UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA

BRUNA BARCELOS SÓ

ANÁLISE DA FORMAÇÃO E DESENVOLVIMENTO DE BIOFILMES EM BLOCOS DE DENTINA HUMANA

Porto Alegre
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Trabalho de Conclusão de Curso apresentado ao Curso de Graduação em Odontologia da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do título de Cirurgiã-Dentista.

Orientador: Prof. Dr. Francisco Montagner

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RESUMO

Micro-organismos e seus produtos são os principais agentes causadores de patologias pulpares e periapicais. Esses micro-organismos podem estar dispostos em biofilmes em infecções primárias ou secundárias do canal radicular. Por isso, é de extrema importância para a endodontia estudar esses biofilmes. No entanto, ainda há muita controvérsia na literatura sobre a metodologia de cultivo e incubação. Portanto, o objetivo desse estudo é caracterizar biofilmes formados *in situ* seguido de diferentes métodos de incubação e meio de cultura. Com isso, o objetivo é também avaliar a influência do meio de cultura e do tempo de incubação na formação de biofilmes *in situ* e desenvolvimento *in vitro*. Para esse estudo, foram selecionados 5 voluntários de acordo com critérios de inclusão. A metodologia foi separada em 2 fases e os mesmos 5 voluntários participaram de ambas. Uma placa intra-oral foi confeccionada para cada voluntário. Em cada uma, foram fixados 4 blocos de dentina humana (2x2x2mm). Os voluntários utilizaram a placa durante 3 dias e então uma amostra de cada placa foi separada para a extração de DNA e hibridização DNA-DNA (*Checkerboard DNA-DNA Hybridization*). As outras 3 amostras de cada voluntário foram incubadas em meio de cultura BHI e estufa microbiológica (37º C). No período de 7, 14 e 21 dias, uma amostra de cada voluntário era removida para extração de DNA e análise de hibridização. Outra amostra foi avaliada quanto ao biovolume de biofilme e quanto a viabilidade das células bacterianas, por meio de microscopia confocal a laser. Após a primeira fase, houve um período de intervalo (*wash-out*) de 7 dias até a segunda fase. A mesma metodologia foi realizada com diferença para as condições de incubação que, dessa vez, foi meio de cultura FAB em jarra de anaerobiose. Os dados foram tabulados e procedeu-se a análise estatística. Os resultados mostraram que houve maior diversidade e quantidade de espécies para o meio FAB em jarra de anaerobiose em comparação ao BHI. A análise imediata mostrou uma maior diversidade de espécies e também maior carga microbiana tanto para o BHI quanto para o FAB. Análises de agrupamento de Ward demostraram que amostras pertencentes ao mesmo participante, mas em diferentes tipos de incubação, não são similares. As amostras de um mesmo período de tempo, mas provenientes de diferentes voluntários também não são similares. As amostras de BHI, de um modo geral, são mais similares entre si do que as do FAB. O biofilme de FAB em 14 dias tem carga microbiana significantemente maior que o período imediato e 7 dias nas mesmas condições, e também foi significantemente maior que o mesmo período em BHI. A carga microbiana das espécies selecionadas em BHI decresce ao longo do tempo, no entanto, o biovolume permanece estatisticamente estável. É possível que haja um crescimento de micro-organismos anaeróbios facultativos, os quais não tem uma significante representatividade nas sondas de DNA selecionadas para esse estudo. Pode-se concluir que há particularidades nos biofilmes formados mesmo em mesmas condições de incubação ou de amostras provenientes de um mesmo paciente. Além disso, não há uma metodologia ideal para a formação de biofilmes pois depende do objetivo de cada estudo o qual definirá o tipo de biofilme que se espera e, com isso, também sua metodologia.

Palavras-chave: Microbiologia. Bactéria. Biofilmes. Hibridização *in situ*. Microscopia confocal.

ABSTRACT

Microorganisms can be disposed in biofilms and cause primary or secondary infections in the root canal system and periapical tissues. Therefore, studying these biofilms is extremely important in Endodontics. However, the current literature presents many controversies with regard to the methodology of biofilm cultivation and incubation. Then, the aim of this study is to characterize the biofilm which is formed *in situ* followed by different incubation and culture media conditions. Additionally, the aim is also to evaluate the influence of the culture media and incubation period in the *in situ* biofilm formation and *in vitro* development. Five volunteers were selected to this study according to the inclusion criteria. The methodology was divided in 2 different stages, and same volunteers participated in both. An intraoral apparatus was made for each individual. Four human dentine blocks (2x2x2mm) were attached to each apparatus. Volunteers used these apparatuses for 3 days and then one sample of each was separated for DNA extraction and Checkerboard Hybridization. Those 3 left dentine blocks of each volunteer were incubated in BHI culture media in microbiological incubator (37ºC). In 7, 14 and 21 days, one sample of each volunteer was removed for DNA extraction and Checkerboard analysis. The sample was evaluated for biofilm biovolume and percentage of viable cells, through confocal microscopy. After this first stage, there was a *washout* period of 7 days until the next stage. Same methodology was performed in the second stage, apart from the incubation conditions which were in FAB culture media and anaerobiosis jar. Data were collected and statistical analysis was carried out. The results showed that there were greater species diversity and higher microbial loads for FAB and anaerobiosis jar in comparison with BHI. Immediate analysis showed greater diversity and higher loads for both BHI and FAB in comparison with the other time periods. Ward's grouping analysis showed that even those samples which belonged from the same patient were not similar to each other when they are incubated in different conditions. Samples from same time period which belong to different patients were not similar to each other as well. BHI incubated samples are more similar among them in comparison with the FAB ones. The biofilms which were incubated in FAB for 14 days are statistically higher in bacterial load than immediate and 7 days in same incubation conditions. Furthermore, samples of FAB in 14 days were statistically higher in bacterial load when comparing to the same time period in BHI. Microbial loads of the selected species seem to decrease over time in BHI conditions whereas the biovolume remained statistically stable. This situation might be associated with the growth of facultative anaerobe species, which did not have a significant representativeness in the selected species for this study. In conclusion, every biofilm, even when they originally belong from the same patient or incubated in same conditions, is unique. Furthermore, there is no ideal methodology for biofilm formation and development since it depends on the aim of each study which define the one which adapts the most according to the type of biofilm that is going to be formed.

Keywords: Microbiology. Biofilm. Bacteria. *In situ* hybridization. Confocal microscopy.

SUMÁRIO

1 INTRODUÇÃO

Micro-organismos e seus produtos são considerados os principais agentes etiológicos das lesões periapicais (KAKEHASHI; STANLEY; FITZGERALD, 1965; SUNDQVIST, 1976) bem como de alterações pulpares (RICUCCI; SIQUEIRA, 2010). Para desencadear lesões periapicais, esses micro-organismos podem estar dispostos sobre a forma de biofilme o qual é encontrado na polpa necrótica em infecções primárias ou secundárias do sistema de canais radiculares (RICUCCI et al., 2009). O biofilme pode ser definido como células bacterianas ligadas a uma superfície incorporado a uma matriz em que o polissacarídeo extracelular (PEC) preenche o espaço entre as células (COSTERTON, 2007).

Segundo Sundqvist et al. (1998), o tratamento endodôntico objetiva eliminar os micro-organismos que infectam o canal radicular, com consequente reparo da região periapical. No entanto, quando eles estão dispostos em um biofilme se torna muito mais difícil eliminá-los, pois eles são resistentes ao estresse alcalino (CHÁVEZ DE PAZ et al., 2007) e tem aumentada resistência a agentes antimicrobianos (DUNAVANT et al., 2006). Por isso, se torna cada vez mais importante o estudo dos biofilmes associados às infecções endodônticas, para que sejam propostas alternativas adequadas para melhor eliminá-los do sistema de canais radiculares, contribuindo para o sucesso do tratamento endodôntico.

Diferentes modelos *in vitro* (CHÁVEZ DE PAZ, 2012; GRÜNDLING et al., 2011; JARAMILLO et al., 2012; OZDEMIR et al., 2010; RIOS et al., 2011; YASUNAGA et al., 2013), *ex vivo* (BERGMANS et al., 2008; SAHAR-HELFT et al., 2013; STOJICIC et al., 2013) e *in vivo* (BONSOR et al., 2006; BURLESON et al., 2007; RÔÇAS; SIQUEIRA, 2011) foram propostos para o estudo de biofilmes sobre a dentina humana e bovina. No entanto, não há um modelo padrão que determine a metodologia para esse tipo de estudo (Tabelas 1 e 2).

Tabela 1 - Revisão da literatura dos estudos de biofilme sobre dentina *in vitro* e *ex vivo*

ARTIGO	CARACTERÍSTICAS DA AMOSTRA				MÉTODO DE INDUÇÃO DE BIOFILME				FORMA DE AVALIAÇÃO	
	Substrato	Número	Forma	Dimensão	Espécie (s)	Tempo (dias)	Meio de cultivo	Temperatura	Método	Parâmetro
NORRINGTON *et al.*, 2008	Dentina humana	69	Anéis	L: - C: - E: 2 mm	Canal radicular	4	Meio de cultura Anaeróbico	37ºC	MEV	Desenvolvimento de cepas bacterianas através de um biofilme na dentina e inibição por antibióticos
GRÜNDLING et al., 2011	Dentina bovina	80	Raízes	L: - C: 15 mm E: -	*E. faecalis*	1	BHI	37ºC	MEV	Escores para categorizar presença de biofilme
ALVES et al., 2013	Dentina humana	55	Raízes	L: - C: 10 mm E: -	*E. faecalis ATCC 29212*	30	TSB	37ºC	Cultura	Contagem microbiana
BACA *et al.*, 2011	Dentina humana	80	Bloco	C: 2 mm L: 2 mm E:1,8 mm	*E. faecalis*	-	BHI	37°c	MEV	Células viáveis
RIOS *et al.*, 2011	Dentina humana		Raiz	L: - C: 12 mm E: -	*E. faecalis*	14	BHI	37ºC	MEV Cultura	Contagem microbiana por UFC.
LI; LIU; XU, 2012	Dentina humana	12	Bloco	C: 4 mm L: 4 mm E:0,2 mm	*E. faecalis*	2	BHI	37ºC	MCL	Percentual de células viáveis

Legenda:

Dimensão:
C=Comprimento
L=Largura
E=espessura

Meios de cultivo:
BHI= Brain Heart Infusion
TSB = Tryptic Soy Broth

Método:
MEV = Microscopia Eletrônica de Varredura
MCL = Microscopia Confocal a Laser

Tabela 1. Revisão da literatura dos estudos de biofilme sobre dentina *in vitro* e *ex vivo* (continuação).

ARTIGO	CARACTERÍSTICAS DA AMOSTRA				MÉTODO DE INDUÇÃO DE BIOFILME				FORMA DE AVALIAÇÃO	
	Substrato	Número	Forma	Dimensão	Espécie (s)	Tempo (dias)	Meio de cultivo	Temperatura	Método	Parâmetro
DA SILVA *et al.*, 2013	Dentina bovina	35	Raiz	L: - C: 4 mm E: -	*E. faecalis*	7	BHI	37ºC	MCL	Análise qualitativa (localização, área) e quantitativa do biofilme.
OZDEMIR *et al.*, 2010	Dentina humana	80	Raiz	L: - C: 4 mm E: -	*E. faecalis*	2	BHI	37ºC	MEV MCL	Adesão do *E. faecalis* na dentina
GUERREIRO-TANOMARU *et al.*, 2013	Dentina humana	-	Bloco	C: 5 mm L: 5 mm E:0,7 mm	*E. faecalis*	14-21	BHI	37ºC	MCL	Células viáveis
HOHSCHEIDT *et al.*, 2013	Dentina bovina	45	Raízes	L: - C: 15 mm E: -	*E. faecalis ATCC 8750*	21	TSB	37ºC	MCL	Análise qualitativa (localização, área) e quantitativa do biofilme.
JARAMILLO *et al.*, 2012	Dentina humana	30	bloco	L: - C: 3 mm E: -	*Saliva*	4	Meio Todd-Hewitt (TH)	37ºC	MEV MCL	Células vivas, Células viáveis e formação de biofilme *in vitro*.

Legenda:

Dimensão:
C=Comprimento
L=Largura
E=espessura

Meios de cultivo:
BHI= Brain Heart Infusion
TSB = Tryptic Soy Broth

Método:
MEV = Microscopia Eletrônica de Varredura
MCL = Microscopia Confocal a Laser

Tabela 1 - Revisão da literatura dos estudos de biofilme sobre dentina *in vitro* e *ex vivo* (conclusão).

ARTIGO	CARACTERÍSTICAS DA AMOSTRA				MÉTODO DE INDUÇÃO DE BIOFILME				FORMA DE AVALIAÇÃO	
	Substrato	Número	Forma	Dimensão	Espécie (s)	Tempo (dias)	Meio de cultivo	Temperatura	Método	Parâmetro
MUHAMMAD *et al.*, 2014	Dentina humana	34	Raiz	L: - C: 14 mm E: -	*P. gingivalis ATCC 33277* *S. salivarius ATCC 7073* *E. faecalis* *P. intermedia*	1 (*E.faecalis/ S. salivarius)* *3 (P. gingivalis)* 5 (*P. intermedia)*	Ágar Sangue Ágar Mueller Hinton (S. salivarius)	37ºC	MEV	Ausência/ Presença de biofilme antes e após os tratamentos.
RAN *et al.*, 2015	Dentina humana	40	Raiz	L: - C: 3 mm E: -	*E. faecalis ATCC 33186*	28	TSB	37ºC	MEV MCL	Distância de penetração dos micro-organismomicro-organismos nos túbulos dentinários
ORDINOLA-ZAPATA *et al.*, 2013	Dentina Bovina	40	Bloco	L: 2 mm C: 2 mm E: 2 mm	*Saliva*	3	BHI	37ºC	MCL	Porcentagem de células vivas.

Legenda:

Dimensão:
C=Comprimento
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E=espessura

Meios de cultivo:
BHI= Brain Heart Infusion
TSB = Tryptic Soy Broth

Método:
MEV = Microscopia Eletrônica de Varredura
MCL = Microscopia Confocal a Laser

Tabela 2 -Revisão da literatura dos estudos de biofilme sobre dentina *in situ*.

	CARACTERÍSTICAS DA AMOSTRA				**MÉTODOS DE INDUÇÃO**				**FORMA DE AVALIAÇÃO**	
Autor	Substrato	Núm. de amostras	Núm. De Voluntários	Tamanho bloco	Tempo (dias)	Meio de cultivo	Tempo de incubação	Condição de incubação	Método de avaliação	O que avaliou
ORDINOLA-ZAPATA, *et al.*, 2012	Dentina bovina	25	5	L: 3 C: 3 E: 2 mm	2	BHI	12h	Estufa 37ºC	MCL	Biovolume em lm3, número de células sobreviventes em lm3, espessura média do biofilme e cobertura do substrato em %.
DEL CARPIO-PEROCHENA *et al.*, 201	Dentina bovina	30	10-15	L: 4 C: 4 E: 2 mm	3	-	-	-	MCL	Biofilme, células incorporadas em uma matriz extracelular ligada à dentina; células isoladas não associadas a matriz; dentina não-colonizada.

Legenda:

Dimensão:
C=Comprimento
L=Largura
E=espessura

Meios de cultivo:
BHI= Brain Heart Infusion
TSB = Tryptic Soy Broth

Método:
MEV = Microscopia Eletrônica de Varredura
MCL = Microscopia Confocal a Laser

O principal método encontrado nos estudos é *in vitro*. A maioria deles emprega o microrganismo *Enterococcus faecalis*, devido a sua presença em infecções endodônticas persistentes. Em alguns estudos, realiza-se uma indução *in situ* em dentina bovina ou humana, podendo ter como via de contaminação a boca, através de placas intra-orais, com blocos de dentina 3x3x2mm ou 4x4x2mm, utilizadas por um tempo não-padrão entre os estudos conforme os estudos revisados na Tabela 1 e 2. O tempo de indução varia de 18h até 3 semanas. Posteriormente são cultivados principalmente em Ágar BHI composto de Infusão de cérebro-coração, (sólidos), hidrolisado péptico de tecido animal, hidrolisado pancreático de caseína, cloreto de sódio, glucose, fosfato dissódico de hidrogénio, o qual é recomendado como meio universal para bactérias aeróbias, poucos estudos utilizam meios mais ricos. Após o cultivo, são incubados por 37ºC em estufa durante tempo que pode variar entre os estudos principalmente entre 24 e 48h. Por meio da microscopia confocal analisam, em grande parte dos estudos, a viabilidade das células bacterianas, e também da microscopia eletrônica de varredura, não só células viáveis mas também morfologia do biofilme.

Modelos *in situ* são utilizados, geralmente, com blocos de dentina colocados em aparelhos intraorais, com a finalidade de se obter a infecção dentinária, a indução. Este tipo de modelo facilita a colonização microbiana (BARTHEL et al., 2002). Logo após a contaminação *in situ*, etapas laboratoriais *in vitro* têm-se centrado na eficácia dos irrigantes e medicamentos para remover biofilmes específicos, cultivados em poços, em filtros de membrana, e em amostras de dentina utilizando uma ou algumas espécies selecionadas encontradas na infecção radicular (SHEN, et al., 2009). Modelos *in vivo* são realizados em animais. A finalidade é avaliar microscopicamente tecidos que não podem ser retirados sem prejuízo dos seres humanos. No mesmo filo existem similaridades anatômicas, morfológicas, fisiológicas e bioquímicas que têm sido demonstradas, por isso os princípios biológicos fundamentais que são delineados nos estudos em animais podem ter aplicação no homem, contudo, como também nos outros tipos de estudos, os estudos em animais jamais podem dar respostas definitivas (BRACKEN, 2009).

Não há um consenso na literatura endodôntica sobre emprego de um método único para a indução e cultivo de um biofilme que possa ser determinado como padrão. Frequentemente, a indução de biofilmes envolve etapas clínicas e laboratoriais. Assim, é necessário que sejam avaliados os fatores que contribuiem

para o estabelecimento de um biofilme complexo, que simule aquele presente no interior dos canais radiculares e que seja passível de estudo. O objetivo do presente estudo será avaliar a influência de meios de cultura e tempo de incubação sobre o desenvolvimento de biofilmes em superfícies de dentina radicular humana.

2 OBJETIVOS

O objetivo geral do presente estudo é caracterizar a presença de biofilmes multiespécie em blocos de dentina em um modelo *in situ*, seguido de imersão em diferentes meios de cultura.

Os objetivos específicos serão:

a) determinar o efeito do meio de cultura no padrão de formação do biofilme, avaliando a composição microbiana, estrutura e a viabilidade celular por meio hibridização de DNA e microscopia confocal a laser, e;

b) determinar o efeito do tempo de imersão do bloco de dentina no padrão de formação do biofilme, avaliando a composição microbiana e a viabilidade celular por meio de hibridização de DNA e microscopia confocal a laser.

3 ARTIGO

(Esse manuscrito será submetido à publicação no Journal of Endodontics)

INTRODUCTION

Microorganisms and their products are considered the main periapical diseases etiologic agents (1,2). These microorganisms can be disposed in biofilms which is found in primary or secondary root canal infections (3). Biofilms are defined as bacterial cells attached to a surface enveloped by extracellular polysaccharide matrix (4).

The arrangement of microorganisms in biofilms may compromise the endodontic treatment since it is more challenging to eliminate them. This occurs because they are resistant to the alkaline stress (5) and to antimicrobial agents (6). Thus, it is important to determine the biofilm composition and structure to find the best method to eliminate it from the root canal system.

Several studies have been conducted to evaluate the effect of chemical substances that are employed in the root canal treatment over biofilms. Different sorts of *in vitro* (7–13), *ex vivo* (14–16) and *in situ* models (17,18) have been suggested to study biofilms on human and bovine dentin. However, regardless of the study model, there is no consensus in the literature in relations to laboratory factors that contribute to a rich biofilm establishment befitting clinical reality. The type of substrate (19,20), microbial species (21,22), growth conditions as nutrient-rich media, nutrient-deprived media, aerobic and anaerobic and incubation period may influence the biofilm characteristics (23). It is known that old biofilms are more resistant to disinfectants that young biofilms (22,24,25). Moreover, it is known that nutrient-rich media could be associated with higher bacterial population (26) and higher bacterial penetration into the dentinal tubules (23) than other culture media. Nevertheless, it is unknown how long the incubation period should be and which culture media could better characterize a complex biofilm.

Thus, the aim of this study was to characterize the biofilms formed *in situ* followed by different cultivation conditions. Furthermore, to evaluate the influence of culture media and incubation period on *in situ* biofilms development.

METHODS

This research was approved by the Research Board from the Dental School (Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil) and by the Ethics Committee in Research (Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil), protocol CAAE 43748015.9.0000.5347.

Dentin Blocks Preparation

Human radicular dentin blocks were made from permanent teeth that were extracted regardless the conduction of this research. Patients who donated their teeth after extraction signed a donation term and an informed consent form. Three dentin blocks (2 mm x 2 mm x 2 mm) were produced from each tooth. Dentin blocks went through a process which involved the elimination of dentin debris that were produced when they were cut 17% EDTA immersion during 3 min was performed. Then, the samples were sterilized in autoclave (121^{o}C, 1atm, 15 minutes).

In situ biofilm induction

Five participants were included, following the inclusion criteria: ≥18 years' old, no use of antibiotics in the 3 previous months in order not to interfere with the oral microbiota; and no systemic disease. Each patient signed an informed consent term before joining the research.

An intraoral impression from the lower arch was taken with a sterilized metallic impression tray and alginate material (Avagel, Brazil). After alginate setting time, the

impression was removed and disinfected 1% NaOCl spray during 10 min. Type III dental plaster was spilled into the alginate impression in order to obtain the plaster model. The intraoral device was made with acrylic resin (JET Clássico, Campo Limpo Paulista, Brasil) and orthodontic clips. Four wells were produced in the intraoral device for attaching the dentin blocks afterwards. The blocks were stuck into the wells by using godiva material (Lysanda, São Paulo, Brasil). They were placed in the lingual flank of the device, 2 blocks for each side.

The participants wore the intraoral device for 3 days, in two moments. A 7 day-washout period was warranted (27). All the volunteers received a form with instructions to be followed during the experiment, a case for the device storage, a toothbrush with soft bristles (Colgate, São Bernardo do Campo, Brasil), a toothpaste (Colgate, São Bernardo do Campo, Brasil) and a dental floss (Sanifill, Rio de Janeiro, Brasil). In this same appointment, the volunteers received instructions concerning how to use the intraoral device and how to hygienize it. The instructions included removing the devices during meals.

After induction period, patients gave the intraoral devices back to the researchers. Inside laminar flow cabinets, researchers removed the dentin blocks from the devices using sterilized Hollemback 3S spatulas (Golgran, São Caetano do Sul, Brasil). The exceeded godiva material was removed with scalpel blades (Solidor, Barueri, Brasil).

Biofilm cultivation

Sample were distributed into groups according to the culture media, incubation environment and the incubation time period (7, 14 or 21 days). Control groups will be composed of samples which will be removed from the intraoral devices and then immediately analyzed by confocal microscopy. Each patient will give one sample to each group. Experimental group descriptions are delineated in **Table 1**. Each group comprised 5 dentin blocks.

Samples from G1, G2 and G3 were stored in sterilized plastic tubes with 5ml of BHI broth (Himedia Laboratories Limited, Ghatkopar West, Mumbai, India) and incubated in 37°C inside a microbiological incubator for 7, 14 or 21 days respectively.

Every 3 days, 2 mL of the contaminated culture media were replaced by 2mL of a fresh and sterilized BHI broth. G4, G5 and G6 samples will be individually stored in sterilized tubes with 5mL of Fastidious Anaerobe Broth (FAB, LabM, Bury, United Kingdom) and incubated in anaerobic jars containing Anaerobiosis generator (GasPak, BD, Franklin Lakes, New Jersey, EUA). This set will be stored in microbiological incubator for 7, 14 or 21 days respectively. Every 3 days, 2 mL of the contaminated culture media were replaced by 2mL of a fresh and sterilized FAB broth. All the procedures were performed inside a laminar flow cabinet (SPLabor, São Paulo, Brasil).

Biofilm analysis

Confocal laser scanning microscopy analysis (CLSM)

Each sample received a code. The images were obtained by a single researcher who was blind for the groups. The Kit of Bacterial Viability BacLightTM 5 (Molecular Probes, Eugene, OR) was used to determine the bacterial viability. The SYTO9 stain (480 nm excitation and maximum 500 nm emission) was able to color all bacteria in green, while the propidium iodine stain (490 nm excitation and 635 nm emission) was able to be incorporated only by those which had some membrane damage. Both stains were placed over the blocks for 5 minutes in a 1:1 volume proportion (50µL of each solution).

The cell fluorescence was visualized by using the CLSM (Olympus Europa Holding GmbH, Hamburg, Germany). The images were used to show the green and red fluorescence. The specimens were observed by using a 60× increase with an additional of 3×. Images were obtained through the Olympus FluorView 1.7 Version Software.

There were 5 topics which were evaluated by analyzing the image captures total biovolume of viable and non-viable bacteria; biovolume of viable and non-viable bacteria separately and their respective proportion to the total expressed by percentage numbers.

Checkerboard DNA-DNA hybridization

The checkerboard DNA-DNA hybridization assay was performed as previously described by Ferreira *et al*. (2015) (28). For DNA isolation, each dentin block was placed in 1.5-ml microcentrifuge tubes containing 1 ml TE buffer (10 mm Tris–HCl, 0.1 mm EDTA, pH 7.6). The samples were boiled for 10 minutes and neutralized with 0.8 mL 5 mol/L ammonium acetate. The released DNA was then placed into extended slots of a Minislot 30 apparatus (Immunetics, Cambridge, MA), concentrated onto a positively charged nylon membrane (Boehringer, Mannheim, Germany), and fixed to the membrane by incubation at 120°C for 20 minutes. A Miniblotter 45 (Immunetics) device was used to hybridize the 40 digoxigenin-labeled whole-genomic DNA probes at right angles to the lanes of the clinical samples. Bound probes were detected by using phosphatase-conjugated antibodies to digoxigenin and chemiluminescence (CDP-Star Detection Reagent; Amersham Biosciences, Chicago, IL). Signals were visually evaluated by comparison with 2 standards. These standards consisted of a mixture of 10^5 and 10^6 cells from each bacteria tested placed in the last 2 lanes of each membrane. The sensitivity of this assay was adjusted to permit the detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe. Considering that, the number (0) was designated for the times when there were no signal detection; (1) when there was a signal less intense than the 10^5 cells control; (2) to approximately 10^5 cells; (3) when it is between 10^5 and 10^6 cells; (4) to approximately 10^6; (5) to more than and 10^6 cells.

Data analysis

Descriptive and inferential statistical analyses were carried out through Prisma 7 for Windows v. 7.02 (GraphPad Software Inc.). Cluster analysis was performed with Past 3 v.3.14 (Hammer et al., 2001) (Øyvind Hammer, Natural History Museum, University of Oslo).

Data for the presence/absence of the targeted bacterial species and the respective score regarding its total load were recorded for each sample. The median score for each species, in each group were determined.

Cluster analysis was performed to determine the similarity/dissimilarity among samples, regarding the presence/absence of the species, as well their specific load in each sample. The Ward's grouping method, with Euclidean distance was employed.

The median value for the microbial load and for the percentage of viable cells for each group was determined. The null hypothesis tested were:

a) There is no difference in the microbial load among the samples that were grown under the same condition at 0, 7, 14, and 21 days (Friedmann Test, α=5%).

b) There is no difference in the microbial load among samples that were grown under different conditions, in the same period of time (Mann-Whitney Test, α=5%)

c) There is no difference in the percentage of viable cells among the samples were grown under the same condition at 0, 7, 14, and 21 days (Friedmann Test, α=5%).

d) There is no difference in the percentage of viable cells among samples that were grown under different conditions, in the same period of time (Mann-Whitney Test, α=5%).

RESULTS

Table 2 shows the median values for the obtained load of each species according to the codes from 0 to 5, as previously described.

Different loads as well as different species can be observed comparing the tested methods. For each period of incubation, higher loads are shown for the FAB and anaerobic jar condition. The highest score found in BHI was 1.5 (*F. nucleatum*, *F. n. spp vincentii, C. orchracea* and *S. gordonii)* and they were found only for the immediate period. However, scores of 3 or 2.5 (*F. n. spp vincenti, S. mitis* and *S. oralis*) were found in immediate period, an higher scores were found in 7 days for FAB. However, for BHI the great majority of the species had detection scores equal to zero, except for *Enterococcus spp*. The same behavior was observed for the other periods when comparing FAB to BHI bacterial load.

Despite the period, for the same incubation method, the highest bacterial loads were found immediately after the sample removal from the intraoral devices. In the FAB group, a few number of species with low cell load were found in the 21 days period, and the score 1 was found only for three species (*F. periodonticum, C. orchracea and Enterococcus spp).* FAB + anaerobic jar for 7 days seemed to harbor more species with also a higher cell load. For BHI, the incubation period with the higher and more frequent scores was 14 days in which the score of 1 was found eight times.

Figure 1 shows the dendogram obtained from the clustering analysis of bacterial profiles in samples with biofilms that were grown under BHI + aerobic conditions or FAB + anaerobic conditions, as detected by checkerboard DNA-DNA hybridization. For both methods, the samples were not grouped in large clusters according to the time of incubation. However, 7-day and 21-day samples seemed to be more similar between each other for both graphics. Samples belonging to the same participant and incubated for different periods were in different clusters. FAB + anaerobic conditions originated two large clusters that had low grouping pattern. The BHI + aerobic condition produced clusters with small distance between them.

Figure 2 shows the biovolume observed after biofilm induction in BHI or FAB, in different periods of time, through CLSM analysis. There were significant statistical

differences between the two different incubation methods and also for a same method but in different periods of incubation. In BHI, the biovolume presented no difference over time. However, in FAB the difference was statistically relevant for the microbial load in 14 days which was higher than immediate period or 7 days. There was no statistical difference for biovolume between 14 days and 21 days in FAB. In comparing the incubation methods in a same period, 14 days was the only one which indicated a relevant difference. The microbial load in FAB was statistically higher than in BHI in the 14-day period.

The percentage of viable cells in the samples, regarding the incubation conditions and the periods of time are shown in **Figure 3**. There is no difference among the percentage of viable cells in the samples for zero, 7 days or 14 days in BHI + aerobic conditions. The only relevant difference for this incubation method was in 21 days, once the percentage of viable cells was higher than time 0. For FAB + anaerobic condition, there was no statistical differences among all the periods. Furthermore, there was no statistically relevant differences between the different culture media in a same period of time.

DISCUSSION

Studies in which the methods include biofilm induction are extremely important for endodontics. They are useful to test irrigation methods and substances, intracanal medications (29–31), antibiotic drugs (31) or to basically analyze how microorganisms behave in endodontic infections (32,33). However, most of them do not present a standardized methodology, they still vary with regard to culture media, whether it is BHI or different types of agar. Furthermore, some studies use bovine dentine, while others use human dentine. There is also a difference on how, anaerobically or aerobically, and how long they are stored for. Little is known whether these different methods would produce different biofilms or not. The aim of this study was to depict the biofilms formed *in situ* followed by different cultivation methods. Furthermore, it was also aimed to define the influence of culture media and incubation period for both the composition and bacterial load, as well as cells viability.

According to the current literature, the biofilm formation protocol might be performed *in vivo*, *ex vivo*, *in situ* or *in vitro*. *In situ* method creates the possibility to analyze biofilms formed by the microorganisms from the oral cavity, which is their natural environment. The microorganisms adhere to a tooth surface, enamel or dentine, and then this could possibly make these studies more relevant as they represent the structure of the microbial communities in this ecosystem. Considering this aspect, this method is more advantageous to study various oral infections as the microorganisms are collected from their natural habitat. *In vivo* biofilms have a typical structure. The external part is more water-rich and contain fewer cells which is correspondent to a more recent biofilm. The deepest content of this biofilm is more densely cellularized and rougher, characteristics of an old biofilm (34).

Previous studies have already tested the effect of irrigants or medicaments over *in situ* biofilms before (17,29,35–37) or after additional laboratorial cultivation (19). However, the effect of further laboratorial development of the biofilm through the immersion in culture media and incubation in specific conditions has not been demonstrated yet. Then, checkerboard DNA-DNA hybridization was utilized for species identification and quantification. Even though its specificity and sensitivity is not that high, they are much higher than culture method. In this study, the presence

of selected species was assessed, and other components of the microbial communities might not be reached. Furthermore, the method did not allow the detection of the targeted species with low DNA load. Additionally, there were more DNA probes for strict anaerobic microorganisms comparing to the facultative anaerobes.

Molecular biology based methods did not allow to determine the viability of the microbial cells. Therefore, the CLSM was employed to describe the proportion of viable and non-viable cells in the biofilm structure. CLSM is performed in several studies with different aims. They can be included as a method to study biofilm growth itself (38), antimicrobial irrigant solutions or intracanal medications effect (17–19,29,39). CLSM can provide information not only of a total volume but also the proportion of viable and non-viable ones. However, it does not allow perceiving the biofilm morphology which is a role of the Electronic Microscopy. Electronic Microscopy would create a static analysis of the biofilm. The association of DNA hybridization and CLSM microscopy allowed determining both the composition and viability of the microbial cells in the biofilm.

In this study, the initial composition of the biofilms was similar in the samples, however, the FAB group had a higher cell load than the BHI group. The species *F. nucleatum*, *P. nigrescens*, *F. periodonticum*, *F. nucleatum spp vincentii*, *C. showae*, *C. ochracea*, all the *Streptococcus* spp., *V. parvula*, *A. odontolyticus*, *T. socranskii*, *E. saburreum*, *E. faecalis* and *E. faecium* were detected in the immediate period. Then, all the species of the yellow complex and almost all the species of the orange complex were detected in the initial samples. For the 7, 14, and 21 days periods, different types of species were found for BHI and FAB as well as different cell loads. *F. nucleatum, F. nucleatum spp vincentii, S. oralis, S. anginosus, S. sanguinis and Enterococcus* spp. were the species with higher score and most frequent for BHI. In FAB, higher scores were found for some species in comparing to BHI and also more frequent species such as *C. gingivalis*, *E. saburreum*, *C. orchracea*, *S. sanguinis* and *V. parvula*. Scores of 1.5 or even 2 were found in FAB group while in BHI the highest score was 1.

The comparison between the microbial communities in endodontic infections and in the induced biofilms are warranted. The limitation of comparing to other studies involve the fact that they frequently do not analyze the same species of

bacteria. Rôças et al. (2011) described the microbial profile in root canal samples from symptomatic and asymptomatic teeth, through DNA-DNA hybridization. However, the detected species with higher counts in their clinical samples were not included in the present research, except for the *Fusobacterium nucleatum.* In symptomatic infections *Streptococcus spp* and *Fusobacterium nucleatum* were found and could be considered more accordingly to the presented results (40). Furthermore, other microbiota analysis of asymptomatic endodontic infections showed low counts for the microorganism *F. nucleatum ss vincenti* (41), which was the main one in this study. It seems that the presented results do not look like the asymptomatic microbiota. There are also some evidence in studies of symptomatic primary endodontic infections which include some similar microbiota (42,43). However, this study showed a small variety of species, then it could be compared to a post-treatment disease as they usually present less species than the primary ones. Generally, the *E. faecalis* is the main pathogen of this type of infections but previous studies have been showing that this can be questioned. Apart from that, there are similar microorganisms in these infections such as *Streptococcus spp.* but other which were not found in this study such as *P. acnes* (44). Therefore, it is complex to affirm to which type of endodontic infection these results are similar to, because in some aspects they are similar to a specific one but in others they are completely different. Besides the clinical condition, previous studies showed that the microbial communities in endodontic infections are also influenced by several factors such as geographic location (45–47) and varies among the individuals and also in different sites from the same patient (48,49).

When performing the *in situ* method, as in this study, the grown biofilm after induction may differ a lot in species, viability and quantity due to the individuality of the volunteers. This situation could be minimized by choosing only one individual as a volunteer, then the samples would be standardized (18). However, according to the results of this study this hypothesis could be questioned. Even for the same volunteers, the biofilms grown in different dentine blocks were different from each other in bacterial load and species. Biofilms with different species and bacterial load represent polymicrobial infections. The structure of these infections is really important to be studied since it is how endodontic infections occur.

By analyzing clusters dendogram, it is possible to conclude that even for same patients, samples are not similar in different periods of time, because culture media and incubation might have modified the primary biofilm. They allow for selective bacterial growth, due to nutritional conditions and different gaseous environments. Samples obtained from different patients, in the same period of time, harbor different bacterial composition. Zaura *et al.* (2009) indicated that the oral microbiome of healthy subjects shares a core of species, but unique components have been also detected for each individual (50). In the present study, each patient formed a different biofilm and then each sample got even more different from each other due to period of time, culture media and incubation. Primary biofilms are various and not similar to each other, therefore even same incubation, culture media and period of time do not allow biofilms to be standardized.

CLSM allowed assessing the percentage of viable cells and also biovolume in the biofilm. Biovolume is the total volume of microbial cells in the biofilm (in μm^3), however it did not provide information on the cell viability. Then, the percentage of viable cells indicates the proportion of the biovolume mass which represents the viable ones. It is relevant to consider the percentage of viable cells, because there might be samples with a similar biovolume but with distinct percentage of viable cells. Another limitation of CLSM is the maximum depth that this method is able to detect. From depths of 5μm, resolution and focus drop drastically. From 15μm, the confocal signal level gets around only 10% of its capacity. Therefore, thick biofilms are not properly analyzed by this method (51).

In the samples that were incubated in BHI and aerobic condition, bacterial load detected through checkerboard seems to be initially higher and decreases over time. The initial bacterial load was formed mainly by strict anaerobes. The reduction for the detection of the targeted species is possibly related to the growth of facultative species which are not well represented in the selected DNA probes. Anaerobic species were not able to grow over time in BHI. Despite the changes in bacterial diversity and load for the DNA-DNA hybridization method in the BHI samples over time, their biovolume remain stable during time. The reason for these findings might be the growth of other facultative ones than the selected for the present in the biofilm matrix. In the BHI samples, there is a high proportion of viable cells in the biofilm matrix, that changed from zero to 21 days. Biofilms induced by the FAB group had a higher bacterial load in 14 days. It might be associated with the growth of both

facultative and especially the strict anaerobic species. There was a change in the bacterial load from 7 to 14 days, with a detectable increase of *F. nucleatum*, *P. intermedia*, *P. nigrescens*, *F. nucleatum* spp *vincentii*, *S. oralis*, bacteria from the purple complex, *Enterococcus* spp. *G. morbillorum*, *C. showae*, *S. constellatus*, *A. actinomycentemcomitans*, and the red complex members *P. gingivalis* and *T. denticola* were detected in 7 day-biofilms but were not detected in 14 days-biofilm. It might be associated to microenvironmental alterations that took place inside the biofilm matrix because of aging. The percentage of viable cells did not change from zero to 21 days.

As other types of biofilms, the oral biofilm, after formation, develops through a process called microbial community succession. This process can be defined as the changes in conditions of an environment leading to changes in microbial species composition (4). In the present study, it was feasible to observe that microbial species composition changed over time according to the environment conditions they were exposed to. In a research of succession of bacteria and interaction, it was shown that bacteria from the same phylum used to appear together in same conditions (52). Similar situation happened in the present study, since bacteria from the same phylum are present in similar conditions such as Firmicutes including *Enterococcus* and *Streptococcus spp*. In addition, Actinobacteria also seem to be correlated. However, in the present study, statistic analysis was not performed in order to make statistically relevant correlations.

An important consequence of these findings is the fact that every biofilm is unique, and different from each other even in the same individual or in different individuals but similar incubation conditions. The cultivation and incubation method might impact on the composition of these biofilms and their cell viability. Even though these conditions influence biofilms characteristics, it is not possible to define which experimental method is the best one to characterize a rich biofilm because it depends on the aim of each study. Furthermore, the microbial succession observed in this study might not be the same which would occur in different sites of the oral cavity.

TABLES

Table 1. Experimental groups

Group	Incubation condition	Period
Control (Phase 1)	-	Immediate
Control (Phase 2)	-	Immediate
G1	BHI Broth + Microbiological incubator	7 days
G2	BHI Broth + Microbiological incubator	14 days
G3	BHI Broth + Microbiological incubator	21 days
G4	FAB + Anaerobic jar	7 days
G5	FAB + Anaerobic jar	14 days
G6	FAB + Anaerobic jar	21 days

Table 2. Median values for the bacterial cell load, according to the incubation conditions and period of time.

Microbial Complex	Species	BHI + Incubator				FAB + Anaerobic Jar			
		0	7d	14d	21d	0	7d	14d	21d
Red	*P. gingivalis*	0	0	0	0	0	0.5	0	0
	T. denticola	0	0	0	0	0.5	1	0	0
	T. forsythia	0	0	1	0	0.5	0.5	0.5	0
Orange	*F. nucleatum*	1.5	0	1	0	1	0	2	0
	P. intermedia	0	0	0	0	1	1	1.5	0
	P. nigrescens	0.5	0	0	0	1.5	0	1.5	0
	P. micros	0	0	0	0	0.5	1	1	0
	E. notadum	0	0	0	0	0	0.5	0.5	0
	S. constellatus	0.5	0	0	0	0.5	0.5	0	0.5
	F. periodonticum	1	0	0	0	1	1	1	1
	F. n. (spp vincentii)	1.5	0	1	0	3	0.5	1	0.5
	C. rectus	0	0	0	0	1	1.5	1	0.5
	C. showae	0.5	0	0.5	0	0.5	0	0	0
Green	*C. gingivalis*	0	0	0.5	0	2	2.5	1	0
	C. sputigena	0	0	0	0	0	0	0.5	0
	E. corrodens	0	0	0	0	0	0	0.5	0
	A. a	0.5	0	0	0	0	0.5	0	0
	C. orchracea	1.5	0	0	0.5	1	1.5	1	1
Yellow	*S. sanguinis*	1	0	1	0	1.5	1.5	1	0.5
	S. intermedius	0.5	0	0	0	0.5	0.5	0.5	0
	S. mitis	1	0	0	0	2.5	0.5	0.5	0.5
	S. oralis	1	0	1	1	3	1	2	0.5
	S. gordonii	1.5	0	1.5	0	1.5	0.5	0.5	0.5
Blue	*A. gerencseriae*	0	0	0	0	0.5	1	0	0
	A. israelli	0	0	0	0	0	0	0.5	0
	A. naeslundii	0	0	0	0	0	0.5	1	0
Purple	*V. parvula*	0.5	0	0	0	2	0.5	1.5	0
	A. odontolyticus	0.5	0	0.5	0	1.5	0.5	1	0
Other	*S. anginosus*	1	0	1	0	0.5	0	1	0
	T. socranskii	1	0	0	0	0.5	1	0.5	0
	E. saburreum	0.5	0	0	0	2	2	1	0
	S. noxia	0	0	0	0	1	0.5	0.5	0
	P. acnes	0	0	0	0	0	0	0	0
	P. melaninogenica	0	0	0	0	0.5	1	0	0
	G. morbillorum	0	0	0	0	0.5	0	0	0
	A. oris	0	0	0	0	0.5	1	0	0
	L. buccalis	0	0	0	0	0	0	0.5	0
Enterococcus spp.	*E. faecalis*	1	0	1	1	1	0.5	1	0.5
	E. faecium	1	0.5	1	0	1	1	2	1

FIGURES

Figure 1. Ward's grouping method dendrogram for samples that were grown in BHI + aerobic conditions (A) or FAB + anaerobic condition. Cophenetic coefficient A = 0.7205; B = 0.7205.

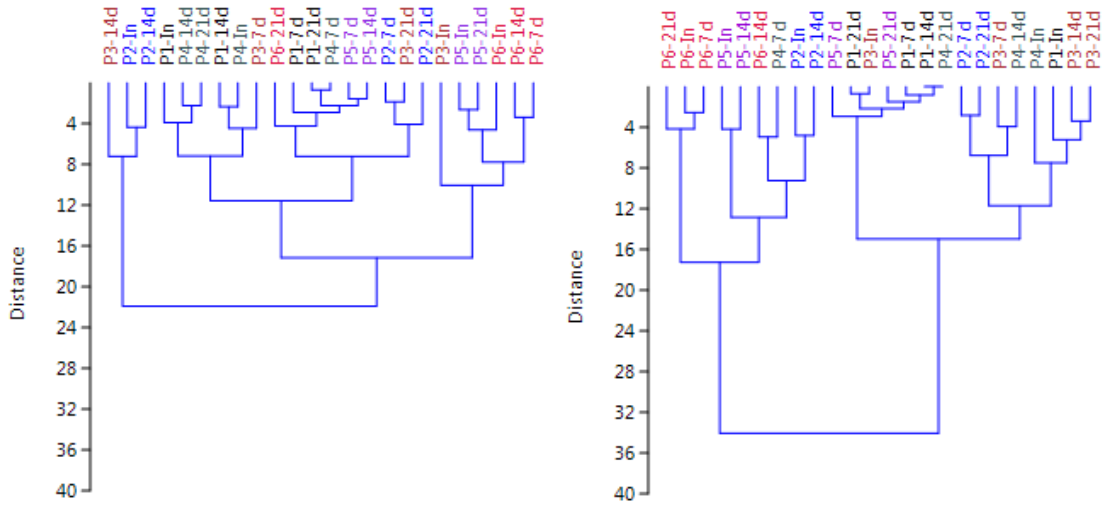


Figure 2. Biofilm biovolume ($\mu m^3/\mu m^2$) observed after biofilm induction in BHI (A) or FAB (B) in different periods of incubation. The red bar indicates the median values of the biofilm biovolume for each period. Lower case letters represent the statistically significant differences for the samples which were incubated in the same conditions over time (Friedmann Test, P<.05). Equal symbols above the letters represent statistically significant differences in microbial load for different incubation method but same period of time. (Mann-Whitney Test, P<0.05).

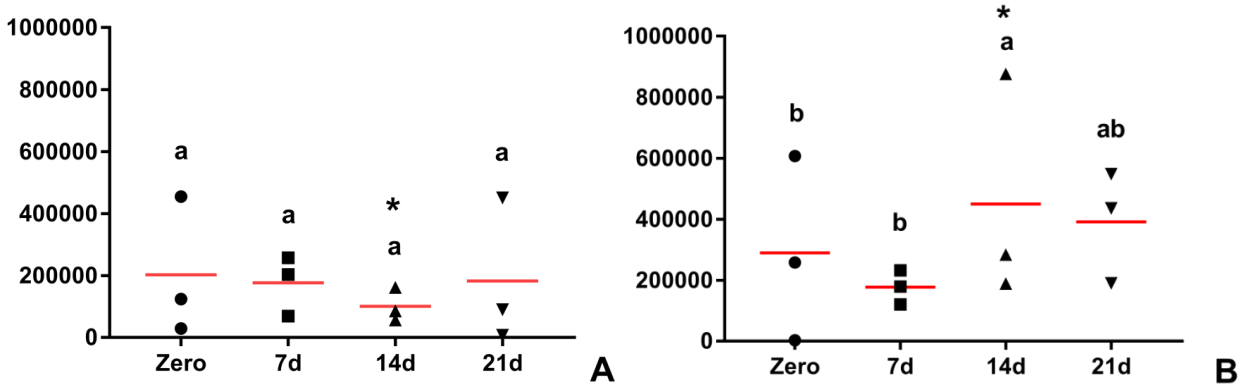
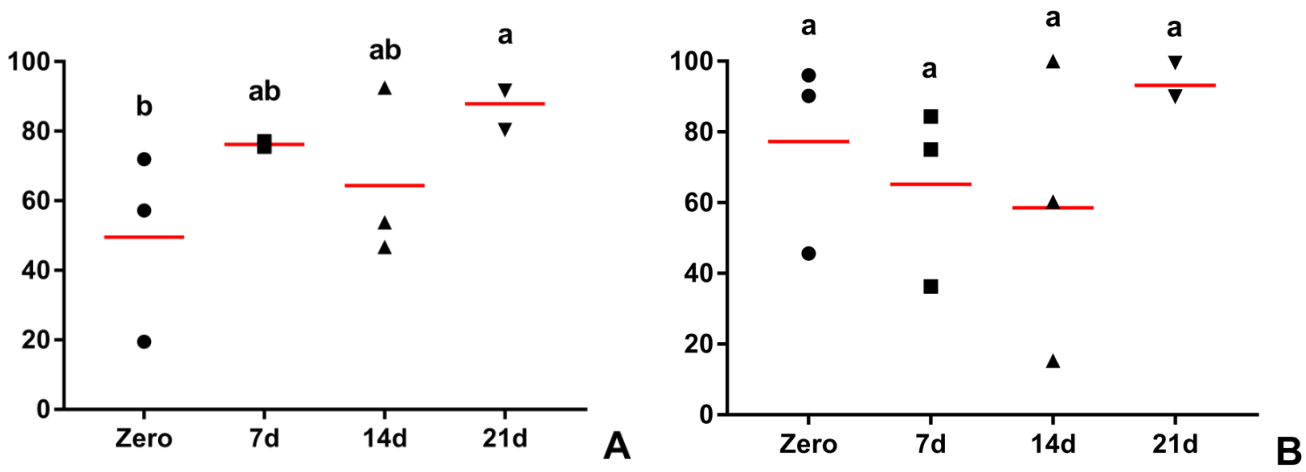


Figure 3. Percentage of viable cells which observed after biofilm induction in BHI (A) or FAB (B), in different periods of time. Different lower case letters indicate a statically relevant difference for the samples which were incubated in the same condition in different periods of time. (Friedmann Test, P<.05).



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4 CONSIDERAÇÕES FINAIS

O estudo dos biofilmes bucais é de extrema importância na área da Odontologia, especialmente em Endodontia. Eles são os principais agentes etiológicos de diversos tipos de patologias que ocorrem na cavidade bucal. O presente trabalho tinha como objetivo sugerir uma metodologia padrão para cultivo e incubação para estudos desses biofilmes. No entanto, todo biofilme produzido possui sua singularidade e peculiaridade, mesmo que formado ou desenvolvido em condições similares. Por esse motivo, ao escolher uma metodologia para estudá-los, é necessário definir, primeiro, o objetivo do estudo. Dessa maneira, é possível conhecer que tipo de biofilme se almeja formar e, com isso, a metodologia adequada.

Tendo em vista a complexidade das comunidades microbianas na cavidade bucal, é necessário que pesquisas científicas continuem a ser realizadas nesse assunto. À medida que se amplia o conhecimento sobre biofilmes dentários, também melhores oportunidades de tratamento e prevenção de patologias pulpares e periapicais poderão ser ofertadas.

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ANEXO A – PARECER CONSUBSTANCIADO DO CEP

UFRGS
UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL / PRÓ-REITORIA DE PESQUISA -

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Análise da formação e desenvolvimento de biofilmes em blocos de dentina humana

Pesquisador: FRANCISCO MONTAGNER
Área Temática:
Versão: 3
CAAE: 43748015.9.0000.5347
Instituição Proponente: UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.221.983

Apresentação do Projeto:
Trata-se de um projeto de pesquisa da Faculdade de Odontologia da UFRGS que visa desenvolver um modelo experimental para estudar o biofilme microbiano existente na boca de humanos.

Objetivo da Pesquisa:
Caracterizar a presença de biofilmes multiespécie em blocos de dentina em um modelo in situ, seguido de imersão em diferentes meios de cultura.

Avaliação dos Riscos e Benefícios:
Riscos e benefícios foram considerados apropriadamente.

Comentários e Considerações sobre a Pesquisa:
Cinco pacientes serão selecionados de acordo com o critério de inclusão e posteriormente a moldagem e confecção dos dispositivos; e a obtenção de dentes (n=15) para a produção dos blocos de dentina que serão incluídos neles. Após a confecção desses aparelhos, a metodologia subsequente se constituirá de 2 fases, denominadas Fase 1 e Fase 2. Na Fase 1 os pacientes serão instruídos a utilizar o dispositivo durante 3 dias e após isso as amostras de dentina serão removidos do dispositivo intrabucal e dividida em grupos para análise de acordo com o meio de cultura (FAB ou BHI), o ambiente de incubação (presença ou ausência de oxigênio) e o tempo que ali permanecerão (3 dias, 7 dias ou 21 dias). As amostras serão divididas em 3 grupos

Endereço: Av. Paulo Gama, 110 - Sala 317 do Prédio Anexo 1 da Reitoria - Campus Centro
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Telefone: (51)3308-3738 **Fax:** (51)3308-4085 **E-mail:** etica@propesq.ufrgs.br

experimentais para cada fase, mais um grupo controle. Na fase 1, a condição de incubação das amostras será BHI Caldo associado a estufa microbiológico em diferentes tempos, sendo que os grupos controle são amostras removidas e imediatamente submetidas à análise. Após um período de 15 dias de wash-out, na Fase 2 a condição de incubação será o meio de cultura FAB + Jarra de Anaerobiose. Depois da incubação, todas as amostras serão submetidas a análise por meio de hibridização DNA-DNA, Microscopia Confocal a Laser e Microscopia Eletrônica de Varredura (MEV). A presença e o número de células de determinadas espécies microbianas será determinada pela hibridização DNA-DNA. A presença de morfotipos microbianos específicos e a quantificação da área coberta pelo biofilme serão analisados por meio de Microscopia eletrônica de varredura. Em Microscopia Confocal a Laser, serão analisados a biomassa de bactérias vivas e total de microrganismos, porcentagem de área colonizada da dentina, biovolume/área (m^3/m^2), porcentagem de células vivas, proporção de células vivas. A análise quantitativa da viabilidade bacteriana será realizada com o software de bio 19 Image_L. O teste estatístico será selecionado após a análise dos dados quanto aos parâmentros de normalidade (Teste de Shapiro-Willk, =5%).

Caso os dados apresentem distribuição normal, será realizado teste de ANOVA. Caso os dados não demonstrem distribuição normal, será empregado o teste de Kruskall-Wallis. O nível de significância determinado para o estudo será de 5%. Para a quantificação dos resultados em MEV foram utilizados escores modificados de Bhuva et. Al. (2010), Escores de 1 a 4 em relação a estrutura do biofilme.

Considerações sobre os Termos de apresentação obrigatória:

- O critério de elegibilidade é ser aluno de graduação ou pós-graduação da Faculdade de Odontologia. Estes serão convidados a participar do estudo por meio de cartazes, O modelo de cartaz foi apresentado pelos pesquisadores e encontra-se em condições de ser aprovado.
- Carta de ciência e concordância do laboratório onde vai ser realizado o estudo foi anexada, bem como formulário da Plataforma Brasil e parecer da COMPESQ.
- O cálculo de tamanho amostral foi devidamente apresentado.
- Como está previsto o uso de dentes humanos, um termo de doação de dente foi apresentado.

Recomendações:

Sem recomendações adicionais.

Conclusões ou Pendências e Lista de Inadequações:

Considerando que as mudanças solicitadas pelo CEP foram atendidas, o parecer é pela aprovação

do projeto.

Considerações Finais a critério do CEP:

Aprovado.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Outros	PARECER COMPESQ.pdf	02/04/2015 14:50:35		Aceito
Folha de Rosto	PLATAFORMA BRA.pdf	07/04/2015 22:59:57		Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_492190.pdf	07/04/2015 23:00:15		Aceito
Outros	SÓ-TCC-ANEXO 1 CartaLabim.pdf	15/06/2015 18:45:35		Aceito
Outros	SÓ-TCC-ANEXO 2- CME.pdf	15/06/2015 18:45:44		Aceito
Outros	SÓ-TCC-ANEXO 3 - CTBMF.pdf	15/06/2015 18:45:57		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	SÓ-TCC-Apêndice 2-TCLE1_CEP.docx	15/06/2015 18:46:26		Aceito
Outros	SÓ-TCC-Apêndice 3 - CARTAZ.pptx	15/06/2015 18:47:10		Aceito
Outros	SÓ-TCC-Apêndice 5-Instrucoes.pdf	15/06/2015 18:47:36		Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_492190.pdf	15/06/2015 19:02:32		Aceito
Projeto Detalhado / Brochura Investigador	SÓ-TCC-Projeto-CORRIGIDO.docx	13/08/2015 17:45:54		Aceito
Declaração de Pesquisadores	SÓ-TCC-Apêndice 1-Doaçao.docx	13/08/2015 17:47:27		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	SÓ-TCC-Apêndice 4-TCLE2_CEP.docx	13/08/2015 17:47:52		Aceito
Outros	RESPOSTA AO CEP 12 AGO.docx	13/08/2015 17:52:29		Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_492190.pdf	13/08/2015 17:53:19		Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:
Não

PORTO ALEGRE, 10 de Setembro de 2015

Assinado por:
MARIA DA GRAÇA CORSO DA MOTTA
(Coordenador)