strain. Thus, our study also addressed a comparison between the phagocytosis and NO production by human macrophages against *E. faecalis* ATCC 4083 from root canal and a strain isolated here from a pulpless tooth, as well as between them and the ATCC 29212 strain, a urine isolate widely used in endodontic-related researches (5,11,27). The comparison between the different strains was done in two conditions: after growth in neutral medium or alkaline medium, simulating a bacterial response after endodontic treatment with calcium hydroxide.

Despite the pathogenicity of enterococcal cells differing among the strains and being influenced by the environment (28,29), several works focusing endodontics used the urine strain ATCC 29212 (8,11,20,27). Indeed, our results revealed differences between root-canal strains and those from urine. In a medium with

normal pH, root-canal strains were less phagocytized by MDM than the urine strain, and triggered lower NO production by these cells. These differences could be partially attributed to different expression of a wide range of virulence factors among the root canal strains and urine enterococci, reflecting in particular capacity to elude immune defenses (28,29) This suggests that researchers should be careful when using urine strains, in order to study the involvement of *E. faecalis* in endodontic lesions. However, after alkaline stress, the number of macrophages that internalized bacteria was similar among the 3 analyzed strains.

When comparing the control group with that after alkaline stress, we observed a significant interference of alkaline stress on phagocytosis of strain isolated here and urine strain, but not enterococci ATCC from root canal. As regards this ATCC 4083 strain (CANAL 1), there was a tendency to be less phagocytized by MDM, although without statistical difference when compared with non-stressed enterococci. On the other hand, the percent of MDM that showed <5 or ≥5 internalized bacteria, as well as the NO production was not altered by stimulation with enterococcal cells submitted to alkaline stress in comparison with bacteria grown in neutral BHI-broth, irrespective of the strain studied.

To confirm that macrophages expressed important receptors for recognition of *E. faecalis*, even the alkaline-stressed bacteria, we verified the expression of two macrophage surface molecules. The CD14, a known surface receptor for the LPS of gram-negative bacteria, together with TLR2, are important in recognizing the peptidoglycans and LTA of gram-positive bacteria (18,19,30). As the expression of both surface molecules was similar between macrophages stimulated with alkaline-stressed or non-stressed enterococcal cells, the impaired phagocytosis observed here is not related to changes in these receptors. Actually, other receptors may recognize *E. faecalis,* as corroborated by Kau et al (31).

Based on our data relating phagocytosis, the present study highlights the fact that enterococci from root canal, submitted to alkaline-stress, might escape from an efficient phagocytosis by host macrophages, which could contribute to their persistence in root canal systems of teeth treated with CH. Possibly, the alkaline stress results in alterations on structure of enterococci, impairing the phagocytosis by defense cells. This finding may be related to studies that claim the ability of *E. faecalis* to survive under harsh environments, including high alkalinity levels, which

could explain their frequent recovery in persisting infections subsequent to endodontic therapy (9,10,11,12,32,33).

CH is well established as an antimicrobial medication applied in endodontic procedures. The hydroxyl ions released in an aqueous environment destroy several bacterial species by damaging the cellular membrane, DNA and proteins. In addition, CH can inactivate and alter some virulence factors in bacteria. We believe that these changes may eventually modify structures important for pathogenicity and enterococcal recognition by immune cells (34), taking the CH some apparently ambiguous properties, as the collagen-binding ability through adhesins expression, which contributes to bacterial persistence on dentinal tubules (7,35). Besides, the damage on lipid moieties of bacterial virulence factors promoted by CH, as occurs in LTA inactivation by deacylation of fatty acids, decrease the TNF-α production by macrophages, attenuating the host response (8,20). Therefore, it's possible that these alterations occur on remanescent enterococci, when CH was not able to completely eliminating *E. faecalis* in the root canal, maybe impairing the host response.

Other cell wall glycopolymers of *E. faecalis,* such as capsular serotypes C and D help in resistance to phagocytosis, since the presence of this capsule will mask virulence factors (17,36). According to Pinheiro et al (23), enterococci from root-filled teeth with periapical lesions belong to lineages associated with capsule expression and production of multiple virulence factors. However, both clinical and experimental studies are needed to understand the relationship between alkaline stress and synthesis of capsule by *E. faecalis* (34).

The percentage of enterococcal cells in failed root-canal procedures is higher and the wide range of *E. faecalis* strains (5) present in root canal pose a challenge to study the pathogenicity of persistent periapical lesions, and reflects the expression of a variety of virulence factors that depend on, and are modulated by the environment, such as intracanal dressings (13,17,35,35,36). Although there are few reports about the effects of alkaline stress on the structural virulence factors of *E. faecalis*, Flahaut et al showed that thirty-seven proteins in *E. faecalis* upregulated by alkaline stress seemed to increase the bacterial resistance to homologous challenge, while other proteins were associated with synthesis of bacterial cell walls (6). Several other bacteria can also be exposed to effects of alkaline environment in the intestine, some foods and polluted waters (7). For example, the disinfection process in food products

promotes alkaline stress of *Listeria monocytogenes,* with upregulation of stress-related proteins, such as NADPH dehydrogenase NamA, a cytoplasmic protein related to detoxification of reactive oxygen species. Expression of these proteins facilitates the survival and persistence of these bacteria in a food processing environment (38).

Several substances, such as camphorated paramonochlorophenol (CMCP), propylene glycol and chlorhexidine may be used associated with CH in intracanal dressings, to improve the antimicrobial effect on persistence apical periodontitis bacteria, as was shown by Silveira et al (39). The in vitro association of CH and CMCP showed evidence of being more effective in eliminating *E. faecalis* (39,40). However, the effects of these substances should be considered on the structure of bacteria, expression of virulence factors and consequently impact on the recognition and action of the immune system against *E. faecalis*. Eliminating these bacteria from root canals is a critical treatment goal. Studies evaluating strains from root-canals and changes in the bacterial structures considered pathogen-associated molecular patterns (PAMPs), caused by alkaline stress, would contribute to the design of treatments that would also improve the immune mechanisms.

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3 Discussion

3 **DISCUSSION**

E. faecalis are commensal bacteria inhabiting the gastrointestinal tract in humans, and are also found as a component of certain foods, such as some cheeses, helping them to ripen and develop flavor (RAVAZI et al., 2007, VIDANA et al., 2016). Some studies have suggested that viable *E. faecalis* from food are capable of leaking into the dental pulp chamber space and persisting in periapical lesions (KAMPFER et al., 2007; RAVAZI et al., 2007), however no foodborne transmission could be shown, based on the similarities of virulence factor patterns in root-canal isolates (VIDANA et al., 2016).

CH, an alkaline substance used as an effective antimicrobial dressing during endodontic therapy, can eliminate several bacterial species after a short period of time. Nevertheless, *E. faecalis* remained viable after relatively extended periods of alkaline stress, as previously shown and confirmed by our results (EVANS et al., 2002; NAJAKO et al., 2004, 2006). *E. faecalis* obtained from oral cavity of various individuals seems to have different responses to alkaline stress, suggesting a distribution of virulence factors amongst isolates (WECKWERTH et al., 2013). In our study, a different response to alkaline stress and antibiotic susceptibility was observed in root-canal strains when compared with urine enterococci. The urine strain tended to have greater ability to withstand an alkaline environment and showed resistance to all antibiotics tested, even after alkaline stress.

Nevertheless, when the nutrient supply was not renewed, all strains would enter into a viable but not cultured (VBNC) state, defined as “a cell that is metabolically active, while being incapable of undergoing the cellular division required for growth in or on a medium normally supporting growth of that cell” (ROWAN, 2004). This is a survival strategy adopted under some adverse conditions, for example starvation and pH stress, especially by bacteria from soil or the marine environment (BREEUWER; ABEE, 2000; ROWAN, 2004). It represents a programmed physiological response to allow survival during a potentially lethal environmental stress (OLIVER, 1995; ROWAN, 2004; CHÁVEZ DE PAZ; HAMILTON; SVENSÄTER, 2008). When the environment returns to a favorable state, the stress response is possibly released and the bacteria resume metabolic activity and cell division. The presence of bacteria in a state of non-growth could

explain the survival of bacteria in persistent infections in root canals, where microorganisms must survive with very low nutrient supplies and exposure to calcium hydroxide intracanal medication (CHÁVEZ DE PAZ et al., 2003, 2007; CHÁVEZ DE PAZ; HAMILTON; SVENSÄTER, 2008). Furthermore, if starvation or exposure to sodium hypochlorite solution or diffusing calcium hydroxide induces *E. faecalis* to enter into a stress response then this might confer cross protection to *E. faecalis* when it is subsequently exposed to a calcium hydroxide dressing (EVANS et al., 2002).

Alterations in virulence factors, related to bacteria under alkaline-stress have been reported by other studies (FLAHAUT et al., 1997; BAIK et al., 2008, 2011). Some *E. faecalis* strains can even produce capsular polysaccharides that contribute to virulence through mechanisms including resistance to opsonophagocytosis and masking of bacterial surface antigens from detection by the host immune system (THURLOW et al., 2009). Indeed, our results showed that phagocytic ability of macrophages was impaired when these cells were challenged with *E. faecalis* stressed with alkaline broth. Even some genes related to virulence factors can be destroyed when hydroxide ions react with bacterial DNA, resulting in denaturation of the strands, lack of virulence and possibly without being recognized by host macrophages (IMLAY et al., 1988).

This inappropriate recognition of the virulence factors, in the first steps of phagocytosis, would reduce the internalization of bacteria and impair the development of the immune response to *E. faecalis* (SAVA et al., 2010). However, neither the NO production nor the expression of TLR-2/CD-14, by macrophages infected with alkaline-stressed bacteria, were altered when compared with non-stressed enterococci. This finding led us to conclude that only the first stages of phagocytosis, *i.e.* recognition, would be changed when the MDM were infected with alkaline-stressed enterococcal bacteria, so that subsequent cellular mechanisms of NO production were not altered.

When root-canal strains were compared with urine enterococci, not only the phagocytic ability, but the NO production also differed during the challenge, emphasizing this possible difference in profile between strains from different sources.

Although the in vitro method does not reflect the conditions of intracanal environment in clinical practice, controlled conditions are required in order to assess the response of bacteria in stress (CHÁVEZ DE PAZ, 2012). The in vitro methods used in this study, with fluorescent markers, allowed to calculate the total number of internalized bacteria, the Intensity of TLR2 and CD14 expression in human MDM, and the total viable bacterial biofilm in restricted environments, simulating stresses on bacteria of in vivo conditions.

In the majority of natural environments, such as root-canal system and apical periodontitis, bacteria are associated in multispecies biofilms; these interspecies interactions can change the physiology of individual species (KOSTAKI et al 2012). Occasionally, single bacterial species, as *E. faecalis,* can be isolated in post-treatment apical periodontitis (TRONSTAD, BARNETT, CERVONE, 1990).

In fact, planktonic enterococci used in most of our experiments behaved differently when they were in the form of a biofilm, that is the natural state in which they are found in the root-canal. This finding is similar to that of other studies published (CHÁVEZ DE PAZ et al., 2007). However, phagocytosis assays require in vitro models using planktonic bacteria, providing a standardized number of bacteria for phagocytic cells.

4 Conclusions

4 CONCLUSIONS

- Urine enterococci are more readily phagocytosed by human macrophages when compared with root-canal strains.
- Alkaline-stressed enterococcal cells are less frequently phagocytosed by human macrophages.
- NO production by macrophages after challenge with root-canal strains differs when compared NO production after challenge with urine enterococci.
- NO production as well as intensity of TLR2 and CD14 expression were not altered in macrophages challenged by alkaline-stressed bacteria.
- Root-canal strains have different features when compared with urine enterococci, with the main differences being evident in their resistance/susceptibility to antibiotics.
- The urine isolate tends to withstand higher alkaline stress when compared with root-canal strains.

We found evidence to establish differences between the root-canal *E. faecalis* and those of urine origin, associated with phagocytic response, NO production, and with different growing conditions and alkaline stresses. Therefore, we suggest that researches that aim to interpret responses related to endodontic treatment should use root-canal isolates as reference.

The association between an alkaline environment and starvation condition can reduce bacterial growth, therefore intracanal medication with CH dressing and coronal restorations to prevent infiltration should be critical in treatments of apical periodontitis.

This impaired phagocytosis of *E. faecalis* could contribute to their persistence in root canal systems that were previously treated with CH.

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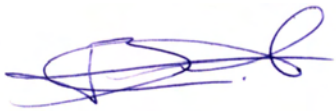
Appendixes

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN THESIS

We hereby declare that we are aware of the article "Response to alkaline stress by *Enterococcus faecalis* from root canal: in vitro evaluation of pH, antimicrobial susceptibility and biofilm formation" will be included in Thesis of the student José Burgos Ponce and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, 24 de Abril de 2016 .

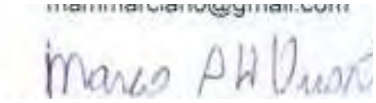


José Burgos Ponce
Author Signature

Raquel Zanin Midena
Author Signature

Marcelo Milanda
Author Signature

Paulo Henrique Weckwerth
Author Signature



Flaviana Bombarda de Andrade
Author Signature

Marco Antonio Hungaro Duarte
Author Signature

Vanessa Soares Lara
Author Signature

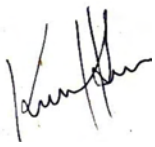
DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN THESIS

We hereby declare that we are aware of the article “Alkaline-stress impairs the phagocytosis of *Enterococcus faecalis* by human macrophages” will be included in Thesis of the student José Burgos Ponce and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, 24 de Abril de 2016 .

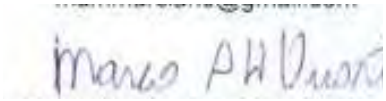
José Burgos Ponce
Author Signature

Raquel Zanin Midena
Author Signature



Karen Henriette Pinke
Author Signature

Paulo Henrique Weckwerth
Author Signature



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Annexes

Anexo A - Parecer consubstanciado do CEP

FACULDADE DE ODONTOLOGIA DE BAURU-USP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Estudo da capacidade fagocítica de macrófagos humanos frente a diferentes cepas de Enterococcus faecalis

Pesquisador: José Burgos Ponce
Área Temática:
Versão: 2
CAAE: 46839215.0.0000.5417
Instituição Proponente: Universidade de São Paulo - Faculdade de Odontologia de Bauru
Patrocinador Principal: MINISTERIO DA EDUCACAO

DADOS DO PARECER

Número do Parecer: 1.198.877

Apresentação do Projeto:
Idem ao parecer 1.173.165 de 29/07/2015.

Objetivo da Pesquisa:
Idem ao parecer 1.173.165 de 29/07/2015.

Avaliação dos Riscos e Benefícios:
Idem ao parecer 1.173.165 de 29/07/2015.

Comentários e Considerações sobre a Pesquisa:
Trata -se de um trabalho que pretende avaliar e comparar a resposta fagocitária e imunomodulatória de macrófagos humanos frente a diferentes cepas de Enterococcus faecalis, submetidos ou não ao estresse alcalino.Não existe problema que torne a pesquisa inviável do ponto de vista ético, no entanto pesquisador não possui todos os documentos necessários para a efetivação de sua pesquisa,como carta de aquiescência da universidade do Sagrado Coração para uso de material citado na pesquisa.

Solicitação atendida e explicada no oficio de encaminhamento anexo na PB.

Endereço: DOUTOR OCTAVIO PINHEIRO BRISOLLA 75 QUADRA 9
Bairro: VILA NOVA CIDADE UNIVERSITARIA **CEP:** 17.012-901
UF: SP **Município:** BAURU
Telefone: (14)3235-8356 **Fax:** (14)3235-8356 **E-mail:** cep@fob.usp.br

FACULDADE DE ODONTOLOGIA DE BAURU- USP

Continuação do Parecer: 1.198.877

Considerações sobre os Termos de apresentação obrigatória:
Atendido.

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:
- O pesquisador não apresentou termo de aquiescência da Universidade do Sagrado Coração (USC-Bauru) para uso da cepa ATCC® 4083TM.
ATENDIDO NO OFICIO RESPOSTA AO CEP.

-Não deixou claro como serão recrutados os sujeitos da pesquisa, para os dois grupos selecionados totalizando 18 participantes.
ATENDIDO, ESCLARECIDO E ELUCIDADO NO PROJETO.

- O cronograma de execução deverá ser ajustado, pois com as datas atuais levam ao entendimento que o projeto está sendo executado na sua fase de coleta sem a aprovação do CEP.
ATENDIDO.

- O TCLE deverá ser elaborado acatando todas as alíneas de A a H conforme a resolução 466/2012, e a linguagem deverá ser apresentada para o sujeito da pesquisa na primeira pessoa em todo seu conteúdo.
ATENDIDO.

-O pesquisador refere que o sujeito da pesquisa receberá o encaminhamento para de tratamento de canal na clínica de Endodontia da FOB. Esclarecer ao CEP em que momento será este encaminhamento e esclarecer ainda que os participantes serão encaminhados ao setor de Triagem para tratamento dentro da Faculdade de Odontologia de Bauru- USP, conforme o fluxo de rotina de agendamentos, sem qualquer prioridade.
ATENDIDO E ESCLARECIDO. TCLE AJUSTADO.

-Esclarecer o apoio financeiro do ministério da cultura conforme citado no documento anexado na plataforma INFORMAÇÕES BÁSICAS DO PROJETO.
APOIO FINANCEIRO BOLSA CAPES, ESCLARECIDO.

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FACULDADE DE ODONTOLOGIA DE BAURU-USP

Continuação do Parecer: 1.198.877

Considerações Finais a critério do CEP:

Esse projeto foi considerado APROVADO na reunião ordinária do CEP de 19.08.2015, com base nas normas éticas da Resolução CNS 466/12. Ao término da pesquisa o CEP-FOB/USP exige a apresentação de relatório final. Os relatórios parciais deverão estar de acordo com o cronograma e/ou parecer emitido pelo CEP. Alterações na metodologia, título, inclusão ou exclusão de autores, cronograma e quaisquer outras mudanças que sejam significativas deverão ser previamente comunicadas a este CEP sob risco de não aprovação do relatório final. Quando da apresentação deste, deverão ser incluídos todos os TCLEs e/ou termos de doação assinados e rubricados, se pertinentes.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Declaração de Instituição e Infraestrutura	TERMO-CIP.pdf	06/05/2015 19:29:46		Aceito
Declaração de Instituição e Infraestrutura	TERMO-ENDO.pdf	06/05/2015 19:30:03		Aceito
Declaração de Instituição e Infraestrutura	TERMO-FARMAC.pdf	06/05/2015 19:30:17		Aceito
Folha de Rosto	FOLHA DE ROSTO.pdf	06/05/2015 19:15:34		Aceito
Outros	CARTA DE ENCAMINHAMENTO.pdf	06/05/2015 19:37:44		Aceito
Projeto Detalhado / Brochura Investigador	PROJETO- COMITE ETICA.docx	06/05/2015 20:41:45		Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_385972.pdf	06/05/2015 20:42:23		Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_385972.pdf	19/05/2015 11:34:37		Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_385972.pdf	06/06/2015 20:05:25		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_CEP_01.docx	03/07/2015 15:44:57		Aceito
TCLE / Termos de Assentimento / Justificativa de	TCLE_CEP_02.docx	03/07/2015 15:45:12		Aceito

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FACULDADE DE ODONTOLOGIA DE BAURU-USP

Continuação do Parecer: 1.198.877

Ausência	TCLE_CEP_02.docx	03/07/2015 15:45:12		Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_385972.pdf	03/07/2015 16:28:45		Aceito
Outros	OFICIO RESPOSTA-FOLHA01.JPG	12/08/2015 01:19:04		Aceito
Outros	OFICIO RESPOSTA-FOLHA02.JPG	12/08/2015 01:19:53		Aceito
Projeto Detalhado / Brochura Investigador	PROJETO- COMITE ETICA-DETALHADO.docx	12/08/2015 01:21:48		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_CEP_01_CORRIGIDO.docx	12/08/2015 01:39:32		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_CEP_02_CORRIGIDO.docx	12/08/2015 01:40:20		Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_385972.pdf	12/08/2015 01:45:46		Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

BAURU, 25 de Agosto de 2015

Assinado por:
Izabel Regina Fischer Rubira Bullen
(Coordenador)

Endereço: DOUTOR OCTAVIO PINHEIRO BRISOLLA 75 QUADRA 9
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