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TAYNARA SANTOS GOULART

# QUANTIFICAÇÃO, ANÁLISE ESTRUTURAL DO BIOFILME MULTIESPÉCIE, MICRODUREZA DA DENTINA PÓS-FORMAÇÃO DO BIOFILME E PENETRAÇÃO MICROBIANA NOS TÚBULOS DENTINÁRIOS EM DENTES IRRADIADOS

Florianópolis
2023

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Dissertação submetida ao Programa de Pós-Graduação em Odontologia, do Centro de Ciências da Saúde da Universidade Federal de Santa Catarina (UFSC), como requisito para obtenção do título de Mestre em Odontologia, área de concentração em Endodontia.
Orientador: Prof. Dr. Lucas da Fonseca Roberti Garcia
Coorientadora: Profa. Dra. Josiane de Almeida Cava da Silveira

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Certificamos que esta é a versão original e final do trabalho de conclusão que foi julgado adequado para obtenção do título de Mestre em Endodontia.


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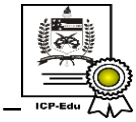


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Prof. Dr. Lucas da Fonseca Roberti Garcia

Orientador

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*Este trabalho é dedicado principalmente*
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## RESUMO

O objetivo deste estudo *in vitro* foi quantificar a presença de microrganismos e a biomassa de biofilme multiespécie, avaliar a microdureza dentinária pós- formação do biofilme, analisar a penetração microbiana e a arquitetura do biofilme multiespécie sobre a dentina radicular irradiada. Foram utilizados 63 cilindros de dentina humana radicular, distribuídos em seis grupos experimentais. Em três grupos não foi realizada a formação de biofilme no canal radicular, sendo as amostras analisadas apenas em Microscopia Eletrônica de Varredura (MEV) (n = 3): G1) dentina não irradiada (DNI); G2) dentina irradiada com 55 Gy (D55); e G3) dentina irradiada com 70 Gy (D70). Nos outros três grupos, um biofilme multiespécie de *Enterococcus faecalis*, *Streptococcus mutans* e *Candida albicans* foi formado no canal radicular (n = 18): G4) DNI + biofilme; G5) D55 + biofilme; e G6) D70 + biofilme. Após 21 dias, o biofilme aderido às paredes do canal radicular (n = 12) foi coletado e quantificado por meio da contagem de unidades formadoras de colônias (UFCs). A arquitetura do biofilme e a superfície dentinária também foram avaliadas em MEV (n = 3). Nos cilindros restantes de cada grupo (n=3), *slices* de dentina foram confeccionados e a penetração microbiana nos túbulos dentinários foi avaliada por meio de Microscopia Confocal de Varredura a Laser (MCVL). Para análise da biomassa do biofilme e da microdureza (Knoop) pós-formação do biofilme, 45 amostras de dentina bovina foram distribuídas em três grupos experimentais (n = 15): G1) DNI + biofilme; G2) D55 + biofilme; e G3) D70 + ,biofilme. Previamente a formação do biofilme, o teste de microdureza inicial (D0) foi realizado. Após 21 dias, a biomassa do biofilme foi mensurada através da coloração com cristal violeta e absorbância. Posteriormente, a microdureza final (D1) foi realizada. Os dados foram estatisticamente analisados (α=5%). As análises em MEV e MCLS foram descritivas. A D70 apresentou quantidade significativamente maior de microrganismos viáveis comparado à DNI (p=0,010) e à D55 (p=0,004). Foi observado um aumento gradual na biomassa do biofilme da DNI, para a D55 (P < 0,001) e para a D70 (P < 0,001). Uma redução na microdureza aproximadamente duas vezes maior foi observada na dentina irradiada com 55 Gy ou com 70 Gy. A dentina irradiada levou à formação de um biofilme mais complexo e à diminuição da microdureza dentinária pós-formação do biofilme. Um número maior de microrganismos foi observado na dentina irradiada com 70 Gy.

**Palavras-chaves:** Biofilme, Radioterapia, Endodontia, Microscopia Eletrônica de Varredura, Microscopia Confocal a Laser.

## ABSTRACT

To investigate the effect of different radiotherapy regimens (55 Gy and 70 Gy) on the multispecies biofilm formation. The primary purpose was to quantify the number of microorganisms, to assess the architecture of a multispecies biofilm and the microbial penetration into the dentinal tubules of non-irradiated and irradiated human root dentine. The secondary purpose was to assess the biomass of the biofilm and the microhardness pre- and post-biofilm formation on non-irradiated and irradiated bovine root dentine. Sixty-three human root dentine cylinders were distributed into six groups. In three groups, no biofilm was formed ($n$=3): G1) non-irradiated dentine; G2) 55 Gy; and G3) 70 Gy. In the other three groups ($n$=18), a 21-day multispecies biofilm composed of the microorganisms *Enterococcus faecalis*, *Streptococcus mutans*, and *Candida albicans* was formed in the root canal lumen: G4) non-irradiated dentine+biofilm; G5) 55 Gy+biofilm; and G6) 70 Gy+biofilm. The biofilm attached to the root canal walls was collected and quantified through colony-forming units (CFUs) counting ($n$=12). The biofilm architecture and dentine surface were assessed under Scanning Electron Microscopy (SEM) ($n$=3). Dentine slices were prepared, and microbial penetration into dentinal tubules was assessed under Confocal Laser Scanning Microscopy (CLSM) ($n$=3). For the analysis of the biofilm biomass and dentine microhardness (Knoop) pre- and after biofilm formation, 45 bovine dentine specimens were distributed into three groups ($n$=15): G1) non-irradiated dentine+biofilm; G2) 55 Gy+biofilm; and G3) 70 Gy+biofilm. Data were statistically analyzed ($\alpha$=5%). SEM and CLSM analyses were descriptive. Specimens irradiated with 70 Gy had a higher quantity of viable microorganisms than non-irradiated ($p$=.010) and 55 Gy ($p$=0.004). A gradual increase in the biofilm biomass from non-irradiated to 55 Gy ($p$<.001) and 70 Gy ($p$<.001) was observed. Irradiated specimens had a greater reduction in dentine microhardness after biofilm formation than non-irradiated specimens. Irradiated dentine led to the formation of a more complex and irregular biofilm. There was microbial penetration into the dentinal tubules, regardless of the radiation dose. Radiation therapy increased the number of viable microorganisms and biofilm biomass and reduced the dentine microhardness. Microbial penetration into dentinal tubules was noticeable, regardless of the radiation dose.



**KEYWORDS**

biofilm, radiotherapy, endodontics, scanning electron microscopy, confocal laser scanning microscopy.

## LISTA DE FIGURAS

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.

## LISTA DE ABREVIATURAS E SIGLAS

ATCC- American Type Culture Collection
BHI – *Brain Heart Infusion*
*C. albicans* – *Candida albicans*
CLSM – Confocal Laser Scanning Microscope
CT – Comprimento de Trabalho
DO – Densidade Ótica
DP – Desvio Padrão
*E. faecalis* – *Enterococcus faecalis*
EDTA – ácido etilenodiamino tetra-cético
et al. – e colaboradores
G1 – Grupo 1
G2 – Grupo 2
G3 – Grupo 3
Gy - Gray
kW – quilowatt
Log10 – Logaritmo na base 10
M – Mol
MEV – Microscopia Eletrônica de Varredura
mL – mililitros
mm – milímetros
n – número de amostras
NaOCl – hipoclorito de sódio
P – Probabilidade de significância
PBS – *Phosphate-buffered saline*
pH – potencial hidrogeniônico
*S. mutans* – *Streptococcus mutans*
SPSS – *Statistical Package for the Social Sciences*
UFC – Unidade Formadora de Colônia
W – watt

## LISTA DE SÍMBOLOS

Å - Ångström

% – porcentagem

µL – microlitro

*µ*m – micrometros

ºC – graus Celsius

# – calibre

< – menor

= – igual

≈ – aproximadamente

**SUMÁRIO**

## 1. INTRODUÇÃO E CONTEXTUALIZAÇÃO

A radioterapia é utilizada como modalidade de tratamento para uma ampla gama de cânceres que envolvem a região de cabeça e pescoço (DIJK et al., 2020). Na radioterapia, ondas eletromagnéticas chamadas de radiações ionizantes criam elétrons livres que podem causar danos diretos e indiretos às células (DA CUNHA et al., 2017). Quanto maior a atividade mitótica de um grupo celular, maior a sua sensibilidade à radiação ionizante (KATAOKA et al., 2012). Portanto, o alvo principal da radiação ionizante é a molécula de DNA das células neoplásicas por se encontrarem em estágio de maior atividade mitótica (HADDAD et al., 2008). O dano indireto é causado pela produção de radicais livres a partir da radiólise de diferentes moléculas, como por exemplo a água, interrompendo ligações de hidrogênio que produzem íons H+ e OH-, altamente instáveis e reativos (KATAOKA et al., 2012). Esses íons se ligarão a outras moléculas, que serão danificadas e perderão a função, ou produzirão mais radicais livres (KATAOKA et al., 2012).

Apesar da utilidade da radioterapia no tratamento dos diversos tipos de câncer de cabeça e pescoço ser indiscutível, além das células neoplásicas, outras estruturas são atingidas (DIJK et al., 2020). Os efeitos colaterais da radioterapia na região de cabeça e pescoço têm impacto determinante nas estruturas próximas a esta região, como glândulas, dentes, ossos e tecidos moles (KIELBASSA et al., 2006; RAY-CHAUDHURI et al., 2013). Quando as glândulas salivares são atingidas (GUPTA et al., 2022) é comum a ocorrência de xerostomia, tornando os pacientes mais susceptíveis ao desenvolvimento de cárie relacionada à radiação (FATTORE et al., 1986, GUPTA et al., 2022), cuja progressão é mais acelerada do que a cárie convencional (AGUIAR et al., 2009).

Devido ao menor fluxo salivar, são estabelecidas condições favoráveis para a adesão e a coagregação dos microrganismos, com consequente formação de biofilme (GUPTA et al., 2022), sendo mais comuns, nestes casos, microrganismos acidogênicos e cariogênicos, como *Streptococcus mutans* e a *Candida spp* (GUPTA et al., 2022). Por ser mais rápida, a progressão da cárie relacionada à radiação pode levar ao comprometimento pulpar em um curto período de tempo (AGUIAR et al., 2009; GUPTA et al., 2022).

A radiólise da água promove efeitos colaterais mais pronunciados em tecidos com concentrações mais altas de conteúdo orgânico (WALKER, 1975). Assim sendo, uma das estruturas mais afetadas pela radioterapia é a dentina (RODRIGUES et al., 2018). A radiação ionizante produzida durante o tratamento radioterápico gera severa desidratação dessa estrutura, levando à formação de trincas e fissuras ao redor dos túbulos dentinários, diminuição da microdureza, destruição dos prolongamentos odontoblásticos, exposição e colapso das fibrilas colágenas da matriz orgânica (GONÇALVES et al., 2014; DE SIQUEIRA MELLARA et al., 2014; MARTINS et al., 2016; RODRIGUES et al., 2018; VELO et al., 2018).

Os microrganismos, quando presentes na dentina intrarradicular, promovem uma ação ácida sobre a hidroxiapatita que é capaz de dissolvê-la (EICK et al., 2021), promovendo a sua precipitação, cujos íons cálcio servirão de substrato primário para a adesão bacteriana e formação de biofilme (OZDEMIR et al., 2010). Tal processo leva a alterações profundas na estrutura dentinária, tornando-a mais frágil e com aspecto mais erodido (BUZALAF et al., 2015). A radiação ionizante é responsável pela formação de radicais livres e pela liberação de íons, dentre os quais, os íons Ca e P (RODRIGUES et al., 2018), que podem se agregar à estrutura do biofilme, tornando-o ainda mais resistente e aderido à dentina intrarradicular (VENEGAS et al., 2006). A presença de íons cálcio no biofilme aumenta a agregação celular, uma vez que esta é estabelecida através de pontes de cálcio (ROSE et al., 2000; VENEGAS et al., 2006).

Todas essas alterações na estrutura dentinária devido à irradiação, aumentam a sua rugosidade, afetando a interação dos microrganismos com a superfície do substrato (TEUGHELS et al., 2006). Uma superfície dentinária desmineralizada, em decorrência da exposição das fibrilas colágenas, garante maior força de adesão aos microrganismos aderentes do que a dentina não irradiada (MAYRAND et al., 1985, XU et al., 2019). O *Enterococcus faecalis*, por exemplo, apresenta enzimas proteolíticas, como a serina protease e a gelatinase (HUBBLE et al., 2003), que são fatores potenciais de virulência, e que lhe conferem grande capacidade de ligação à dentina (MAYRAND et al., 1985; HUBBLE et al., 2003; XU et al., 2019).

Outro fator a ser considerado pela ação da radioterapia é a destruição da dentina peritubular e intratubular (WAGNER et al., 2014; RODRIGUES et al., 2018), levando a obliteração total ou parcial da entrada dos túbulos dentinários (VELO et al., 2018). Diante de uma estrutura dentinária sem alterações, os túbulos dentinários são facilmente invadidos e colonizados pelos microrganismos (PARMAR et al., 2011), servindo como um ambiente único e protetor, e representando um desafio para o tratamento endodôntico (PARMAR et al., 2011, BALDASSO et al., 2012). Quando uma dose de 55 Gy de radiação é entregue, observa-se uma obliteração parcial dos túbulos dentinários (VELO et al., 2018). Já com 70 Gy, as aberturas dos túbulos desaparecem completamente, em decorrência da sua obliteração total pelo processo de radiólise da dentina (VELO et al., 2018).

Todas essas alterações são dependentes da quantidade de radiação entregue aos tecidos-alvo e adjacentes (LIESHOUT & BOTS, 2014; DOUCHY et al., 2022). A localização e o tipo de câncer determinam tanto o método mais apropriado de tratamento, como a dose de radiação necessária (RODRIGUES et al., 2018). Em casos de câncer de cabeça e pescoço, normalmente, o núcleo da massa tumoral recebe uma dose total que pode variar entre 55 Gy e 70 Gy, fracionadas diariamente, por um período de 5 a 7 semanas (JHAM, et al., 2006). Lieshout & Bots (2014) em uma revisão sistemática, identificaram três níveis de danos dentários dependendo da intensidade da dose de radiação administrada: 0-30 Gy resultou em dano dentário mínimo, 30-60 Gy aumentou a relação dente-dose-dano em duas ou três vezes, e 60 Gy aumentou essa relação em dez vezes (LIESHOUT & BOTS, 2014). Assim, pode-se afirmar que, apesar de fundamental para o tratamento, a radioterapia afeta de forma significativa a função oral dos pacientes, podendo esta ser permanente e com grande impacto na qualidade de vida (DOUCHY et al., 2022).

## 2. OBJETIVOS

*Objetivo geral*

- Investigar o efeito de diferentes doses de radioterapia (55 Gy e 70 Gy) na formação de biofilme multiespécie em dentina humana e bovina.

*Objetivos Específicos*

- Quantificar o número de microrganismos, avaliar a arquitetura de um biofilme multiespécie e a penetração microbiana nos túbulos dentinários da dentina radicular humana não irradiada e irradiada.
- Avaliar a biomassa do biofilme e a microdureza pré e pós-formação do biofilme em dentina radicular bovina não irradiada e irradiada.

## 3. ARTIGO

**Effect of different radiation therapy regimens on multispecies biofilm formation - a laboratory investigation**

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## Abstract

**Aim:** To investigate the effect of different radiotherapy regimens (55 Gy and 70 Gy) on the multispecies biofilm formation. The primary purpose was to quantify the number of microorganisms, to assess the architecture of a multispecies biofilm and the microbial penetration into the dentinal tubules of non-irradiated and irradiated human root dentine. The secondary purpose was to assess the biomass of the biofilm and the microhardness pre- and post-biofilm formation on non-irradiated and irradiated bovine root dentine. **Methodology:** Sixty-three human root dentine cylinders were distributed into six groups. In three groups, no biofilm was formed ($n$=3): G1) non-irradiated dentine; G2) 55 Gy; and G3) 70 Gy. In the other three groups ($n$=18), a 21-day multispecies biofilm composed of the microorganisms *Enterococcus faecalis*, *Streptococcus mutans*, and *Candida albicans* was formed in the root canal lumen: G4) non-irradiated dentine+biofilm; G5) 55 Gy+biofilm; and G6) 70 Gy+biofilm. The biofilm attached to the root canal walls was collected and quantified through colony-forming units (CFUs) counting ($n$=12). The biofilm architecture and dentine surface were assessed under Scanning Electron Microscopy (SEM) ($n$=3). Dentine slices were prepared, and microbial penetration into dentinal tubules was assessed under Confocal Laser Scanning Microscopy (CLSM) ($n$=3). For the analysis of the biofilm biomass and dentine microhardness (Knoop) pre- and after biofilm formation, 45 bovine dentine specimens were distributed into three groups ($n$=15): G1) non-irradiated dentine+biofilm; G2) 55 Gy+biofilm; and G3) 70 Gy+biofilm. Data were statistically analyzed ($\alpha$=5%). SEM and CLSM analyses were descriptive. **Results:** Specimens irradiated with 70 Gy had a higher quantity of viable microorganisms than non-irradiated ($p$=.010) and 55 Gy ($p$=0.004). A gradual increase in the biofilm biomass from non-irradiated to 55 Gy ($p$<.001) and 70 Gy ($p$<.001) was observed. Irradiated specimens had a greater reduction in dentine microhardness after biofilm formation than non-irradiated specimens. Irradiated dentine led to the formation of a more complex and irregular biofilm. There was microbial penetration into the dentinal tubules, regardless of the radiation dose. **Conclusions:** Radiation therapy increased the number of viable microorganisms and biofilm biomass and reduced the dentine microhardness. Microbial penetration into dentinal tubules was noticeable, regardless of the radiation dose.

**KEYWORDS**

biofilm, radiotherapy, endodontics, scanning electron microscopy, confocal laser scanning microscopy.

## INTRODUCTION

Radiation therapy is widely used in the treatment of several types of head and neck cancer (Jham et al., 2006; Dijk et al., 2020). Although it has a high success rate, irradiated patients are susceptible to side effects, including oral complications that may negatively impact their life quality (Sroussi et al., 2017; De Felice et al., 2018). The current literature reports that changes in the chemical and mechanical properties of enamel and dentine are evident after radiation therapy (Velo et al., 2018; Rodrigues et al., 2018; Douchya et al., 2022). The ionizing radiation generates highly unstable and reactive free radicals due to a phenomenon named radiolysis (de Barros et al., 2017; Douchya et al., 2022). Organic tissues are the most affected by radiolysis (de Barros et al., 2017; Douchya et al., 2022), and dentine, due to its high organic content, is severely compromised (Douchya et al., 2022).

Radiolysis tends to affect the calcium ions dissociation of dentine (Rodrigues et al., 2018; Fonseca et al., 2020). As a consequence of chemical changes, structural alterations are also likely to manifest in dentine, encompassing reduced microhardness, the propagation of microcracks, the disruption of odontoblastic processes, heightened surface roughness, and the disintegration of the collagen fibrils network (Douchya et al., 2022). Xerostomia is also a common side effect of radiation therapy in cases of head and neck cancer (Gupta et al., 2015). This condition decreases the salivary flow, compromising its buffering and cleaning potential (Gupta et al., 2015). Therefore, changes in the pH and oral microbiota create favourable conditions for biofilm formation (Fattore et al., 1986; Gupta et al., 2015), with a greater proliferation of acidogenic and cariogenic microorganisms, such as *Candida spp* and *Streptococcus mutans* (Gupta et al., 2015).

Ionizing radiation plays a role in generating and releasing calcium and phosphorus ions (Rodrigues et al., 2018). These ions may potentially interact with the biofilm structure, enhancing its resilience and adhesion to the root dentine. (Venegas et al., 2006). The presence of calcium ions in the biofilm increases cell aggregation, as it is established through calcium bonds (Rose et al., 2000; Venegas et al., 2006). Furthermore, the heightened dentine roughness may favour microorganism aggregation, as the demineralized dentine surface

provides a stronger adhesion force to microorganisms due to the collagen fibrils network exposure (Teughels et al., 2006). Another detrimental effect of radiation therapy is the total or partial obliteration of dentinal tubules (Velo et al., 2018). In the absence of structural changes, dentinal tubules are easily invaded and colonized by microorganisms (Parmar et al., 2011), representing a challenge for endodontic treatment (Parmar et al., 2011; Baldasso et al., 2012). Partial obliteration of dentinal tubule entrances is observed when a 55 Gy radiation dose is administered (Velo et al., 2018). Conversely, complete obliteration of the tubule entrances occurs when exposed to 70 Gy (Velo et al., 2018). In such scenarios, it is conceivable that microbial infiltration into dentinal tubules is restricted, simplifying the sanitization of the root canal system. Nevertheless, it is plausible to hypothesize that radiation therapy, apart from weakening the dentine substrate, may also contribute to the development of a more organized and intricate biofilm, potentially intensifying the demineralization process.

Therefore, this *in vitro* study investigated the effect of different radiotherapy regimens (55 Gy and 70 Gy) on the multispecies biofilm formation. The primary purpose was to quantify the number of microorganisms, to assess the architecture of a multispecies biofilm and the microbial penetration into the dentinal tubules of non-irradiated and irradiated human root dentine. The secondary purpose was to assess the biomass of the biofilm and the microhardness pre- and post-biofilm formation on non-irradiated and irradiated bovine root dentine. The null hypotheses tested were: (I) there would be no difference in the number of microorganisms present in the root canal lumen of non-irradiated, and irradiated human dentine; (II) there would be no difference in the biofilm architecture and microbial penetration into dentinal tubules of non-irradiated and irradiated human dentine; (III) there would be no difference in the biofilm biomass formed on non-irradiated and irradiated bovine dentine; and (IV) there would be no difference in the Knoop microhardness of non-irradiated and irradiated bovine dentine post-biofilm formation.

## MATERIAL AND METHODS

The manuscript of this laboratory study has been written according to Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) 2021 guidelines (Nagendrababu et al., 2021) (Figure 1).

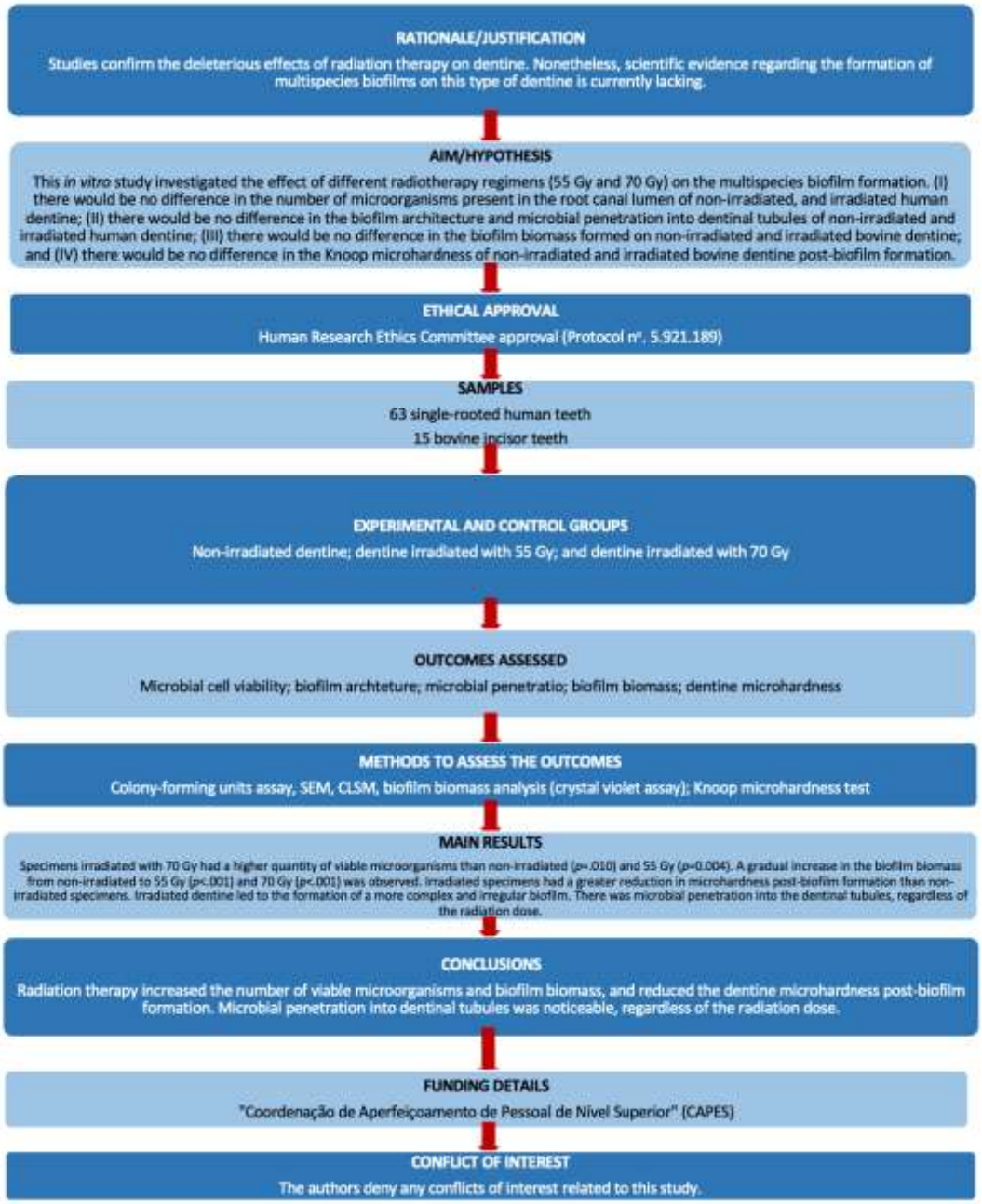


**FIGURE 1.** PRILE 2021 flowchart.

This *in vitro* experimental study was previously approved by the Human Research Ethics Committee at the Federal University of Santa Catarina (UFSC) (Protocol nº. 5.921.189). Freshly extracted single-rooted human teeth (upper incisors and canines) and bovine incisors teeth were used. Considering the institutional guidelines in which this study was performed, ethical approval for

using bovine teeth was not required. The study design was conducted following the ethical standards laid down in the 2008 Declaration of Helsinki.

The laboratory phases mentioned below were conducted in the facilities of the Laboratory of Molecular Genetics of Bacteria (GeMBac - UFSC), Laboratory of Biomechanics and Dental Materials, Central Laboratory of Electron Microscopy (LCME - UFSC), Multi-User Laboratory for Biology Studies (LaMEB - UFSC), and the Laboratory of Endodontics (UFSC). The microbial species used were obtained from the Integrated Laboratories of Biological Engineering at the Federal University of Santa Catarina (LiEB - UFSC). The irradiation of the teeth was performed at the Department of Radiotherapy of the Oncological Research Centre (CEPON), under the supervision of a physicist and a medical radio-oncologist.

**Sample size calculation**

The minimum number of repetitions required per group for each experiment was calculated to detect a significant difference of 5% among them (significance level). Based on previous studies (Donnermeyer et al., 2018; Kabil et al., 2021; Saghiri et al., 2021) and an effect size f = 0.80, the sample size was calculated using the G*Power software, version 3.1.9.6. (http://www.psycho.uni-duesseldorf.de/abteilungen/aap/gpower3/).

**Human teeth analysis**

**Selection and specimen preparation**

Sixty-three sound single-rooted human teeth, including upper incisors and canines, freshly extracted for reasons unrelated to this research, were used. The teeth were initially subjected to clinical inspection under magnification (×4) and radiographic analysis. Teeth with a total length ranging from 22 mm to 25 mm, a fully formed root, a single straight root canal, and a closed apex were included in the final specimen pool. Teeth with internal calcifications or resorptions, cracks, fractures, and previous root canal treatment were discarded and replaced. The selected teeth were cleaned with periodontal curettes and placed in a 0.1% thymol solution for 48 hours for disinfection, followed by rinsing with running water

for 24 hours. The teeth were stored in plastic flasks containing distilled water and kept in an incubator at 37°C until the beginning of the experiments.

The teeth were prepared following the methodology of Hillesheim et al. (2017), with adaptation. Initially, the teeth were decoronated at the cementoenamel junction using a double-sided diamond disc (Microdont) to standardize the root length at 15 mm. The working length was determined by inserting a size 15 K-file (Dentsply Maillefer, Ballaigues, Switzerland) into the root canal until the tip was visible at the apical foramen. The mechanical preparation was performed with a reciprocating instrument (Reciproc R50; VDW GmbH, Munich, Germany) coupled to a 6:1 reduction handpiece, powered by an electric motor (X-Smart Plus; Dentsply Sirona, São Paulo, SP, Brazil) in the RECIPROC ALL mode, following the manufacturer's recommendations. Root canal preparation was performed by thirds until reaching the working length. After each advancement and removal of the instrument for cleaning, the root canal was irrigated with 3 mL of 0.85% sterile saline solution using a plastic syringe (Ultradent, South Jordan, UT, USA) and a NaviTip 30ga needle (Ultradent).

Next, the apical third of the roots (≈ 5 mm) was sectioned using a double-sided diamond disc (Microdont, Ribeirão Preto, SP, Brazil), resulting in 10 mm long cylinders (cervical and middle thirds of the root) (Figure 2). The outer diameter of each cylinder was standardized at 2 mm using diamond burs (KG Sorensen, São Paulo, SP, Brazil). The cylinders were immersed in an ultrasonic bath containing 17% EDTA solution (Biodinâmica, Ibiporã, PR, Brazil), followed by a 2.5% NaOCl solution (Asfer Indústria Química, São Caetano do Sul, SP, Brazil), both for 3 minutes, to remove the smear layer. Finally, an ultrasonic bath in distilled water was performed to remove any residues of the NaOCl solution.

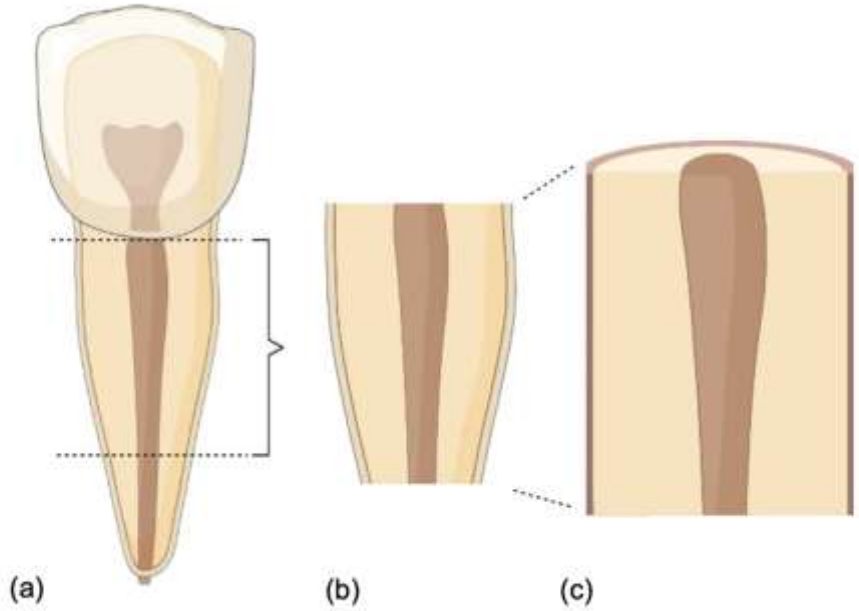


**FIGURE 2.** Schematic representation of specimen preparation. (a) Single-rooted human tooth. (b) Dentine cylinder after decoronation and apical third sectioning. (c) Dentine cylinders after outer diameter standardization.

## Experimental groups distribution

The specimens were randomly distributed (www.random.org) into six groups. In three groups, no biofilm was formed ($n$ = 3): G1) non-irradiated dentine; G2) dentine irradiated with 55 Gy; and G3) dentine irradiated with 70 Gy. In the other three groups, a multispecies biofilm was formed on the root canal lumen of the dentin cylinders ($n$ = 18): G4) non-irradiated dentine + biofilm; G5) dentine irradiated with 55 Gy + biofilm; and G6) dentine irradiated with 70 Gy + biofilm (Figure 3a).

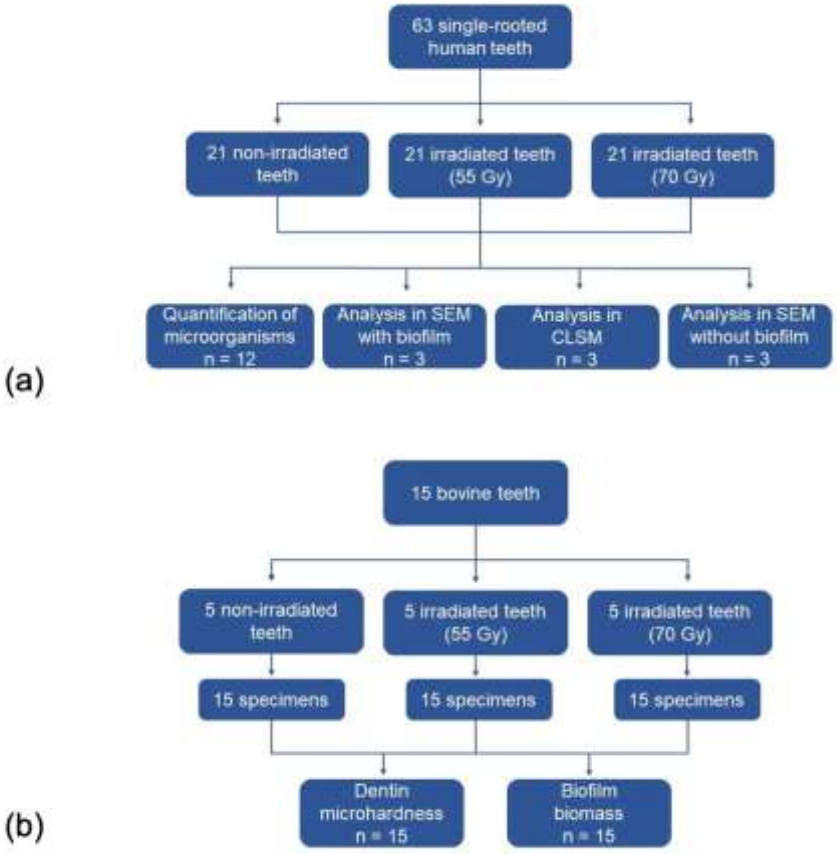


**FIGURE 3.** Flowchart of groups distribution. (a) Single rooted-human teeth. (b) Bovine incisor teeth.

## Specimens irradiation

The specimens were irradiated by the intensity-modulated radiation therapy (IMRT) technique with dynamic multileaf collimators (Dynamic Multileaf Collimator - DMLC) on a linear accelerator (Clinac 2100C; Varian Medical Systems, Inc., Palo Alto, CA, USA). The irradiation was conducted at the CEPON. The specimens were fully immersed in distilled and deionized water within a plastic holder, aligned and equidistant from the radiation source, ensuring a standardized distribution of doses (400 UM/min) (Da Cunha et al., 2016). The irradiation followed the protocol for head and neck cancer treatment, using 6MV energy (photons) with a total administration of 55 Gy, fractionated into 2 Gy daily doses, 5 days a week, over 5 weeks and 3 days; and 70 Gy, fractionated into 2 Gy daily doses, 5 days a week, over 7 weeks (Yamin et al., 2018; Cancelier et al., 2023; Coelho et al., 2023). At the end of each irradiation cycle, the distilled

and deionized water was replaced with artificial saliva, and the teeth were kept at 37°C to simulate the oral conditions (Cancelier et al., 2023; Coelho et al., 2023). With each new irradiation cycle, the artificial saliva was replaced with distilled and deionized water. On completion of the irradiation protocol, the specimens were once again stored in artificial saliva at 37°C until use.

**Microbial species and growth condition**

The facultative anaerobic microbial species used for the multispecies biofilm formation were *Streptococcus mutans* (ATCC 25175), *Enterococcus faecalis* (ATCC 29212), and *Candida albicans* (ATCC 10231). The microorganisms were obtained from a -80°C stock (LiEB). A fresh culture of each species was obtained by incubating 500 µL from the stock in 10 mL of Brain Heart Infusion (BHI) broth, pH 7.1, containing 0.2% glucose, in aerobic conditions at 37°C. Before the start of the experiment, the optical density of each culture was adjusted to DO600 ≈ 0.5.

**Multispecies biofilm formation and dentine cylinders inoculation**

Before biofilm formation, the specimens were sterilized in an autoclave at 121°C for 20 minutes. Subsequently, the specimens were placed in Eppendorf tubes containing sterile culture medium (BHI) and subjected to an ultrasonic bath for 10 minutes to maximize the penetration of the culture medium into the dentinal tubules, according to the methodology of Neto et al. (2021). Then, the specimens were immersed into the Eppendorf tubes containing 1500 µL of a mixed culture inoculum (*E. faecalis* 1:100 (CF ≈ 106 CFU/mL), *S. mutans* 1:100 (CF ≈ 106 CFU/mL), and *C. albicans* (CF ≈ 104 CFU/mL)) and centrifuged at 1,400, 2,000, 3,600, and 5,600g (Hsiangtai Machinery Ind.CO; Taisan Hsiang, Taipei Hsien, Taiwan) for 2 cycles of 5 minutes each (Albuquerque et al., 2017). Between each centrifugation cycle, a fresh microbial inoculum was added to the Eppendorf tubes. At the end of the centrifugation cycles, the specimens were incubated for 21 days, with medium renewal every two days, under aerobic conditions at 37°C for biofilm formation. Finally, to remove non-attached microbial cells, the specimens were transferred to new Eppendorf tubes with 1500 µL of phosphate-buffered saline (PBS) solution.

**Biofilm sampling and colony-forming units counting**

The dentine cylinders (*n* = 12) were aseptically removed from the Eppendorf tubes, and the intracanal biofilm attached to the root canal walls was collected using a sterile size 45 Hedströem file (Dentsply Maillefer) (Azim et al., 2016; Alfadda et al., 2021). To standardize the biofilm sampling, the active part of the file was vigorously pressed against the root canal walls 6 times, ensuring contact with all surfaces of the canal (Figure 4). This process was performed twice. Subsequently, the files were individually placed in plastic flasks containing 2 mL of PBS, which were subjected to sonication for 15 minutes at an amplitude of 40 W to detach the biofilm. Then, the microbial suspensions were vortexed, and after serial dilution, 10 µL aliquots were plated in duplicate on BHI agar (CFUs). The plates were incubated aerobically at 37°C for 48 hours. The number of colony-forming units/mL (CFUs/mL) was then determined. The experiment was conducted at three different time points.

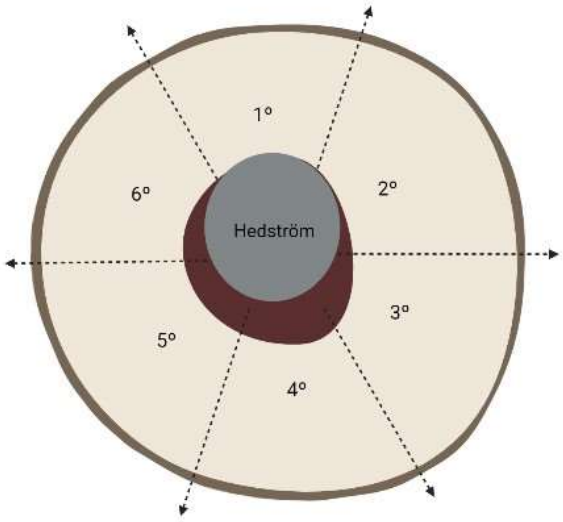


**FIGURE 4.** Schematic representation of the multispecies biofilm sampling. Axial section of the dentine cylinder with the Hedström file touching the root canal walls by region (1st to 6th region) in a clockwise direction.

**Biofilm architecture and dentine surface - Scanning Electron Microscopy (SEM)**

The architecture of the multispecies biofilm and the dentine surface was analyzed under SEM (JEOL JSM 6390 LV, Akishima, Japan) (*n* = 3). The dentine

cylinders were aseptically removed from the Eppendorf tubes and longitudinally cleaved to obtain hemisections, which allowed access to the root canal lumen. The hemisections were fixed in 2.5% glutaraldehyde buffered with 0.2 M cacodylate for 12 hours at 4°C, followed by washing with cacodylate buffer for 1 hour, and dehydration with increasing alcohol gradients (25%, 50%, 75%, and 95% for 20 minutes each, and 100% for 1 hour). Subsequently, they were mounted on metal stubs with the canal surface facing upwards, air-dried, and sputter-coated with a gold-palladium thin layer (300 Å). The images were obtained with the SEM operating at 10 kV. Two representative areas per hemisection, corresponding to the cervical and middle thirds, were selected to assess the dentine microstructure of uncontaminated specimens, and the architecture of the multispecies biofilm on the dentine surface of contaminated specimens. The specimens were examined under magnifications ranging from 1000× to 10,000×.

**Microbial penetration into dentinal tubules - Confocal Laser Scanning Microscopy (CLSM)**

**Specimens preparation**

Before biofilm formation, dentine cylinders (*n* = 3) were individually embedded in colourless self-curing acrylic resin (Dencrilay; Dencril, Vaieiras, SP, Brazil). The root canal entrance was protected with utility wax. After curing, the acrylic resin block containing the dentine cylinder was sectioned transversally in relation to its longitudinal axis with a double-sided diamond disc (Buehler, Lake Forest, IL, USA), under constant water cooling, to obtain 0.6-mm-thick dentine slices. The thickness of each dentine slice was measured using a digital caliper (Nagano, São Paulo, SP, Brazil). Next, the specimens were washed and sonicated in distilled water for 15 minutes at an amplitude of 40 W to remove debris formed during the cutting process.

**CLSM analysis**

After biofilm formation, microbial penetration into the dentinal tubules was assessed under CLSM (*n* = 3). The dentine slices were placed on a glass coverslip (0.17 mm thick). To determine bacterial viability, the "Live/Dead" kit

(BacLightTM L-13152 kit; Molecular Probes Inc., Eugene, OR, USA) was used. The biofilm was stained with SYTO 9 and propidium iodide dyes, which were applied to the specimens for 5 minutes at a 1:1 ratio (total volume = 200 µL), in the absence of light. The CLSM (Olympus Europa Holding GmbH, Hamburg, Germany) was set at excitation/emission wavelengths of 488 nm for SYTO 9 for live microorganisms (viable), and 514 nm for propidium iodide for dead microorganisms (non-viable).

The specimens were examined under 200×, 680×, and 1000× magnifications, with a numerical opening of 1.4, and the confocal pinhole set to a diameter of 60 µm. The fluorescence of the stained microorganisms was visualized, and the images were processed using the LAS AF Lite software (Leica Microsystems, Wetzlar, Germany) with a resolution of 1024 pixels and a zoom factor of 1.0, resulting in a final pixel resolution of 0.41 mm/pixel. Scans with a depth of 35 µm (1 µm step size, 35 slices/scan) were obtained from each specimen. A descriptive analysis of microbial penetration into the dentinal tubules was performed by a previously trained and calibrated examiner, blindly. Microorganisms stained in green (SYTO 9) indicate viable microbial cells. Microorganisms stained in red (propidium iodide) indicate non-viable microbial cells.

**Bovine teeth analysis**

Fifteen bovine incisors, which had been freshly extracted and were approximately three years old, were generously provided by a local slaughterhouse. The teeth underwent a 48-hour disinfection process in a 0.5% chloramine solution, followed by a thorough 24-hour rinse under running water. The removal of periodontal ligaments was carried out using scalpels and gauze pads. Subsequently, a meticulous clinical examination at 4× magnification with a magnifying lens was performed. The bovine teeth were stored in plastic flasks containing distilled water and maintained in an incubator at 37°C until the beginning of the experiments.

**Experimental groups distribution**

Before specimen preparation, the teeth were randomly allocated (www.random.org) into three groups (*n* = 5): G1) non-irradiated teeth; G2) teeth irradiated with 55 Gy; and G3) teeth irradiated with 70 Gy. The teeth were irradiated according to the protocol described above (Figure 3b).

**Specimens preparation**

The teeth had their crowns and apical portions removed using a double-sided diamond disc (Microdont) under constant water cooling to obtain root segments 20 mm in length. The pulp tissue was removed with a size 35 K-file (Dentsply Maillefer), under constant irrigation (5 mL) with 0.85% sterile saline solution using a plastic syringe (Ultradent) and a 30-gauge NaviTip needle (Ultradent). Subsequently, the root segments were cross-sectioned with a double-sided diamond disc (Microdont), resulting in root dentine fragments (8 mm in height x 4 mm in width x 2 mm in thickness), totalling 15 specimens per group. The specimens were meticulously polished with abrasive sandpaper (Norton, São Paulo, SP, Brazil) in decreasing order of abrasiveness (grit 220, 400, 600, and 1200), under constant water cooling, followed by polishing with alumina-based paste in decreasing order of abrasiveness (0.3, 0.1, and 0.05 μm). Between the use of each sandpaper and paste, the specimens were carefully washed under copious running water. Finally, the specimens were immersed in an ultrasonic bath containing distilled water for 3 minutes for debris removal. Afterwards, the initial microhardness test (D0 - baseline) was performed.

**Multispecies biofilm formation**

As previously described, the same microbial species and growth conditions were performed. Before biofilm formation, the dentine specimens were positioned in the Amsterdam Active Attachment biofilm model (AAA-model) (Exterkate et al., 2010; de Almeida et al., 2018), following a previously published protocol (de Almeida et al., 2017), and then sterilized with ethylene gas.

A 24-well culture plate was inoculated with 1.5 mL/well of growth medium and inoculated with the diluted mixed culture of *E. faecalis*, *S. mutans*, and *C. albicans* at a 1:100 ratio (OD ≈ 1 nm). The plate was sealed with the AAA-model.

The bovine dentine specimens were suspended, serving as a substrate for passive biofilm growth. The setup was incubated for 21 days under aerobic conditions at 37°C, with medium renewal every two days. In the end, to remove non-attached microbial cells, the specimens were transferred to new Eppendorf tubes with 1200 µL of phosphate-buffered saline (PBS) solution.

**Biofilm biomass analysis**

After the incubation period, the multispecies biofilm formed on bovine dentine specimens was stained with 0.01% crystal violet for 10 minutes. The specimens were removed from the Eppendorf tubes containing PBS and immersed in 1 mL of crystal violet solution. Next, they were consecutively immersed (3x) in Eppendorf tubes containing PBS to remove the excess dye solution. Then, the crystal violet was solubilized by immersing the specimens in 1 mL of 2% sodium deoxycholate in Eppendorf tubes for 10 minutes. Four 200 µL aliquots of the stained solution were removed from each tube and added to the wells of a 96-well plate. The biofilm biomass quantification was performed by measuring absorbance at 620 nm (ThermoPlate; TP-Reader 501122026FSE, Curitiba, PR, Brazil).

**Knoop microhardness test**

The initial Knoop microhardness (Shimadzu HMV-2; Shimadzu, Tokyo, Japan) of the dentine specimens was designated as "D0" (baseline). Microhardness was calculated from the average of three equidistant indentations at a depth of 150 µm, applied with a 50 g load for 10 seconds. The area of the indentations was measured at 400× magnification using the Newage C.A.M.S (Computer Assisted Measurement System; Newage Testing Instruments Inc., Southampton, PA, USA) software. After biofilm formation and the colourimetric test with the crystal violet, the specimens underwent a second microhardness assessment, designated as "D1." The calculation for "D1" followed the same protocol used for obtaining the initial microhardness values (D0).

### Statistical analysis

For all analyses, the homogeneity of variances was assessed using the Levene test, and the normality of residuals was assessed using the Shapiro-Wilk test. The significance level was set at 5%. All analyses were conducted with the aid of the SPSS software version 21.0 (IBM, Armonk, NY, USA). For the multispecies biofilm cell viability (CFU counting), the mean values of CFU/mL were determined, and the data were normalized by taking the base-10 logarithm ($log^{10}$) of each CFU/mL value. Considering parametric data for biofilm cell viability and biofilm biomass, the One-way ANOVA and Tukey's post hoc tests were applied. For the microhardness test, a paired t-test was used. The analysis of the biofilm architecture on dentine (SEM) and the penetration of microorganisms into the dentinal tubules (CLSM) was descriptive.

## RESULTS

### Biofilm cell viability - CFU counting

The mean values ($log^{10}$) of CFUs/mL, corresponding to viable microorganisms attached to the root canal walls, are expressed in Table 1.

**TABLE 1.** Mean values ($log^{10}$) and standard deviation (SD) of CFUs/mL corresponding to viable microorganisms attached to the root canal walls.

| Groups | Log CFUs/mL ± SD |
|---|---|
| Non-irradiated | 6.02 ± 0.35 [a] |
| 55 Gy | 5.96 ± 0.52 [a] |
| 70 Gy | 6.58 ± 0.37 [b] |

Different lowercase letters in the same column indicates a significant difference.
One-way ANOVA and Tukey's post hoc tests ($\alpha$ = 5%).
$n$ = 12

Specimens irradiated with 70 Gy had greater multispecies biofilm formation, with a higher number of viable microorganisms in comparison with non-irradiated ($p$ = .010) and specimens irradiated with 55 Gy ($p$ = .004).

**SEM analysis**

Representative SEM images may be seen in Figures 5-8. The analysis of specimens without biofilm showed a sound non-irradiated dentine surface (Figure 5a). Specimens irradiated with 55 Gy showed microcracks around the dentinal tubules and an eroded appearance (Figure 5b). In the specimens irradiated with 70 Gy, the dentine surface displayed a more eroded surface, with morphologically altered and partially obliterated dentinal tubules (Figure 5c).

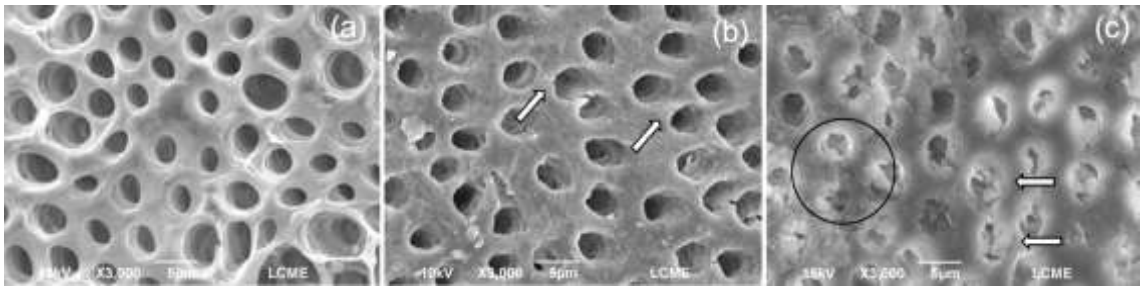


**FIGURE 5.** Representative SEM images of specimens without biofilm formation. (a) Non-irradiated specimen. Non-obstructed dentinal tubules. (b) 55 Gy. Root canal dentine with the presence of microcracks (arrows) and dentinal tubule entrances partially obliterated. (c) 70 Gy. Root canal dentine with dentinal tubule entrances completely obliterated (arrow). Note the disruption of the inter- and peritubular dentine, with different levels of erosion caused by irradiation (circle). Magnification of 3,000×.

After the 21-day incubation period, it was possible to observe a dense, mature, homogeneous, and well-structured multispecies biofilm covering the entire length of the root canal for all experimental conditions (non-irradiated- Figure 6; 55 Gy - Figure 7; and 70 Gy - Figure 8). However, in the specimens irradiated with 55 Gy and 70 Gy, the presence of a biofilm with a higher concentration of microorganisms and more complex microbial colonies was noted when compared to non-irradiated dentine.

In all experimental conditions (non-irradiated and irradiated), microbial penetration into the dentinal tubules was observed. The microorganisms *E. faecalis* and *S. mutans* were able to penetrate and colonize the dentinal tubules of the non-irradiated specimens (Figure 6e-f), specimens irradiated with 55 Gy (Figure 7f), and specimens irradiated with 70 Gy (Figure 8f) more easily than the *C. albicans*.

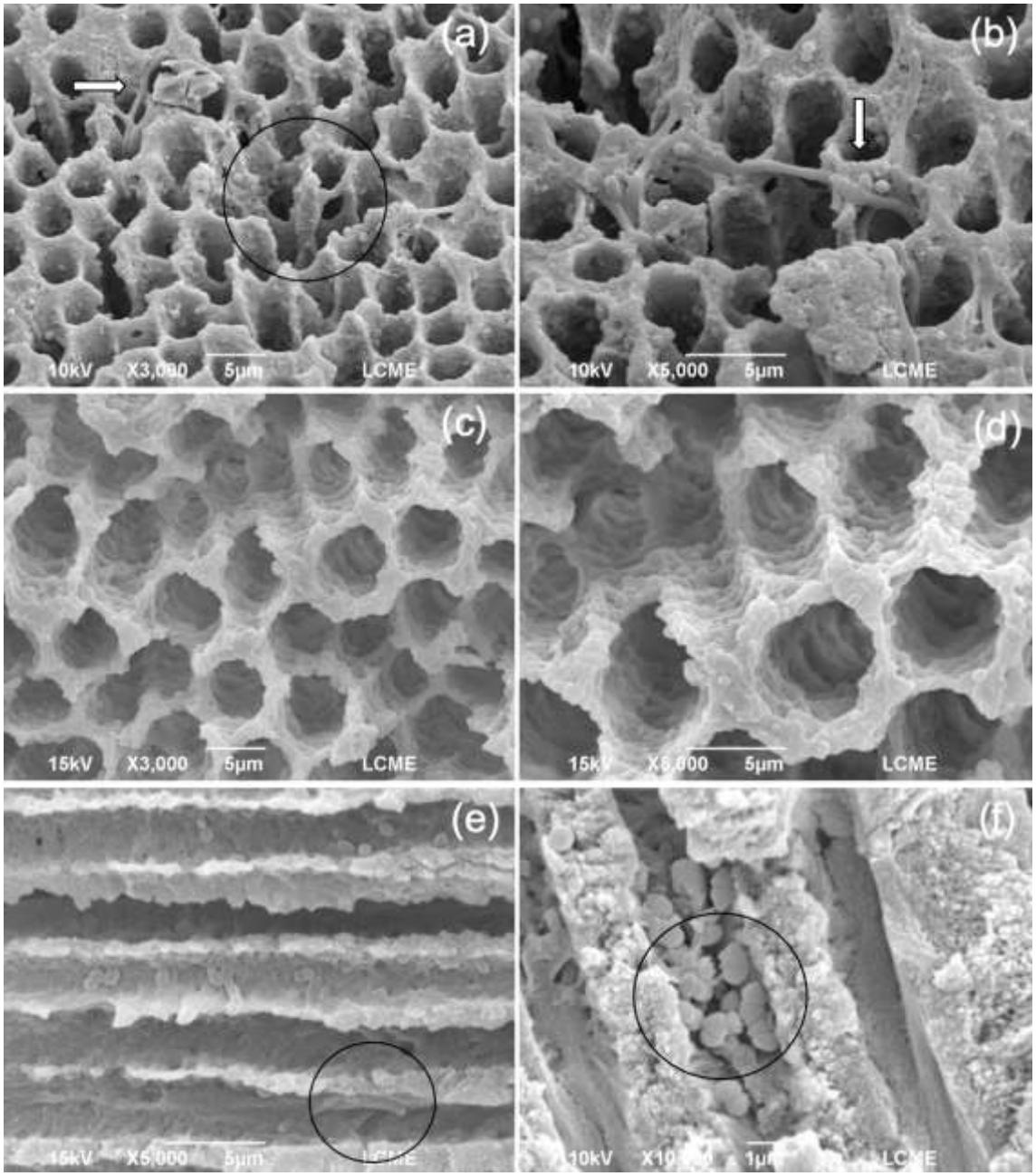


**FIGURE 6.** Representative SEM images of non-irradiated specimens after the 21-day incubation period for multispecies biofilm formation. (a) Note the presence of an eroded dentine caused by the biofilm (circle). (a) Collapse of the cell walls of *C. albicans* (arrow). (b) Presence of hyphae in part of the dentin substrate (arrow). (c and d) Architecture of a uniform and smooth multispecies biofilm covering the surface of the root canal lumen. (e) Note the presence of *C. albicans* hyphae (circle), and (f) *S. mutans* and *E. faecalis* microorganisms (circle) within the dentinal tubules. Magnifications of 3,000×, 5,000×, and 10,000×.

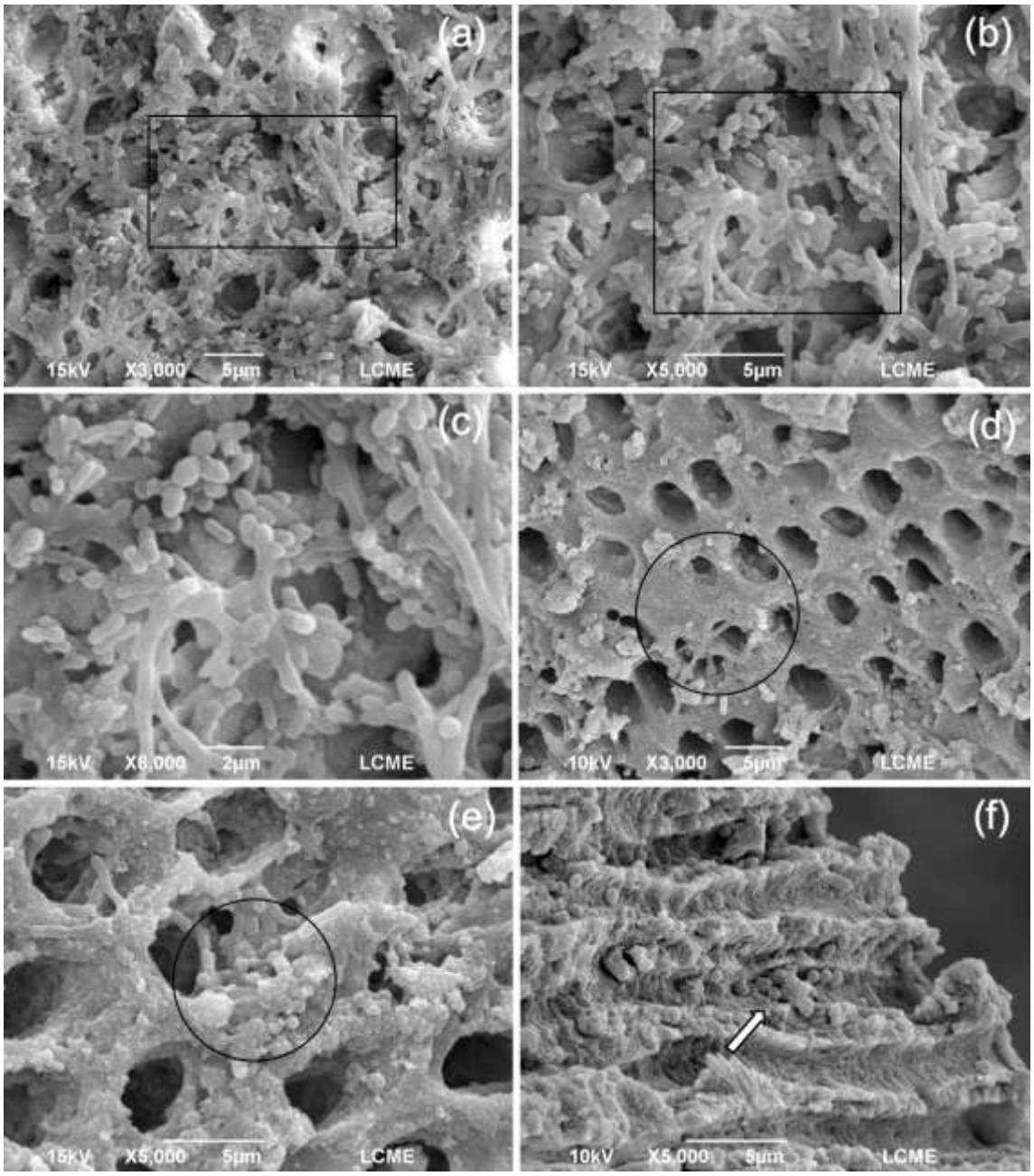


**FIGURE 7.** Representative SEM images of specimens irradiated with 55 Gy after the 21-day incubation period for multispecies biofilm formation. (a-c) Architecture of a more complex and irregular multispecies biofilm covering the entire surface of the root dentine. Note the presence of *S. mutans*, *E. faecalis* and *C. albicans* hyphae (detail). (d and e) Eroded dentine and dentinal tubules entrance partially obliterated. It is possible to observe the penetration of microorganisms into the dentinal tubules (circle). (f) Microorganisms within the dentinal tubules (arrow). Magnifications of 3,000×, 5,000×, and 8,000×.

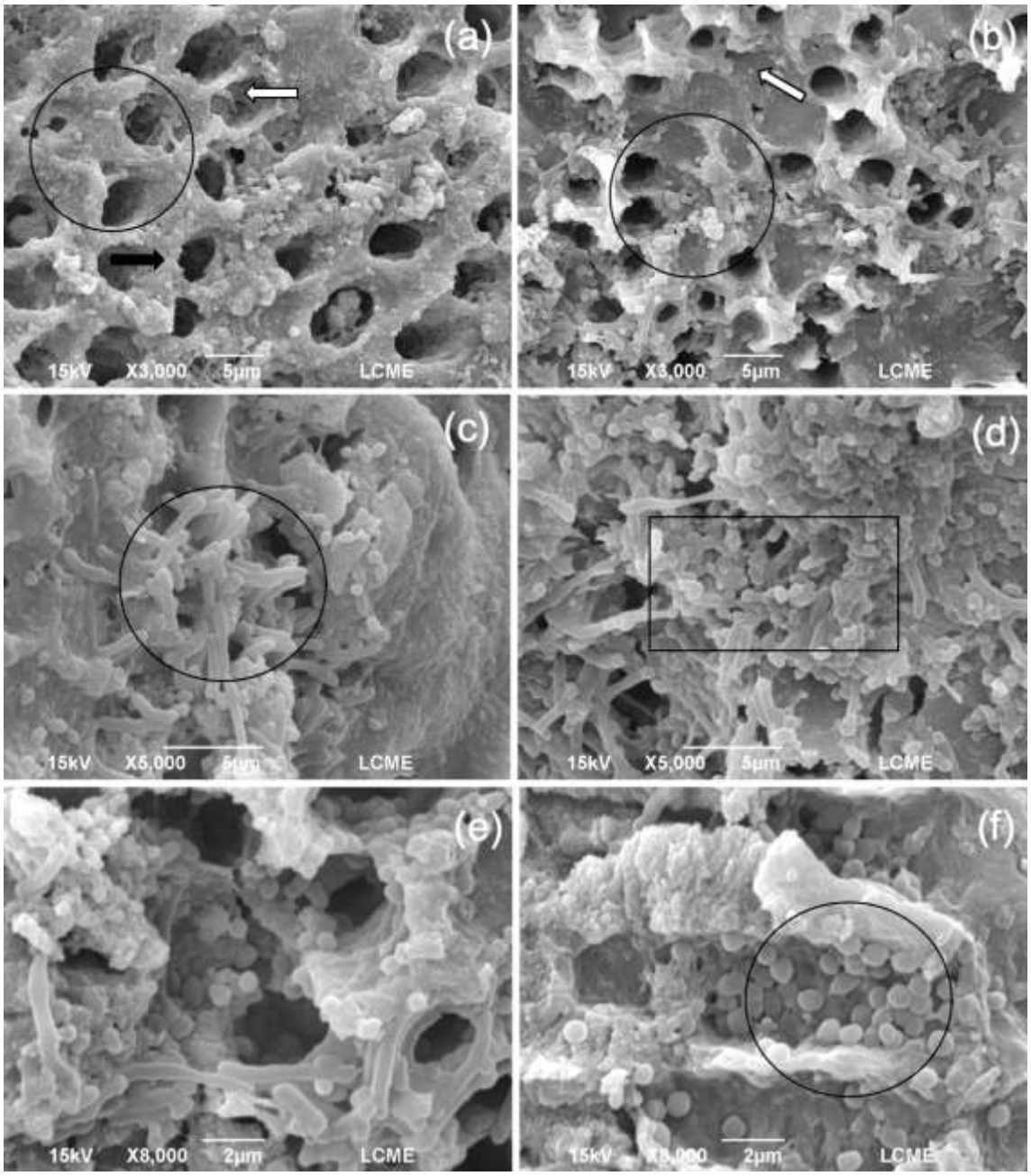


**FIGURE 8.** Representative SEM images of specimens irradiated with 70 Gy after the 21-day incubation period for multispecies biofilm formation. (a and b) Dentin substrate highly eroded (circle). Note the extensive disruption of the peri- and intertubular dentine (arrow). (c) Hyphae formation covering the dentine substrate (circle). (d) Multispecies biofilm covering the entrance of the dentinal tubules. Note a more complex formation, with greater microorganisms' aggregation (detail) (e) *C. albicans* interaction with the other microorganisms (*S. mutans* and *E. faecalis*). (f) *S. mutans* and *E. faecalis* within the dentinal tubules. Note the morphological characteristics of both microorganisms (circle). Magnifications of 3,000×, 5,000×, and 8,000×.

## CLSM analysis

Representative CLSM images may be seen in Figure 8. The three-dimensional images showed the presence of viable and non-viable microorganisms in the root canal lumen and inside the dentinal tubules after the 21-day incubation period (Figure 9). A similar amount of microorganisms was observed inside the dentinal tubules, regardless of the experimental condition (non-irradiated - Figure 9a; 55 Gy - Figure 9b; and 70 Gy - Figure 9c).

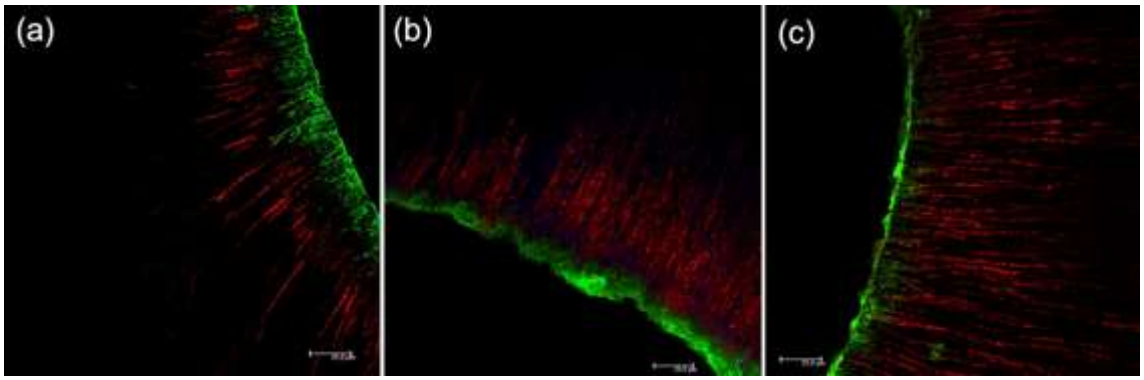


**FIGURE 9.** Representative CLSM images of the root dentine after the 21-day incubation period for multispecies biofilm formation. Note the *E. faecalis* and *S. mutans* penetrating the dentinal tubules of non-irradiated specimens (a), irradiated with 55 Gy (b), and 70 Gy (c). Green colour (SYTO 9) indicates viable microbial cells and red colour (propidium iodide) indicates non-viable cells. Note that in the non-irradiated specimens, there was a greater penetration of viable microorganisms inside the dentinal tubules compared to the irradiated ones. In the irradiated specimens, the majority of viable microorganisms remained confined to the root canal lumen. Microorganisms inside the dentinal tubules may be clearly identified by their coccus morphology under 630× magnification. All bars represent 25 μm.

## Biofilm biomass assay

Table 2 presents the values of biofilm biomass formed in non-irradiated dentine specimens, irradiated with 55 Gy, and 70 Gy. The highest amount of biofilm biomass was observed in the specimens irradiated with 70 Gy, in comparison with the biomass formed in the specimens irradiated with 55 Gy ($p < .001$) and non-irradiated ($p < .001$), which had the lowest amount.

**TABLE 2.** Mean values and standard deviation (SD) of the multispecies biofilm mass formed on the dentine specimens and stained with crystal violet.

| Groups | Biofilm biomass (DO620 nm) ± SD |
|---|---|
| Non-irradiated | 0.026467 ± 0.00807 [a] |
| 55 Gy | 0.043767 ± 0.0083305 [b] |
| 70 Gy | 0.063021 ± 0.0103756 [c] |

Different lowercase letters in the same column indicates a significant difference.
(OD) optical density.
One-way ANOVA and Tukey's post hoc tests ($\alpha$ = 5%).
$n$ = 15

**Knoop microhardness**

The Knoop microhardness values are shown in Table 3.

After the 21-day incubation period, a significant reduction in the microhardness was observed in the non-irradiated specimens, irradiated with 55 Gy, and 70 Gy ($p$ < .001). The irradiated specimens (55 and 70 Gy) had a significantly greater reduction in microhardness post-biofilm formation compared to non-irradiated specimens ($p$ < .001).

**TABLE 3.** Mean values and standard deviation (SD) of Knoop microhardness of non-irradiated and irradiated (55 Gy and 70 Gy) specimens after 21 days of multi-species biofilm formation.

| Groups | Microdureza | |
|---|---|---|
| | D0 | D1 |
| Non-irradiated | 29.55 ± 6.57 | 18.88 ± 5.22 * |
| 55 Gy | 39.16 ± 5.99 | 20.66 ± 6.76 * |
| 70 Gy | 39.93 ± 6.27 | 14.42 ± 2.38 * |

(*) in the same row indicates a significant difference between D0 and D1.
paired t-test ($\alpha$ = 5%).
$n$ = 15

**DISCUSSION**

Studies confirm the deleterious effects of radiation therapy on dentine. Nonetheless, scientific evidence regarding the formation of multispecies biofilms on this type of dentine is currently lacking. Therefore, the present study bridged a gap in the current scientific literature.

Based on the results obtained, the first null hypothesis was partially accepted, as only specimens irradiated with 50 Gy showed a similar number of microorganisms to non-irradiated dentine. When irradiated with 70 Gy, the root dentine specimens had a significantly higher number of microorganisms than the other experimental groups. The second null hypothesis was also partially accepted. Specimens irradiated with 55 Gy and 70 Gy displayed a more complex biofilm architecture than non-irradiated dentine. However, there was similar microbial penetration into the dentinal tubules for all experimental conditions.

The third and fourth null hypotheses were both rejected, as the biofilm biomass was greater in dentine irradiated with 55 Gy and 70 Gy. Furthermore, there was a significant reduction in the dentine microhardness post-biofilm formation in the irradiated specimens.

In this study, the cervical and middle thirds of human single-rooted teeth were selected to create dentine cylinders. This choice was influenced by the anatomical complexity of the apical third (Ricucci et al., 2010). It is considered a critical zone due to the presence of root curvatures, ramifications, and lateral canals, which may often remain untouched by endodontic instruments during mechanical preparation (Ricucci et al., 2010). For this reason, dentine cylinders were employed to ensure a standardized approach for biofilm formation and sampling (Azim et al., 2016; Alfadda et al., 2021).

Since endodontic infections are typically polymicrobial, a multispecies biofilm was established using the microorganisms *E. faecalis*, *S. mutans*, and *C. albicans*. These microorganisms may readily flourish in patients undergoing head and neck cancer radiotherapy (Epstein et al., 1998). Furthermore, as previously reported (Alfadda et al., 2021), this study employed Hedström files for biofilm sampling, enabling the active part of the instrument to scrape the root canal walls. This biofilm sampling method overcomes the limitations associated with sterile paper points sampling, where microorganisms in hard-to-reach areas are not effectively collected (Siqueira et al., 2013). Additionally, paper points cannot disrupt the biofilm attached to the root canal walls (Siqueira et al., 2013).

Due to methodological reasons, bovine incisor teeth were used for biofilm biomass quantification and dentine microhardness assessment. The larger size of bovine incisor teeth, in comparison to single-rooted human teeth, facilitated the

preparation of dentine specimens with suitable flat surfaces for use in the AAA-model and for conducting the microhardness test. Acquiring a significant number of human teeth in suitable conditions for experimentation may raise substantial ethical concerns (Inagati et al. 2021). Both human and bovine teeth have been used in several studies (Silva et al. 2019; De Souza et al. 2022), especially due to their morphological and histological similarities (Neves et al., 2018), and the consistency of findings across these two models enhances the credibility of employing bovine teeth in laboratory investigations.

Depending on the location and type of tumour, the cumulative radiation doses reaching dental structures may be as high as the final dose delivered directly to the tumour (Polce et al., 2021). Due to changes in its organic and inorganic components, dentine is severely affected by radiation (Velo et al., 2018). Among these changes, it is worth mentioning the reduction in calcium (Ca) and phosphorus (P) ions (Velo et al., 2018). The Ca/P ratio determines the hydroxyapatite mineralization rate, a parameter directly related to the mechanical properties of the dentine and its biodegradation rate (Slosarczyk et al., 1999). Lower Ca/P values indicate that irradiated root dentine is less mineralized, resulting in a hydroxyapatite content more susceptible to degradation (Velo et al., 2018). These changes in dentine structure may have an additional impact on biofilm formation and dentine microhardness post-biofilm formation, as noted in the present study.

Biofilm is the most common form of microbial growth to withstand the environment (Lin et al., 2017). It consists of three basic components: microorganisms, extracellular polymeric substances (EPS), or self-produced matrix, and substrate, each playing unique roles in the overall biofilm maturation (Xie et al., 2017). During biofilm formation, microorganisms may dissolve the dentine structure and induce the precipitation of calcium carbonate and calcium phosphate (van der Waal et al., 2012). When incorporated into the biofilm structure, calcium may lead to an increase in its cohesive strength and, consequently, its stability (van der Waal et al., 2012). The lack of calcium during biofilm attachment to the substrate results in a weak structure that cannot support the development of additional layers during its formation (Huang et al., 1994). Although calcium induces extracellular matrix formation, it does not have a

stimulating effect on microbial growth rate (van der Waal et al., 2012). However, it may change the rate and extent of biofilm accumulation as calcium concentration increases (Patrauchan et al., 2005). Therefore, changes in dentine irradiated with 70 Gy may have contributed to the increase in the extent and thickness of the multispecies biofilm, and consequently, in the number of microorganisms, however, not in the cell density within the biofilm.

Biomass is a pathogenic characteristic of biofilms (Lin et al., 2017). It is composed of viable and non-viable cells, the matrix, and all metabolites produced by microorganisms (Lin et al., 2017). EPS are essential for biofilm formation, and their complexity provides functions that allow biofilm expansion and protection for microorganisms (Lin et al., 2017). A high concentration of calcium ions within the biofilm allows for an increase in its biomass and the strengthening of its structure (Lin et al., 2017). Calcium ions facilitate binding between polysaccharide-cell and polysaccharide-polysaccharide (Huang et al., 1994; Lin et al., 2017), as well as increase the production of exopolysaccharides that compose biofilms (Huang et al., 1994). Therefore, this might be a possible explanation for the results found in this study, as a gradual and significant increase in biofilm biomass was observed from non-irradiated dentine to dentine irradiated with 55 Gy and 70 Gy.

Additionally, calcium concentration possibly had a direct effect on the biofilm's surface. Lower calcium concentration is related to a biofilm with a smooth surface appearance (van der Waal et al., 2012; Körstgens et al., 2001). Conversely, higher calcium concentration results in a biofilm with a rough surface due to aggregate formation (van der Waal et al., 2012; Körstgens et al., 2001). SEM images showed noticeable differences in the biofilm formed on non-irradiated dentine, which appeared smoother and more delicate. Dentine specimens irradiated with 55 Gy and 70 Gy displayed a biofilm with a denser, thicker, and irregular appearance. Furthermore, the SEM images confirmed the growth of microorganisms *S. mutans*, *E. faecalis*, and *C. albicans* within the biofilm, based on their morphological characteristics.

The irradiated dentine led to the formation of a more complex biofilm, characterized by the presence of hyphae. *C. albicans* may grow structurally in the root canal lumen in the form of yeast cells, germ tubes, and hyphae, as previously demonstrated in several studies (Gow et al., 1982; Yokoyama et al., 1983; Gow

et al., 1984; Brawner et al., 1985). The formation of germ tubes occurs when the cytoplasmic content of the cell is extruded, giving rise to hyphae (Sem et al., 1997). The SEM images of this study revealed few yeast cells due to the collapse of the cell walls. This collapse results from the sample preparation procedures for SEM analysis (Sem et al., 1997). Nevertheless, the significant presence of hyphae indicates that the cells were actively growing (Gow et al., 1984). Furthermore, in the presence of a typical mixed endodontic infection, *C. albicans* may interact with other microorganisms and microbial products, leading to coaggregation reactions and the formation of a complex biofilm, as observed in the current study (Bagg et al., 1986; Jenkinson et al., 1990).

Although some studies have reported partial or total obliteration of the dentinal tubules entrance after irradiation (Velo et al., 2018), this phenomenon did not affect the penetration of microorganisms into the dentinal tubules, as shown in the CLSM and SEM images. In the CLSM analysis, the presence of bacteria in the dentinal tubules is identifiable due to their coccal structure. Due to the changes caused to dentine by irradiation, leading to increased biofilm complexity, it may be hypothesized that a proper sanitization of dentinal tubules may be hindered. Therefore, further studies are needed to confirm this theory.

The teeth were exposed to radiation once a day at a dose of 2 Gy/fraction, administered 5 days a week, similar to the standard protocol used for the clinical treatment of head and neck cancer (Jham et al., 2006). Typically, therapeutic radiation reaches approximately 55 Gy in 5 weeks of treatment. Our results showed that the gradual exposure to radiation up to 70 Gy directly influenced the findings, demonstrating that the side effects of radiation therapy are cumulative and irreversible, and they directly impact biofilm formation on dentine. These changes may compromise the success of endodontic treatment in oncological patients undergoing head and neck radiotherapy. Consequently, additional *in vitro* and *in vivo* research should be undertaken to explore and establish suitable clinical guidelines for addressing the requirements of these patients.

## CONCLUSION

Despite the inherent limitations of an *in vitro* study, based on the results obtained, it is possible to state that radiation therapy gradually led to the formation

of a more complex biofilm and a reduction in dentine microhardness post-biofilm formation. Dentine irradiated with 70 Gy allowed the formation of a multispecies biofilm with a greater number of viable microorganisms attached to the root canal lumen and greater biomass. Microbial penetration into dentinal tubules was noticeable, regardless of the radiation dose. However, non-irradiated specimens showed greater penetration of viable microorganisms inside the dentinal tubules compared to the irradiated ones. In the irradiated specimens, viable microorganisms were found predominantly in the root canal lumen.

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## CONFLICT OF INTEREST STATEMENT

The authors do not have any conflict of interest to declare.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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## 4. CONCLUSÃO

Apesar das limitações inerentes de um estudo *in vitro*, e com base nos resultados obtidos, é possível afirmar que a dentina irradiada levou à formação de um biofilme mais complexo e à diminuição da microdureza dentinária pós-formação do biofilme. Além disso, houve penetração microbiana nos túbulos dentinários, independentemente da dose de radiação. Um número maior de microrganismos foi observado na dentina irradiada com 70 Gy.

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# ANEXO A - PARECER DO COMITÊ DE ÉTICA EM PESQUISA COM SERES HUMANOS

UNIVERSIDADE FEDERAL DE SANTA CATARINA - UFSC

Plataforma Brasil

## PARECER CONSUBSTANCIADO DO CEP

**DADOS DO PROJETO DE PESQUISA**

**Título da Pesquisa:** ANÁLISE ESTRUTURAL DO BIOFILME MULTIESPÉCIE E DA PENETRAÇÃO MICROBIANA NOS TÚBULOS DENTINÁRIOS EM DENTES IRRADIADOS

**Pesquisador:** LUCAS DA FONSECA ROBERTI GARCIA

**Área Temática:**

**Versão:** 2

**CAAE:** 66264122.2.0000.0121

**Instituição Proponente:** CENTRO DE CIÊNCIAS DA SAÚDE

**Patrocinador Principal:** Financiamento Próprio

**DADOS DO PARECER**

**Número do Parecer:** 5.921.189

**Apresentação do Projeto:**

As informações que seguem e as elencadas nos campos "Objetivo da pesquisa" e "Avaliação dos riscos e benefícios" foram retiradas do arquivo PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_...pdf, de 18/02/2023, preenchido pelos pesquisadores.

Segundo os pesquisadores:

Resumo:

O objetivo deste estudo in vitro será avaliar a estrutura de um biofilme multiespécie sobre a dentina intrarradicular irradiada e a penetração dos microrganismos nos túbulos dentinários. Serão utilizados 72 cilindros de dentina radicular, obtidos a partir de dentes unirradiculares humanos. Os cilindros de dentina distribuídos aleatoriamente em seis grupos experimentais. Três grupos não terão a presença de biofilme no canal radicular e vão ser analisados apenas em Microscopia Eletrônica de Varredura (MEV) (n = 3): G1) dentina hígida; G2) dentina irradiada com 55 Gy; e G3) dentina irradiada com 70 Gy. Nos outros três grupos, um biofilme multiespécie de Enterococcus faecalis, Streptococcus mutans e Candida albicans será formado no canal radicular (n = 21): G1) dentina hígida + biofilme; G2) dentina

**Endereço:** Universidade Federal de Santa Catarina, Prédio Reitoria II, R: Desembargador Vitor Lima, nº 222, sala 701
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| Projeto Detalhado / Brochura Investigador | Projeto_de_Pesquisa.pdf | 18/02/2023 22:26:46 | LUCAS DA FONSECA ROBERTI GARCIA | Aceito |
| TCLE / Termos de Assentimento / Justificativa de Ausência | TCLE.pdf | 15/12/2022 16:23:03 | LUCAS DA FONSECA ROBERTI GARCIA | Aceito |
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| Declaração de Instituição e Infraestrutura | Declaracao_Infraestrutura_UFSC_assinado.pdf | 15/12/2022 15:50:02 | LUCAS DA FONSECA ROBERTI GARCIA | Aceito |

**Situação do Parecer:**
Aprovado

**Necessita Apreciação da CONEP:**
Não