

Do different strains of *E. faecalis* have the same behavior towards intracanal medications in *in vitro* research?

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Abstract: The aim of this study was to evaluate the antimicrobial action of different endodontic pastes against *Enterococcus faecalis* ATCC 29212, isolated from the urinary tract, and compare the action with *E. faecalis* ATCC 4083, isolated from the root canal. For this purpose, dentin blocks were infected for 21 days with both bacteria at different time-intervals to ensure there would be no cross contamination. After this period, blocks were immersed in the test medications for 7 days, according to the following groups: CH/S, CH/P, CH/CMCP, CH/CHX, CH/DAP and TAP. Images of the samples were captured with a confocal microscope and the percentage of live cells was computed by means of the Bioimage program. The ATCC 29212 strain was shown to be more resistant to CH/SS, Calen, CH/DAP, and TAP than the ATCC 4083 strain. The antimicrobial action of the medications against each strain were divergent concerning the order of susceptibility. The authors concluded that the strains behaved in a different manner: in general, those extracted from the urinary tract were more resistant to the tested medications. Therefore, when *E. faecalis* must be used for *in vitro* research in endodontics, we suggest the use of ATCC 4083 strain to obtain results that are closer to the clinical reality.

Keywords: *Enterococcus faecalis*; Calcium Hydroxide; Chlorhexidine; Anti-Bacterial Agents; Endodontics.

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Introduction

Inflammatory lesions of the peri-radicular tissues result from an infection caused by the aggressive invasion of microorganisms from the root canal system that resisted the antibodies and immune cells of the host.¹ *Enterococcus faecalis* are the bacteria most frequently found in root canals of re-treatment and persistent infection cases, since they are capable of adapting to adverse environments and have a high level of pathogenicity that leads to perpetuation of the endodontic infection.²

The application of intracanal medications is one of the strategies used for root canal antiseptics and the most frequently used medication is calcium hydroxide (CH), which releases hydroxyl ions in an aqueous solution, providing a highly alkaline environment that affects most microorganisms in infected root canals. However, microorganisms resistant to CH such as *E. faecalis*,³ *Candida* species,⁴ and *Actinomyces radidentis*⁵ have been associated with endodontic failure.

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Therefore, to improve its antimicrobial effect, active substances are mixed with CH. Camphorated paramonochlorophenol (CMCP) was suggested as a vehicle in 1966,⁶ extending the CH action to facultative and anaerobic bacteria and improving its ability to penetrate into dentinal tubules.⁷ Chlorhexidine gluconate (CHX) is used as an intracanal irrigant and medicament because of its broad spectrum of activity against Gram-positive and Gram-negative bacteria found in endodontic infections.⁸ Studies have shown the effectiveness of CHX against microorganisms that are resistant to CH, and the antimicrobial benefits of the mixture of the two products (CH+CMCP).^{9,10}

In cases of endodontic failure, the Triple Antibiotic Paste (TAP) containing metronidazole, minocycline, and ciprofloxacin has been suggested. This composition provides powerful action against microorganisms of the oral cavity,¹¹ however, minocycline can cause tooth discoloration. Thus, its exclusion from the paste has been indicated, and the product was called Double Antibiotic Past (DAP).¹² An alternative that has not been extensively studied is the interaction of the antimicrobial action of DAP with the biological benefits of CH.

The easiness of inducing *E. faecalis* biofilm *in vitro*¹³ has increased the interest in studying the resistance of this bacteria to the different substances used in root canal treatment.^{14,15,16} However, the strain used most often is ATCC 29212, isolated from the urinary tract.^{17,18,19,20} With the concern of conducting *in vitro* research as close as possible to clinical reality, the aim of this study was to evaluate the susceptibility of ATCC 29212 to different intracanal pastes and compare with that of ATCC 4083, which is isolated from the root canal.

The null hypothesis tested were:

- a. The strains have the same behavior towards the different pastes;
- b. The pastes present similar antimicrobial action.

Methodology

Obtaining dentin blocks

Fourteen bovine central incisors with completely developed roots were placed on the bench in a lateral position to provide access to their flattest portion. The roots were then perforated in the cervical and middle thirds by means of a 4 mm trephine bur

(Neodent, Curitiba, Brazil) coupled to a contra-angle placed perpendicular to the tooth to obtain 4 dentin blocks per tooth. Subsequently, the dentin surfaces were polished with a polishing machine (Arotec, São Paulo, Brazil) and 300 and 600 grit abrasive papers (Buehler, Lake Bluff, USA). Organic residues and dentin scrapings from the cutting procedure were removed with 1% sodium hypochlorite (Rioquímica Ltda., São José do Rio Preto, Brazil) for 15 minutes, followed by 17% EDTA (Biodinâmica, Ibitiporã, Brazil) for 5 minutes and sterilization in autoclave at 121°C.

Bacterial growth

The antimicrobial activity tests were conducted under aseptic conditions in a laminar flow chamber (VecoFlow Ltda, Campinas, SP, Brazil). Each bacterium was manipulated at different time intervals so that there would be no contamination between strains. To activate bacteria, 15 µL of each strain (American Type Culture Collection [ATCC] 4083 and [ATCC] 29212) were inserted into 3 mL of sterile brain-heart infusion (BHI) (Oxoid, Basingstoke, UK) and kept in an oven (model Q816M20; Composto de Quimis Científicos Ltda, Diadema, SP, Brazil) at 37°C for overnight growth. Afterwards, the bacterial density was adjusted to 10⁸ cells/mL with a spectrophotometer (Bel Photonics do Brazil Ltda, Osasco, Brazil) at an optical density of 1 at 600 nm according to the 0.5 MacFarland standard.

Contamination of the specimens

Each well of 24-well plates contained 1 dentin block + 0.9 mL sterile BHI + 0.1 mL of the inoculum; plates were kept in an oven (model Q816M20; Composto de Quimis Científicos Ltda, Diadema, SP, Brazil) at 37°C for 21 days. To avoid nutrient deficiency, the BHI culture medium (Oxoid, Basingstoke, UK) was completely replaced every 48 hours, without the addition of new microorganisms.

Treatment of specimens

After biofilm maturation, the infected samples were washed with 1 mL of distilled water to remove loosely adherent and planktonic bacteria. Subsequently, the dentin blocks were immersed in the test medications (n = 5) and remained in an oven at 37°C for a period of 7 days. The control group received no treatment.

Intracanal dressings

- a. G1 (CH/S): CH (calcium hydroxide) (Merck & Co, Kenilworth, NJ, USA)/ saline (1 g/0.8 mL);
- b. G2 (CH/P): CH and polyethylene glycol (Calen; SS White Artigos Dentarios Ltd, Rio de Janeiro, Brazil);
- c. G3 (CH/CMCP): CH, polyethylene glycol and camphorated paramonochlorophenol (CMCP) (Calen PMCC, SS White Artigos Dentarios Ltd, Rio de Janeiro, Brazil);
- d. G4 (CH/CHX): CH (Merck & Co), 2% chlorhexidine gluconate (FGM, Joinville, Brazil) and propylene glycol (1 g/0.5 mL/0.3 mL);
- e. G5 (CH/DAP): CH (Merck & Co), Double Antibiotic Paste (Metronidazole, Ciprofloxacin) - Brainfarma Indústria Química e Farmacêutica S.A., São Paulo, Brazil) and Saline (500 mg of each antibiotic/ 500 mg CH /1mL);
- f. G6 (TAP): Triple Antibiotic Paste (Metronidazole, Ciprofloxacin, Minocycline - Brainfarma Indústria Química e Farmacêutica S.A., São Paulo, Brazil) and Saline (500 mg of each antibiotic/ 1 mL).

Microbiological Analysis

The samples were washed with PBS (phosphate buffer solution) to remove the pastes and residues and then stained with 15 µL of SYTO9 and propidium iodide solution (Live/Dead BaLight Viability Kit; Molecular Probes, Eugene, USA) for 15 minutes. SYTO9¹¹ is a selective nucleic acid fluorescent stain, indicated for staining live and dead cells (general stain) and propidium iodide identifies microbial populations with an affected cellular membrane or dead cells, and presents a red fluorescence. On entering the cells, the red fluorescence diminishes the fluorescence of SYTO9 leaving the dead cells with a red or yellowish fluorescence. After staining, 4 images per dentin block totaling 20 images per group were captured by means of a Laser Scanning Confocal Microscope (LSCM) (Laica, Mannheim, Germany) at 40x magnification. The percentage of live and dead bacteria was obtained by means of Bioimage v2-1 software (www.bioImageL.com).

Results

Figure shows confocal laser scanning microscopy of the biofilms treated with the tested intracanal dressings: (CH/S) calcium hydroxide + saline solution, (CH/P)

calcium hydroxide + polyethylene glycol, (CH/CMCP) calcium hydroxide + polyethylene glycol + CMCP, (CH/CHX) calcium hydroxide + propylene glycol + chlorhexidine, (CH/DAP) calcium hydroxide + Double Antibiotic Paste, (TAP) Triple Antibiotic Paste and (CG) control group. *Enterococcus faecalis* biofilm (ATCC 29212) and *Enterococcus faecalis* biofilm (ATCC 4083) are identified by numbers 1 and 2, respectively. Live cells are indicated in green, and dead cells are indicated in red.

Table 1 shows the percentage of viable cells of different biofilms after a one-week treatment with the medications. The ATCC 29212 strain showed more resistance to the CH/S, CH/P, CH/DAP, and TAP pastes than the ATCC 4083 strain, without statistical differences among the other groups.

The order of susceptibility to the medications from high to low was TAP, CH/S, CH/CMCP, CH/CHX, CH/P, and CH/DAP for ATCC 29212 and TAP, CH/S, CH/CHX, CH/P, CH/CMCP, and CH/DAP for ATCC 4083.

Discussion

The null hypotheses were rejected because:

- a. Differences were observed in the susceptibility of *E. faecalis* ATCC 4083 and ATCC 29212 to the studied pastes;
- b. The pastes presented different antimicrobial action.

Enterococcus faecalis is a Gram-positive, facultative anaerobic bacterium that presents in pairs in the coccus form, or in short chains in the planktonic form.^{2,21,22} It is also capable of easily organizing itself into a biofilm, which increases its resistance to chemical-physical root canal treatments and alkaline environments, as in the case of intracanal medications with CH.¹⁶ When these bacteria are exposed to unfavorable conditions, such as nutritional limitation, they go into “starvation phase/mode” and are capable of resisting for up to 12 months within dentinal tubules, even after the filling of the root canal system.^{23,24}

Endodontic pathogens are micro-organisms able to penetrate enamel, dentin and pulp, and establish and survive in the root canal space.²⁵ The ability of *E. faecalis* to adapt to external factors¹ and its high virulence result in a high degree of pathogenicity,^{13,26} with an advantage over other microorganisms found

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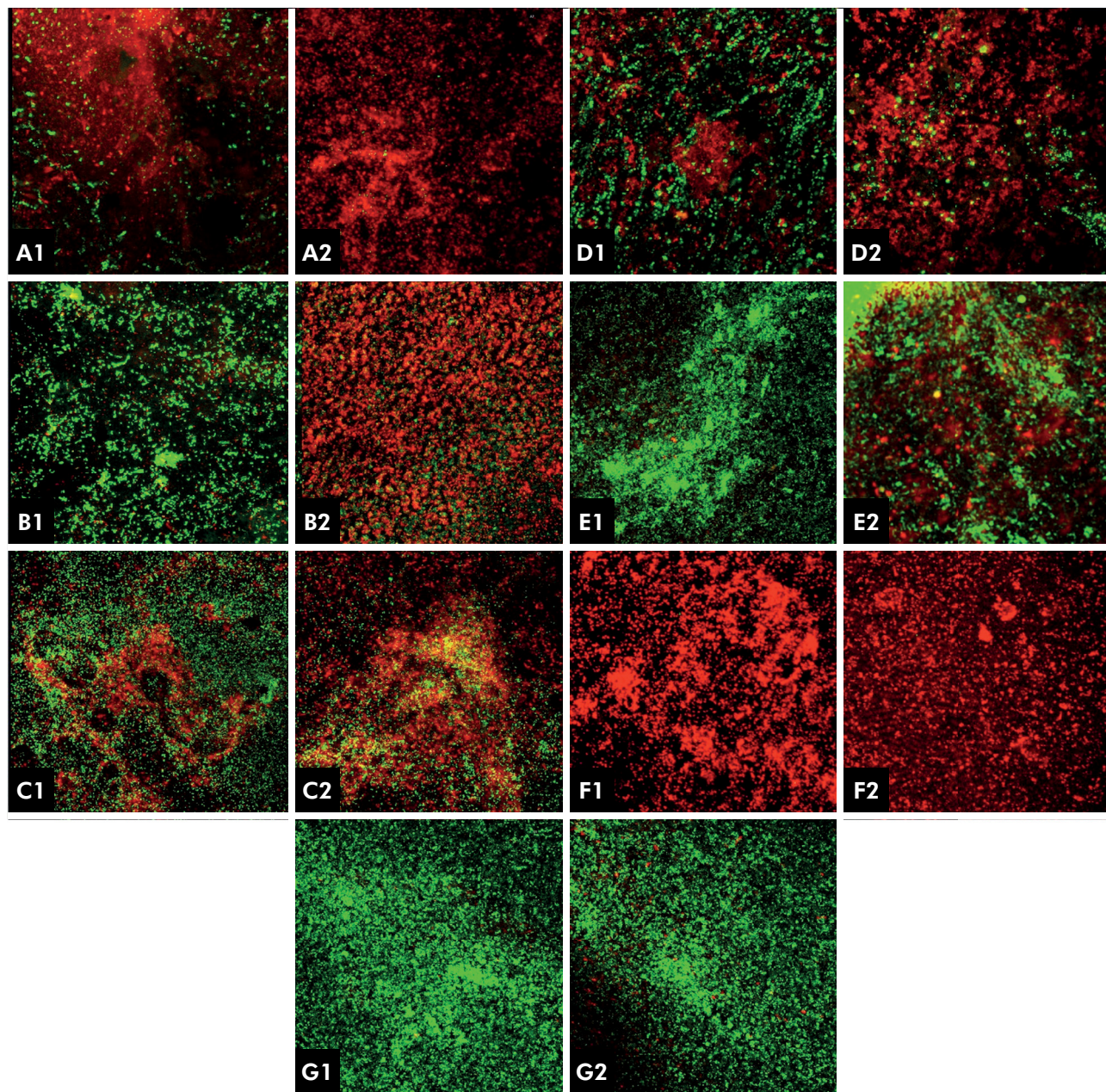


Figure.- Confocal laser scanning microscopy of biofilms treated with (A) calcium hydroxide + saline solution, (B) calcium hydroxide + polyethylene glycol, (C) calcium hydroxide + polyethylene glycol + CMCP, and (D) calcium hydroxide + propylene glycol + CHX, (E) DAP + CH, (F) TAP, and (G) control group. Number 1 corresponds to *Enterococcus faecalis* biofilm (ATCC 29212) and number 2 to *Enterococcus faecalis* biofilm (ATCC 4083). Live cells are indicated in green, and dead cells are indicated in red. Each picture represents an area of 275 x 275 μm .

Table. Median (Med), minimum and maximum (Min–Max) values of the percentage of live cells of different biofilms after contact with the experimental medicaments for a week.

ATCC	Control	CH/S	CH/P	CH/CMCP	CH/CHX 2%	CH/DAP	TAP
4083	75.59 (42.9–96.5) ^{aA}	13.15 (0.21–53.10) ^{bCD}	44.64 (4.08–92) ^{bABC}	52.94 (8.14–82.4) ^{aAB}	35 (8.44–77.6) ^{aBC}	59.98 (11.46–80.3) ^{bAB}	0.06 (0.001–1.23) ^{bD}
29212	86.75 (26.9–99.9) ^{aA}	34.52 (3.99–92.9) ^{aCD}	71.9 (5.54–96.91) ^{aAC}	58.26 (0–98.95) ^{aBCD}	45.8 (7.1–98) ^{aBC}	83.73 (24.02–93.4) ^{aAB}	10.42 (0.2–47.84) ^{aD}

*Kruskal-Wallis with Dunn's post-hoc. P-value < 0.05; Different capital letters in rows indicate statistically significant intergroup differences in the same biofilm. Different lower letters in columns indicate statistically significant intragroup differences between biofilms.

during the different stages of endodontic treatment; thus, it is considered “the villain” of endodontics.² Indeed, although primary endodontic infection is caused by a mixed community of microorganisms such as *Firmicutes*, *Bacteroidetes*, *Spirochaetes*, *Fusobacteria*, *Actinobacteria*, *Proteobacteria*, *Synergistes*, TM7 and SR1,^{27,28} the main microorganism isolated in cases of endodontic treatment failure is *E. faecalis*, with a prevalence of 24 to 77%.^{29,30,31}

Given the resistance of *E. faecalis* to endodontic treatment, this microorganism has frequently been used in *in vitro* research in the planktonic or biofilm form.^{16,17} A biofilm is a thin layer of microorganisms adhering to the surface of a structure enclosed in a extracellular matrix (ECM) that increases its resistance to environment stresses. All strains of *E. faecalis* produce ECM in aerobic conditions; however, the amount produced varies depending on the isolate, environment, biofilm complexity, and substrate.²⁵ The ATCC (American Type Culture Collection) lists 69 commercially available strains of *E. faecalis*. A strain is a descriptive subdivision of a species and when it undergoes significant mutations or as it adapts to new environmental conditions, the descendants may form new strains, which are clonally related isolates, since they have common phenotypes and genotypes.^{32,33} Each of these isolates has an ATCC number and different hereditary characteristics arising from absorption of molecules or their fragments of DNA disposed in their respective environments.²

Distinct prevalence patterns of virulence genes of *Enterococcus* spp. have been isolated from root canals with treatment failure. The different expression profiles of these virulence factors with intermediate/total resistance to several antimicrobial agents can be explained by environmental factors such as geographic differences and dietary habits.^{34,35} Moreover, endodontic bacterial species are arranged close to one another, which is highly conducive to establishing interactions such as food chains, quorum-sensing systems, and exchange of genes related to virulence and antibiotic resistance.³⁶ Thus, the infection of teeth can have an influence on the microbiota and consequently on the type of the virulence exchanged by bacteria. Associations have been reported between endodontic bacterial species

and antibiotic resistance, including *Prevotella* species from dentoalveolar abscesses³⁷ and *Enterococcus faecalis* from teeth with post-treatment apical periodontitis.³⁸ This is important for understanding the pathogenicity of virulence factors and their effects on the host.

Due to the concern related to the accuracy of *in vitro* research and the different patterns of virulence and resistance genes of *E. faecalis* strains, their monitoring should be encouraged for establishing the most effective drug. In this study, the behavior of a *E. faecalis* lineage originally isolated from root canal infection - ATCC 4083^{15,16} - was compared with that of ATCC 29212 (isolated from urine), which is used in most studies.^{17,18,19,39}

In general, we observed that ATCC 29212 was more resistant than ATCC 4083. (Table). One of the main mechanisms of CH antimicrobial action - within an inert vehicle - is the alkalization of the medium.⁶ Many diseases, diets, and medications make urine acidic or basic, which may justify the greater resistance of ATCC 29212 (isolated from urine) to changes in pH caused by the CH/SS and CH/P pastes in comparison with ATCC 4083 (isolated from the root canal).

Camphorated paramonochlorophenol (CMCP) is a widely used vehicle that increases the CH paste spectrum of action, improving the ability of Ca²⁺ and OH ions to penetrate dentinal tubules.⁴⁰ Chlorhexidine (CHX) presents a broad antimicrobial spectrum and has excellent antimicrobial activity at pH between 5.5 and 7.¹⁴ However, in an alkaline environment, CHX may decompose into reactive by-products such as reactive oxygen compounds that may also kill *E. faecalis*.⁴¹ In the groups with active vehicles (camphorated paramonochlorophenol and chlorhexidine) added to CH pastes, there was no statistical difference between the two bacterial strains relative to the percentage of live cells. However, when we compared the susceptibility of bacteria to the different pastes, results were divergent, since the antimicrobial action of the CH/CMCP paste was better than CH/CHX for ATCC 29212, and the association of CHX showed advantages over CMCP for ATCC 4083.

A higher prevalence of live cells was found for ATCC 29212 when the two bacteria were compared regarding the antibiotic pastes. The indiscriminate use of antimicrobial agents is responsible for the

development of microbial resistance.⁴² Antibiotics are frequently taken by the systemic route and excreted through urine, which may justify the results.⁴³ The TAP paste is used in the topical form in root canal systems, and contains ciprofloxacin, metronidazole, and minocycline. It is indicated in cases of antimicrobial resistance because of its broad spectrum of action.⁴⁴ As minocycline can cause discoloration of teeth, the combination of metronidazole and ciprofloxacin, called DAP, has solved this problem.⁴⁵ Researchers have found no difference between antimicrobial activities of these antibiotic pastes.^{44,46}

However, in this study, the DAP paste was used in association with CH. When we compared the order of bacterial resistance in relation to the pastes, we found that TAP and CH/DAP had the best and worst antimicrobial result, respectively. TAP significantly reduced cell viability, differently from CH/DAP that showed results similar to the control. The alkaline

environment provided by the addition of CH to DAP probably influenced the ability of the antibiotic to penetrate into the bacterial cells.

Nevertheless, the authors concluded in general that the strain isolated from the urinary tract (ATCC 29212) was more resistant than that isolated from the root canal (ATCC 4083) and behaved in a different manner in relation to the order of resistance to medications, as was the case of CH/CHX and CH/CMCP treatments. Therefore, evaluation of the susceptibility to antibiotics is important to understand and control the propagation of endodontic diseases. For *in vitro* research, the use of strains isolated from the root canal is recommended, since different strains of the same species may differ due to acquired characteristics.

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■ Do different strains of *E. faecalis* have the same behavior towards intracanal medications in *in vitro* research?

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