UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE ODONTOLOGIA DE PIRACICABA

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FARMACÊUTICA

AVALIAÇÃO *IN VITRO* DOS EFEITOS DA NICOTINA E COTININA SOBRE A EXPRESSÃO DE PROTEÍNAS E CAPACIDADE DE ADESÃO E INVASÃO DE *PORPHYROMONAS GINGIVALIS*.

Tese apresentada à Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas, para obtenção de título de Doutora em Odontologia, Área de Concentração em Farmacologia, Anestesiologia e Terapêutica.

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

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Não reclames nem te faças de vítima.

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

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RESUMO

O uso do cigarro tem sido associado com a progressão da periodontite bem como com a redução da resposta à terapia aplicada a essa doença. *Porphyromonas gingivalis* é um importante colonizador do biofilme subgengival além de ser um dos principais patógenos envolvidos no estabelecimento e progressão da doença periodontal. No entanto, os possíveis efeitos dos principais derivados do cigarro sobre *P. gingivalis* ainda não foram totalmente investigados. Dessa forma, os objetivos deste estudo foram avaliar os efeitos da nicotina e cotinina sobre a expressão de proteínas e sobre a capacidade de adesão e invasão celular de *P. gingivalis*. A fim de avaliar a expressão de proteínas, culturas de *P. gingivalis* W83 foram expostas à nicotina e cotinina nas concentrações de 6 e 600μg/mL, as proteínas foram extraídas, separadas por eletroforese bidimensional em gel de poliacrilamida (12.5% SDS-PAGE) e identificadas por LC-MS/MS. Os géis e suas corridas eletroforéticas foram feitas em triplicatas e a detecção de proteínas nos mesmos foi feita através de coloração com corante Coomassie. Proteínas diferentemente expressas foram digeridas com tripsina e as amostras de peptídeos sequenciadas utilizando um sistema Q-TOF API LC-MS/MS. A busca MS/MS foi realizada utilizando os bancos de dados MSDB e NCBI através do programa Mascot. Para examinar a capacidade de adesão e invasão de *P. gingivalis*, monocamadas de células KB e culturas de *P. gingivalis* ATCC 33277 foram expostas às concentrações de 0.1, 10 e 100 μg/mL de nicotina e cotinina. As células epiteliais foram incubadas por 24 h enquanto *P. gingivalis* foi exposta a essas substâncias até atingir a fase logarítmica. Após o período de incubação, *P. gingivalis* foi submetida aos ensaios de adesão e invasão às células KB. O número de bactérias associadas às células foi obtido através de contagem de unidades formadoras de colônia. Os resultados obtidos da análise expressão de proteínas mostraram que a adição de nicotina e cotinina promoveram alterações no proteoma de *P. gingivalis*. Entre os ± 430 spots de proteínas reproduzíveis detectados em cada gel, 20 proteínas foram menos expressas e 42 foram mais expressas em pelo menos um dos tratamentos ($p<0.05$; ANOVA - Tukey). Entre as proteínas identificadas, muitas estavam envolvidas em processos como produção de energia celular, síntese de proteínas, estresse oxidativo, virulência, transporte, etc. Em relação aos resultados obtidos nos ensaios de adesão e invasão, foi evidenciado que, quando as células epiteliais foram inoculadas com nicotina e cotinina, nenhuma diferença significativa na colonização de *P. gingivalis* foi encontrada. Quando *P. gingivalis* foi exposta à maior concentração de cotinina, sua capacidade de adesão e invasão às células epiteliais aumentou de forma expressiva ($p<0.05$; ANOVA - Tukey). No entanto, a nicotina e as outras concentrações de cotinina testadas não alteraram a capacidade de colonização. Esses achados indicam que a nicotina e a cotinina podem afetar a expressão de proteínas de *P.*

gingivalis. Ainda, a cotinina pode alterar positivamente a eficiência de adesão e invasão de *P. gingivalis.*

PALAVRAS-CHAVE: *Porphyromonas gingivalis,* nicotina, cotinina, 2-DE, espectrometria de massas, adesão bacteriana, invasão bacteriana, células epiteliais.

ABSTRACT

Cigarette smoking is associated with the development of periodontitis and the decreased response to periodontal therapy. *P. gingivalis* is an important colonizer of the subgingival biofilm and is one of the major pathogens involved in the initiation and progression of periodontal disease. However, the possible effects of major cigarette's derivatives on *P. gingivalis* were not fully investigated. Thus, the purpose of the present study was to evaluate the effects of nicotine and cotinine on the protein expression and cellular adhesion and invasion abilities of *P. gingivalis*. To evaluate protein expression, *P. gingivalis* W83 cultures were exposed to nicotine and cotinine 6 and 600μg/mL concentrations, the proteins were extracted, separated by two-dimensional polyacrylamide gel electrophoresis (12.5% PAGE) and identified with LC-MS/MS. The gels were run in triplicates and detection of proteins was obtained by staining the gels with Coomassie blue. Proteins differentially expressed were digested with trypsin, and the peptide samples sequenced using a Q-TOF API LC-MS/MS system. The MS/MS was searched against the MSDB and NCBI databank using Mascot program. In order to assess *P. gingivalis* adhesion and invasion abilities, KB cells monolayers and *P. gingivalis* ATCC 33277 cultures were exposed to 0.1, 10 and 100 μg/mL nicotine and cotinine concentrations. The epithelial cells were incubated for 24 h while *P. gingivalis* was exposed to these substances until early logarithmic phase. After incubation period, *P. gingivalis* were submitted to assays to evaluate adhesion to and invasion of KB cells. The number of bacteria associated with these cells was assessed by counting the colony-forming unities. The results from protein expression analyses showed that addition of nicotine and cotinine promoted alterations in proteome profile of *P. gingivalis.* Among ± 430 protein spots reproducibly detected on each gel, 20 protein spots were downregulated, and 42 were upregulated at least in one treatment ($p<0.05$; ANOVA – Tukey test). The identified proteins are involved in several processes, *i.e.* energy production, protein synthesis, oxidative stress, virulence, transport and binding activities. Data obtained from adhesion and invasion assays evidenced that epithelial cells inoculated with nicotine and cotinine did not show any significant differences in *P. gingivalis* colonization. When *P. gingivalis* was exposed to the higher concentration of cotinine, adherence and invasion of this bacterium to the epithelial cells markedly increased ($p<0.05$; ANOVA – Tukey test). However, nicotine and the other concentrations of cotinine did not alter the colonization ability. These findings indicate that nicotine and cotinine may affect *P. gingivalis* protein expression. In addition, cotinine may alter positively *P. gingivalis* adhesion and invasion efficiencies.

Key-words: *Porphyromonas gingivalis,* nicotine, cotinine, 2-DE, mass spectrometry, bacterial adhesion, bacterial invasion, cells, epithelial.

SUMÁRIO

1. INTRODUÇÃO

A relação do fumo, nicotina e cotinina com a doença periodontal e a microbiota subgengival

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. Esses formam um biofilme patogênico que se adere sobre a superfície dental, de modo a produzir substâncias citotóxicas que levam à inflamação gengival (Socransky & Haffajee, 2002). Uma das principais causas da instalação dessas doenças é o tabagismo (Bergström *et al*., 2000; Machuca *et al*., 2000).

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 que nunca fumaram, independente de sexo, idade ou índice de placa. A severidade dos sinais clínicos dessa doença é maior em fumantes do que em ex-fumantes, sendo menor nos que nunca fumaram (Haber *et al.,* 1993; Grossi *et al.,* 1994, 1995; Bergström *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 (Bergström & Preber, 1994; Grossi *et al.*, 1994, 1995; Schenkein *et al.*, 1995; Gunsolley *et al*., 1998; Elter *et al*., 1999; Haffajee & Socransky, 2001 (1); Calsina *et al*., 2002; Baharin *et al*., 2006). A terapia periodontal em fumantes é menos eficaz do que em não fumantes, apresentando menor

melhoria da profundidade de sondagem, dos níveis de inserção periodontal e resultados menos promissores em cirurgias de implantes (Johnson & Hill, 2004).

A relação entre o uso do tabaco e a microbiota oral ainda não foi bem esclarecida. Estudos examinaram a prevalência, a proporção e os níveis 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 (2001, (2)) relataram maior prevalência, em fumantes, de algumas espécies bacterianas que compõem o biofilme oral incluindo *Porphyromonas gingivalis.* No estudo conduzido por Zambon *et al*. (1996), pacientes fumantes apresentaram maiores proporções de espécies como *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans* (antiga *Actinobacillus actinomycetemcomitans*) e *P. gingivalis.* Van Winkelhoff *et al*. (2001) encontraram uma maior prevalência de *Prevotella intermedia* e *Prevotella nigrescens* e maiores níveis de *Peptostreptococcus micros* e *Fusobacterium 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., estão a nicotina e a cotinina (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, 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). A nicotina pode intensificar a perda óssea periodontal induzida por trauma oclusal (Nogueira-Filho *et al*., 2004).

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), redução da liberação de citocinas inflamatórias por monócitos (Mariggió *et al*., 2001; Pabst *et al*., 1995) e uma maior liberação pelos osteoblastos (Kamer *et al*., 2006), indução de apoptose de leucócitos polimorfonucleares (Mariggiò *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 (Lahmouzi et al., 2000), alteração da expressão de β1-integrina (Austin et al., 2001) 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). A diferenciação de miofibroblastos também é inibida pela nicotina (Fang & Svoboda, 2005).

Alguns autores relataram 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. Num estudo prévio conduzido por Cogo *et al.* (2008), a nicotina e cotinina pareceram não interferir no perfil de crescimento e na viabilidade da espécie *P. gingivalis*, tanto na forma planctônica, quanto na forma de biofilme. Esse achado está de acordo com o

estudo de Teughels *et al.* (2005), que verificaram que *P. gingivalis* não tem sua viabilidade alterada na presença dessas substâncias. No entanto, esse mesmo estudo, verificou que a adesão de *P. gingivalis* às células epiteliais, essas últimas expostas à nicotina e cotinina, foi alterada, com uma redução significante.

Porphyromonas gingivalis, suas características e seu papel na doença periodontal

Entre os microrganismos conhecidos como patógenos periodontais está a espécie *P. gingivalis,* cuja prevalência é maior em sítios com doença do que em sítios saudáveis de pacientes com periodontite (Riviere *et al*., 1996). *P. gingivalis* é um bacilo Gram negativo, não fermentador de carboidratos, anaeróbio estrito e que utiliza ferro na forma de hemina para promover o seu crescimento (Lamont & Jenkinson, 1998). No biofilme subgengival, *P. gingivalis* atua como um colonizador tardio, um processo facilitado por outras espécies de microrganismos que colonizam primariamente o biofilme, provendo um ambiente mais adequado para a adesão, suprindo com substratos para o crescimento e reduzindo a tensão de oxigênio para valores adequados para o crescimento de *P. gingivalis*. Entre os microrganismos em que *P. gingivalis* se adere estão os colonizadores primários como os estreptococos orais (Lamont *et al*., 1994), *Actinomyces naeslundii* (Goulbourne & Ellen, 1991) e os colonizadores tardios como *F. nucleatum* (Kinder & Holt, 1989), *Treponema denticola* (Grenier, 1992) e *Tannerella forsythia* (previamente *Tannerella forsythensis*) (Yao *et al.*, 1996). *P. gingivalis* tem sido extensivamente estudado, inclusive o genoma de uma cepa, a W83, já foi completamente seqüenciado (Nelson *et al*., 2003). A fim de aperfeiçoar a colonização, a obtenção de nutrientes e a adesão as bactérias e células do hospedeiro, *P. gingivalis* produz adesinas, fímbrias, proteinases e hemaglutininas. Esses fatores de virulência estão envolvidos no processo de destruição do tecido do hospedeiro e de sua defesa imunológica, contribuindo profundamente para a instalação e desenvolvimento da doença periodontal (Lamont & Jenkinson, 2000).

Entre as principais proteinases estão as gingipaínas, que são endopeptidases do tipo cisteína, responsáveis por pelo menos 85% da atividade proteolítica da bactéria (Potempa *et al.*, 1997). As gingipaínas são especificadas como gingipaína K e gingipaína R e são capazes de hidrolisar ligações peptídicas de Lis-Xaa ou Arg-Xaa, respectivamente

(Potempa *et al.,* 1995). Entre as gingipaínas R, estão a HRgpA e a RgpB, produtos de 2 genes distintos porém relacionados, os genes *rgpA* e *rgpB*. A gingipaína K, ou Kgp, é um produto específico do gene *kgp* (Imamura *et al.,* 2003).

Além das proteases RgpA e Kgp que estão envolvidas no processo de hemaglutinação, *Pa. gingivalis* produz diferentes proteínas associadas a essa atividade, as hemaglutininas. Essa atividade é essencial para a obtenção de heme proteínas, que servem como fonte nutricional para a bactéria (Lepine & Progulske, 1996). Essas proteínas são expressas por 5 genes, *hagA, hagD* e *hagE*, que codificam polipeptídeos entre 73% e 93% de seqüências idênticas de aminoácidos, e *hagB* e *hagC*, cujos polipeptídeos tem 98.6% de homologia (Progulske-Fox *et al.,*1995; Lepine & Progulske, 1996).

A fímbria é um dos fatores mais importantes responsáveis por mediar a adesão de *P. gingivalis* (Lamont & Jenkinson, 1998, 2000). Além disso, esse fator parece estar associado à capacidade de invasão dessa bactéria as células epiteliais e endoteliais (Dorn *et al.,* 2000). Os genes responsáveis pela produção das fímbrias, a *fimA,* são classificados em 6 variantes (I, Ib, II, III, IV e V) (Nakagawa *et al.,* 2000; 2002). Apesar de *P. gingivalis* W83 (*fimA* IV) ser considerada uma das mais virulentas da sua espécie, essa cepa é considerada pouco invasiva e enquanto a ATCC 33277 (*fimA* I) possui maior capacidade de invasão às células (Dorn *et al*., 2000; Umeda *et al*., 2006).

Para adaptação fisiológica ao ambiente do biofilme subgengival, *P. gingivalis* conta com o fenômeno conhecido como *quorum sensing*, que envolve a regulação da expressão de genes específicos através do acúmulo de compostos sinalizadores, mediando a comunicação intercelular (Yuan *et al.,* 2005). Estudos prévios reportaram que *P. gingivalis* tem um sistema de *quorum sensing* regulado pelo gene *Lux-S* (Chung *et al.,* 2001; Frias *et al.,* 2001; Burgess *et al.,* 2002), que além de modular a expressão de fatores de virulência (Burgess *et al.,* 2002), está envolvido também na expressão de genes relacionados ao estresse e aos genes de produção de proteínas de membrana de efluxo e antígenos imunoreativos (Yuan *et al.,* 2005).

Em resposta ao estresse, *P. gingivalis* produz uma série de proteínas, a fim de se adequar a esse ambiente hostil (Lopatin *et al*., 2000). Apesar de várias dessas proteínas serem sintetizadas em resposta ao choque térmico, uma variedade de outros fatores de

estresse ambiental, como o estresse oxidativo, metais pesados e toxinas, podem também induzir a produção dessas proteínas (Lopatin *et al*., 2000). Entre as principais proteínas que participam do processo de proteção causados pelos radicais oxidativos estão HtpG, GroEL, DnaK, AhpC, TPR, Trigger factor e Super óxido dismutase (SOD) (Okano *et al*., 2006).

Existem evidências na literatura de que o aumento ou a redução de algumas substâncias ou mesmo alterações no meio de crescimento de *P. gingivalis* podem levar as modificações nos padrões de virulência, resposta ao estresse oxidativo e *quorum sensing* dessa espécie periodontal. A presença de alguns antimicrobianos em doses abaixo da concentração inibitória mínima altera esses padrões de atividade. A clorexidina, por exemplo, é capaz de reduzir a adesão de *P. gingivalis* às células epiteliais, a hemaglutinação e a coagregação (Grenier, 1996; Lee, 2001). A doxiciclina, tetraciclina e minociclina, em doses subterapêuticas, podem inibir as *gingipaínas* (Imamura *et al.,*2001) e reduzir a atividade proteolítica (Grenier *et al*., 2003). Em temperaturas de crescimento elevadas, a síntese de fímbrias, *gingipains*, RagA e RagB foram reduzidas significantemente (Murakami *et al*., 2004). Em condições limitadas de hemina, *P. gingivalis* apresentou alterações no perfil de transcrição de vários genes (Kiyama-Kishikawa *et al.*, 2005).

Porphyromonas gingivalis e a sua adesão e invasão às células epiteliais

P. gingivalis é um patógeno periodontal que adere e invade as células epiteliais da gengiva, permanecendo no seu interior por períodos prolongados sem causar morte às células do hospedeiro (Xia *et al*., 2007). Essa invasão às células epiteliais é considerada como um fator essencial para a colonização das bolsas periodontais e para o início e progressão e severidade da periodontite (Pathirana *et al*., 2007). Após a invasão, esses microrganismos mantém sua viabilidade celular, expandindo a colonização em outras células epiteliais, o que permite sua sobrevivência na cavidade oral (Rudney *et al.,* 2001, Vitkov *et al*., 2005; Xia *et al*., 2007). Existe uma forte correlação *in vivo* do número de bactérias aderidas ao epitélio da gengiva e a severidade da inflamação (Vaahtoniemi *et al*., 1993). Além de se manter viável no interior das células epiteliais, *P. gingivalis* modula a resposta inflamatória dessas células, com um aumento da produção de citocinas

inflamatórias como a IL-8, IL-1, IL-6 e TNFα, o que pode contribuir para a manutenção de uma infecção crônica (Eick *et al.,* 2006). Na presença de componentes das células epiteliais, *P. gingivalis* tem sua expressão protéica alterada. Entre as proteínas que tem sua produção modificada, estão uma proteína homóloga a proteína de internalização da espécie bacteriana *Listeria monocytogenes* e subunidades da Clp protease ATP-dependente, que também é responsável pela adesão e invasão nessa mesma espécie. Além disso, a síntese da fímbria FimA, essencial para o mecanismo de invasão de *P. gingivalis* é reduzida, o que significa que, depois de internalizada na célula do hospedeiro, essa bactéria não necessita mais expressar essa fímbria em grandes quantidades (Zhang *et al*., 2005).

Entre as variáveis da fímbria FimA, as cepas que possuem a tipo II, são as que possuem maior capacidade de invasão, quando comparada com cepas que possuem outros genótipos dessa fímbria (I, III, IV e V) (Amano *et al*., 2004). Apesar de *P. gingivalis* W83 (*fimA* IV) ser considerada uma das mais virulentas da sua espécie, essa cepa é considerada pouco invasiva e outras cepas como a ATCC 33277 (*fimA* I) possuem uma maior capacidade de invasão às células (Umeda *et al*., 2006).

Recentemente foi sugerido que as integrinas, uma super família de proteínas heterodiméricas transmembrana, são as primeiras proteínas que inicializam o processo de internalização de bactérias, pois funcionam como receptores para fímbrias de diferentes bactérias, inclusive para a FimA (Yilmaz *et al.*, 2002). Yilmaz *et al.* (2002) reportaram que, existe uma interação física entre a FimA e os heterodímeros β1 e α5β1 das integrinas expressas nas células epiteliais gengivais. As proteinases produzidas pela *P. gingivalis*, em especial a *gingipaína* RgpA e RgpB, estão envolvidas na adesão às células epiteliais, através de ligação aos receptores celulares pelos domínios catalíticos dessas proteinases (Chen & Duncan, 2004).

Justificativa do estudo

O fumo ainda é uma prática comum e aceita na sociedade contemporânea e vem acarretando sérios riscos a integridade dos indivíduos que fazem uso do tabaco. A doença periodontal é uma das doenças entre uma variedade de enfermidades causadas pelo tabaco.

Vários estudos já evidenciaram a relação entre a nicotina e a cotinina e a incidência e severidade dessas doenças. No entanto, pouco se sabe sobre os efeitos dessas substâncias nas bactérias da microbiota oral, em especial sobre *P. gingivalis*.

Dessa forma, se faz necessário pesquisar as possíveis alterações que a nicotina e a cotinina podem causar sobre *P. gingivalis*, como uma forma de contribuir para o entendimento da relação entre as patologias periodontais e estas substâncias. Assim, uma vez que estas substâncias estão normalmente presentes na saliva e no fluído gengival de fumantes, são necessários novos estudos para o conhecimento dos seus efeitos sobre um importante patógeno periodontal, *P. gingivalis*.

2. PROPOSIÇÃO

As propostas desse estudo foram:

1. Verificar por eletroforese bidimensional, possíveis alterações no padrão de produção de proteínas totais da *P. gingivalis* W83 na presença de nicotina e cotinina, e através de espectrometria de massas, seqüenciar as proteínas que tiveram seu padrão de produção alterado;

2. Avaliar a capacidade de adesão e invasão da *P. gingivalis* ATCC 33277 às células KB, após exposição à nicotina e cotinina.

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 tese de Doutorado e permite a inserção de artigos científicos de autoria do candidato.

Assim sendo, esta tese é composta de dois capítulos contendo artigos que se encontram em fase de submissão para publicação em revista científica, conforme descrito a seguir:

Capítulo 1

Artigo "*Changes in the proteome of Porphyromonas gingivalis promotted by nicotine and cotinine"*

Este artigo será submetido ao periódico: *Proteomics*.

Capítulo 2

Artigo "*The effects of nicotine and cotinine on Porphyromonas gingivalis adherence and invasion of epithelial cells"*

Este artigo foi submetido ao periódico: *Archives of Oral Biology.*

3.1. CAPÍTULO 1

Changes in the proteome of *Porphyromonas gingivalis* promotted by nicotine and cotinine

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A**bstract:**

Porphyromonas gingivalis, a Gram-negative oral anaerobic bacterium, is considered an important pathogen for chronic periodontitis. To observe the effect of nicotine and cotinine on *P. gingivalis* adaptative ability, changes in the protein expression were analyzed using a proteomic approach. Proteins of *P. gingivalis* exposed to nicotine (6 and 600 μg/L) and cotinine (6 and 600 μg/L) were extracted, separated by two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D SDS-PAGE) and identified through liquid chromatography-mass spectrometry (LC-MS/MS). Proteins were separated in the first dimension using immobilized pH gradient strips (IPG strip; pH 4-7), and in the second-dimension by a homogeneous 12.5% polyacrylamide SDS-PAGE. The gels were run in triplicate and protein was detected by staining the gels with colloidal Coomassie blue brilliant (CBB) G-250. Results showed that nicotine and cotinine altered the proteome profile of *P. gingivalis.* Of ~430 protein spots reproducibly detected on each gel, 20 were downregulated, 42 upregulated, and 60 exclusively expressed at least in one of the treatments or in control. Differentially-expressed proteins were digested overnight with trypsin at 37ºC, and then the peptide samples were sequenced by means of liquid chromatography coupled with a quadrupole time of flight mass spectrometer (Q-TOF API LC-MS/MS system). MS/MS data were submitted to Mascot database to search for protein identification. The proteins identified were observed to be involved in several biological processes, such as energy production, protein synthesis, oxidative stress, virulence, and molecule transport and binding activities. These results might help understand the effects and mechanisms of nicotine and cotinine on *P. gingivalis* behavior.

1. Introduction

Periodontal diseases are complex, multifactorial, polymicrobial infections characterized by inflammatory conditions that cause destruction of tooth supporting tissues. Furthermore, a possible connection is emerging between chronic periodontitis and serious systemic conditions such as cardiovascular diseases (Beck *et al.*, 1998; Miyakawa *et al.*, 2004; Williams *et al.*, 2008), spontaneous preterm low birth weight (Offenbacher *et al.*, 1998; Marakoglu *et al.*, 2008), rheumatoid arthritis (Mercado *et al.*, 2003; Pischon *et al.*, 2008), diabetes (Pucher and Stewart *et al.*, 2004) and respiratory infections (Scannapieco and Genco *et al.*, 1999). The development of periodontal diseases is a consequence of intricate interactions between bacteria on periodontal sites and the hosts' immune and inflammatory reactions. Among a group of periodontal bacteria associated with the etiology of periodontitis is the anaerobic *Porphyromonas gingivalis*, which is a Gram-negative, non-motile, short rod residing predominantly in the subgingival biofilms (Yilmaz, 2008). This bacterium expresses several virulence factors as proteases, fimbriae, lipopolysaccharide, adhesins, which may cause tissue destruction and host inflammatory and immune responses (Imamura, 2006).

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 (Johnson and Hill, 2004). 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 (Schenkein *et al.*, 1995; Elter *et al.*, 1999; Hafajee and Socransky, 2001). Tobacco smoke contains more than 4000 substances. Nicotine, which is one the most important tobacco substance, has a short blood half life (±2 h), while cotinine is the main nicotine metabolite and has a longer blood half life (±19 h) (Tonetti, 1998).

It is well known that nicotine and cotinine have deleterious effects in the oral cavity of smokers, especially on periodontal tissues. However, very few *in vitro* studies evaluated the effects of nicotine and cotinine on oral bacteria. Although nicotine and cotinine are not able to neither reduce nor increase the *in vitro* viability of *P. gingivalis*

(Cogo *et al.*, 2008), these substances may have some other effects on these bacteria. Sayers *et al.* (1997, 1999) reported that a synergic interaction between *P. gingivalis'* toxins and nicotine or cotinine can occur. The colonization of epithelial cells by *P. gingivalis*, could be also altered in the presence of nicotine or cotinine (Teughels *et al.*, 2005). Clearly, further studies are needed to understand the effects of nicotine and cotinine on *P. gingivalis* metabolism.

We had taken a proteomic approach to analyze the effects of nicotine and cotinine on *P. gingivalis* W83, using two-dimensional gel electrophoresis and LC-MS/MS to characterize the changes in protein levels. This strain was chosen for the study because of the availability of its genomic DNA sequence from the website of The Institute for Genomic Research (TIGR;http:www.tigr.org). In addition, the W83 strain has been described as one of the most virulent and disease-promoting among others in the same species. We observed significant changes in the expression levels of many proteins.

2. Material and methods

2.1. Bacterial culture conditions and treatments

P. gingivalis W83 was gently provided by Dr Max J. Goodson (the Forsyth Institute, Boston, MA, USA). For total protein analyses, *P. gingivalis* W83 was cultured in 120 mL of brain heart infusion supplemented with hemin (5 µg/mL) (Sigma Chemical Co, Poole, UK) and menadione (1 µg/mL) (Sigma), under anaerobic conditions (10% CO_2, 10% H_2 and 80% N_2 - MiniMacs Anaerobic Workstation, Don Whitley Scientific, Shipley, UK), at 37 ^oC. Five groups concerning bacterial growth were considered as follows: 1) nicotine 600 µg/mL (Ni600); 2) nicotine 6 µg/mL (Ni6); 3) cotinine 600 µg/mL (Co600); 4) cotinine 6 µg/mL (Co6); and 5) control (bacterial growth whithout any substance). Both nicotine and cotinine were purchased from Sigma. *P. gingivalis* were grown in the presence of nicotine or cotinine until an optical density of 1.4 at 660 nm was reached (late-logarithmic phase, approximately 18 h of growth). For growth evaluation, *P. gingivalis* was cultured in the same conditions mentioned above. The growth evaluation was performed in triplicate. The optical density obtained in growth assays was analyzed by using ANOVA.

Statistical differences between control and concentration groups were determined by the Dunnet test ($p<0.05$).

2.2. Total protein extraction

The method for extraction of total proteins was adapted from a previously described method (Damerval *et al.*, 1986). Briefly, *P. gingivalis* W83 was centrifuged at 8.000 *g,* 4 ^{o}C, for 16 min and the supernatant was discarded. The proteins of the pellet were precipitated by adding 10% trichloroacetic acid (Sigma) and 0.07% 2-mercaptoethanol in cold-iced acetone (Merck & Co., Inc., Whitehouse Station, N.J. USA) and stored at -20 °C for 1h. After centrifuged at 13.000 *g*, 4 ^{o}C, for 15 min, the protein pellets were rinsed twice (1 h each rinsing at -20 °C) with cold-iced acetone containing 0.07% 2-mercaptoethanol. Precipitated pellets were centrifuged at 16.000 g, 4 ^{o}C, for 20 min. The supernatant was removed and the protein pellet air-dried and solubilized in 1 mL of buffer (7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), and 100 mM dithiothreitol (DTT)). Proteins were quantified using the Bradford method (Bradford, 1976).

2.3. Two-Dimensional Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (2D SDS- PAGE)

Protein samples (750 μg of total protein) were solubilized in 1% ampholytes (IPG buffer pH 4.0 – 7.0), 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, and 100 mM dithiothreitol (DTT) up to a volume of 400 μL and then centrifuged at 8.000 *g* for 8 min. The supernatant obtained was applied onto 4-7 linear immobilized pH gradient strips (18 cm, GE Healthcare, Piscataway, NJ, USA), which were submitted to isoelectric focalization (IEF) using an IPGphor apparatus (GE Amersham Biosciences) at 75 KVh. The strips were rehydrated at 50 mA for 1 h at room temperature. The IEF process was initiated at 30 V, gradually increasing to 8.000 V and kept at this voltage until reaching 75.000 V of accumulated voltage (1 h at 0 V; 1 h at 30 V; 1 h at 200 V; 1 h at 400 V; 1 h at 700 V; 1 h at 1.000 V, 10 h at 5.000 V, and remaining hours at 8000 V). After the IEF, the strips were kept at -80 ºC. Before running the second dimension electrophoresis, strips were kept at

room temperature for 15 min in equilibration buffer (6 M urea, 2% (w/v) sodium dodecyl sulfate (SDS), 50 mM Tris-HCl pH 6.8, 30% (v/v) glycerol, 0.001% (w/v) bromophenol blue) with 2% (w/v) DTT, and then, another 15 min in equilibration buffer with 4% (w/v) iodoacetamide (IAA). The second dimension electrophoresis was performed in vertical gradient using 12.5% polyacrylamide gels at 16 mA for 30 min and at 24 mA until the dye reached the bottom of the gel. Triplicates were performed for each sample. Proteins were stained with Coomassie Brilliant Blue G-250 (CBB - GE Health Care) (Candiano *et al*., 2004). Protein fixation was carried out using a solution containing 40% ethanol and 10% acetic acid for 60 min and washed with water twice (10 min each time). The gels were then kept overnight in a staining solution containing 20% (v/v) methanol, 10% (w/v) ammonium sulfate, 2% (v/v) phosphoric acid and 0.1% (w/v) CBB. These gels were submitted to three washes 10 min each in water and then stored in 1% (v/v) acetic acid for gel image analysis.

2.4. Gel image analysis and spot detection

Gels were scanned (UTA-1100 scanner, Labscan V5.0 software, GE Amersham Biosciences) and submitted to image analysis using the ImageMaster 2D software V4 (GE Amersham Biosciences). Each gel was analyzed separately and the differences between gels were quantified. For each sample analyzed, the average spot volume of the three replicate gels was determined and normalized using the "total spot volume normalization" parameter (individual spot volume/total spots volume x 100). Spot volumes obtained for the control were compared to those observed for the other groups (nicotine and cotinine). ANOVA and Tukey test were used for the statistical analysis at a significance level of $p<0.05$. The statistically different spots were excised from the gels and identified by means of LC-MS/MS.

2.5. *In-gel* protein digestion

The protein digestion was carried out according to a method previously described by Fiorani Celedon *et al.*, 2007. Protein spots that were statistically different and spots exclusively expressed in the treatments or in control were removed from the gels, cut into small cubes (±1 mm) and washed with ultrapure water for 15 min. The gel pieces were

discolored (5 washes) using a solution of 50% (v/v) acetronitrile (ACN) and 50 mM ammonium bicarbonate, until complete removal of the CBB. The two dimensional (2-DE) gel spots were completely dehydrated with 100% (v/v) ACN, rehydrated with 20 mM DTT, and kept at 60 °C for 40 minutes. This solution was then discarded, replaced with 55 mM IAA, and protected from the light for 30 min; the gel pieces were dehydrated with 100% ACN. Right after the gel pieces were totally dried, the ACN was removed and the tubes were air-dried for complete removal of the solvent. The gel pieces were rehydrated with a solution containing 10 ng/μL trypsin (Promega, Madison, WI, USA) and 25 mM ammonium bicarbonate to perform the protein digestion. The tubes were incubated at 37°C for 12 h. After digestion, the peptides were extracted from the gel pieces through two washes using 50 μL of 60% (v/v) ACN and 1% (v/v) formic acid (FA), and then another wash with 50 μL of ACN. The supernatants from the three gel replicates were mixed and vacuum dried. Peptides were then suspended in 12 μL of 1% (v/v) FA for LC-MS/MS analysis.

2.6. Protein identification and analysis by LC-MS/MS

The LC-MS/MS protein identification was carried out according to a method described by Fiorani-Celedon *et al.* (2007). Peptides obtained from the protein digestion were identified by using a Capillary liquid chromatography (Cap-LC) coupled to a Quadrupole Time of Flight Mass Spectrometer (Q-TOF Ultima API mass spectrometer - Waters, Milford, MA, USA). Five microliters of the peptide solution was loaded onto a Nanoease trapping column (0.18 x 23.5 mm - Waters) for pre-concentration, followed by peptide separation in a LC nanoease column Symmetry 300 C18 (3.5 μm, 75 x 100 mm - Waters). Peptides were eluted in a 60-min linear gradient of solvent A (5 % (v/v) ACN, 0.1% (v/v) formic acid in water) and solvent B (95 % (v/v) ACN, 0.1% (v/v) formic acid in water), at a flow rate of 0.25 μL/min. A positive ion mode at a 3 kV needle voltage was used. The mass limit was set from 300 to 2000 m/z, and the MS/MS spectra were obtained for the most intense peaks (≥ 15 counts). Multiply charged precursor ions were selected for fragmentation and automated data dependent acquisition (DDA) was used for peptide sequencing through the MassLynx software (Waters), switching from the MS to MS/MS

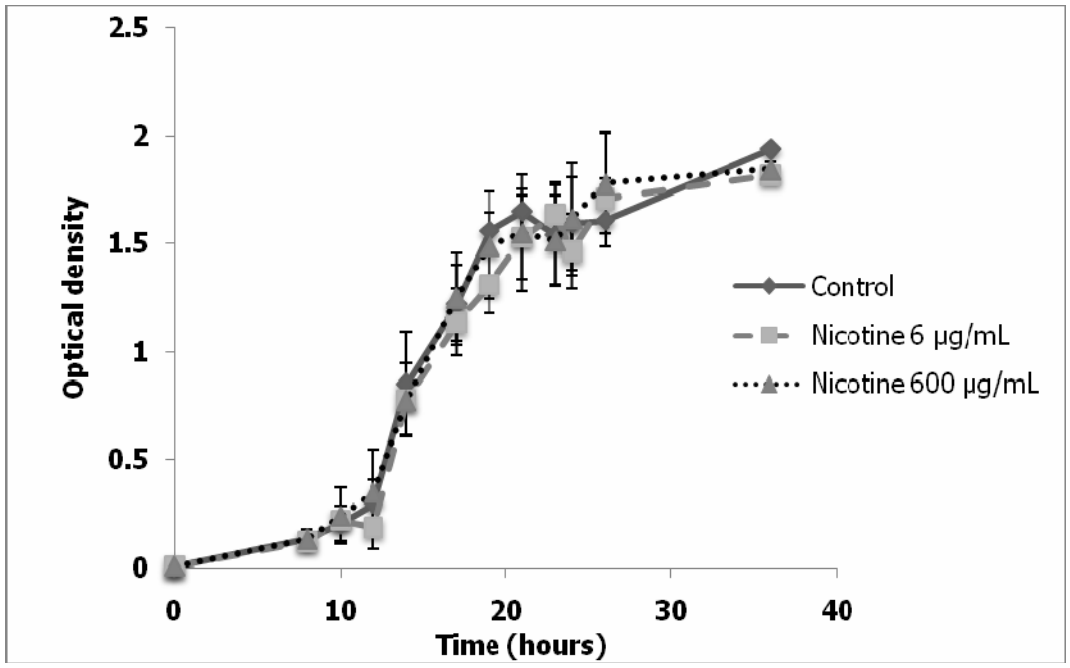
mode and then returning to the MS. The resulting fragmented spectra were processed using the ProteinLynx (V4.0 software, Waters) and the MASCOT MS/MS Ion Search (www.matrixscience.com); the latter was used to compare the sequences by using the MSDB, SwissProt and NCBInr databank. Combined MS-MS/MS searches were conducted with the following parameters: MS/MS mass tolerance at 0.5 Da; trypsin as the enzyme used; peptide tolerance at 100 ppm; carbamidomethylation of cysteine (fixed modification); and methionine oxidation (variable modification). According to the MASCOT probability analysis, only significant (p<0.05) hits were accepted. After protein identification, their gene ontology and cellular role category were verified in Uniprot (www.uniprot.org) and TIGR (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi) data bases.

3. Results and Discussion

3.1. Culture growth

P. gingivalis W83 exposed or not to nicotine and cotinine showed similar growth patterns; such finding is in accordance with previous data (Cogo *et al.,* 2008). Figure 1 shows the bacterial growth (optical density at 660 nm wavelength) for *P. gingivalis*, exposed to different concentrations of each substance. Growth control is also shown in figure 1. No statistically significant difference was observed among control and the 6 and 600 µg/mL nicotine/cotinine groups (ANOVA, Dunnet Test, p>0.05).

A



B

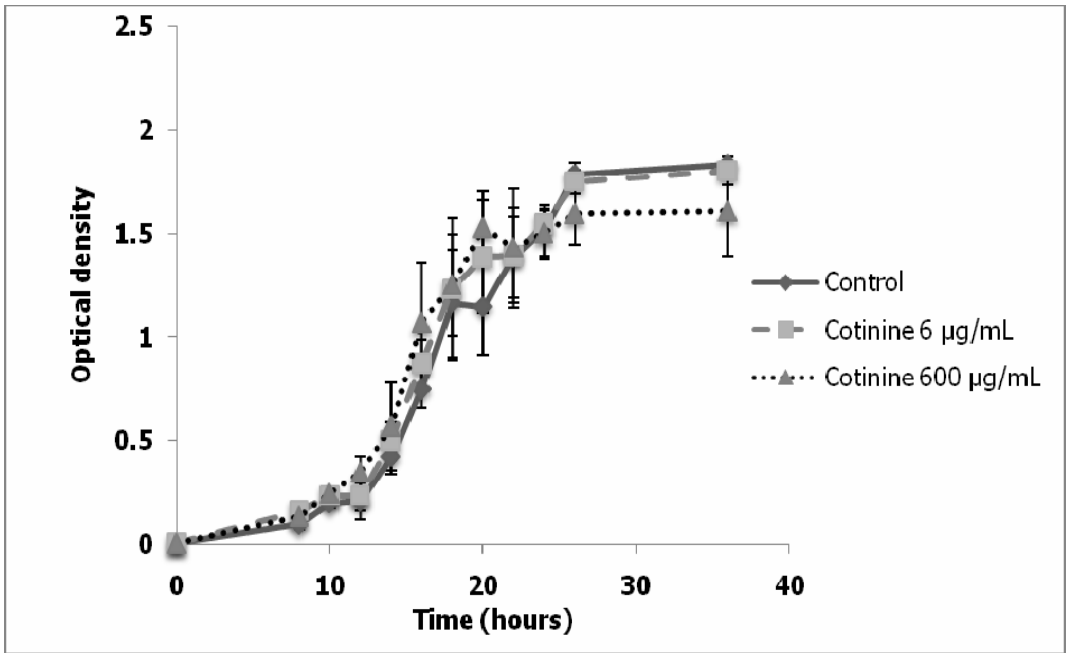


Figure 1. Growth curves of *P. gingivalis* W83 exposed to cotinine (A) and nicotine (B) (O.D. at 660 nm) during 38 hours. *P. gingivalis* not exposed to any substance was used as control.

3.2. Characterization of *P. gingivalis* proteome with 2D SDS-PAGE and protein identification by MS

The protocol for sample preparation and subsequent 2D SDS-PAGE of *P. gingivalis* proteome showed well-resolved spots, with high reproducibility, without striking, as previously observed by Damerval *et al*. (1986) and Fiorani-Celedon *et al*. (2007).

Initial analyses using IPG strips of pH 3–10 and 4–7 showed that most of the protein spots were concentrated within the pH range 4–7 (data not shown). Consequently, all analyses of *P. gingivalis* proteins were carried out within the pH range 4–7.

The comparison among control and experimental groups showed that many spot volumes increased or decreased significantly (ANOVA, Tukey Test $p<0.05$). In addition, some spots were exclusively expressed only in the treatments or in the control. The total number of spots, not expressed spots, exclusively expressed spots, and down- and upregulated spots regarding the treatment groups are shown in table 1.

Table 1. Total number of protein spots, not expressed protein spots, exclusively expressed protein spots, and down- and upregulated protein spots concerning the treatment groups.

Group	Total spots	Protein spots not Expressed[1]	Protein spots Exclusively Expressed[1]	Downregulated Protein Spots[1]	Upregulated Protein Spots[1]
Ni 6 µg/mL	431	25	37	20	26
Ni 600 µg/mL	470	9	28	22	22
Co 6 µg/mL	413	51	44	8	29
Co 600 µg/mL	471	27	43	12	14

[1] number of spots obtained by comparing to that found for the control (443 spots)

The induction factor in response to the treatments for each protein spot is shown in table 2. Table 3 shows the proteins exclusively expressed for treatments and control groups. Although no changes in the growth of *P. gingivalis* in culture (with or without nicotine and cotinine) were found, its protein expression profile was considerably altered. Protein expression changes without altering *P. gingivalis* growth was previously observed when this bacterium was exposed to epithelial cells (Zhang *et al*., 2005).

Figure 2 illustrates protein separation using the IPG strips (pH 4 to 7 – linear strips) and 12.5% polyacrylamide gel SDS-PAGE of *P. gingivalis* protein gel. The image analysis showed the detection of more than 430 protein spots which matched among all gels. This result is in accordance to the one previously found by Nakano *et al*. (2005). These authors observed around 446 protein spots from whole-cell lysates of *P. gingivalis* W83 through image analysis of 2-DE gels, using pH 4.0–7.0 gel strips and SDS-PAGE with 12.5% acrylamide.

In order to compare protein expression, reference maps were generated using proteins extracted from all experimental groups, including control. These reference maps illustrate spot proteins, especially those differentially (fig. 3) or exclusively (fig. 4) expressed.

Only differentially or exclusively expressed proteins (n=118) were identified by MS. Data concerning the proteins with expression levels different from those observed for the control, as well as those regarding the exclusively expressed proteins found for the treatment groups are shown in tables 2 and 3, respectively. Such proteins were grouped according to their molecular functions. Tables 4 - 5 and 6 - 7 show supplementary data for tables 2 and 3 (these tables will not be shown in the published paper version, but they must be sent to the Journal as supplementary data). Identification of all proteins showed a good correlation between theoretical and experimental values for both isoeletric point (pI) and relative molecular mass (Mr).

Most of the proteins identified have role as key enzymes for metabolic functions such as energy production, amino acid biosynthesis, etc. Other proteins responsible for bacterial homeostasis and virulence were also found. A number of isoforms were identified in all treatments.

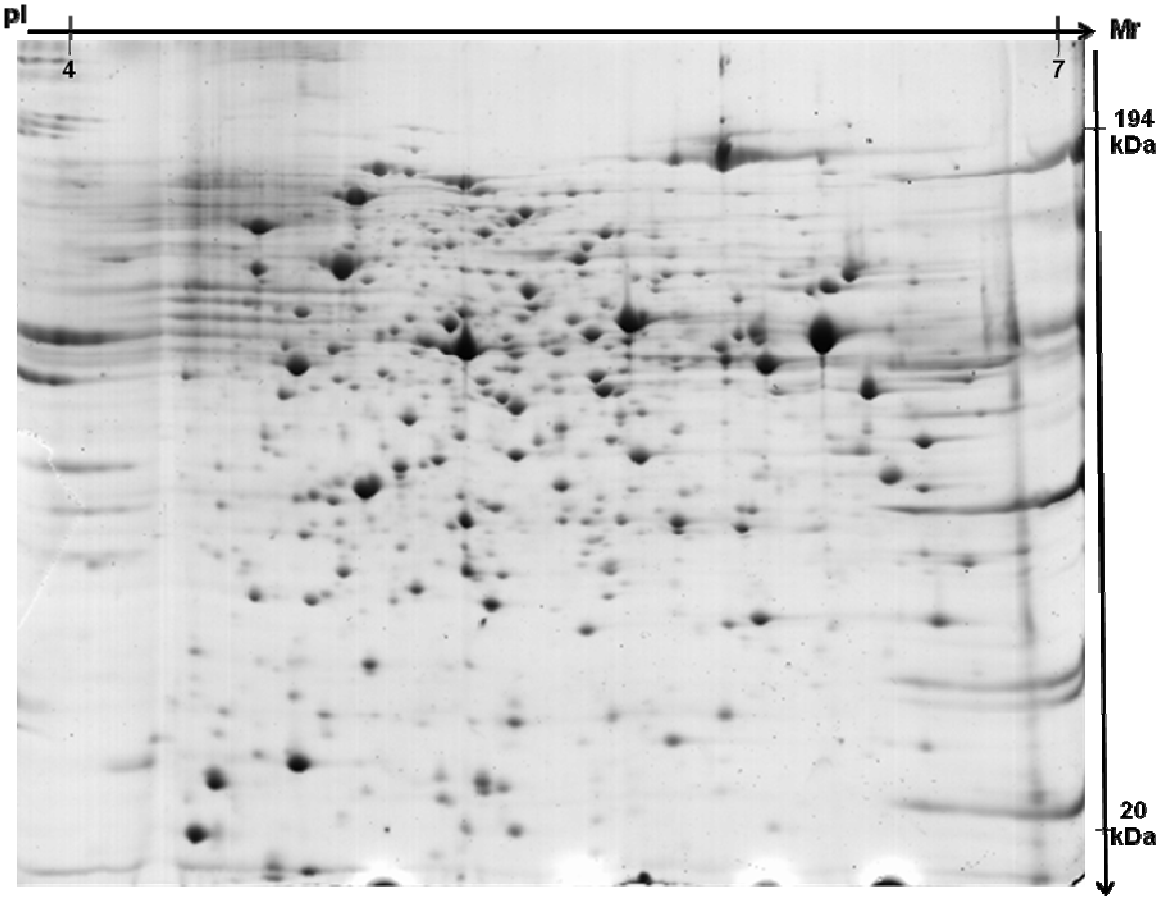


Figure 2. Representative 2-DE gel using the IPG strips pH (4 to 7) and 12.5% polyacrylamide gel SDS-PAGE of *P. gingivalis* protein gel.

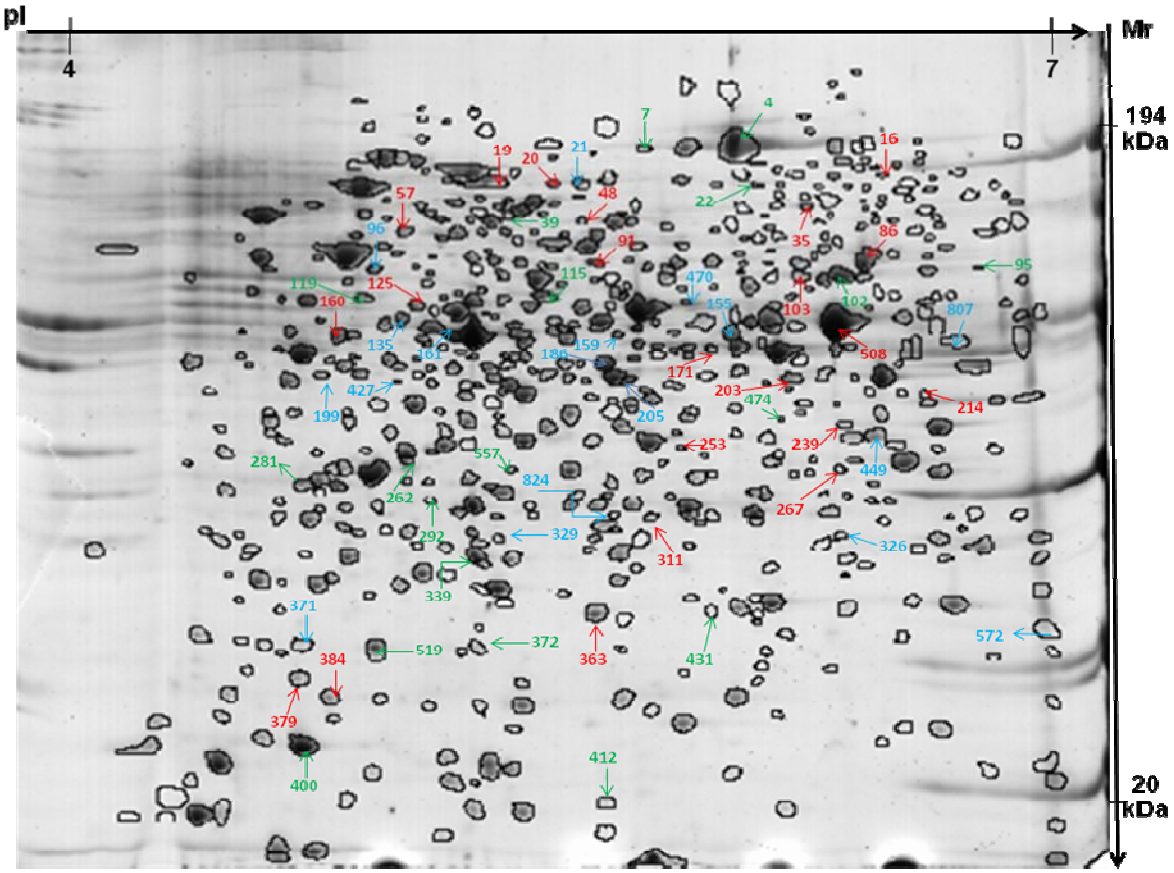


Figure 3. Reference map representing spots differentially expressed in the treatments. Spots are circled and the numbers indicate the respective proteins identified in Tables 2, 4 and 6. Colored numbers represent the treatment that the statistical difference (p<0.05) in spot volume was found: Red – Nicotine; Blue – Cotinine and Green – Both nicotine and cotinine.

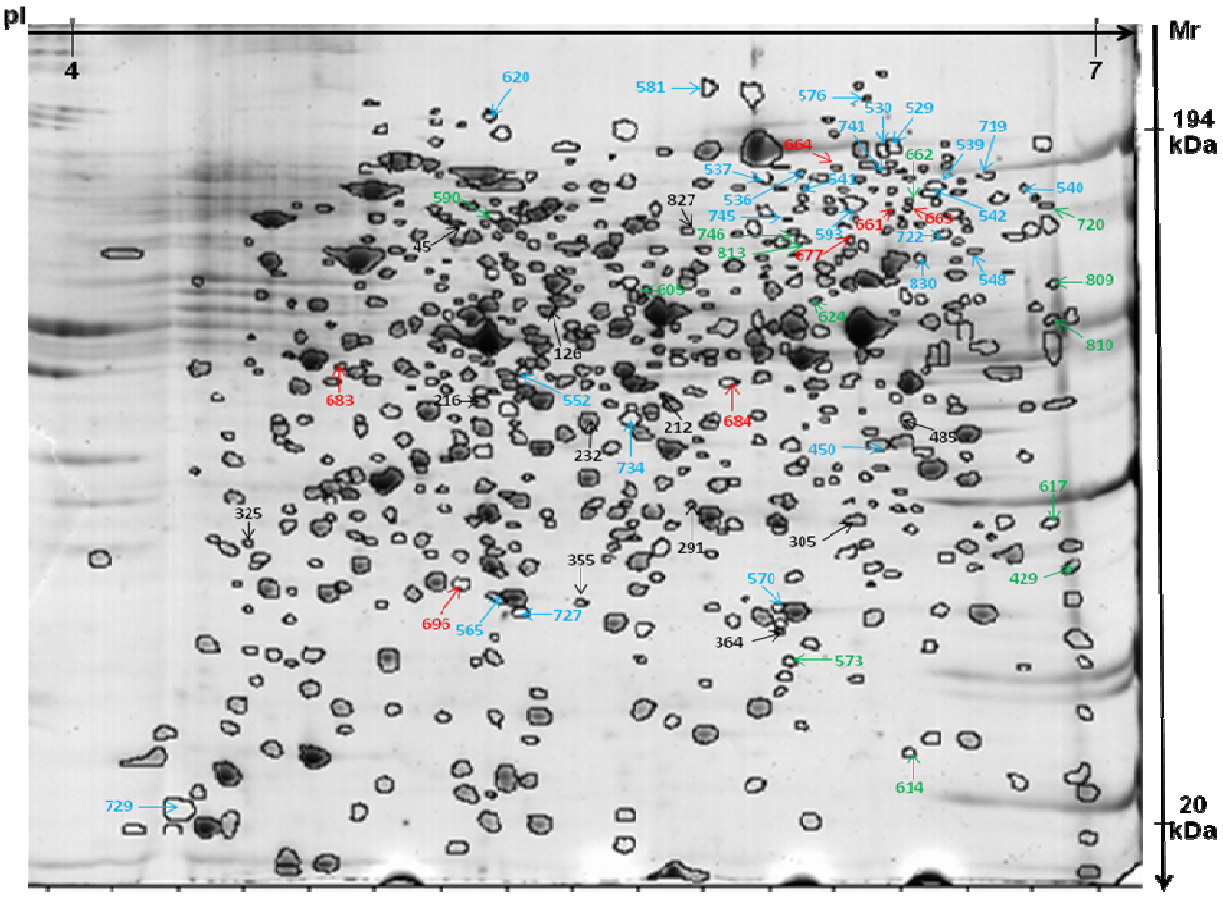


Figure 4. Reference map representing spots exclusively expressed in the treatments or in control. Spots are circled and the numbers indicate the respective proteins identified in Tables 3, 5 and 7. Colored numbers represent the experimental group that exclusively expressed the protein spot: Red – Nicotine; Blue – Cotinine, Green – Both nicotine and cotinine and Black – control.

Table 2. Proteins from *P. gingivalis* differently expressed in the treatments and their corresponding induction factor (volume of spot in treatment/volume of spot in control)[1]

Protein	Spot number	TIGR locus	Induction Factor			
			Co6	Co600	Ni6	Ni600
Cellular Processes						
Cell envelope biosynthesis						
Phosphomannomutase, putative	57	PG 2010	0.85	0.65	0.44[a]	0.52
Oxidative stress						
Dps family protein	384	PG 0090	0.98	0.86	0.74[a]	0.73[a]
Redox-sensitive transcriptional activator OxyR (OxyR)	474	PG 0270	2.50[a]	2.91[a]	2.61[b]	1.75[b]
Rubrerythrin	372	PG 0195	2.23[a]	1.94[a]	2.03[a]	1.79[a]
Thiol peroxidase	371	PG 1729	1.55[b]	0.99	0.74	0.92
Thiol peroxidase	519	PG 1729	1.22[b]	0.99	0.90	0.79[a]
TPR domain protein	125	PG 0449	0.94	0.99	1.08	1.52[b]
TPR domain protein	135	PG 0449	1.48[a]	1.15	0.96	0.89
Transport and binding activity						
RagA protein	4	PG 0185	0.55[a]	0.71[a]	1.47[b]	0.46[b]
RagA protein	7	PG 0185	3.30[b]	0.88	3.33[a]	3.10[a]
Copper homeostasis protein cutC	311	PG 0714	–	0.72	0.94	1.70[b]
Hypothetical tonB-linked outer membrane receptor PG50	20	PG 0707	0.75	1.43	0.96	2.79[b]
Hypothetical tonB-linked outer membrane receptor PG50	21	PG 0707	0.50[a]	1.06	0.92	1.05
Virulence and acquisition of peptides						
Dipeptidyl aminopeptidase IV, putative	35	PG 1361	1.10	1.07	2.04[b]	1.20
Hypothetical protein	16	PG 2029	–	–	4.82[b]	2.26
Peptidase, M20/M25/M40 family	115	PG 0561	0.47[b]	0.75	0.82	0.52[b]
Peptidase, M24 family	262	PG 1210	–	0.54[a]	0.56[a]	0.55[a]
Information storage and processing						
DNA replication, recombination and repair						

Hypothetical protein	160	PG 1591	1.12	0.79	0.92	0.54[b]
Protein synthesis and folding						
Elongation factor Ts	292	PG 0378	2.16[a]	1.46[a]	1.03	2.89[b]
Ribosome recycling factor (Ribosome-releasing factor) (RRF)	363	PG 1901	0.93	0.80	0.41[b]	0.73[b]
Translation elongation factor G, putative	19	PG 0933	-	1.28	2.09[b]	0.94
Tyrosyl-tRNA synthetase	159	PG 1824	1.94[b]	1.18	1.14	1.12
Transcription						
Transcription termination factor Rho	22	PG 0332	-	2.32[a]	2.21[a]	2.23[a]
Metabolism						
Amino acid biosynthesis						
3-dehydroquinate dehydratase, type II	412	PG 1731	-	0.62[a]	-	0.72[a]
Phosphoserine aminotransferase	203	PG 1278	0.92	0.73	1.03	1.47[b]
Serine hydroxymethyltransferase	807	PG 0042	1.37[a]	2.9[a]	-	-
Central intermediary metabolism						
Acetyl-CoA hydrolase/transferase family protein	95	PG 1013	2.72[a]	2.84[a]	2.14[a]	1.9[a]
Coenzyme, prosthetic groups and carriers biosynthesis						
ATP:cob(I)alamin adenosyltransferase, putative	431	PG 1124	0.971	1.43[b]	0.74	1.66[b]
Oxidoreductase, putative	214	PG 0430	1.25	1.10	2.52[a]	1.28
Pyridoxal phosphate biosynthetic protein PdxJ	824	PG 0630	2.52[b]	1.04	1.22	1.39
Riboflavin synthase, alpha subunit	339	PG 0733	0.50[b]	0.88	0.57[a]	0.71[a]
Energy production: Amino acids and amines						
2-amino-3-ketobutyrate CoA ligase	155	PG 0481	2.50[b]	1.56[b]	1.22	0.94
Delta-1-pyrroline-5-carboxylate dehydrogenase	86	PG 1269	0.72	1.21	2.1[b]	1.18
D-lysine 5,6-aminomutase, alpha subunit	96	PG 1073	1.75[b]	0.98	0.96	0.85

Formiminotransferase-cyclodeaminase-related protein	557	PG 0329	1.86[b]	1.17	3.13[a]	3.77[a]
Glutamate dehydrogenase	508	PG 1232	0.94	0.99	1.46[b]	1.20
L-asparaginase	199	PG 2121	0.69[a]	0.64[a]	0.87	0.89
Methionine gamma-lyase	186	PG 0343	1.45[b]	1.03	0.96	0.94
Energy production: Electron transport						
Electron transfer flavoprotein, beta subunit	267	PG 0777	1.45	1.03	0.95	1.54[b]
Electron transport complex, RnfABCDGE type, G subunit	281	PG 0306	0.64[a]	0.63[a]	0.64[a]	0.66[a]
Putative NADPH-NAD transhydrogenase	171	-	1.19	0.77	1.05	1.7[b]
Pyruvate synthase	48	PG 0429	-	0.83	0.91	1.57[b]
Energy production: Fermentation						
4-hydroxybutyrate CoA-transferase	470	PG 0690	1.19	1.92[a]	1.83	1.28
4-hydroxybutyryl-CoA dehydratase	102	PG 0692	1.50	1.70[a]	2.33[a]	1.45[a]
4-hydroxybutyryl-CoA dehydratase	103	PG 0692	1.02	1.06	1.20	1.63[a]
4-hydroxybutyryl-CoA dehydratase	383	PG 0692	-	-	-	0.50
NAD-dependent 4-hydroxybutyrate dehydrogenase	205	PG 0689	1.50[b]	0.98	1.02	1.17
Energy production: Glycolysis/gluconeogenesis						
Malate dehydrogenase	239	PG 1949	-	1.08	1.09	1.45[a]
Phosphopyruvate hydratase	572	PG 1824	1.31	0.56[b]	1.30	0.98
Phosphopyruvate hydratase	161	PG 1824	1.30[a]	1.05	1.15	1.11
Energy production: Pentose phosphate pathway						
Ribulose-phosphate 3-epimerase	329	PG 1595	1.53[b]	1.12	0.77	1.07
Fatty acid and phospholid catabolism						
Acyl-CoA dehydrogenase, short-chain specific	449	PG 1076	0.40[a]	-	-	0.82
Nucleotide biosynthesis, transport and catabolism						

Dihydroorotate dehydrogenase	253	PG 1065	2.33[b]	1.02	1.42	0.93
Phosphoribosylaminoimidazolecarboxamide formyltransferase	91	PG 1397	0.63	0.75	0.63[a]	0.72[a]
Unknown function						
Conserved domain protein	326	PG 0645	1.80[b]	1.03	1.01	0.88
GTP-binding protein TypA	39	PG 0615	0.53[a]	0.7[a]	0.76[a]	0.90
Immunoreactive 42 kDa antigen PG33	427	PG 0694	5.32[b]	1.18	0.62	1.15
Immunoreactive 53 kD antigen PG123	119	PG 2167	0.50	0.29[a]	0.32[a]	0.66
POYPRTH NID	400	-	1.36[a]	0.90	0.18[b]	0.75[b]
ThiJ/PfpI family protein	379	PG 0634	1.19	0.88	0.66[b]	0.99

1 – ([1]) According to Mascot probability analysis ($p < 0.05$), only significant hits were accepted;
2 – ([a]) Differences statically significant between one treatment and control; ([b]) Differences statically significant among one treatment, the other concentration of the same treatment and control. Spots were concluded to be significantly up- or down-regulated when $p< 0.05$ (ANOVA - Tukey test).

Table 3. Proteins from *P. gingivalis* exclusively expressed in the treatments or in control[1]

Protein	Spot number	TIGR locus	co6	co600	Ni6	Ni600	Control
Cellular Processes							
Cell envelope biosynthesis							
DTDP-4-dehydrorhamnose 3,5-epimerase	325	PG 1562	-	-	-	-	exclusive
Phosphomannomutase	605	PG 1094	-	exclusive[2]	exclusive	-	-
Oxidative stress/Phosphate starvation							
Alkyl hydroperoxide reductase, C subunit	727	PG 0618	exclusive	-	-	-	-
Dps family protein	683	PG 0090	-	-	-	exclusive	-
Fe superoxide dismutase	570a	PG 1545	exclusive	-	-	-	-
PhoH family protein	126	PG 1323	-	-	-	-	exclusive
Rubrerythrin (RR)	565	PG 0195	exclusive	-	-	-	-
TPR domain protein	664	PG 0449	-	-	exclusive	-	-
Transport and binding activity							

RagA protein	581	PG 0185	-	exclusive	-	-	-
Virulence and acquisition of peptides							
Aminopeptidase C	810	PG 1605	-	exclusive	exclusive	-	-
Arg-gingipain-1 proteinase	590	P28784	-	exclusive	-	-	-
Lysine-specific cysteine proteinase	827	-	-	-	-	-	exclusive
Outer membrane protein 40 precursor	540	PG 0694	exclusive	-	-	-	-
Uracil phosphoribosyltransferase	291	PG 0752	-	-	-	-	exclusive
Information storage and processing							
DNA replication, recombination and repair							
Holliday junction ATP-dependent DNA helicase ruvA	355	PG 0811	-	-	-	-	exclusive
Hypothetical protein	661	PG 1591	-	-	exclusive	-	-
Single-stranded binding protein	364	PG 0271	-	-	-	-	exclusive
Single-stranded binding protein	573	PG 0271	exclusive	-	exclusive	-	-
Protein synthesis and folding							
50S ribosomal protein L25/general stress protein Ctc	429	PG 0167	-	exclusive	exclusive	exclusive	-
50S ribosomal protein L7/L12	729	PG 0393	exclusive	-	-	-	-
50S ribosomal protein L9	696	PG 0597	-	-	-	exclusive	-
Heme uptake protein A and B (Ribosomal protein S1)	45	PG 1297	-	-	-	-	exclusive
Peptidyl-prolyl cis-trans isomerase	617	PG 1226	-	exclusive	exclusive	exclusive	-
Transcription							
Metabolism							
Amino acid biosynthesis							
Phosphoserine aminotransferase	662	PG 1278	exclusive	-	exclusive	-	-
Phosphoserine aminotransferase	830	PG 1278	-	exclusive	-	-	-
Phosphoserine aminotransferase	684	PG 1278	-	-	-	exclusive	-

Central intermediary metabolism							
Alkaline phosphatase, putative	746	PG 0890	-	exclusive	exclusive	-	-
Coenzyme, prosthetic groups and carriers biosynthesis							
Aminotransferase, putative	536	PG 1327	exclusive	-	-	-	-
Aminotransferase, putative	745	PG 1327	-	exclusive	-	-	-
D-isomer specific 2-hydroxyacid dehydrogenase family protein	722	PG 1279	exclusive	-	-	-	-
Pantoate--beta-alanine ligase	216	PG 0477	-	-	-	-	exclusive
Riboflavin synthase, alpha subunit	663	PG 0733	-	-	exclusive	-	-
Riboflavin synthase, alpha subunit	663	PG 0733	-	-	exclusive	-	-
Energy production: Amino acids and amines							
Delta-1-pyrroline-5-carboxylate dehydrogenase	529	PG 1269	exclusive	-	-	-	-
Glutamate dehydrogenase	593	PG 1232	-	exclusive	-	-	-
Methionine gamma-lyase	576	PG 0343	exclusive	exclusive	-	-	-
Energy production: Electron transport							
Electron transfer flavoprotein, alpha subunit	305	PG 1078	-	-	-	-	exclusive
Electron transfer flavoprotein, alpha subunit	541	PG 1078	exclusive	-	-	-	-
Electron transfer flavoprotein, alpha subunit	719	PG 1078	exclusive	-	-	-	-
Putative NADPH-NAD transhydrogenase	677	not found	-	-	exclusive	exclusive	-
Energy production: Fermentation							
2-oxoglutarate oxidoreductase, alpha subunit	232	PG 1812	-	-	-	-	exclusive
2-oxoglutarate oxidoreductase, alpha subunit	537	PG 1812	exclusive	-	-	-	-
2-oxoglutarate oxidoreductase, gamma subunit	548	PG 1809	exclusive	exclusive	-	-	-
4-hydroxybutyrate CoA-transferase	624	PG 1956	-	exclusive	exclusive	exclusive	-
4-hydroxybutyryl-CoA dehydratase	620	PG 0692	-	exclusive	-	-	-
Fumarate hydratase class I, anaerobic	813	PG 1417	-	exclusive	exclusive	-	-

Energy production: Glycolysis/gluconeogenesis							
Glyceraldehyde 3-phosphate dehydrogenase, type I	720	PG 2124	exclusive	-	exclusive	-	-
Glyceraldehyde 3-phosphate dehydrogenase, type I	809	PG 2124	-	exclusive	exclusive	-	-
Malate dehydrogenase	450	PG 1949	exclusive	-	-	-	-
Phosphoenolpyruvate carboxykinase	741	PG 1676	-	exclusive	-	-	-
Phosphoenolpyruvate carboxykinase	530	PG 1676	exclusive	-	-	-	-
Energy production: Pentose phosphate pathway							
Aldose 1-epimerase	552	PG 1632	exclusive	-	-	-	-
Fatty acid and phospholid catabolism							
Acyl-CoA dehydrogenase, short-chain specific	539	PG 1076	exclusive	-	-	-	-
Nucleotide biosynthesis, transport and catabolism							
Aspartate carbamoyltransferase catalytic subunit	212	PG 0357	-	-	-	-	exclusive
Dihydroorotate dehydrogenase family protein	734	PG 2055	exclusive	-	-	-	-
Ribose-phosphate pyrophosphokinase	485	PG 2097	-	-	-	-	exclusive
Unknown function							
Immunoreactive 42 kDa antigen PG33	542	PG 0694	exclusive	-	-	-	-
Thioesterase family protein	614	PG 1174	exclusive	exclusive	-	exclusive	-

1 - According to Mascot probability analysis ($p < 0.05$), only significant hits were accepted;
2 – Exclusive – spots exclusively expressed in the treatment or in control gels.

Supplementary table (4). Proteins from *P. gingivalis* differently expressed in the treatments

Protein	Spot number	TIGR locus	Accession number	Score [1]	M_r [2]	pI [3]	Coverage [4]	Peptides matched [5]
Cellular Processes								
Cell envelope biosynthesis								
Phosphomannomutase, putative	57	PG 2010	Q7MTF4 PORGI	217	61587	5.23	14	7
Oxidative stress								
Dps family protein	384	PG 0090	Q7MXS1 PORGI	571	17910	4.96	79	40
Redox-sensitive transcriptional activator OxyR (OxyR)	474	PG 0270	Q7MXD3 PORGI	55	35408	5.88	14	4
Rubrerythrin	372	PG 0195	RUBY PORGI	293	22670	5.83	28	15
Thiol peroxidase	371	PG 1729	Q7MU37 PORGI	112	19461	5.26	33	6
Thiol peroxidase	519	PG 1729	Q7MU37 PORGI	335	19461	5.26	76	26
TPR domain protein	125	PG 0449	Q7MWY0 PORGI	858	52557	7.92	52	52
TPR domain protein	135	PG 0449	Q7MWY0 PORGI	356	52557	7.92	32	14
Transport and binding activity								
RagA protein	4	PG 0185	Q7MXJ7 PORGI	774	112362	6.07	22	37
RagA protein	7	PG 0185	Q7MXJ7 PORGI	851	112362	6.07	28	40
Copper homeostasis protein cutC	311	PG 0714	CUTC PORGI	73	27057	5.73	8	2
Hypothetical tonB-linked outer membrane receptor PG50	20	PG 0707	Q9KIB4 PORGI	719	94510	5.58	29	38
Hypothetical tonB-linked outer membrane receptor PG50	21	PG 0707	Q9KIB4 PORGI	93	94510	5.58	4	4
Virulence and acquisition of peptides								
Dipeptidyl aminopeptidase IV, putative	35	PG 1361	Q7MUW6 PORGI	992	82670	6.16	45	63
Hypothetical protein	16	PG 2029	Q7MTD8 PORGI	137	98046	8.36	10	8
Peptidase, M20/M25/M40 family	115	PG 0561	Q7MWN9 PORGI	259	50677	5.42	20	10
Peptidase, M24 family	262	PG 1210	Q7MV80 PORGI	242	66825	5.56	23	14

Information storage and processing								
DNA replication, recombination and repair								
Hypothetical protein	160	PG 1591	Q7MUD8 PORGI	809	42720	5.02	47	49
Protein synthesis and folding								
Elongation factor Ts	292	PG 0378	gi34540207	637	30282	5.31	59	22
Ribosome recycling factor (Ribosome-releasing factor) (RRF)	363	PG 1901	RRF PORGI	263	20773	5.61	20	11
Translation elongation factor G, putative	19	PG 0933	Q7MVV0 PORGI	636	80533	5.33	31	20
Tyrosyl-tRNA synthetase	159	PG 1824	gi34540106	416	48197	5.65	36	15
Transcription								
Transcription termination factor Rho	22	PG 0332	Q7MX79 PORGI	665	72309	5.89	35	54
Metabolism								
Amino acid biosynthesis								
3-dehydroquinate dehydratase, type II	412	PG 1731	Q7MU35PORGI	130	15665	5.55	26	10
Phosphoserine aminotransferase	203	PG 1278		884	40320	6.13	57	40
Serine hydroxymethyltransferase	807	PG 0042	gi34539916	505	46815	6.33	20	20
Central intermediary metabolism								
Acetyl-CoA hydrolase/transferase family protein	95	PG 1013	Q7MVN7 PORGI	62	54983	6.18	11	8
Coenzyme, prosthetic groups and carriers biosynthesis								
ATP:cob(I)alamin adenosyltransferase, putative	431	PG 1124	Q7MVE3 PORGI	283	21419	5.63	45	10
Oxidoreductase, putative	214	PG 0430	Q7MWZ8 PORGI	52	37409	6.27	16	4
Pyridoxal phosphate biosynthetic protein PdxJ	824	PG 0630	gi34540436	77	27047	5.58	26	4
Riboflavin synthase, alpha subunit	339	PG 0733	Q7MW99 PORGI	624	22636	5.30	75	34
Energy production: Amino acids and amines								
2-amino-3-ketobutyrate CoA ligase	155	PG 0481	Q7MWV5 PORGI	354	44490	5.70	19	7

Delta-1-pyrroline-5-carboxylate dehydrogenase	86	PG 1269	Q7MV36 PORGI	807	60211	6.20	40	53
D-lysine 5,6-aminomutase, alpha subunit	96	PG 1073	Q7MVI8 PORGI	339	57602	5.06	17	10
Formiminotransferase-cyclodeaminase-related protein	557	PG 0329	Q7MX81 PORGI	89	33345	5.50	20	6
Glutamate dehydrogenase	508	PG 1232	gi34540940	548	49623	5.98	30	20
L-asparaginase	199	PG 2121	gi34541699	241	39971	5.01	20	6
Methionine gamma-lyase	186	PG 0343	Q7MX71 PORGI	871	43060	5.54	50	31
Energy production: Electron transport								
Electron transfer flavoprotein, beta subunit	267	PG 0777	Q7MW68 PORGI	249	31061	6.25	16	8
Electron transport complex, RnfABCDGE type, G subunit	281	PG 0306	Q7MXA3 PORGI	309	23136	4.77	50	16
Putative NADPH-NAD transhydrogenase	171	not found	gi188994984	801	41501	5.89	65	46
Pyruvate synthase	48	PG 0429	Q7MWZ9 PORGI	756	67846	5.6	32	23
Energy production: Fermentation								
4-hydroxybutyrate CoA-transferase	470	PG 0690	Q7MTJ6 PORGI	55	47725	5.64	9	3
4-hydroxybutyryl-CoA dehydratase	102	PG 0692	Q7MWD1 PORGI	640	54513	6.00	54	44
4-hydroxybutyryl-CoA dehydratase	103	PG 0692	Q7MWD1 PORGI	756	54513	6.00	54	35
4-hydroxybutyryl-CoA dehydratase	383	PG 0692	Q7MWD1 PORGI	96	54513	6.00	13	5
NAD-dependent 4-hydroxybutyrate dehydrogenase	205	PG 0689	Q7MWD4 PORGI	169	42431	5.60	23	9
Energy production: Glycolysis/gluconeogenesis								
Malate dehydrogenase	239	PG 1949	Q7MTK2 PORGI	199	36449	6.34	19	8
Phosphopyruvate hydratase	572	PG 1824	gi 34541315	229	46248	5.25	33	13
Phosphopyruvate hydratase	161	PG 1824	gi 34541315	1082	46248	5.25	60	36
Energy production: Pentose phosphate pathway								
Ribulose-phosphate 3-epimerase	329	PG 1595	Q7MUA4 PORGI	105	24080	5.34	29	5
Fatty acid and phospholid catabolism								
Acyl-CoA dehydrogenase, short-chain specific	449	PG 1076	Q7MVI5 PORGI	243	42283	6.21	26	9

Nucleotide biosynthesis, transport and catabolism								
Dihydroorotate dehydrogenase	253	PG 1065	Q7MVJ6 PORGI	86	33327	5.69	11	3
Phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	91	PG 1397	Q7MUT5 PORGI	760	56166	5.61	33	45
Unknown function								
Conserved domain protein	326	PG 0645	Q7MWH2 PORGI	68	39270	6.52	9	3
GTP-binding protein TypA	39	PG 0615	Q7MWJ5 PORGI	66	67609	5.35	10	4
Immunoreactive 42 kDa antigen PG33	427	PG 0694	gi34540489	229	42596	7.68	22	8
Immunoreactive 53 kD antigen PG123	119	PG 2167	Q9X6S8 PORGI	69	53605	9.00	11	5
POYPRTH NID	400	not found	AAA51298	257	111251	8.58	10	10
ThiJ/PfpI family protein	379	PG 0634	Q7MWH8 PORGI	227	19346	4.93	36	6

1- Score given tot the results obtained from Mascot Search ;
2- Mr- Relative mass of protein in Da;
3- pI – isoeletric point of the protein;
4- Coverage of peptides sequenced;
5- Number of peptides matches for MS/MS.

Supplementary table (5). Proteins from *P. gingivalis* exclusively expressed in the treatments or in control

Protein	Spot number	TIGR locus	Accession number	Score [1]	M_r [2]	pI [3]	Coverage [4]	Peptides matched [5]
Cellular Processes								
Cell envelope biosynthesis								
DTDP-4-dehydrorhamnose 3,5-epimerase	325	PG 1562	Q7MUG1 PORGI	97	22798	4.8	17	4
Phosphomannomutase	605	PG 1094	Q7MVH0 PORGI	47	50236	5.62	6	2
Oxidative stress/ Phosphate starvation								
Alkyl hydroperoxide reductase, C subunit	727	PG 0618	Q7MWJ2 PORGI	348	21292	5.37	66	13
Dps family protein	683	PG 0090	Q7MXS1 PORGI	161	17910	4.96	52	11
Fe superoxide dismutase	570a	PG 1545	gi227251	220	21560	5.80	46	9

PhoH family protein	126	PG 1323	Q7MUZ7 PORGI	729	49904	5.37	36	14
Rubrerythrin (RR)	565	PG 0195	gi27734543	224	22670	5.83	23	7
TPR domain protein	664	PG 0449	gi34540270	52	52557	7.92	2	1
Transport and binding activity								
RagA protein	581	PG 0185	Q7MXJ7 PORGI	110	112362	6.07	9	9
Virulence and acquisition of peptides								
Aminopeptidase C	810	PG 1605	Q7MUC5 PORGI	180	51265	7.03	16	9
Arg-gingipain-1 proteinase	590	P28784	gi557068	57	186575	4.99	3	4
Lysine-specific cysteine proteinase	827		O07442 PORGI	57	188823	5.03	1	2
Outer membrane protein 40 precursor	540	PG 0694	OMP40 PORGI	37	42596	7.68	6	3
Uracil phosphoribosyltransferase	291	PG 0752	gi34540541	51	24470	5.74	15	4
Information storage and processing								
DNA replication, recombination and repair								
Holliday junction ATP-dependent DNA helicase ruvA	355	PG 0811	RUVA PORGI	44	21501	5.53	17	2
Hypothetical protein	661	PG 1591	Q7MUD8 PORGI	80	42720	5.02	10	5
Single-stranded binding protein	364	PG 0271	Q7MXD2 PORGI	60	17523	5.95	57	6
Single-stranded binding protein	573	PG 0271	Q7MXD2_PORGI	173	17523	5.95	48	9
Protein synthesis and folding								
50S ribosomal protein L25/general stress protein Ctc	429	PG 0167	gi34540029	159	21041	9.06	34	6
50S ribosomal protein L7/L12	729	PG 0393	gi34540220	184	12736	4.71	25	3
50S ribosomal protein L9	696	PG 0597	gi34540405	79	19450	5.31	17	3
Heme uptake protein A and B (Ribosomal protein S1)	45	PG 1297	Q9X6Q9 PORGI	160	67481	5.26	12	7
Peptidyl-prolyl cis-trans isomerase	617	PG 1226	Q7MV65PORGI	103	26868	8.90	8	3
Transcription								

Metabolism								
Amino acid biosynthesis								
Phosphoserine aminotransferase	662	PG 1278	gi34540980	507	40320	6.13	54	20
Phosphoserine aminotransferase	830	PG 1278		368	40320	6.13	47	17
Phosphoserine aminotransferase	684	PG 1278	gi34540980	327	40320	6.13	46	21
Central intermediary metabolism								
Alkaline phosphatase, putative	746	PG 0890	Q7MVY1PORGI	72	63069	6.48	14	8
Coenzyme, prosthetic groups and carriers biosynthesis								
Aminotransferase, putative	536	PG 1327	Q7MUZ3PORGI	99	49772	5.92	6	3
Aminotransferase, putative	745	PG 1327	gi34541019	55	49772	5.92	4	2
D-isomer specific 2-hydroxyacid dehydrogenase family protein	722	PG 1279	Q7MV29 PORGI	62	33508	6.25	16	4
Pantoate--beta-alanine ligase	216	PG 0477	Q7MWV8 PORGI	279	31459	5.3	29	11
Riboflavin synthase, alpha subunit	663	PG 0733	Q7MW99 PORGI	230	22636	5.30	38	8
Riboflavin synthase, alpha subunit	663	PG 0733	Q7MW99 PORGI	230	22636	5.30	38	8
Energy production: Amino acids and amines								
Delta-1-pyrroline-5-carboxylate dehydrogenase	529	PG 1269	Q7MV36 PORGI	110	60211	6.20	13	6
Glutamate dehydrogenase	593	PG 1232	gi34540940	714	49623	5.98	44	2
Methionine gamma-lyase	576	PG 0343	Q7MX71 PORGI	302	43060	5.54	35	13
Energy production: Electron transport								
Electron transfer flavoprotein, alpha subunit	305	PG 1078	Q7MVI3 PORGI	359	36894	6.35	17	8
Electron transfer flavoprotein, alpha subunit	541	PG 1078	Q7MVI3 PORGI	371	36894	6.35	33	10
Electron transfer flavoprotein, alpha subunit	719	PG 1078	Q7MVI3 PORGI	88	36894	6.35	24	6
Putative NADPH-NAD transhydrogenase	677	not found	gi188994984	220	41501	5.89	22	8
Energy production: Fermentation								

2-oxoglutarate oxidoreductase, alpha subunit	232	PG 1812	Q7MTW9 PORGI	303	39005	5.95	25	17
2-oxoglutarate oxidoreductase, alpha subunit	537	PG 1812	Q7MTW9 PORGI	141	39000	5.95	28	8
2-oxoglutarate oxidoreductase, gamma subunit	548	PG 1809	gi34541429	75	20101	6.32	23	4
4-hydroxybutyrate CoA-transferase	624	PG 1956	Q7MTJ6 PORGI	45	48243	5.95	5	2
4-hydroxybutyryl-CoA dehydratase	620	PG 0692	Q7MWD1 PORGI	312	54513	6.00	21	9
Fumarate hydratase class I, anaerobic	813	PG 1417	Q7MUS2 PORGI	61099	61	5.97	5	3
Energy production: Glycolysis/gluconeogenesis								
Glyceraldehyde 3-phosphate dehydrogenase, type I	720	PG 2124	Q7MT61 PORGI	92	36278	6.84	16	5
Glyceraldehyde 3-phosphate dehydrogenase, type I	809	PG 2124	Q7MT61PORGI	163	36278	6.84	24	9
Malate dehydrogenase	450	PG 1949	Q7MTK2 PORGI	108	36449	6.34	6	6
Phosphoenolpyruvate carboxykinase	741	PG 1676	gi 34541315	392	59583	6.10	33	14
Phosphoenolpyruvate carboxykinase	530	PG 1676	gi 34541315	616	59583	6.10	42	24
Energy production: Pentose phosphate pathway								
Aldose 1-epimerase	552	PG 1632	Q7MUA4 PORGI	217	39291	5.28	29	12
Fatty acid and phospholid catabolism								
Acyl-CoA dehydrogenase, short-chain specific	539	PG 1076	Q7MVI5 PORGI	48	42283	6.21	10	3
Nucleotide biosynthesis, transport and catabolism								
Aspartate carbamoyltransferase catalytic subunit	212	PG 0357	gi34540189	117	34923	5.65	15	3
Dihydroorotate dehydrogenase family protein	734	PG 2055	Q7MTB7 PORGI	203	35967	5.54	33	10
Ribose-phosphate pyrophosphokinase	485	PG 2097	gi34541678	409	35100	6.15	38	20
Unknown function								
Immunoreactive 42 kDa antigen PG33	542	PG 0694	gi34540489	105	42596	7.68	18	6
Thioesterase family protein	614	PG 1174	Q7MVA3 PORGI	93	18462	6.04	13	2

1- Score given tot the results obtained from Mascot Search ;

2- Mr- Relative mass of protein in Da;
3- pI – isoeletric point of the protein;
4- Coverage of peptides sequenced;
5- Number of peptides matches for MS/MS.

Supplementary table (6). Mean normalized volume and standard deviation of proteins spots from *P. gingivalis* differently expressed in the treatments.

Protein	Spot number	Co6		Co600		Ni6		Ni600		Control	
		Vol	SD	Vol	SD	Vol	SD	Vol	SD	Vol	SD
Cellular Processes											
Cell envelope biosynthesis											
Phosphomannomutase, putative	57	0.108	0.028	0.082	0.001	0.056[a]	0.007	0.066	0.009	0.127	0.026
Oxidative stress											
Dps family protein	384	0.237	0.020	0.207	0.042	0.180[a]	0.025	0.176[a]	0.027	0.242	0.008
Redox-sensitive transcriptional activator OxyR (OxyR)	474	0.032[a]	0.004	0.037[a]	0.009	0.034[b]	0.006	0.023[b]	0.002	0.013	0.003
Rubrerythrin	372	0.278[a]	0.043	0.243[a]	0.003	0.254[a]	0.051	0.224[a]	0.070	0.125	0.013
Thiol peroxidase	371	0.253[b]	0.051	0.162	0.030	0.121	0.014	0.150	0.007	0.163	0.008
Thiol peroxidase	519	0.610[b]	0.067	0.494	0.020	0.450	0.046	0.395[a]	0.010	0.500	0.006
TPR domain protein	125	0.183	0.018	0.194	0.011	0.210	0.042	0.294[b]	0.021	0.194	0.013
TPR domain protein	135	0.291[a]	0.019	0.227	0.036	0.189	0.011	0.175	0.042	0.197	0.035
Transport and binding activity		-	-	-	-	-	-	-	-	-	-
RagA protein	4	1.222[a]	0.566	1.560[a]	0.045	3.244[b]	0.497	1.017[b]	0.171	2.204	0.168
RagA protein	7	0.287[b]	0.213	0.077	0.000	0.290[a]	0.086	0.270[a]	0.051	0.087	0.001
Copper homeostasis protein cutC	311	-	-	0.047	0.005	0.061	0.003	0.111[b]	0.018	0.065	0.007
Hypothetical tonB-linked outer membrane receptor PG50	20	0.045	0.001	0.085	0.021	0.057	0.006	0.166[b]	0.010	0.060	0.017
Hypothetical tonB-linked outer membrane receptor PG50	21	0.091[a]	0.019	0.195	0.030	0.170	0.006	0.193	0.017	0.184	0.033
Virulence and acquisition of peptides											
Dipeptidyl aminopeptidase IV, putative	35	0.130	0.036	0.127	0.052	0.242[b]	0.011	0.142	0.026	0.119	0.023
Hypothetical protein	16	-	-	-	-	0.154[b]	0.031	0.072	0.025	0.032	0.002
Peptidase, M20/M25/M40 family	115	0.160[b]	0.043	0.258	0.025	0.281	0.031	0.178[b]	0.027	0.341	0.031
Peptidase, M24 family	262	-	-	0.078[a]	0.017	0.081[a]	0.001	0.0798[a]	0.019	0.144	0.012

Information storage and processing											
DNA replication, recombination and repair											
Hypothetical protein	160	0.421	0.036	0.299	0.053	0.348	0.023	0.204[b]	0.011	0.377	0.010
Protein synthesis and folding											
Elongation factor Ts	292	0.128[a]	0.035	0.087[a]	0.003	0.061	0.011	0.171[b]	0.029	0.059	0.007
Ribosome recycling factor (Ribosome-releasing factor) (RRF)	363	0.330	0.036	0.287	0.017	0.145[b]	0.023	0.258[b]	0.014	0.356	0.010
Translation elongation factor G, putative	19	-	-	0.609	0.104	0.988[b]	0.091	0.443	0.122	0.473	0.237
Tyrosyl-tRNA synthetase	159	0.142[b]	0.015	0.086	0.011	0.084	0.011	0.082	0.014	0.073	0.019
Transcription											
Transcription termination factor Rho	22	-	-	0.180[a]	0.016	0.172[a]	0.028	0.173[a]	0.026	0.077	0.014
Metabolism											
Amino acid biosynthesis											
3-dehydroquinate dehydratase, type II	412	-	-	0.066[a]	0.006	-	-	0.077[a]	0.001	0.107	0.007
Phosphoserine aminotransferase	203	0.263	0.009	0.208	0.018	0.293	0.049	0.418[b]	0.044	0.285	0.033
Serine hydroxymethyltransferase	807	0.261[a]	0.038	0.551[a]	0.071	-	-	-	-	0.190	0.056
Central intermediary metabolism											
Acetyl-CoA hydrolase/transferase family protein	95	0.160[a]	0.026	0.167[a]	0.024	0.126[a]	0.009	0.112[a]	0.036	0.059	0.020
Coenzyme, prosthetic groups and carriers biosynthesis											
ATP:cob(I)alamin adenosyltransferase, putative	431	0.060	0.009	0.089[b]	0.009	0.046	0.006	0.103[b]	0.007	0.062	0.005
Oxidoreductase, putative	214	0.098	0.025	0.086	0.015	0.198[a]	0.057	0.100	0.031	0.078	0.021
Pyridoxal phosphate biosynthetic protein PdxJ	824	0.088[b]	0.022	0.036	0.005	0.043	0.010	0.049	0.013	0.035	0.009
Riboflavin synthase, alpha subunit	339	0.218[b]	0.021	0.382	0.038	0.249[a]	0.073	0.308[a]	0.024	0.436	0.017
Energy production: Amino acids and amines											

2-amino-3-ketobutyrate CoA ligase	155	0.946[b]	0.016	0.590[b]	0.086	0.463	0.099	0.354	0.015	0.378	0.024
Delta-1-pyrroline-5-carboxylate dehydrogenase	86	0.614	0.074	1.035	0.245	1.797[b]	0.377	1.012	0.112	0.855	0.109
D-lysine 5,6-aminomutase, alpha subunit	96	0.283[b]	0.047	0.158	0.004	0.155	0.016	0.137	0.011	0.162	0.008
Formiminotransferase-cyclodeaminase-related protein	557	0.041[b]	0.002	0.026	0.004	0.069[a]	0.009	0.083[a]	0.010	0.022	0.001
Glutamate dehydrogenase	508	3.189	0.429	3.386	0.207	4.982[b]	0.485	4.089	0.253	3.408	0.383
L-asparaginase	199	0.072[a]	0.006	0.067[a]	0.002	0.091	0.006	0.093	0.003	0.105	0.015
Methionine gamma-lyase	186	0.621[b]	0.085	0.443	0.033	0.416	0.009	0.407	0.031	0.431	0.043
Energy production: Electron transport											
Electron transfer flavoprotein, beta subunit	267	0.089	0.013	0.063	0.018	0.058	0.012	0.095[b]	0.012	0.062	0.013
Electron transport complex, RnfABCDGE type, G subunit	281	0.134[a]	0.012	0.133[a]	0.019	0.134[a]	0.023	0.138[a]	0.018	0.209	0.023
Putative NADPH-NAD transhydrogenase	171	0.326	0.071	0.212	0.046	0.288	0.047	0.465[b]	0.055	0.274	0.028
Pyruvate synthase	48	-	-	0.050	0.013	0.055	0.005	0.094[b]	0.011	0.060	0.009
Energy production: Fermentation											
4-hydroxybutyrate CoA-transferase	470	0.072	0.017	0.116[a]	0.012	0.110	0.011	0.077	0.015	0.060	0.012
4-hydroxybutyryl-CoA dehydratase	102	0.808	0.139	0.915[a]	0.104	1.252[a]	0.204	0.779[a]	0.067	0.538	0.116
4-hydroxybutyryl-CoA dehydratase	103	0.186	0.015	0.192	0.009	0.218	0.010	0.295[a]	0.022	0.181	0.065
4-hydroxybutyryl-CoA dehydratase	383	-	-	-	-	-	-	0.160	0.112	0.321	0.059
NAD-dependent 4-hydroxybutyrate dehydrogenase	205	0.290[a]	0.029	0.189	0.027	0.197	0.026	0.225	0.012	0.193	0.039
Energy production: Glycolysis/gluconeogenesis											
Malate dehydrogenase	239	-	-	0.170	0.028	0.171	0.027	0.227[a]	0.015	0.157	0.032
Phosphopyruvate hydratase	572	0.413	0.115	0.176[b]	0.006	0.411	0.095	0.310	0.021	0.315	0.075
Phosphopyruvate hydratase	161	0.504[a]	0.056	0.410	0.042	0.449	0.059	0.431	0.047	0.389	0.031
Energy production: Pentose phosphate pathway											
Ribulose-phosphate 3-epimerase	329	0.109[b]	0.014	0.080	0.006	0.055	0.012	0.076	0.012	0.071	0.003
Fatty acid and phospholid catabolism											

Acyl-CoA dehydrogenase, short-chain specific	449	0.165[a]	0.012	-	-	-	-	0.344	0.132	0.419	0.071
Nucleotide biosynthesis, transport and catabolism											
Dihydroorotate dehydrogenase	253	0.082[b]	0.018	0.036	0.006	0.050	0.014	0.033	0.008	0.035	0.004
Phosphoribosylaminoimidazolecarboxamide formyltransferase	91	0.072	0.011	0.086	0.016	0.072[a]	0.008	0.082[a]	0.013	0.114	0.013
Unknown function											
Conserved domain protein	326	0.095[b]	0.014	0.055	0.008	0.054	0.011	0.047	0.005	0.053	0.012
GTP-binding protein TypA	39	0.050[a]	0.014	0.066[a]	0.015	0.071[a]	0.004	0.085	0.006	0.094	0.010
Immunoreactive 42 kDa antigen PG33	427	0.173[b]	0.010	0.039	0.006	0.020	0.009	0.037	0.005	0.033	0.011
Immunoreactive 53 kD antigen PG123	119	0.142	0.018	0.084[a]	0.005	0.091[a]	0.008	0.190	0.084	0.285	0.123
POYPRTH NID	400	1.422[a]	0.104	0.944	0.053	0.194[b]	0.079	0.788[b]	0.019	1.045	0.026
ThiJ/PfpI family protein	379	0.274	0.045	0.201	0.024	0.152[b]	0.013	0.229	0.010	0.230	0.011

1 – ([a]) Differences statically significant between one treatment and control; ([b]) Differences statically significant among one treatment, the other concentration of the same treatment and control. Spots were concluded to be significantly up- or down-regulated when $p < 0.05$ (ANOVA - Tukey test).

Supplementary table (7). Mean normalized volume and standard deviation of proteins spots from *P. gingivalis* exclusively expressed in the treatments.

Protein	Spot number	Co6		Co600		Ni6		Ni600		Control	
		Vol	SD	Vol	SD	Vol	SD	Vol	SD	Vol	SD
Cellular Processes											
Cell envelope biosynthesis											
DTDP-4-dehydrorhamnose 3,5-epimerase	325	-	-	-	-	-	-	-	-	0.045	0.010
Phosphomannomutase	605	-	-	0.055	0.005	0.058	0.012	-	-	-	-
Oxidative stress/ Phosphate starvation											
Alkyl hydroperoxide reductase, C subunit	727	0.112	0.030	-	-	-	-	-	-	-	-

Dps family protein	683	-	-	-	-	-	-	0.084	0.017	-	-
Fe superoxide dismutase	570a	0.063	0.009	-	-	-	-	-	-	-	-
PhoH family protein	126	-	-	-	-	-	-	-	-	0.205	0.041
Rubrerythrin (RR)	565	0.126	0.026	-	-	-	-	-	-	-	-
TPR domain protein	664	-	-	-	-	0.030	0.004	-	-	-	-
Transport and binding activity		-	-	-	-	-	-	-	-	-	-
RagA protein	581	-	-	0.188	0.038	-	-	-	-	-	-
Virulence and acquisition of peptides											
Aminopeptidase C	810	-	-	0.234	0.060	0.216	0.257	-	-	-	-
Arg-gingipain-1 proteinase	590	-	-	0.332	0.076	-	-	-	-	-	-
Lysine-specific cysteine proteinase	827	-	-	-	-	-	-	-	-	0.162	0.044
Outer membrane protein 40 precursor	540	0.071	0.032	-	-	-	-	-	-	-	-
Uracil phosphoribosyltransferase	291	-	-	-	-	-	-	-	-	0.059	0.008
Information storage and processing											
DNA replication, recombination and repair											
Holliday junction ATP-dependent DNA helicase ruvA	355	-	-	-	-	-	-	-	-	0.045	0.003
Hypothetical protein	661	-	-	-	-	0.025	0.007	-	-	-	-
Single-stranded binding protein	364	-	-	-	-	-	-	-	-	0.042	0.011
Single-stranded binding protein	573	0.054	0.009	-	-	0.089	0.012	-	-	-	-
Protein synthesis and folding											
50S ribosomal protein L25/general stress protein Ctc	429	-	-	0.184	0.037	0.185	0.041	0.177	0.028	-	-
50S ribosomal protein L7/L12	729	0.570	0.138	-	-	-	-	-	-	-	-
50S ribosomal protein L9	696	-	-	-	-	-	-	0.124	0.025	-	-
Heme uptake protein A and B (Ribosomal protein S1)	45	-	-	-	-	-	-	-	-	0.095	0.028
Peptidyl-prolyl cis-trans isomerase	617	-	-	0.160	0.053	0.145	0.045	0.158	0.106	-	-

Transcription

Metabolism

Amino acid biosynthesis

Phosphoserine aminotransferase	662	0.325	0.032	-	-	0.027	0.011	-	-	-	-
Phosphoserine aminotransferase	830	-	-	0.168	0.032	-	-	-	-	-	-
Phosphoserine aminotransferase	684	-	-	-	-	-	-	0.134	0.009	-	-

Central intermediary metabolism

Alkaline phosphatase, putative	746	-	-	0.064	0.018	0.106	0.006	-	-	-	-

Coenzyme, prosthetic groups and carriers biosynthesis

Aminotransferase, putative	536	0.044	0.008	-	-	-	-	-	-	-	-
Aminotransferase, putative	745	-	-	0.043	0.016	-	-	-	-	-	-
D-isomer specific 2-hydroxyacid dehydrogenase family protein	722	0.095	0.035	-	-	-	-	-	-	-	-
Pantoate--beta-alanine ligase	216	-	-	-	-	-	-	-	-	0.132	0.009
Riboflavin synthase, alpha subunit	663	-	-	-	-	0.019	0.007	-	-	-	-
Riboflavin synthase, alpha subunit	663	-	-	-	-	0.019	0.007	-	-	-	-

Energy production: Amino acids and amines

Delta-1-pyrroline-5-carboxylate dehydrogenase	529	0.131	0.079	-	-	-	-	-	-	-	-
Glutamate dehydrogenase	593	-	-	0.372	0.063	-	-	-	-	-	-
Methionine gamma-lyase	576	0.019	0.004	0.037	0.003	-	-	-	-	-	-

Energy production: Electron transport

Electron transfer flavoprotein, alpha subunit	305	-	-	-	-	-	-	-	-	0.259	0.052
Electron transfer flavoprotein, alpha subunit	541	0.030	0.004	-	-	-	-	-	-	-	-
Electron transfer flavoprotein, alpha subunit	719	0.111	0.006	-	-	-	-	-	-	-	-
Putative NADPH-NAD transhydrogenase	677	-	-	-	-	0.073	0.007	0.032	0.008	-	-

Energy production: Fermentation											
2-oxoglutarate oxidoreductase, alpha subunit	232	-	-	-	-	-	-	-	-	0.464	0.123
2-oxoglutarate oxidoreductase, alpha subunit	537	0.128	0.022	-	-	-	-	-	-	-	-
2-oxoglutarate oxidoreductase, gamma subunit	548	0.078	0.029	0.048	0.011	-	-	-	-	-	-
4-hydroxybutyrate CoA-transferase	624	-	-	0.039	0.007	0.049	0.007	0.032	0.010	-	-
4-hydroxybutyryl-CoA dehydratase	620	-	-	0.102	0.025	-	-	-	-	-	-
Fumarate hydratase class I, anaerobic	813	-	-	0.102	0.013	0.085	0.004	-	-	-	-
Energy production: Glycolysis/gluconeogenesis											
Glyceraldehyde 3-phosphate dehydrogenase, type I	720	0.109	0.018	-	-	0.193	0.051	-	-	-	-
Glyceraldehyde 3-phosphate dehydrogenase, type I	809	-	-	0.163	0.110	0.111	0.065	-	-	-	-
Malate dehydrogenase	450	0.197	0.013	-	-	-	-	-	-	-	-
Phosphoenolpyruvate carboxykinase	741	-	-	0.141	0.039	-	-	-	-	-	-
Phosphoenolpyruvate carboxykinase	530	0.199	0.028	-	-	-	-	-	-	-	-
Energy production: Pentose phosphate pathway											
Aldose 1-epimerase	552	0.145	0.030	-	-	-	-	-	-	-	-
Fatty acid and phospholid catabolism											
Acyl-CoA dehydrogenase, short-chain specific	539	0.079	0.058	-	-	-	-	-	-	-	-
Nucleotide biosynthesis, transport and catabolism											
Aspartate carbamoyltransferase catalytic subunit	212	-	-	-	-	-	-	-	-	0.065	0.001
Dihydroorotate dehydrogenase family protein	734	0.158	0.015	-	-	-	-	-	-	-	-
Ribose-phosphate pyrophosphokinase	485	-	-	-	-	-	-	-	-	0.214	0.044
Unknown function											
Immunoreactive 42 kDa antigen PG33	542	0.073	0.049	-	-	-	-	-	-	-	-
Thioesterase family protein	614	0.053	0.005	0.049	0.001	-	-	0.060	0.009	-	-

3.3. Protein changes and functional classification

Cell envelope biosynthesis

The bacterial cell surface and its components are essential for *P. gingivalis* integrity, assisting its growth, nutrient acquisition, colonization, biofilm formation and evasion of host defense (Yoshimura *et al.*, 2008). This bacterium releases outer membrane vesicles containing lipopolysaccharides (LPS), which can penetrate (Grenier *et al.*, 1995) and destroy the periodontal tissue (Moore *et al.,* 1986) leading in many cases to periodontitis. This LPS and other polysaccharides such as capsular polysaccharides may also contribute to *P. gingivalis* coaggregation with *Fusobacterium nucleatum*, which is a colonizer of oral biofilm related to periodontal diseases (Moore *et al.*, 1982, Moore *et al.,* 1985).

Phosphomannomutase (PMM) plays a crucial role in the synthesis of cell envelope components of several bacterial species, such as surface polysaccharides (SPS) and LPS. This enzyme was reported to participate in the LPS biosynthetic pathways of *Vibrio furnissii* (Kim *et al.*, 2003) and *Pseudomonas aeruginosa* (Regni *et al.*, 2002). Thus, PMM may contribute to *P. gingivalis* LPS production, as well as participates in the biosynthesis of some other surface polysaccharides.

DTDP-4-dehydrorhamnose 3,5-epimerase (DHR) is a key enzyme in the biosynthesis of dTDP-L-rhamnose (Schnaitman and Klena, 1993). This carbohydrate is found in polysaccharides produced by important human pathogens such as *Salmonella* (Graninger *et al.*, 1999). Lipopolysaccharide of *P. gingivalis* also contains rhamnose (Bramanti *et al.*, 1989), and DHR may participate in this carbohydrate biosynthesis pathway.

Despite their importance, in the presence of nicotine and cotinine, the PMM enzyme was observed to be downregulated (spot 57 – locus PG 2010), especially with 6 μg/mL nicotine. In addition, the other spot (605 – locus PG 1094) identified as PMM protein was only expressed in Ni6 and Co600 groups. The production of PMM – PG 1094 in these two treatments may have occurred to supply the reduction of the production of PMM – PG 2010. DHR was not expressed in any of the treatments. Such reduction in the expression of these proteins might represent an alternative to reduce the energy waste.

Nevertheless, low levels of PMM and the absence of DHR could cause a decrease in bacterial viability and in evasion from host cells.

Oxidative Stress and Phosphate Starvation

Most of the spots identified as proteins belonging to the oxidative stress class, were upregulated in the presence of nicotine and cotinine. Only two bacterial enzymes (Dps family protein and Thiol peroxidase) had their expressions decreased as they were exposed to nicotine. Other proteins, such as Redox-sensitive transcriptional activator OxyR (OxyR), Rubrerythrin, Thiol peroxidase and TPR domain protein, involved in the process of aerotolerance of *P. gingivalis,* were more abundant in the experimental groups than in the control. In addition, other essential proteins such as Alkyl hydroperoxide reductase, Fe superoxide dismutase, Rubrerythrin and TPR domain protein were exclusively expressed in at least one treatment, indicating also a stimulation of the production of these proteins. All the proteins mentioned above play an essential role in the oxidative stress protection of *P. gingivalis* (Nakayama *et al.*, 1990; Sztukowska *et al.*, 2002; Ueshima *et al.*; 2003; Johnson *et al.*, 2004; Diaz *et al.*, 2006; Okano *et al.*, 2006; Meuric *et al.*, 2008) enabling this bacterium to survive within periodontal pockets despite occasional exposures to aerobic conditions (Ueshima *et al.*; 2003). In the present study, the bacterial cultures were not exposed to an aerobic environment; nicotine and cotinine were observed to enhance the oxidative stress response of *P. gingivalis*.

Some previous studies reported nicotine as an oxidative agent. Nicotine at very low concentration, as observed in oral tissues of smokers, can induce oxidative stress in epithelial cells isolated from colorectal carcinoma - HCT-116 (Crowley-Weber *et al.*, 2003). These authors suggested that nicotine can induce an enhancement in oxidative stress due to a possible increase in H_2O_2 concentration. Nicotine can also cause oxidative damage to developing neural cells both *in vitro* and *in vivo* (Qiao *et al.*, 2005). Rat's mesencephalic cells exposed to different concentrations of nicotine may increase the reactive oxygen species (ROS) in a dose dependent manner (Barr *et al.*, 2007). This study also demonstrated nicotine activating the transcription of nuclear factor kappa B, a family of DNA binding

proteins that regulates transcription of many genes and control many cellular processes (Widera *et al.*, 2006; Barr *et al.*, 2007).

Nicotine was found to increase ROS generation in murine embryo tissues (Zhao and Reece *et al.*, 2006). Therefore, nicotine might play a role as an oxidative substance into the bacterial cells, inducing ROS production. ROS play a critical role in many human and bacterial cells causing DNA injury, genomic instability, and hypomethylation of DNA (Barr *et al.*, 2007). The up-regulation of oxidative stress proteins in *P. gingivalis* could be used as a strategy to neutralize damaging oxidants. No information about cotinine effects on DNA was found in the scientific literature.

PhoH family protein, which controls cellular response to phosphate starvation, was only expressed in the control. However, the control bacterial cultures were not deprived of phosphate.

Transport and binding activity

RagA, previously known as immunodominant surface antigen, was identified in sera of patients with periodontal disease (Curtis *et al.*, 1991). RagA protein expression was recently found to be altered depending on the aeration condition (Okano *et al.*, 2006), although its function in the oxidative stress process is not clear. This protein has homology to TonB-linked outer-membrane receptors, which are involved in the recognition and active uptake of a specific carbohydrate or glycoprotein and iron acquisition in *P. gingivalis* (Curtis *et al.*, 1999). In the present study, three possible isoforms of these proteins were found. The spot 548 was exclusively expressed with cotinine at 600 µg/mL. Spots 7 and 4 were more and less abundant, respectively, when compared to the control. The same pattern of expression was found considering other proteins linked to the outer membrane transport activity, the hypothetical tonB-linked outer membrane receptor PG50. The expression of this protein in spot 20 was upregulated by nicotine at 600 µg/mL and the protein in spot 21 was downregulated by cotinine at 6 µg/mL. Only the copper homeostasis protein - cutC, which is a protein that possibly accomplishes the adequate balance between copper requirement and copper toxicity (Gupta *et al.*, 1995), had a distinctive protein

expression profile. In the present study, the number of some cutC isoforms was observed to increase or decrease depending on the treatment.

The reason for this increasing/decreasing behavior of these proteins (tonB-linked outer membrane receptor PG50 and cutC) remains unclear. In the presente study, it was found that nicotine and cotinine can induce the production of proteins involved in the oxidative stress response probably due to the presence of reactive oxygen species (ROS). The increased oxidative stress in the bacteria during the treatments might have reduced the expression of RagA protein. It is possible that high concentrations of ROS lead to a decrease of RagA production (spot 4), converting the bacteria into a less inflammatory phenotype. Consequently, this *P. gingivalis* phenotype might be able to evade the host immune response in the periodontal biofilm. The reduction of RagA synthesis in hostile conditions, as high temperatures, was also observed previously (Murakami *et al*., 2004). In addition, the incresead production of the one RagA isoform (spot 7) might have occurred to compensate the reduction of the other RagA isoform (spot 4). This possible different isoform (spot 7) might also contribute to transform *P. gingivalis* into a less antigenic bacterial phenotype. However, such hypothesis has to be confirmed.

Virulence and acquisition of peptides

Virulence factors are indispensable to the establishment and survival of bacteria in the gingival crevice and successive invasion into host cells. Proteases, considered as the most important virulence factors of *P. gingivalis*, may enable the degradation of host proteins, as well as the adherence of this bacterium to host tissues and to other oral microorganisms (Nelson *et al.*, 2003). Some of the proteins identified in the present study have been recently investigated. Arg- and Lys-specific gingipains are proteins capable of disturbing local immunity and impairing the host's inflammatory response. These proteinases may favor adherence and proteolytic properties of *P. gingivalis* and its ability to invade deeper periodontal tissues (Imamura *et al.*, 2003; Chen and Duncan, 2004). The gingipains are also essential to the process of haem acquisition from haemoglobin (Okamoto *et al.*, 1998; Smalley *et al.*, 2004); they have been reported as major factors to protect *P. gingivalis* against the human complement system, providing

resistance to the bactericidal lytic activity of human serum (Okuda *et al.*, 1986). Recently, Arg-gingipain was mentioned as the main component responsible for the enhancement of RANKL expression in human gingival fibroblasts and periodontal ligament cells (Belibasakis *et al.*, 2007), causing osteoclast stimulation and further bone resorption in cases of periodontitis. The outer membrane protein 40 kDa (40 kDa OMP) is a key virulence factor involved in the coaggregation activity of *P. gingivalis* (Hamajima *et al.*, 2007) and in the hemin-binding process (Shibata *et al.*, 2003). Uracil phosphoribosyltransferase, encoded by prtH gene, can hydrolyze the C3 protein of the complement system (Fletcher *et al.*, 1994).

In the present study, both nicotine and cotinine interfered with the intensity of expression of some proteases, as well as in their exclusive production in comparison to the control. Reduced levels of proteins of the peptidase M20/M25/M40 family and the peptidase M24 family were found, in contrast to the higher levels of dipeptidyl aminopeptidase IV putative protein and a hypothetical protein with proteolytic function. Aminopeptidase C, arg-gingipain-1 proteinase (arg-gingipain), and outer membrane protein 40 precursor (Omp40) were exclusively expressed in some of the treatments, while lysine-specific cysteine proteinases (lys-gingipain) and uracil phosphoribosyltransferase were only found in control gels. In summary, nicotine and cotinine seemed to have an ambiguous effect on this class of proteins, stimulating or reducing some virulence factors.

The downregulation of some virulence factors of *P. gingivalis* in different culture conditions was previously reported. *P. gingivalis* internalized in gingival epithelial cells *in vitro* underexpressed some proteases to avoid host cells damage and apoptotic cell death (Xia *et al.*, 2007). Under aerobic conditions, *P. gingivalis* overexpressed SOD gene (related to the oxidative stress response), but repressed FimA expression, which is a virulence factor responsible for cell adhesion and invasion (Wu *et al.*, 2008). As mentioned in these previous studies, the down expression of some genes or proteins might occur to preserve bacterial energy or to prepare it to a new condition of life. In the present study, the presence of nicotine and cotinine may be a stressful factor, and *P. gingivalis* had to downregulate some apparently unessential proteins to preserve energy. In contrast, some other proteins associated with virulence were over or exclusively expressed. Probably these

proteins were upregulated to carry out proteolysis and to recycle amino acids for protein synthesis, as well as to control the bacterial response to a stressful condition. In fact, the effect of both nicotine and cotinine on proteases expression may not cause an extremely change in the proteolytic activity of *P. gingivalis*, since 42 proteinases were identified in its genome sequence (Nelson *et al.*, 2003).

Information storage and processing

Most proteins involved in the transcription, protein synthesis and folding were upregulated or exclusively expressed in the experimental groups, except the ribosome recycling factor (RRF). This up-regulation profile was expected, since many bacterial proteins were increased due to the treatments. RRF was also supposed to be increased, since this protein dissociates the ribosome complex after translation and releases it to start another cycle (Janosi *et al.*, 1994). This protein works simultaneously with the elongation factor G (EF-G) (Hirashima and Kaji, 1973), which was upregulated in one of the treatments in the present study. The translation elongation factors (EF-Ts), which regulates the translation elongation, could act as a steric chaperone for folding of the translation elongation factors (EF-Tu), protecting against growth inhibition as previously described in *Escherichia coli* (Krab *et al.*, 2001). Translation elongation factor G, another upregulated protein, was also reported in *E. coli* as a chaperone and might be implicated in protein folding and protection against stress, besides its role in translation (Caldas *et al.*, 2000). EF-Tu, EF-Ts, and EF-G are involved in the conduction of the aminoacyl tRNAs to the ribosome and in the translocation of the ribosome along the mRNA (Cooper, 2000). The possible role of EF-Ts and EF-G as chaperone may explain their higher levels under the stressful conditions of the bacterial exposure to nicotine and cotinine.

Some proteins identified and classified in the cell role category as participating in the biological process of DNA replication, recombination and repair were exclusively expressed in the nicotine and/or cotinine groups. Only a hypothetical protein, which was predicted to be involved in the DNA modification process, was expressed in all groups (including control), but it was less abundant after the 600 μg/mL nicotine treatment. Stressful conditions can induce overexpression of proteins related to DNA processes, as

observed in *S. mutans* exposed to low pH conditions (Len *et al.*, 2004). However, none of the proteins exclusively expressed in the present study are essential to DNA repairing processes, which may represent that no drastic DNA damage occurred in *P. gingivalis* during its exposure to nicotine and cotinine. To the best of our knowledge, there is no study about nicotine and cotinine direct outcomes on the structure and processes of DNA from bacterial cells. However, there are some controversial results concerning the mutagenicity effects of nicotine and cotinine on mammalian cells. In a study using Chinese hamster ovary cells (CHO), neither nicotine nor cotinine was able to generate genotoxic effects (Doolittle *et al.*, 1995). In contrast, nicotine was reported to modestly stimulate DNA damage and sister chromatide exchange in a dose and time dependent manner in CHO cells (Trivedi *et al.*, 1990). DNA from upper aerodigestive tract epithelia (Sassen *et al.*, 2005) and human salivary glands cells (Ginzkey *et al.*, 2008) may also be impaired by nicotine exposure. Further studies are needed to elucidate if nicotine and cotinine may have genotoxic effects on bacterial cells.

Metabolism

The addition of nicotine or cotinine in *P. gingivalis* cultures promoted a significant alteration in the enzymes involved in metabolism, causing an up-regulation or exclusive expression in most of the proteins, compared to the control. These enzymes are part of the metabolism of amino acids, coenzymes, prosthetic groups and carriers, fatty acids, phospholipids and nucleotides synthesis, and production of energy. Special attention must be given to the proteins involved in the energy production. They play an important role in bacterial metabolic pathways such as amino acids and amines metabolism, electron transport, fermentation, glycolysis, pentose phosphate pathway, and the Krebs cycle. Their up-regulation assists the bacteria to produce more energy to preserve all bacterial processes, including virulence. When bacteria are subject to certain stressful conditions, an alteration in the redox state is observed. The increase in enzymes such as malate dehydrogenase (spot 239) serves as a redox valve for using excess NADPH to convert oxaloacetic acid into malate which is essential to keep homeostasis in stressed bacteria. Phosphopyruvate hydratase (spot 161 and 572) and glyceraldehyde-3-phosphate-dehydrogenase (spot 720 and 809) were strongly upregulated in the presence of nicotine. These are abundant and key

glycolytic enzymes, essential for the degradation of carbohydrates via glycolysis. However, glucose is poorly used as energy source for *P. gingivalis*, and carbohydrates are not the most preferable source to readily maintain growth (Shah and Williams, 1987). In fact, pentose phosphate pathway has an essential role in providing precursor metabolites to support anaerobic growth (Nelson *et al.*, 2003), and their enzymes were also in high levels in the present study.

Enzymes from fermentation pathways were also important to produce energy, and their expression was upregulated in the experimental groups. However, many fermentation products are possibly harmful to host cells (Nelson *et al.*, 2003) and can damage much more the host tissue cells exposed to the deleterious effects of nicotine and cotinine.

Therefore, abundance of proteins from metabolism is consistent with a greater demand for energy, which is essential for bacterial adaptation to a new condition: the presence of nicotine and cotinine.

4. Concluding remarks

This work is the first report of proteomic evaluation of nicotine and cotinine effects in *Porphyromonas gingivalis.* The proteomics-based analysis carried out in the present study showed that many proteins were altered in the presence of nicotine and cotinine. Concentrations of nicotine and cotinine used in this study were adequate for the evaluation of their effects. Mean nicotine yield in smoke can vary from 1.10 to 3.40 mg/cigarette, based on the Massachusetts smoking regimen for all cigarette brand styles and major market categories in 1997–2005 (Connolly *et al.*, 2007). It is difficult to measure total nicotine concentration from cigarette smoking in saliva and crevicular fluid due to its short half life. Therefore, most of the studies have been describe cotinine levels of smokers. The mean levels of cotinine reported in saliva and crevicular fluid ranged from 7.978 μg/mL to 15.027 μg/mL and 2.259 μg/mL to 3.186 μg/mL, respectively (Chen *et al.*, 2001). In the present study, the nicotine and cotinine low concentration was in agreement with those physiological levels while the other concentration was higher than those found

in saliva and crevicular fluid. Nevertheless, previous studies used high concentrations of these substances to induce cellular effects (Sayers *et al.* 1997; Teughels *et al.* 2005)

Probably, more genes may have their protein abundance patterns changed when *P. gingivalis* is exposed to nicotine and cotinine in the host environment than in the conditions described here. Nonetheless, despite *in vitro* conditions are not the same as *in vivo* environment, these findings are a prediction of what may occur biologically. When exposed to these substances, *P. gingivalis* downregulated production or exclusively expressed some virulence factors, upregulated the expression of proteins involved in oxidative stress response, produced more energy while dispensed with production of some energetically costly molecules to overcome the probable changes provoked by nicotine and cotinine and to maintain homeostasis. These changes in protein expression may enable *P. gingivalis* to adapt to nicotine and cotinine exposure. In conclusion, these findings suggest that nicotine and cotinine can affect the protein expression of *P. gingivalis*.

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6. References

1. Barr, J., Sharma, C.S., Sarkar, S., Wise, K., *et al*., Nicotine induces oxidative stress and activates nuclear transcription factor kappa B in rat mesencephalic cells. *Mol. Cell. Biochem*. 2007, *297*, 93-9.
2. Beck, J. D., Offenbacher, S., Williams, R., Gibbs, P., Garcia, R. Periodontitis: a risk factor for coronary heart disease? *Ann. Periodontol*. 1998, *3*, 127–141.
3. Belibasakis, G. N., Bostanci, N., Hashim, A., Johansson, A., *et al*., Regulation of RANKL and OPG gene expression in human gingival fibroblasts and periodontal ligament cells by *Porphyromonas gingivalis*: a putative role of the Arg-gingipains, *Microb. Pathog*. 2007, *43*, 46-53.

4. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. 1976, *72*, 248-54.
5. Bramanti, T. E., Wong, G. G., Weintraub, S. T., Holt, S. C., Chemical characterization and biologic properties of lipopolysaccharide from *Bacteroides gingivalis* strains W50, W83, and ATCC 33277. *Oral Microbiol. Immunol*. 1989, *4*, 183-92.
6. Caldas, T., Laalami, S., Richarme, G., Chaperone properties of bacterial elongation factor EF-G and initiation factor IF2. *J. Biol. Chem*. 2000, *275*, 855-60.
7. Candiano, G., Bruschi, M., Musante, L., Santucci, L., *et al*., Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. *Electrophoresis* 2004, *25*, 1327-33.
8. Chen, T., Duncan, M. J., Gingipain adhesin domains mediate *Porphyromonas gingivalis* adherence to epithelial cells. *Microb. Pathog*. 2004, *36*, 205-9.
9. Chen, X., Wolff, L., Aeppli, D., Guo, Z., *et al*., Cigarette smoking, salivary/gingival crevicular fluid cotinine and periodontal status. A 10-year longitudinal study. *J. Clin. Periodontol*. 2001, *28*, 331-9.
10. Cogo, K., Montan, M. F., Bergamaschi, C. C., Andrade, E. D., *et al*., *In vitro* evaluation of the effect of nicotine, cotinine, and caffeine on oral microorganisms. *Can. J. Microbiol*. 2008, *54*, 501-8.
11. Connolly, G. N., Alpert, H. R., Wayne, G. F., Koh, H. Trends in nicotine yield in smoke and its relationship with design characteristics among popular US cigarette brands, 1997-2005. *Tob. Control*. 2007, *16*, e5.
12. Cooper, G. M. The Cell: a Molecular Approach, 2nd ed, American Society for Microbiology, Washington, DC, 2000, pp. 273–290.
13. Crowley-Weber, C. L., Dvorakova, K., Crowley, C., Bernstein, H., *et al*., Nicotine increases oxidative stress, activates NF-kappaB and GRP78, induces apoptosis and sensitizes cells to genotoxic/xenobiotic stresses by a multiple stress inducer, deoxycholate: relevance to colon carcinogenesis. *Chem. Biol. Interact*. 2003, *145*, 53-66.

14. Curtis, M. A, Slaney, J. M., Carman, R. J., Johnson, N. W., Identification of the major surface protein antigens of *Porphyromonas gingivalis* using IgG antibody reactivity of periodontal case-control serum. *Oral Microbiol. Immunol.* 1991, *6*, 321–326.
15. Curtis, M. A., Hanley, S. A., Aduse-Opoku, J. The rag locus of *Porphyromonas gingivalis*: a novel pathogenicity island. *J. Periodontal. Res*. 1999, *34*, 400-5.
16. Damerval, C., de Vienne. D., Zivy. M., Thiellement, H. Technical improvements in two-dimensional electrophoresis increase the level of genetic variation detected in wheat-seedling proteins. *Electrophoresis* 1986, *7*, 52 – 54.
17. Diaz, P. I., Slakeski, N., Reynolds, E. C., Morona, R., *et al*., Role of oxyR in the oral anaerobe *Porphyromonas gingivalis*. *J. Bacteriol*. 2006, *188*, 2454-62.
18. Doolittle, D. J., Winegar, R., Lee, C. K., Caldwell, W. S., Hayes, A.W, de Bethizy JD. The genotoxic potential of nicotine and its major metabolites. Mutat Res. 1995 Oct;344(3-4):95-102.Links
19. Elter, J. R., Beck, J. D., Slade, G. D., Offenbacher, S., Etiologic models for incident periodontal attachment loss in older adults. *J. Clin. Periodontol.* 1999, *26*, 113-123.
20. Fiorani Celedon, P. A., de Andrade, A., Meireles, K. G., Gallo de Carvalho, M. C., Proteomic analysis of the cambial region in juvenile *Eucalyptus grandis* at three ages. *Proteomics* 2007, *7*, 2258-74.
21. Fletcher, H. M., Schenkein, H. A., Macrina, F. L., Cloning and characterization of a new protease gene (prtH) from *Porphyromonas gingivalis*. Infect Immun. 1994, *62*, 4279-86.
22. Ginzkey, C., Kampfinger, K., Friehs, G., Köhler, C., *et al*., Nicotine induces DNA damage in human salivary glands. *Toxicol. Lett*. 2008 *in press*
23. Gupta, S. D., Lee, B. T. O., Camakaris, J., Wu, H. C., Identification of cutC and cutF (nlpE) genes involved in copper tolerance in *Escherichia coli*. *Journal of Bacteriology*, 1995, p. 4207–4215 Vol. 177, No. 15
24. Graninger, M., Nidetzky, B., Heinrichs, D. E., Whitfield, C., Messner, P., Characterization of dTDP-4-dehydrorhamnose 3,5-epimerase and dTDP-4-dehydrorhamnose reductase, required for dTDP-L-rhamnose biosynthesis in *Salmonella enterica* serovar Typhimurium LT2. *J. Biol. Chem*. 1999, *35*, 25069-77.

25. Grenier, D., Bertrand, J., Mayrand, D. *Porphyromonas gingivalis* outer membrane vesicles promote bacterial resistance to chlorhexidine. *Oral Microbiol. Immunol*. 1995, *10*, 319-20.
26. Haffajee, A. D., Socransky, S. S. Relationship of cigarette smoking to attachment level profiles. *J. Clin. Periodontol.* 2001, *28*, 283-295.
27. Hamajima, S., Maruyama, M., Hijiya, T., Hatta, H., Abiko, Y., Egg yolk-derived immunoglobulin (IgY) against *Porphyromonas gingivalis* 40-kDa outer membrane protein inhibits coaggregation activity. *Arch. Oral Biol*. 2007, *52*, 697-704.
28. Hirashima, A., Kaji, A., Role of elongation factor G and a protein factor on the release of ribosomes from messenger ribonucleic acid. *J. Biol. Chem*., 1973, *248*, 7580-7.
29. Imamura, T., The role of gingipains in the pathogenesis of periodontal disease. *J. Periodontol*. 2003, *74,* 111-8.
30. Janosi, L., Shimizu, I., Kaji, A., Ribosome recycling factor (ribosome releasing factor) is essential for bacterial growth. *Proc. Natl. Acad. Sci. U S A*. 1994, *91*, 4249-53.
31. Johnson, G. K., Hill, M., Cigarette smoking and the periodontal patient. *J. Periodontol*. 2004, *75*, 196-209.
32. Johnson, N. A., Liu, Y., Fletcher, H, M., Alkyl hydroperoxide peroxidase subunit C (ahpC) protects against organic peroxides but does not affect the virulence of *Porphyromonas gingivalis* W83. *Oral Microbiol. Immunol*., 2004, *19*, 233-9.
33. Krab, I. M., te Biesebeke, R., Bernardi, A., Parmeggiani, A., Elongation factor Ts can act as a steric chaperone by increasing the solubility of nucleotide binding-impaired elongation factor-Tu. *Biochemistry*. 2001, *40*, 8531-5.
34. Kim, S. H., Ahn, S. H., Lee, J. H., Lee, EM, Kim NH, Park KJ, Kong IS. Genetic analysis of phosphomannomutase/phosphoglucomutase from *Vibrio furnissii* and characterization of its role in virulence. *Arch. Microbiol*., 2003, *180*, 240-50.
35. Len, A. C., Harty, D. W., Jacques, N. A., Stress-responsive proteins are upregulated in *Streptococcus mutans* during acid tolerance. *Microbiology*. 2004, *150*, 1339-51.
36. Marakoglu, I., Gursoy, U. K., Marakoglu, K., Cakmak, H., Ataoglu, T., Periodontitis as a risk factor for preterm low birth weight. *Yonsei Med. J*., 2008, *49*, 200-3.

37. Mercado, F. B., Marshall, R. I., Bartold, P. M., Inter-relationships between rheumatoid arthritis and periodontal disease. A review. *J. Clin. Periodontol*. 2003, *30*, 761–772.
38. Meuric, V., Gracieux, P., Tamanai-Shacoori, Z., Perez-Chaparro, J., Bonnaure-Mallet, M. Expression patterns of genes induced by oxidative stress in *Porphyromonas gingivalis*. *Oral Microbiol. Immunol*., 2008, *23*, 308-14.
39. Miyakawa, H., K. Honma, M. Qi, Kuramitsu H. K., Interaction of *Porphyromonas gingivalis* with low-density lipoproteins: implications for a role for periodontitis in atherosclerosis. *J. Periodontal Res*. 2004, 39, 1–9.
40. Moore, J., Wilson, M., Kieser, J. B., The distribution of bacterial lipopolysaccharide (endotoxin) in relation to periodontally involved root surfaces. *J. Clin. Periodontol*., 1986, *13*, 748-51.
41. Moore, W. E., Holdeman, L. V., Cato, E. P., Smibert, R. M., *et al*., Comparative bacteriology of juvenile periodontitis. *Infect. Immun*. 1985, *48*, 507-19.
42. Murakami Y, Masuda T, Imai M, Iwami J, Nakamura H, Noguchi T, Yoshimura F. Analysis of major virulence factors in *Porphyromonas gingivalis* under various culture temperatures using specific antibodies. Microbiol Immunol. 2004; 48 (8): 561-9.
43. Moore, W. E., Holdeman, L. V., Smibert, R. M. , Hash DE, *et al*., Bacteriology of severe periodontitis in young adult humans. *Infect. Immun*. 1982, *38*, 1137-48.
44. Nakano, Y., Shibata, Y., Kawada, M., Kojima, M., A searchable database for proteomes of oral microorganisms. *Oral Microbiol Immunol*. 2005, *20*, 344-8.
45. Nakayama, K., The superoxide dismutase-encoding gene of the obligately anaerobic bacterium *Bacteroides gingivalis*. *Gene*. 1990, *96*, 149-50.
46. Nelson KE, Fleischmann RD, DeBoy RT, Paulsen IT, *et al*., Complete genome sequence of the oral pathogenic Bacterium *Porphyromonas gingivalis* strain W83. *J. Bacteriol*. 2003, *185*, 5591-601.
47. Okamoto, K., Nakayama, K., Kadowaki, T., Abe, N., *et al*., Involvement of a lysine-specific cysteine proteinase in hemoglobin adsorption and heme accumulation by *Porphyromonas gingivalis. J. Biol. Chem*., 1998, *273*, 21225-31.
48. Okano, S., Shibata, Y., Shiroza, T., Abiko, Y., Proteomics-based analysis of a counter-oxidative stress system in *Porphyromonas gingivalis*. *Proteomics* 2006, *6*, 251–258.

49. Okuda, K., Kato, T., Naito, Y., Ono, M., *et al*., Susceptibility of *Bacteroides gingivalis* to bactericidal activity of human serum. *J. Dent. Res*. 1986, *65*, 1024-7.
50. Offenbacher, S., Jared, H. L., O'Reilly, P. G., Wells, *et al*., Potential pathogenic mechanisms of periodontitis associated pregnancy complications. *Ann. Periodontol*. 1998, *3*, 233–250.
51. Pischon N, Pischon T, Kröger J, Gülmez E, *et al*., Association among rheumatoid arthritis, oral hygiene, and periodontitis. *J. Periodontol*. 2008, *79*, 979-86.
52. Pucher, J., Stewart, J., Periodontal disease and diabetes mellitus. *Curr. Diab. Rep*. 2004, *4*, 46-50
53. Qiao, D., Seidler, F. J., Slotkin, T. A. Oxidative mechanisms contributing to the developmental neurotoxicity of nicotine and chlorpyrifos. *Toxicol. Appl. Pharmacol*. 2005, *206*, 17-26.
54. Regni, C., Tipton, P. A., Beamer, L. J., Crystal structure of PMM/PGM: an enzyme in the biosynthetic pathway of *P. aeruginosa* virulence factors. *Structure* 2002, *10*, 269-79.
55. Sassen, A. W., Richter, E., Semmler, M. P., *et al*., Genotoxicity of nicotine in mini-organ cultures of human upper aerodigestive tract epithelia. *Toxicol. Sci*. 2005, *88*, 134-41.
56. Sayers, N. M., Gomes, B. P., Drucker, D. B., Blinkhorn, A. S., Possible lethal enhancement of toxins from putative periodontopathogens by nicotine: implications for periodontal disease. *J. Clin. Pathol*., 1997, *50*, 245-9.
57. Sayers, N.M., James, J.A., Drucker, D.B., Blinkhorn, A.S., Possible potentiation of toxins from *Prevotella intermedia*, *Prevotella nigrescens*, and *Porphyromonas gingivalis* by cotinine. *J. Periodontol*. 1999, *70*, 1269-75.
58. Scannapieco, F. A., Genco, R. J. Association of periodontal infections with atherosclerotic and pulmonary diseases. *J. Periodontal. Res*. 1999, *34*, 340-5.
59. Schenkein, H. A., Gunsolley, J. C., Koertge, T. E., Schenkein, J. G., Tew, J. G. Smoking and its effects on early-onset periodontitis. *J. Am. Dent. Assoc.* 1995, *126*, 1107-1113.

60. Schnaitman, C. A., Klena, J. D., Genetics of lipopolysaccharide biosynthesis in enteric bacteria. *Microbiol. Rev*. 1993, *57*, 655-82.
61. Shah, H. N., Williams, R. A. D., Utilization of glucose and amino acids by *Bacteroides intermedius* and *Bacteroides gingivalis*. *Curr. Microbiol*. 1987, *15*, 241–246
62. Shibata, Y., Hiratsuka, K., Hayakawa, M., Shiroza, T., *et al*., A 35-kDa co-aggregation factor is a hemin binding protein in *Porphyromonas gingivalis*. *Biochem. Biophys. Res. Commun*. 2003, 300, 351-356.
63. Smalley, J. W., Thomas, M. F., Birss A. J., Withnall, R., Silver, J., A combination of both arginine- and lysine-specific gingipain activity of *Porphyromonas gingivalis* is necessary for the generation of the micro-oxo bishaem-containing pigment from haemoglobin. *Biochem. J*. 2004, *379*, 833-40.
64. Sztukowska, M., Bugno, M., Potempa, J., Travis, J., Kurtz Jr., D. M. Role of rubrerythrin in the oxidative stress response of *Porphyromonas gingivalis*. *Mol. Microbiol*. 2002, *44*, 479-88.
65. Teughels, W., Van Eldere, J., van Steenberghe, D., Cassiman, J. J., Influence of nicotine and cotinine on epithelial colonization by periodontopathogens. *J. Periodontol*. 2005, *76*, 1315-22.
66. Tonetti, M. S. Cigarette smoking and periodontal diseases: etiology and management of disease. *Ann. Periodontol*. 1998, *3*, 88-101.
67. Trivedi, A. H., Dave, B. J., Adhvaryu, S. G., Assessment of genotoxicity of nicotine employing in vitro mammalian test system. *Cancer. Lett*. 1990, *54*, 89-94.
68. Ueshima, J., Shoji, M., Ratnayake, D. B., Abe, K., *et al*., Purification, gene cloning, gene expression, and mutants of Dps from the obligate anaerobe *Porphyromonas gingivalis*. *Infect. Immun*. 2003, *71*, 1170-8.
69. Widera, D., Mikenberg, I., Kaltschmidt, B., Kaltschmidt, C., Potential role of NF-kappaB in adult neural stem cells: the underrated steersman? *Int. J. Dev. Neurosci*. 2006, *24*, 91-102.
70. Williams, R. C., Barnett, A. H., Claffey, N., Davis, M., *et al*., The potential impact of periodontal disease on general health: a consensus view. *Curr. Med. Res. Opin*. 2008, 24, 1635-43

71. Wu, J., Lin, X., Xie, H., OxyR is involved in coordinate regulation of expression of *fim*A and sod genes in *Porphyromonas gingivalis*. *FEMS Microbiol. Lett*. 2008, *282*, 188-95.
72. Xia, Q., Wang, T., Taub, F., Park, Y., *et al*., Quantitative proteomics of intracellular *Porphyromonas gingivalis*. *Proteomics*. 2007, *7*, 4323-37.
73. Yoshimura, F., Murakami, Y., Nishikawa, K., Hasegawa, Y., Kawaminami, S., Surface components of *Porphyromonas gingivalis*. *J. Periodontal. Res*. 2008 *in press.*
74. Yilmaz, O., The chronicles of *Porphyromonas gingivalis*: the microbium, the human oral epithelium and their interplay. *Microbiology*. 2008, *154*, 2897-903.
75. Zhang, Y., Wang, T., Chen, W., Yilmaz, O., *et al*., Differential protein expression by *Porphyromonas gingivalis* in response to secreted epithelial cell components. *Proteomics*. 2005, *5,* 198-211.
76. Zhao, Z., Reece, E. A., Nicotine-induced embryonic malformations mediated by apoptosis from increasing intracellular calcium and oxidative stress. *Birth Defects. Res. B. Dev. Reprod. Toxicol*. 2005, *74*, 383-91.

3.1. CAPÍTULO 2

The effects of nicotine and cotinine on *Porphyromonas gingivalis* adherence and invasion of epithelial cells

Running title: Nicotine and cotinine on *P. gingivalis*

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Abstract

Smoking is risk factor for development of periodontitis. *P. gingivalis* is an important colonizer of the subgingival crevice and is a major pathogenic agent in the initiation and progression of severe forms of periodontal disease. However, the effect of derivatives of tobacco and tobacco products on *P. gingivalis* is poorly understood. Therefore, the aim of this study to determine the influence of nicotine and cotinine on bacterial adherence and invasion of epithelial cells. KB cells monolayers and *P. gingivalis* ATCC 33277 were exposed to 0.1, 10 and 100 μg/mL of nicotine and cotinine concentrations. The epithelial cells were incubated for 24 h while *P. gingivalis* was exposed to these substances to early logarithmic phase. After the incubation period, *P. gingivalis* ability to adhere and invade KB cells was assayed. The number of bacteria associated with KB cells was assessed by counting the colony-forming unities. Cotinine at 100 μg/mL significantly increased *P. gingivalis* adherence and invasion of epithelial cells, when the bacteria was exposed to this substance ($p<0.05$; ANOVA – Tukey test). No other condition or drug altered the bacteria colonization ability ($p>0.05$). These data indicated that cotinine might interfere on *P. gingivalis* ability to invade and adhere to epithelial cells. Further studies are needed to evaluate the clinical relevancy of this study, analyzing whether oral cells might be more susceptible to be colonized by *P. gingivalis* in smokers.

Keywords: bacterial adhesion; bacterial invasion; epithelial cells; cotinine; nicotine; *Porphyromonas gingivalis.*

1. Introduction

Periodontal disease is an inflammatory disorder that comprises a group of infective agents that leads to periodontal attachment loss, gingival swelling and bleeding, alveolar bone injury and even tooth loss if not treated (Page, 1991). Associations between periodontitis and cardiovascular diseases have been recognized (Arbes *et al.*, 1999; Persson and Persson, 2008). Furthermore, this disease may increase the risk for preterm or low birth weight deliveries (Gibbs, 2001; Agueda *et al.*, 2008) and less controlled diabetes (Lalla *et al.*, 2006; Ebersole *et al.*, 2008).

Epidemiological studies indicate that cigarette smoking is also associated with the incidence and prevalence of a number of medical illnesses including low birth weight, cancer, pulmonary, cardiovascular, gastrointestinal, and periodontal diseases (Georgia and Margaret, 2004). Smokers are about three times more likely to develop periodontitis and the outcomes following non-surgical or surgical therapy are less promising (Johnson and Hill, 2004; Laxman and Annaji, 2008).

Both nicotine, one of the major tobacco smoking constituents, and cotinine, one of the most important metabolites of nicotine, have been recently indicated as participating on periodontal diseases pathways, impairing the host response by different mechanisms. Nicotine has been shown to induce cytokines release from cultured murine osteoblasts (Kamer *et al.*, 2006) as well as from in mouse blood cells (Makino *et al.*, 2008), affecting various functions of the human gingival fibroblasts (Lahmouzi *et al.*, 2000; Tanur *et al.*, 2000; Ho and Chang, 2006), influencing the alveolar bone loss by occlusal trauma (Nogueira-Filho *et al.*, 2004), and up-regulating the lipopolysaccharide-mediated monocyte secretion of PGE_2 (Payne *et al.*, 1996). Cotinine also inhibits the attachment and the growth of human fibroblasts (James *et al.*, 1999). However, few studies have shown direct or indirect effects of nicotine and cotinine on oral pathogens.

Porphyromonas gingivalis, a Gram negative, anaerobic bacterium, has been strongly associated with the etiology of periodontitis. This pathogen expresses several virulence factors including extracellular proteases, which damage host tissues, and adherence proteins that assist bacterial colonization (Lamont and Jenkinson, 1998). *P.*

gingivalis has been reported as having the ability to invade and survive into human epithelial and endothelial cells (Dorn *et al.*, 2000).

The adherence and invasion of bacteria to the host oral cells precede the development of many infectious processes. Some studies showed that bacterial colonization to epithelial cells was improved by tobacco smoke and its components (El Ahmer *et al.*, 1999; Chen *et al.*, 2002; Teughels *et al.*, 2005). The adherence and invasion of pathogenic bacteria, such as *Porphyromonas gingivalis*, to oral epithelial cells may play an important role in the induction and progression of chronic periodontitis (Lamont and Jenkinson, 2000) and the severity of inflammation (Vaahtoniemi *et al.*, 1993).

The possible relationship between increased bacterial cell colonization in host tissues and nicotine and cotinine exposure has been poorly investigated. The aim of the present study was to evaluate the ability of *P. gingivalis* to adhere and invade epithelial cells after bacterial and cellular exposure to different concentrations of nicotine and cotinine.

2. Materials and Methods

2.1. Bacterial strain and growth conditions

P. gingivalis ATCC 33277 was subcultured on brain heart agar - BHA (Difco Co., Detroit, MI, USA) supplemented with 7% defibrinated sheep blood, 5 μg/mL haemin and 1 μg/mL menadione (Sigma Chemical Co., St Louis, MO, USA). For the adherence and invasion tests, *P. gingivalis* were grown in brain heart infusion broth – BHI (Difco), also complemented with 5 μg/mL haemin and 1 μg/mL menadione. The bacterial cultures were incubated under anaerobic conditions (10% CO_2, 10% H_2 and 80% N_2 - MiniMacs Anaerobic Workstation, Don Whitley Scientific, Shipley, UK) at 37 ^{o}C.

2.2. Experimental design

For adherence and invasion tests, bacteria or cells, in independent experiments, were previously incubated with different concentrations of nicotine and

cotinine (Sigma). KB cells were exposed 24 h to the substances, while *P. gingivalis* were incubated until the cultures reached the optical density (OD) of 1.2 (logarithmic phase, approximately 16 h) at 660 nm wave-length. Cells or bacteria that were not previously incubated with nicotine or cotinine were used as controls. Nicotine was diluted in distilled and sterilized water and cotinine in ethanol (0.8% v/v). These substances were assayed at concentrations of 100, 10 and 0.1 μg/mL.

2.3. Adherence and Invasion assays

Adherence and invasion of *P. gingivalis* were assessed in human epithelial cells (KB cells), kindly provided by Professor MPA Mayer (Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo). These tests were carried out according to the methods previously described (Lamont *et al.*, 1995; Umeda *et al.*, 2006) with slight modifications. The KB cells were kept in DMEM-PS medium comprised of Dulbecco's modified Eagle medium (DMEM) (Gibco, United Kingdom), 10% fetal calf serum (Cultilab, Campinas, Brazil), pen/strep/glut solution (100 units/mL penicillin, 100 μg/mL streptomycin and 0.292 mg/mL L-glutamine - Gibco), MEM non essential and essential amino acids solutions (Gibco) and 2.20 g/L sodium bicarbonate (Sigma). KB cells were cultured in 25 cm^2 tissue culture flasks (Corning Inc., Corning, NY, USA) at 37 ^oC in an atmosphere of 5% CO_2. From these cultures flasks, about 2 x 10^5 KB cells were inoculated into each well of 24-well tissue culture plates (Corning) and incubated at 37 ^oC for 24 h to reach a confluent monolayer (approximately 2.5 x 10^5 KB cells) in DMEM-PS. *P. gingivalis* were cultivated as mentioned before (OD at A_{660nm} = 1.2). The bacterial cultures were centrifuged at 5000 rpm, for 10 min and resuspended in DMEM without antibiotics and serum at 1 x 10^8 cfu/mL. Prior to bacterial infection, the wells containing KB cells were washed three times with phosphate-buffered saline (PBS) (pH 7.5, 0.8% NaCl).

For adherence assays, the bacterial suspensions were added to the confluent KB monolayers at a multiplicity of infection of 1:200 and incubated at 37 ^oC in 5% CO_2 for 90 min. After incubation, the wells were washed three times with PBS to remove unattached

bacteria. The KB cells were then detached from the wells by means of incubation using with 1 mL trypsin-EDTA (Gibco) diluted in PBS at 37 °C for 2 min. The suspensions were serially diluted, plated on BHA plates with 7% defibrinated sheep blood, 5 μg/mL haemin and 1 μg/mL menadione, and incubated anaerobically at 37 ^{o}C for 5 days. The colony forming units (cfu) concerning adhered bacteria were determined by viable counting.

For invasion assays, the *P. gingivalis* suspensions were added to wells containing KB cell monolayers at the same multiplicity of infection of 1:200. Tissue culture plates were centrifuged at 2000 *g*, at 4 ^{o}C, for 10 min, before incubating at 37 ^{o}C in 5% CO_2 atmosphere for 3 h. Unattached bacteria were then removed by washing the wells three times with PBS. External adherent bacteria were killed by incubating the infected monolayers with DMEM containing metronidazole 0.1 mg/mL (Sigma) and gentamicin 0.5 mg/mL (Gibco) for 60 min. The wells were washed three times with PBS, and KB cells were lysed by means of incubation in 1 mL of cold sterile water for 20 min. Cell lysates were recovered, plated on to BHA medium and grown at 37 ^{o}C for 5 days. The cfu of invasive bacteria was determined by colony counting. All assays were performed in triplicate, at least in two different experiments.

2.4. Statistical analyses

Data concerning adhered and invasive *P. gingivalis* were analyzed by using ANOVA while statistical differences among control and each concentration group were determined by Tukey's post hoc test. All statistical analyses were carried out at a significance level of 5% (BioEstat version 5.0, Mamiraua/CNPq, Belém, PA, Brazil).

3. Results

3.1. Nicotine and cotinine effects on bacterial adherence

Figure 1A summarizes the effects of nicotine and cotinine on bacterial adherence, when *P. gingivalis* was exposed to either nicotine or cotinine. Non-exposed bacteria were used as control. In relation to the nicotine exposure, no statistically

significant differences (p>0.05) were observed in the number of bacteria adhered to the epithelial cells. When exposed to the cotinine at 0.1 and 10 μg/mL concentrations, no differences were also observed. However, at the concentration of 100 μg/mL, cotinine increased the bacterial adherence significantly (p<0.05).

Figure 1B shows the results concerning *P. gingivalis* adherence when the epithelial cells were incubated with the substances. The cfu/mL of bacteria associated to the cells did not differ among treatments and control (p>0.05).

3.2. Nicotine and cotinine effects on bacterial invasion

Mean values regarding *P. gingivalis* invasion into epithelial cells after exposing bacteria to nicotine and cotinine are shown in figure 2A. A significant increase (p<0.01) in bacterial invasion was observed in the 100 μg/mL cotinine group. No statistically significant differences (p>0.05) were observed considering the other groups.

Comparing bacterial invasion among experimental groups and their controls when KB cells were exposed (24 h) to nicotine and cotinine, the mean values of cfu/mL of the experimental groups were similar to those obtained in control. As shown in figure 2B, no statistically significant differences were found (p>0.05).

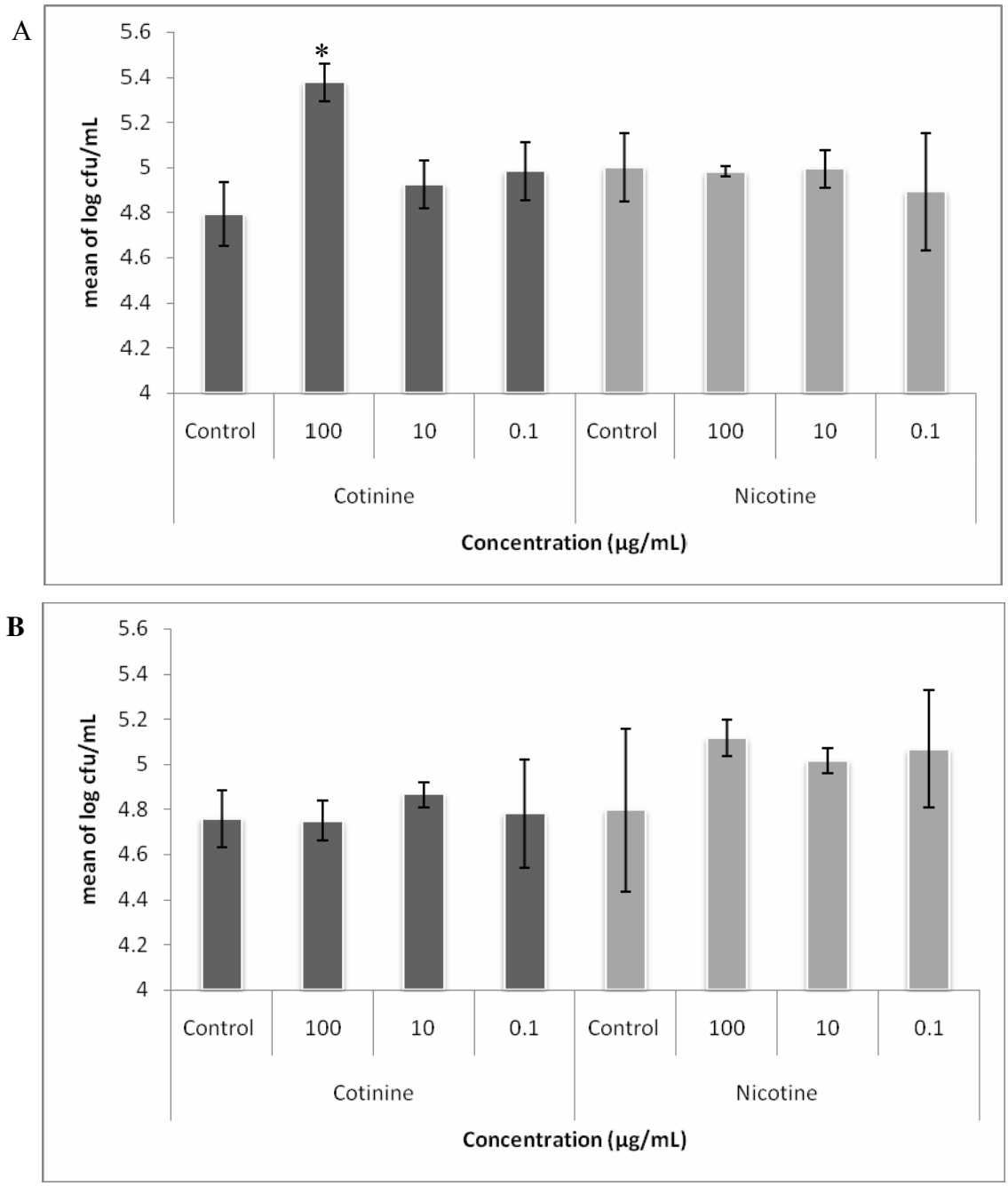

Figure 1. Mean of LOG cfu/mL and standard deviation of bacterial adherence to epithelial cells when *P. gingivalis* (A) or KB cells (B) were exposed to nicotine and cotinine. Non-exposed bacteria or cells were used as controls. Statistically significant *p* values are shown (*) above the columns.

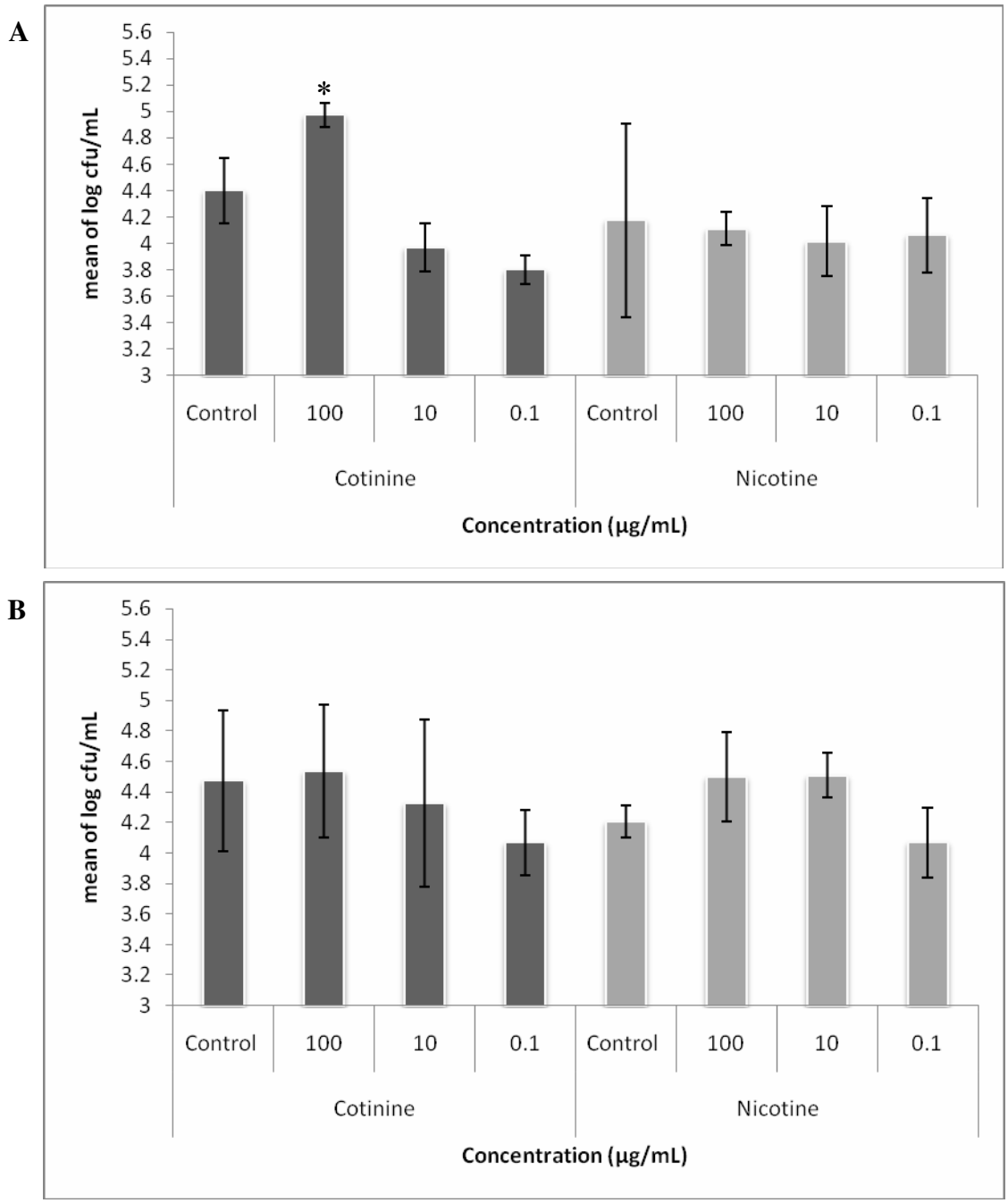

Figure 2. Mean of LOG cfu/mL and standard deviation of bacterial invasion of epithelial cells when *P. gingivalis* (A) or KB cells (B) were exposed to nicotine and cotinine. Non-exposed bacteria or cells were used as controls. Statistically significant *p* values are shown (*) above the columns.

3.3. Epithelial cells viability after exposure to nicotine and cotinine

The epithelial cells were incubated with nicotine and cotinine for 24 h. A cell viability assay was performed using Trypan blue staining. Table 1 shows the number of cells/mL of KB cells after incubation. No statistically significant differences (p>0.05) were observed in any group.

Table 2. Effect of cotinine and nicotine on KB cells viability

Experimental Group	Mean number of cells/mL	SD
cotinine control	2.13×10^5	1.25×10^4
cotinine 100 µg/mL	2.12×10^5	2.14×10^4
cotinine 10 µg/mL	2.33×10^5	2.60×10^4
cotinine 0.1 µg/mL	2.21×10^5	4.01×10^4
nicotine control	2.81×10^5	7.92×10^4
nicotine 100 µg/mL	2.63×10^5	5.30×10^4
nicotine 10 µg/mL	2.13×10^5	5.30×10^4
nicotine 0.1 µg/mL	2.75×10^5	3.54×10^4

4. Discussion

P. gingivalis is an important pathogen involved in the onset and development of periodontal diseases, mainly due to its ability to invade and survive within the host cells (Dorn *et al.*, 2000). The tobacco derivatives, nicotine and cotinine, have also been reported as playing an important role in the progression of periodontitis (Johnson and Hill, 2004; Laxman and Annaji, 2008). Nicotine and cotinine have also been pointed out as important tobacco's substances that may be implicated in periodontitis progression (Leow *et al*., 2006; Makino *et al*., 2008). Nevertheless, the biological mechanisms of this interaction are not well understood. Therefore, was aimed at investigating whether nicotine and cotinine could interfere with the susceptibility of epithelial cells to be colonized by *P. gingivalis*.

In the present study, only the highest concentration of cotinine interfered with *P. gingivalis* adherence and invasion abilities. Cotinine in lower concentrations and

nicotine have not showed any significant effect on bacterial colonization of the epithelial cells. Furthermore, cotinine affected the colonization of KB cells by *P. gingivalis* only when the bacterium was first exposed to cotinine, i.e., *P. gingivalis* must be directly exposed to cotinine to increase its colonization of epithelial cells. The previous epithelial cells exposure to cotinine or nicotine did not change the adherence/invasion properties of *P. gingivalis.* In a proteomic study, it was also observed that, when *P. gingivalis* cultures were exposed to cotinine 600 µg/mL, one Arg-gingipain protein isoform was exclusively expressed by this bacterium (data not published). This proteinase is one of the most important factors that contribute to *P. gingivalis* adherence to mammalian cells (Lamont and Jenkinson, 1999).

Other studies have investigated the effect of nicotine and cotinine on the bacterial adherence and invasion abilities. Nicotine effects were tested on *Escherichia coli* invasion into human brain microvascular endothelial cells (HBMEC) (Chen *et al.*, 2002). After HBMEC incubation with 10^{-5} to 10^{-7} M nicotine tartrate during 24 h, the levels of *E. coli* invasion were higher when compared to HBMEC control. In the present study, however, nicotine had no influence on invasion of *P. gingivalis* in the epithelial cells. Considering these contrasting findings, it is possible to assume that nicotine effects on bacterial colonization to the host cells can be species-specific or dependent on the human cell structure.

Nicotine and cotinine effects on epithelial colonization by periodontopathogens were previously studied by Teughels *et al.* (2005). *P. gingivalis* colonization of primary epithelial was significantly decreased when exposed to 1mg/mL nicotine. However, when HeLa cells were incubated with a range of cigarette smoke extract (CSE), no effect was detected in the bacterial association to these cells. Such findings are in disaccord with those observed in the present study, in which no correlation was found between *P. gingivalis* colonization to epithelial cells and nicotine considering all concentrations tested. Thus, the divergent outcomes could be due to the different nicotine concentrations tested. In addition, Teughels *et al.* incubated only the epithelial cells with nicotine and cotinine, while

in the present study both epithelial cells and *P. gingivalis* were exposed to nicotine and cotinine.

There are few reports regarding the effect of cigarette smoking on the adherence of respiratory tract bacteria to epithelial cells. El Ahmer *et al.* (1999) studied the bacterial binding to the epithelial cells of smokers and non-smokers by using flow cytometry. They observed that buccal epithelial cells (BEC) from smokers were more vulnerable to be colonized by respiratory pathogens as *Neisseria meningitidis*, *Neisseria lactamica*, *Streptococcus pneumoniae*, *Bordetella pertussis*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Staphylococcus aureus* than BEC from non-smokers. In addition, BEC of non-smokers treated with CSE were also more susceptible to the bacterial binding. Adherence of bacteria to pharyngeal cells obtained from non-smokers and smokers were studied by Fainstein and Musher (1979). These authors reported an increased adherence for some species such as *S. pneumoniae*, *S. aureus* and *H. influenza* to the cells derived from smokers.

Piatti *et al.* (1997) investigated the adherence of some respiratory pathogens to buccal cells and observed an increased pneumococcal adherence in smokers compared to non-smokers. No differences were found to *S. aureus*, *H. influenzae* and *Pseudomonas aeruginosa* adherence, showing that these abilities could be cell dependent. Ozlu *et al.* (2008) showed changes in the *Streptococcus pneumoniae* adherence to BEC in rats chronically exposed to high levels of cigarette smoke. Based on these previous investigations, cigarette smoke can also alter bacterial colonization to the epithelial cells. These findings are in accord with the results from the present work, despite the differences in methodologies and experimental design.

To evaluate nicotine and cotinine effect on cell viability, KB cells were exposed to these substances for 24 h and the number of viable cells was counted and compared to the cells that were not exposed. Neither nicotine nor cotinine was observed to affect cell viability. The nicotine and cotinine effect on bacterial viability were not assessed in this study due to the previous findings in literature showing that these substance did not alter *P. gingivalis* viability and growth. Teughels *et al.* (2005) investigated the effect of nicotine

and cotinine at concentrations ranging from 10 to 1000 μg/mL on *P. gingivalis* viability and no significant differences were found. In a previous study, these smoke substances showed no influence on *P. gingivalis* growth in both planktonic and biofilm forms (Cogo *et al.*, 2008).

P. gingivalis prevalence and proportions in subgingival microbiota from smokers have been studied. Some studies have reported no differences in the prevalence of subgingival species of this pathogen between smokers and non-smokers with periodontitis (Van der Velden *et al.*, 2003; Apatzidou *et al.*, 2005; Salvi *et al.*, 2005). However, some authors showed that smoking increases the likelihood of prevalence and proportions of this microorganism (Zambon *et al.*, 1996; Haffajee and Socransky, 2001).

KB cells, used in the present study to test bacterial adherence and invasion, were considered for many years as cells derived from oral cancer, but actually they originated from a glandular cancer of the cervix (Masters *et al.*, 2002). These cells have been widely used in biomedical research to study many pathways, including the interaction between *P. gingivalis* and cells from oral cavity.

The concentrations of nicotine and cotinine used in this study were satisfactory for the assessment of their effects, since it is difficult to measure total nicotine concentration from cigarette smoking in saliva and crevicular fluid due to its short half life. Based on the Massachusetts smoking regimen for all cigarette brand styles and major market categories from 1997 to 2005, nicotine ranges from 1.10 to 3.40 mg/cigarette (Connolly *et al.*, 2007). The mean levels of cotinine described in saliva and crevicular fluid vary from 7.978 μg/mL to 15.027 μg/mL and 2.259 μg/mL to 3.186 μg/mL, respectively (Chen *et al.*, 2001). Although the low concentration of nicotine and cotinine used in the present study are close to the levels found in human saliva and gingival fluid, the others are higher. However, some studies have used high nicotine and cotinine levels in *P. gingivalis* experiments to induce cellular effects (Sayers *et al.,* 1997; Teughels *et al.,* 2005).

In conclusion, cotinine at a high concentration might improve *P. gingivalis* adherence as well as its invasion into epithelial cells; however, such outcome was observed only when the pathogen tested was previously exposed to cotinine.

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6. References:

1. Agueda A, Echeverría A, Manau C. Association between periodontitis in pregnancy and preterm or low birth weight: Review of the literature. *Med Oral Patol Oral Cir Bucal* 2008; **13**: 609-15.
2. Apatzidou DA, Riggio MP, Kinane DF. Impact of smoking on the clinical, microbiological and immunological parameters of adult patients with periodontitis. *J Clin Periodontol* 2005; **32**: 973-83.
3. Arbes SJ Jr, Slade GD, Beck JD. Association between extent of periodontal attachment loss and self-reported history of heart attack: an analysis of NHANES III data. *J Dent Res* 1999; **78**: 1777-82.
4. Chen YH, Chen SH, Jong A, Zhou ZY, Li W, Suzuki K, *et al.* Enhanced *Escherichia coli* invasion of human brain microvascular endothelial cells is associated with alternations in cytoskeleton induced by nicotine. *Cell Microbiol* 2002; **4**: 503-14.
5. Chen X, Wolff L, Aeppli D, Guo Z, Luan W, Baelum V, Fejeskov O. Cigarette smoking, salivary/gingival crevicular fluid cotinine and periodontal status. A 10-year longitudinal study. *J Clin Periodontol* 2001; **28**: 331-9.
6. Cogo K, Montan MF, Bergamaschi C de C, D Andrade E, Rosalen PL, Groppo FC. In vitro evaluation of the effect of nicotine, cotinine, and caffeine on oral microorganisms. *Can J Microbiol* 2008; **54**: 501-8.

7. Connolly GN, Alpert HR, Wayne GF, Koh H. Trends in nicotine yield in smoke and its relationship with design characteristics among popular US cigarette brands, 1997-2005. *Tob Control* 2007; **16**: e5.
8. Dorn BR, Burks JN, Seifert KN, Progulske-Fox A. Invasion of endothelial and epithelial cells by strains of *Porphyromonas gingivalis*. *FEMS Microbiol Lett* 2000; **187**: 139–144.
9. Ebersole JL, Holt SC, Hansard R, Novak MJ. Microbiologic and immunologic characteristics of periodontal disease in Hispanic Americans with type 2 diabetes. *J Periodontol* 2008; **79**: 637–646.
10. El Ahmer OR, Essery SD, Saadi AT, Raza MW, Ogilvie MM, Weir DM, *et al.* The effect of cigarette smoke on adherence of respiratory pathogens to buccal epithelial cells. *FEMS Immunol Med Microbiol* 1999; **23**: 27-36.
11. Fainstein V, Musher DM Bacterial adherence to pharyngeal cells in smokers, nonsmokers, and chronic bronchitics. *Infect Immun* 1979; **26**: 178-82.
12. Georgia KJ, Margaret H. Cigarette smoking and the periodontal patient. State of the Art Review. *J Periodontol* 2004; **75**: 196-209.
13. Gibbs RS. The relationship between infections and adverse pregnancy outcomes: an overview. *Ann Periodontol* 2001; **6**: 153-63.
14. Haffajee AD, Socransky SS Relationship of cigarette smoking to the subgingival microbiota. *J Clin Periodontol* 2001; **28**: 377-88.
15. Ho YC, Chang YC. Regulation of nicotine-induced cyclooxygenase-2 protein expression in human gingival fibroblasts. *Acta Pharmacol Sin* 2006; **27**: 409-13.
16. James JA, Sayers NM, Drucker DB, Hull PS. Effects of tobacco products on the attachment and growth of periodontal ligament fibroblasts. *J Periodontol* 1999; **70**: 518-25.
17. Johnson GK, Hill M. Cigarette smoking and the periodontal patient. *J Periodontol* 2004; **75**: 196-209.

18. Kamer AR, El-Ghorab N, Marzec N, Margarone JE 3rd, Dziak R. Nicotine induced proliferation and cytokine release in osteoblastic cells. *Int J Mol Med* 2006; **17**: 121-7**.**
19. Lalla E, Kaplan S, Chang SM, Roth GA, Celenti R, Hinckley K, *et al.* Periodontal infection profiles in type 1 diabetes. *J Clin Periodontol* 2006; **33**: 855-62.
20. Lamont RJ, Chan A, Belton CM, Izutsu KT, Vasel D, Weinberg A. *Porphyromonas gingivalis* invasion of gingival epithelial cells. *Infect Immun* 1995; **63**: 3878–3885.
21. Lamont RJ, Jenkinson HF. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. *Microbiol Mol Biol Rev* 1998; **62**: 1244–1263.
22. Lamont RJ, Jenkinson HF. Subgingival colonization by *Porphyromonas gingivalis*.*Oral Microbiol Immunol* 2000; **15**: 341-9.
23. Lahmouzi J, Simain-Sato F, Defresne MP, De Pauw MC, Heinen E, Grisar T, *et al.* Effect of nicotine on rat gingival fibroblasts in vitro. *Connect Tissue Res* 2000; **41**: 69-80.
24. Laxman VK, Annaji S. Tobacco use and its effects on the periodontium and periodontal therapy. *J Contemp Dent Pract* 2008; **9**: 97-107.
25. Leow NM, Morel-Kopp MC, Woodward M, Chen Q, Tofler GH, Taylor BA. The relationship between cotinine and periodontal disease. *Ann R Australas Coll Dent Surg* 2006; **18**: 51-2.
26. Makino A, Yamada S, Okuda K, Kato T. Nicotine involved in periodontal disease through influence on cytokine levels. *FEMS Immunol Med Microbiol* 2008; **52**: 282-6.
27. Masters JR. HeLa cells 50 years on: the good, the bad and the ugly. *Nat Rev Cancer* 2002; **2**: 315-9.
28. Nogueira-Filho GR, Fróes Neto EB, Casati MZ, Reis SR, Tunes RS, Tunes UR, *et al.* Nicotine effects on alveolar bone changes induced by occlusal trauma: a histometric study in rats. *J Periodontol* 2004; **75**: 348-52.

29. Ozlu T, Celik I, Oztuna F, Bulbul Y, Ozsu S. *Streptococcus pneumoniae* adherence in rats under different degrees and durations of cigarette smoke. *Respiration* 2008; **75**: 339-44.
30. Page RC. The role of inflammatory mediators in the pathogenesis of periodontal disease. *J Periodont Res* 1991; **26**: 230–42.
31. Payne JB, Johnson GK, Reinhardt RA, Dyer JK, Maze CA, Dunning DG. Nicotine effects on PGE2 and IL-1 beta release by LPS-treated human monocytes. *J Periodontal Res* 1996; **1**: 99-104.
32. Persson GR, Persson RE. Cardiovascular disease and periodontitis: an update on the associations and risk. *J Clin Periodontol* 2008; **35**: 362-79.
33. Piatti G, Gazzola T, Allegra L. Bacterial adherence in smokers and non-smokers. *Pharmacol Res* 1997; **36**: 481-4.
34. Salvi GE, Ramseier CA, Kandylaki M, Sigrist L, Awedowa E, Lang NP. Experimental gingivitis in cigarette smokers: a clinical and microbiological study. *J Clin Periodontol* 2005; **32**: 441-7.
35. Sayers, N.M., James, J.A., Drucker, D.B., and Blinkhorn A.S. Possible potentiation of toxins from *Prevotella intermedia*, *Prevotella nigrescens*, and *Porphyromonas gingivalis* by cotinine. *J. Periodontol* 1999; **70**: 1269-75.
36. Tanur E, McQuade MJ, McPherson JC, Al-Hashimi IH, Rivera-Hidalgo F. Effects of nicotine on the strength of attachment of gingival fibroblasts to glass and non-diseased human root surfaces. *J Periodontol* 2000; **71**: 717-22.
37. Teughels W, Van Eldere J, van Steenberghe D, Cassiman JJ, Fives-Taylor P, Quirynen M. Influence of nicotine and cotinine on epithelial colonization by periodontopathogens. *J Periodontol* 2005; **76**: 1315-22.
38. Umeda JE, Missailidis C, Longo PL, Anzai D, Wikström M, Mayer MP. Adhesion and invasion to epithelial cells by *fimA* genotypes of *Porphyromonas gingivalis*. *Oral Microbiol Immunol* 2006; **21**: 415-9.

39. Vaahtoniemi LH, Räisänen S, Stenfors LE. Attachment of bacteria to oral epithelial cells in vivo: a possible correlation to gingival health status. *J Periodontal Res* 1993; **28**: 308-11.
40. Van der Velden U, Varoufaki A, Hutter JW, Xu L, Timmerman MF, Van Winkelhoff AJ, *et al.* Effect of smoking and periodontal treatment on the subgingival microflora. *J Clin Periodontol* 2003; **30**: 603-10.
41. Zambon JJ, Grossi SG, Machtei EE, Ho AW, Dunford R, Genco RJ. Cigarette smoking increases the risk for subgingival infection with periodontal pathogens. *J Periodontol* 1996; **67**: 1050-4.

4. CONSIDERAÇÕES GERAIS

P. gingivalis é um importante patógeno envolvido no início e progressão da doença periodontal, principalmente pela sua capacidade de invadir e sobreviver no interior das células (Dorn *et al*., 2000), além de possuir vários fatores de virulência que possibilitam a destruição dos tecidos e das defesas imunológicas do hospedeiro (Lamont & Jenkinson, 2000). A relação entre o hábito de fumar e o desenvolvimento das periodontites já está bem estabelecida (Johnson and Hill, 2004; Laxman and Annaji, 2008). A nicotina e a cotinina têm sido apontadas como importantes substâncias derivadas do cigarro que podem participar do processo que leva ao aparecimento e desenvolvimento das doenças que atingem o periodonto. No entanto, os mecanismos biológicos dessa possível interação bactéria – nicotina/cotinina foram muito pouco estudados. Dessa forma, o presente estudo teve como objetivo avaliar os possíveis mecanismos pelos quais a nicotina e a cotinina podem interagir com a *P. gingivalis*, através da análise de expressão de proteínas dessa bactéria e da sua capacidade de adesão e invasão às células epiteliais.

Em relação à análise de expressão de proteínas, foi observado que a nicotina e a cotinina puderam alterar consideravelmente a produção das mesmas. Na presença dessas substâncias derivadas do cigarro, *P. gingivalis* reduziu ou produziu exclusivamente alguns fatores de virulência, aumentou a expressão de proteínas relacionadas ao estresse oxidativo, produziu em grande quantidade proteínas envolvidas no processo de energia celular enquanto deixou de expressar algumas proteínas que não eram essenciais para a viabilidade bacteriana, provavelmente para poupar energia. Talvez, em condições experimentais *in vivo* ou até mesmo no ambiente do hospedeiro, *P. gingivalis* tivesse outro perfil de síntese de proteínas na presença dessas substâncias. No entanto, apesar das condições *in vitro* não representarem todo o universo de situações que ocorrem *in vivo*, esses achados são uma previsão do que poderia ocorrer biologicamente, ou seja, uma adaptação bacteriana a uma nova situação, a presença da nicotina e cotinina.

Quanto à capacidade de adesão e invasão de *P. gingivalis* às células epiteliais, somente a cotinina na maior concentração testada foi capaz de aumentá-la. Ainda, essa alteração ocorreu somente quando a bactéria foi exposta a cotinina. Esses achados indicam que, essa substância pode causar alguma alteração diretamente na bactéria, sem alterar a sua viabilidade.

Apesar de a cotinina estimular a colonização de *P. gingivalis* às células epiteliais, somente uma proteína relacionada à função de adesão e invasão, a Arg-gingipain, foi expressa exclusivamente no tratamento cotinina 600 μg/mL, como observado nos ensaios de avaliação proteômica de *P. gingivalis*. No estudo do proteoma, a bactéria foi exposta à cotinina, mas não às células epiteliais. Já no estudo de adesão e invasão, *P. gingivalis* foi incubada com a cotinina e com as células epiteliais. Possivelmente, a presença das próprias células epiteliais somada às altas concentrações de cotinina seja um estímulo maior para a produção de fatores de colonização do que somente a exposição à essa substância derivada do fumo.

Para as avaliações desse estudo, foram utilizadas duas cepas distintas de *P. gingivalis*. Na análise proteômica foi utilizada a cepa W83, cujo genoma foi recentemente seqüenciado (Nelson et al., 2003). Nos ensaios de adesão e invasão às células epiteliais, a cepa escolhida para as análises foi a ATCC 33277. A opção por essa cepa foi feita devido a sua maior capacidade de invasão. Inclusive, em testes pilotos realizados previamente ao presente estudo, foi verificada a baixa capacidade de invasão de *P. gingivalis* W83. Apesar de *P. gingivalis* W83 (*fimA* IV) ser considerada uma das mais virulentas da sua espécie, essa cepa é considerada pouco invasiva, enquanto a ATCC 33277 (*fimA* I) possui maior capacidade de invasão às células (Dorn *et al*., 2000; Umeda *et al*., 2006). De qualquer forma, a utilização de cepas diferentes não deixa de permitir comparações entre os dois trabalhos conduzidos nesse estudo.

As concentrações de nicotina e cotinina utilizadas nos dois estudos conduzidos foram selecionadas de acordo com resultados obtidos em estudos pilotos. Para o primeiro estudo conduzido, a avaliação proteômica, culturas de *P. gingivalis* foram expostas à

nicotina e cotinina, e em concentrações superiores a 700 μg/mL, a viabilidade bacteriana começava a mostrar leves sinais de alteração. Portanto, foram selecionadas duas concentrações de trabalho: 600 μg/mL e 6 μg/mL, essa última sendo uma concentração próxima à encontrada na saliva de fumantes. Para o segundo estudo, quando as células epiteliais foram expostas à concentração de 600 μg/mL, houve uma redução da viabilidade das mesmas. Dessa forma, concentrações mais baixas foram utilizadas no estudo de adesão e invasão de *P. gingivalis* às células epiteliais.

Por fim, o presente estudo, através dos resultados encontrados, pode trazer uma considerável contribuição para elucidar os mecanismos que envolvem a interação da nicotina, cotinina e a *P. gingivalis* e talvez ainda, fornecer subsídios para entender a relação dessas com a doença periodontal.

5. CONCLUSÃO

Os resultados do presente estudo indicam que a nicotina e a cotinina podem interferir na expressão de proteínas da *Porphyromonas gingivalis*. Além disso, a cotinina pode alterar a capacidade de adesão e invasão de *P. gingivalis* às células epiteliais, quando a bactéria é exposta à essa substância. Estudos futuros são necessários para saber o mecanismo pelos quais essas substâncias atuam e para conhecer a relevância fisiológica desses achados.

6. REFERÊNCIAS[2]

1. Amano A, Nakagawa I, Okahashi N, Hamada N. Variations of *Porphyromonas gingivalis* fimbriae in relation to microbial pathogenesis. J Periodontal Res. 2004; 39(2): 136-42.
2. Apatzidou DA, Riggio MP, Kinane DF. Impact of smoking on the clinical, microbiological and immunological parameters of adult patients with periodontitis. J Clin Periodontol. 2005; 32(9): 973-83.
3. Armitage AK, Turner DM. Absorption of nicotine in cigarette and cigar smoke through the oral mucosa. Nature. 1970; 226(5252): 1231-2.
4. Austin GW, Cuenin MF, Hokett SD, Peacock ME, Sutherland DE, Erbland JF *et al*. Effect of nicotine on fibroblast beta 1 integrin expression and distribution *in vitro*. J Periodontol. 2001; 72(4): 438-44.
5. Baharin B, Palmer RM, Coward P, Wilson RF. Investigation of periodontal destruction patterns in smokers and non-smokers. J Clin Periodontol. 2006 Jul;33(7):485-90.
6. Bergström J, Eliasson S, Dock J. A 10-year prospective study of tobacco smoking and periodontal health. J Periodontol. 2000; 71(8): 1338-47.
7. Bergström J, Preber H. Tobacco use as a risk factor. J Periodontol. 1994; 65(5 Suppl): 545-50.
8. Bostrom L, Bergstrom J, Dahlen G, Linder LE. Smoking and subgingival microflora in periodontal disease. J Clin Periodontol. 2001; 28(3): 212-9.
9. Burgess NA, Kirke DF, Williams P, Winzer K, Hardie KR, Meyers NL, Aduse-Opoku J, Curtis MA, Camara M. LuxS-dependent quorum sensing in *Porphyromonas gingivalis* modulates protease and haemagglutinin activities but is not essential for virulence. Microbiology. 2002; 148 (Pt 3): 763-72.

[2] De acordo com a norma da FOP/UNICAMP, baseadas na norma do International Committee of Medical Journal Editors – Grupo de Vancouver. Abreviatura dos periódicos em conformidade com o Medline.

10. Calsina G, Ramon JM, Echeverria JJ. Effects of smoking on periodontal tissues. J Clin Periodontol. 2002; 29(8): 771-6.
11. Chang YC, Huang FM, Tai KW, Yang LC, Chou MY. Mechanisms of cytotoxicity of nicotine in human periodontal ligament fibroblast cultures *in vitro*. J Periodontal Res. 2002; 37(4): 279-85.
12. Chen T, Duncan MJ. Gingipain adhesin domains mediate *Porphyromonas gingivalis* adherence to epithelial cells. Microb Pathog 2004. 36:205-209.
13. Chung WO, Park Y, Lamont RJ, McNab R, Barbieri B, Demuth DR. Signaling system in *Porphyromonas gingivalis* based on a LuxS protein. J Bacteriol. 2001; 183 (13): 3903-9.
14. Cogo K, Montan MF, Bergamaschi C de C, D Andrade E, Rosalen PL, Groppo FC. In vitro evaluation of the effect of nicotine, cotinine, and caffeine on oral microorganisms. Can J Microbiol. 2008; 54(6):501-8.
15. Dorn BR, Burks JN, Seifert KN, Progulske-Fox A. Invasion of endothelial and epithelial cells by strains of *Porphyromonas gingivalis*. FEMS Microbiol Lett. 2000; 187 (2): 139-44.
16. Eick S, Reissmann A, Rödel J, Schmidt KH, Pfister W. *Porphyromonas gingivalis* survives within KB cells and modulates inflammatory response. Oral Microbiol Immunol. 2006; 21 (4): 231-7.
17. Elter JR, Beck JD, Slade GD, Offenbacher S. Etiologic models for incident periodontal attachment loss in older adults. J Clin Periodontol. 1999; 26(2): 113-23.
18. Eramo S, Tassi C, Negri P, Manta M, Fraschini M, Pedetta F. ELISA analysis of salivary cotinine in smokers. Minerva Stomatol. 2000; 49(4): 163-8.
19. Fang Y, Svoboda KK. Nicotine inhibits myofibroblast differentiation in human gingival fibroblasts. J Cell Biochem. 2005; 95(6): 1108-19.
20. Frias J, Olle E, Alsina M. Periodontal pathogens produce quorum sensing signal molecules. Infect Immun. 2001; 69 (5): 3431-4.

21. Ghosheh OA, Browne D, Rogers T, de Leon J, Dwoskin LP, Crooks PA. A simple high performance liquid chromatographic method for the quantification of total cotinine, total 3'-hydroxycotinine and caffeine in the plasma of smokers. J Pharm Biomed Anal. 2000; 23(2-3): 543-9.
22. Goulbourne PA, Ellen RP. Evidence that *Porphyromonas* (*Bacteroides*) *gingivalis* fimbriae function in adhesion to *Actinomyces viscosus.* J. Bacteriol. 1991, 173 (17): 5266–5274. Grenier D. Demonstration of a bimodal coaggregation reaction between *Porphyromonas gingivalis* and *Treponema denticola*. Oral Microbiol Immunol. 1992, 7 (5): 280-4.
23. Grenier D. Demonstration of a bimodal coaggregation reaction between *Porphyromonas gingivalis* and *Treponema denticola*. Oral Microbiol Immunol. 1992, 7 (5): 280-4.
24. Grenier D. Effect of chlorhexidine on the adherence properties of *Porphyromonas gingivalis*.J Clin Periodontol. 1996; 23 (2): 140-2.
25. Grenier D, Roy E, Mayrand D. Modulation of *Porphyromonas gingivalis* proteínase activity by suboptimal doses of antimicrobial agents. J Periodontol. 2003; 74 (9): 1316-9.
26. Grossi SG, Zambon JJ, Ho AW, Koch G, Dunford RG, Machtei EE, *et al*. Assessment of risk for periodontal disease. I. Risk indicators for attachment loss. J Periodontol. 1994; 65(3): 260-7.
27. Grossi SG, Genco RJ, Machtei EE, Ho AW, Koch G, Dunford R, *et al*. Assessment of risk for periodontal disease. II. Risk indicators for alveolar bone loss. J Periodontol. 1995; 66(1): 23-9.
28. Gunsolley JC, Quinn SM, Tew J, Gooss CM, Brooks CN, Schenkein HA. The effect of smoking on individuals with minimal periodontal destruction. J Periodontol. 1998; 69(2): 165-70.

29. Haber J, Wattles J, Crowley M, Mandell R, Joshipura K, Kent RL. Evidence for cigarette smoking as a major risk factor for periodontitis. J Periodontol. 1993; 64(1): 16-23.
30. Haffajee AD, Socransky SS. Microbial etiological agents of destructive periodontal diseases. Periodontol 2000. 1994; 5: 78-111.
31. Haffajee AD, Socransky SS (1). Relationship of cigarette smoking to attachment level profiles. J Clin Periodontol. 2001; 28(4): 283-95.
32. Haffajee AD, Socransky SS (2). Relationship of cigarette smoking to the subgingival microbiota. J Clin Periodontol. 2001; 28(5): 377-88.
33. Henemyre CL, Scales DK, Hokett SD, Cuenin MF, Peacock ME, Parker MH, *et al.* Nicotine stimulates osteoclast resorption in a porcine marrow cell model. J Periodontol. 2003; 74(10): 1440-6.
34. Imamura T, Matsushita K, Travis J, Potempa J. Inhibition of trypsin-like cysteine proteinases (gingipains) from *Porphyromonas gingivalis* by tetracycline and its analogues. Antimicrob Agents Chemother. 2001; 45 (10): 2871-6.
35. Imamura T. The role of gingipains in the pathogenesis of periodontal disease. J Periodontol. 2003;74 (1): 111-8.
36. Johnson GK, Hill M. Cigarette smoking and the periodontal patient. J Periodontol. 2004; 75(2):196-209.
37. Kamer AR, El-Ghorab N, Marzec N, Margarone JE 3rd, Dziak R. Nicotine induced proliferation and cytokine release in osteoblastic cells. Int J Mol Med 2006; 17: 121-7
38. Keene K, Johnson RB. The effect of nicotine on growth of *Streptococcus mutans*. Miss Dent Assoc J. 1999; 55(4): 38-9.
39. Kinder SA, Holt SC. Characterization of coaggregation between *Bacteroides gingivalis* T22 and *Fusobacterium nucleatum* T18. Infect Immun. 1989; 57 (11): 3425-33.

40. Kiyama-Kishikawa M, Hiratsuka K, Abiko Y. Gene expression profiling and characterization under hemin limitation in *Porphyromonas gingivalis.* J Oral Sci. 2005; 47 (4): 191-7.
41. Lahmouzi J, Simain-Sato F, Defresne MP, De Pauw MC, Heinen E, Grisar T, *et al*. Effect of nicotine on rat gingival fibroblasts *in vitro*. Connect Tissue Res. 2000; 41(1): 69-8.
42. Lamont RJ, Gil S, Demuth DR, Malamud D, Rosan B. Molecules of *Streptococcus gordonii* that bind to *Porphyromonas gingivalis*. Microbiology 1994, 140 (Pt. 4): 867–872.
43. Lamont RJ, Jenkinson HF. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. Microbiol Mol Biol Rev. 1998; 62 (4): 1244-63.
44. Lamont RJ, Jenkinson HF. Subgingival colonization by *Porphyromonas gingivalis*. Oral Microbiol Immunol. 2000 Dec;15(6):341-9.
45. Laxman VK, Annaji S. Tobacco use and its effects on the periodontium and periodontal therapy. J Contemp Dent Pract. 2008 Nov 1;9(7):97-107.
46. Lee SY. Effects of chlorhexidine digluconate and hydrogen peroxide on *Porphyromonas gingivalis* hemin binding and coaggregation with oral streptococci. J Oral Sci. 2001; 43 (1): 1-7.
47. Lepine G, Progulske-Fox A. Duplication and differential expression of hemagglutinin genes in *Porphyromonas gingivalis*. Oral Microbiol Immunol. 1996;11 (2): 65-78.
48. Lopatin DE, Combs A, Sweier DG, Fenno JC, Dhamija S. Characterization of heat-inducible expression and cloning of HtpG (Hsp90 homologue) of *Porphyromonas gingivalis*. Infect Immun. 2000; 68 (4): 1980-7.
49. Machuca G, Rosales I, Lacalle JR, Machuca C, Bullon P. Effect of cigarette smoking on periodontal status of healthy young adults. J Periodontol. 2000; 71 (1): 73-8.

50. Mager DL, Haffajee AD, Socransky SS. Effects of periodontitis and smoking on the microbiota of oral mucous membranes and saliva in systemically healthy subjects. J Clin Periodontol. 2003; 30(12): 1031-7.
51. Makino A, Yamada S, Okuda K, Kato T. Nicotine involved in periodontal disease through influence on cytokine levels. FEMS Immunol Med Microbiol 2008; 52: 282-6.
52. Mariggio MA, Guida L, Laforgia A, Santacroce R, Curci E, Montemurro P, *et al*. Nicotine effects on polymorphonuclear cell apoptosis and lipopolysaccharide-induced monocyte functions. A possible role in periodontal disease? J Periodontal Res. 2001; 36(1): 32-9.
53. Marsh PD, Martin M. Oral microbiology. 1992 . 3.ed. London, Chaoman & Hall.
54. Murakami Y, Masuda T, Imai M, Iwami J, Nakamura H, Noguchi T, Yoshimura F. Analysis of major virulence factors in *Porphyromonas gingivalis* under various culture temperatures using specific antibodies. Microbiol Immunol. 2004; 48 (8): 561-9.
55. Nakagawa I, Amano A, Kimura RK, Nakamura T, Kawabata S, Hamada S. Distribution and molecular characterization of *Porphyromonas gingivalis* carrying a new type of *fimA* gene. Clin Microbiol. 2000; 38 (5): 1909-14.
56. Nakagawa I, Amano A, Ohara-Nemoto Y, Endoh N, Morisaki I, Kimura S, Kawabata S, Hamada S. Identification of a new variant of *fimA* gene of *Porphyromonas gingivalis* and its distribution in adults and disabled populations with periodontitis. J Periodontal Res. 2002; 37 (6): 425-32.
57. Nelson KE, Fleischmann RD, DeBoy RT, Paulsen IT, Fouts DE, Eisen JA, *et al*. Complete genome sequence of the oral pathogenic Bacterium *Porphyromonas gingivalis* strain W83. J Bacteriol. 2003; 185 (18): 5591-601.
58. Nogueira-Filho GR, Froes Neto EB, Casati MZ, Reis SR, Tunes RS, Tunes UR, *et al*. Nicotine effects on alveolar bone changes induced by occlusal trauma: a histometric study in rats. J Periodontol. 2004; 75(3): 348-52.

59. Okano S, Shibata Y, Shiroza T, Abiko Y.Proteomics-based analysis of a counter-oxidative stress system in Porphyromonas gingivalis. Proteomics. 2006 Jan;6(1):251-8

60. Pabst MJ, Pabst KM, Collier JA, Coleman TC, Lemons-Prince ML, Godat MS, *et al*. Inhibition of neutrophil and monocyte defensive functions by nicotine. J Periodontol. 1995; 66(12): 1047-55.

61. Pathirana RD, O'Brien-Simpson NM, Brammar GC, Slakeski N, Reynolds EC. Kgp and RgpB, but not RgpA, are important for *Porphyromonas gingivalis* virulence in the murine periodontitis model. Infect Immun. 2007; 75(3):1436-42

62. Pavia CS, Pierre A, Nowakowski J. Antimicrobial activity of nicotine against a spectrum of bacterial and fungal pathogens. J Med Microbiol. 2000; 49(7): 675-6.

63. Payne JB, Johnson GK, Reinhardt RA, Dyer JK, Maze CA, Dunning DG. Nicotine effects on PGE2 and IL-1 beta release by LPS-treated human monocytes. J Periodontal Res. 1996; 31(2): 99-104.

64. Potempa J, Pavloff N, Travis J. *Porphyromonas gingivalis*: a proteínase/gene accounting audit. Trends Microbiol. 1995; 3 (11): 430-4.

65. Potempa J, Pike R, Travis J. Titration and mapping of the active site of cysteine proteinases from *Porphyromonas gingivalis* (gingipains) using peptidyl chloromethanes. Biol Chem. 1997; 378 (3-4): 223-30.

66. Progulske-Fox A, Tumwasorn S, Lepine G, Whitlock J, Savett D, Ferretti JJ, Banas JA. The cloning, expression and sequence analysis of a second *P. gingivalis* gene that codes for a protein involved in hemagglutination. Oral Microbiol Immunol. 1995; 10 (5): 311-8.

67. Riviere GR, Smith KS, Tzagaroulaki E, et al. Periodontal status and detection frequency of bacteria at sites of periodontal health and gingivitis. J Periodontol. 1996; 67(2):109-15.

68. Rudney JD, Chen R, Sedgewick GJ, Intracellular *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in buccal epithelial cells collected from human bjects. Infect. Immun. 2001, *69*, 2700–2707.
69. Salvi GE, Ramseier CA, Kandylaki M, Sigrist L, Awedowa E, Lang NP. Experimental gingivitis in cigarette smokers: a clinical and microbiological study. J Clin Periodontol. 2005; 32(5): 441-7.
70. Sayers NM, Gomes BP, Drucker DB, Blinkhorn AS. Possible lethal enhancement of toxins from putative periodontopathogens by nicotine: implications for periodontal disease. J Clin Pathol. 1997; 50(3): 245-9
71. Sayers NM, James JA, Drucker DB, Blinkhorn AS. Possible potentiation of toxins from *Prevotella intermedia*, *Prevotella nigrescens*, and *Porphyromonas gingivalis* by cotinine. J Periodontol. 1999; 70(11): 1269-75.
72. Schenkein HA, Gunsolley JC, Koertge TE, Schenkein JG, Tew JG. Smoking and its effects on early-onset periodontitis. J Am Dent Assoc. 1995; 126(8): 1107-13.
73. Socransky SS, Haffajee AD. Dental biofilms: difficult therapeutic targets. Periodontol 2000. 2002; 28: 12-55.
74. Teughels W, Van Eldere J, van Steenberghe D, Cassiman JJ, Fives-Taylor P, Quirynen M. Influence of nicotine and cotinine on epithelial colonization by periodontopathogens. J Periodontol. 2005; 76(8): 1315-22.
75. Tonetti MS. Cigarette smoking and periodontal diseases: etiology and management of disease. Ann Periodontol. 1998; 3(1): 88-101.
76. Umeda JE, Missailidis C, Longo PL, Anzai D, Wikström M, Mayer MP. Adhesion and invasion to epithelial cells by *fimA* genotypes of Porphyromonas gingivalis. Oral Microbiol Immunol. 2006; 21(6):415-9.
77. Vaahtoniemi LH, Raisanen S, Stenfors LE. Attachment of bacteria to oral epithelial cells in vivo: a possible correlation to gingival health status. J Periodontal Res. 1993; 28:308-311

78. van der Velden U, Varoufaki A, Hutter JW, Xu L, Timmerman MF, Van Winkelhoff AJ, *et al*. Effect of smoking and periodontal treatment on the subgingival microflora. J Clin Periodontol. 2003; 30(7): 603-10.
79. van Winkelhoff AJ, Bosch-Tijhof CJ, Winkel EG, van der Reijden WA. Smoking affects the subgingival microflora in periodontitis. J Periodontol. 2001; 72(5): 666-71.
80. Vitkov L, Krautgartner WD, Hannig M. Bacterial internalization in periodontitis. Oral Microbiol. Immunol. 2005, *20*, 317–321.
81. Wendell KJ, Stein SH. Regulation of cytokine production in human gingival fibroblasts following treatment with nicotine and lipopolysaccharide. J Periodontol. 2001; 72(8): 1038-44.
82. Xia Q, Wang T, Taub F, Park Y, Capestany CA, Lamont RJ, Hackett M. Quantitative proteomics of intracellular *Porphyromonas gingivalis*. Proteomics. 2007; 7 (23): 4323-37.
83. Yamamoto Y, Nishida N, Tanaka M, Hayashi N, Matsuse R, Nakayama K, *et al*. Association between passive and active smoking evaluated by salivary cotinine and periodontitis. J Clin Periodontol. 2005; 32(10): 1041-6.
84. Yao ES, Lamont RJ, Leu SP, Weinberg A. Interbacterial binding among strains of pathogenic and commensal oral bacterial species. Oral Microbiol Immunol. 1996; 11 (1): 35–41.
85. Yilmaz O, Young PA, Lamont RJ, Kenny GE. Gingival epithelial cell signalling and cytoskeletal responses to Porphyromonas gingivalis invasion. Microbiology. 2003; 149 (9): 2417-26.
86. Yuan L, Hillman JD, Progulske-Fox A. Microarray analysis of quorum-sensing-regulated genes in *Porphyromonas gingivalis*. Infect Immun. 2005; 73 (7): 4146-54
87. Zambon JJ, Grossi SG, Machtei EE, Ho AW, Dunford R, Genco RJ. Cigarette smoking increases the risk for subgingival infection with periodontal pathogens. J Periodontol. 1996; 67 (10 Suppl): 1050-4.

88. Zhang Y, Wang T, Chen W, Yilmaz O, Park Y, Jung I-Y, Hackett M, Lamont RJ Differential protein expression by *Porphyromonas gingivalis* in response to secreted epithelial cell components. Proteomics 2005, 5: 198–211.

7. ANEXO

Abaixo, encontra-se o comprovante de submissão de um dos artigos científicos que compõem essa tese (*The effects of nicotine and cotinine on Porphyromonas gingivalis adhesion and invasion of epithelial cells*) para a Revista *Archives of Oral Biology*.

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