UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

KARINA COGO
FARMACÊUTICA

AVALIAÇÃO *IN VITRO* DO EFEITO DA NICOTINA, COTININA E CAFEÍNA SOBRE MICRORGANISMOS ORAIS.

Dissertação apresentada à Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas, para obtenção do título de Mestre em Odontologia, na Área de Farmacologia, Anestesiologia e Terapêutica.

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"... teu destino está constantemente sob teu controle.

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Teus pensamentos e vontades são a chave de teus atos e atitudes..

São as fontes de atração e repulsão na tua jornada de vivência.

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A mudança está em tuas mãos.

Reprogrames tua meta, busques o bem e viverás melhor.

Embora ninguém possa voltar atrás e fazer um novo começo, qualquer um pode começar agora e fazer um novo fim."

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SUMÁRIO

RESUMO

Existem evidências de que o biofilme presente na região subgengival é o principal agente etiológico das doenças periodontais. O uso do cigarro tem sido associado com a progressão da doença periodontal bem como com a redução da resposta à terapia aplicada a essa doença. Alguns estudos têm indicado uma forte relação entre o hábito de fumar e o consumo de café. No entanto, os mecanismos pelos quais o cigarro e o consumo de café podem afetar o tecido periodontal ainda não foram totalmente esclarecidos. Dessa forma, o objetivo do presente estudo foi avaliar *in vitro* os efeitos da nicotina, cotinina e cafeína na viabilidade de algumas espécies bacterianas da microbiota subgengival. Biofilmes mono-espécie de *Streptococcus gordonii*, *Porphyromonas gingivalis* e *Fusobacterium nucleatum* e as combinações de biofilme com duas espécies, *S. gordonii* + *F. nucleatum* e *F. nucleatum* + *P. gingivalis* foram desenvolvidos em discos de hidroxiapatita banhados em saliva artificial. Um total de sete espécies foi avaliado como células planctônicas, incluindo as espécies acima mencionadas e o *Streptococcus oralis*, *Streptococcus mitis*, *Propionibacterium acnes* e *Actinomyces naeslundii.* As espécies bacterianas foram incubadas com ou sem nicotina, cotinina e cafeína nas concentrações que variaram de 0,37 a 400 μg/mL para células planctônicas e 400 μg/mL para biofilme. O crescimento das células planctônicas e do biofilme foi avaliado pelos testes de susceptibilidade e "time-kill", respectivamente. Os resultados do teste de susceptibilidade mostraram que a nicotina reduziu o crescimento da *S. gordonii* (400 μg/mL) e *S. oralis* (0,37-400 μg/mL); a cotinina estimulou o crescimento das espécies *A. naeslundii* (0,37 μg/mL) e *F.* nucleatum (0,37-400 μg/mL) e reduziu o crescimento da *S. oralis* (400 μg/mL); e a cafeína estimulou o crescimento da *F. nucleatum* (400 μg/mL). Nos testes de "time-kill" foram observados um aumento do crescimento do biofilme mono-espécie de *F. nucleatum* e uma redução da viabilidade do biofilme mono-espécie de *S. gordonii,* após 24 horas e 48 horas de exposição à cotinina e à cafeína, respectivamente. Esses resultados indicam que a nicotina, cotinina e cafeína podem afetar, embora em pequena extensão, o crescimento e a viabilidade das espécies bacterianas orais estudadas.

ABSTRACT

There are significant evidences that subgingival accumulation of bacterial biofilm is the etiologic agent in periodontal diseases. Cigarette smoking might result in progression of periodontitis and in impaired response to periodontal therapy. Some studies indicated a strong relationship between cigarette smoking and coffee drinking. However, the mechanisms by which smoking and coffee consumption affect the periodontium are not clear. The purpose of this *in-vitro* study was to evaluate the effects of nicotine, cotinine, and caffeine on the viability of some bacterial species from the oral microbiota. Single-species biofilms of *Streptococcus gordonii*, *Porphyromonas gingivalis*, *and Fusobacterium nucleatum* and dual-species biofilms of *S. gordonii* + *F. nucleatum* and *F. nucleatum* + *P. gingivalis* were grown on hydroxyapatite discs. Seven species were studied as planktonic cells, including *Streptococcus oralis*, *Streptococcus mitis*, *Propionibacterium acnes*, *Actinomyces naeslundii*, and the species mentioned above. Bacteria were incubated in either 0 or 0.37 to 400 μg/mL of nicotine, cotinine or caffeine for planktonic cells and 0 or 400 μg/mL for biofilm. The viability of planktonic cells and biofilms was analyzed by susceptibility tests and time-kill assays, respectively. "Susceptibility Test" showed that nicotine reduced the growth of *S. gordonii* (400 μg/mL) and *S. oralis* (0.37-400 μg/mL); cotinine stimulated the growth of *A. naeslundii* (0.37 μg/mL) and *F.* nucleatum (0.37-400 μg/mL) and reduced the growth of *S. oralis* (400 μg/mL), and caffeine stimulated the growth of *F. nucleatum* (400 μg/mL). Results of "Killing Assays" showed an enhanced growth of *F. nucleatum* in single-species biofilm and a reduced viability of *S. gordonii* in single-species biofilm, 24 h and 48 h after exposure to cotinine and caffeine, respectively. These findings indicated that nicotine, cotinine and caffeine could slightly affect the growth and viability of some oral bacterial strains.

1. INTRODUÇÃO

A cavidade bucal, de modo similar a outros sítios do corpo humano, apresenta uma microbiota natural com composição característica, a qual coexiste de modo harmônico com o hospedeiro. Entretanto, a maioria dos indivíduos sofre, em algum período de sua vida, episódios localizados de doenças bucais que são causados por um desequilíbrio na composição da microbiota bucal residente (Marsh & Martin, 1992). As manifestações clínicas deste desequilíbrio incluem a cárie dental e a doença periodontal.

As doenças periodontais são infecções causadas por microrganismos que colonizam a superfície dental na margem gengival ou abaixo dela. Colonizadores primários do biofilme dental, a espécie *Actinomyces naeslundii* e os estreptococos do grupo mitis (entre eles o *Streptococcus oralis, Streptococcus gordonii* e *Streptococcus mitis*), dão suporte à colonização do biofilme por outras espécies bacterianas, além de formar a base estrutural do biofilme (Socransky & Haffajee, 2005). A espécie *A. naeslundii* é considerada umas das mais importantes na construção do biofilme subgengival e supragengival (Mager *et al.*, 2003), predominando tanto nos biofilmes formados na cavidade oral de pacientes saudáveis, quanto nos pacientes com doença periodontal (Ximenez *et al., 2000*). As espécies de estreptococos, de maneira diferente das outras espécies, são capazes de co-agregarem entre si, o que contribui para serem numericamente abundantes no biofilme dental (Frandsen *et al.,* 1991; Kolenbrander & Andersen, 1989).

Após a formação inicial do biofilme por camadas de colonizadores primários, um novo ambiente nutricional e uma nova superfície para aderência de outras espécies são formados, favorecendo a colonização dos chamados colonizadores tardios, como o *Fusobacterium nucleatum* e a *Porphyromonas gingivalis*. A espécie bacteriana *F. nucleatum* é a de maior prevalência entre os microorganismos Gram negativos no biofilme tardio, além de ter sido considerada um possível patógeno no processo da doença periodontal (Socransky & Haffajee, 2002). Essa espécie bacteriana parece ter a capacidade de dar suporte ao crescimento de outras

espécies anaeróbicas estritas, como por exemplo, a *P. gingivalis,* pela sua capacidade de se adaptar e reduzir ambientes com oxigênio, além de suprir o meio com dióxido de carbono (Diaz *et al.*, 2002).

A *P. gingivalis* tem sido reconhecida como um importante patógeno periodontal. Estudos reportaram sua maior prevalência em sítios com periodontite do que em sítios saudáveis dos mesmos pacientes (Riviere *et al.*, 1996). Uma outra espécie colonizadora do biofilme supra e subgengival de pacientes saudáveis e de pacientes com periodontite é a espécie *Propionibacterium acnes* (Socransky & Haffajee, 2002).

Esses microrganismos podem formar um biofilme patogênico que se adere sobre a superfície dental, de modo a produzir produtos citotóxicos que levam à inflamação gengival e a periodontites (Socransky & Haffajee, 2002). Um dos principais fatores que influenciam na evolução dessas doenças é o tabagismo.

Existem evidências substanciais sobre o efeito prejudicial do fumo na saúde periodontal. Em um trabalho conduzido por Calsina *et al.* (2002), indivíduos fumantes e ex-fumantes tiveram incidência de doença periodontal de, respectivamente, 2,7 e 2,3 vezes maior do que indivíduos não fumantes, independente de sexo, idade ou índice de placa. A severidade de alguns sinais clínicos dessa doença é maior em fumantes do que em ex-fumantes, sendo menor nos não fumantes (Haber *et al.,* 1993; Grossi *et al.,* 1994, 1995; Bergström *et al.*, 2000; Machuca *et al.*, 2000). Estudos têm mostrado a associação entre o cigarro e a perda óssea alveolar, redução da inserção periodontal, aumento da profundidade de sondagem, bem como o aumento da prevalência e a severidade das periodontites (Bergstrom & Preber, 1994; Brown *et al.*, 1994; Grossi *et al.*, 1994, 1995; Schenkein *et al.*, 1995; Gunsolley *et al.*, 1998; Elter *et al.*, 1999; Haffajee & Socransky, 2001a; Calsina *et al.*, 2002). A terapia periodontal em fumantes é menos eficaz do que em não fumantes, apresentando menor redução da profundidade de sondagem, dos níveis de inserção periodontal e resultados menos promissores em cirurgias de implantes (Johnson & Hill, 2004).

Indivíduos fumantes apresentam as respostas inflamatórias e imunes aos patógenos periodontais prejudicadas (Obeid & Bercy, 2000). Pode ocorrer deficiência das funções neutrofílicas como na fagocitose (Macfarlane *et al.*, 1992), na produção de superóxido, peróxido de hidrogênio (Ryder *et al.*, 1998) e na produção de inibidor de protease (Persson *et al.*, 2001). Além dessas alterações, a produção de citocinas inflamatórias, como a IL-1b e IL-8, está aumentada no fluido gengival crevicular de pacientes fumantes com gengivites (Giannopoulou *et al.*, 2003). O aumento de citocinas como a IL-1b e TNF-α também foi observado em um estudo *in vitro* que avaliou a secreção dessas substâncias por células mononucleares expostas à fumaça de tabaco (Ryder *et al.*, 2002). A exposição ao tabaco também promove alterações no tecido periodontal como modificações na inserção e crescimento dos fibroblastos (James *et al.*, 1999; Gamal & Bayomy, 2002) e redução da sua capacidade de recuperação (Benatti *et al.*, 2005).

A relação entre o consumo de cigarros e a microbiota oral ainda não foi bem esclarecida. Estudos examinaram a prevalência e a proporção de espécies bacterianas subgengivais em adultos fumantes e não fumantes, e não encontraram diferença estatisticamente significante (Boström *et al.*, 2001; Mager *et al.*, 2003; van der Velden *et al.*, 2003; Apatzidou *et al.*, 2005; Salvi *et al.*, 2005). No entanto, outras investigações encontraram resultados conflitantes. Haffajje & Socransky (2001b) relataram maior prevalência, em fumantes, de algumas espécies como *Eubacterium nodatum, Fusobacterium nucleatum ss vicentii, Prevotella intermedia, Prevotella nigrescens, Prevotella micros, Bacteroides forsythus, Porphyromonas gingivalis* e *Treponema denticola*. No estudo conduzido por Zambon *et al.* (1996), pacientes fumantes apresentaram maiores proporções de espécies como *B. forsythus*, *Actinomyces actinomycetemcomitans* e *P. gingivalis*. Van Winkelhoff *et al.* (2001) encontraram uma maior prevalência de *P. intermedia* e *P. nigrescens* e maiores níveis de *Peptostreptococcus micros* e *F. nucleatum* em fumantes.

Entre as mais de 4000 substâncias resultantes da combustão do tabaco e presentes na fumaça do cigarro, como o monóxido de carbono, radicais oxidativos, carcinógenos (ex.: nitrosaminas), etc., está uma substância psicoativa viciante, a

nicotina. (Tonetti, 1998). A nicotina é considerada a substância farmacologicamente mais ativa do tabaco. A maior parte dela é absorvida pela mucosa alveolar, mas a sua absorção pode ocorrer também, de forma mais lenta, através da mucosa oral, em quantidades suficientes para induzir efeitos farmacológicos (Armitage & Turner, 1970). Sua metabolização ocorre rapidamente no organismo, tendo uma meia vida plasmática de aproximadamente 2 horas (Eramo *et al.*, 2000). A cotinina, um dos principais metabólitos da nicotina, possui uma meia-vida bem mais longa, aproximadamente de 19 horas, o que faz com que sua presença seja pesquisada como indicador de exposição à nicotina (Eramo *et al.*, 2000). Devido à rápida metabolização da nicotina, a cotinina é encontrada em maior quantidade do que a nicotina nos fluídos biológicos, inclusive na saliva (Ghosheh *et al.*, 2000). A determinação salivar de cotinina confirma a relação entre a sua concentração, a incidência de periodontite e a exposição à fumaça do cigarro (Yamamoto *et al.*, 2005).

Muitos dos efeitos indesejáveis do cigarro são atribuídos à nicotina relacionando-a com os processos patológicos do tecido periodontal. A perda óssea alveolar que ocorre em indivíduos fumantes pode estar relacionada a estímulos dos osteoclastos pela nicotina (Henemyre *et al.*, 2003).

Têm sido reportadas alterações no processo inflamatório e na resposta imune pela nicotina, como a estimulação da secreção de prostaglandina E_2 por monócitos plasmáticos (Payne *et al.*, 1996), inibição da liberação de IL-1β por monócitos (Pabst *et al.*, 1995; Mariggió *et al.*, 2001), indução de apoptose de leucócitos polimorfonucleares (Mariggió *et al.*, 2001), inibição da ação bactericida de neutrófilos, redução da produção de ânion superóxido, peróxido de hidrogênio e da absorção de oxigênio pelos neutrófilos (Pabst *et al.*, 1995).

Muitos estudos têm observado modificações nos fibroblastos induzidas pela nicotina como redução da viabilidade, proliferação e inserção dos fibroblastos (Tanur *et al.*, 2000; Lahmouzi *et al.*, 2000), alteração da expressão de β1-integrina (Austin *et al.*, 2001), aumento da produção de colagenase e redução da produção de colágeno (Tipton & Dabbous, 1995) e aumento da apoptose (Lahmouzi *et al.*, 2000). Culturas

de fibroblastos quando expostas a nicotina apresentam maior produção de citocinas pró-inflamatórias como a IL-6 e IL-8 (Wendell & Stein, 2001) e maior ativação de COX-2 (Chang *et al.*, 2003). Na presença de nicotina, queratinócitos produzem maiores quantidades de IL-1 (Johnson & Organ, 1997). A diferenciação de miofibroblastos também é inibida pela nicotina (Fang & Svoboda, 2005).

Alguns estudos relataram interações entre a nicotina e bactérias da microbiota periodontal. Sayers *et al.* (1997) avaliaram o potencial de toxinas produzidas por cinco periodontopatógenos (*Prevotela intermedia*, *P. gingivalis*, *Porphyromonas asacharolytica*, *Fusobacterium necrophorum* e *F. nucleatum)* na presença da nicotina. Os resultados mostraram que a nicotina em combinação com toxinas extracelulares livres pode levar a um aumento do potencial letal dessas toxinas. Num estudo similar, Sayers *et al.* (1999) indicaram que a cotinina também pode agir sinergicamente com toxinas bacterianas. A nicotina e a cotinina, em concentrações elevadas, podem afetar a susceptibilidade de células epiteliais à colonização de patógenos periodontais como *A. actinomycetemcomitans* e *P. gingivalis* (Teughels *et al.*, 2005).

Existem poucas investigações na literatura científica relatando os possíveis efeitos da nicotina e da cotinina sobre a viabilidade e o crescimento de bactérias da microbiota oral. Pavia *et al.* (2000) relataram que estreptococos do grupo viridans tiveram o seu crescimento inibido na presença de nicotina. Em outro estudo, conduzido por Keene & Johnson (1999), concentrações de nicotina inibiram ou estimularam o crescimento de *Streptococcus mutans*, de forma dose dependente. Em contraste, Teughles *et al.* (2005) reportaram que a viabilidade de dois patógenos orais, *A. actinomycetemcomitans e P. gingivalis,* não foi alterada na presença de nicotina e cotinina.

Além da nicotina e da cotinina, a cafeína está presente em maior quantidade nos líquidos biológicos de indivíduos fumantes (Swason *et al.*, 1994). Essa evidência foi relatada em estudos epidemiológicos que constataram que os fumantes tendem a consumir em maior quantidade, bebidas e outros produtos que contenham cafeína do

que não fumantes. Esses estudos têm demonstrado uma associação positiva entre o fumo e o hábito de beber café (Swason *et al.*, 1994).

Mesmo tendo o conhecimento de que substâncias como a nicotina, cotinina estão presentes na cavidade oral de fumantes e que estão intimamente ligadas à causa das doenças periodontais, pouco se sabe sobre os seus efeitos na microbiota bacteriana subgengival. Pouco se sabe também sobre os efeitos da cafeína nessa microbiota, com possível contribuição para a evolução do processo infeccioso periodontal.

Desse modo, o presente estudo pretende avaliar o efeito da nicotina, cotinina e cafeína sobre o crescimento e a viabilidade de algumas espécies de bactérias da microbiota oral, nas formas planctônicas e em biofilme.

2. PROPOSIÇÃO

O objetivo deste estudo foi avaliar *in vitro* o efeito da nicotina, cotinina e cafeína sobre o crescimento e a viabilidade das seguintes espécies bacterianas da microbiota oral: *Streptococcus gordonii*, *Streptococcus oralis*, *Streptococcus mitis*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum* nucleatum, *Propionibacterium acnes* e *Actinomyces naeslundii* nas formas planctônica e biofilme.

3. CAPÍTULOS

Esta dissertação está baseada na Deliberação CCPG/001/98/Unicamp e na aprovação pela Congregação da Faculdade de Odontologia de Piracicaba em sua 105ª Reunião Ordinária em 17/12/2003, que regulamenta o formato alternativo para dissertação de Mestrado e permite a inserção de artigos científicos de autoria do candidato.

Assim sendo, esta dissertação é composta de um capítulo contendo um artigo que foi submetido à publicação em revista científica, conforme descrito a seguir:

Capítulo 1

Artigo "*In vitro evaluation of the effect of nicotine, cotinine and caffeine on oral microrganisms.*"

Este artigo foi submetido ao periódico: Archives of Oral Microbiology.

3.1 Capítulo 1

In-vitro evaluation of the effect of nicotine, cotinine and caffeine on oral microorganisms.

Running Title - Nicotine, cotinine, caffeine on oral bacteria

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Abreviations:
BHI- Brain Heart Infusion
HA- Hydroxyapatite

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ABSTRACT

Objective: The purpose of this in-vitro study was to evaluate the effects of nicotine, cotinine, and caffeine on the growth and viability of some bacterial species from the oral microbiota

Design: Single-species biofilms of *Streptococcus gordonii*, *Porphyromonas gingivalis*, *and Fusobacterium nucleatum* and dual-species biofilms of *S. gordonii* + *F. nucleatum* and *F. nucleatum* + *P. gingivalis* were grown on hydroxyapatite discs. Seven species were studied as planktonic cells, including *Streptococcus oralis*, *Streptococcus mitis*, *Propionibacterium acnes*, *Actinomyces naeslundii*, and the species mentioned above. Bacteria were incubated in either 0 or 0.37 to 400 μg/mL of nicotine, cotinine or caffeine for planktonic cells and 0 or 400 μg/mL for biofilm. The viability of planktonic cells and biofilms was analyzed by susceptibility tests and time-kill assays, respectively.

Results: "Susceptibility Test" showed that nicotine reduced the growth of *S. gordonii* (400 μg/mL) and *S. oralis* (0.37-400 μg/mL); cotinine stimulated the growth of *A. naeslundii* (0.37 μg/mL) and *F.* nucleatum (0.37-400 μg/mL) and reduced the growth of *S. oralis* (400 μg/mL) and caffeine stimulated the growth of *F. nucleatum* (400 μg/mL). Results of "Killing Assays" showed an enhanced growth of *F. nucleatum* in single-species biofilm and a reduced viability of *S. gordonii* in single-species biofilm, 24 h and 48 h after exposure to cotinine and caffeine, respectively.

Conclusions: These findings indicate that nicotine, cotinine and caffeine can slightly affect the growth and viability of some oral bacterial strains.

Keywords: nicotine, cotinine, caffeine, oral microorganisms

INTRODUCTION

Subgingival accumulation of bacterial biofilm has been considered an etiologic agent of periodontal diseases. Pathogenic biofilm is known to produce cytotoxic substances, resulting in gingival inflammation.[1]

Some bacterial strains are important colonizers of the dental biofilm. Early colonizers of biofilm—*A. naeslundii*, and members of the mitis group of streptococci (*S. oralis, S. gordonii and S. mitis*)—promote further bacterial colonization and support biofilm structure.[2] Actinomyces species were in high proportions in biofilm samples from the tooth surfaces[3] and predominate in both periodontally healthy and periodontitis subjects.[4] Mitis group of streptococci is also numerically abundant in dental plaque.[5] *F. nucleatum* is the most prevalent in the gram negative species in the biofilm at later stage[6] and have been considered a possible pathogen in periodontal diseases.[1] *P. gingivalis* has been well recognized as a periodontopathogen and was reported to be more prevalent in diseased sites of subjects with periodontitis than in healthy sites of diseased subjects.[7] Another species that colonizes subgingival biofilm of healthy and periodontally diseased patients is *P. acnes*.[1] The substances that interfere with the viability of these bacteria could be able to modify biofilm formation.

The use of tobacco is recognized as one of the most important risk factors responsible for the development and progression of periodontal diseases as well as for a further reduction in the response to the periodontal therapy.[8] Several studies comparing smokers to non-smokers have shown that smokers have more alveolar bone loss, deeper periodontal pockets, and higher levels of attachment and tooth loss. [9-15]

The relationship between cigarette smoking and the subgingival microbiota is not clear. Some studies have reported no difference in the prevalence of subgingival species of microorganisms between smokers and non-smokers with periodontitis.[3, 16-19] However, some authors showed that smoking increases the likelihood of prevalence and proportions of certain periodontal pathogens.[20-22]

Tobacco smoke contains more than 4,000 substances, including carbon monoxide, oxidating radicals, carcinogens (e.g., nitrosamines) and addictive substances such as nicotine. Cotinine is the main nicotine metabolite.[23] Nicotine has a short blood half life (±2 h), while cotinine has a longer (±19 h) serum half life.[24] Therefore, cotinine has been used as a chemical marker of cigarette exposure in studies relating smoking to many diseases.[25] These substances have been found in saliva and gingival crevicular fluid of smokers.[26]

Cigarette smoking is strongly associated with coffee drinking. Epidemiological studies have shown that 86.4% of smokers versus 77.2% of nonsmokers consume coffee, which is rich in caffeine. Former smokers drink more coffee than never smokers, but somewhat less than smokers.[27]

Very few in vitro studies were found to evaluate the effects of nicotine and cotinine on growth and viability of oral bacteria. Pavia et al.[28] showed that nicotine caused a dose-dependent growth inhibition of viridans streptococci. Keene and Johnson[29] reported that nicotine could stimulate or inhibit *Streptococcus mutans* growth in a biphasic dosage dependent manner. Sayers et al.[30-31] reported that synergy between nicotine or cotinine and toxins from periodontopathogens can occur. The colonization of epithelial cells by two periodontopathogens, *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*, could be altered in the presence of nicotine or cotinine. However, the viability of these bacteria was not affected when exposed to high concentrations of nicotine and cotinine.[32]

Although nicotine and cotinine are known to have some effect on smokers' oral cavity, especially on periodontal tissues, nothing is known about the effects of caffeine on periodontal diseases. Besides, the relationship between these substances and the subgingival microbiota remains unclear.

The purpose of the present study was to evaluate the effects of nicotine, cotinine, and caffeine on the growth and viability of planktonic cells and biofilms in an in-vitro assay.

MATERIAL AND METHODS

Bacteria strains and Culture Conditions

The susceptibility of seven oral bacteria species (*Streptococcus oralis* PB182, *Streptococcus mitis* ATCC903, *Streptococcus gordonii* ATCC10558, *Porphyromonas*

gingivalis 381, *Fusobacterium nucleatum* nucleatum ATCC25586, *Propionibacterium acnes* ATCC11827, and *Actinomyces naeslundii* I ATCC12104) was examined by using the macrodilution broth test. *S. gordonii, F. nucleatum*, and *P. gingivalis* were also used to produce single-species and dual-species biofilms, which were analyzed in time-kill assays.

Oral streptococci, *F. nucleatum*, *P. acnes* and *A. naeslundii* were grown in Brain Heart Infusion Broth - BHI (Difco Co., Detroit, MI, USA). For cultivation of *P. gingivalis*, 5 μg/mL haemin (Difco Co.) and 1 μg/mL menadione (Difco Co.) were added to BHI. Streptococci were grown in an incubator (Jouan IG150, Jouan, France) with atmosphere enriched with 10% CO_2, at 37°C. The other bacterial species were grown under anaerobic conditions (10% CO_2, 10% H_2 and 80% N_2) using an anaerobic incubator (MiniMacs Anaerobic Workstation, Don Whitley Scientific, Shipley, UK), at 37°C.

Susceptibility Test (macrodilution broth test)

Susceptibility was assayed by using the macrodilution broth test described by Koneman et al.[33] The test was carried out in tubes (in triplicate) containing 5 mL of BHI broth. All the substances tested were purchased from Sigma Chemical Co (Poole, UK). Nicotine (nicotine hydrogen tartrate) and caffeine were diluted in distilled and sterilized water and cotinine ((-)- cotinine) in ethanol (0.8% v/v). These substances were assayed at concentrations of 400, 100, 25, 6.25, 1.5 and 0.4 μg/mL.

After obtaining the concentrations mentioned above, all tubes received a 75 µL of a standardized inoculum (10^7 CFU/mL), containing bacterial suspension prepared in the sterile saline solution (0.9% NaCl), and adjusted with a spectrophotometer to a cell density of 0.5 McFarland standard (1 x 10^8 CFU/mL) at a wavelength of 660 nm. This suspension was diluted in saline solution to obtain a final inoculum of 1 x 10^7 CFU/mL.

The tubes were then incubated at 37°C, in an atmosphere containing 10% CO_2 for 18 h (streptococci), or under anaerobic for 48 h (other bacteria). Bacterial growth was assessed microbiologically. Three tubes with inoculum but without any of the above substances were used as positive control. The vehicle control contained ethanol 0.8% (v/v).

Samples from each tube were serially diluted (10^{-2} to 10^{-5}) in saline solution and spirally plated (Spiral Plater System, Don Whitley Scientific, Shipley, UK) in a logarithmic distribution on brain heart infusion agar with 5% defibrinated sheep blood. For *P. gingivalis*, 5 µg/mL haemin and 1 µg/mL menadione were added to BHI. Petri dishes were then incubated at 37°C, in 10% CO_2 for 18 h (streptococci), and under anaerobic conditions for 48 h (other bacteria). After incubation, colonies were counted to determine colony-forming units per mL.

Biofilm assays

The biofilm assay method was adapted from a previously described method.[34] Single-species and dual-species biofilms, such as 1- *S. gordonii;* 2 - *P. gingivalis;* 3 -

F. nucleatum; 4 - *S. gordonii* + *F. nucleatum;* 5 - *P. gingivalis* + *F. nucleatum,* were grown on surface of sintered hydroxyapatite discs (Ceramic – Calcium Hydroxyapatite, 0.5" diameter ceramic – Clarkson Calcium Phosphates, Williamsport, PA, USA). These discs were previously autoclaved and placed in a vertical position into the 50 mL polystyrene tubes containing artificial saliva, except for *S. gordonii* single-species biofilm. Artificial saliva was prepared as described Pratten et al.[35] *S. gordonii* single-species biofilm were grown in BHI and 0.5% (w/v) sucrose. For *P. gingivalis* biofilms, 5 μg/mL haemin and 1 μg/mL menadione were added to the artificial saliva.

For single-species biofilms, the inoculum was standardized to 10^8 CFU/mL. For *P. gingivalis* + *F. nucleatum* biofilms, the inoculum was 10^8 CFU/mL for *P. gingivalis* and 10^5 CFU/mL for *F. nucleatum*. For *S. gordonii* + *F. nucleatum* biofilms, the inoculum was 10^8 CFU/mL for *S. gordonii* and 10^5 CFU/mL. *F. nucleatum* inoculum was prepared with sterile saline solution and then standardized by using a spectrophotometer at a wavelength of 660 nm.

The tubes were incubated for 24 h, at 37°C in 10% CO_2 (*S. gordonii* single-species biofilm) or under anaerobic conditions (other biofilms). After this period, the medium was replaced with BHI, which was renewed every 24 hours. BHI was added by 5 μg/mL haemin and 1 μg/mL menadione for *P. gingivalis* biofilms. Gram's stain was used to check culture purity. Biofilms were grown for 5 days (*S. gordonii* single-species biofilm) and 7 days (dual-species biofilms and *P. gingivalis* and *F. nucleatum* single-species biofilms) and then submitted to killing assays.

Killing assays

The killing assays were carried out as described by Duarte et al.[34] Tests were carried out in triplicates. The biofilms were transferred to their respective media containing caffeine, nicotine, cotinine, all at 400 μg/mL; or ethanol 0.8% [vehicle control (v/v)] and then incubated at 37°C, in 10% CO_2 (*S. gordonii* single-species biofilm) or in anaerobic conditions (other biofilms). At specific time intervals (0, 2, 5, 8, 18, 24 and 48 h), HA discs were removed from the tubes and rinsed in a 7.5-mL sterile saline solution twice for 10 seconds each. Each disc was transferred to a tube containing sterile saline solution and biofilm was dispersed by means of sonication (Vibra Cell 400w, Sonics & Materials Inc, Newtown, CT, USA) at 4°C, with 5% amplitude, and 6 pulses (9.9 s each pulse and a 5-sec interval). Biofilm suspensions were logarithmic plated on brain heart infusion agar with 5% defibrinated sheep blood. For *P. gingivalis*, 5 μg/mL haemin and 1 μg/mL menadione were added. The plates were incubated at 37°C, in 10% CO_2 for 48 h (*S. gordoni* single-species biofilm) or under anaerobic conditions for 72 h (other biofilms). After the incubation period, colony-forming units were quantified. Colonies differentiation was carried out using Gram's stain.

Data analysis

The number of colony forming units obtained in the susceptibility assays was analyzed by using ANOVA. Statistical differences between control and concentration

groups were determined by the Dunnet test. Data obtained from biofilm killing assays were performed by using the Mann Whitney U test. Statistical software (BioEstat version 3.0, Mamirauá/CNPq, Belém, PA, Brazil) was used to carry out the analysis. The significant level was set at 5%.

RESULTS

Susceptibility test

The effects of nicotine, cotinine and caffeine on the growth of bacterial strains tested are shown in Figure 1. The figure summarizes the bacterial growth in log of colony-forming units/mL (CFU/mL) when exposed to different concentrations of the substances tested (μg/mL). The growth controls are represented as 0 μg/mL.

Slight differences between the growth control and the groups tested were found. However, some of these differences were statistically significant ($p<0.05$). When exposed to nicotine, *S. gordonii* (400 μg/mL) and *S. oralis* (from 0.37 to 400 μg/mL) had their growth decreased. Susceptibility test results also showed that cotinine enhanced growth of *A. naeslundii* (0.37 μg/mL) and *F. nucleatum* (from 0.37 to 400 μg/mL) and reduced growth of *S. oralis* (400 μg/mL). Caffeine stimulated the growth of *F. nucleatum* (400 μg/mL).

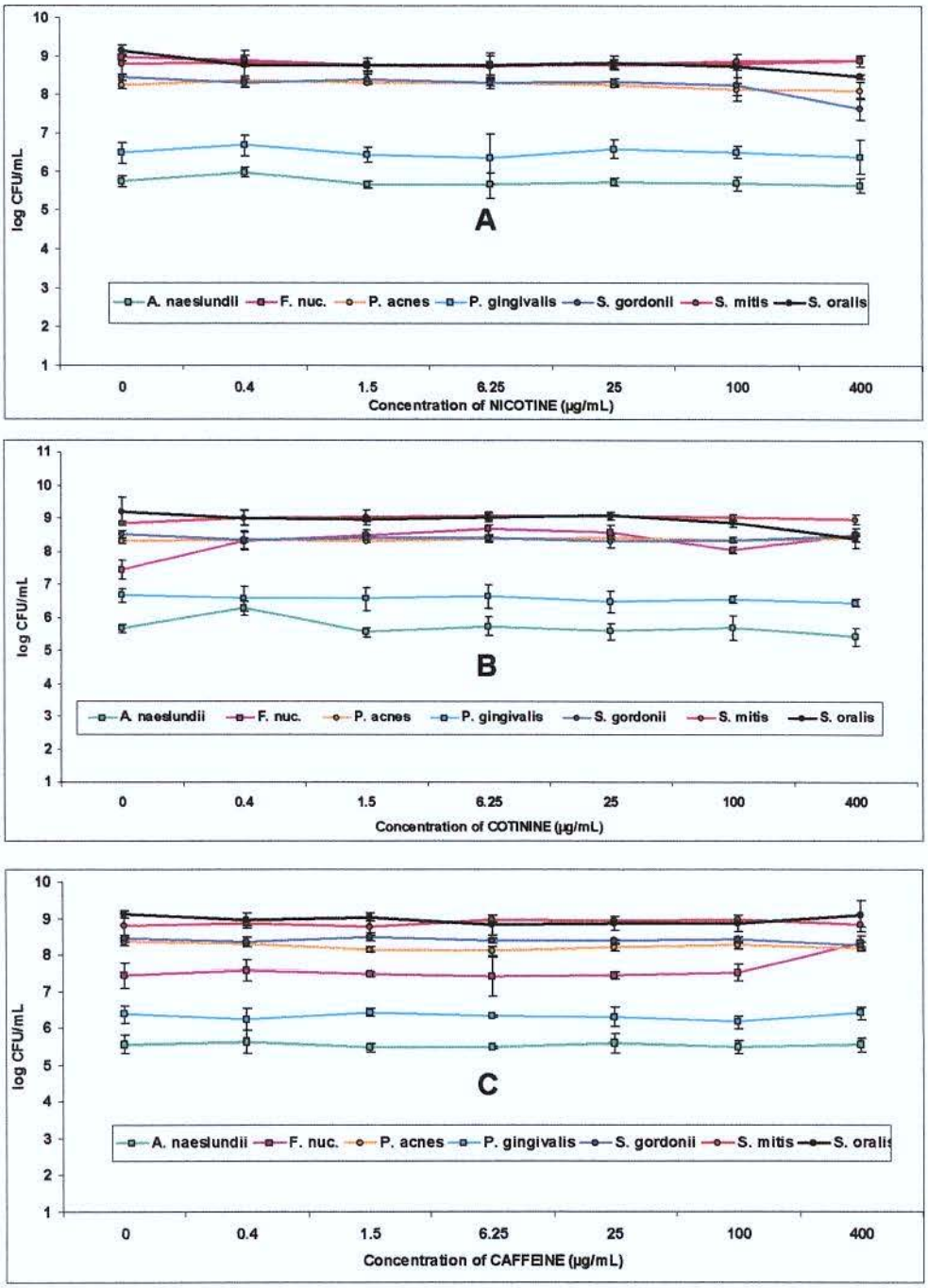

Figure 1. Log CFU/mL of the bacteria strains tested 18 h (streptococci) and 48 h (other bacteria) after exposure to different concentrations of nicotine (A), cotinine (B) and caffeine (C).

Killing Assays

The results of the influence of nicotine, cotinine and caffeine on bacterial single-species biofilms are shown in Figure 2. These substances did not show any activity against single-species biofilms during the 18-h treatment period. However, after a 24-h and 48-h exposure to caffeine, *S. gordonii* had its viability reduced in comparison to growth control ($p<0.05$). In contrast, after a 24-h and 48-h exposure to cotinine, viable counts were observed to be higher for *F. nucleatum* when compared to growth control ($p<0.05$). In dual-species biofilms, the viability of *F. nucleatum*, *P. gingivalis* and *S. gordonii* was not altered in the presence of any substances tested (Figure 3).

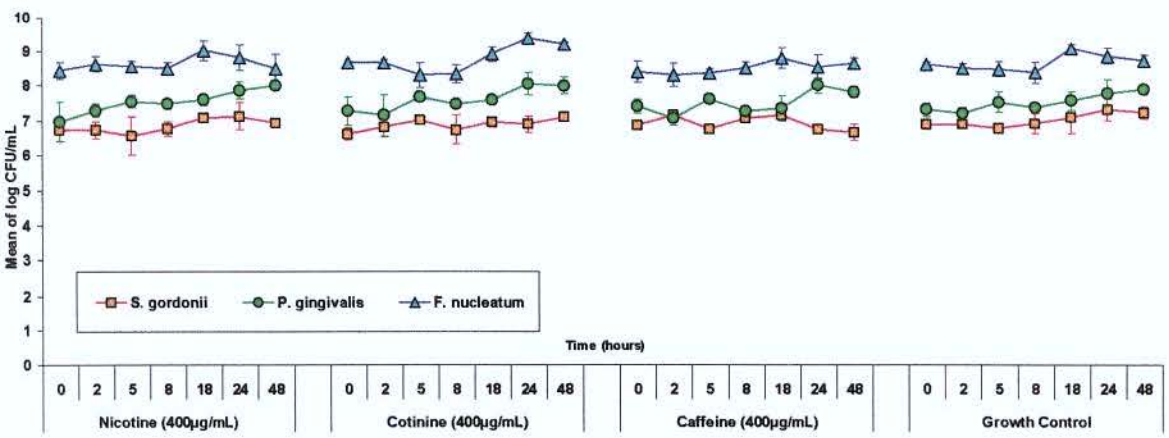

Figure 2. Time kill curves for *S. gordonii*, *F. nucleatum* and *P. gingivalis* biofilms (single-species) exposed (48 h) to nicotine, cotinine and caffeine.

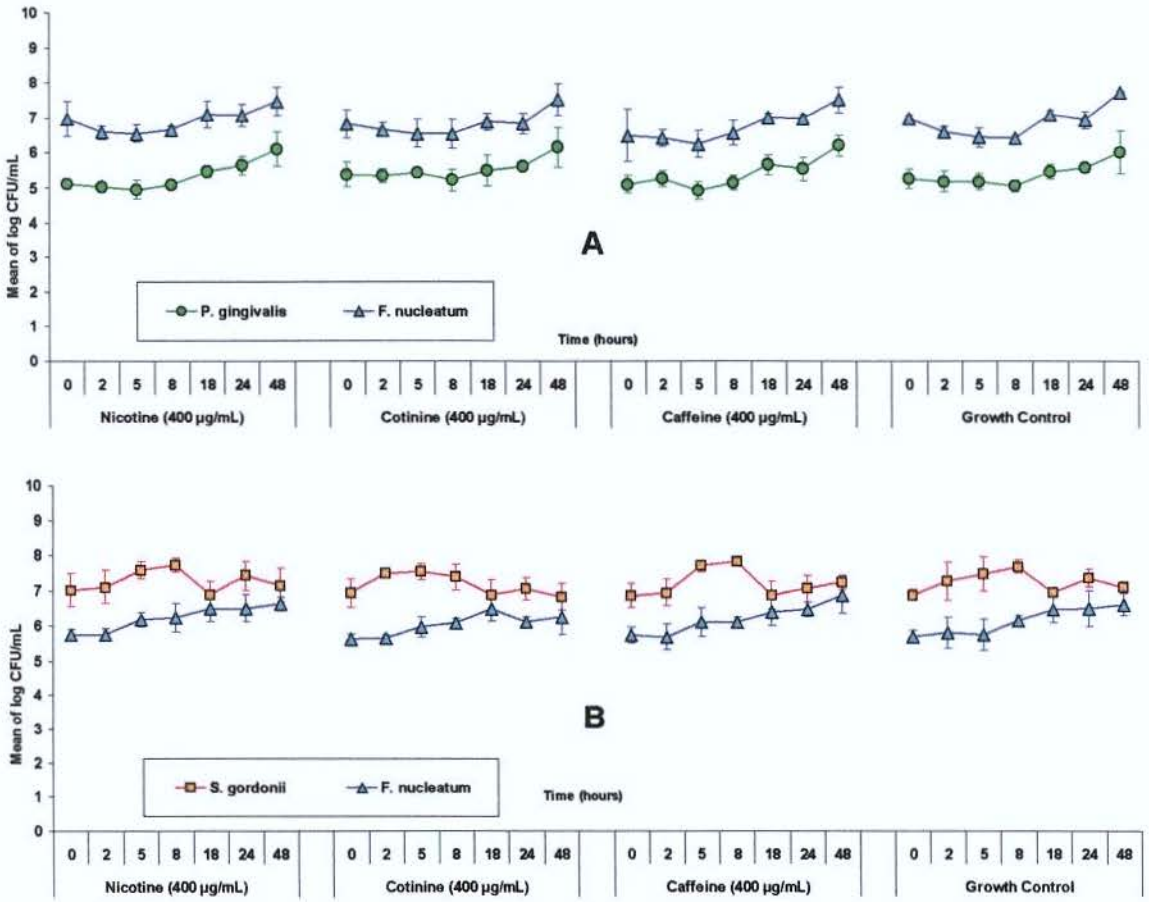

Figure 3. Time kill curves for dual-species biofilms of *P. gingivalis* + *F. nucleatum* (A) and *S. gordonii* + *F. nucleatum* (B) exposed (48 h) to nicotine, cotinine and caffeine.

DISCUSSION

Previous studies established the impact of cigarette smoking in the progression of periodontal diseases and in healing following periodontal therapy.[8] Coffee drinking is reported to be strongly associated with cigarette smoking.[27] However, the precise

role of cigarette smoking constituents (nicotine and cotinine) in the etiology of periodontal disease is not fully studied. Many mechanisms of interactions between these substances and periodontal diseases have been proposed. However, few studies have investigated the effect of nicotine, cotinine and caffeine in the development of periodontal microorganisms. The present study investigated the effect of the previously mentioned substances on the viability and growth of some oral microorganisms.

The present study showed that nicotine, cotinine and caffeine could interfere with the development of different species of oral bacteria, slightly inhibiting or stimulating their growth or interfering with cell viability. Similar results were previously observed by Pavia et al.,[28] who showed that nicotine at concentrations varying from 100 to 250 μg/ml could reduce the growth of a broad spectrum of microorganisms, such as *Escherichia coli, Klebsiella pneumoniae, Listeria monocytogenes, Cryptococcus neoformans and Candida albicans*, while only slightly inhibited or unaffected *Staphylococcus aureus* and *Borrelia burgdoferi*. Viridans streptococci were also susceptible to nicotine, which markedly reduced the bacterial cells viable counts. However, these authors did not mention which species of viridans streptococci they studied. We also observed a species-dependent effect since the concentrations of each cell-affecting substance differed among the species. However, our results did not show a very strong activity of nicotine against streptococci.

Roberts and Cole [36] reported a prolific growth of *Haemophilus influenzae* in the presence of tobacco or nicotine added to a phosphate-buffered saline agar. This

assay was performed using a culture medium that poorly supported the growth of *Haemophilus influenzae.* In the present study, the assays were carried out using rich culture media, which promoted a proper bacterial growth, suggesting that, in a nutrient scarce condition, bacteria might use nicotine as a nutrient source. Probably, bacterial growth stimulation by nicotine could be higher in a nutrient-poor environment. Moreover, the bacterial growth on Roberts and Cole [36] study was analyzed qualitatively through visual analyses and not quantitatively - calculating numbers of colony-unit forming per mL or measuring the optical density - making difficult comparisons to this study. Comparisons between our results and those reported in previous studies are also difficult due to differences in methodology, nicotine concentrations and species of microorganisms tested; most of them were not from oral microbiota.

Keene and Johnson [29] observed a biphasic dose-dependent effect of nicotine on *Streptococcus mutans* growth. Concentrations of 0.1M (16.22mg/mL) and 0.01M (1.622mg/mL) of nicotine inhibited the bacteria growth, 10^{-3}M (162.2µg/mL) and 10^{-4}M (16.22µg/mL) stimulated it, while 10^{-6}M (162.2ng/mL) and 10^{-7}M (16.2ng/mL) reduced the number of viable cells. The present study did not support this nicotine dose-dependent profile; streptococci had their growth reduced by nicotine in concentrations ranging from 0.37µg/mL to 400µg/mL for *S. oralis* and 400µg/mL for *S. gordonii* while Keene and Johnson [29] showed a stimulated growth of *S. mutans* in similar nicotine concentrations. This difference might have occurred because of the different species studied.

Teughels et al.[32] evaluated the bacterial viability of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* when exposed to 10, 100 and 1000μg/mL of nicotine or cotinine, during 0, 2, 4 and 6 hours of inoculation. These authors concluded that neither nicotine nor cotinine significantly affected bacterial viability. These latter findings are in agreement with the current investigation, suggesting that nicotine has no potential activity on growth of *P. gingivalis.*

The influence of caffeine on strains of *Escherichia coli* growth was investigated by Sandlie et al.[37] Concentrations up to 8 mM (1553 μg/ml) had little effect on the growth of these bacteria, while in higher concentrations, the growth strongly decreased. A 50% inhibition of growth was found with a concentration of 20 mM (3883 μg/ml). In the present study, even at the highest concentration tested (400 μg/mL) no significant reduction on bacterial growth was found considering planktonic cells. Only after 24 hours of exposition to caffeine, the viability of *S. gordonii* biofilm was slightly reduced.

Differences between planktonic cells and biofilms were observed in the present study. While nicotine slightly reduced the growth of planktonic cells of *S. gordonii,* no major activity was observed on its biofilm. Caffeine slightly reduced the viability of *S. gordonii* biofilm after 24 h of exposure and had no interference with its planktonic cells. For single-species biofilms, caffeine and cotinine interfered with the viability of *S. gordonii* and *F. nucleatum*, respectively. For dual-species biofilm, no influence on viability was found, either when *S. gordonii* was exposed to caffeine or when *F. nucleatum* was exposed to cotinine. These findings show the relevance of using

biofilm models to determine the effects of any substance. For the comparison between biofilms and planktonic cells, it was hypothesized that biofilms respond differently to some antimicrobial agents, because of their different characteristics as incomplete or slow substance penetration into biofilms, quorum sensing among biofilm cells inducing resistance and low growth rate.[1]

Although the results of the present study showed no significant increases or decreases in some oral bacterial growth, previous studies reported different mechanisms by which nicotine and/or cotinine could affect oral bacteria. An investigation conducted by Wendell and Stein[38] showed that the combination of nicotine and lipopolysaccharide produced by *P. gingivalis* induced the production of inflammatory cytokines IL-6 and IL-8. Sayers et al.[30] assayed the lethal potential of toxins produced by five periodontopathogens in chick embryo (*Prevotela intermedia*, *P. gingivalis*, *Porphyromonas asacharolytica*, *Fusobacterium necrophorum* and *F. nucleatum)* in the presence of nicotine. The results showed that nicotine combined with cell-free extra cellular toxins or cell lysates may result in a possible lethal enhancement. A similar study conducted by Sayers et al.[31] indicated that cotinine had the same potential as that of nicotine to potentiate bacterial toxins from *P. intermedia*, *Prevotela nigrescens* and *P. gingivalis*. Nicotine and cotinine in high concentrations might affect the susceptibility of epithelial cells to be colonized by *A. actinomycetemcomitans* and *P. gingivalis*.[32] The mechanisms mentioned above may be more relevant than the influence of nicotine and cotinine with cell growth and viability.

Some studies have reported that concentration levels of nicotine in saliva range from 70µg to 1560µg/mL, [39] with a mean level of approximately 115ng/mL.[40] The mean levels of cotinine reported in saliva and crevicular fluid ranged from 424ng to 3.6µg/mL and 2.5µg/mL to 15.0µg/mL, respectively.[26, 41] In the present study, the nicotine and some of the cotinine concentrations used were in agreement with physiological levels while some concentrations were higher than those found in saliva and crevicular fluid. Nevertheless, previous studies used high concentrations of these substances to induce cellular effects.[31-32] Concentrations of nicotine and cotinine used in this study were adequate for the evaluation of their effects. No investigation was found reporting caffeine saliva or crevicular fluid concentrations.

We concluded that nicotine, cotinine and caffeine could slightly interfere with the growth of some of the oral bacterial strains evaluated. However, further studies are required to elucidate the exact role of these substances and their physiological relevancy in oral bacteria.

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4. CONCLUSÃO

Os resultados do presente estudo indicam que a nicotina, cotinina e cafeína podem interferir, em pequena extensão, no crescimento e na viabilidade de bactérias da microbiota oral. Essa interferência é espécie e dose dependentes. Estudos futuros são necessários para saber o mecanismo pelos quais essas substâncias atuam e para conhecer a relevância fisiológica desses achados.

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6. ANEXOS

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